BEFORE THE CONTROLLER OF PATENTS,

New Delhi

PRE GRANT OPPOSITION UNDER SECTION 25 (1) AGAINST

PATENT APPLICATION NO. 9322/DELNP/2007

Fresenius Kabi Oncology Ltd. .....Opponent

-Vs-

Novartis AG, Switzerland .....Applicant

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(Opponent)

Dr. Prachi Tiwari

Fresenius Kabi Oncology Ltd.

The Controller of Patents

The Patent Office, New Delhi

BEFORE THE CONTROLLER OF PATENTS

THE PATENT OFFICE

NEW DELHI

In the matter of Pre grant opposition under section 25(1) of The Patents Act, 1970 *as amended by* The Patents (Amendment) Act 2013,

-And-

In the matter of The Patents Rules, 2003 as *amended by* The Patent (Amendment) Rules 2016

-And-

IN THE MATTER of Patent Application No. 9322/DELNP/2007 dated 18 July 2006 and nationalized in India on 03 December 2007 made by Novartis AG, Lichtstrasse 35 CH-4056 Basel, Switzerland.

……Applicant

-And-

IN THE MATTER of opposition to the grant of a patent thereto under Section 25(1) by.....

…….Opponent

REPRESENTATION UNDER SECTION 25 (1)

We, FRESENIUS KABI ONCOLOGY LIMITED B-310, Som Datt Chambers-I Bhikaji Cama Place, New Delhi -110 066, India; (hereinafter called 'opponent') make the following representation under Section 25(1) of the Act in opposing the grant of patent on the application indicated in the cause title.

Opponent's business and activities

The opponent is a Company incorporated under the Companies Act, 1956, and having its principal office FRESENIUS KABI ONCOLOGY LIMITED, B-310, Som Datt Chambers-I Bhikaji Cama Place, New Delhi -110 066, India; carrying on business, inter alia, of manufacture, research and sale of anticancer agents.

Locus standi

Locus standi is not a condition precedent for an opposition under Section 25(1). In any event it is stated that the application under opposition relates to an alleged invention in the field of pharmaceutical products. The opponent being engaged in the research and development as well as in the manufacture of drugs / medicinal compositions for many years has interest in opposing the grant of patent to the application under opposition and is a person interested and therefore has locus standi to initiate the present proceedings.

1. GROUNDS OF OPPOSITION

The application is opposed on the following grounds:

a. Section 25(1) (b) -Prior Publication

b. Section 25(1) (d) -Prior Public Knowledge/Public Use In India

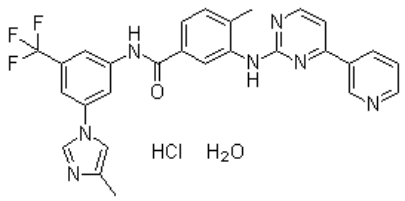
c. Section 25(1) (e) -Obviousness/Lack of Inventive Step

d. Section 25(1) (f) -Not an Invention

e. Section 25(1) (g) -Insufficiency of disclosure

2. Review of the Applicant's claims:

The opposed application is directed to Nilotinib hydrochloride monohydrate represented by following formula:



It also seeks to protect a method of preparing the Nilotinib hydrochloride monohydrate. Further, it seeks to protect a pharmaceutical composition comprising Nilotinib hydrochloride monohydrate and at least one pharmaceutically acceptable carrier, diluents, vehicle or excipient.

3. Claims of the opposed application: The claims of the opposed application have been amended on 18 March 2013. Presently, there are total three (3) claims, presented herein below for ready reference:

Claim 1*: A salt of 4-methyl-N-[3-(4-methyl-imidazol-1-yl)-5-trifluoromethyl-phenyl]-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-benzamide, which is 4-methyl-N-[3-(4-methyl-imidazol-1-yl)-5-trifluoromethyl-phenyl]-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-benzamide monohydrochloride monohydrate.*

Claim 2*: A method of preparing 4-methyl-N-[3-(4-methyl-imidazol-1-yl)-5-trifluoromethyl-phenyl]-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-benzamide monohydrochloride monohydrate comprising the steps of:*

a. *combining 4-methyl-n-[3-(4-methyl-imidazol-1-yl)-5-trifluoromethyl-phenyl]-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-benzamide free base and hydrochloric acid in methanol under a nitrogen atmosphere;*

b. *heating the reaction mixture to a temperature ranging from about 42-50 °C;*

c. *stirring the reaction mixture;*

d. *filtering the reaction mixture while maintaining the temperature above 40 °C to obtain a clear solution;*

e. *cooling the clear solution to about 30 °C while stirring under nitrogen atmosphere;*

f. *seeding the solution;*

g. *cooling the seeded solution to about 23 °C;*

h. *stirring the solution to obtain a suspension;*

i. *cooling the suspension to about -10 °C;*

j. *stirring the suspension;*

k. *filtering solids;*

l. *rinsing the solids with cold methanol; and*

m. *drying the solids at about 50 -55 °C and 10-20 torr to obtain 4-methyl-n-[3-(4-methyl-imidazol-1-yl)-5-trifluoromethyl-phenyl]-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-benzamide monohydrochloride monohydrate salt.*

Claim 3: *A pharmaceutical composition comprising:*

a) *a therapeutically effective amount of a salt as claimed in claim 1; and*

b) *at least one pharmaceutically acceptable carrier, diluents, vehicle or excipient.*

4. There is no notification of grant of patent on the opposed application as per the current status at Indian Patent office website. The present representation is framed on the basis of above claims of the Indian patent application no. 9322/DELNP/2007. In the event that the claims are further amended and the present representation does not automatically address the subject matter of the amended claims, the opponent craves leave to file a fresh or supplementary representation, if the amendments warrant so. The opponent has examined and carefully considered the complete specification of the opposed application and wishes to draw the attention of the Ld. Controller to the most salient features therein under the grounds of opposition discussed herein.

5. <u>DOCUMENTS RELIED ON:</u>

- WO2004/005281 entitled "Inhibitors of tyrosine kinase" published on 15 Jan 2004; referred herein as **D1** and annexed hereto as **Exhibit 1**.

- Medical Review of Nilotinib downloaded from USFDA published on 29 Oct 2007; referred herein as **D2** and annexed hereto as **Exhibit 2**. Can be downloaded from the link https://www.accessdata.fda.gov/drugsatfda_docs/nda/2007/022068TOC.cfm

- Terese Bergfors et. Al "Seeds to crystals" published in Journal of Structural Biology 2003; 142: 66–76; referred herein as **D3** and annexed hereto as **Exhibit 3**.

- Thomas et. al "In vitro activity of Bcr-Abl inhibitors AMN107 and BMS-354825 against clinically relevant imatinib-resistant Abl kinase domain mutants" published in Cancer Res 2005; 65 (11): 4500-4505; referred herein as **D4** and annexed hereto as **Exhibit 4**.

- Mirna Golemovic et. al "AMN107, a Novel Aminopyrimidine Inhibitor of Bcr-Abl, Has In vitro Activity against Imatinib-Resistant Chronic Myeloid Leukemia, Clin Cancer Res July 1, 2005; 11 (13): 4941-4947; referred herein as **D5** and annexed hereto as **Exhibit 5**.

- Weisberg et. al "Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl" published in Cancer Cell: February 2005, Vol. 7, 129-141; referred herein as **D6** and annexed hereto as **Exhibit 6**.

- ❖ Hynes et.al "Hydrate formation in NH+ containing salts of pharmaceutically acceptable anions: A CSD survey" published in *Cryst Eng Comm*, 2005, 7(55), 342-345; referred herein as **D7** and annexed hereto as **Exhibit 7**.

- ❖ P.H. Stahl and C.G. Wermuth "Handbook of Pharmaceutical Salts (2002)- Chapter II"; referred herein as **D8** and annexed hereto as **Exhibit 8**.

- ❖ Stephen Byrn et. al "Pharmaceutical solids: A strategic approach to regulatory consideration" published in *Pharmaceutical Research, Vol 12, No. 7, 1995*; referred herein as **D9** and annexed hereto as **Exhibit 9**.

- ❖ WO2005/054235 entitled "Process for the Preparing Ziprasidone Monohydrochloride Hydrate" published on 16 June 2005; referred herein as **D10** and annexed hereto as **Exhibit 10**.

- ❖ Pharmacological Review of Nilotinib downloaded from USFDA published on 29 Oct. 2007; annexed hereto as **Exhibit 11**. Can be downloaded from the link https://www.accessdata.fda.gov/drugsatfda_docs/nda/2007/022068TOC.cfm

- ❖ Dog Experimentation Data submitted by Applicant in Response of First Examination Report from Indian Patent Office along with a declaration from a Scientist Tsu-Han declaration; annexed hereto as **Exhibit 12**.

- ❖ Product Monograph of Tasigna® from Health Canada annexed hereto as **Exhibit 13**. Can be downloaded from the link https://health-products.canada.ca/dpd-bdpp/info.do?lang=en&code=80138

GROUNDS OF OPPOSITION

6. ANTICIPATION/LACK OF NOVELTY -section 25 (1)(b) and section 25 (1)(d) & OBVIOUSNESS/LACK OF INVENTIVE STEP -section 25 (1)(e)

The claims of opposed application lack novelty and are not patentable under section 25(1)(b) and (d) of the Indian patents act, 1970 (as amended in 2005, hereinafter referred to as “the Act”).

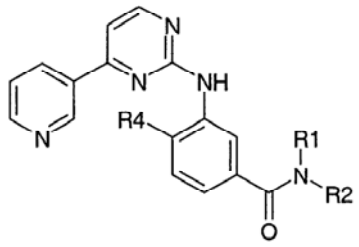
The claims of opposed application also lack inventive step and are not patentable under section 25(1)(e) of the Indian patents act, 1970 (as amended in 2005, hereinafter referred to as “the Act”).

6.1 Claim 1: Lack of Novelty in view of D1:

The subject matter of claim 1 relates to a hydrated form of an acid addition salt of Nilotinib. More particularly, it relates to hydrochloric acid salt of Nilotinib in the hydrated form.

The opponent states that the subject matter of claim 1 was well known in the art before the priority date of the opposed application therefore it lacks novelty.

D1 discloses compounds of formula (I) or a pharmaceutically acceptable salt of such compounds. The compound of formula (I) is represented below:



Formula (I)

Also, D1 specifically describes processes for the preparation of such compounds. In an embodiment, D1 states that *“a compound of formula (I) is prepared according to or in analogy to the processes and process steps defined in the Examples”*

More particularly, example 92 of D1 describes a process for the preparation of a compound *viz.* “4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]aminol-*N*-[5-(4-methyl-1 H-imidazol-1-yl)-3-(trifluoromethyl)phenyl]benzamide”, which is IUPAC name of the compound Nilotinib.

Therefore, it is clear that the compound of formula (I) encompass Nilotinib. In general, claim 9 provides specific definitions of various substituents for compound of formula (I) to result the compound Nilotinib.

Related to acid addition salt of Nilotinib, D1 discloses that *"Salts are especially the pharmaceutically acceptable salts of compounds of formula (I). Such salts are formed, for example, as acid addition salts, preferably with organic or inorganic acids, from compounds of formula I with a basic nitrogen atom, especially the pharmaceutically acceptable salts. Suitable inorganic acids are, for example, halogen acids, such as hydrochloric acid, sulfuric acid, or phosphoric acid* (see page 08, paragraph 1).

D1 further provides method for the preparation of the salts of compound of formula (I), *"…. Salts of a compound of formula I with a salt-forming group may be prepared in a manner known per se. Acid addition salts of compounds of formula I may thus be obtained by treatment with an acid or with a suitable anion exchange reagent*" (see Page 29, paragraph 4)

In view of above, it is clear that acid addition salt or more particularly hydrochloride salt of Nilotinib was prepared in D1.

Related to hydrate form of Nilotinib hydrochloride, D1 discloses that "*The compounds of formula I, including their salts, are also obtainable in the form of hydrates, or their crystals can include for example the solvent used for crystallization ……* (see page 31, paragraph 6). In other words, hydrated form of Nilotinib hydrochloride was obtained in D1.

In view of above, it is clear that each and every limitation of the claimed invention was disclosed in D1. Therefore, the subject matter of claim 1 cannot be considered novel and hence ought to be rejected.

6.1.1 Claim 1: Lack of Inventive Step in view of D1:

Opponent submits that subject matter of claim 1 do not involve inventive step and ought to be rejected on this ground as well. As explained above, Nilotinib was specifically prepared in example 92 of D1. Opponent states that starting from Nilotinib and making hydrochloride (HCl) salt and hydrate thereof is well within the skill set of a person having ordinary skill in the art. Therefore the obtained product cannot be considered inventive.

As evident from the disclosure from the opposed application, the applicant has followed a standard process of making the HCl salt by treating the Nilotinib with

hydrochloric acid. Also, the applicant failed to show any comparative examples to substantiate the difference from the product obtained in D1 w.r.t. the product of claim 1 of the opposed application. Therefore, the subject matter of the instant claim cannot be considered inventive in the absence of such comparative data. Hence ought to be rejected at its outset.

6.2 Claim 1: Lack of Novelty in view of D2:

D2 is a Medical Review of Nilotinib hydrochloride monohydrate, submitted by Novartis before the USFDA.

D2 provides chemical structure of nilotinib hydrochloride monohydrate under code AMN107 along with chemical name (see page 16, section 2, Introduction and background). D2 also provides all the regulatory activities with respect to nilotinib hydrochloride monohydrate in a chronological order (see page 17, section 2.5, Presubmission Regulatory activities). D2 discloses that investigational new drug application for nilotinib hydrochloride monohydrate was submitted on 24 April 2004. Therefore it is clear that nilotinib hydrochloride monohydrate was known before the priority date of the opposed application. Hence, subject matter of claim 1 lacks novelty.

6.3 Claim 1: Lack of Novelty in view of D4

The claim 1 lacks Novelty in view of D4, the D4 published on 01 June 2005 which is before the priority date of opposed application.

D4 discloses use of "***NVP-AMN107-AA***; *4-methyl-N-[3-(4-methyl-1H-imidazol)-5-(trifluoromethyl) phenyl]-3-(3-pyridinyl)-2pyridinyl]amino] benzamide, hydrochloride*" (see page 4501, first column, under heading "Reagents"), which is IUPAC name of nilotinib hydrochloride, for the study of in vitro activity of Bcr-Abl kinase inhibitors against clinically relevant imatinib-resistant Abl kinase domain mutants.

The opponent states that D4 discloses the use of nilotinib hydrochloride without providing information about its hydration state. The applicant of the opposed application, D4 and product Tasigna® at USFDA are same, the product Tasigna® has been approved by FDA on 29 Oct. 2007 as nilotinib hydrochloride monohydrate for the treatment of chronic myelogenous leukaemia. During pharmacological review **(Attached as Exhibit 11)** of the Nilotinib at FDA, applicant (Novartis) himself provides the ***NVP-AMN107-AA code for nilotinib hydrochloride monohydrate.*** Opponent is aware of the fact that Exhibit 11 is not a prior art document; it is

presented herein to show that code NVP-AMN107-AA, known from prior art document D4, is used for nilotinib hydrochloride monohydrate.

From the above diclosure, it is evident that nilotinib hydrochloride monohydrate was known prior to priority date of opposed applicatiion. Hence the claim 1 of the opposed application lacks novelty in view of D4.

6.4 Claim 2: Lack of Novelty in view of D1:

The subject matter of claim 2 relates to a process for the preparation of nilotinib hydrochloride monohydrate by preparing a solution of nilotinib free base with hydrochloric acid in methanol. The solution was filtered, seeded and dried.

Opponent states that such process forms the part of prior art D1. D1 describes a method for the preparation of the salts of compound of formula (I) [i.e. nilotinib], *"…. Salts of a compound of formula I with a salt-forming group may be prepared in a manner known per se. Acid addition salts of compounds of formula I may thus be obtained by treatment with an acid or with a suitable anion exchange reagent*" (see Page 29, paragraph 4)

D1 further discloses that "*The compounds of formula I, including their salts, are also obtainable in the form of hydrates, or their crystals can include for example the solvent used for crystallization ……* (see page 31, paragraph 6).

In view of above disclosure, it is clear that all the steps of process claim 2 were disclosed in D1, therefore it lacks novelty.

6.4.1 Claim 2: Lack of inventive step in view of D1:

It is submitted that the applicant failed to substantiate the difference from the process described in D1 w.r.t. the process of the instant claim, therefore process of claim 2 lacks inventive step as well.

It is further submitted that the use of seeds for crystallization is well known in the art. To this effect, opponent rely upon D3 which describes applications and recommendations for seeding. D3 suggest that "*Seeding is a powerful tool for the separation of nucleation and growth. In this technique, previously nucleated crystals are used as seeds and introduced into new drops equilibrated at lower levels of supersaturation.* Therefore, in view of disclosure of D1 and D3, the subject matter of claim 2 cannot be considered inventive and hence ought to be rejected on this ground as well.

6.5 Claim 3: Lack of Novelty in view of D1:

D1 discloses "*The present invention relates especially to pharmaceutical composition that comprise a* compound of formula I*, ... or a pharmaceutically acceptable salts, or a hydrate or solvate thereof, and at least one pharmaceutically acceptable carrier*" see page 32, paragraph 4), and "*a pharmaceutical preparation ..... Comprises an effective quantity of a compound of formula I or N-oxides thereof .... , or a pharmaceutically acceptable salt thereof, if salt-forming groups are present, together with at least one pharmaceutically acceptable carrier*." (see page 33, 1st paragraph).

Further, D1 provides "*Pharmaceutical compositions for oral administration can be obtained, ..., by combining the active ingredient with one or more solid carriers, ... , by the inclusion of additional excipients, ...*" (see page 34, 3rd paragraph).

Thus, D1 discloses pharmaceutical composition comprising an effective amount of compound I, which includes nilotinib hydrochloride monohydrate (as decribed under Lack of Novelty- claim 1) and at least one pharmaceutically acceptable carrier or excipients, correponding to subject matter of claim 3. Thefore claim 3 lacks novelty in view of D1.

7. NOT AN INVENTION/NOT PATENTABLE -section 25 (1)(f)

7.1 Invention not patentable as per Section 2(1) (ja)

The opponent states that claimed invention falls under the mischief of section 2(1)(ja). According to the definition of inventive step "*the invention should be a technical advancement over the prior art or it should show economical significance or both and should not be obvious to a person skilled in the art"*. Opponent states that there is no technical advancement of the compound of claim 1 is disclosed or demonstrated in the specifications over the compounds disclosed in D1.

Opponent states that the applicant is trying to claim an already known compound and therefore is not entitled to a patent and ought to be rejected in toto.

7.2 Invention not patentable as per section 3(d)

The opponent states that the nilotinib hydrochloride monohydrate as claimed in the opposed application is not an invention within the meaning of section 3(d) of the Act.

It is submitted that in the case of Novartis Vs. Union of India [AIR 2013 SC 1311], the Supreme Court especially in paragraph 187 has held that *"In whatever way*

therapeutic efficacy may be interpreted, this much absolutely clear that the physical-chemical properties of beta crystalline form of Imatinib Mesylate, namely

(i) more beneficial flow properties, (ii) better thermodynamic stability, and (iii) lower hygroscopicity, may be otherwise beneficial but these properties cannot even be taken into account for the purpose of the test of Section 3(d) of the Act, since these properties have nothing to do with therapeutic efficacy".

The Supreme Court has also held in paragraph 188 that "*Bioavailability falls outside the area of efficacy in case of a medicine .... ".*

Further, in paragraph 189, the Court held that " ... *the position that emerges is that just increased bioavailability alone may not necessarily lead to an enhancement of therapeutic efficacy ....* ".

The Supreme Court has also held that *"... whether or not an increased in bioavailability leads to enhancement of therapeutic efficacy in any given case must be specifically claimed and established by research data .... "*.

However, in the case at hand, the subject matter of claim 1 fails to pass the test of Section 3(d) as interpreted by the Supreme Court because:

(i) No material on record to establish enhanced therapeutic efficacy:

It is submitted that there is no document or material on record to establish therapeutic efficacy as required by the Supreme Court. No research data is presented to fortify the conclusion that compound of claim 1 has better therapeutic efficacy. There is nothing in the patent application to support this conclusion. As per Supreme Court, therapeutic efficacy must be specifically established through research data, which has not been put forth in the present case.

(ii) Bioavailability does not automatically mean therapeutic efficacy:

It is submitted that therapeutic efficacy and bioavailability are two different parameters and are required to be specifically demonstrated through research data. Bioavailability is pharmacokinetic parameter whereas therapeutic efficacy is a pharmacodynamic parameter. Even as per the directions of the Hon'ble Supreme Court, therapeutic efficacy must be specifically established through research data, which has not been put forth in the present case.

(iii) Comparison of nilotinib versus nilotinib hydrochloride monohydrate: Not proper and does not satisfy Section 3(d) requirements

The applicant has compared the bioavailability of nilotinib with nilotinib hydrochloride monohydrate (see Exhibit 12).

In this regard, as mentioned earlier, the bioavailability data alone is not enough to fulfill the requirements of Sec. 3(d), as per the directions laid by the Supreme Court.

The opponent further submits that nilotinib free base is not the closest prior art compound in the instant case, therefore the comparison with nilotinib free base is not proper.

7.3 Invention Not patentable as per section 3(e)

Claim 3 of the opposed application is not patentable because the composition claimed is a mere admixture resulting only in the aggregation of the properties of the components.

Opponent submits that it is required to be shown that a composition comprising the components provides not only the aggregation of properties expected from the components, but an unexpected property resulting from the combination. In the present case no such data has been presented therefore cannot be considered patentable under the act.

8. LACK OF INVENTIVE STEP AND OBVIOUSNESS under Section 25 (1)(e)

8.1 Claim 1: Lack of inventive step and obviousness:

The focus of the opposed application lies in the formation of an alternative solid form of the known compound nilotinib hydrochloride or hydrate thereof known from D1, D5, D6 and Exhibit 11. Due to different physical properties e.g. concerning storage stability or solubility characteristics, such solid forms are of interest in the pharmaceutical field. The existence of different solid forms of chemical compounds such hydrates; solvates etc. is well known in the art. It is thus not surprising that different forms do also exist of nilotinib hydrochloride. An inventive step may only be considered if surprising or unexpected effect in view of the closest prior art originates.

- ✓ D1 discloses nilotinib, its hydrochloride salt and hydrates thereof.

- ✓ D5 mentions "*Imatinib and AMN107 monohydrochloride were provided by (Novartis, East Hanover, NJ)* (see page 4942, 2nd column, under heading "drugs"). Thus D5 discloses nilotinib mono-hydrochloride.

- ✓ D6 relates to characterization of AMN 107 as a selective inhibitor of native and mutant Bcr-Abl. Further, it provides pharmacokinetic parameters of *AMN107/AA-salt*, following single oral administration of either 20 mg/kg or 75 mg/kg to naïve mice (See page 135, title of Table 2). The code *AMN107/AA-salt* is defined as mono-hydrochloride salt of nilotinib in product monograph of Tasigna®, which is submitted by the applicant at Health Canada (see page 54, section Detailed Pharmacology of Exhibit 13). The applicant of the opposed application, D6 and product Tasigna® at Health Canada are same therefore applicant submits that code *AMN107/AA-salt* of D6 is mono-hydrochloride salt of nilotinib, which is known before the priority date of the opposed application.

- ✓ Exhibit 11 discloses Toxicology study of *AMN107 Hydrochloride,* which is Nilotinib mono-hydrochloride. As per details, the Toxicology study has been initiated on 26 Nov. 2003 which is before the priority date of the opposed application (See page 91 of Exhibit 11).

8.1.1 Nilotinib Hydrochloride was known from D5 and/or D6 and/or Exhibit 11 and its hydrate was known from D1. All applicant has done is the formation of a monohydrate of the compound known from D5 and/or D6 and/or Exhibit 11 and verification of physicochemical parameters such as solubilty and/or stabilty. It is well known in the art to try the different solid form of a drug compound and verify its physiochemical properties.

8.1.2 There are several references in the art that shows hydrate formation is common in the pharmaceutical field and most importantly is an inherent property of certain salts like hydrochloride. For instance three such references are provided herein:

D7 dicloses hydrate formation tendency in NH+ containing salts of pharmaceutically acceptable anions. It provides that "*there is high occurence of halides and carboxylates salts in all the cation groups ..... there is increased occurence of halides salts in tertiary amine and cyclic amines at the expense of the other anion groups*" (see page 342, second column, heading "Anion Profiles". With regard to hydrate formation, it dicloses "*within the group of anions studies,* ***halides tend to be more highly hydrated*** *than carboxylates*" (see page 344, second column, first two lines). It is evident from diclosure of

D6 that drug having amine (NH+) functionality are more easily crystallised with halide anion and are more highly hydrated. In the present case, halide salt of nilotinib i.e. nilotinib hydrochloride is known in the art and teaching of D7 provides motivation to a person skilled in the art to look for hydrate formation in hydrochloride salt of nilotinib.

D8 provides a general method for the preparation of phamaceutically acceptable salts including hydrochloride salts. It discloses "*hydrochlorides require stoichiometric amount of H_2O to be present in order to yield hydrated crystals*". It shows that there is autonomus occurence of hydrates in case of hydrochoride salts of basic drug substance.

D9 discloses a conceptual approach to the development of pharmaceutical solids and presents a step by step approach for the solid state research of a drug. D9 provides four decision trees to follow, so that a desired form of a drug molecule can be achieved. The decision trees relate to polymorphs, **hydrates**, desolvated solvates, and amorphous forms. ***D9 also advises to investigate the drug substance for the existence of*** *polymorphs and* ***hydrates*** *due to various reasons* (see page 946, column first). The first step mentioned is the crystallisation of the substance from a number of different solvents (See page 946, second column). Next step after the preliminary crystallisation is to check for hydrate formation using a solvent-water mixture (See page 949, first column).

8.1.3 Further, opponent states that hydrate formation is a common phenomenon of drug molecules and industry make wide spread use of it: Many drugs were approved in the US in the form of hydrochloride monohydrates prior to the priority date of the opposed application i.e. 18 July 2005. Some of them are mentioned here with the approval year **(taken from http://www.fda.gov/default.htm):**

- Lincomycin hydrochloride monohydrate (Prior to 01 Jan. 1982)
- Ropivacaine hydrochloride monohydrate (24 Sep. 1996)
- Sibutramine hydrochloride monohydrate (22 Nov. 1997)
- Tirofiban hydrochloride monohydrate (17 May 2002)
- Ciprofloxacin hydrochloride monohydrate (26 Sep. 1997)
- Cephalexin hydrochloride monohydrate (Prior to 01 Jan. 1982)
- Cefepime hydrochloride monohydrate (18 Jan. 1996)

8.1.4 The teachings of cited prior art references are summarized below:

- Nilotinib, its hydrochloride salt and hydrate thereof is known from D1.
- Nilotinib hydrochloride is known from D5, D6 and Exhibit 11.
- Higher percentage of hydrate formation in halide salts of drug having amine (NH+) functionality is known from D7.
- Monohydrate formation is common among hydrochloride salts of pharmaceutical compounds and widely used in industry as evident from the list of FDA approved drugs.
- Teaching of the prior art put emphasis on the investigation of the existence of polymorphs and hydrates (D8 and D9) and screening methodology to obtain a suitable solid state of a drug molecule is well known in the prior art such as D9.

8.1.5 Based on the above prior arts, it is obvious for the person skilled in art to follow the recommended strategies for the study of the solid state properties to arrive at a desired solid state of the known compound i.e. nilotinib hydrochloride. Thus, claim of the opposed application lack substantial technical advancement and therefore lacks inventiveness.

8.1.6 It is also mentioned in "Manual of Patent office Practice and Procedure 2011" page 79 under handing "Determination of inventive step"

"***If the invention is predictable based on the available prior art, merely requiring workshop improvement by a person skilled in the art, the inventive step is lacking***"

Monohydrate form claimed in the opposed application is a result of routine experimentation and is predictable based on the prior art, thus does not involve any inventive merit. Therefore, the claims of the opposed application are prima facie obvious to a person skilled in the art. Thus, as per the submissions made under this section, the Opponent contends that the claims of the opposed application are obvious and devoid of inventive merit on the basis of D1 or D4 or D5 combined with D7 and/or D8 and/or D9.

8.2 Claim 2: Lack of inventive step in view of D5/D6 combined with D1 or D8- D10:

Claim 2 define process for the production of Nilotinib hydrochloride monohydrate using methanol a solvent. However, applicant does not present any specific technical feature of the process claimed and are hence not inventive.

With regard to process for preparing salts, specification of opposed application mentioned "*Generally, as used herein, "salt" refers to a compound prepared by the reaction of an organic acid or base drug with a pharmaceutically acceptable mineral or organic acid or base*" (see page 4 paragraph [0015]). Same process methodology has been claimed in claim 2 of opposed application and also known from prior art references D1, D8, D9 and D10.

D1 discloses that "*salts of a compound of formula I with a salt forming group may be prepared in a manner known per se. Acid addition salts of compound of formula I thus may be obtained by the treatment with an acid or with a suitable anion exchange reagent"* (see page 29, 4th paragraph). D1 further discloses nilotinib, its hydrochloride salt and hydrates thereof.

D8 discloses a general process for the hydrochloride formation of basic drug substances, by "*a typical procedure is to dissolve the organic base in the minimal amount of hot isopropanol and to add calculated amount of concentrated HCl*" (see page 250, point 2.1). It provides various examples with drug molecules using different solvent such as diethyl ether (procedure [1]), *methanol* (procedure [2]) and ethanol (procedure [3]).

D9 provides solvents for the crystallization steps – "*water, methanol, ethanol ..... New crystal forms can be obtained by cooling hot saturated solution...*" (see page 946, column second, third paragraph).

D10 provides the process for the preparation of thiazol compound (ziprasidone) by the *reaction of free base with hydrogen chloride in aqueous organic solvent*, wherein solvent include *methanol* (see page 7, lines 5-10).

In view of above, the process provided by applicant as well as solvent used in the process are already disclosed in prior art references. So the subject matter of claim 2 of the opposed application lacks inventive step and is thus liable to be rejected.

9. Insufficiency of disclosure -Section 25 (1)(g)

At the onset, the opponent states that the whole objective of a patent grant is that a quid pro quo system is followed, whereby the Patent Office grants a patent to an inventor when he discloses the mode and method of performing an invention, along with details pertaining to the invention such as prior art, description etc. The very basis of granting a patent is to provide monopoly right to the inventor/applicant in

lieu of disclosure of the working of the invention to enable an unimaginative individual having sufficient skill in the art, to perform the invention in its best embodiment.

The opponent regrets to state that the applicant has attempted to mock the institution of patenting itself by filing the subject application. It is absolutely distressing to state that the opposed application does not adhere to the aforementioned criteria in addition to that of non- obviousness, and inventive merit.

Opponent states that opposed application fails to provide a method to obtain compound 'nilotinib hydrochloride monohydrate'

It is submitted that the process outlined in example 1 *vis-a-vis* claim 2 of the opposed application has not been described in such a precise manner as to allow the person skilled in the art to rework it.

The opposed application describes a method for preparing nilotinib monohydrochloride monohydrate salt only in Example 1. In this example seeds were added at specific temperature and also claim 2 defines "seeding the solution" in step f) as mandatory step. However, there is no disclosure as how these seeds are prepared so as to follow the process of claim 2, so as to prepare nilotinib hydrochloride monohydrate. Therefore, there is insufficiency of disclosure for claim 1 and claim 2.

Further, without having nilotinib hydrochloride in hands, it will be impossible for a person skilled in the art to prepare its pharmaceutical composition (subject matter of claim 3). Hence, there is insufficiency of disclosure with regard to subject product as well as its composition in the absence of sufficiency of the process to prepare the product.

All claims of the opposed application lack sufficiency of disclosure, therefore application in *toto* ought to be rejected on the basis of insufficiency of disclosure as well.

10. RELIEF SOUGHT

The opponent has established and made out a case on each of the aforesaid grounds of opposition and pray to the Ld. Controller for the following relief(s).

1) take on record the present representation ;

2) leave to file additional evidence(s);
3) forward copy of reply of applicant and evidence if any and any amendments filed;
4) leave to file a replication to the reply of the applicant and evidence
5) refusal of the application in *toto*;
6) such other relief or reliefs as the Controller may deem appropriate.
7) Grant of Hearing

Dated this the 15th day of June 2017

Dr. Prachi Tiwari (IN/PA-2045)
Of Fresenius Kabi Oncology Ltd.

(Opponent)

To

The Controller of Patents

The Patent Office Branch

New Delhi

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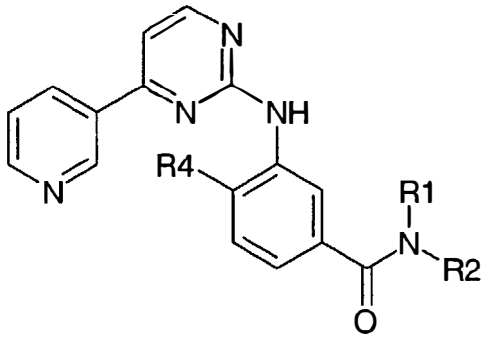
(54) Title: INHIBITORS OF TYROSINE KINASES



(I)

(57) Abstract: The invention relates to compounds of formula (I) Wherein the substituents R1, R2 and R4 have the meaning as set forth and explained in the description of the invention, to processes for the preparation of these compounds, pharmaceutical compositions containing same, the use thereof optionally in combination with one or more other pharmaceutically active compounds for the therapy of a disease which responds to an inhibition of protein kinase activity, especially a neoplastic disease, in particular leukaemia, and a method for the treatment of such disease.

Inhibitors of Tyrosine Kinases

The invention relates to novel substituted pyrimidinylaminobenzamides, processes for the preparation thereof, pharmaceutical compositions containing same, the use thereof optionally in combination with one or more other pharmaceutically active compounds for the therapy of a disease which responds to an inhibition of protein kinase activity, especially a neoplastic disease, in particular leukaemia, and a method for the treatment of such a disease.

Background of the invention

Protein kinases (PKs) are enzymes which catalyze the phosphorylation of specific serine, threonine or tyrosine residues in cellular proteins. These post-translational modifications of substrate proteins act as molecular switches regulating cell proliferation, activation and/or differentiation. Aberrant or excessive PK activity has been observed in many disease states including benign and malignant proliferative disorders. In a number of cases, it has been possible to treat diseases, such as proliferative disorders, by making use of PK inhibitors *in vitro* and *in vivo.*

In view of the large number of protein kinase inhibitors and the multitude of proliferative and other PK-related diseases, there is an ever-existing need to provide novel classes of compounds that are useful as PK inhibitors and thus in the treatment of these PTK related diseases. What is required are new classes of pharmaceutically advantageous PK inhibiting compounds.

The Philadelphia Chromosome is a hallmark for chronic myelogenous leukaemia (CML) and carries a hybrid gene that contains N-terminal exons of the bcr gene and the major C-terminal part (exons 2–11) of the c-abl gene. The gene product is a 210 kD protein (p210 Bcr-Abl). The Abl-part of the Bcr-Abl protein contains the abl-tyrosine kinase which is tightly regulated in the wild type c-abl, but constitutively activated in the Bcr-Abl fusion protein. This deregulated tyrosine kinase interacts with multiple cellular signalling pathways leading to transformation and deregulated proliferation of the cells (Lugo *et al.*, Science 247, 1079 [1990]).

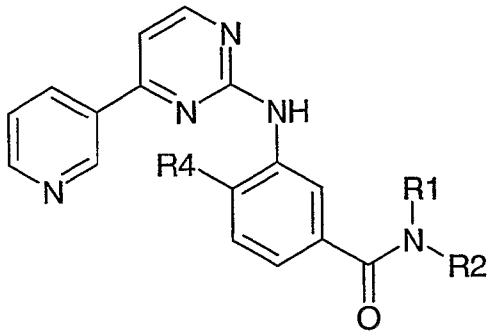
General description of the invention

It has now been found that various compounds of the pyrimidinylaminobenzamide class show inhibition of protein kinase activity. The compounds of formula I, described below in

more detail, especially show inhibition of one or more tyrosine kinases, such as c-Abl, Bcr-Abl, the receptor tyrosine kinases PDGF-R, Flt3, VEGF-R, EGF-R, and c-Kit, as well as combinations of two or more of these; in the case of novel pyrimidinylaminobenzamides according to the invention, the compounds are appropriate for the inhibition of these and/or other protein kinases, especially those mentioned above and/or for the inhibition of mutants of these enzymes, especially of Bcr-Abl, for example the Glu255 -> Valine mutant. In view of these activities, the compounds can be used for the treatment of diseases related to especially aberrant or excessive activity of such types of kinases, especially those mentioned.

Detailed description of the invention

The invention relates to a compound of formula I,



(I)

wherein

R_1 represents hydrogen, lower alkyl, lower alkoxy-lower alkyl, acyloxy-lower alkyl, carboxy-lower alkyl, lower alkoxycarbonyl-lower alkyl, or phenyl-lower alkyl;

R_2 represents hydrogen, lower alkyl, optionally substituted by one or more identical or different radicals R_3, cycloalkyl, benzcycloalkyl, heterocyclyl, an aryl group, or a mono- or bicyclic heteroaryl group comprising zero, one, two or three ring nitrogen atoms and zero or one oxygen atom and zero or one sulfur atom, which groups in each case are unsubstituted or mono- or polysubstituted;
and R_3 represents hydroxy, lower alkoxy, acyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, N-mono- or N,N-disubstituted carbamoyl, amino, mono- or disubstituted amino, cycloalkyl, heterocyclyl, an aryl group, or a mono- or bicyclic heteroaryl group comprising zero, one, two

or three ring nitrogen atoms and zero or one oxygen atom and zero or one sulfur atom, which groups in each case are unsubstituted or mono- or polysubstituted;

or wherein R_1 and R_2 together represent alkylene with four, five or six carbon atoms optionally mono- or disubstituted by lower alkyl, cycloalkyl, heterocyclyl, phenyl, hydroxy, lower alkoxy, amino, mono- or disubstituted amino, oxo, pyridyl, pyrazinyl or pyrimidinyl; benzalkylene with four or five carbon atoms; oxaalkylene with one oxygen and three or four carbon atoms; or azaalkylene with one nitrogen and three or four carbon atoms wherein nitrogen is unsubstituted or substituted by lower alkyl, phenyl-lower alkyl, lower alkoxycarbonyl-lower alkyl, carboxy-lower alkyl, carbamoyl-lower alkyl, N-mono- or N,N-disubstituted carbamoyl-lower alkyl, cycloalkyl, lower alkoxycarbonyl, carboxy, phenyl, substituted phenyl, pyridinyl, pyrimidinyl, or pyrazinyl;

R_4 represents hydrogen, lower alkyl, or halogen;

and a N-oxide or a pharmaceutically acceptable salt of such a compound.

The general terms used hereinbefore and hereinafter preferably have within the context of this disclosure the following meanings, unless otherwise indicated:

The prefix "lower" denotes a radical having up to and including a maximum of 7, especially up to and including a maximum of 4 carbon atoms, the radicals in question being either linear or branched with single or multiple branching.

Where the plural form is used for compounds, salts, and the like, this is taken to mean also a single compound, salt, or the like.

Any asymmetric carbon atoms may be present in the (R)-, (S)- or (R,S)-configuration, preferably in the (R)- or (S)-configuration. The compounds may thus be present as mixtures of isomers or as pure isomers, preferably as enantiomer-pure diastereomers.

The invention relates also to possible tautomers of the compounds of formula I.

Lower alkyl is preferably alkyl with from and including 1 up to and including 7, preferably from

and including 1 to and including 4, and is linear or branched; preferably, lower alkyl is butyl, such as n-butyl, sec-butyl, isobutyl, tert-butyl, propyl, such as n-propyl or isopropyl, ethyl or methyl. Preferably lower alkyl is methyl, propyl or tert-butyl.

Lower acyl is preferably formyl or lower alkylcarbonyl, in particular acetyl.

An aryl group is an aromatic radical which is bound to the molecule via a bond located at an aromatic ring carbon atom of the radical. In a preferred embodiment, aryl is an aromatic radical having 6 to 14 carbon atoms, especially phenyl, naphthyl, tetrahydronaphthyl, fluorenyl or phenanthrenyl, and is unsubstituted or substituted by one or more, preferably up to three, especially one or two substituents, especially selected from amino, mono- or disubstituted amino, halogen, lower alkyl, substituted lower alkyl, lower alkenyl, lower alkynyl, phenyl, hydroxy, etherified or esterified hydroxy, nitro, cyano, carboxy, esterified carboxy, alkanoyl, benzoyl, carbamoyl, N-mono- or N,N-disubstituted carbamoyl, amidino, guanidino, ureido, mercapto, sulfo, lower alkylthio, phenylthio, phenyl-lower alkylthio, lower alkylphenylthio, lower alkylsulfinyl, phenylsulfinyl, phenyl-lower alkylsulfinyl, lower alkylphenylsulfinyl, lower alkylsulfonyl, phenylsulfonyl, phenyl-lower alkylsulfonyl, lower alkylphenylsulfonyl, halogen-lower alkylmercapto, halogen-lower alkylsulfonyl, such as especially trifluoromethanesulfonyl, dihydroxybora (-$B(OH)_2$), heterocyclyl, a mono- or bicyclic heteroaryl group and lower alkylene dioxy bound at adjacent C-atoms of the ring, such as methylene dioxy. Aryl is more preferably phenyl, naphthyl or tetrahydronaphthyl, which in each case is either unsubstituted or independently substituted by one or two substituents selected from the group comprising halogen, especially fluorine, chlorine, or bromine; hydroxy; hydroxy etherified by lower alkyl, e.g. by methyl, by halogen-lower alkyl, e.g. trifluoromethyl, or by phenyl; lower alkylene dioxy bound to two adjacent C-atoms, e.g. methylenedioxy, lower alkyl, e.g. methyl or propyl; halogen-lower alkyl, e.g. trifluoromethyl; hydroxy-lower alkyl, e.g. hydroxymethyl or 2-hydroxy-2-propyl; lower alkoxy-lower alkyl; e.g. methoxymethyl or 2-methoxyethyl; lower alkoxycarbonyl-lower alkyl, e.g. methoxy-carbonylmethyl; lower alkynyl, such as 1-propynyl; esterified carboxy, especially lower alkoxycarbonyl, e.g. methoxycarbonyl, n-propoxy carbonyl or iso-propoxy carbonyl; N-mono-substituted carbamoyl, in particular carbamoyl monosubstituted by lower alkyl, e.g. methyl, n-propyl or iso-propyl; amino; lower alkylamino, e.g. methylamino; di-lower alkylamino, e.g. dimethylamino or diethylamino; lower alkylene-amino, e.g. pyrrolidino or piperidino; lower oxaalkylene-amino, e.g. morpholino, lower azaalkylene-amino, e.g. piperazino, acylamino,

e.g. acetylamino or benzoylamino; lower alkylsulfonyl, e.g. methylsulfonyl; sulfamoyl; or phenylsulfonyl.

A cycloalkyl group is preferably cyclopropyl, cyclopentyl, cyclohexyl or cycloheptyl, and may be unsubstituted or substituted by one or more, especially one or two, substitutents selected from the group defined above as substitutents for aryl, most preferably by lower alkyl, such as methyl, lower alkoxy, such as methoxy or ethoxy, or hydroxy, and further by oxo or fused to a benzo ring, such as in benzcyclopentyl or benzcyclohexyl.

Substituted alkyl is alkyl as last defined, especially lower alkyl, preferably methyl; where one or more, especially up to three, substituents may be present, primarily from the group selected from halogen, especially fluorine, amino, N-lower alkylamino, N,N-di-lower alkylamino, N-lower alkanoylamino, hydroxy, cyano, carboxy, lower alkoxycarbonyl, and phenyl-lower alkoxycarbonyl. Trifluoromethyl is especially preferred.

Mono- or disubstituted amino is especially amino substituted by one or two radicals selected independently of one another from lower alkyl, such as methyl; hydroxy-lower alkyl, such as 2-hydroxyethyl; lower alkoxy lower alkyl, such as methoxy ethyl; phenyl-lower alkyl, such as benzyl or 2-phenylethyl; lower alkanoyl, such as acetyl; benzoyl; substituted benzoyl, wherein the phenyl radical is especially substituted by one or more, preferably one or two, substituents selected from nitro, amino, halogen, N-lower alkylamino, N,N-di-lower alkylamino, hydroxy, cyano, carboxy, lower alkoxycarbonyl, lower alkanoyl, and carbamoyl; and phenyl-lower alkoxycarbonyl, wherein the phenyl radical is unsubstituted or especially substituted by one or more, preferably one or two, substituents selected from nitro, amino, halogen, N-lower alkylamino, N,N-di-lower alkylamino, hydroxy, cyano, carboxy, lower alkoxycarbonyl, lower alkanoyl, and carbamoyl; and is preferably N-lower alkylamino, such as N-methylamino, hydroxy-lower alkylamino, such as 2-hydroxyethylamino or 2-hydroxypropyl, lower alkoxy lower alkyl, such as methoxy ethyl, phenyl-lower alkylamino, such as benzylamino, N,N-di-lower alkylamino, N-phenyl-lower alkyl-N-lower alkylamino, N,N-di-lower alkylphenylamino, lower alkanoylamino, such as acetylamino, or a substituent selected from the group comprising benzoylamino and phenyl-lower alkoxycarbonylamino, wherein the phenyl radical in each case is unsubstituted or especially substituted by nitro or amino, or also by halogen, amino, N-lower alkylamino, N,N-di-lower alkylamino, hydroxy, cyano, carboxy, lower alkoxycarbonyl, lower alkanoyl, carbamoyl or aminocarbonylamino.

Disubstituted amino is also lower alkylene-amino, e.g. pyrrolidino, 2-oxopyrrolidino or piperidino; lower oxaalkylene-amino, e.g. morpholino, or lower azaalkylene-amino, e.g. piperazino or N-substituted piperazino, such as N-methylpiperazino or N-methoxycarbonylpiperazino.

Halogen is especially fluorine, chlorine, bromine, or iodine, especially fluorine, chlorine, or bromine.

Etherified hydroxy is especially C_8-C_{20}alkyloxy, such as n-decyloxy, lower alkoxy (preferred), such as methoxy, ethoxy, isopropyloxy, or tert-butyloxy, phenyl-lower alkoxy, such as benzyloxy, phenyloxy, halogen-lower alkoxy, such as trifluoromethoxy, 2,2,2-trifluoroethoxy or 1,1,2,2-tetrafluoroethoxy, or lower alkoxy which is substituted by mono- or bicyclic heteroaryl comprising one or two nitrogen atoms, preferably lower alkoxy which is substituted by imidazolyl, such as 1H-imidazol-1-yl, pyrrolyl, benzimidazolyl, such as 1-benzimidazolyl, pyridyl, especially 2-, 3- or 4-pyridyl, pyrimidinyl, especially 2-pyrimidinyl, pyrazinyl, isoquinolinyl, especially 3-isoquinolinyl, quinolinyl, indolyl or thiazolyl.

Esterified hydroxy is especially lower alkanoyloxy, benzoyloxy, lower alkoxycarbonyloxy, such as tert-butoxycarbonyloxy, or phenyl-lower alkoxycarbonyloxy, such as benzyloxycarbonyloxy.

Esterified carboxy is especially lower alkoxycarbonyl, such as tert-butoxycarbonyl, isopropoxycarbonyl, methoxycarbonyl or ethoxycarbonyl, phenyl-lower alkoxycarbonyl, or phenyloxycarbonyl.

Alkanoyl is primarily alkylcarbonyl, especially lower alkanoyl, e.g. acetyl.

N-Mono- or N,N-disubstituted carbamoyl is especially substituted by one or two substituents independently selected from lower alkyl, phenyl-lower alkyl and hydroxy-lower alkyl, or lower alkylene, oxa-lower alkylene or aza-lower alkylene optionally substituted at the terminal nitrogen atom.

A mono- or bicyclic heteroaryl group comprising zero, one, two or three ring nitrogen atoms and zero or one oxygen atom and zero or one sulfur atom, which groups in each case are

unsubstituted or mono- or polysubstituted, refers to a heterocyclic moiety that is unsaturated in the ring binding the heteroaryl radical to the rest of the molecule in formula I and is preferably a ring, where in the binding ring, but optionally also in any annealed ring, at least one carbon atom is replaced by a heteroatom selected from the group consisting of nitrogen, oxygen and sulfur; where the binding ring preferably has 5 to 12, more preferably 5 or 6 ring atoms; and which may be unsubstituted or substituted by one or more, especially one or two, substitutents selected from the group defined above as substitutents for aryl, most preferably by lower alkyl, such as methyl, lower alkoxy, such as methoxy or ethoxy, or hydroxy. Preferably the mono- or bicyclic heteroaryl group is selected from 2H-pyrrolyl, pyrrolyl, imidazolyl, benzimidazolyl, pyrazolyl, indazolyl, purinyl, pyridyl, pyrazinyl, pyrimidinyl, pyridazinyl, 4H-quinolizinyl, isoquinolyl, quinolyl, phthalazinyl, naphthyridinyl, quinoxalyl, quinazolinyl, quinnolinyl, pteridinyl, indolizinyl, 3H-indolyl, indolyl, isoindolyl, oxazolyl, isoxazolyl, thiazolyl, isothiazolyl, triazolyl, tetrazolyl, furazanyl, benzo[d]pyrazolyl, thienyl and furanyl. More preferably the mono- or bicyclic heteroaryl group is selected from the group consisting of pyrrolyl, imidazolyl, such as 1H-imidazol-1-yl, benzimidazolyl, such as 1-benzimidazolyl, indazolyl, especially 5-indazolyl, pyridyl, especially 2-, 3- or 4-pyridyl, pyrimidinyl, especially 2-pyrimidinyl, pyrazinyl, isoquinolinyl, especially 3-isoquinolinyl, quinolinyl, especially 4- or 8-quinolinyl, indolyl, especially 3-indolyl, thiazolyl, benzo[d]pyrazolyl, thienyl, and furanyl. In one preferred embodiment of the invention the pyridyl radical is substituted by hydroxy in ortho position to the nitrogen atom and hence exists at least partially in the form of the corresponding tautomer which is pyridin-(1H)2-one. In another preferred embodiment, the pyrimidinyl radical is substituted by hydroxy both in position 2 and 4 and hence exists in several tautomeric forms, e.g. as pyrimidine-(1H, 3H)2,4-dione.

Heterocyclyl is especially a five, six or seven-membered heterocyclic system with one or two heteroatoms selected from the group comprising nitrogen, oxygen, and sulfur, which may be unsaturated or wholly or partly saturated, and is unsubstituted or substituted especially by lower alkyl, such as methyl, phenyl-lower alkyl, such as benzyl, oxo, or heteroaryl, such as 2-piperazinyl; heterocyclyl is especially 2- or 3-pyrrolidinyl, 2-oxo-5-pyrrolidinyl, piperidinyl, N-benzyl-4-piperidinyl, N-lower alkyl-4-piperidinyl, N-lower alkyl-piperazinyl, morpholinyl, e.g. 2- or 3-morpholinyl, 2-oxo-1H-azepin-3-yl, 2-tetrahydrofuranyl, or 2-methyl-1,3-dioxolan-2-yl.

Salts are especially the pharmaceutically acceptable salts of compounds of formula I.

Such salts are formed, for example, as acid addition salts, preferably with organic or inorganic acids, from compounds of formula I with a basic nitrogen atom, especially the pharmaceutically acceptable salts. Suitable inorganic acids are, for example, halogen acids, such as hydrochloric acid, sulfuric acid, or phosphoric acid. Suitable organic acids are, for example, carboxylic, phosphonic, sulfonic or sulfamic acids, for example acetic acid, propionic acid, octanoic acid, decanoic acid, dodecanoic acid, glycolic acid, lactic acid, fumaric acid, succinic acid, adipic acid, pimelic acid, suberic acid, azelaic acid, malic acid, tartaric acid, citric acid, amino acids, such as glutamic acid or aspartic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, cyclohexanecarboxylic acid, adamantanecarboxylic acid, benzoic acid, salicylic acid, 4-aminosalicylic acid, phthalic acid, phenylacetic acid, mandelic acid, cinnamic acid, methane- or ethane-sulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 2-naphthalenesulfonic acid, 1,5-naphthalene-disulfonic acid, 2-, 3- or 4-methylbenzenesulfonic acid, methylsulfuric acid, ethylsulfuric acid, dodecylsulfuric acid, N-cyclohexylsulfamic acid, N-methyl-, N-ethyl- or N-propyl-sulfamic acid, or other organic protonic acids, such as ascorbic acid.

In the presence of negatively charged radicals, such as carboxy or sulfo, salts may also be formed with bases, e.g. metal or ammonium salts, such as alkali metal or alkaline earth metal salts, for example sodium, potassium, magnesium or calcium salts, or ammonium salts with ammonia or suitable organic amines, such as tertiary monoamines, for example triethylamine or tri(2-hydroxyethyl)amine, or heterocyclic bases, for example N-ethyl-piperidine or N,N'-dimethylpiperazine.

When a basic group and an acid group are present in the same molecule, a compound of formula I may also form internal salts.

For isolation or purification purposes it is also possible to use pharmaceutically unacceptable salts, for example picrates or perchlorates. For therapeutic use, only pharmaceutically acceptable salts or free compounds are employed (where applicable in the form of pharmaceutical preparations), and these are therefore preferred.

In view of the close relationship between the novel compounds in free form and those in the form of their salts, including those salts that can be used as intermediates, for example in the

purification or identification of the novel compounds, any reference to the free compounds hereinbefore and hereinafter is to be understood as referring also to the corresponding salts, as appropriate and expedient.

The compounds of formula I and N-oxides thereof have valuable pharmacological properties, as described hereinbefore and hereinafter.

The efficacy of the compounds of the invention as inhibitors of c-Abl, Bcr-Abl, and VEGF-receptor tyrosine kinase activity can be demonstrated as follows:

Test for activity against c-Abl protein tyrosine kinase. The test is conducted as a filter binding assay as follows: The His-tagged kinase domain of c-Abl is cloned and expressed in the baculovirus/Sf9 system as described by Bhat *et al.*, J Biol Chem. 272, 16170-5 (1997). A protein of 37 kD (c-Abl kinase) is purified by a two-step procedure over a Cobalt metal chelate column followed by an anion exchange column with a yield of 1-2 mg/L of Sf9 cells. The purity of the c-Abl kinase is >90% as judged by SDS-PAGE after Coomassie blue staining. The assay contains: c-Abl kinase (50 ng), 20 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 10 μM Na_3VO_4, 1 mM DTT and 0.06 μCi/assay [γ^{33} P]-ATP (5 μM ATP) using 30 μg/mL poly-Ala,Glu,Lys,Tyr-6:2:5:1 (Poly-AEKY, Sigma P1152) in the presence of 1% DMSO, total volume of 30 μL. Reactions are terminated by adding 10 μL of 250 mM EDTA, and 30 μL of the reaction mixture is transferred onto Immobilon-PVDF membrane (Millipore, Bedford, MA, USA) previously soaked for 5 min with methanol, rinsed with water, then soaked for 5 min with 0.5% H_3PO_4 and mounted on vacuum manifold with disconnected vacuum source. After spotting all samples, vacuum is connected and each well rinsed with 200 μL 0.5 % H_3PO_4. Membranes are removed and washed on a shaker with 0.5% H_3PO_4 (4 times) and once with ethanol. Membranes are counted after drying at ambient temperature, mounting in Packard TopCount 96-well frame, and addition of 10 μL/well of Microscint TM (Packard).

Test for activity against Bcr-Abl. The murine myeloid progenitor cell line 32Dcl3 transfected with the p210 Bcr-Abl expression vector pGDp210Bcr/Abl (32D-bcr/abl) was obtained from J. Griffin (Dana Faber Cancer Institue, Bosten, MA, USA). The cells express the fusion Bcr-Abl protein with a constitutively active abl kinase and proliferate growth factor independent. The cells are expanded in RPMI 1640 (AMIMED), 10% fetal calf serum, 2 mM glutamine (Gibco) („complete medium"), and a working stock is prepared by freezing aliquots of 2 x 10^6 cells

per vial in freezing medium (95% FCS, 5% DMSO (SIGMA)). After thawing, the cells are used during maximally 10 –12 passages for the experiments.

For cellular assays, compounds are dissolved in DMSO and diluted with complete medium to yield a starting concentration of 10 μM followed by preparation of serial 3-fold dilutions in complete medium. 200'000 32D-Bcr/Abl cells in 50 μL complete medium are seeded per well in 96 well round bottom tissue culture plates. 50 μL per well of serial 3-fold dilutions of the test compound are added to the cells in triplicates. Untreated cells are used as control. The compound is incubated together with the cells for 90 min at 37°C, 5% CO_2, followed by centrifugation of the tissue culture plates at 1300 rpm (Beckmann GPR centrifuge) and removal of the supernatants by careful aspiration taking care not to remove any of the pelleted cells. The cell pellets are lysed by addition of 150 μL lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM sodium chloride, 5 mM EDTA, 1 mM EGTA, 1% NP-40, 2 mM sodium ortho-vanadate, 1 mM PMSF, 50 μg/mL aprotinin and 80 μg/mL leupeptin) and either used immediatedly for the ELISA or stored frozen in the plates at –20°C until usage.

Black ELISA plates (Packard HTRF-96 black plates) are precoated over night at 4°C with 50 ng/well of the rabbit polyclonal anti-abl-SH3 domain Ab 06-466 from Upstate in 50 μL PBS. After washing 3 times with 200 μL/well PBS containing 0.05% Tween20 (PBST) and 0.5% TopBlock (Juro), residual protein binding sites are blocked with 200 μL/well PBST, 3% TopBlock for 4 h at room temperature followed by incubation with 50 μL lysates of untreated or compound-treated cells (20 μg total protein per well) for 3-4 h at 4°C. After 3 washings, 50 μL/well anti-phosphotyrosine Ab PY20(AP) labeled with alkaline phosphatase (Zymed) diluted to 0.2 μg/mL in blocking buffer is added and incubated over night (4°C). For all incubation steps the plates are covered with plate sealers (Costar). Finally, the plates are washed another three times with washing buffer and once with deionized water before addition of 90 μL/well of the AP-substrate CDPStar RTU with Emerald II. The plates, now sealed with Packard TopSeal™-A plate sealers, are incubated for 45 min at room temperature in the dark and luminescence is quantified by measuring counts per second (CPS) with a Packard Top Count Microplate Scintillation Counter (Top Count).

The difference between the ELISA-readout (CPS) obtained for with the lysates of the untreated 32D-Bcr/Abl cells and the readout for the assay-background (all components, but without cell lysate) is calculated and taken as 100% reflecting the constitutively phosphorylated Bcr-Abl protein present in these cells. The activity of the compound on the Bcr-Abl kinase activity is expressed as percent reduction of the Bcr-Abl phosphorylation. The

values for the IC_{50} and IC_{90} are determined from the dose response curves by graphical extrapolation.

Test for activity against VEGF-receptor tyrosine kinase. The test is conducted using Flt-1 VEGF-receptor tyrosine kinase. The detailed procedure is as follows: 30 µL kinase solution (10 ng of the kinase domain of Flt-1, Shibuya *et al.*, Oncogene 5, 519-24 [1990]) in 20 mM Tris•HCl pH 7.5, 3 mM manganese dichloride ($MnCl_2$), 3 mM magnesium chloride ($MgCl_2$), 10 µM sodium vanadate, 0.25 mg/mL polyethylenglycol (PEG) 20000, 1mM dithiothreitol and 3 µg/µL poly(Glu,Tyr) 4:1 (Sigma, Buchs, Switzerland), 8 µM [^{33}P]-ATP (0.2 µCi) , 1% DMSO, and 0 to 100 µM of the compound to be tested are incubated together for 10 minutes at room temperature. The reaction is then terminated by the addition of 10 µL 0.25 M ethylenediaminetetraacetate (EDTA) pH 7. Using a multichannel dispenser (LAB SYSTEMS, USA), an aliquot of 20 µL is applied to a PVDF (= polyvinyl difluoride) Immobilon P membrane (Millipore, Bedford, USA), through a Gibco-BRL microtiter filter manifold and connected to a vacuum. Following complete elimination of the liquid, the membrane is washed 4 times successively in a bath containing 0.5% phosphoric acid (H_3PO_4) and once with ethanol, incubated for 10 minutes each time while shaking, then mounted in a Hewlett Packard TopCount Manifold and the radioactivity measured after the addition of 10 µL Microscint® (ß-scintillation counter liquid). IC_{50}-values are determined by linear regression analysis of the percentages for the inhibition of each compound in at least four concentrations (as a rule 0.01, 0.1, 1.0 and 10 µmol). The IC_{50}-values that can be found with compounds of formula I are in the range of 1 to 10'000 nM, preferably in the range of 1 to 100 nM.

The inhibition of VEGF-induced KDR-receptor autophosphorylation can be confirmed with a further in vitro experiment in cells: transfected CHO cells, which permanently express human VEGF receptor (KDR), are seeded in complete culture medium with 10% fetal calf serum (FCS) in 6-well cell-culture plates and incubated at 37°C under 5% CO_2 until they show about 80% confluency. The compounds to be tested are then diluted in culture medium (without FCS, with 0.1% bovine serum albumin) and added to the cells. (Controls comprise medium without test compounds). After two hours of incubation at 37°C, recombinant VEGF is added; the final VEGF concentration is 20 ng/mL). After a further five minute incubation at 37°C, the cells are washed twice with ice-cold PBS (phosphate-buffered saline) and immediately lysed in 100 µL

lysis buffer per well. The lysates are then centrifuged to remove the cell nuclei, and the protein concentrations of the supernatants are determined using a commercial protein assay (BIORAD). The lysates can then either be immediately used or, if necessary, stored at –20°C.

A sandwich ELISA is carried out to measure the KDR-receptor phosphorylation: a monoclonal antibody to KDR (for example Mab 1495.12.14) is immobilized on black ELISA plates (OptiPlate™ HTRF-96 from Packard). The plates are then washed and the remaining free protein-binding sites are saturated with 1% BSA in PBS. The cell lysates (20 μg protein per well) are then incubated in these plates overnight at 4°C together with an anti-phosphotyrosine antibody coupled with alkaline phosphatase (PY20:AP from Transduction Laboratories). The plates are washed again and the binding of the antiphosphotyrosine antibody to the captured phosphorylated receptor is then demonstrated using a luminescent AP substrate (CDP-Star, ready to use, with Emerald II; TROPIX). The luminescence is measured in a Packard Top Count Microplate Scintillation Counter (Top Count). The difference between the signal of the positive control (stimulated with VEGF) and that of the negative control (not stimulated with VEGF) corresponds to VEGF-induced KDR-receptor phosphorylation (= 100 %). The activity of the tested substances is calculated as % inhibition of VEGF-induced KDR-receptor phosphorylation, wherein the concentration of substance that induces half the maximum inhibition is defined as the ED50 (effective dose for 50% inhibition). Compounds of formula I here preferably show ED50 values in the range of 0.25 nM to 1000 nM, preferably 0.25 to 250 nM.

A compound of formula I or a N-oxide thereof inhibits to varying degrees also other tyrosine kinases involved in signal transduction which are mediated by trophic factors, for example Bcr-Abl and Abl kinase, Arg, kinases from the Src family, especially c-Src kinase, Lck, and Fyn; also kinases of the EGF family, for example, c-erbB2 kinase (HER-2), c-erbB3 kinase, c-erbB4 kinase; insulin-like growth factor receptor kinase (IGF-1 kinase), especially members of the PDGF-receptor tyrosine kinase family, such as PDGF-receptor kinase, CSF-1-receptor kinase, Kit-receptor kinase and VEGF-receptor kinase; and also serine/threonine kinases, all of which play a role in growth regulation and transformation in mammalian cells, including human cells.

The inhibition of c-erbB2 tyrosine kinase (HER-2) can be measured, for example, in the same way as the inhibition of EGF-R protein kinase, using known procedures.

On the basis of these studies, a compound of formula I according to the invention shows therapeutic efficacy especially against disorders dependent on protein kinase, especially proliferative diseases.

On the basis of their efficacy as inhibitors of VEGF-receptor tyrosine kinase activity, the compounds of the formula I primarily inhibit the growth of blood vessels and are thus, for example, effective against a number of diseases associated with deregulated angiogenesis, especially diseases caused by ocular neovascularisation, especially retinopathies, such as diabetic retinopathy or age-related macula degeneration, psoriasis, haemangioblastoma, such as haemangioma, mesangial cell proliferative disorders, such as chronic or acute renal diseases, e.g. diabetic nephropathy, malignant nephrosclerosis, thrombotic microangiopathy syndromes or transplant rejection, or especially inflammatory renal disease, such as glomerulonephritis, especially mesangioproliferative glomerulonephritis, haemolytic-uraemic syndrome, diabetic nephropathy, hypertensive nephrosclerosis, atheroma, arterial restenosis, autoimmune diseases, diabetes, endometriosis, chronic asthma, and especially neoplastic diseases (solid tumors, but also leukemias and other "liquid tumors", especially those expressing c-kit, KDR, Flt-1 or Flt-3), such as especially breast cancer, cancer of the colon, lung cancer (especially small-cell lung cancer), cancer of the prostate or Kaposi's sarcoma. A compound of formula I (or an N-oxide thereof) inhibits the growth of tumours and is especially suited to preventing the metastatic spread of tumors and the growth of micrometastases.

A compound of formula I can be administered alone or in combination with one or more other therapeutic agents, possible combination therapy taking the form of fixed combinations or the administration of a compound of the invention and one or more other therapeutic agents being staggered or given independently of one another, or the combined administration of fixed combinations and one or more other therapeutic agents. A compound of formula I can besides or in addition be administered especially for tumor therapy, such as leukaemia therapy, in combination with chemotherapy, radiotherapy, immunotherapy, surgical intervention, or a combination of these. Long-term therapy is equally possible as is adjuvant therapy in the context of other treatment strategies, as described above. Other possible treatments are therapy to maintain the patient's status after tumor regression, or even chemopreventive therapy, for example in patients at risk.

Therapeutic agents for possible combination are especially one or more cytostatic or cytotoxic compounds, for example a chemotherapeutic agent or several selected from the group comprising indarubicin, cytarabine, interferon, hydroxyurea, bisulfan, or an inhibitor of polyamine biosynthesis, an inhibitor of protein kinase, especially of serine/threonine protein kinase, such as protein kinase C, or of tyrosine protein kinase, such as epidermal growth factor receptor tyrosine kinase, a cytokine, a negative growth regulator, such as TGF-ß or IFN-ß, an aromatase inhibitor, a classical cytostatic, and an inhibitor of the interaction of an SH2 domain with a phosphorylated protein.

A compound according to the invention is not only for the (prophylactic and preferably therapeutic) management of humans, but also for the treatment of other warm-blooded animals, for example of commercially useful animals, for example rodents, such as mice, rabbits or rats, or guinea-pigs. Such a compound may also be used as a reference standard in the test systems described above to permit a comparison with other compounds.

In general, the invention relates also to the use of a compound of formula I or a N-oxide thereof for the inhibition of tyrosine kinase activity, either in vitro or in vivo.

With the groups of preferred compounds of formula I and N-oxides thereof mentioned hereinafter, definitions of substituents from the general definitions mentioned hereinbefore may reasonably be used, for example, to replace more general definitions with more specific definitions or especially with definitions characterized as being preferred.

In particular, the invention relates to compounds of formula I, wherein

R_1 represents hydrogen, lower alkyl, lower alkoxy-lower alkyl, acyloxy-lower alkyl, carboxy-lower alkyl, lower alkoxycarbonyl-lower alkyl, or phenyl-lower alkyl;

R_2 represents hydrogen, lower alkyl, optionally substituted by one or two identical or different radicals R_3, cycloalkyl, benzcycloalkyl, heterocyclyl, an aryl group, or a mono- or bicyclic heteroaryl group comprising one, two or three nitrogen atoms or one sulfur atom, which aryl and heteroaryl groups in each case are unsubstituted or mono- or polysubstituted;

and R_3 represents hydroxy, lower alkoxy, acyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, N-mono- or N,N-disubstituted carbamoyl, amino, mono- or disubstituted amino, cycloalkyl, heterocyclyl, an aryl group, furanoyl, thienoyl, or a mono- or bicyclic heteroaryl group comprising one, two or three ring nitrogen atoms, zero or one ring oxygen atom and zero or one ring sulphur atom, which aryl and heteroaryl groups in each case are unsubstituted or mono- or polysubstituted;

or wherein R_1 and R_2 together represent alkylene with four or five carbon atoms, optionally mono- or disubstituted by lower alkyl, cycloalkyl, heterocyclyl, phenyl, hydroxy, lower alkoxy, amino, mono- or disubstituted amino, pyridyl, pyrazinyl or pyrimidinyl; benzalkylene with four or five carbon atoms in the alkylene group; oxaalkylene with one oxygen and three or four carbon atoms, or azaalkylene with one nitrogen and three or four carbon atoms wherein nitrogen is unsubstituted or substituted by lower alkyl, phenyl-lower alkyl, lower alkoxycarbonyl-lower alkyl, carboxy-lower alkyl, carbamoyl-lower alkyl, N-mono- or N,N-disubstituted carbamoyl-lower alkyl, cycloalkyl, lower alkoxycarbonyl, phenyl, substituted phenyl, pyridinyl, pyrimidinyl, or pyrazinyl;

R_4 represents hydrogen, lower alkyl, or halogen;

and a N-oxide or a pharmaceutically acceptable salt of such a compound.

More particular, the invention relates to compounds of formula I, wherein

R_1 represents hydrogen, lower alkyl, lower alkoxy-lower alkyl, lower alkoxycarbonyl-lower alkyl, or phenyl-lower alkyl;

R_2 represents hydrogen, lower alkyl, optionally substituted by one or two identical or different radicals R_3, cyclopentyl, benzcyclopentyl, cylcohexyl, pyrrolidinyl, oxazolinyl, piperidinyl, N-substituted piperidinyl, morpholinyl, azepinyl, oxo-azepinyl, oxazepinyl, phenyl, naphthalinyl, tetrahydronaphthalinyl or a mono- or bicyclic heteroaryl group comprising one or two nitrogen atoms, which phenyl, naphthalinyl and heteroaryl groups in each case are unsubstituted or mono- or polysubstituted, thienyl, or lower alkoxycarbonyl-lower alkylthienyl;

and R_3 represents hydroxy, lower alkoxy, acyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, N-mono- or N,N-disubstituted carbamoyl, amino, lower alkylamino, di-lower alkylamino, phenylamino, N-lower alkyl-N-phenylamino, pyrrolidino, oxopyrrolidino, piperidino, morpholino, imidazolino, oxoimidazolino, cycloalkyl, heterocyclyl, furyl, phenyl, naphthalinyl, tetrahydronaphthalinyl, or a mono- or bicyclic heteroaryl group comprising one or two nitrogen atoms, which phenyl, naphthalinyl and heteroaryl group are unsubstituted or mono- or polysubstituted;

or wherein R_1 and R_2 together represent alkylene with four or five carbon atoms, optionally mono- or disubstituted by lower alkyl, cycloalkyl, heterocyclyl, phenyl, hydroxy, lower alkoxy, amino, mono- or disubstituted amino, pyridyl, pyrazinyl or pyrimidinyl; benzalkylene with four or five carbon atoms in the alkylene group; oxaalkylene with one oxygen and four carbon atoms; or azaalkylene with one nitrogen and four carbon atoms wherein nitrogen is unsubstituted or substituted by lower alkyl, phenyl-lower alkyl, lower alkoxycarbonyl-lower alkyl, carboxy-lower alkyl, carbamoyl-lower alkyl, N-mono- or N,N-disubstituted carbamoyl-lower alkyl, cycloalkyl, lower alkoxycarbonyl, phenyl, substituted phenyl, pyridinyl, pyrimidinyl, or pyrazinyl;

R_4 represents hydrogen, lower alkyl, or halogen;

and a N-oxide or a pharmaceutically acceptable salt of such a compound.

More particular, the invention relates to compounds of formula I, wherein

R_1 represents hydrogen, lower alkyl, lower alkoxy-lower alkyl, lower alkoxycarbonyl-lower alkyl, or phenyl-lower alkyl;

R_2 represents hydrogen; lower alkyl, optionally substituted by one radical R_3, by two phenyl groups, by two lower alkoxycarbonyl groups, by phenyl and lower alkoxycarbonyl, or by hydroxyphenyl and lower alkoxycarbonyl; cyclopentyl; benzcyclopentyl; cylcohexyl; pyrrolidinyl; oxazolinyl; piperidinyl; N-lower alkylpiperidinyl; N-benzylpiperidinyl; N-pyrimidinylpiperidinyl; morpholinyl; azepinyl; oxo-azepinyl; oxazepinyl; phenyl, naphthalinyl, tetrahydronaphthalinyl or a mono- or bicyclic heteroaryl group comprising one or two nitrogen atoms, which phenyl, naphthalinyl and heteroaryl groups in each case are unsubstituted or

substituted by one or two substituents selected from the group consisting of lower alkyl, trifluoro-lower alkyl, hydroxy-lower alkyl, lower alkoxy-lower alkyl, amino-lower alkyl, lower alkylamino-lower alkyl, di-lower alkylamino-lower alkyl, N-cyclohexyl-N-lower alkylamino-lower alkyl, lower alkoxycarbonylpiperidino-lower alkyl, N-lower alkylpiperazino-lower alkyl, lower alkoxycarbonyl-lower alkyl, hydroxy, lower alkoxy, trifluoro-lower alkoxy, 1H-imidazolyl-lower alkoxy, lower alkanoyloxy, benzoyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, lower alkyl carbamoyl, amino, lower alkanoylamino, benzoylamino, amino mono- or disubstituted by lower alkyl, by hydroxy-lower alkyl or by lower alkoxy-lower alkyl, 1H-imidazolyl, mono- or di-lower alkyl-1H-imidazolyl, pyrrolidino, piperidino, piperazino, N-lower alkylpiperazino, morpholino, sulfamoyl, lower alkylsulfonyl, phenylsulfonyl, lower alkylsulfinyl, phenylsulfinyl, lower alkylthio, phenylthio, phenyl, pyridyl, halogenyl, or benzoyl; thienyl; or lower alkoxycarbonyl-lower alkylthienyl; and

R_3 represents hydroxy, lower alkoxy, acyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, carbamoyl mono-or disubstituted by lower alkyl, phenyl or lower alkylene, amino, lower alkylamino, di-lower alkylamino, phenylamino, N-lower alkyl-N-phenylamino, pyrrolidino, oxopyrrolidino, piperidino, morpholino, imidazolino, oxoimidazolino, cycloalkyl, heterocyclyl, furyl; phenyl, naphthalinyl, tetrahydronaphthalinyl, or a mono- or bicyclic heteroaryl group comprising one or two nitrogen atoms, which phenyl, naphthalinyl and heteroaryl group is unsubstituted or substituted by one or two substituents selected from the group consisting of lower alkyl, trifluoro-lower alkyl, lower alkoxycarbonyl-lower alkyl, hydroxy, lower alkoxy, trifluoro-lower alkoxy, lower alkanoyloxy, benzoyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, amino, lower alkanoylamino, benzoylamino, amino mono- or disubstituted by lower alkyl, by hydroxy-lower alkyl or by loweralkoxy-lower alkyl, pyrrolidino, piperidino, morpholino, piperazino, N-lower alkylpiperazino, N-lower alkoxycarbonylpiperazino, phenyl, pyridyl, 1H-imidazolyl, lower alkyl-1H-imidazolyl, sulfamoyl, lower alkylsulfonyl, phenylsulfonyl, lower alkylsulfinyl, phenylsulfinyl, lower alkylthio, phenylthio, halogenyl, or benzoyl;

or wherein R_1 and R_2 together represent alkylene with four or five carbon atoms, optionally mono- or disubstituted by lower alkyl, cycloalkyl, phenyl, hydroxy, lower alkoxy, amino, benzoylamino, piperidino, pyridyl, pyrazinyl or pyrimidinyl; benzalkylene with four or five carbon atoms in the alkylene group; oxaalkylene with one oxygen and four carbon atoms; or azaalkylene with one nitrogen and four carbon atoms wherein nitrogen is unsubstituted or

substituted by lower alkyl, phenyl-lower alkyl, lower alkoxycarbonyl-lower alkyl, carboxy-lower alkyl, carbamoyl-lower alkyl, carbamoyl-lower alkyl N-mono- or N,N-disubstituted by lower alkyl, phenyl, lower alkylene or oxa-lower alkylene, cycloalkyl, lower alkoxycarbonyl, phenyl, methoxyphenyl, trifluoromethylphenyl, trifluoromethoxyphenyl, pyridinyl, pyrimidinyl, or pyrazinyl;

R_4 represents hydrogen or lower alkyl;

and a N-oxide or a pharmaceutically acceptable salt of such a compound.

In a preferred group of compounds of formula I,

R_1 represents hydrogen, lower alkyl, lower alkoxy-lower alkyl, or benzyl;

R_2 represents lower alkyl, optionally substituted by one radical R_3, by two phenyl groups, by two lower alkoycarbonyl groups, by phenyl and lower alkoxycarbonyl, or by hydroxyphenyl and lower alkoxycarbonyl; cyclopentyl; benzcyclopentyl; cylcohexyl; pyrrolidinyl; piperidinyl; N-lower alkylpiperidinyl; N-benzylpiperidinyl; N-pyrimidinylpiperidinyl; morpholinyl; azepinyl; oxoazepinyl; phenyl; naphthalinyl; tetrahydronaphthalinyl; pyridyl; lower alkyl-pyridyl; quinolinyl; thienyl; lower alkoxycarbonylmethylthienyl; or phenyl substituted by one or two substituents selected from the group consisting of lower alkyl, trifluoro-lower alkyl, hydroxy-lower alkyl, amino-lower alkyl, lower alkylamino-lower alkyl, di-lower alkylamino-lower alkyl, N-cyclohexyl-N-lower alkylamino-lower alkyl, lower alkoxycarbonylpiperidino-lower alkyl, N-lower alkylpiperazino-lower alkyl, lower alkoxycarbonyl-lower alkyl, hydroxy, lower alkoxy, trifluoro-lower alkoxy, 1H-imidazolyl-lower alkoxy, lower alkanoyloxy, benzoyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, lower alkylcarbamoyl, amino, lower alkanoylamino, benzoylamino, amino mono- or disubstituted by lower alkyl, by hydroxy-lower alkyl or by loweralkoxy-lower alkyl, 1H-imidazolyl, lower alkyl-1H-imidazolyl, pyrrolidino, piperidino, piperazino, N-lower alkylpiperazino, morpholino, sulfamoyl, lower alkylsulfonyl, phenyl, pyridyl, halogenyl, or benzoyl;

and R_3 represents hydroxy, lower alkoxy, lower alkanoyloxy, benzoyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, amino, lower alkylamino, di-lower alkylamino, phenylamino, N-lower alkyl-N-phenylamino, pyrrolidino, oxopyrrolidino, piperidino, morpholino, imidazolino,

oxoimidazolino, cyclopropyl, cyclopentyl, cyclohexyl, tetrahydrofuranyl, phenyl, naphthalinyl, tetrahydronaphthalinyl, furyl, a mono- or bicyclic heteroaryl group comprising one or two nitrogen atoms, which heteroaryl group is unsubstituted or mono- or disubstituted by lower alkyl, hydroxy and lower alkoxy, or phenyl substituted by one or two substituents selected from the group consisting of lower alkyl, trifluoro-lower alkyl, lower alkoxycarbonyl-lower alkyl, hydroxy, lower alkoxy, trifluoro-lower alkoxy, lower alkanoyloxy, benzoyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, amino, lower alkanoylamino, benzoylamino, amino mono- or disubstituted by lower alkyl, by hydroxy-lower alkyl or by loweralkoxy-lower alkyl, pyrrolidino, piperidino, morpholino, piperazino, N-lower alkylpiperazino, N-lower alkoxycarbonylpiperazino, phenyl, pyridyl, 1H-imidazolyl, lower alkyl-1H-imidazolyl, sulfamoyl, lower alkylsulfonyl, halogenyl, or benzoyl;

or wherein R_1 and R_2 together represent alkylene with four or five carbon atoms, optionally mono- or disubstituted by phenyl, hydroxy, amino, benzoylamino, or piperidino; benzalkylene with four or five carbon atoms in the alkylene group; oxaalkylene with one oxygen and four carbon atoms; or azaalkylene with one nitrogen and four carbon atoms wherein nitrogen is unsubstituted or substituted by lower alkyl, phenyl-lower alkyl, lower alkoxycarbonyl-lower alkyl, carbamoyl-lower alkyl, pyrrolidinocarbonyl-lower alkyl, morpholinocarbonyl-lower alkyl, cyclopentyl, lower alkoxycarbonyl, phenyl, methoxyphenyl, trifluoromethylphenyl, pyridinyl; pyrimidinyl, or pyrazinyl;

R_4 represents hydrogen or methyl;

and a N-oxide or a pharmaceutically acceptable salt of such a compound.

A specially preferred group of compounds comprises compounds of formula I wherein
R_1 represents hydrogen, and
R_2 represents phenyl substituted by trifluoromethyl, especially 3-trifluoromethylphenyl, and optionally a further substituent selected from the group consisting of hydroxy-lower alkyl, e.g. 1-hydroxy-1-methylethyl, lower alkylamino, e.g. methyl- or ethylamino, hydroxy-lower alkylamino, e.g. 2-hydroxy-1-propylamino or 2-hydroxy-2-propylamino, di-lower alkylamino, e.g. diethylamino, 1H-imidazolyl, lower alkyl-1H-imidazolyl, e.g. 2- or 4-methyl-1H-imidazolyl, carbamoyl, lower alkylcarbamoyl, e.g. methylcarbamoyl, pyrrolidino, piperidino, piperazino, lower alkylpiperazino, e.g. 4-methylpiperazino, morpholino, lower alkoxy, e.g. methoxy,

fluoro-lower alkoxy, e.g. trifluoromethoxy or 2,2,2-trifluoroethoxy, phenyl, pyridyl, e.g. 2-, 3- or 4-pyridyl, and halogenyl, e.g.chloro or fluoro;
R_4 represents methyl;
and a N-oxide or a pharmaceutically acceptable salt of such a compound.

One preferred embodiment of the invention relates to compounds of formula I wherein
R_1 is hydrogen,
R_2 represents phenyl which is mono- or disubstituted by imidazol-lower alkoxy, lower alkyl amino, trifluoromethyl, hydroxy lower alkyl amino, bis-(lower alkoxy lower alkyl) amino, lower alkyl piperazinyl, piperidinyl, pyrrolidinyl, morpholinyl, phenyl, pyridyl, imidazolyl which is unsubstituted or mono- or disubstituted by lower alkyl or N-lower alkyl carbamoyl;
R_4 is lower alkyl;
and to the N-oxides and pharmaceutically acceptable salts of such compounds.

Particularly preferred are the compounds of the Examples.

Other compounds which are particularly preferred are:
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzanilide,
4-Methyl-*N*-(3-pyridinyl)-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
N-(4-Chlorophenyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
2(R)- and 2(S)-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoylamino]propanoic acid,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-(8-quinolinyl)benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-(3-[trifluoromethoxy]phenyl)benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-(2-pyrrolidinoethyl)benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-(3-pyrrolidinophenyl)benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-(1-[2-pyrimidinyl]-4-piperidinyl)benzamide,
N-(4-Di-[2-methoxyethyl]amino-3-trifluoromethylphenyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
N-(4-[1H-Imidazolyl]-3-trifluoromethylphenyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-(2-pyrrolidino-5-trifluoromethylphenyl)benzamide,
N-(3,4-difluorophenyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,

4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-(3-trifluoromethylbenzyl)benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-(3-trifluoromethylphenyl)benzamide,
N-(3-Chloro-5-trifluoromethylphenyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
N-(4-Dimethylaminobutyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
4-Methyl-*N*-[4-(4-methyl-1-piperazinyl)-3-trifluoromethylphenyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(2,2,2-trifluoroethoxy)-3-trifluoromethylphenyl]benzamide,
4-Methyl-*N*-[4-(2-methyl-1H-imidazolyl)-3-trifluoromethylphenyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
4-Methyl-*N*-(4-phenyl-3-trifluoromethylphenyl)-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
4-Methyl-*N*-[4-(4-methyl-1H-imidazolyl)-3-trifluoromethylphenyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
Methyl 2(R)- and 2(S)-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoylamio]-3-[4-hydroxyphenyl]propanoate,
N-[2-(*N*-Cyclohexyl-*N*-methylaminomethyl)phenyl]-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
N-[3-[2-(1H-Imidazolyl)ethoxy]phenyl]-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
4-Methyl-*N*-[3-morpholino-5-trifluoromethylphenyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-(4-pyrrolidino-3-trifluoromethylphenyl)benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-(4-piperidino-3-trifluoromethylphenyl)benzamide,
4-Methyl-*N*-[4-morpholino-3-trifluoromethylphenyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
N-(4-Ethylamino-3-trifluoromethylphenyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-(3-trifluoromethoxyphenyl)benzamide,
N-[4-(2-Hydroxypropylamino)-3-trifluoromethylphenyl]-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,

N-(4-Diethylamino-3-trifluoromethylphenyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[3-(3-pyridinyl)-5-trifluorophenyl]benzamide,
N-[3-[3-(1H-Imidazolyl)propoxy]phenyl]-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(3-pyridinyl)-3-trifluorophenyl]benzamide,
4-Methyl-*N*-[3-(4-methyl-1-piperazinyl)-5-trifluorophenyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
4-Methyl-*N*-[3-methylcarbamoyl-5-trifluorophenyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide,
4-Methyl-*N*-[3-methylcarbamoyl-5-morpholino]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide.

Further compounds which are particularly preferred are:
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[3-[3-(1H-imidazol-1-yl)propoxy]-phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[3-[2-(1H-imidazol-1-yl)ethoxy]phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(ethylamino)-3-(trifluoromethyl)phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(diethylamino)-3-(trifluoromethyl)phenyl]benzamide,
(±)-4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-[(2-hydroxypropyl)amino]-3-(trifluoromethyl)phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-[bis(2-methoxyethyl)amino]-3-(trifluoromethyl)phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(4-methyl-1-piperazinyl)-3-(trifluoromethyl)phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(1-piperidinyl)-3-(trifluoromethyl)phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(1-pyrrolidinyl)-3-(trifluoromethyl)phenyl]benzamide,

4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(4-morpholinyl)-3-(trifluoromethyl)phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-phenyl-3-(trifluoromethyl)phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-N-[3-[4-(3-pyridinyl)-3-(trifluoromethyl)phenyl]methyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(1H-imidazol-1-yl)-3-(trifluoromethyl)phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(2,4-dimethyl-1H-imidazol-1-yl)-3-(trifluoromethyl)phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(4-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(2-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[3-(4-morpholinyl)-5-[(methylamino)carbonyl]phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[3-[(methylamino)carbonyl]-5-(trifluoromethyl)phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[5-(3-pyridinyl)-3-(trifluoromethyl)phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[5-(4-morpholinyl)-3-(trifluoromethyl)phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[5-(2-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[5-(4-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[5-(5-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)phenyl]benzamide,
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[3-(4-methyl-1-piperazinyl)-5-(trifluoromethyl)phenyl]benzamide, and
4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[2-(1-pyrrolidinyl)-5-(trifluoromethyl)phenyl]benzamide.

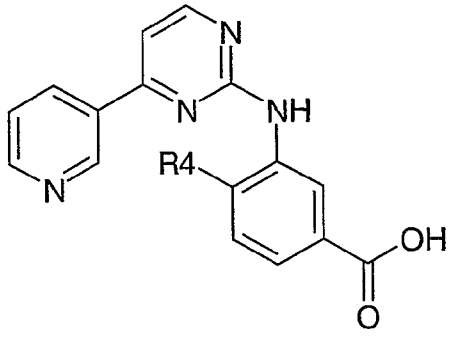
The invention relates also to 4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoic acid and to 3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoic acid; intermediates for the formation of the preferred amides of the invention.

Especially, the invention relates to the use of a compound of formula I or of a N-oxide or a possible tautomer thereof or of a pharmaceutically acceptable salt of such a compound for the preparation of a pharmaceutical composition for the treatment of a disease which responds to an inhibition of protein kinase activity, wherein the disease is a neoplastic disease.

More particularly, the invention relates to the use of a compound of the formula I or of a N-oxide or a possible tautomer thereof; or of a pharmaceutically acceptable salt of such a compound for the preparation of a pharmaceutical composition for the treatment of leukaemia which responds to an inhibition of the Abl tyrosine kinase activity.

Furthermore, the invention provides a method for the treatment of a disease which responds to an inhibition of protein kinase activity, which comprises administering a compound of formula I or a N-oxide or a pharmaceutically acceptable salt thereof, wherein the radicals and symbols have the meanings as defined above, in a quantity effective against said disease, to a warm-blooded animal requiring such treatment.

A compound of the invention may be prepared by processes that, though not applied hitherto for the new compounds of the present invention, are known per se, especially a process characterized in that for the synthesis of a compound of the formula I wherein the symbols R_1, R_2 and R_4 are as defined for a compound of the formula I, a 4- R_4-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzoic acid of formula II



(II)

wherein R_4 is as defined for a compound of formula I, or a derivative thereof wherein the carboxy group –COOH is in activated form, is reacted with an amine of the formula III

$$R_1\text{-NH-}R_2 \qquad \text{(III)}$$

wherein R_1 and R_2 are as defined for a compound of the formula I, optionally in the presence of a dehydrating agent and an inert base and/or a suitable catalyst, and optionally in the presence of an inert solvent;

where the above starting compounds II and III may also be present with functional groups in protected form if necessary and/or in the form of salts, provided a salt-forming group is present and the reaction in salt form is possible;

any protecting groups in a protected derivative of a compound of the formula I are removed;

and, if so desired, an obtainable compound of formula I is converted into another compound of formula I or a N-oxide thereof, a free compound of formula I is converted into a salt, an obtainable salt of a compound of formula I is converted into the free compound or another salt, and/or a mixture of isomeric compounds of formula I is separated into the individual isomers.

Detailed description of the process:

A derivative of the compound of formula II wherein the carboxy group is in activated form is especially a reactive ester, a reactive anhydride or a reactive cyclic amide.

Reactive esters of the acid of formula II are especially esters unsaturated at the linking carbon atom of the esterifying radical, for example esters of the vinyl ester type, such as actual vinyl esters (obtainable, for example, by transesterification of a corresponding ester with vinyl acetate; activated vinyl ester method), carbamoylvinyl esters (obtainable, for example, by treatment of the corresponding acid with an isoxazolium reagent; 1,2-oxazolium or Woodward method), or 1-lower alkoxyvinyl esters (obtainable, for example, by treatment of the corresponding acid with a lower alkoxyacetylene; ethoxyacetylene method), or esters of the amidino type, such as N,N'-disubstituted amidino esters (obtainable, for example, by

treatment of the corresponding acid with a suitable N,N'-disubstituted carbodiimide, for example N,N'-dicyclohexylcarbodiimide; carbodiimide method), or N,N-disubstituted amidino esters (obtainable, for example, by treatment of the corresponding acid with an N,N-disubstituted cyanamide; cyanamide method), suitable aryl esters, especially phenyl esters suitably substituted by electron-attracting substituents (obtainable, for example, by treatment of the corresponding acid with a suitably substituted phenol, for example 4-nitrophenol, 4-methylsulfonyl-phenol, 2,4,5-trichlorophenol, 2,3,4,5,6-pentachloro-phenol or 4-phenyldiazophenol, in the presence of a condensation agent, such as N,N'-dicyclohexyl-carbodiimide; activated aryl esters method), cyanomethyl esters (obtainable, for example, by treatment of the corresponding acid with chloroacetonitrile in the presence of a base; cyanomethyl esters method), thio esters, especially unsubstituted or substituted, for example nitro-substituted, phenylthio esters (obtainable, for example, by treatment of the corresponding acid with unsubstituted or substituted, for example nitro-substituted, thiophenols, inter alia by the anhydride or carbodiimide method; activated thiol esters method), amino or amido esters (obtainable, for example, by treatment of the corresponding acid with an N-hydroxy-amino or N-hydroxy-amido compound, for example N-hydroxy-succinimide, N-hydroxy-piperidine, N-hydroxy-phthalimide or 1-hydroxy-benzotriazole, for example by the anhydride or carbodiimide method; activated N-hydroxy esters method), or silyl esters (which are obtainable, for example, by treatment of the corresponding acid with a silylating agent, for example hexamethyl disilazane, and react readily with hydroxy groups but not with amino groups).

Anhydrides of the acid of formula II may be symmetric or preferably mixed anhydrides of that acid, for example anhydrides with inorganic acids, such as acid halides, especially acid chlorides (obtainable, for example, by treatment of the corresponding acid with thionyl chloride, phosphorus pentachloride or oxalyl chloride; acid chloride method), azides (obtainable, for example, from a corresponding acid ester via the corresponding hydrazide and treatment thereof with nitrous acid; azide method), anhydrides with carbonic acid semiderivatives, such as corresponding esters, for example carbonic acid lower alkyl semiesters (obtainable, for example, by treatment of the corresponding acid with haloformic, such as chloroformic, acid lower alkyl esters or with a 1-lower alkoxycarbonyl-2-lower alkoxy-1,2-dihydroquinoline, for example 1-lower alkoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; mixed O-alkylcarbonic acid anhydrides method), or anhydrides with dihalogenated, especially dichlorinated, phosphoric acid (obtainable, for example, by treatment of the

corresponding acid with phosphorus oxychloride; phosphorus oxychloride method), or anhydrides with organic acids, such as mixed anhydrides with organic carboxylic acids (obtainable, for example, by treatment of the corresponding acid with an unsubstituted or substituted lower alkane- or phenylalkane-carboxylic acid halide, for example phenylacetic acid chloride, pivalic acid chloride or trifluoroacetic acid chloride; mixed carboxylic acid anhydrides method), with organic sulfonic acids (obtainable, for example, by treatment of a salt, such as an alkali metal salt, of the corresponding acid, with a suitable organic sulfonic acid halide, such as lower alkane- or aryl-, for example methane- or p-toluene-sulfonic acid chloride; mixed sulfonic acid anhydrides method), or with organic phosphonic acids (obtainable, for example, by treatment of the corresponding acid with a suitable organic phosphonic anhydride or phosphonic cyanide; mixed phosphonic acid anhydrides methhod), and symmetric anhydrides (obtainable, for example, by condensation of the corresponding acid in the presence of a carbodiimide or of 1-diethylaminopropyne; symmetric anhydrides method).

Suitable cyclic amides are especially amides with five-membered diazacycles of aromatic character, such as amides with imidazoles, for example imidazole (obtainable, for example, by treatment of the corresponding acid with N,N'-carbonyldiimidazole; imidazolide method), or pyrazoles, for example 3,5-dimethyl-pyrazole (obtainable, for example, by way of the acid hydrazide by treatment with acetylacetone; pyrazolide method).

Derivatives of the acid of formula II wherein the carboxy group is in activated form are preferably formed in situ. For example, N,N'-disubstituted amidino esters can be formed in situ by reacting a mixture of the acid of formula II and the amine of formula III in the presence of a suitable N,N-disubstituted carbodiimide, for example N,N'-dicyclohexylcarbodiimide. Reactive mixed anhydrides of the acid of formula II with an organic phosphonic acid may be formed in situ by reaction with e.g. propylphosphonic anhydride or diethylcyanophosphonate in the presence of suitable base, preferably a tertiary amine, e.g. triethylamine or dimethylaminopyridine.

The reaction can be carried out in a manner known per se, the reaction conditions being dependent especially on whether, and if so how, the carboxy group of the carboxylic acid of formula II has been activated, usually in the presence of a suitable solvent or diluent or of a mixture thereof and, if necessary, in the presence of a condensation agent, which, for

example when the carboxy group participating in the reaction is in the form of an anhydride, may also be an acid-binding agent, with cooling or heating, for example in a temperature range from approximately -30 °C to approximately +150 °C, especially approximately from 0 °C to +100 °C, preferably from room temperature (approx. +20 °C) to +70 °C, in an open or closed reaction vessel and/or in the atmosphere of an inert gas, for example nitrogen. Customary condensation agents are, for example, carbodiimides, for example N,N'-diethyl-, N,N'-dipropyl-, N,N'-dicyclohexyl- or N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide, suitable carbonyl compounds, for example carbonyldiimidazole, or 1,2-oxazolium compounds, for example 2-ethyl-5-phenyl-1,2-oxazolium 3'-sulfonate and 2-tert-butyl-5-methyl-isoxazolium perchlorate, or a suitable acylamino compound, for example 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline. Customary acid-binding condensation agents are, for example, alkali metal carbonates or hydrogen carbonates, for example sodium or potassium carbonate or hydrogen carbonate (customarily together with a sulfate), or organic bases, such as, customarily, pyridine or triethylamine, or sterically hindered tri-lower alkylamines, for example N,N-diisopropyl-N-ethyl-amine.

In a preferred variant, the carboxylic acid of formula II is reacted with an amine of formula III in a suitable solvent, such as e.g. N,N-dimethylformamide, in the presence of propyl-phosphonic anhydride or diethylcyanophosphanate and triethylamine, between 1 and 48 hours at between 0°C and around 50°C, preferably at room temperature.

Protecting groups

If one or more other functional groups, for example carboxy, hydroxy, amino, or mercapto, are or need to be protected in a compound of formula III, because they should not take part in the reaction, these are such groups as are usually used in the synthesis of amides, in particular peptide compounds, and also of cephalosporins and penicillins, as well as nucleic acid derivatives and sugars.

The protecting groups may already be present in precursors and should protect the functional groups concerned against unwanted secondary reactions, such as acylations, etherifications, esterifications, oxidations, solvolysis, and similar reactions. It is a characteristic of protecting groups that they lend themselves readily, i.e. without undesired secondary reactions, to removal, typically by solvolysis, reduction, photolysis or also by

enzyme activity, for example under conditions analogous to physiological conditions, and that they are not present in the end-products.The specialist knows, or can easily establish, which protecting groups are suitable with the reactions mentioned hereinabove and hereinafter.

The protection of such functional groups by such protecting groups, the protecting groups themselves, and their removal reactions are described for example in standard reference books for peptide synthesis as cited hereinbefore, and in special books on protective groups such as J. F. W. McOmie, "Protective Groups in Organic Chemistry", Plenum Press, London and New York 1973, in "Methoden der organischen Chemie" (Methods of organic chemistry), Houben-Weyl, 4th edition, Volume 15/I, Georg Thieme Verlag, Stuttgart 1974, and in T. W. Greene, "Protective Groups in Organic Synthesis", Wiley, New York.

Additional process steps

In the additional process steps, carried out as desired, functional groups of the starting compounds which should not take part in the reaction may be present in unprotected form or may be protected for example by one or more of the protecting groups mentioned hereinabove under "protecting groups". The protecting groups are then wholly or partly removed according to one of the methods described there.

Salts of a compound of formula I with a salt-forming group may be prepared in a manner known per se. Acid addition salts of compounds of formula I may thus be obtained by treatment with an acid or with a suitable anion exchange reagent.

Salts can usually be converted to free compounds, e.g. by treating with suitable basic agents, for example with alkali metal carbonates, alkali metal hydrogencarbonates, or alkali metal hydroxides, typically potassium carbonate or sodium hydroxide.

Stereoisomeric mixtures, e.g. mixtures of diastereomers, can be separated into their corresponding isomers in a manner known per se by means of suitable separation methods. Diastereomeric mixtures for example may be separated into their individual diastereomers by means of fractionated crystallization, chromatography, solvent distribution, and similar procedures. This separation may take place either at the level of a starting compound or in a compound of formula I itself. Enantiomers may be separated through the formation of

diastereomeric salts, for example by salt formation with an enantiomer-pure chiral acid, or by means of chromatography, for example by HPLC, using chromatographic substrates with chiral ligands.

A compound of the formula I wherein R_1 is hydrogen can be converted to the respective compound wherein R_1 is lower alkyl by reaction e.g. with a diazo lower alkyl compound, especially diazomethane, in an inert solvent, preferably in the presence of a noble metal catalyst, especially in dispersed form, e.g. copper, or a noble metal salt, e.g. copper(I)-chloride or copper(II)-sulfate. Also reaction with lower alkylhalogenides is possible, or with other leaving group carrying lower alkanes, e.g. lower alkyl alcohols esterified by a strong organic sulfonic acid, such as a lower alkanesulfonic acid (optionally substituted by halogen, such as fluoro), an aromatic sulfonic acid, for example unsubstituted or substituted benzenesulfonic acid, the substituents preferably being selected from lower alkyl, such as methyl, halogen, such as bromo, and/or nitro, e.g. esterified by methanesulfonic acid, or p-toluene sulfonic acid. The alkylation takes place under usual conditions for alkylation of amides, especially in aqueous solution and/or in the presence of polar solvents, typically alcohols, for example methanol, ethanol, isopropanol, or ethylene glycol, or dipolar aprotic solvents, e.g. tetrahydrofuran, dioxane, or dimethylformamide, where applicable in the presence of acidic or basic catalysts, generally at temperatures from about 0°C to the boiling temperature of the corresponding reaction mixture, preferably between 20°C and reflux temperature, if necessary under increased pressure, e.g. in a sealed tube, and/or under inert gas, typically nitrogen or argon.

It should be emphasized that reactions analogous to the conversions mentioned in this chapter may also take place at the level of appropriate intermediates.

General process conditions

All process steps described here can be carried out under known reaction conditions, preferably under those specifically mentioned, in the absence of or usually in the presence of solvents or diluents, preferably such as are inert to the reagents used and able to dissolve these, in the absence or presence of catalysts, condensing agents or neutralisiing agents, for example ion exchangers, typically cation exchangers, for example in the H^+ form, depending on the type of reaction and/or reactants at reduced, normal, or elevated temperature, for

example in the range from -100°C to about 190°C, preferably from about -80°C to about 150°C, for example at -80 to -60°C, at room temperature, at - 20 to 40°C or at the boiling point of the solvent used, under atmospheric pressure or in a closed vessel, where appropriate under pressure, and/or in an inert atmosphere, for example under argon or nitrogen.

Salts may be present in all starting compounds and transients, if these contain salt-forming groups. Salts may also be present during the reaction of such compounds, provided the reaction is not thereby disturbed.

At all reaction stages, isomeric mixtures that occur can be separated into their individual isomers, e.g. diastereomers or enantiomers, or into any mixtures of isomers, e.g. racemates or diastereomeric mixtures.

The invention relates also to those forms of the process in which one starts from a compound obtainable at any stage as a transient and carries out the missing steps, or breaks off the process at any stage, or forms a starting material under the reaction conditions, or uses said starting material in the form of a reactive derivative or salt, or produces a compound obtainable by means of the process according to the invention and processes the said compound in situ. In the preferred embodiment, one starts from those starting materials which lead to the compounds described hereinabove as preferred, particularly as especially preferred, primarily preferred, and/or preferred above all.

In the preferred embodiment, a compound of formula I is prepared according to or in analogy to the processes and process steps defined in the Examples.

The compounds of formula I, including their salts, are also obtainable in the form of hydrates, or their crystals can include for example the solvent used for crystallization (present as solvates).

Pharmaceutical preparations, methods, and uses

The present invention relates furthermore to a method for the treatment of a neoplastic disease which responds to an inhibition of a protein kinase activity, which comprises

administering a compound of formula I or a N-oxide or a pharmaceutically acceptable salt thereof, wherein the radicals and symbols have the meanings as defined above for formula I, in a quantity effective against said disease, to a warm-blooded animal requiring such treatment.

In particular the invention relates to a method for the treatment of leukaemia which responds to an inhibition of the Abl tyrosine kinase activity, which comprises administering a compound of formula I or a N-oxide or a pharmaceutically acceptable salt thereof, wherein the radicals and symbols have the meanings as defined above for formula I, in a quantity effective against said leukaemia, to a warm-blooded animal requiring such treatment.

The present invention relates also to pharmaceutical compositions that comprise a compound of formula I or a N-oxide thereof as active ingredient and that can be used especially in the treatment of the diseases mentioned at the beginning. Compositions for enteral administration, such as nasal, buccal, rectal or, especially, oral administration, and for parenteral administration, such as intravenous, intramuscular or subcutaneous administration, to warm-blooded animals, especially humans, are especially preferred. The compositions comprise the active ingredient alone or, preferably, together with a pharmaceutically acceptable carrier. The dosage of the active ingredient depends upon the disease to be treated and upon the species, its age, weight, and individual condition, the individual pharmacokinetic data, and the mode of administration.

The present invention relates especially to pharmaceutical compositions that comprise a compound of formula I, a tautomer, a N-oxide or a pharmaceutically acceptable salt, or a hydrate or solvate thereof, and at least one pharmaceutically acceptable carrier.

The invention relates also to pharmaceutical compositions for use in a method for the prophylactic or especially therapeutic management of the human or animal body, to a process for the preparation thereof (especially in the form of compositions for the treatment of tumors) and to a method of treating tumor diseases, especially those mentioned hereinabove.

The invention relates also to processes and to the use of compounds of formula I or N-oxides thereof for the preparation of pharmaceutical preparations which comprise compounds of formula I or N-oxides thereof as active component (active ingredient).

In the preferred embodiment, a pharmaceutical preparation is suitable for administration to a warm-blooded animal, especially humans or commercially useful mammals suffering from a disease responsive to an inhibition of the Abl tyrosine kinase, for example chronic myelogenous leukaemia (CML), and comprises an effective quantity of a compound of formula I or N-oxides thereof for the inhibition of the Bcr-Abl fusion protein, or a pharmaceutically acceptable salt thereof, if salt-forming groups are present, together with at least one pharmaceutically acceptable carrier.

A pharmaceutical composition for the prophylactic or especially therapeutic management of neoplastic and other proliferative diseases of a warm-blooded animal, especially a human or a commercially useful mammal requiring such treatment, especially suffering from such a disease, comprising as active ingredient in a quantity that is prophylactically or especially therapeutically active against the said diseases a novel compound of formula I or N-oxides thereof, is likewise preferred.

The pharmaceutical compositions comprise from approximately 1% to approximately 95% active ingredient, single-dose administration forms comprising in the preferred embodiment from approximately 20% to approximately 90% active ingredient and forms that are not of single-dose type comprising in the preferred embodiment from approximately 5% to approximately 20% active ingredient. Unit dose forms are, for example, coated and uncoated tablets, ampoules, vials, suppositories, or capsules. Further dosage forms are, for example, ointments, creams, pastes, foams, tinctures, sprays, etc. Examples are capsules containing from about 0.05 g to about 1.0 g active ingredient.

The pharmaceutical compositions of the present invention are prepared in a manner known per se, for example by means of conventional mixing, granulating, coating, dissolving or lyophilizing processes.

Preference is given to the use of solutions of the active ingredient, and also suspensions or dispersions, especially isotonic aqueous solutions, dispersions or suspensions which, for example in the case of lyophilized compositions comprising the active ingredient alone or together with a carrier can be made up before use. The pharmaceutical compositions may be sterilized and/or may comprise excipients, for example preservatives, stabilizers, wetting

agents and/or emulsifiers, solubilizers, salts for regulating osmotic pressure and/or buffers and are prepared in a manner known per se, for example by means of conventional dissolving and lyophilizing processes. The said solutions or suspensions may comprise viscosity-increasing agents or solubilizers.

Suspensions in oil comprise as the oil component the vegetable, synthetic, or semi-synthetic oils customary for injection purposes. In respect of such, special mention may be made of liquid fatty acid esters that contain as the acid component a long-chained fatty acid having from 8 to 22 carbon atoms. The alcohol component of these fatty acid esters has a maximum of 6 carbon atoms and is a monovalent or polyvalent, for example a mono-, di- or trivalent, alcohol, especially glycol and glycerol.

Pharmaceutical compositions for oral administration can be obtained, for example, by combining the active ingredient with one or more solid carriers, if desired granulating a resulting mixture, and processing the mixture or granules, if desired or necessary, by the inclusion of additional excipients, to form tablets or tablet cores.

Suitable carriers are especially fillers, such as sugars, cellulose preparations, and/or calcium phosphates, and also binders, such as starches, and/or polyvinylpyrrolidone, and/or, if desired, disintegrators. Additional excipients are especially flow conditioners and lubricants.

Tablet cores can be provided with suitable, optionally enteric, coatings through the use of, inter alia, concentrated sugar solutions which may comprise gum arabic, talc, polyvinylpyrrolidone, polyethylene glycol and/or titanium dioxide, or coating solutions in suitable organic solvents or solvent mixtures, or, for the preparation of enteric coatings, solutions of suitable cellulose preparations.

Pharmaceutical compositions for oral administration also include hard capsules consisting of gelatin, and also soft, sealed capsules consisting of gelatin and a plasticizer. The hard capsules may contain the active ingredient in the form of granules, for example in admixture with fillers, binders, and/or glidants, and optionally stabilizers. In soft capsules, the active ingredient is preferably dissolved or suspended in suitable liquid excipients, to which stabilizers and detergents may also be added.

Pharmaceutical compositions suitable for rectal administration are, for example, suppositories that consist of a combination of the active ingredient and a suppository base.

For parenteral administration, aqueous solutions of an active ingredient in water-soluble form, for example of a water-soluble salt, or aqueous injection suspensions that contain viscosity-increasing substances, for example sodium carboxymethylcellulose, sorbitol and/or dextran, and, if desired, stabilizers, are especially suitable. The active ingredient, optionally together with excipients, can also be in the form of a lyophilizate and can be made into a solution before parenteral administration by the addition of suitable solvents.

Solutions such as are used, for example, for parenteral administration can also be employed as infusion solutions.

Preferred preservatives are, for example, antioxidants, such as ascorbic acid, or microbicides, such as sorbic acid or benzoic acid.

The invention relates likewise to a process or a method for the treatment of one of the pathological conditions mentioned hereinabove, especially a disease which responds to an inhibition of a tyrosine kinase, especially a corresponding neoplastic disease. The compounds of formula I or N-oxides thereof can be administered as such or especially in the form of pharmaceutical compositions, prophylactically or therapeutically, preferably in an amount effective against the said diseases, to a warm-blooded animal, for example a human, requiring such treatment. In the case of an individual having a bodyweight of about 70 kg the daily dose administered is from approximately 0.05 g to approximately 5 g, preferably from approximately 0.25 g to approximately 1.5 g, of a compound of the present invention.

The present invention relates especially also to the use of a compound of formula I or N-oxides thereof, or a pharmaceutically acceptable salt thereof, especially a compound of formula I which is said to be preferred, or a pharmaceutically acceptable salt thereof, as such or in the form of a pharmaceutical formulation with at least one pharmaceutically acceptable carrier for the therapeutic and also prophylactic management of one or more of the diseases mentioned hereinabove, preferably a disease which responds to an inhibition of a protein kinase, especially a neoplastic disease, more especially leukaemia which responds to an inhibition of the Abl tyrosine kinase.

The preferred dose quantity, composition, and preparation of pharmaceutical formulations (medicines) which are to be used in each case are described above.

Starting materials

New starting materials and/or intermediates, as well as processes for the preparation thereof, are likewise the subject of this invention. In the preferred embodiment, such starting materials are used and reaction conditions so selected as to enable the preferred compounds to be obtained.

The substituted aminobenzoic acid of formula II, for example, can be obtained by reaction of an ester of 3-amino-4-R_4-benzoic acid, e.g. 3-amino-4-methylbenzoic acid, with cyanamide and condensing the obtainable guanidine with 3-(dimethylamino)-1-(3-pyridinyl)-2-propen-1-one, and finally hyrolysing the ester function.

Starting materials of the formula III are known, commercially available, or can be synthesized in analogy to or according to methods that are known in the art.

The following Examples serve to illustrate the invention without limiting the invention in its scope.

Abbreviations

DMSO	dimethylsulfoxide
HPLC/MS-MS	high-pressure liquid chromatography/ tandem mass spectrometry
min	minutes
m.p.	melting point
NMP	N-methyl-pyrrolidone
NMR	nuclear magnetic resonance
PEG	polyethylen glycol
THF	tetrahydrofuran

Examples

Example 1: *N*-(2-Furanylmethyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide

A solution containing ~50% of propylphosphonic anhydride in *N,N*-dimethylformamide (Fluka, Buchs, Switzerland; 674 µL, ~1 mmol) is added within 20 minutes to a stirred mixture of 4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzoic acid (214.4 mg, 0.7 mmol), furfurylamine (Aldrich, Buchs, Switzerland; 61.8 µL, 0.7 mmol) and triethylamine (776 µL, 5.6 mmol) in 2 mL *N,N*-dimethylformamide. After stirring for 24 hours at room temperature, the mixture is treated with a half-saturated aqueous solution of sodium hydrogen carbonate and extracted three times with ethyl acetate. The solvent is evaporated off under reduced pressure and the residue dried *in vacuo*. The crude product is crystallised from dichloromethane to give the title compound as a crystalline solid.

^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.28 (s, 3H); 4.43 (d, 2H); 6.23 (m, 1H); 6.33-6.37 (m, 1H); 7.30 (d,1H); 7.42 (d, 1H); 7.49 (ddd, 1H); 7.53 (m, 1H); 7.59 (dd, 1H); 8.11 (d, 1H); 8.38 (m, 1H); 8.49 (d, 1H); 8.66 (dd, 1H); 8.87 (t, 1H); 9.05 (s, 1H); 9.22 (m, 1H).

The starting material is prepared as follows:

Example 1a: 3-[(Aminoiminomethyl)amino]-4-methyl-benzoic acid ethyl ester mononitrate

Cyanamide (Fluka, Buchs, Switzerland; 77.4 g, 1.842 mol) is added to a solution of 3-amino-4-methylbenzoic acid ethyl ester (J. Med. Chem. 16, 118-122, 1973; 150 g, 0.837 mol) in 850 mL of ethanol. Hydrochloric acid (Fluka, Buchs, Switzerland; 108 mL of 12M, 1.27 mol) is then added dropwise over 15 min and the reaction mixture is then stirred at 90°C (bath temperature) for 15 hours. The solvent is evaporated off under reduced pressure to give a residue which is treated with water (1000 mL) and stirred with cooling at 5-10°C. A solution of ammonium nitrate (Merck, Darmstadt, Germany; 134.8 g, 1.68 mol) in water (400 mL) is added dropwise over 30 min. followed by ice-water (1200 mL). After stirring for an additional 30 min. the product is filtered off, washed with ice-water (3 x 1000 mL) and air-dried. The residue is washed with diethyl ether (2 x 2000 m L) and dried *in vacuo* at 50° to give the title compound as a crystalline solid, m.p.195-197°C.

Example 1b: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzoic acid ethyl ester

A stirred mixture of the intermediate Example 1a (164 g, 0.577 mol), 3-(dimethylamino)-1-(3-pyridinyl)-2-propen-1-one (113.8 g, 0.646 mol) and powdered NaOH (99%; Merck,

Darmstadt, Germany; 26.6 g, 0.658 mol) in ethanol (2200 mL) is heated under reflux for 68 h. The reaction solvent is evaporated off under reduced pressure and the residue partitioned between ethyl acetate and water. The organic layer is separated and the aqueous phase extracted twice with ethyl acetate. The combined organic extracts are washed with water and brine, dried (Na_2SO_4) and the solvent is evaporated off under reduced pressure to give a residue, which is crystallised from diethyl ether to give the title compound as a crystalline solid, m.p. 95-96°C.

Example 1c: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzoic acid

Aqueous sodium hydroxide (500 mL of 2M) is added dropwise to a stirred suspension of the intermediate Example 1b (132.8 g, 0.397 mol) in ethanol (1200 m L) and water (1200 mL). The reaction mixture is stirred at 45°C for 2.5 h and then treated dropwise with aqueous HCl (1000 mL of 1M) over 1.5 hours. After addition of water (1000 mL) the precipitate is filtered off, washed with water (4 x 500 mL) and dried at room temperature. Residual water present in the air-dried product is removed by azeotropic distillation with toluene under reduced pressure. The dried toluene suspension is diluted with diethyl ether and filtered. The solid residue is washed with diethyl ether and dried *in vacuo* at 80°C to give the title compound, m.p. 277-278°C.

Example 2: *N*-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]-4-[(4-methyl-1-piperazinyl)methyl]benzeneamine

A solution containing ~50% of propylphosphonic anhydride in *N,N*-dimethylformamide (Fluka, Buchs, Switzerland; 875 µL, ~1.5 mmol) is added within 20 minutes to a stirred mixture of 4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzoic acid (306 mg, 1.0 mmol), 4-[(4-methyl-1-piperazinyl)methyl]benzeneamine (Chem. Abstr. Reg. Number: 70261-82-4; 205 mg, 1.0 mmol) and triethylamine (830 µL, 6.0 mmol) in 8 mL *N,N*-dimethylformamide. After stirring for 24 hours at room temperature, the mixture is treated with a saturated aqueous ammonium chloride and extracted three times with ethyl acetate. The solvent is evaporated off under reduced pressure and the residue dried *in vacuo*. The crude product is crystallised from ethanol-ethyl acetate to give the title compound as a crystalline solid, m.p. 153-155°C.

Example 3: 1-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]-4-(2-pyridinyl)-piperazine

A solution containing ~50% of propylphosphonic anhydride in *N,N*-dimethylformamide (Fluka, Buchs, Switzerland; 674 µL, ~1 mmol) is added within 20 minutes to a stirred mixture of 4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzoic acid (214.4 mg, 0.7 mmol), 1-(2-pyridyl)piperazine (Aldrich, Buchs, Switzerland; 114.3 mg, 0.7 mmol) and triethylamine (776 µL, 5.6 mmol) in 2 mL *N,N*-dimethylformamide. After stirring for 24 hours at room temperature, the mixture is treated with a half-saturated aqueous solution of sodium hydrogen carbonate and extracted three times with ethyl acetate. The solvent is evaporated off under reduced pressure and the residue dried *in vacuo*. The crude product is purified by column chromatography on silica gel, eluent 5-10% methanol in dichloromethane, to give the title compound as a solid. ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.31 (s, 3H); 3.35-3.74 (m, 8H); 6.65 (ddd, 1H); 6.79 (d, 1H); 7.13 (dd, 1H); 7.32 (d, 1H); 7.44 (d, 1H); 7.49-7.56 (m, 2H); 7.69 (m, 1H); 8.11 (m, 1H); 8.40 (m, 1H); 8.52 (d, 1H); 8.66 (dd, 1H); 9.06 (s, 1H); 9.24 (m, 1H).

The following compounds are prepared analogously by utilising the appropriate amine (supplier in parenthesis):

Example 4: 4-Methyl-*N*-[2-(2-pyridinyl)ethyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide utilising 2-(2-aminoethyl)pyridine (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.27 (s, 3H); 2.97 (t, 2H); 3.58 (m, 2H); 7.18 (ddd, 1H); 7.25 (m, 1H); 7.29 (d,1H); 7.42 (d, 1H); 7.47-7.56 (m, 2H); 7.65 (m, 1H); 8.06 (d, 1H); 8.39 (m, 1H); 8.44-8.51 (m, 3H); 8.66 (dd, 1H); 9.04 (s, 1H); 9.22 (m, 1H).

Example 5: 4-Methyl-*N*-[1-(phenylmethyl)-4-piperidinyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]-amino]benzamide utilising 4-amino-1-benzylpiperidine (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.47-1.63 (m, 2H); 1.69-1.80 (m, 2H); 1.92-2.05 (m, 2H); 2.27 (s, 3H); 2.73-2.83 (m, 2H); 3.43 (s, 2H); 3.68-3.83 (m, 1H); 7.18-7.33 (m ,6H); 7.42 (d, 1H); 7.49 (ddd, 1H); 7.55 (dd, 1H); 8.10 (m, 1H); 8.14 (d, 1H); 8.37 (m, 1H); 8.49 (d, 1H); 8.65 (dd, 1H); 9.04 (s, 1H); 9.21 (m, 1H).

Example 6: 4-Methyl-*N*-(4-pyridinylmethyl)-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide utilising 4-(aminomethyl)pyridine (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.30 (s, 3H); 4.46 (d, 2H); 7.26 (m, 2H); 7.33 (d, 1H); 7.43 (d, 1H); 7.47 (ddd, 1H);7.62 (dd, 1H); 8.16 (d, 1H); 8.38 (m, 1H); 8.45 (m, 2H); 8.50 (d, 1H); 8.66 (dd, 1H); 9.03 (t, 1H); 9.08 (s, 1H); 9.23 (m, 1H).

Example 7: 4-Methyl-*N*-[2-(1-methyl-1H-pyrrol-2-yl)ethyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]-amino]benzamide utilising 2-(2-aminoethyl)-1-methylpyrrol [Chem. Abstr. Reg. Number: 83732-75-6]. ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.28 (s, 3H); 2.75 (t, 2H); 3.42 (m, 2H); 3.51 (s, 3H); 5.76-5.85 (m, 2H); 6.57 (m,1H); 7.30 (d,1H); 7.43 (d, 1H); 7.46-7.58 (m, 2H); 8.10 (br. 1H); 8.40 (m, 1H); 8.48-8.55 (m, 2H); 8.64-8.69 (m, 1H); 9.05 (s, 1H); 9.23 (m, 1H).

Example 8: *N*-[(4-Methoxyphenyl)methyl]-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide utilising 4-methoxybenzylamine (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.28 (s, 3H); 3.69 (s, 3H); 4.37 (d, 2H); 6.80-6.87 (m, 2H); 7.17-7.23 (m, 2H); 7.31 (d,1H); 7.42 (d, 1H); 7.47 (ddd, 1H); 7.59 (dd, 1H); 8.11 (d, 1H); 8.38 (m, 1H); 8.49 (d, 1H); 8.66 (dd, 1H); 8.87 (t, 1H); 9.05 (s, 1H); 9.23 (m, 1H).

Example 9: 4-Methyl-*N*-(2-methylpropyl)-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide utilising isobutylamine (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 0.85 (d, 6H); 1.81 (m, 1H); 2.27 (s, 3H); 3.04 (m, 2H); 7.29 (d,1H); 7.42 (d, 1H); 7.48 (dd, 1H); 7.55 (dd, 1H); 8.07 (d, 1H); 8.31-8.41 (m, 2H); 8.49 (d, 1H); 8.65 (dd, 1H); 9.05 (s, 1H); 9.22 (m, 1H).

Example 10: 4-Methyl-*N*-(2-morpholinoethyl)-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide utilising 4-(2-aminoethyl)morpholine (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.28 (s, 3H); 2.33-2.46 (m, 6H); 3.30-3.40 (m, 2H); 3.53 (m, 4H); 7.30 (d,1H); 7.42 (d, 1H); 7.46-7.57 (m, 2H); 8.06 (d, 1H); 8.30 (m, 1H); 8.38 (m, 1H); 8.49 (d, 1H); 8.66 (dd, 1H); 9.05 (s, 1H); 9.22 (m, 1H).

Example 11: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[(tetrahydro-2-furanyl)-methyl]benzamide utilising tetrahydrofurfurylamine (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.49-1.63 (m, 1H); 1.70-1.93 (m, 3H); 2.27 (s, 3H); 3.27 (m, 2H); 3.58 (m, 1H); 3.72 (m, 1H); 3.94 (m, 1H); 7.29 (d,1H); 7.42 (d, 1H); 7.49 (ddd, 1H); 7.56 (dd, 1H); 8.08 (d, 1H); 8.35-8.45 (m, 2H); 8.49 (d, 1H); 8.66 (dd, 1H); 9.04 (s, 1H); 9.21 (m, 1H).

Example 12: *N*-[2-(2,4-Dihydroxy-5-pyrimidinyl)ethyl]-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide utilising 5-(2-aminoethyl)-2,4(1H,3H)-pyrimidinedione [Chem. Abstr. Reg. Number: 221170-25-8]. ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.27 (s, 3H); 2.40 (t,

2H); 3.34 (m, 2H); 7.15 (m,1H); 7.29 (d,1H); 7.42 (d, 1H); 7.47-7.55 (m, 2H); 8.07 (d, 1H); 8.35-8.42 (m, 2H); 8.49 (d, 1H); 8.66 (dd, 1H); 9.04 (s, 1H); 9.22 (m, 1H); 10.59 (s, 1H); 11.01 (s, 1H).

Example 13: *N*-Cyclohexyl-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide utilising cyclohexylamine (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.00-1.16 (m, 1H); 1.18-1.36 (m, 4H); 1.52-1.85 (m, 5H); 2.27 (s, 3H); 3.66-3.82 (m, 1H); 7.28 (d, 1H); 7.41 (d, 1H); 7.48 (m, 1H); 7.55 (dd, 1H); 8.06-8.12 (m, 2H); 8.37 (m, 1H); 8.49 (d, 1H); 8.66 (dd, 1H); 9.04 (s, 1H); 9.21 (m, 1H).

Example 14: *N*-[(3*S*)-Hexahydro-2-oxo-1H-azepin-3-yl]-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide utilising L(-)-alpha-amino-epsilon-caprolactam [Chem. Abstr. Reg. Number: 21568-87-6]. ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.11-1.31 (m, 1H); 1.37-1.82 (m, 3H); 1.83-1.96 (m, 2H); 2.28 (s, 3H); 3.00-3.13 (m, 1H); 3.15-3.30 (m, 1H); 4.58 (m, 1H); 7.32 (d,1H); 7.43 (d, 1H); 7.51 (ddd, 1H); 7.55 (dd, 1H); 7.84 (m, 1H); 8.08 (d, 1H); 8.13 (d, 1H); 8.40 (m, 1H); 8.50 (d, 1H); 8.66 (dd, 1H); 9.06 (s, 1H); 9.22 (m, 1H).

Example 15: *N*-[2-(3,4-Dimethoxyphenyl)ethyl]-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]-amino]benzamide utilising 2-(3,4-dimethoxyphenyl)ethylamine (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.27 (s, 3H); 2.75 (t, 2H); 3.43 (m, 2H); 3.67 (s, 6H); 6.70 (dd, 1H); 6.77-6.83 (m, 2H); 7.30 (d,1H); 7.42 (d, 1H); 7.46-7.57 (m, 2H); 8.07 (d, 1H); 8.36-8.46 (m, 2H); 8.49 (d, 1H); 8.66 (dd, 1H); 9.05 (s, 1H); 9.22 (m, 1H).

Example 16: 2-[[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]amino]-4-thiazole-acetic acid ethyl ester utilising ethyl 2-amino-4-thiazoleacetate (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.16 (t, 3H); 2.32 (s, 3H); 3.70 (s, 2H); 4.06 (q, 2H); 7.01 (s, 1H); 7.36 (d,1H); 7.42-7.54 (m, 2H); 7.82 (d, 1H); 8.34-8.47 (m, 2H); 8.52 (d, 1H); 8.66 (m, 1H); 9.08 (s, 1H); 9.24 (m, 1H); 12.57 (br., 1H).

Example 17: *N*-[3-(1H-Imidazol-1-yl)propyl]-4-methyl-3-[[4-(3-pyridinyl)-2pyrimidinyl]amino]-benzamide utilising 1-(3-aminopropyl)imidazole (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.96 (qui, 2H); 2.30 (s, 3H); 3.24 (m, 2H); 4.01 (t, 2H); 6.91 (s,1H); 7.22 (m, 1H); 7.34 (d,1H); 7.45 (d, 1H); 7.51 (ddd, 1H); 7.59 (dd, 1H); 7.70 (s, 1H); 8.14 (d, 1H); 8.42 (m, 1H); 8.47 (t, 1H); 8.52 (d, 1H); 8.68 (dd, 1H); 9.10 (s, 1H); 9.25 (m, 1H).

Example 18: *N*-(Cyclopropylmethyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide utilising cyclopropanemethylamine (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 0.17-0.22 (m, 2H); 0.36-0.42 (m, 2H); 0.96-1.06 (m, 1H); 2.28 (s, 3H); 3.11 (m, 2H); 7.31 (d,1H); 7.43 (d, 1H); 7.50 (ddd, 1H); 7.58 (dd, 1H); 8.10 (d, 1H); 8.40 (m, 1H); 8.47 (t, 1H); 8.50 (d, 1H); 8.67 (dd, 1H); 9.07 (s, 1H); 9.23 (m, 1H).

Example 19: *N*-(2-methoxyethyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide utilising 2-methoxyethylamine (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.28 (s, 3H); 3.23 (s, 3H); 3.36-3.46 (m, 4H); 7.31 (d,1H); 7.43 (d, 1H); 7.51 (ddd, 1H); 7.57 (dd, 1H); 8.10 (d, 1H); 8.38-8.47 (m, 2H); 8.50 (d, 1H); 8.68 (dd, 1H); 9.07 (s, 1H); 9.23 (m, 1H).

Example 20: 4-Methyl-*N*-[3-(2-oxo-1-pyrrolidinyl)propyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]-amino]benzamide utilising 1-(3-aminopropyl)-2-pyrrolidinone (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.67 (m, 2H); 1.89 (m, 2H); 2.18 (t, 2H); 2.28 (s, 3H); 3.19 (m, 4H); 3.32 (m, 2H); 7.30 (d, 1H); 7.42 (d, 1H); 7.49 (ddd, 1H); 7.54 (dd, 1H); 8.09 (d, 1H); 8.31-8.42 (m, 2H); 8.49 (d, 1H); 8.66 (dd, 1H); 9.04 (s, 1H); 9.22 (m, 1H).

Example 21: *N*,4-Dimethyl-N-(phenylmethyl)-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide utilising N-benzylmethylamine (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.28 (s, 3H); 2.86 (s, 3H); 4.51-4.68 (m, 2H); 7.08-7.35 (m,7H); 7.43 (d, 1H); 7.48 (m, 1H); 7.71 (s, 1H); 8.35-8.54 (m, 2H); 8.67 (m, 1H); 8.97-9.09 (m, 1H); 9.24 (m, 1H).

Example 22: *N*-[4-(Acetylamino)phenyl]-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide utilising 4-aminoacetanilide (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.01 (s, 3H); 2.32 (s, 3H); 7.38 (d,1H); 7.45 (d, 1H); 7.47-7.54 (m, 3H); 7.63-7.71 (m, 3H); 8.22 (m, 1H); 8.43 (m, 1H); 8.52 (d, 1H); 8.67 (dd, 1H); 9.13 (s, 1H); 9.25 (m, 1H); 9.90 (s, 1H); 10.11 (s, 1H).

Example 23: *N*-(4-Methoxy-2-methylphenyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide utilising 4-methoxy-2-methylaniline (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.16 (s, 3H); 2.32 (s, 3H); 3.73 (s, 3H); 6.75 (dd, 1H); 6.82 (m, 1H); 7.16

(d, 1H); 7.37 (d,1H); 7.45 (d, 1H); 7.49 (ddd, 1H); 7.69 (dd, 1H); 8.25 (d, 1H); 8.41 (m, 1H); 8.52 (d, 1H); 8.67 (dd, 1H); 9.12 (s, 1H); 9.25 (m, 1H); 9.69 (s, 1H).

Example 24: 4-Methyl-*N*-[4-(methylsulfonyl)benzyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide utilising 4-methylsulfonylbenzylamine hydrochloride (Acros, Morris Plains, NJ). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.30 (s, 3H); 3.16 (s, 3H); 4.54 (d, 2H); 7.34 (d,1H); 7.44 (d, 1H); 7.49 (ddd, 1H); 7.55 (m, 2H); 7.63 (dd, 1H); 7.86 (m, 2H); 8.16 (d, 1H); 8.40 (m, 1H); 8.51 (d, 1H); 8.67 (dd, 1H); 9.10 (m, 2H); 9.24 (m, 1H).

Example 25: *N*-[[4-(Dimethylamino)phenyl]methyl]-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]-amino]benzamide utilising 4-(dimethylamino)benzylamine dihydrochloride (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.28 (s, 3H); 2.82 (s, 6H); 4.32 (d, 2H); 6.64 (m, 2H); 7.11 (m, 2H); 7.31 (d, 1H); 7.43 (d, 1H); 7.48 (ddd, 1H); 7.59 (dd, 1H); 8.12 (d, 1H); 8.39 (m, 1H); 8.50 (d, 1H); 8.68 (dd, 1H); 8.81 (t, 1H); 9.07 (s, 1H); 9.24 (m, 1H).

Example 26: *N*-(2-Amino-2-oxoethyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide utilising glycinamide hydrochloride (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.29 (s, 3H); 3.78 (d, 2H); 7.02 (s, 1H); 7.30-7.36 (m, 2H); 7.44 (d, 1H); 7.53 (ddd, 1H); 7.61 (dd, 1H); 8.11 (m, 1H); 8.41 (m, 1H); 8.50 (d, 1H); 8.57 (t, 1H); 8.67 (dd, 1H); 9.08 (s, 1H); 9.24 (m, 1H).

Example 27: *N*-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]glycine methyl ester utilising glycine methylester hydrochloride (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.29 (s, 3H); 3.63 (s, 3H); 3.98 (d, 2H); 7.34 (d,1H); 7.44 (d, 1H); 7.52 (ddd, 1H); 7.59 (dd, 1H); 8.11 (d, 1H); 8.41 (m, 1H); 8.50 (d, 1H); 8.67 (dd, 1H); 8.87 (t, 1H); 9.09 (s, 1H); 9.23 (m, 1H).

Example 28: *N*-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]beta-alanine methyl ester utilising beta-alanine methylester hydrochloride (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.27 (s, 3H); 2.57 (t, 2H); 3.46 (m, 2H); 3.57 (s, 3H); 7.31 (d, 1H); 7.43 (d, 1H); 7.50-7.55 (m, 2H); 8.07 (d, 1H); 8.40 (m, 1H); 8.47 (t, 1H); 8.50 (d, 1H); 8.67 (dd, 1H); 9.07 (s, 1H); 9.23 (m, 1H).

Example 29: *N*-[[4-(Aminosulfonyl)phenyl]methyl]-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]-amino]benzamide utilising p-(aminomethyl)benzenesulfonamide hydrochloride (Sigma, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.29 (s, 3H); 4.51 (d, 2H); 7.30 (s, 2H); 7.34 (d, 1H); 7.43-7.50 (m, 4H); 7.62 (dd, 1H); 7.75 (m, 2H); 8.16 (d, 1H); 8.40 (m, 1H); 8.51 (d, 1H); 8.68 (dd, 1H); 9.06 (t, 1H); 9.09 (s, 1H); 9.24 (m, 1H).

Example 30: *N*-(3-Hydroxypropyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide utilising 3-amino-1-propanol (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.65 (qui, 2H); 2.28 (s, 3H); 3.29 (m, 2H); 3.42 (m, 2H); 4.50 (m, 1H); 7.30 (d, 1H); 7.43 (d, 1H); 7.51 (ddd, 1H); 7.56 (dd, 1H); 8.09 (d, 1H); 8.36-8.43 (m, 2H); 8.50 (d, 1H); 8.67 (dd, 1H); 9.07 (s, 1H); 9.23 (m, 1H).

Example 31: *N,N*-Diethyl-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide utilising diethylamine (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.04 (m, 6H); 2.28 (s, 3H); 3.31 (m, 4H); 7.02 (dd, 1H); 7.27 (d, 1H); 7.44 (d, 1H); 7.51 (ddd, 1H); 7.61 (m, 1H); 8.39 (m, 1H); 8.51 (d, 1H); 8.68 (dd, 1H); 9.01 (s, 1H); 9.23 (m, 1H).

Example 32: *N*-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]-(L)-phenylalanine 1,1-dimethylethyl ester utilising L-phenylalanine t-butylester hydrochloride (Novabiochem (Juro), Lucerne, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.32 (s, 9H); 2.28 (s, 3H); 3.07 (m, 2H); 4.53 (m, 1H); 7.13-7.29 (m, 5H); 7.32 (d, 1H); 7.44 (d, 1H); 7.50 (ddd, 1H); 7.55 (dd, 1H); 8.05 (m, 1H); 8.39 (m, 1H); 8.49 (d, 1H); 8.63 (d, 1H); 8.67 (dd, 1H); 9.08 (s, 1H); 9.23 (m, 1H).

Example 33: *N*-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]-(D)-alanine 1,1-dimethylethyl ester utilising D-alanine t-butylester hydrochloride (Novabiochem (Juro), Lucerne, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.34 (d, 3H); 1.38 (s, 9H); 2.28 (s, 3H); 4.32 (m, 1H); 7.33 (d, 1H); 7.43 (d, 1H); 7.51 (ddd, 1H); 7.61 (dd, 1H); 8.14 (m, 1H); 8.40 (m, 1H); 8.50 (m, 1H); 8.58 (d, 1H); 8.67 (dd, 1H); 9.08 (s, 1H); 9.23 (m, 1H).

Example 34: *N*-[1-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]-4-piperidinyl]-benzamide utilising N-4-piperidinyl-benzamide (Maybridge Chemical Co. Ltd). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.49 (m, 2H); 1.68-1.94 (m, 2H); 2.30 (s, 3H); 2.92 (m, 1H); 3.16 (m, 1H); 3.79 (m, 1H); 4.05 (m, 1H); 4.42 (m, 1H); 7.08 (dd, 1H); 7.31 (d, 1H); 7.41-7.54 (m, 5H); 7.63

(m, 1H); 7.79-7.84 (m, 2H); 8.28 (d, 1H); 8.40 (m, 1H); 8.51 (d, 1H); 8.66 (dd, 1H); 9.06 (s, 1H); 9.24 (m, 1H).

Example 35: 4-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]-morpholine utilising morpholine (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.29 (s, 3H); 3.47 (m, 8H); 7.10 (dd, 1H); 7.30 (d, 1H); 7.44 (m, 1H); 7.52 (ddd, 1H); 7.65 (m, 1H); 8.40 (m, 1H); 8.51 (d, 1H); 8.69 (dd, 1H); 9.05 (s, 1H); 9.23 (m, 1H).

Example 36: 1-(4-Methoxyphenyl)-4-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzoyl]piperazine utilising 1-(4-methoxyphenyl)-piperazine (Emka Chemie, Neufahrn, Germany). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.30 (s, 3H); 2.87-3.08 (m, 4H); 3.50-3.75 (m, 4H); 3.67 (s, 3H); 6.78-6.88 (m, 4H); 7.12 (dd, 1H); 7.31 (d, 1H); 7.44 (m, 1H); 7.51 (ddd, 1H); 7.67 (m, 1H); 8.38 (m, 1H); 8.52 (m, 1H); 8.67 (dd, 1H); 9.06 (s, 1H); 9.23 (m, 1H).

Example 37: 1-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]-4-(4-pyridinyl)-piperazine utilising 1-(4-pyridyl)-piperazine (Emka Chemie, Neufahrn, Germany). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.31 (s, 3H); 3.30 (m, 4H); 3.59 (m, 4H); 6.77 (m, 2H); 7.14 (dd, 1H); 7.32 (d, 1H); 7.45 (d, 1H); 7.52 (ddd, 1H); 7.70 (m, 1H); 8.16 (m, 2H); 8.41 (m, 1H); 8.53 (d, 1H); 8.67 (dd, 1H); 9.07 (s, 1H); 9.24 (m, 1H).

Example 38: 1-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]-4-(pyrazinyl)-piperazine utilising 1-(2-pyrazinyl)-piperazine (Emka Chemie, Neufahrn, Germany). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.31 (s, 3H); 3.57 (m, 8H); 7.14 (dd, 1H); 7.32 (d, 1H); 7.45 (d, 1H); 7.51 (ddd, 1H); 7.72 (m, 1H); 7.85 (d, 1H); 8.08 (d, 1H); 8.29 (d, 1H); 8.40 (m, 1H); 8.53 (d, 1H); 8.65 (dd, 1H); 9.06 (s, 1H); 9.24 (m, 1H).

Example 39: 1-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]-4-(phenylmethyl)-piperazine utilising 1-benzyl-piperazine (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.21-2.42 (m, 4H); 2.28 (s, 3H); 3.34-3.63 (m, 6H); 7.07 (dd, 1H); 7.21-7.34 (m, 6H); 7.43-7.50 (m, 2H); 7.63 (m, 1H); 8.38 (m, 1H); 8.50 (d, 1H); 8.65 (dd, 1H); 9.03 (s, 1H); 9.22 (m, 1H).

Example 40: 1-Cyclopentyl-4-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]-piperazine utilising 1-cyclopentyl-piperazine (Emka Chemie, Neufahrn, Germany). ^{1}H-NMR

(400 MHz, DMSO-d_6, δ): 1.20-1.31 (m, 2H); 1.39-1.62 (m, 4H); 1.65-1.75 (m, 2H); 2.18-2.47 (m, 8H); 3.27-3.62 (m, 4H); 7.08 (dd, 1H); 7.29 (d, 1H); 7.44 (d, 1H); 7.51 (ddd, 1H); 7.62 (m, 1H); 8.38 (m, 1H); 8.51 (d, 1H); 8.68 (dd, 1H); 9.04 (s, 1H); 9.22 (m, 1H).

Example 41: 4-{{4-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]-1-piperazinyl}-acetyl}morpholine utilising 4-[2-(piperazin-1-yl)-acetyl]-morpholine (Emka Chemie, Neufahrn, Germany). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.29 (s, 3H); 2.31-2.49 (m, 4H); 3.16 (s, 2H); 3.37-3.60 (m, 12H); 7.07 (dd, 1H); 7.29 (d,1H); 7.45 (d, 1H); 7.52 (ddd, 1H); 7.65 (m, 1H); 8.39 (m, 1H); 8.51 (d, 1H); 8.68 (dd, 1H); 9.04 (s, 1H); 9.23 (m, 1H).

Example 42: 1-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]-4-[2-oxo-2-(1-pyrrolidinyl)ethyl]piperazine utilising 1-[2-(piperazin-1-yl)-acetyl-pyrrolidine (Emka Chemie, Neufahrn, Germany). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.73 (m, 2H); 1.83 (m, 2H); 2.29 (s, 3H); 2.43 (m, 4H); 3.09 (s, 2H); 3.25 (m, 2H); 3.34-3.63 (m, 6H); 7.07 (dd, 1H); 7.29 (d, 1H); 7.45 (d, 1H); 7.52 (ddd, 1H); 7.64 (m, 1H); 8.39 (m, 1H); 8.51 (d, 1H); 8.68 (dd, 1H); 9.04 (s, 1H); 9.22 (m, 1H).

Example 43: 4-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]-1-piperazine-carboxylic acid ethyl ester utilising ethyl 1-piperazinecarboxylate (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.16 (t, 3H); 2.29 (s, 3H); 3.19-3.63 (m, 8H); 4.02 (q, 2H); 7.10 (dd, 1H); 7.30 (d, 1H); 7.45 (d, 1H); 7.52 (ddd, 1H); 7.66 (m, 1H); 8.40 (m, 1H); 8.51 (d, 1H); 8.68 (dd, 1H); 9.06 (s, 1H); 9.23 (m, 1H).

Example 44: 2-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]-1,2,3,4-tetrahydro-isoquinoline utilising 1,2,3,4-tetrahydroisoquinoline (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.31 (s, 3H); 2.79 (m, 2H); 3.57-3.90 (m, 2H); 4.58-4.79 (m, 2H); 7.08-7.23 (m, 5H); 7.32 (d, 1H); 7.42-7.50 (m, 2H); 7.70 (m, 1H); 8.39 (m, 1H); 8.51 (d, 1H); 8.67 (dd, 1H); 9.05 (s, 1H); 9.24 (m, 1H).

Example 45: *N,N*-bis(2-Methoxyethyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide utilising bis(2-methoxyethyl)amine (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.28 (s, 3H); 3.09 (br.s, 3H); 3.23 (br.s, 3H); 3.47 (m, 8H); 7.04 (dd, 1H); 7.27 (d, 1H); 7.44 (d, 1H); 7.51 (ddd, 1H); 7.62 (m, 1H); 8.39 (m, 1H); 8.51 (d, 1H); 8.68 (dd, 1H); 9.01 (s, 1H); 9.23 (m, 1H).

Example 46: 1'-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]-1,4'-bipiperidine utilising 4-piperidinopiperidine (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.21-1.50 (m, 8H); 1.51-1.83 (m, 2H); 2.29 (s, 3H); 2.39 (m, 4H); 2.68 (m, 1H); 2.95 (m, 1H); 3.71 (m, 1H); 4.42 (m, 1H); 7.07 (dd, 1H); 7.28 (d, 1H); 7.45 (d, 1H); 7.52 (ddd, 1H); 7.63 (m, 1H); 8.40 (m, 1H); 8.51 (d, 1H); 8.67 (dd, 1H); 9.03 (s, 1H); 9.23 (m, 1H).

Example 47: *N*-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]-N-(phenylmethyl)-glycine ethyl ester utilising N-benzylglycine ethyl ester (Fluka, Buchs, Switzerland). ^{1}H-NMR (300 MHz, DMSO-d_6, δ): 0.97-1.20 (m, 3H); 2.27 (s, 3H); 3.90-4.12 (m, 4H); 4.58-4.68 (m, 2H); 7.07 (m, 1H); 7.15-7.34 (m, 6H); 7.38-7.53 (m, 2H); 7.65-7.74 (m, 1H); 8.35-8.51 (m, 2H); 8.66 (dd, 1H); 8.96-9.04 (m, 1H); 9.22 (m, 1H).

Example 48: *N*-(3-Chlorophenyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide utilising 3-chlor-aniline (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.33 (s, 3H); 7.14 (m, 1H); 7.36 (m, 1H); 7.41 (d, 1H); 7.46 (d, 1H); 7.49 (ddd, 1H); 7.68-7.73 (m, 2H); 7.95 (m, 1H); 8.25 (m, 1H); 8.43 (m, 1H); 8.53 (d, 1H); 8.66 (dd, 1H); 9.15 (s, 1H); 9.26 (m, 1H); 10.33 (s, 1H).

Example 49: *N*-(2,2-Diphenylethyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide utilising 2,2-diphenylethylamine (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.24 (s, 3H); 3.87 (m, 2H); 4.41 (m, 1H); 7.12-7.17 (m, 2H); 7.23-7.31 (m, 9H); 7.41-7.44 (m, 2H); 7.51 (ddd, 1H); 7.97 (m, 1H); 8.37-8.44 (m, 2H); 8.48 (d, 1H); 8.68 (dd, 1H); 9.05 (s, 1H); 9.23 (m, 1H).

Example 50: *N*-(2,3-Dihydro-1H-inden-1-yl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide utilising 1-Aminoindane (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.90-2.01 (m, 1H); 2.29 (s, 3H); 2.43 (m, 1H); 2.77-2.86 (m, 1H); 2.91-2.98 (m, 1H); 5,56 (m, 1H); 7.08-7.25 (m, 4H); 7.31 (d, 1H); 7.43 (d, 1H); 7.50 (ddd, 1H); 7.64 (dd, 1H); 8.20 (m, 1H); 8.40 (m, 1H); 8.50 (d, 1H); 8.68-8.72 (m, 2H); 9.08 (s, 1H); 9.24 (m, 1H).

Example 51: *N*-(Diphenylmethyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide utilising alpha-aminodiphenylmethane (Fluka, Buchs, Switzerland) ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.29 (s, 3H); 6.41 (d, 1H); 7.20-7.36 (m, 11H); 7.43 (d, 1H); 7.46 (ddd, 1H);

7.67 (dd, 1H); 8.18 (m, 1H); 8.38 (m, 1H); 8.50 (d, 1H); 8.68 (dd, 1H); 9.10 (s, 1H); 9.20 (d, 1H); 9.24 (m, 1H).

Example 52: 4-Methyl-*N*-[2-(1-piperidinyl)ethyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide utilising 1-(2-aminoethyl)piperidine (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.30-1.38 (m, 2H); 1.41-1.48 (m, 4H); 2.28 (s, 3H); 2.31-2.41 (m, 6H); 3.33 (m, 2H); 7.31 (d, 1H); 7.44 (d, 1H); 7.51 (ddd, 1H); 7.55 (dd, 1H); 8.08 (m, 1H); 8.28 (t, 1H); 8.40 (m, 1H); 8.51 (d, 1H); 8.67 (dd, 1H); 9.07 (s, 1H); 9.24 (m, 1H).

Example 53: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-N-(5,6,7,8-tetrahydro-1-naphthalenyl)benzamide utilising 5,6,7,8-tetrahydro-1-naphthylamine (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.63-1.71 (m, 4H); 2.32 (s, 3H); 2.60 (m, 2H); 2.74 (m, 2H); 6.96 dd, 1H); 7.07-7.14 (m, 2H); 7.37 (d, 1H); 7.45 (d, 1H); 7.49 (ddd, 1H); 7.69 (dd, 1H); 8.25 (m, 1H); 8.41 (m, 1H); 8.52 (d, 1H); 8.67 (dd, 1H); 9.12 (s, 1H); 9.25 (m, 1H); 9.65 (br.s).

Example 54: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-N-[[4-(trifluoromethyl)phenyl]-methyl]benzamide utilising 4-(trifluoromethyl)benzylamine (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.30 (s, 3H); 4.53 (d, 2H); 7.34 (d,1H); 7.44 (d, 1H); 7.46-7.53 (m, 3H); 7.62 (dd, 1H); 7.66 (m, 2H); 8.16 (m, 1H); 8.40 (m, 1H); 8.51 (d, 1H); 8.67 (dd, 1H); 9.08 (t, 1H); 9.10 (s, 1H); 9.24 (m, 1H).

Example 55: 4-Methyl-*N*-[(5-methylpyrazinyl)methyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide utilising 2-(aminomethyl)-5-methylpyrazine (TCI-JP, Distrib. Zürich, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.29 (s, 3H); 2.45 (s, 3H); 4.54 (d, 2H); 7.33 (d, 1H); 7.44 (d, 1H); 7.49 (ddd, 1H); 7.62 (dd, 1H); 8.14 (m, 1H); 8.40 (m, 1H); 8.45 (m, 2H); 8.50 (d, 1H); 8.66 (dd, 1H); 9.07 (t, 1H); 9.09 (s, 1H); 9.23 (m, 1H).

Example 56: *N*-(2-Ethoxyethyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide utilising 2-ethoxyethylamine (TCI-JP, Distrib. Zurich, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ):1.07 (t, 3H); 2.28 (s, 3H); 3.30-3.49 (m, 6H); 7.31 (d, 1H); 7.43 (d, 1H); 7.51 (ddd, 1H); 7.57 (dd, 1H); 8.09 (m, 1H); 8.38-8.45 (m, 2H); 8.50 (d, 1H); 8.67 (dd, 1H); 9.07 (s, 1H); 9.24 (m, 1H).

Example 57: 4-Methyl-*N*-[2-(2-oxo-1-imidazolidinyl)ethyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]-amino]benzamide utilising 1-(2-aminoethyl)imidazolidin-2-one [Chem. Abstr. Reg. Number: 6281-42-1]. ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.27 (s, 3H); 3.13-3.22 (m, 4H); 3.30-3.40 (m, 4H); 6.27 (br.s, 1H); 7.30 (d, 1H); 7.43 (d, 1H); 7.49-7.56 (m, 2H); 8.08 (d, 1H); 8.40 (m, 1H); 8.45 (t, 1H); 8.50 (d, 1H); 8.67 (dd, 1H); 9.06 (s, 1H); 9.23 (m, 1H).

Example 58: 4-Methyl-*N*-(5-methyl-2-pyridinyl)-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide utilising 2-amino-5-picoline (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.26 (s, 3H); 2.32 (s, 3H); 7.35 (d, 1H); 7.45 (d, 1H); 7.49 (ddd, 1H); 7.64 (dd, 1H); 7.77 (dd, 1H); 8.07 (d, 1H); 8.18 (m, 1H); 8.31 (d, 1H); 8.43 (m, 1H); 8.52 (d, 1H); 8.66 (dd, 1H); 9.08 (s, 1H); 9.25 (m, 1H); 10.58 (s, 1H).

Example 59: 1-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]-4-phenyl-4-piperidinol utilising 4-hydroxy-4-phenylpiperidine (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.45-1.73 (m, 2H); 1.88 (m, 2H); 2.28 (s, 3H); 3.15 (m, 1H); 3.47 (m, 1H); 3.64 (m, 1H); 4.39 (m, 1H); 5.14 (s, 1H); 7.14 (dd, 1H); 7.19 (m, 1H); 7.26-7.31 (m, 3H); 7.43 (d, 1H); 7.45-7.51 (m, 3H); 7.69 (d, 1H); 8.40 (m, 1H); 8.48 (d, 1H); 8.67 (dd, 1H); 9.03 (s, 1H); 9.24 (m, 1H).

Example 60: *N*-(3-Benzoylphenyl)-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzamide utilising 3-aminobenzophenone (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.32 (s, 3H); 7.39 (d, 1H); 7.43-7.58 (m, 6H); 7.67 (m, 1H); 7.70-7.77 (m, 3H); 8.13 (m, 1H); 8.20 (m, 1H); 8.27 (m, 1H); 8.42 (m, 1H); 8.52 (d, 1H); 8.66 (dd, 1H); 9.14 (s, 1H); 9.25 (m, 1H); 10.41 (s, 1H).

Example 61: *N*-[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]-glycine 1,1-dimethylethyl ester utilising glycine t-butyl ester hydrochloride (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.40 (s, 9H); 2.29 (s, 3H); 3.86 (d, 2H); 7.33 (d, 1H); 7.43 (d, 1H); 7.51 (ddd, 1H); 7.58 (dd, 1H); 8.10 (d, 1H); 8.40 (m, 1H); 8.50 (d, 1H); 8.67 (dd, 1H); 8.75 (t, 1H); 9.08 (s, 1H); 9.23 (m, 1H).

Example 62: 4-[[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]amino]benzene-acetic acid ethyl ester utilising ethyl 4-aminophenylacetate (Maybridge Chemical Co. Ltd.). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.16 (t, 3H); 2.32 (s, 3H); 3.60 (s, 2H); 4.06 (q, 2H); 7.21

(m, 2H); 7.38 (d, 1H); 7.45 (d, 1H); 7.48 (ddd, 1H); 7.70 (m, 3H); 8.23 (m, 1H); 8.41 (m, 1H); 8.52 (d, 1H); 8.66 (dd, 1H); 9.13 (s, 1H); 9.25 (m, 1H); 10.16 (s, 1H).

Example 63: 4-Methyl-*N*-[3-(methylphenylamino)propyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]-amino]benzamide utilising N-(3-aminopropyl)-N-methylaniline (TCI-JP, Distrib. Zürich, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.73 (qui, 2H); 2.28 (s, 3H); 2.84 (s, 3H); 3.24-3.37 (m, 4H); 6.55 (m, 1H); 6.65 (m, 2H); 7.10 (m, 2H); 7.31 (d, 1H); 7.43 (d, 1H); 7.47 (ddd, 1H); 7.55 (dd, 1H); 8.10 (d, 1H); 8.37-8.44 (m, 2H); 8.50 (d, 1H); 8.65 (dd, 1H); 9.06 (s, 1H); 9.23 (m, 1H).

Example 64: 1-[[3-[[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]amino]phenyl]-methyl]-4-piperidinecarboxylic acid ethyl ester utilising ethyl 1-(3-aminobenzyl)piperidine-4-carboxylate (Maybridge Chemical Co. Ltd.). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.14 (t, 3H); 1.49-1.61 (m, 2H); 1.72-1.80 (m, 2H); 1.92-2.02 (m, 2H); 2.27 (m, 1H); 2.32 (s, 3H); 2.74 (m, 2H); 3.40 (s, 2H); 4.03 (q, 2H); 6.98 (d, 1H); 7.25 (m, 1H); 7.38 (d, 1H); 7.43-7.51 (m, 2H); 7.66-7.73 (m, 3H); 8.25 (s, 1H); 8.42 (m, 1H); 8.52 (d, 1H); 8.65 (dd, 1H); 9.12 (s, 1H); 9.25 (m, 1H); 10.14 (s, 1H).

Example 65: [[4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoyl]amino]propanedioic acid diethyl ester utilising diethyl aminomalonate hydrochloride (Aldrich, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.19 (t, 6H); 2.30 (s, 3H); 4.10-4.22 (m, 4H); 5.27 (d, 1H); 7.35 (d, 1H); 7.44 (d, 1H); 7.51 (ddd, 1H); 7.63 (dd, 1H); 8.15 (m, 1H); 8.40 (m, 1H); 8.50 (d, 1H); 8.67 (dd, 1H); 9.11 (s, 1H); 9.21-9.25 (m, 2H).

Example 66: *N*-[2-[bis(1-Methylethyl)amino]ethyl]-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]-amino]benzamide utilising 2-diisopropylamino-ethylamine (Fluka, Buchs, Switzerland). ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 0.95 (m, 12H); 2.28 (s, 3H); 2.49 (m, 2H); 2.94 (m, 2H); 3.17 (m, 2H); 7.30 (d, 1H); 7.43 (d, 1H); 7.50 (ddd, 1H); 7.54 (dd, 1H); 8.09 (br.s, 1H); 8.27 (m, 1H); 8.40 (m, 1H); 8.50 (d, 1H); 8.67 (dd, 1H); 9.06 (s, 1H); 9.23 (m, 1H).

Example 67: *N*-[3-(Diethylamino)phenyl]-4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide

A solution containing ~50% of propylphosphonic anhydride in *N,N*-dimethylformamide (Fluka, Buchs, Switzerland; 674 µL, ~1.05 mmol) is added within 20 minutes to a stirred mixture of 4-

methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzoic acid (214.4 mg, 0.7 mmol), *N,N*-diethyl-1,3-benzenediamine (115 mg, 0.7 mmol) and triethylamine (776 µL, 5.6 mmol) in 2 mL *N,N*-dimethylformamide. After stirring for 24 hours at room temperature, the mixture is treated with a half-saturated aqueous solution of sodium hydrogen carbonate and extracted three times with ethyl acetate. The solvent is evaporated off under reduced pressure and the residue dried *in vacuo*. The crude product is purified by chromatography on silica gel, eluent 2% methanol in dichloromethane and crystallised from acetone to give the title compound as a crystalline solid. ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 1.07 (t, 6H); 2.31 (s, 3H); 3.29 (m, 4H); 6.38 (m, 1H); 7.06 (m, 2H); 7.11 (m, 1H); 7.36 (d, 1H); 7.43-7.50 (m, 2H); 7.67 (m, 1H); 8.21 (m, 1H); 8.43 (m, 1H); 8.51 (d, 1H); 8.66 (dd, 1H); 9.12 (s, 1H); 9.24 (m, 1H); 9.90 (s, 1H).

Example 68: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[[3-[(1-hydroxy-1-methyl-ethyl)]-5-(1,1,1-trifluoromethyl)phenyl]methyl]benzamide

Diethylcyanophosphonate (Aldrich, Buchs, Switzerland; 0.33 mL, 2.0 mmol) is added to a stirred mixture of 4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzoic acid (306 mg, 1.0 mmol), 3-[(1-hydroxy-1-methylethyl)]-5-(1,1,1-trifluoromethyl)benzeneamine (220 mg, 1.0 mmol) and triethylamine (560 µL, 4.0 mmol) in 5 mL *N,N*-dimethylformamide at 10°C. After stirring for 3 hours at 60°C, the mixture is treated with saturated aqueous solution of sodium hydrogen carbonate and extracted three times with ethyl acetate. The combined extracts are dried ($MgSO_4$), filtered and the solvent is evaporated off under reduced pressure to afford a crude product which is recrystallised from ethylacetate to give the title compound as a crystalline solid, m.p. 253-258°C.

Example 69: 3-[[4-(3-Pyridinyl)-2-pyrimidinyl]amino]-*N*-[(4-methyl-1-piperazinyl)methyl]-benzamide

Diethylcyanophosphonate (Aldrich, Buchs, Switzerland; 0.50 mL, 3.0 mmol) is added to a stirred mixture of 3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzoic acid (438 mg, 1.5 mmol), 4-[(4-methyl-1-piperazinyl)methyl]benzeneamine (308 mg, 1.5 mmol) and triethylamine (840 µL, 3.0 mmol) in 10 mL *N,N*-dimethylformamide at 10°C. After stirring for 12 hours at 60°C, the mixture is treated with an aqueous solution of sodium hydrogen carbonate and extracted three times with ethyl acetate. The combined extracts are washed with water, and the solvent is evaporated off under reduced pressure to give a residue. The residue is resuspended in water and filtered to afford the crude product which is recrystallised from tetrahydrofuran-

ethyl acetate to give *N*-[3-[[4-(3-Pyridinyl)-2-pyrimidinyl]amino]-*N*-[(4-methyl-1-piperazinyl)-methyl]benzamide as a crystalline solid, m.p. 220-224°C.

Example 69a: 3-[(Aminoiminomethyl)amino]-4-methylbenzoic acid methyl ester mononitrate
Utilising the procedure described in Example 1a, but with 3-aminobenzoic acid methyl ester (Fluka, Buchs, Switzerland) in lieu of 3-amino-4-methylbenzoic acid ethyl ester, afforded the title compound as a crystalline solid, m.p. 170-172°C.

Example 69b: 3-[[4-(3-Pyridinyl)-2-pyrimidinyl]amino]benzoic acid methyl ester
Utilising the procedure described in Example 1b, but with the intermediate of Example 69a in lieu of 4-methyl-3-[(aminoiminomethyl)amino]-4-methylbenzoic acid ethyl ester mononitrate, afforded the title compound as a crystalline solid, m.p. 195-200°C.

Example 69c: 3-[[4-(3-Pyridinyl)-2-pyrimidinyl]amino]benzoic acid
Utilising the procedure described in Example 1c, but with the intermediate of Example 69b in lieu of 4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]benzoic acid ethyl ester, afforded the title compound as a crystalline solid, m.p. 285-293°C.

Example 70: 3-[[4-(3-Pyridinyl)-2-pyrimidinyl]amino]-*N*-[(3-(1-hydroxy-1-methylethyl)-5-(1,1,1-trifluoromethyl)phenyl]benzamide
Utilising the procedure described in Example 69, but with 3-(1-hydroxy-1-methylethyl)-5-(1,1,1-trifluoromethyl)benzeneamine in lieu of 4-[(4-methyl-1-piperazinyl)methyl]benzene-amine, afforded 3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[(3-(1-hydroxy-1-methylethyl)-5-(1,1,1-trifluoromethyl)phenyl]benzamide as a crystalline solid, m.p. 213-215°C.

Example 71: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[3-[3-(1H-imidazol-1-yl)propoxy]-phenyl]benzamide
Utilising the procedure described in Example 3, but employing 3-[3-(1H-imidazol-1-yl)propoxy]-benzenamine (Takao Nishi et al., JP 10182459) in lieu of 1-(2-pyridyl)piperazine, afforded the title compound as a solid. ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.12-2.21 (m, 2H); 2.33 (s, 3H); 3.87 (t, 2H); 4.13 (t, 2H); 6.66 (dd, 1H); 6.87 (s, 1H); 7.15-7.26 (m, 2H); 7.32-7.42 (m, 2H); 7.44-7.52 (m, 3H); 7.61 (s, 1H); 7.70 (d, 1H); 8.24 (s, 1H); 8.43 (d, 1H); 8.53 (d, 1H); 8.67 (d, 1H); 9.13 (s, 1H); 9.26 (br. s, 1H); 10.13 (s, 1H).

Example 72: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[3-[2-(1H-imidazol-1-yl)ethoxy]phenyl]benzamide

Utilising the procedure described in Example 3, but employing 3-[2-(1H-imidazol-1-yl)ethoxy]-benzenamine (Rolf Paul et al., Journal of Medicinal Chemistry (1993), 36(19), 2716-25) in lieu of 1-(2-pyridyl)piperazine, afforded the title compound as a crystalline solid. ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 2.34 (s, 3H); 4.22 (t, 2H); 4.37 (t, 2H); 6.68 (dd, 1H); 6.90 (s, 1H); 7.21-7.27 (m, 2H); 7.36-7.43 (m, 2H); 7.46-7.53 (m, 3H); 7.67-7.74 (m, 2H); 8.25 (br. s, 1H); 8.44 (dt, 1H); 8.54 (d, 1H); 8.68 (dd, 1H); 9.15 (s, 1H); 9.27 (br. d, 1H); 10.15 (s, 1H).

Example 73: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(ethylamino)-3-(trifluoromethyl)phenyl]benzamide

Utilising the procedure described in Example 69, but employing N-ethyl-2-(trifluoromethyl)-1,4-benzenediamine in lieu of 3-[(1-hydroxy-1-methylethyl)]-5-(1,1,1-trifluoromethyl)-benzeneamine, afforded the title compound as a crystalline solid, m.p. 178–180°C.

The aniline is prepared as follows:

Example 73a: N-ethyl-2-(trifluoromethyl)-1,4-benzenediamine

A mixture of 2-bromo-5-nitrobenzotrifuoride (Lancaster Synthesis, GmbH; 5.4 g, 20 mmol) and a solution of ethylamine in ethanol (50 mL of 2M, 100 mmol) is heated at 80°C for 18 hours in a steel pressure vessel. The mixture is then cooled and the solvent is evaopated off under reduced pressure to yield the crude product which is purified by column chromatography (silica gel, eluent 20% ethyl acetate in hexane) to afford N-ethyl-4-nitro-6-(trifluoromethyl)-benzenamine as yellow oil. This product is dissolved in ethanol (180 mL) and hydrogenated at atmospheric pressure over Raney nickel (0.5 g) at 45°C. The calculated amount of hydrogen is taken up in 50 hours. The mixture is then filtered and the solvent is evaporated off under reduced pressure to yield the crude product which is purified by chromatography (silica gel; eluent 50% ethyl acetate in hexane) and recrystallised from ether – hexane to give the title compound as a beige crystalline solid. ^{1}H-NMR (400 MHz, DMSO-d_6): 1.11 (t, 3H), 3.05 (m, 2H), 4.18 (br t, 1H), 4.66 (br.s, 2H), 6.58 – 6.64 (m, 1H) and 6.68 – 6.75 (m, 2H).

Example 74: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(diethylamino)-3-(trifluoromethyl)phenyl]benzamide

Utilising the procedure described in Example 3, but employing N,N-diethyl-2-(trifluoromethyl)-1,4-benzenediamine (Toshio Niwa, DE 3524519) in lieu of 1-(2-pyridyl)piperazine, afforded the title compound as a crystalline solid, m.p. 128–131°C.

Example 75: (±)-4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-[(2-hydroxy-propyl)amino]-3-(trifluoromethyl)phenyl]benzamide

Utilising the procedure described in Example 3, but employing (±)-1-[[4-amino-2-(trifluoromethyl)phenyl]amino]-2-propanol (Tsutomu Mano, EP 299497) in lieu of 1-(2-pyridyl)piperazine, afforded the title compound as a crystalline solid, m.p. 184–186°C.

Example 76: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-[bis(2-methoxy-ethyl)amino]-3-(trifluoromethyl)phenyl]benzamide

Utilising the procedure described in Example 3, but employing *N,N*-bis(2-methoxyethyl)-2-(trifluoromethyl)-1,4-benzenediamine (Toshio Niwa, DE 3524519) in lieu of 1-(2-pyridyl)piperazine, afforded the title compound as a crystalline solid, m.p. 156–157°C.

Example 77: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(4-methyl-1-piperazinyl)-3-(trifluoromethyl)phenyl]benzamide

Utilising the procedure described in Example 3, but employing 4-(4-methyl-1-piperazinyl)-3-(trifluoromethyl)-benzenamine (Anthony David Baxter, WO 0119800) in lieu of 1-(2-pyridyl)piperazine, afforded the title compound as a crystalline solid, m.p. 214–217°C.

Example 78: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(1-piperidinyl)-3-(trifluoromethyl)phenyl]benzamide

Utilising the procedure described in Example 3, but employing 4-(1-piperidinyl)-3-(trifluoromethyl)-benzenamine (Leping Li, WO 0151456) in lieu of 1-(2-pyridyl)piperazine, afforded the title compound as a crystalline solid, m.p. 201–202°C.

Example 79: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(1-pyrrolidinyl)-3-(trifluoromethyl)phenyl]benzamide

Utilising the procedure described in Example 3, but employing 4-(1-pyrrolidinyl)-3-(trifluoromethyl)-benzenamine (Steven Lee Bender WO 0153274) in lieu of 1-(2-pyridyl)piperazine afforded the title compound as a crystalline solid, m.p. 129–130°C.

Example 80: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(4-morpholinyl)-3-(trifluoromethyl)phenyl]benzamide

Utilising the procedure described in Example 69, but employing 4-(4-morpholinyl)-3-(trifluoromethyl)-benzenamine (Steven Lee Bender WO 0153274) in lieu of 3-[(1-hydroxy-1-methylethyl)]-5-(1,1,1-trifluoromethyl)benzeneamine, afforded the title compound as a crystalline solid, m.p. 216–218°C.

Example 81: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-phenyl-3-(trifluoromethyl)phenyl]benzamide

Utilising the procedure described in Example 3, but employing 4-(phenyl)-3-(trifluoromethyl)-benzenamine in lieu of 1-(2-pyridyl)piperazine afforded the title compound as a crystalline solid, m.p. 172–174°C.

The aniline is prepared as follows:

Example 81a: 4-(Phenyl)-3-(trifluoromethyl)benzenamine

Phenyl boronic acid (Aldrich, Buchs, Switzerland; 2.7 g, 22 mmol), Palladium II acetate (0.225 g, 1 mmol), tri-o-tolylphosphine (0.608 g, 2 mmol) and aqueous potassium carbonate solution (50 mL of 1 M) is added to a stirred solution of 2-bromo-5-nitrobenzotrifuoride (Lancaster Synthesis, GmbH; 5.4 g, 20 mmol) in dimethylformamide (200 mL) and heated at 120°C under an argon atmosphere for 1 h. The mixture is then evaporated to dryness under reduced pressure and the residue is treated with water (100 mL) and extracted with ethyl acetate (3 x 80 mL). The combined extracts are washed (brine), dried (MgSO4), filltered and the solvent is evorated off under reduced pressure to afford 4'-nitro-2'-(trifluoromethyl)-[1,1'-Biphenyl]. The biphenyl is dissolved in ethanol (200 mL) and hydrogenated at atmospheric pressure over Raney nickel (2 g) at 22°C. The calculated amount of hydrogen is taken up in 11 hours. The mixture is then filtered and the solvent is evaporated off under reduced pressure to yield the crude product which is purified by chromatograghy (silica gel; eluent ethyl acetate) to give the title compound as a brown oil. ^{1}H-NMR (400 MHz, DMSO-d_6): 5.62 (br.s, 2H), 6.80 (dd, 1H), 6.96 (d, 1H), 6.99 (d, 1H), 7.19 – 7.23 (m, 2H), and 7.29 – 7.39 (m, 3H).

Example 82: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-N-[3-[4-(3-pyridinyl)-3-(trifluoromethyl)phenyl]methyl]benzamide

Utilising the procedure described in Example 69, but employing 4-(3-pyridinyl)-3-(trifluoromethyl)-benzenamine in lieu of 3-[(1-hydroxy-1-methylethyl)]-5-(1,1,1-trifluoromethyl)benzeneamine, afforded the title compound as a crystalline solid, m.p. 276–280°C.

The aniline is prepared as follows:

Example 82a: 4-(3-Pyridinyl)-3-(trifluoromethyl)benzenamine
A stirred solution of 2-bromo-5-nitrobenzotrifuoride (Lancaster Synthesis, GmbH; 3.37 g, 12.5 mmol) and 3-(tri-n-butylstannyl)pyridine (Maybridge Chemical Co. Ltd., England; 5.0 g, 13.6 mmol) in xylene (75 mL) was purged with argon for 10 minutes at 20°C. Tetrakis(triphenylphosphine)palladium (0) (1.4 g, 1.25 mmol) is then added and the resulting mixture is heated at 130°C for 24 hours under an argon atmosphere. The mixture is then cooled, treated with an aqueous solution of sodium hydroxide (150 mL of 0.1 M) and purged with air for 2 hours. The resulting mixture is then diluted with ethylacetate (200 mL) and filtered. The orgainic phase is then sequentially washed with water (2 x 80 mL) and saturated aqueous sodium chloride (1 x 80 mL), dried ($MgSO_4$), filtered and the solvent is evaporated off under reduced pressure to yield the crude product which is purified by column chromatography (silica gel, eluent 50% ethyl acetate in hexane) to afford 3-[(4-nitro-3-(trifluoromethyl)phenyl]pyridine. This product is dissolved in ethanol (200 mL) and hydrogenated at atmospheric pressure over Raney nickel (0.23 g) at 22°C. The calculated amount of hydrogen is taken up in 24 hours. The mixture is then filtered and the solvent is evaporated off under reduced pressure to yield the crude product which is purified by chromatography (silica gel; eluent 50% ethyl acetate in hexane) and recrystallised from ether – hexane to give the title compound as a colourless crystalline solid, m.p. 92–93°C.

Example 83: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(1H-imidazol-1-yl)-3-(trifluoromethyl)phenyl]benzamide
Utilising the procedure described in Example 3, but employing 4-(1H-imidazol-1-yl)-3-(trifluoromethyl)-benzenamine (Steven Lee Bender WO 0153274) in lieu of 1-(2-pyridyl)piperazine afforded the title compound as a crystalline solid, m.p. 226–229°C.

Example 84: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(2,4-dimethyl-1H-imidazol-1-yl)-3-(trifluoromethyl)phenyl]benzamide

Utilising the procedure described in Example 69, but employing 4-(2,4-dimethyl-1H-imidazol-1-yl)-3-(trifluoromethyl)-benzenamine in lieu of 3-[(1-hydroxy-1-methylethyl)]-5-(1,1,1-trifluoromethyl)benzeneamine, afforded the title compound as an amorphous solid.

The aniline is prepared as follows:

Example 84a: 4-(2,4-dimethyl-1H-imidazol-1-yl)-3-(trifluoromethyl)-benzenamine
A mixture of 2-bromo-5-nitrobenzotrifuoride (Lancaster Synthesis, GmbH; 6.0 g, 22 mmol) and 2,4-dimethylimidazole (10.6, 110 mmol) is heated at 120°C for 36 hours under an argon atmosphere. The mixture is then cooled and the residue is treated with water (150 mL) and extracted with ethyl acetate (3 x 80 mL). The combined extracts are washed (brine), dried ($MgSO_4$), filtered and the solvent is evaporated off under reduced pressure to yield the crude product which is purified by column chromatography (silica gel, eluent ethyl acetate) to afford 1-[4-nitro-2-(trifluoromethyl)phenyl]-1H-imidazole as yellow crystalline solid. This product is dissolved in ethanol (290 mL) and hydrogenated at atmospheric pressure over Raney nickel (1.15 g) at 25°C. The calculated amount of hydrogen is taken up in 14 hours. The mixture is then filtered and the solvent is evaporated off under reduced pressure to yield the crude product which is purified by recrystallisation from ether – hexane to give the title compound as a crystalline solid, m.p. 163–164°C.

Example 85: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(4-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)phenyl]benzamide
Utilising the procedure described in Example 69, but employing 4-(4-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)-benzenamine in lieu of 3-[(1-hydroxy-1-methylethyl)]-5-(1,1,1-trifluoromethyl)benzeneamine, afforded the title compound as a crystalline solid, m.p. 154–163°C.

The aniline is prepared as follows:

Example 85a: 4-(4-Methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)-benzenamine
Utilising the procedure described in Example 84a, but employing 4(5)-methyl-1H-imidazole in lieu of 2,4-dimethylimidazole, afforded the title compound as a beige crystalline solid, m.p. 141–143°C.

Example 86: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[4-(2-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)phenyl]benzamide

Utilising the procedure described in Example 69, but employing 4-(2-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)-benzenamine in lieu of 3-[(1-hydroxy-1-methylethyl)]-5-(1,1,1-trifluoromethyl)benzeneamine, afforded the title compound as a crystalline solid, m.p. 154–163°C.

The aniline is prepared as follows:

Example 86a: 4-(2-Methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)-benzenamine

Utilising the procedure described in Example 84a, but employing 2-methyl-1H-imidazole in lieu of 2,4-dimethylimidazole, afforded the title compound as a colourless crystalline solid, m.p. 117–119°C.

Example 87: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[3-(4-morpholinyl)-5-[(methylamino)carbonyl]phenyl]benzamide

Utilising the procedure described in Example 69, but employing 3-amino-5-(4-morpholinyl)-N-(methyl)-benzamide in lieu of 3-[(1-hydroxy-1-methylethyl)]-5-(1,1,1-trifluoromethyl)-benzeneamine, afforded the title compound as a crystalline solid, m.p. 153–156°C.

The aniline is prepared as follows:

Example 87a: 3-Bromo-5-nitro-benzoic acid, 1,1-dimethylethyl ester

A solution of *n*-butyllithium in hexane (12.8 mL of 2.5 M, 32 mmol) is added with stirring to *t*-butanol (46 mL) at 25°C under an argon atmosphere. After 30 min the mixture is treated dropwise with a solution of 3-bromo-5-nitro-benzoyl chloride (J. Mindl, Collect. Czech. Chem. Commun. (1973), 38, 3496-505; 32 mmol) in dry THF (40 mL) and stirred for a further 17 h. The mixture is then treated with ether (250 mL) and washed with brine. The ether solution was dried ($MgSO_4$) and the solvent is evaporated off under reduced pressure to give the crude product which is purified by column chromatography (silica gel, eluent 20% ethyl acetate in hexane) and recrystallised from ether – hexane to afford the title compound as colourless crystalline solid, m.p. 77–78°C.

Example 87b: 3-(4-Morpholinyl)-5-nitro-benzoic acid, 1,1-dimethylethyl ester

A stirred mixture of 3-bromo-5-nitro-benzoic acid, 1,1-dimethylethyl ester (example 86a; 3.02 g, 10 mmol) and morpholine (1.22 mL, 14 mmol) in toluene (50 mL) is treated with sodium *t*-butylate (1.34 g, 14 mmol), tri-*t*-butylphosphine (3 mL, 1.5 mmol) and tris-(dibenzylidene-acetone)dipalladium[0] (0.45 g, 0.5 mmol) under an argon atmosphere, and then heated at 60°C for 18 h. The mixture is diluted with ethyl acetate (150 mL), filtered, washed with brine (2 x 50 mL), dried ($MgSO_4$) and the solvent is evaporated off under reduced pressure to give the crude product which is purified by column chromatography (silica gel, eluent 15% ethyl acetate in hexane) and recrystallised from ethyl acetate – hexane to afford the title compound as colourless crystalline solid, m.p. 116–118°C.

Example 87c: 3-(4-Morpholinyl)-5-nitro-benzoic acid, methyl ester
A mixture of 3-(4-morpholinyl)-5-nitro-benzoic acid, 1,1-dimethylethyl ester (Example 87b; 0.77 g, 2.5 mmol), 1,8-diazabicyclo[5,4,0]undec-7-ene (o.56 mL, 3.75 mL), and potassium bromide (1.09 g, 12.5 mmol) in methanol (25 mL) is stirred at 90 °C for 250 min. The cooled mixture is then added to hydrochloric acid (50 mL of 0.1 M) and extracted with ethyl acetate (3 x 100 mL). The combined extacts are washed with saturated aqueous sodium hydro-carbonate (2 x 25 mL), water (2 x 25 mL) and brine (2 x 50 mL), dried ($MgSO_4$) and the solvent is evaporated off under reduced pressure to give the crude product which is purified by recrystallisation from ethyl acetate – hexane to afford the title compound as yellow crystalline solid.

Example 87d: 3-(4-Morpholinyl)-5-nitro-N-(methyl)-benzamide
A stirred solution of 3-(4-morpholinyl)-5-nitro-benzoic acid, methyl ester (Example 86c; 0.53 g, 2 mmol) in toluene (5 mL) under an argon atmosphere, is treated with a mixture of methylamine hydrochloride (0.27 g, 4 mmol), trimethylaluminium (2 mL of a 2 M solution in toluene, 4 mmol) in toluene (5 mL) and heated at 60 °C for 18 h. The cooled mixture is then treated with hydrochloric acid (10 mL of 2 M), stirred for 5 min and then treated with aqueous sodium hydroxide (5 mL of 4 M). The mixture is then treated with water (100 mL) and extracted with ethyl acetate (3 x 100 mL). The combined extacts are washed with brine (2 x 50 mL), dried ($MgSO_4$) and the solvent is evaporated off under reduced pressure to give the crude product which is purified by recrystallisation from ethyl acetate to afford the title compound as yellow crystalline solid, m.p. 204–207°C.

Example 87e: 3-Amino-5-(4-Morpholinyl)-N-(methyl)-benzamide

A solution of 3-(4-morpholinyl)-5-nitro-N-(methyl)-benzamide (Example 86d; 300 mg, 1.12 mmol) in ethanol (20 mL) is hydrogenated at atmospheric pressure over Raney nickel (0.2 g) at 25°C. The calculated amount of hydrogen is taken up in 19 hours. The mixture is then filtered and the solvent is evaporated off under reduced pressure to yield the crude product which is purified by recrystallisation from ethyl acetate to give the title compound as a beige crystalline solid, m.p. 201–204°C.

Example 88: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[3-[(methylamino)carbonyl]-5-(trifluoromethyl)phenyl]benzamide

Utilising the procedure described in Example 69, but employing 3-amino-5-(trifluoromethyl)-N-(methyl)-benzamide in lieu of 3-[(1-hydroxy-1-methylethyl)]-5-(1,1,1-trifluoromethyl)-benzeneamine, afforded the title compound as a crystalline solid, m.p. 245–249°C.

Example 88a: 3-Amino-5-(trifluoromethyl)-N-(methyl)-benzamide

Utilising the procedure described in Example 86e, but employing α,α,α-trifluoro-N-methyl-5-nitro-m-toluamide (Dean E. Welch, J. Med. Chem. (1969), 12, 299-303) in lieu of 3-(4-morpholinyl)-5-nitro-N-(methyl)-benzamide, afforded the title compound as a beige crystalline solid, m.p. 113-115°C.

Example 89: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[5-(3-pyridinyl)-3-(trifluoromethyl)phenyl]benzamide

Utilising the procedure described in Example 69, but employing 5-(3-pyridinyl)-3-(trifluoro-methyl)-benzenamine in lieu of 3-[(1-hydroxy-1-methylethyl)]-5-(1,1,1-trifluoromethyl)-benzeneamine, afforded the title compound as a crystalline solid, m.p. 275–279°C.

The aniline is prepared as follows:

Example 89a: 5-(3-Pyridinyl)-3-(trifluoromethyl)benzenamine

A stirred solution of 3-amino-5-bromo-benzotrifuoride (Apollo, England; 1.12 g, 5 mmol) and 3-(tri-n-butylstannyl)pyridine (Maybridge Chemical Co. Ltd., England; 2.0 g, 5.4 mmol) in xylene (30 mL) was purged with argon for 10 minutes at 20°C. Tetrakis(triphenylphosphine)-palladium (0) (1.16 g, 1.0 mmol) is then added and the resulting mixture is heated at 140°C for 36 hours under an argon atmosphere. The mixture is then cooled, treated with an aqueous solution of sodium hydroxide (100 mL of 0.1 M) and purged with air for 2 hours. The

resulting mixture is then diluted with ethylacetate (200 mL) and filtered. The orgainic phase is then sequentially washed with water (2 x 80 mL) and saturated aqueous sodium chloride (1 x 80 mL), dried ($MgSO_4$), filtered and the solvent is evaporated off under reduced pressure to yield the crude product which is purified by column chromatography (silica gel, eluent ethyl acetate) to afford the title compound as a brown oil. ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 5.73 (br s, 2H), 6.83 (dd, 1H), 6.99 (d, 1H), 7.04 (d, 1H), 7.39 (dd, 1H), 7.64 (d, 1H), 8.42 (m, 1H) and 8.53 (dd, 1H).

Example 90: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[5-(4-morpholinyl)-3-(trifluoromethyl)phenyl]benzamide

Utilising the procedure described in Example 69, but employing 5-(4-morpholinyl)-3-(trifluoromethyl)-benzenamine in lieu of 3-[(1-hydroxy-1-methylethyl)]-5-(1,1,1-trifluoro-methyl)benzeneamine, afforded the title compound as a crystalline solid, m.p. 208–211°C.

The aniline is prepared as follows:

Example 90a: [3-Bromo-5-(trifluoromethyl)phenyl]-carbamic acid, 1,1-dimethylethyl ester

A mixture of 3-amino-5-bromo-benzotrifuoride (Apollo, England; 12 g, 50 mmol), di-*t*-butyl-dicarbonate (12 g, 55 mmol) and 4-dimethylaminopyridine (0.61 g, 5 mmol) in acetonitrile (100 mL) is stirred at 60°C for 8 h. The solvent is then evaporated off under reduced pressure to yield the crude product which is purified by column chromatography (silica gel, eluent 10% ethyl acetate in hexane) and recrystallised from hexane to afford the title compound as a colourless crystalline solid, m.p. 113-115°C.

Example 90b: [3-(4-Morpholinyl)-5-(trifluoromethyl)phenyl]-carbamic acid, 1,1-dimethylethyl ester

Utilising the procedure described in Example 86b but employing [3-bromo-5-(trifluoromethyl)-phenyl]-carbamic acid, 1,1-dimethylethyl ester (Example 90a) in lieu of 3-bromo-5-nitro-benzoic acid, 1,1-dimethylethyl ester, afforded the title compound as a crystalline solid, m.p. 146–148°C.

Example 90c: 5-(4-Morpholinyl)-3-(trifluoromethyl)-benzenamine

[3-(4-morpholinyl)-5-(trifluoromethyl)phenyl]-carbamic acid, 1,1-dimethylethyl ester (Example 90b; 1.7 g, 5 mmol) is treated with a solution of hydrogen chloride in isopropanol (30 mL of 4

M) and heated at 60°C for 5 h. The solvent is evaporated off under reduced pressure and the residue is treated with aqueous sodium hydrogen carbonate solution (80 mL) and extracted with ethyl acetate (3 x 80 mL). The combined extacts are washed with brine (2 x 50 mL), dried ($MgSO_4$) and the solvent is evaporated off under reduced pressure to give the crude product which is purified by recrystallisation from ether - hexane to afford the title compound as yellow crystalline solid, m.p. 96-97°C.

Example 91: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[5-(2-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)phenyl]benzamide

Utilising the procedure described in Example 69, but employing 5-(2-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)-benzenamine in lieu of 3-[(1-hydroxy-1-methylethyl)]-5-(1,1,1-trifluoromethyl)benzeneamine, afforded the title compound as a crystalline solid, m.p. 242–247°C.

The was aniline is prepared as follows:

Example 91a: 3-(2-Methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)-benzonitrile

A mixture of 3-fluoro-5-(trifluoromethyl)-benzonitrile (Lancaster Synthesis GmbH; 17 g, 89 mmol) and 2-methylimidazole (Fluka, Buchs, Switzerland; 22.2 g, 270 mmol) in *N,N*-dimethylacetamide (80 mL) is stirred at 145°C for 19 h. The solvent is evaporated off under reduced pressure and the residue is dissolved in ethyl acetate (200 mL). The solution is washed with brine (200 mL), dried (Na_2SO_4) and the solvent is evaporated off under reduced pressure to give the crude product which is purified by recrystallisation from ether - hexane to afford the title compound as yellow crystalline solid, m.p. 132-134°C.

Example 91b: 3-(2-Methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)-benzoic acid

A solution of 3-(2-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)-benzonitrile (Example 91a; 16.7 g, 66 mmol) in dioxane (300 mL) is added to an aqueous solution of sodium hydroxide (275 mL of 1 M) and the mixture is heated at 95°C for 18 h.The solvent is evaporated off under reduced pressure and the residue is neutralised with hydrochloric acid (1 M) and extracted with butanol (2 x 250 mL). The solvent is evaporated of under reduced pressure to give the title compound. ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 7.17 (s, 1H); 8.03 (s, 1H); 8.12 (s, 1H); 8.35 (s, 1H); 8.41 (s, 1H); 8.53 (s, 1H); 13.90 (br., 1H).

Example 91c: [3-(2-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)phenyl]-carbamic acid, 1,1-dimethylethyl ester

Triethylamine (5.23 mL, 37.5 mmol) is added to a stirred suspension of 3-(2-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)-benzoic acid (Example 91b; 6.8 g, 25 mmol) in *t*-butanol (200 mL). Diphenylphosphorylazide (7.6 g, 27.5 mmol) is added to the resulting solution and the mixture is heated 80°C for 16 h. The solvent is evaporated off under reduced pressure and the residue is treated with water (100 mL) and extracted with ethyl acetate (2 x 100 mL). The combined extracts are washed with brine (100 mL), dried (Na_2SO_4) and the solvent is evaporated off under reduced pressure to give the crude product which is purified by column chromatography (silica gel, eluent 2% ethanol in ethyl acetate) and recrystallised from ether - hexane to afford the title compound as a colourless crystalline solid, m.p. 203-208°C.

Example 91d: 5-(2-Methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)-benzenamine

Utilising the procedure described in Example 90c but employing [3-(2-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)phenyl]-carbamic acid, 1,1-dimethylethyl ester (Example 91c) in lieu of [3-(4-morpholinyl)-5-(trifluoromethyl)phenyl]-carbamic acid, 1,1-dimethylethyl ester, afforded the title compound as a yellow crystalline solid, m.p. 130-133°C.

Example 92: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[5-(4-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)phenyl]benzamide

Utilising the procedure described in Example 69, but employing 5-(4-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)-benzenamine in lieu of 3-[(1-hydroxy-1-methylethyl)]-5-(1,1,1-trifluoromethyl)benzeneamine, afforded the title compound as a crystalline solid, m.p. 235–236°C.

The was aniline is prepared as follows:

Example 92a: 3-(4-Methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)-benzonitrile

Utilising the procedure described in Example 91a, but employing 4-methyl-1H-imidazole in lieu of 2-methylimidazole, afforded the title compound as a crystalline solid, m.p. 127–128°C.

Example 92b: 3-(4-Methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)-benzoic acid

Utilising the procedure described in Example 91b, but employing 3-(4-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)-benzonitrile (Example 92a) in lieu of 3-(2-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)-benzonitrile, afforded the title compound as a crystalline solid, m.p. > 300°C.

Example 92c: [3-(4-Methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)phenyl]-carbamic acid, 1,1-dimethylethyl ester
Utilising the procedure described in Example 91c, but employing 3-(4-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)-benzoic acid (Example 92b) in lieu of 3-(2-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)-benzoic acid, afforded the title compound as a crystalline solid, m.p. 186-188°C.

Example 92d: 5-(2-Methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)-benzenamine
Utilising the procedure described in Example 91d, but employing [3-(4-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)phenyl]-carbamic acid, 1,1-dimethylethyl ester (Example 92c) in lieu of [3-(2-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)phenyl]-carbamic acid, 1,1-dimethylethyl ester, afforded the title compound as a colourless crystalline solid, m.p. 127-131°C.

Example 93: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[5-(5-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)phenyl]benzamide
Utilising the procedure described in Example 3, but employing 5-(5-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)-benzenamine in lieu of 1-(2-pyridyl)piperazine, afforded the title compound as a crystalline solid, m.p. 231-233°C.

The aniline is prepared as follows:

Example 93a: 3-(5-Methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)-benzonitrile
Utilising the procedure described in Example 91a, but employing 4-methyl-1H-imidazole in lieu of 2-methylimidazole, afforded the title compound as a crystalline solid, m.p. 99–101°C.

Example 93b: 3-(5-Methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)-benzoic acid
Utilising the procedure described in Example 91b, but employing 3-(5-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)-benzonitrile (Example 93a) in lieu of 3-(2-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)-benzonitrile, afforded the title compound as a colourless crystalline solid, m.p. 243-245°C.

Example 93c: [3-(5-Methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)phenyl]-carbamic acid, 1,1-dimethylethyl ester

Utilising the procedure described in Example 91c, but employing 3-(5-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)-benzoic acid (Example 93b) in lieu of 3-(2-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)-benzoic acid, afforded the title compound as a crystalline solid, m.p. 169-171°C.

Example 93d: 5-(5-Methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)-benzenamine
Utilising the procedure described in Example 91d, but employing [3-(5-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)phenyl]-carbamic acid, 1,1-dimethylethyl ester (Example 93c) in lieu of [3-(2-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)phenyl]-carbamic acid, 1,1-dimethylethyl ester, afforded the title compound as a colourless crystalline solid, m.p. 131-133°C.

Example 94: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[3-(4-methyl-1-piperazinyl)-5-(trifluoromethyl)phenyl]benzamide
Utilising the procedure described in Example 69, but employing 3-(4-methyl-1-piperazinyl)-5-(trifluoromethyl)-benzenamine in lieu of 3-[(1-hydroxy-1-methylethyl)]-5-(1,1,1-trifluoro-methyl)benzeneamine, afforded the title compound as a crystalline solid, m.p. 192–194°C.

The aniline is prepared as follows:

Example 94a: [3-(4-methyl-1-piperazinyl)-5-(trifluoromethyl)phenyl]-carbamic acid, 1,1-dimethylethyl ester
Utilising the procedure described in Example 87b, but employing 1-methyl-1-piperazine in lieu of morpholine, afforded the title compound as a crystalline solid, m.p. 225°C.

Example 94b: 3-(4-methyl-1-piperazinyl)-5-(trifluoromethyl)-benzenamine
Utilising the procedure described in Example 90c, but employing [3-(4-methyl-1-piperazinyl)-5-(trifluoromethyl)phenyl]-carbamic acid, 1,1-dimethylethyl ester (Example 94a) in lieu of [3-(4-morpholinyl)-5-(trifluoromethyl)phenyl]-carbamic acid, 1,1-dimethylethyl ester, afforded the title compound as oil. ^{1}H-NMR (400 MHz, DMSO-d_6): 2.20 (s, 3H), 2.42 (m, 4H), 3.07 (m, 4H), 3.32 (br s, 2H), 5.34 (s, 1H) and 6.31 (s, 2H).

Example 95: 4-Methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[2-(1-pyrrolidinyl)-5-(trifluoromethyl)phenyl]benzamide

Utilising the procedure described in Example 3, but employing 2-(1-pyrrolidinyl)-5-(trifluoromethyl)-benzenamine (Lancaster Synthesis Ltd.; Yasuhiro Ohtake et al., WO 9965874) in lieu of 1-(2-pyridyl)piperazine, afforded the title compound as a crystalline solid. ^{1}H-NMR (400 MHz, DMSO-d_6): 1.77-1.82 (m, 4H); 2.34 (s, 3H); 3.31-3.37 (m, 4H); 6.86 (d, 1H); 7.34-7.44 (m, 2H); 7.47 (d, 1H); 7.49-7.53 (m, 1H); 7.73 (dd, 1H); 8.27 (d, 1H); 8.43 (dt, 1H); 8.53 (d, 1H); 8.69 (dd, 1H); 9.13 (s, 1H); 9.27 (d, 1H); 9.96 (s, 1H).

Example 96: 3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-*N*-[5-(4-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)phenyl]benzamide
Utilising the procedure described in example 1, but employing 3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzoic acid in lieu of 4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzoic acid and 5-(4-methyl-1H-imidazol-1-yl)-3-(trifluoromethyl)-benzenamine in lieu of furfurylamine, afforded the title compound as as pale-yellow crystalline solid, m.p. 264-266°C.

Example 96a: 3-[(Aminoiminomethyl)amino]-benzoic acid ethyl ester mononitrate
Utilising the procedure described in example 1a but employing 3-amino-benzoic acid ethyl ester (Fluka, Buchs, Switzerland) in lieu of 3-amino-4-methylbenzoic acid ethyl ester, afforded the title compound as a crystalline solid, m.p. 170-172°C.

Example 96b: 3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzoic acid ethyl ester
Utilising the procedure described in example 1b but employing 3-[(aminoiminomethyl)amino]-benzoic acid ethyl ester mononitrate in lieu of 3-[(aminoiminomethyl)amino]-4-methyl-benzoic acid ethyl ester mononitrate, afforded the title compound as a crystalline solid, m.p. 197-199°C.

Example 96c: 3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzoic acid
Utilising the procedure described in example 1c but employing 3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzoic acid ethyl ester in lieu of 4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzoic acid ethyl ester, afforded the title compound as a crystalline solid, m.p. 291-295°C.

Example 97: Soft Capsules
5000 soft gelatin capsules, each comprising as active ingredient 0.05 g of one of the compounds of formula I mentioned in the preceding Examples, are prepared as follows:
250 g pulverized active ingredient is suspended in 2L Lauroglykol® (propylene glycol laurate, Gattefossé S.A., Saint Priest, France) and ground in a wet pulverizer to produce a particle

size of about 1 to 3 μm. 0.419 g portions of the mixture are then introduced into soft gelatin capsules using a capsule-filling machine.

Example 98: Pharmacokinetic data:

The compound of formula I to be tested is formulated for administration to female OF1 mice from IFACREDO, France, by first dissolving in NMP, and then by diluting with PEG300 to a final concentration of 10 % v/v NMP: 90 % v/v PEG300, producing a clear solution of the compound. The concentrations were adjusted to deliver a constant volume of 10 mL/kg body weight. The compound is prepared immediately before use. The formulated compound is administered perorally by gavage to provide dosages of 50 mg/kg. At the allotted time points mice (4 at each time) are anesthetized with 3 % isoflurane in medical oxygen and blood samples are obtained by heart puncture into heparinized tubes (ca. 30 IU/mL). The animals are subsequently killed without recovering from the anesthetic. Plasma is prepared from the blood by centrifugation (10,000 g, 5 min) and either analyzed immediately or stored frozen at – 70 °C.

The plasma samples (10 – 250 μL) are e.g. spiked with 5 μL of internal standard, mixed with 200 μL 0.1 M NaOH and 500 μL chloroform in a 1.5 mL Eppendorf tube and shaken vigorously for 10 minutes on an Eppendorf mixer. Thereafter, the mixture is centrifuged (3 min at 10'000xg), the organic phase transferred to a second Eppendorf tube and evaporated to dryness in a vacuum centrifuge (Speedvac 5301). The dry residue e.g. is dissolved in 250 μL of 10 % v/v Acetonitrile in water containing 0.1 % formic acid. The subsequent analysis is carried out e.g. by HPLC/MS-MS using an Agilent 1100 Series (Agilent, Palo Alto, CA, USA) HPLC system with vacuum degasser, binary pump, and thermostated column compartment combined with a cooled autosampler system (HTS PAL, CTC Analytics, Zwingen, Switzerland). The sample (5-15 μL) is injected e.g. onto an Ultra Phenyl column (particle size 3 μm, 50 x1 mm; Restek, Bellefonte, USA) with a guard column (4 x 2 mm) of the same material (Phenomenex, Torrance, USA). After equilibration e.g. with water and a latency period of 1 min the sample is eluted e.g. by a linear gradient of 0 – 100 % acetonitrile in water containing 0.2 % v/v formic acid over a period of 11 min at a flow rate of 60 μL/min. The column is prepared for the next sample e.g. by re-equilibrating for 3 min with 100 % water to the starting conditions. The separation is performed e.g. at a column temperature of 40 °C. The column effluent is introduced e.g. directly into the ion source of a triple stage quadropole mass spectrometer (Quattro Ultima™, Micromass, Manchester, UK) controlled by Masslynx™ 3.5 software (Micromass, Manchester, UK) using as ionization technique

electrospray ionization positive mode (ESI +). The compound is detected by MS/MS following fragmentation of the parent ions. The limit of quantitation is determined at e.g. 0.002 nmol/L. A calibration curve is constructed with known amounts of compound including a fixed amount of internal standard in plasma which is processed as described above. The concentration of unknown samples is calculated from a plot of the peak area ratio of the selected daughter ion of the analyte to the product of its internal standard (ordinate) against the nominal concentration (abscissa). Regression analysis is performed using Quanlynx ™, Masslynx™ software 3.5 (Micromass, Manchester, UK).

Example 99: In vitro inhibition data:

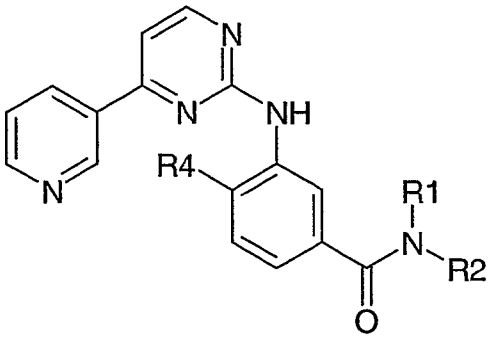
Enzymatic (c-Abl, KDR, Flt3) in vitro inhibition data are presented as % inhibition at 10 μM. The measurements are made as described above in the general description.

Example	Abl%@10μM	KDR%@10μM	Flt3%@10μM
1	51	57	
2	97	73	
3	66	71	
4	77	46	
5	71	60	
6	51	56	
7	72	45	
8	70	81	
9	44	39	
10	57	48	
11	53	41	
12	22	33	
13	78	48	
14	49	54	
15	60	23	
16	42	10	
17	54	62	
18	56	62	
19	41	33	
20	56	22	
21	30	93	
22	59	7	
23	90	67	
24	80	70	
25	44	73	
26	55	56	
27	54	51	
28	73	61	
29	78		
30	57	37	
31	68	83	
32	90	37	
33	97	51	
34	73	89	
35	27	47	
36	57	77	
37	28	82	
38	74	91	
39	64	74	
40	65	78	

Example	Abl%@10μM	KDR%@10μM	Flt3%@10μM
41	13	52	
42	32	56	
43	37	63	
44	75	97	
45	34	61	
46	1	43	
47	39	74	
48	90	50	
49	72	37	
50	87	83	
51	92	52	
52	78	37	
53	88	79	
54	69	74	
55	43	54	
56	40	44	
57	8	42	
58	40	26	
59	75	83	
60	79	36	
61	95	65	
62	59	44	
63	74	82	
64	56	59	
65	96	60	
66	67	23	
67	98	88	41
68	99	96	

WHAT IS CLAIMED IS:

1. A compound of formula



(I)

wherein

R_1 represents hydrogen, lower alkyl, lower alkoxy-lower alkyl, acyloxy-lower alkyl, carboxy-lower alkyl, lower alkoxycarbonyl-lower alkyl, or phenyl-lower alkyl;

R_2 represents hydrogen, lower alkyl, optionally substituted by one or more identical or different radicals R_3, cycloalkyl, benzcycloalkyl, heterocyclyl, an aryl group, or a mono- or bicyclic heteroaryl group comprising zero, one, two or three ring nitrogen atoms and zero or one oxygen atom and zero or one sulfur atom, which groups in each case are unsubstituted or mono- or polysubstituted; and

R_3 represents hydroxy, lower alkoxy, acyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, N-mono- or N,N-disubstituted carbamoyl, amino, mono- or disubstituted amino, cycloalkyl, heterocyclyl, an aryl group, or a mono- or bicyclic heteroaryl group comprising zero, one, two or three ring nitrogen atoms and zero or one oxygen atom and zero or one sulfur atom, which groups in each case are unsubstituted or mono- or polysubstituted; or wherein

R_1 and R_2 together represent alkylene with four, five or six carbon atoms optionally mono- or disubstituted by lower alkyl, cycloalkyl, heterocyclyl, phenyl, hydroxy, lower alkoxy, amino, mono- or disubstituted amino, oxo, pyridyl, pyrazinyl or pyrimidinyl; benzalkylene with four or five carbon atoms; oxaalkylene with one oxygen and three or four carbon atoms; or azaalkylene with one nitrogen and three or four carbon atoms wherein nitrogen is unsubstituted or substituted by lower alkyl, phenyl-lower alkyl, lower alkoxycarbonyl-lower alkyl, carboxy-lower alkyl, carbamoyl-lower alkyl, N-mono- or N,N-disubstituted carbamoyl-lower alkyl, cycloalkyl, lower alkoxycarbonyl, carboxy, phenyl, substituted phenyl, pyridinyl, pyrimidinyl, or pyrazinyl;

R_4 represents hydrogen, lower alkyl, or halogen;

and a N-oxide or a pharmaceutically acceptable salt of such a compound.

2. A compound of formula I according to claim 1 wherein

R_1 represents hydrogen, lower alkyl, lower alkoxy-lower alkyl, acyloxy-lower alkyl, carboxy-lower alkyl, lower alkoxycarbonyl-lower alkyl, or phenyl-lower alkyl;

R_2 represents hydrogen, lower alkyl, optionally substituted by one or two identical or different radicals R_3, cycloalkyl, benzcycloalkyl, heterocyclyl, an aryl group, or a mono- or bicyclic heteroaryl group comprising one, two or three nitrogen atoms or one sulfur atom, which aryl and heteroaryl groups in each case are unsubstituted or mono- or polysubstituted; and

R_3 represents hydroxy, lower alkoxy, acyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, N-mono- or N,N-disubstituted carbamoyl, amino, mono- or disubstituted amino, cycloalkyl, heterocyclyl, an aryl group, furanoyl, thienoyl, or a mono- or bicyclic heteroaryl group comprising one, two or three ring nitrogen atoms, zero or one ring oxygen atom and zero or one ring sulphur atom, which aryl and heteroaryl groups in each case are unsubstituted or mono- or polysubstituted; or wherein

R_1 and R_2 together represent alkylene with four or five carbon atoms, optionally mono- or disubstituted by lower alkyl, cycloalkyl, heterocyclyl, phenyl, hydroxy, lower alkoxy, amino, mono- or disubstituted amino, pyridyl, pyrazinyl or pyrimidinyl; benzalkylene with four or five carbon atoms in the alkylene group; oxaalkylene with one oxygen and three or four carbon atoms, or azaalkylene with one nitrogen and three or four carbon atoms wherein nitrogen is unsubstituted or substituted by lower alkyl, phenyl-lower alkyl, lower alkoxycarbonyl-lower alkyl, carboxy-lower alkyl, carbamoyl-lower alkyl, N-mono- or N,N-disubstituted carbamoyl-lower alkyl, cycloalkyl, lower alkoxycarbonyl, phenyl, substituted phenyl, pyridinyl, pyrimidinyl, or pyrazinyl;

R_4 represents hydrogen, lower alkyl, or halogen;

and a N-oxide or a pharmaceutically acceptable salt of such a compound.

3. A compound of formula I according to claim 1 wherein

R_1 represents hydrogen, lower alkyl, lower alkoxy-lower alkyl, lower alkoxycarbonyl-lower alkyl, or phenyl-lower alkyl;

R_2 represents hydrogen, lower alkyl, optionally substituted by one or two identical or different radicals R_3, cyclopentyl, benzcyclopentyl, cylcohexyl, pyrrolidinyl, oxazolinyl, piperidinyl, N-substituted piperidinyl, morpholinyl, azepinyl, oxo-azepinyl, oxazepinyl, phenyl, naphthalinyl, tetrahydronaphthalinyl or a mono- or bicyclic heteroaryl group comprising one

or two nitrogen atoms, which phenyl, naphthalinyl and heteroaryl groups in each case are unsubstituted or mono- or polysubstituted, thienyl, or lower alkoxycarbonyl-lower alkylthienyl; and

R_3 represents hydroxy, lower alkoxy, acyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, N-mono- or N,N-disubstituted carbamoyl, amino, lower alkylamino, di-lower alkylamino, phenylamino, N-lower alkyl-N-phenylamino, pyrrolidino, oxopyrrolidino, piperidino, morpholino, imidazolino, oxoimidazolino, cycloalkyl, heterocyclyl, furyl, phenyl, naphthalinyl, tetrahydronaphthalinyl, or a mono- or bicyclic heteroaryl group comprising one or two nitrogen atoms, which phenyl, naphthalinyl and heteroaryl group are unsubstituted or mono- or polysubstituted; or wherein

R_1 and R_2 together represent alkylene with four or five carbon atoms, optionally mono- or disubstituted by lower alkyl, cycloalkyl, heterocyclyl, phenyl, hydroxy, lower alkoxy, amino, mono- or disubstituted amino, pyridyl, pyrazinyl or pyrimidinyl; benzalkylene with four or five carbon atoms in the alkylene group; oxaalkylene with one oxygen and four carbon atoms; or azaalkylene with one nitrogen and four carbon atoms wherein nitrogen is unsubstituted or substituted by lower alkyl, phenyl-lower alkyl, lower alkoxycarbonyl-lower alkyl, carboxy-lower alkyl, carbamoyl-lower alkyl, N-mono- or N,N-disubstituted carbamoyl-lower alkyl, cycloalkyl, lower alkoxycarbonyl, phenyl, substituted phenyl, pyridinyl, pyrimidinyl, or pyrazinyl;

R_4 represents hydrogen, lower alkyl, or halogen;

and a N-oxide or a pharmaceutically acceptable salt of such a compound.

4. A compound of formula I according to claim 1 wherein

R_1 represents hydrogen, lower alkyl, lower alkoxy-lower alkyl, lower alkoxycarbonyl-lower alkyl, or phenyl-lower alkyl;

R_2 represents hydrogen; lower alkyl, optionally substituted by one radical R_3, by two phenyl groups, by two lower alkoycarbonyl groups, by phenyl and lower alkoxycarbonyl, or by hydroxyphenyl and lower alkoxycarbonyl; cyclopentyl; benzcyclopentyl; cylcohexyl; pyrrolidinyl; oxazolinyl; piperidinyl; N-lower alkylpiperidinyl; N-benzylpiperidinyl; N-pyrimidinylpiperidinyl; morpholinyl; azepinyl; oxo-azepinyl; oxazepinyl; phenyl, naphthalinyl, tetrahydronaphthalinyl or a mono- or bicyclic heteroaryl group comprising one or two nitrogen atoms, which phenyl, naphthalinyl and heteroaryl groups in each case are unsubstituted or substituted by one or two substituents selected from the group consisting of lower alkyl, trifluoro-lower alkyl, hydroxy-lower alkyl, lower alkoxy-lower alkyl, amino-

lower alkyl, lower alkylamino-lower alkyl, di-lower alkylamino-lower alkyl, N-cyclohexyl-N-lower alkylamino-lower alkyl, lower alkoxycarbonylpiperidino-lower alkyl, N-lower alkylpiperazino-lower alkyl, lower alkoxycarbonyl-lower alkyl, hydroxy, lower alkoxy, trifluoro-lower alkoxy, 1H-imidazolyl-lower alkoxy, lower alkanoyloxy, benzoyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, lower alkyl carbamoyl, amino, lower alkanoylamino, benzoylamino, amino mono- or disubstituted by lower alkyl, by hydroxy-lower alkyl or by lower alkoxy-lower alkyl, 1H-imidazolyl, mono- or di-lower alkyl-1H-imidazolyl, pyrrolidino, piperidino, piperazino, N-lower alkylpiperazino, morpholino, sulfamoyl, lower alkylsulfonyl, phenylsulfonyl, lower alkylsulfinyl, phenylsulfinyl, lower alkylthio, phenylthio, phenyl, pyridyl, halogenyl, or benzoyl; thienyl; or lower alkoxycarbonyl-lower alkylthienyl; and

R_3 represents hydroxy, lower alkoxy, acyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, carbamoyl mono-or disubstituted by lower alkyl, phenyl or lower alkylene, amino, lower alkylamino, di-lower alkylamino, phenylamino, N-lower alkyl-N-phenylamino, pyrrolidino, oxopyrrolidino, piperidino, morpholino, imidazolino, oxoimidazolino, cycloalkyl, heterocyclyl, furyl; phenyl, naphthalinyl, tetrahydronaphthalinyl, or a mono- or bicyclic heteroaryl group comprising one or two nitrogen atoms, which phenyl, naphthalinyl and heteroaryl group is unsubstituted or substituted by one or two substituents selected from the group consisting of lower alkyl, trifluoro-lower alkyl, lower alkoxycarbonyl-lower alkyl, hydroxy, lower alkoxy, trifluoro-lower alkoxy, lower alkanoyloxy, benzoyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, amino, lower alkanoylamino, benzoylamino, amino mono- or disubstituted by lower alkyl, by hydroxy-lower alkyl or by loweralkoxy-lower alkyl, pyrrolidino, piperidino, morpholino, piperazino, N-lower alkylpiperazino, N-lower alkoxycarbonylpiperazino, phenyl, pyridyl, 1H-imidazolyl, lower alkyl-1H-imidazolyl, sulfamoyl, lower alkylsulfonyl, phenylsulfonyl, lower alkylsulfinyl, phenylsulfinyl, lower alkylthio, phenylthio, halogenyl, or benzoyl; or wherein

R_1 and R_2 together represent alkylene with four or five carbon atoms, optionally mono- or disubstituted by lower alkyl, cycloalkyl, phenyl, hydroxy, lower alkoxy, amino, benzoylamino, piperidino, pyridyl, pyrazinyl or pyrimidinyl; benzalkylene with four or five carbon atoms in the alkylene group; oxaalkylene with one oxygen and four carbon atoms; or azaalkylene with one nitrogen and four carbon atoms wherein nitrogen is unsubstituted or substituted by lower alkyl, phenyl-lower alkyl, lower alkoxycarbonyl-lower alkyl, carboxy-lower alkyl, carbamoyl-lower alkyl, carbamoyl-lower alkyl N-mono- or N,N-disubstituted by lower alkyl, phenyl, lower alkylene or oxa-lower alkylene, cycloalkyl, lower alkoxycarbonyl,

phenyl, methoxyphenyl, trifluoromethylphenyl, trifluoromethoxyphenyl, pyridinyl, pyrimidinyl, or pyrazinyl;

R_4 represents hydrogen or lower alkyl;

and a N-oxide or a pharmaceutically acceptable salt of such a compound.

5. A compound of formula I according to claim 1 wherein

R_1 represents hydrogen, lower alkyl, lower alkoxy-lower alkyl, or benzyl;

R_2 represents lower alkyl, optionally substituted by one radical R_3, by two phenyl groups, by two lower alkoycarbonyl groups, by phenyl and lower alkoxycarbonyl, or by hydroxyphenyl and lower alkoxycarbonyl; cyclopentyl; benzcyclopentyl; cylcohexyl; pyrrolidinyl; piperidinyl; N-lower alkylpiperidinyl; N-benzylpiperidinyl; N-pyrimidinylpiperidinyl; morpholinyl; azepinyl; oxoazepinyl; phenyl; naphthalinyl; tetrahydronaphthalinyl; pyridyl; lower alkyl-pyridyl; quinolinyl; thienyl; lower alkoxycarbonylmethylthienyl; or phenyl substituted by one or two substituents selected from the group consisting of lower alkyl, trifluoro-lower alkyl, hydroxy-lower alkyl, amino-lower alkyl, lower alkylamino-lower alkyl, di-lower alkylamino-lower alkyl, N-cyclohexyl-N-lower alkylamino-lower alkyl, lower alkoxycarbonylpiperidino-lower alkyl, N-lower alkylpiperazino-lower alkyl, lower alkoxycarbonyl-lower alkyl, hydroxy, lower alkoxy, trifluoro-lower alkoxy, 1H-imidazolyl-lower alkoxy, lower alkanoyloxy, benzoyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, lower alkylcarbamoyl, amino, lower alkanoylamino, benzoylamino, amino mono- or disubstituted by lower alkyl, by hydroxy-lower alkyl or by loweralkoxy-lower alkyl, 1H-imidazolyl, lower alkyl-1H-imidazolyl, pyrrolidino, piperidino, piperazino, N-lower alkylpiperazino, morpholino, sulfamoyl, lower alkylsulfonyl, phenyl, pyridyl, halogenyl, or benzoyl; and

R_3 represents hydroxy, lower alkoxy, lower alkanoyloxy, benzoyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, amino, lower alkylamino, di-lower alkylamino, phenylamino, N-lower alkyl-N-phenylamino, pyrrolidino, oxopyrrolidino, piperidino, morpholino, imidazolino, oxoimidazolino, cyclopropyl, cyclopentyl, cyclohexyl, tetrahydrofuranyl, phenyl, naphthalinyl, tetrahydronaphthalinyl, furyl, a mono- or bicyclic heteroaryl group comprising one or two nitrogen atoms, which heteroaryl group is unsubstituted or mono- or disubstituted by lower alkyl, hydroxy and lower alkoxy, or phenyl substituted by one or two substituents selected from the group consisting of lower alkyl, trifluoro-lower alkyl, lower alkoxycarbonyl-lower alkyl, hydroxy, lower alkoxy, trifluoro-lower alkoxy, lower alkanoyloxy, benzoyloxy, carboxy, lower alkoxycarbonyl, carbamoyl, amino, lower alkanoylamino, benzoylamino, amino mono- or disubstituted by lower alkyl, by hydroxy-lower alkyl or by

loweralkoxy-lower alkyl, pyrrolidino, piperidino, morpholino, piperazino, N-lower alkylpiperazino, N-lower alkoxycarbonylpiperazino, phenyl, pyridyl, 1H-imidazolyl, lower alkyl-1H-imidazolyl, sulfamoyl, lower alkylsulfonyl, halogenyl, or benzoyl; or wherein

R_1 and R_2 together represent alkylene with four or five carbon atoms, optionally mono- or disubstituted by phenyl, hydroxy, amino, benzoylamino, or piperidino; benzalkylene with four or five carbon atoms in the alkylene group; oxaalkylene with one oxygen and four carbon atoms; or azaalkylene with one nitrogen and four carbon atoms wherein nitrogen is unsubstituted or substituted by lower alkyl, phenyl-lower alkyl, lower alkoxycarbonyl-lower alkyl, carbamoyl-lower alkyl, pyrrolidinocarbonyl-lower alkyl, morpholinocarbonyl-lower alkyl, cyclopentyl, lower alkoxycarbonyl, phenyl, methoxyphenyl, trifluoromethylphenyl, pyridinyl; pyrimidinyl, or pyrazinyl;

R_4 represents hydrogen or methyl;

and a N-oxide or a pharmaceutically acceptable salt of such a compound.

6. A compound of formula I according to claim 1 wherein

R_1 represents hydrogen;

R_2 represents phenyl substituted by trifluoromethyl and optionally a further substituent selected from the group consisting of hydroxy-lower alkyl, lower alkylamino, hydroxy-lower alkylamino, di-lower alkylamino, 1H-imidazolyl, lower alkyl-1H-imidazolyl, carbamoyl, lower alkylcarbamoyl, pyrrolidino, piperidino, piperazino, lower alkylpiperazino, morpholino, lower alkoxy, trifluoro-lower alkoxy, phenyl, pyridyl, and halogenyl;

R_4 represents methyl;

and a N-oxide or a pharmaceutically acceptable salt of such a compound.

7. A compound of formula I according to claim 1 wherein

R_1 represents hydrogen;

R_2 represents phenyl substituted by 3-trifluoromethyl and optionally a further substituent selected from the group consisting of 1-hydroxy-1-methylethyl, methylamino, ethylamino, 2-hydroxy-1-propylamino, 2-hydroxy-2-propylamino, diethylamino, 1H-imidazolyl, 2- and 4-methyl-1H-imidazolyl, carbamoyl, methylcarbamoyl, pyrrolidino, piperidino, piperazino, 4-methylpiperazino, morpholino, methoxy, trifluoromethoxy, 2,2,2-trifluoroethoxy, phenyl, 2-, 3- and 4-pyridyl, chloro, and fluoro;

R_4 represents methyl;

and a N-oxide or a pharmaceutically acceptable salt of such a compound.

8. The compound of formula I according to claim 1 wherein

R_1 represents hydrogen;

R_2 represents 3-(1-hydroxy-1-methylethyl)-5-(trifluoromethyl)phenyl;

R_4 represents methyl;

and a N-oxide or a pharmaceutically acceptable salt of such a compound.

9. A compound according to any one of claim 1 wherein

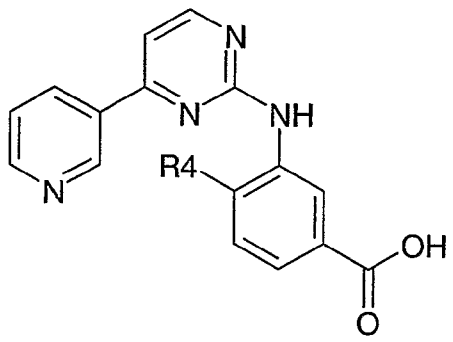
R_1 is hydrogen;

R_2 represents phenyl which is mono- or disubstituted by imidazol-lower alkoxy, lower alkyl amino, trifluoromethyl, hydroxy lower alkyl amino, bis-(lower alkoxy lower alkyl) amino, lower alkyl piperazinyl, piperidinyl, pyrrolidinyl, morpholinyl, phenyl, pyridyl, imidazolyl which is unsubstituted or mono- or disubstituted by lower alkyl or N-lower alkyl carbamoyl;

R_4 is lower alkyl;

and a N-oxide or a pharmaceutically acceptable salt of such a compound.

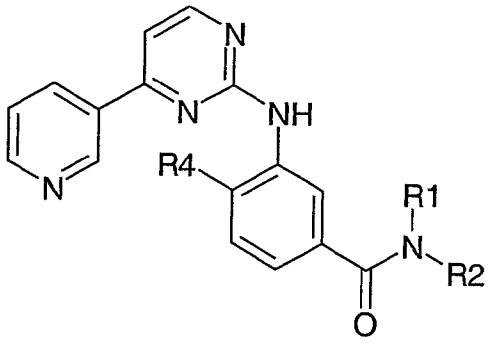
10. A compound of formula



(II)

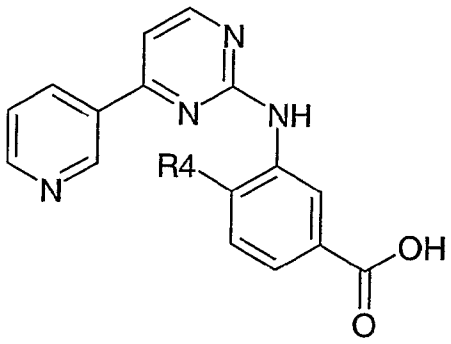
wherein R_4 is methyl or hydrogen.

11. A process for the synthesis of a compound of the formula



(I)

or an N-oxide or a salt thereof, wherein the symbols R_1, R_2 and R_4 are as defined in claim 1, characterized in that a compound of formula II



(II)

wherein R_4 is as defined for a compound of formula I, or a derivative thereof wherein the carboxy group –COOH is in activated form, is reacted with an amine of the formula III

$$R_1\text{-NH-}R_2 \qquad \text{(III)}$$

wherein R_1 and R_2 are as defined for a compound of the formula I, optionally in the presence of a dehydrating agent and an inert base and/or a suitable catalyst, and optionally in the presence of an inert solvent;

where the above starting compounds II and III may also be present with functional groups in protected form if necessary and/or in the form of salts, provided a salt-forming group is present and the reaction in salt form is possible;

any protecting groups in a protected derivative of a compound of the formula I are removed;

and, if so desired, an obtainable compound of formula I is converted into another compound of formula I or a N-oxide thereof, a free compound of formula I is converted into a salt, an obtainable salt of a compound of formula I is converted into the free compound or another

salt, and/or a mixture of isomeric compounds of formula I is separated into the individual isomers.

12. A pharmaceutical composition comprising as an active ingredient a compound of formula I according to any one of claims 1 to 10 or a N-oxide or a pharmaceutically acceptable salt thereof together with a pharmaceutically acceptable carrier.

13. A method for the treatment of a disease which responds to an inhibition of protein kinase activity, which comprises administering a compound of formula I according to any one of claims 1 to 10 or a N-oxide or a pharmaceutically acceptable salt thereof.

14. The use of a compound of formula I according to any one of claims 1 to 10 or a N-oxide or a possible tautomer thereof or of a pharmaceutically acceptable salt thereof for the preparation of a pharmaceutical composition for the treatment of a disease which responds to an inhibition of protein kinase activity.

INTERNATIONAL SEARCH REPORT

In ›nal Application No
PCT/EP 03/07198

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07D401/14 C07D405/14 A61K31/506 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CHEM ABS Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZIMMERMANN J ET AL: "Potent and selective inhibitors of the Abl-kinase: phenylamino-pyrimidine (PAP) derivatives" BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, OXFORD, GB, vol. 7, no. 2, 21 January 1997 (1997-01-21), pages 187-192, XP004135990 ISSN: 0960-894X examples 17,24; table 1	10
Y	the whole document	1-14

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 December 2003	09/12/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Timmermans, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int nal Application No
PCT/EP 03/07198

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	ZIMMERMANN J ET AL: "Phenylamino-pyrimidine (PAP) - derivatives: a new class of potent and highly selective PDGF-receptor autophosphorylation inhibitors" BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, OXFORD, GB, vol. 6, no. 11, 4 June 1996 (1996-06-04), pages 1221-1226, XP004134858 ISSN: 0960-894X examples 3,11; table 1 ---	1-14
Y	EP 0 588 762 A (CIBA GEIGY AG) 23 March 1994 (1994-03-23) page 3, line 52 - line 56; claim 1 ---	1-14
Y	PAUL R ET AL: "Preparation of substituted N-phenyl-4-aryl-2-pyrimidinamines as mediator release inhibitors" JOURNAL OF MEDICINAL CHEMISTRY, AMERICAN CHEMICAL SOCIETY. WASHINGTON, US, vol. 36, no. 19, 17 September 1993 (1993-09-17), pages 2716-2725, XP002134022 ISSN: 0022-2623 examples 2-7,2-22; table II ---	1-14
Y	WO 95 09853 A (CIBA GEIGY AG ;ZIMMERMANN JUERG (CH)) 13 April 1995 (1995-04-13) the whole document -----	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/EP 03/07198

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WO 9509853 A	13-04-1995	AT 208772 T	15-11-2001
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		EP 0672041 A1	20-09-1995
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		TW 378208 B	01-01-2000
		US 5728708 A	17-03-1998
		ZA 9407657 A	03-04-1995

CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER:

22-068

MEDICAL REVIEW

CLINICAL REVIEW

Application Type	NDA
Submission Number	22-068
Submission Code	SN-000
Letter Date	09/29/06
Stamp Date	10/04/06
PDUFA Goal Date	10/29/07 (After Extension)
Clinical Reviewer	Maitreyee Hazarika, MD
Team leader	Ramzi Dagher, MD
Statistical Reviewer	Xiaoping (Janet) Jiang, PhD
Team leader	Rajeshwari Sridhara, PhD
Review Completion Date	10/19/2007
Established Name	Nilotinib
(Proposed) Trade Name	Tasigna
Therapeutic Class	Antineoplastic
Applicant	Novartis
Priority Designation	Standard
Formulation	Capsules
Dosing Regimen	Oral
Indication	chronic phase and accelerated phase Philadelphia chromosome positive chronic myelogenous leukemia (CML) in adult patients resistant or intolerant to prior therapy including imatinib

Table of Contents

Table of Tables

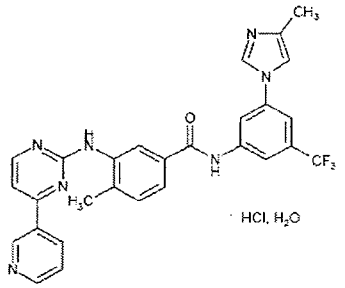
2 INTRODUCTION AND BACKGROUND

2.1 Product Information

Nilotinib (AMN107), a synthetic aminopyrimidine, is a highly selective inhibitor of the kinase activity of the Bcr-Abl oncoprotein. This protein is the product of the BCR-ABL fusion gene, which results from a reciprocal chromosomal translocation in a bone marrow hematopoietic stem cell. Nilotinib is an inhibitor of the Abl tyrosine kinase activity of the Bcr-Abl oncoprotein both in cell lines and in primary Philadelphia-chromosome positive leukemia cells. The drug binds tightly to the inactive conformation of the kinase domain in such a manner that it is an inhibitor of wild-type Bcr-Abl and maintains activity against 32/33 imatinib-resistant mutant forms of Bcr-Abl.

Established name: Nilotinib (AMN107)

Chemical structure of AMN107



4-Methyl-N-[3-(4-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)phenyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide, monohydrochloride, monohydrate

Proposed trade name: Tasigna®

Chemical class: Bcr-Abl tyrosine kinase inhibitor

Pharmacological class: Aminopyrimidine

Proposed indication: Chronic Myelogenous Leukemia resistant to or intolerant of imatinib mesylate

Dosing regimen: 400 mg orally twice daily

2.2 Currently Available Treatment for Indications

Dasatinib (Sprycel®) received accelerated approval in June 2006 for use in the treatment of adults with chronic phase (CP), accelerated phase (AP), or myeloid or lymphoid blast (MB or LB) phase chronic myeloid leukemia (CML) with resistance or intolerance to prior therapy including imatinib mesylate.

2.3 Availability of Proposed Active Ingredient in the United States

Nilotinib is not currently marketed in this country. Nilotinib is available under an expanded access protocol for patients with AP- or BP CML or Ph+ ALL who are resistant to or intolerant of imatinib mesylate.

2.4 Important Issues With Pharmacologically Related Products

Nilotinib is pharmacologically related to imatinib mesylate (Gleevec®) and dasatinib (Sprycel®), both of which are inhibitors of Bcr-Abl tyrosine kinase.

Imatinib mesylate (Gleevec®) was approved on May 10, 2001 for the treatment of CML in three clinical settings: CML-BC, CML-AP and CML-CP [1]. The most frequently reported drug related adverse events were edema, nausea and vomiting, muscle cramps, musculoskeletal pain, diarrhea and rash (cutaneous toxicity). A variety of adverse events represented local or general fluid retention including pleural effusion, ascites, pulmonary edema and rapid weight gain with or without superficial edema. Cytopenias included neutropenia and thrombocytopenia. Severe hepatotoxicity including elevations of transaminases or bilirubin lead to liver failure or death. Post marketing safety reports included cardiotoxicity including severe congestive heart failure in ten patients [2], hypophosphatemia, with associated changes in bone and mineral metabolism [3] [4] and fatal hepatitis [5].

Dasatinib was approved on June 28, 2006 for the treatment of adult patients with imatinib resistant/intolerant CML-CP, CML-AP and Ph + ALL [6]. The most common severe toxicities associated with dasatinib were hematologic, including neutropenia thrombocytopenia and anemia. Others included neutropenic fever, bleeding events, pyrexia, dyspnea, pleural effusion, and diarrhea. Other significant less commonly occurring adverse reactions included cardiac failure, QTc prolongation and CNS hemorrhages, most of which were fatal.

2.5 Presubmission Regulatory Activity

April 24, 2004	Initial investigational new drug application submitted
Dec 9, 2004	End-of-phase 1 meeting for resistant CML CP, AP, BC.

	In the absence of a controlled trial in the CP cohort, FDA agreed that a literature-based comparison to Interferon from large published randomized interferon studies would be reasonable.
Sept 8, 2005	End-of-phase 2 meeting for second indication, newly diagnosed CML CP.
Feb 13, 2006	End-of-phase 1-2 meeting follow-up meeting.
	[redacted] FDA stated that its acceptability will depend on the magnitude of the benefit seen, with the duration of the response being crucial. FDA recommended that the sample size in CML-AP not be decreased. FDA recommended presenting additional efficacy data prior to approval in order to provide additional follow-up validating the demonstration of and providing better precision about the durability of response.
Feb 3, 2006	Agency informed of sudden deaths and clinical information amendment submitted
Feb 27, 2006	Thorough QT study proposal submitted.
March 20, 2006	QT e-mail regarding responses to their QT study proposal : asked justification of conducting healthy volunteer instead of patient study; told that if higher doses are not tolerated, the study will not provide information on the QT effects at the proposed clinical doses; not all subjects may achieve steady state on day 3.
March 23, 2006	QT question e-mail we are concerned that at completion of your proposed QT study you will not have a definite answer regarding the potential for QT prolongation at the clinical doses; you may also need to perform a QT study in patients (e.g., your planned phase 3 trial) ; the sample size calculations could be based on detecting a larger change in QT (e.g., 10-15 msec).
April 27, 2006	Orphan drug designation for nilotinib in CML granted.
May 11, 2006	Fast Track designation for the treatment of imatinib-resistant or intolerant Ph + CML granted.

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Seeds to crystals

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Abstract

Seeding has been critical for obtaining diffraction-quality crystals for many structures. In this article, applications and recommendations for seeding are presented based on examples from our laboratory and other groups. The implementation of seeding in high-throughput crystallization, robotics, and other emerging technologies is also discussed.

Keywords: Crystallization; Macroseeding; Microseeding; Nucleation; Ostwald ripening; Seeding; Streak-seeding

1. Introduction

One of the paradoxes in crystallization is that the optimal solution conditions for nucleation of the crystals are not the ideal ones to support their subsequent growth. This is because spontaneous nucleation is quite simply more likely to occur when the levels of supersaturation are high, whereas slow, ordered growth of large crystals is favored by lower levels. The ideal experiment therefore must somehow uncouple nucleation from growth to satisfy the distinctly different requirements of the two events.

Seeding is a powerful tool for the separation of nucleation and growth. In this technique, previously nucleated crystals are used as seeds and introduced into new drops equilibrated at lower levels of supersaturation. Seeding techniques can be classified into two categories based on the size of the seeds:

- Microseeding—transfer of submicroscopic seeds, too small to be distinguished individually.
- Macroseeding—transfer of a single crystal, usually 5–50 μm.

Seeding can be used in conjunction with other optimization measures such as fine-tuning the precipitant and protein concentrations, adjusting the pH, and screening additives. The course of one such optimization is shown in Fig. 1.

Apart from a role in optimization, seeds can also be used as heterogeneous nucleants to facilitate the crystallization of similarly related proteins. This is often done to ensure that the target molecules will crystallize isomorphously with the native crystals. When one molecule not only nucleates on another but also shares some of the same structural features as the substrate (e.g., the same or very similar crystallographic lattice), the growth is said to be epitaxial. Proteins that have been crystallized by cross-seeding include mutants, selenomethionyl-substituted proteins, heavy atom derivatives, complexes with DNA- and RNA-mers, substrates, inhibitors or other ligands, different monoclonal antibody fragments, and homologous proteins from a different organism.

2. How to seed

Microseeding is an easy method and therefore the type of seeding to try first. Macroseeding (also called seed transfer) is much more labor intensive. It requires tedious transfers of the parent crystal through multiple washes, although the effort can be worth it, especially in terms of increasing the size of the crystal. However, in most seeding situations, a whole seed is not required; microseeds will suffice. The disadvantage to microseeding is that it is difficult to control the number of seeds that are transferred.

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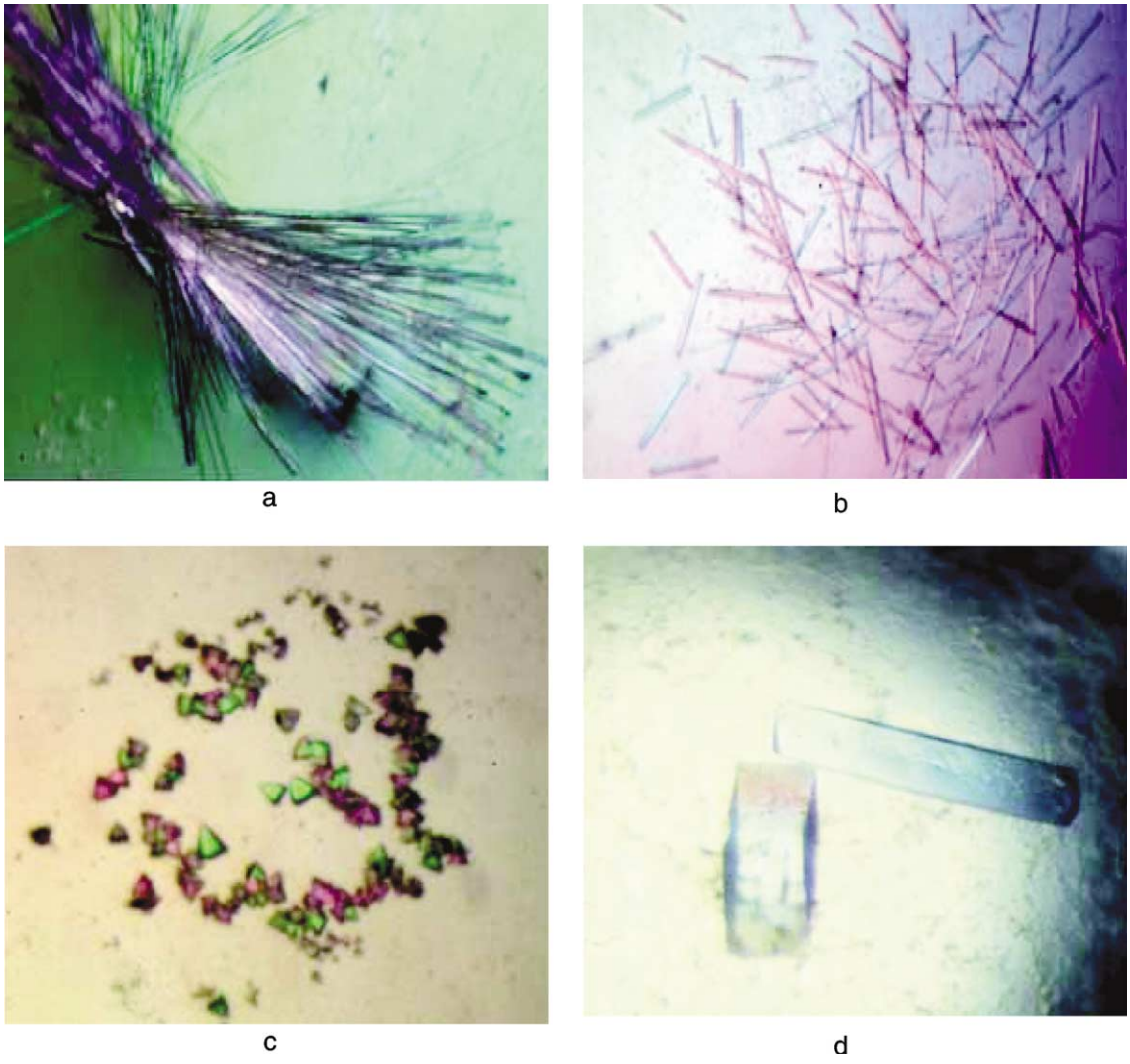

Fig. 1. Optimization of bovine acyl-CoA binding protein crystals (van Aalten et al., 2001). Microseeding was necessary in all the optimization steps (b–d) to obtain crystals that grew singly. Protein concentration in (a) is 18 mg/ml and 10 mg/ml in (b–d). (a) Crystals from the initial screen. (b) Single, tetragonal crystals after microseeding with the crystals in (a). (c) Bipyramidal crystals, $0.2 \times 0.3 \times 0.3$ mm, as a result of including nickel sulfate in the crystallization conditions. (d) Orthorhombic crystals of the complex of the protein and its ligand palmitoyl-CoA.

2.1. Handling microseeds

Microseeds can be made and introduced into the new drop in many different ways. The crystals can be pulverized (smashed) into crystalline particles by tissue homogenizers, sonication, vortexing, seed beads, glass rods, or other utensils. An article which specifically discusses the advantages and disadvantages of different pulverization methods is one by Luft and DeTitta (1999). Preparing a dilution series of the seed stock in a stabilizing mother liquor will give solutions with different amounts of nuclei. The dilution with the optimum number of nuclei has to be determined experimentally but is usually somewhere between 10^{-2} and 10^{-7}. The nuclei can be pipetted from the stock as a small aliquot or transferred with a seeding wand which is dipped into the microseed mixture to pick up seeds and then touched, stirred, or streaked across the surface of the new drop.

Preparing a dilution series of the seed stock makes it more quantitative, but by far the easiest and fastest method for seed transfer is streak seeding (Stura and Wilson, 1990). This method uses an animal whisker (usually cat or rabbit) as a seeding wand, which is touched or stroked over the surface of the parent crystal to dislodge and trap the nuclei. The whisker is then drawn through the new drop, depositing the seeds in a streak line. Although seeding wands have been made from thin glass rods, platinum-wire inoculation loops, and acupuncture needles, animal whiskers or hairs offer a definite advantage. The overlapping cuticles (Fig. 2a) capture the seeds effectively. Here we would like to bring attention to an animal source (Fig. 2b) of material for making seeding wands which, at least to our knowledge,

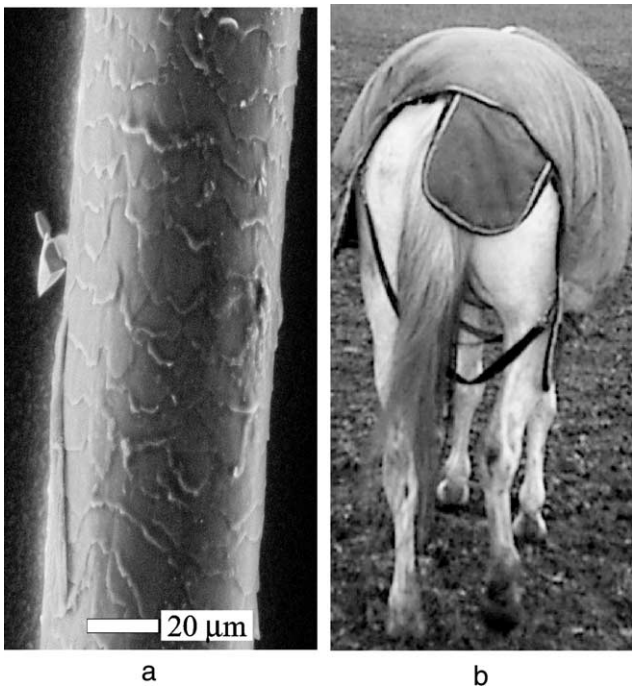

Fig. 2. Material for seeding wands. (a) Scanning electron micrograph of a horse tail hair showing the overlapping cuticles. Note the entrapped crystal on the left. (Micrograph courtesy of G. Vrdoljak, University of California at Berkeley Electron Microscope Laboratory.) (b) An abundant source of material for seeding wands can be found at the flexible C-terminus of this animal.

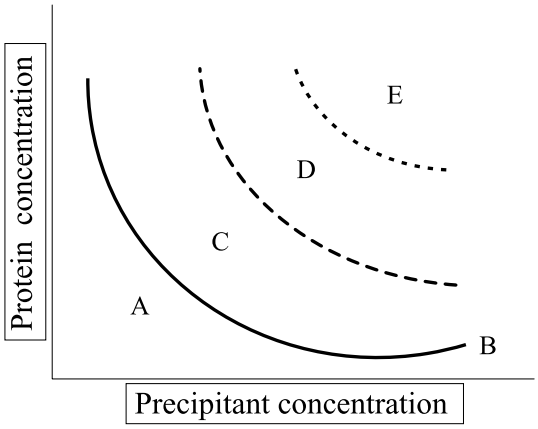

Fig. 3. Two-dimensional, theoretical phase diagram. (A) Undersaturation zone. Undersaturated drops remain clear. (B) Limit of solubility. The solid phase of the protein is in equilibrium with the liquid phase. (C) Metastable zone of supersaturation. The level of supersaturation is high enough to support growth of the solid phase of the protein but not high enough to initiate spontaneous nucleation. This is the best zone for seeding. Note that drops in the metastable zone are also clear, making it difficult to distinguish them from undersaturated drops. One diagnostic is to add a seed: seeds will melt if the solution is undersaturated. The metastable zone pictured here is wide. When the metastable zone is narrow it is difficult to accurately seed into it. (D) Labile supersaturation. Ordered aggregation (spontaneous nucleation) can occur here. (E) Precipitation zone. This region is highly supersaturated, leading to disordered aggregation (amorphous precipitate).

has not been exploited much by the crystallization community. Horse tail hair is much more abundant than most animal whiskers, which makes it useful when organizing large-scale laboratory sessions. In addition, horse tail hairs maintain the same thickness throughout their length (which is why violin bow makers use it), and this property may be useful in its own right for reproducibility in seed transfer. On the other hand, a cat whisker can be cut into three or four segments to obtain wands of different thickness. We use both horse tail hairs and cat whiskers because of their complementary properties. We find that rabbit whiskers, being thinner than both horse hair and cat whiskers, are too flexible.

2.2. Finding the metastable zone

After the dilution series of microcrystals has been made or a parent seed selected for macroseeding, the seeding wand or pipette (if using the aliquot method) is used to place the seed(s) into a new experiment equilibrated at the metastable zone of supersaturation. This is the area in the phase diagram where crystal growth is supported but no nucleation occurs (Fig. 3). A protocol for the determination of a phase diagram for microseeding can be found at http://www.douglas.co.uk (Research Report 1, August 1995). This microseeding method was used for the successful crystallization of two alcohol dehydrogenases (Korkhin et al., 1996).

In practice, most seeding experiments are done without knowledge of the protein's phase diagram since determining the phase diagram is expensive in terms of the researcher's time and protein consumption. If it is not convenient to determine the phase diagram for the protein, some rules of thumb, derived from our own empirical observations, can be applied instead. To move to the metastable zone of supersaturation, the concentration of at least one of the components (precipitant, protein, or both) must be lowered. (See Figs. 1 and 4 for examples.) Our first choice is to begin by lowering the protein concentration. Protein concentration is inversely and linearly proportional to its solubility. Hence, if lowering the protein concentration too much puts the experiment below the solubility limit and the seeds dissolve, only a simple linear interpolation is required for the next round of drops. The protein concentration is raised to some level between where spontaneous nucleation occurred in the first drop and where the seeds melted in the second experiment. The relationship between protein solubility and the concentration of polymeric precipitants (of which polyethylene glycol (PEG) is the most commonly used) is also linear, i.e., protein solubility decreases proportionally as the polymer concentration increases (Atha and Ingham, 1981). This is not true for salt precipitants for which the relationship between salt concentration and protein solubility is

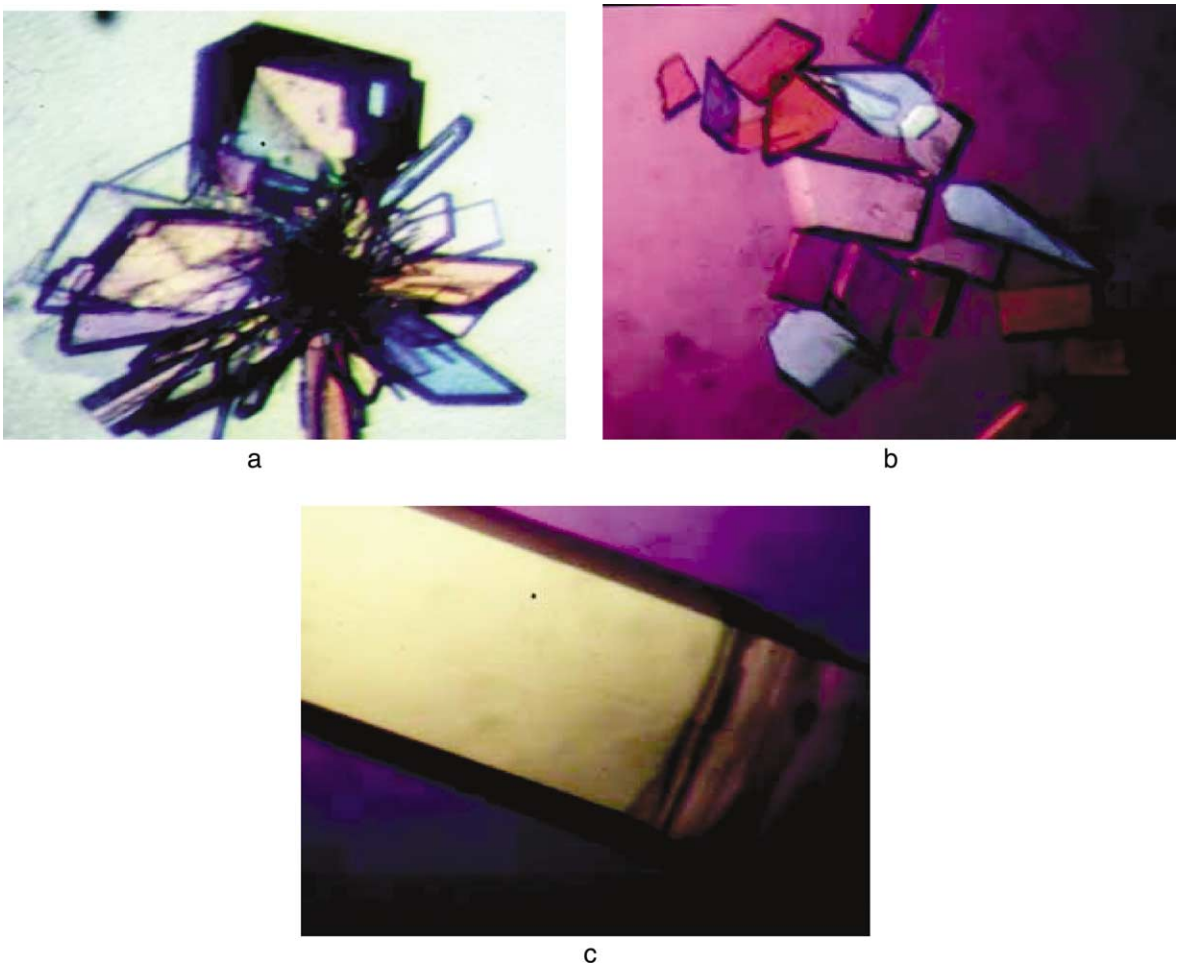

Fig. 4. Improvements through serial seeding. (a) Crystals of CBH2 resulting from spontaneous nucleation after 1 month. (b) Microseeding with the crystals of (a) into a new drop at lower protein concentration gave crystals that grew singly. (c) Result from the second round of seeding, using the crystals in (b) as microseeds. Magnification is the same in (a–c).

more complex, and the nature of the salt affects the solubility, independent of the ionic strength. Thus, it can be more difficult to correctly localize the metastable zone by lowering the salt concentration and we therefore prefer to lower the protein concentration instead.

Lowering the protein concentration also gives a more efficient use of protein. The structure of the complex of tissue factor, factor VIIa, and an inhibitor (Tf.VIIa. 5L15) was obtained with only 1.5 mg of protein (prior to the advent of nanocrystallization methods) (Stura et al., 1996). By judicious use of streak-seeding, the crystals which self-nucleated at a protein concentration of 21 mg/ml could be grown at 7 mg/ml instead.

Nevertheless, it should be pointed out that different results can be obtained depending on which of the concentrations is lowered: protein or precipitant. For example, the cell dimensions and diffraction quality of Tf.VIIa. 5L15 complex crystals were different depending on whether the seeds were grown in the metastable zone at high-protein, low-precipitant concentrations or at low-protein, high-precipitant concentrations. Protocols are available for obtaining polymorphs by changing the precipitant concentration with or without seeding (Stura, 1999).

An option completely different from lowering the protein/precipitant concentration(s) is to seed into a mother liquor totally unrelated to that in which the seeds originated. Transfer of crystals to solvents drastically different from the original mother liquor has been done in many cases out of necessity to facilitate heavy atom or substrate binding. For instance, triose phosphate isomerase crystals grown at 2.4 M ammonium sulfate were moved to 44% PEG 6000 (Wierenga et al., 1992). In our laboratory we use whatever crystals are obtained in the initial screen to streak-seed any of the drops that may have remained clear. We also typically streak-seed the screens for cryoconditions.

2.3. In situ seeding in the metastable zone

Developments in dynamic light scattering technology (DLS) may soon make it possible to decouple nucleation from growth in a more quantitative fashion, be it for

seeding or other optimization technique. The formation of the first nuclei is an event which occurs long before they become visible in the microscope. Saridakis et al. (2002) have recently reported using DLS to identify this onset of nucleation. This information is then used to determine the optimal time at which to dilute the drops. Dilution of the drops lowers the supersaturation, thereby shifting the experiment from the labile to the metastable zone, where no nuclei form but the existing ones will continue to grow.

In a sense this could be described as in situ seeding, i.e., rather than transferring seeds to a new drop at metastable supersaturation, the drop itself is altered so that the nuclei are in a metastable environment. The drops are diluted with water, buffer, or mother liquor (minus the protein). Carboxypeptidase G_2 crystals were grown in this way (Saridakis et al., 1994). In situ seeding is also used for growing crystals for atomic force microscopy (AFM), which requires that the crystals are fastened to a surface that can be inserted into the AFM sample chamber (Kuznetsov et al., 1997).

Other ways to manipulate the solution instead of the seeds include feeding and weeding. In feeding, more protein is added to the drop. The effects are twofold: the drop becomes diluted, lowering the supersaturation, while at the same time more protein becomes available to the growing nuclei. This is an effective approach if the crystals nucleated in the labile zone have exhausted all the dissolved protein. In weeding (Han and Lin, 1996) excess crystals are removed from the drop so that the protein in solution is available to the selected few crystals that remain. Care must be exercised to avoid evaporation in the drop during the weeding. Since this is difficult to do, weeding is not often successful.

3. Some variations and applications

3.1. Improving the seed quality

The selection of the seed crystal is critical in macroseeding, but the origin of the seed should also be taken into consideration for microseeding. Defects accumulate in crystals as they grow and so the oldest, largest crystals should be avoided as sources of seeds. If no small, freshly grown crystals are available, a seed can be chipped from the growing edge of the parent crystal. The fragment can be used as a macroseed or pulverized into microcrystals. If the fragment is to be used as a macroseed, it must be soaked briefly in an etching solution (i.e., undersaturated conditions). This will slightly melt the crystal and generate a fresh surface. The procedure for macroseeding has been described in detail for ribose binding protein and can be used as a guideline in designing a macroseeding protocol for other proteins (Mowbray, 1999).

The quality of the seed crystals, whether for macro- or microseeding, can be improved by serial seeding (also called repeated seeding). In serial seeding, crystals resulting from the first round of experiments are used to seed new drops. When crystals have appeared in these second drops, they are now used as seeds in their own turn for a third set-up, and so on. The seeds can be introduced into the new drops as macroseeds, streak-seeded, or crushed and introduced as microseeds, although the latter two are the most common methods. Fig. 4 shows how the quality of cellobiohydrolase 2 (CBH2) crystals improved with two rounds of seeding. The spontaneously nucleated crystals (Fig. 4a) appeared after 1 month in drops with 20 mg/ml protein, as rosette formations, i.e., many different crystals growing from a single nucleus. To eliminate both the long nucleation time and the tendency of these crystals to grow in rosettes, the crystals were crushed and used as microseeds in new drops at 10 mg/ml protein concentration. At this protein concentration no nucleation ever occurred spontaneously. Single crystals appeared after some days (Fig. 4b). These crystals were then used in turn as microseeds for yet another series of drops. The protein concentration was maintained at 10 mg/ml. This second round of seeding yielded crystals that grew to over 2 mm in 1 week and the 2.0 Å structure was solved with them (Bergfors et al., 1989; Rouvinen et al., 1990).

Here only 2 rounds of reiterative seeding were necessary, but sometimes as many as 7–10 rounds may be required (Thaller et al., 1981). If any improvement is apparent after 1 round of seeding, a second or more passes should always be attempted. This has been found to be of benefit in many of our projects.

3.2. If no crystals are available—seeding with oils, spherulites, and precipitates

3.2.1. Oils

If no crystals or microcrystals are forthcoming in the initial crystallization screening, seeding can be attempted with any solid phase that has resulted. It is obvious that spherulites represent some kind of semi-ordered aggregation, but even oils and precipitates can exhibit short-range order and therefore act as nuclei (ordered aggregates) for further crystal growth (Stura and Wilson, 1991). For these solid states of the protein, streak-seeding is the method of transfer.

The crystallization of the cytochrome domain of cellobiose dehydrogenase (CDH) can be used as an example (Hallberg et al., 2000). This protein was particularly recalcitrant in crystallizing, with the only result being an oily precipitate (Fig. 5), which was found after 1 year in the screens. Despite their semiliquid appearance, these protein oils are quite distinct from free protein in solution. Their exact nature is still under investigation but they are known to be a protein-rich

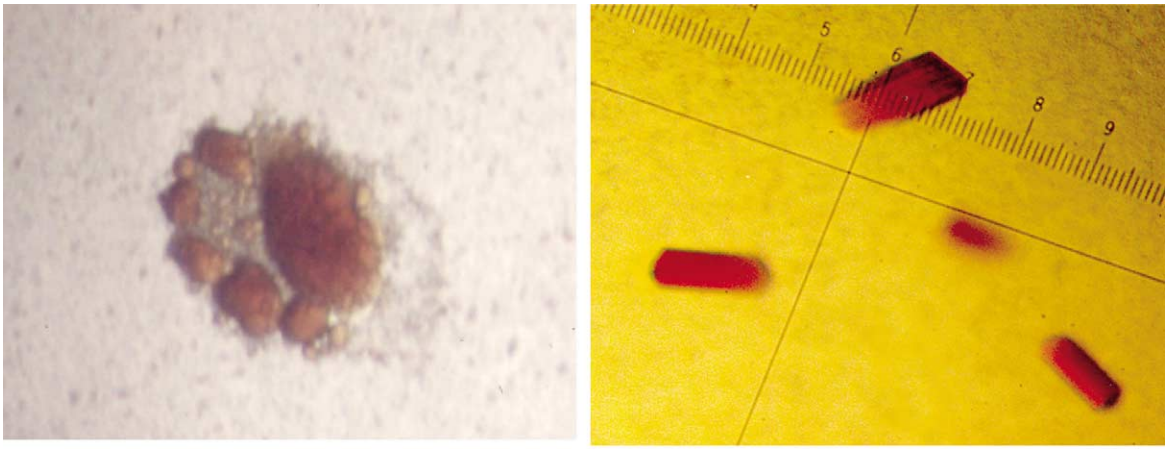

Fig. 5. Crystallization of CDH. (a) CDH precipitated from solution as the oil seen here. (b) The crystals used in determining the structure. Longest dimension is 0.1 mm (courtesy of B.M. Hallberg).

phase (Kuznetsov et al., 2001). The CDH protein is inherently red (due to its heme group), making it easy to identify the red oil as concentrated protein. The oil was used to streak-seed new drops, resulting in microcrystals. Although these were not the crystals ultimately used to solve the structure, they provided the necessary impetus to continue what initially seemed a hopeless project.

3.2.2. Precipitates

Even precipitates can be utilized in combination with seeding:

- Precipitate can be assayed for microcrystallinity by streak-seeding (Figs. 6a and b).
- Drops that have precipitated can be seeded to provoke Ostwald ripening (Fig. 6c).

Not all precipitates are equal: it is important to distinguish the precipitates of denatured protein from amorphous and nonamorphous precipitates and recognize submicroscopic crystals which look like amorphous precipitate. One quick assay for microcrystallinity of precipitates is streak-seeding (Stura and Wilson, 1991). In this method, the precipitate is streak-seeded into a new drop and the results are examined after 2–7 days. Amorphous precipitates will generate only more precipitate along the streak line (Fig. 6a), whereas crystalline precipitates will generate microcrystals. Sometimes, as in Fig. 6b, the seeds generated along the streak line are large enough to use without further refinements.

Precipitation occurs at high levels of supersaturation, far from the metastable zone, so generally seeds are not introduced into precipitate. For some proteins, however, the metastable and labile zones are so narrow that it is difficult to pinpoint the area between precipitation and undersaturation. The seeds are therefore introduced, either inadvertently or of necessity, into the precipitate or the labile phase. This can sometimes provoke a positive response via a mechanism known as Ostwald ripening.

This interesting process is a result of the thermodynamic advantage which large crystals have with respect to smaller ones. Kinetically, it is easier to form many small crystals, i.e., they nucleate more easily. However, large crystals, with their greater volume-to-surface area ratio, represent a lower energy state. In Ostwald ripening the many small crystals are driven thermodynamically to obtain a lower energy state by becoming incorporated into larger crystals (Boistelle and Astier, 1988; Ng et al., 1996). Thus, a seed crystal introduced into a shower of many small nuclei or a precipitate can grow at their expense via Ostwald ripening.

Sousa et al. (1991) report that crystals of T7 RNA polymerase seeded into an amorphous precipitate phase deteriorated into precipitate. This was not expected since the precipitate phase is at a level of supersaturation high enough to support a solid phase of the protein. On the other hand, precipitate that they put into drops in the metastable zone transformed into crystals. The labile and precipitated zones in the phase diagram are both states far from the limit of solubility, i.e., equilibrium, and it is not always possible to predict what will happen kinetically when seeds are introduced. It may provide some comfort to researchers looking at precipitated crystallization drops to keep in mind that "in systems far from equilibrium, order can arise from chaos" (Prigogine, 2001).

3.3. Seeding and additives

Another way to manipulate the level of nucleation through seeding is suggested by the work of Baker and Stewart (2000). Needle crystals of Mog1p (a protein that binds GTPase Ran/Gsp1p) were obtained under several conditions and therefore an array of additives was

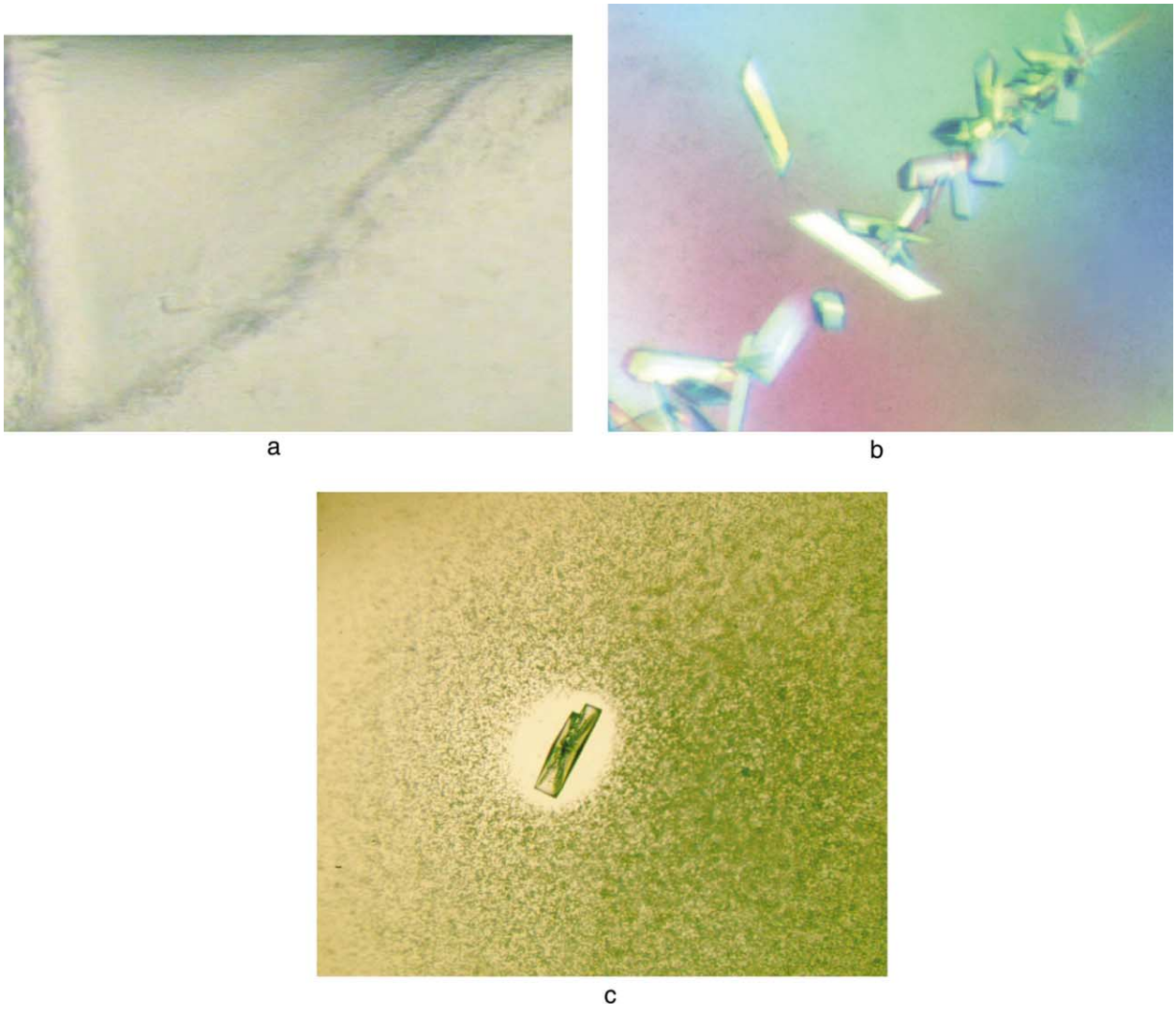

Fig. 6. Seeding and precipitates. (a) Precipitate obtained in the initial crystallization screen for this protein (*Mycobacterium tuberculosis* RV 2465c) was assayed for microcrystallinity by streak-seeding it into a new drop. Only more precipitate grew along the streak line. (b) By contrast, if the precipitate is microcrystalline, crystals will appear along the streak line in the drop. These crystals of *Aspergillus niger* epoxide hydrolase were obtained after several rounds of improvement by streak-seeding (Zou et al., 2000). (c) Ostwald ripening. As the crystal grows, a local concentration gradient with respect to the protein develops. Precipitated protein redissolves, creating the halo or depletion zone seen around the growing crystal.

screened in the subsequent round of optimization. One of the additives, 0.1–0.25% β-octylglucoside, was found to inhibit nucleation completely, i.e., the drops remained clear. These clear drops were streak-seeded to supply the nuclei and crystals appeared within 12 h. After 1 week they had achieved their ultimate size, producing large crystals which diffracted to 2.5 Å. Here the additive functioned as a "poison" in the drop to inhibit nucleation, then seeds were added to provide a controlled number of nuclei.

Glycerol is another commonly used additive in crystallization mother liquors since it offers cryoprotection and also increases the stability of many proteins (Sousa, 1995). (It should be noted that another effect of glycerol is that it increases protein solubility, which then affects the phase diagram of the protein.) The viscosity of glycerol can be exploited in combination with microseeding to titrate the number of nuclei. For example, in the crystallization of T7 RNA polymerase, Sousa et al. (1991) injected microseeds into one end of a large drop (100 μl). The normal convective mixing which occurs in liquids was reduced because of the viscosity of the glycerol and therefore a concentration gradient of microcrystals developed across the drop. Many small crystals appeared at the seed inoculation site but they became fewer and larger at the far end of the drop (Fig. 7). Obviously this effect can be utilized with other viscous mother liquors if the drop is large enough to permit a significant gradient to develop.

Another variation for titration of microseeds which we use in our laboratory is the following. We add the microseeds as a 0.2-μl droplet beside the new drop to be

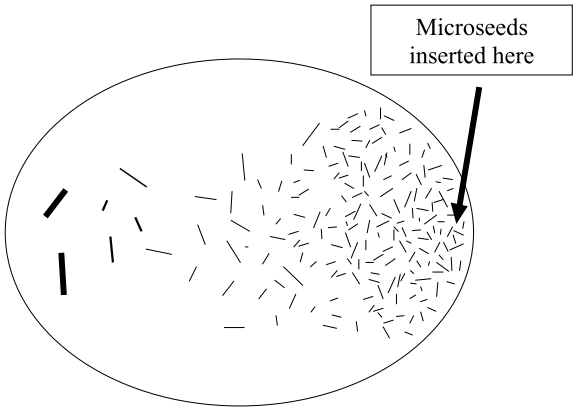

Fig. 7. Effect of microseeding into a viscous mother liquor. Schematic representation of the concentration gradient of microseeds that develops when they are introduced at one end of a 100-μl drop containing 30–40% glycerol in the mother liquor. Adapted from Sousa et al. (1991); used by permission of the publisher.

seeded. A thin liquid bridge is then drawn between the two. This works with or without high-viscosity mother liquors. A greater titration effect can be achieved though, when the liquid is viscid and when there is a large difference between the volumes of the two drops.

Crystallization in gels can also filter away excess nuclei, whether they have occurred spontaneously or have been introduced by seeding.

3.4. Filtration

If excess nuclei are a problem, the simplest recourse is to filter the protein through a 0.22 μm filter to remove dust, precipitated protein, and other particles. (Usually the stock solutions for the mother liquor components will have been filtered previously, but often the proteins are not.) This will help ensure that the seed or seeds are placed in an environment free of unintentional nucleants. In fact, filtering the solutions may be sufficient in itself to improve the quality of the crystals without seeding (Blow et al., 1994).

3.5. Cross-seeding selenomethionyl-substituted proteins: Synergism between microseeds and a heterogeneous nucleant

Incorporation of anomalous scatterers such as Se, Xe, or Br into proteins for multiwavelength anomalous diffraction (MAD) is the phasing method most used in high-throughput protein crystallography. Selenomethionyl proteins crystallize under essentially the same conditions as their native counterparts, although the protein concentration may be different because the Se proteins are sometimes less soluble. Seeding with native protein crystals is frequently done (Doublie and Carter, 1992).

An example from our lab, Fig. 8, shows crystals of selenomethionyl limonene epoxide hydrolase (SeMet-LEH) (Arand et al., submitted). Although it was possible to obtain crystals of the native protein, the selenomethionyl protein was difficult to crystallize. Microseeding with the native crystals sometimes helped but was unreliable. Interestingly, it was found that adding a single grain of sand in combination with the microseeds somehow enhanced the effect of the microseeding so that crystals did grow in most drops. (Sand alone without microseeds had no effect.) The first description of a structure solved with MAD, *Escherichia coli* thioredoxin, also reports using a grain of sand with the microseeds to promote the crystallization of the SeMet-protein (Hendrickson et al., 1990).

The main ingredient in sand is SiO_2. It may be that there are no unique properties of sand which account for its success in the SeMet-LEH and SeMet-thioredoxin crystallizations. Perhaps the addition of any nucleant would have enhanced the microseeding but this was not tested. The effects of heterogeneous nucleants in synergism with homogeneous nucleants (i.e., seeds of the native protein) have not been much investigated, although the use of minerals alone has been the subject of a paper (McPherson and Shlichta, 1988). Currently there is an intense investigation of materials such as silicons (Chayen et al., 2001; Sanjoh et al., 2001), Langmuir–Schaeffer films (Pechkova and Nicolini, 2001, 2002), and polymeric films (Fermani et al., 2001) as surface catalysts. These surfaces promote nucleation but without epitaxy, i.e., there is no lattice match between the nucleant and the protein as in cross-seeding. Synergistic effects between homogeneous and heterogeneous nucleation will need to be explored as this research area develops.

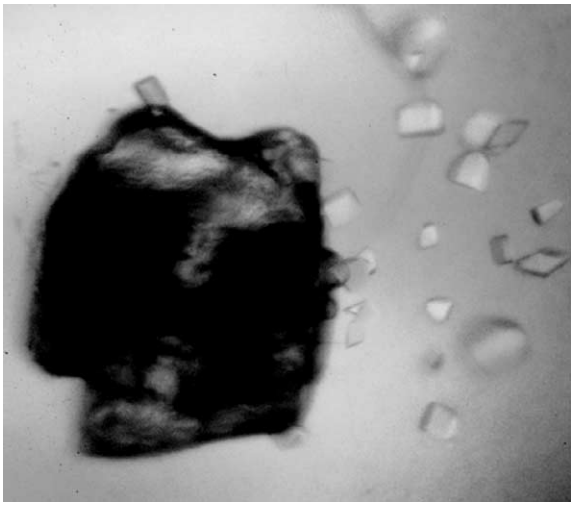

Fig. 8. Crystals of SeMet-LEH growing on and in the vicinity of a grain of sand. The large object to the left is the grain of sand. It is speculated that the crystals seen growing to the right may have nucleated on the sand and floated away later. The typical crystal size is 0.25 mm^3.

4. When seeding will not or does not work

4.1. If the protein is not pure: Effects of seeding into impure solutions

Crystallization is often used in the process industry as a purification step to recover proteins from the highly impure fermentation broth. That this is possible is evidence of the crystal surface's remarkable capacity to select appropriate molecules for incorporation. Nonetheless, purity of the protein solution is among the most critical factors in determining the outcome of the crystallization experiment and many studies have been conducted on the effects of contaminants on crystal quality. Caylor et al. (1999) have specifically looked at the role of seeding and protein purity for hen egg-white lysozyme solutions contaminated with (a) a structurally unrelated protein and (b) a structurally similar one. The result of their study (see Table 1) is probably equally applicable to seeding experiments with other proteins. It seems to indicate that problems resulting from microheterogeneity in the protein solution are not likely to be resolved by seeding.

4.2. Alternatives to seeding

Seeding is only one of many possible optimization methods. Some alternatives to separate nucleation from growth, or to slow down the growth after nucleation has occurred, are presented in Table 2. This table should not be regarded as a comprehensive list of all optimization methods. Rather, the suggestions have been selected on the basis of their simplicity of implementation.

5. Perspectives

The advent of microfocusing synchrotron beams now makes it possible to collect data from crystals of less than 100 μm but the need for large crystals still exists (see Rayment, 2002, for a discussion). Seeding can be used to grow larger crystals or get them to grow singly, improve their diffraction quality, save protein, reduce long waits for spontaneous nucleation, and cross-seed other proteins. We foresee that the usefulness of seeding will continue in the era of high-throughput crystallography. New developments that will affect how seeding is implemented in the future include automation of crystallization, use of laser beams for seed handling, DLS for determining when nucleation has occurred, and new materials for the promotion of nucleation.

5.1. Automation

Large-scale screening for initial crystallization conditions discovers more polymorphs. To some extent the

Table 1
Impurity effects with and without seeding (adapted from Caylor et al., 1999)

Type of contaminant added to the hen egg-white lysozyme (HEWL)	Effect without seeding	Effect when seeded with a well-ordered HEWL seed
20% ovotransferrin (an unrelated protein)	Spontaneous nucleation is difficult	The seeded crystal grows without defect
	Disordered crystal core resulting in cracking and disorder	Spherulites appear in the same drop
20% turkey egg-white lysozyme (structurally similar protein)	Lengthened *c* axis	No improvements in crystal quality
	Crystal cracking	

Table 2
Easy optimization alternatives to seeding

Method	Comments	Reference
Filter the protein	Removes dust, precipitated protein, and particles that could be unintentional nucleation sites	Blow et al. (1994)
Cover the reservoir with oil (in vapor diffusion)	Slows the rate of equilibration	Chayen (1997)
Evaporative dialysis	Useful for proteins with a narrow metastable zone	Bunick et al. (2000)
Temperature shifts	Can work for proteins that exhibit temperature-sensitive solubility	Blow et al. (1994)
"Backing off" (in vapor diffusion)	After nucleation has occurred, the coverslip is moved to a different reservoir for equilibration at metastable conditions	Saridakis and Chayen (2000)

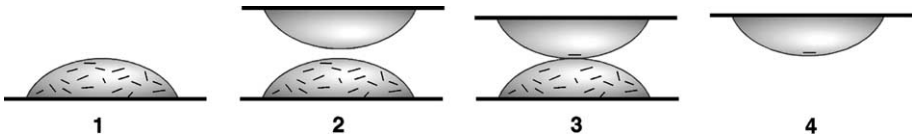

Fig. 9. Sandwich drop set-up for seed transfer (macroseeding) with a laser beam, adapted from Bancel et al. (1998); (1) Sitting drop with microcrystals. (2) A new drop is suspended over the sitting drop. (3) A sandwich interface is made to connect the two drops. A laser beam of some few milliwatts is used to select and propel a single seed to the surface of the sitting drop. (4) After the seed is transferred, the sandwich interface is interrupted to leave a hanging drop with the single seed. (Graphics by Pierre Filing, Biomedical Center Photo Department, Uppsala University.)

availability of more crystals to choose from in the initial screen may lessen the need for optimization. Still, most of the initial trials will produce only microcrystals or crystals that require further improvement. Optimization is difficult to automate since it must be adapted for each case individually. Among the efforts to automate optimization is the program Streak, developed by Douglas Instruments (London, UK). Streak uses a cat whisker mounted into the Douglas Instruments robot Impax, to inoculate microbatch drops with seed dilutions ranging from 10^{-1} to 10^{-6}. It has been tested on racGDP, a small G protein (Haire et al., 2001).

Even without automation, microseeding and streak-seeding are simple, quick, and cheap.

5.2. Laser beams for single-seed transfer (macroseeding)

Macroseeding, however, is more difficult because it requires many manipulations. During all these manipulations, it can happen that the macroseed gets damaged, causing unintentional showers of microcrystals. To address this problem, Bancel et al. (1998) constructed a tabletop laser beam for macroseeding individual seeds (1–25 μm) of tomato bushy stunt virus. The beam levitates the seed crystal from a sitting drop and propels it via a sandwich interface to the new drop. After the transfer is completed, the sandwich interface is interrupted, leaving a hanging drop with the single macroseed (Fig. 9). This method greatly simplifies macroseeding and makes it possible to use extremely small, but individually distinguishable, seeds.

5.3. DLS to determine the metastable zone

Applications using DLS will make it easier in the future to pinpoint the metastable zone or to find the optimal nucleus size (Saridakis, 2000) and thereby increase the accuracy of the seeding.

5.4. Heterogeneous nucleants

The research on nucleation-inducing materials for protein crystallization is currently just getting under way. The role of these heterogeneous nucleants in promoting nucleation alone and in synergism with seeding needs to be explored.

6. Summary

Spontaneous nucleation is a rare and unusual event. No other step in crystallization requires as much energy as the formation of a nucleus. In the experiment that uses a seed, this difficult kinetic barrier to spontaneous nucleation is bypassed. It is hoped that the techniques and applications which have been presented here will make it easier for crystal growers to exploit the valuable resource of seeds. "From a small seed a mighty trunk may grow" (Aeschylus, 526–456 BC).

7. Technical note: obtaining whiskers or horse tail hairs for seeding

Cat whiskers are shed naturally by cats and can be recovered from the floors and carpets of cat owners. Under no circumstance should they be yanked from a living cat: the whiskers are extremely sensitive sensory organs in the feline. Whiskers from dead cats can be bought from Charles River Suppliers. Horse tail hair can be simply cut off using scissors. Do not use horse tail from mares since estrous females spray urine.

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Priority Report

In vitro Activity of Bcr-Abl Inhibitors AMN107 and BMS-354825 against Clinically Relevant Imatinib-Resistant Abl Kinase Domain Mutants

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Abstract

Imatinib, a Bcr-Abl tyrosine kinase inhibitor, is a highly effective therapy for patients with chronic myelogenous leukemia (CML). Despite durable responses in most chronic phase patients, relapses have been observed and are much more prevalent in patients with advanced disease. The most common mechanism of acquired imatinib resistance has been traced to Bcr-Abl kinase domain mutations with decreased imatinib sensitivity. Thus, alternate Bcr-Abl kinase inhibitors that have activity against imatinib-resistant mutants would be useful for patients who relapse on imatinib therapy. Two such Bcr-Abl inhibitors are currently being evaluated in clinical trials: the improved potency, selective Abl inhibitor AMN107 and the highly potent dual Src/Abl inhibitor BMS-354825. In the current article, we compared imatinib, AMN107, and BMS-354825 in cellular and biochemical assays against a panel of 16 kinase domain mutants representing >90% of clinical isolates. We report that AMN107 and BMS-354825 are 20-fold and 325-fold more potent than imatinib against cells expressing wild-type Bcr-Abl and that similar improvements are maintained for all imatinib-resistant mutants tested, with the exception of T315I. Thus, both inhibitors hold promise for treating imatinib-refractory CML. (Cancer Res 2005; 65(11): 4500-5)

Introduction

Imatinib (STI571, Gleevec), an Abl kinase inhibitor, is now the first-line treatment for patients with chronic myelogenous leukemia (CML; ref. 1). Its success is predicated on the efficient inhibition of the deregulated, oncogenic tyrosine kinase Bcr-Abl, which plays a critical role in the pathogenesis of CML (2, 3). Most newly diagnosed CML patients with chronic phase disease treated with imatinib achieve durable responses (4); however, a small percentage of these patients and most advanced-phase patients relapse on imatinib therapy (4–6).

In patients who relapse on imatinib therapy, the Bcr-Abl kinase is reactivated, emphasizing the importance of the kinase activity of this protein to disease pathogenesis. The most common mechanism of resistance, occurring in 60% to 90% of patients who acquire imatinib resistance, involves specific mutations in the kinase domain of Bcr-Abl that interfere with imatinib binding without eliminating ATP binding or kinase activity (reviewed in refs. 7, 8). Clinically observed mutations identified within the Bcr-Abl kinase domain span a range of residual imatinib sensitivities (IC_{50}: 900-4,400 nmol/L) and encompass several functionally distinct kinase domain regions, including the nucleotide binding P-loop, imatinib contact residues, and the activation loop (7, 9, 10).

An understanding of the mechanism of imatinib resistance has prompted the search for alternate Bcr-Abl inhibitors that are effective against clinically observed Bcr-Abl mutants. Two promising new Bcr-Abl inhibitors for treating imatinib-resistant CML are currently being evaluated in clinical trials: the selective Abl inhibitor AMN107 and the dual Src/Abl inhibitor BMS-354825 (Fig. 1*A*, *top*). AMN107 was developed by rational drug design based on the crystal structure of an Abl-imatinib complex, whereas BMS-354825 is a Src inhibitor that was found to exhibit Abl inhibitory properties.

The crystal structure of the Abl kinase domain in complex with imatinib indicates that few changes to the imatinib scaffold are likely to be tolerated (7, 11). Imatinib binds to the canonical ATP site lining the groove between the N and C lobes of the protein. The drug penetrates into the central cleft of the kinase and the high topological congruency between the aniline-pyrimidine substructure and the surface of the distorted ATP-binding pocket suggest that making changes within this region of imatinib might not be very productive. However, the methylpiperazinyl group of imatinib (Fig. 1*A*, *top*) lies along a surface-exposed pocket of the Abl kinase and is potentially more amenable to modification. Replacement of this heterocycle and further rational design to optimize drug-like properties led to the discovery of AMN107 (Novartis Pharmaceuticals, Basel, Switzerland; Fig. 1*A*), which, as predicted, possesses substantially increased binding affinity and selectivity for the Abl kinase compared with imatinib (12).

Another approach to counteract imatinib resistance is to use inhibitors that bind Bcr-Abl with less stringent conformational requirements than imatinib. Imatinib selectively targets an inactive conformation of the Abl kinase domain in which the activation loop is in a nonphosphorylated, closed position that is incompatible with substrate binding (13). Specific differences between the inactive conformations of Abl and Src provide a structural basis for the initially surprising finding that Src family kinases are not imatinib targets, despite a high degree of sequence homology (11). The Abl and Src active conformations are more similar and many inhibitors that bind to the active conformation of Src are also capable of inhibiting Abl (11). BMS-354825 is a dual Src-Abl kinase

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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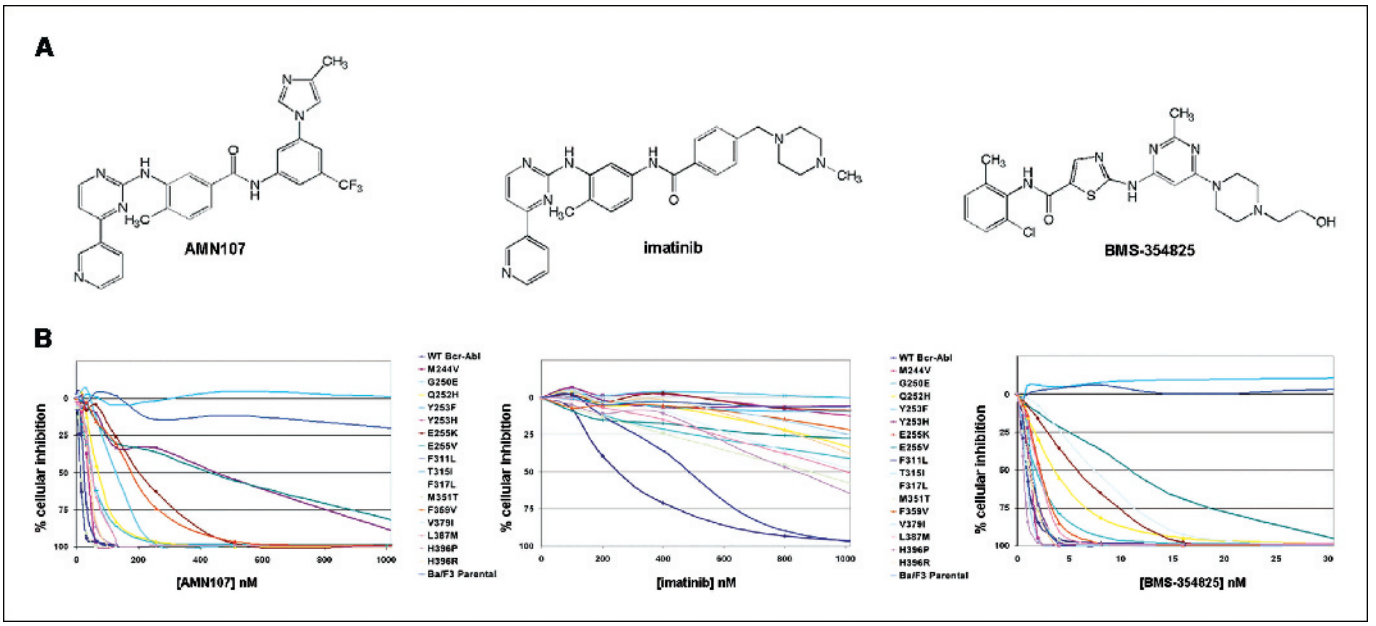

Figure 1. AMN107 and BMS-354825 are more effective than imatinib in inhibiting proliferation of Ba/F3 cells expressing wild-type Bcr-Abl or all Bcr-Abl mutants except T315I. *A*, structures of AMN107, imatinib, and BMS-354825. *B*, Ba/F3 cells supplemented with IL-3 or Ba/F3 cells expressing wild-type or mutant Bcr-Abl protein were plated in quadruplicate at 5×10^3 cells/well in 96-well plates with escalating concentrations of imatinib (0-2,000 nmol/L), AMN107 (0-2,000 nmol/L), or BMS-354825 (0-32 nmol/L) included in the media. Results from day 3 methanethiosulfonate assays were used to construct best-fit curves and calculate the cellular IC_{50} and IC_{90} values. For imatinib, only the IC_{50} value is reported. The mean based on four replicates was calculated in the absence of inhibitor and for each concentration of inhibitor. Means ± SE were generated from three independent experiments and reported as the percentage inhibition of cellular proliferation relative to control. Error bars are omitted for clarity. Note that the scale of the abscissa for the BMS-354825 cellular proliferation graph differs from that of imatinib and AMN107.

inhibitor that inhibits all tested Bcr-Abl kinase domain mutants observed in relapsed patients with the exception of T315I. The drug was also highly effective in a mouse model of imatinib-resistant, Bcr-Abl–dependent disease (14).

Although some data regarding the ability of AMN107 to inhibit various imatinib-resistant mutants is available, a number of the common mutants have not been included in the published data. Further, given differences in cell lines used, variations in levels of Bcr-Abl expression, and differing assay conditions, a direct comparison of AMN107 and BMS-354825 based on published results is not possible. Given that both of these drugs are in clinical trials, knowledge of the relative sensitivity of a particular mutant could assist in determining which drug would be more appropriate for a particular patient. Therefore, in this report, we present a complete profile of AMN107 against imatinib-resistant mutants and a direct cellular and biochemical comparison between imatinib, AMN107, and BMS-354825.

Materials and Methods

Reagents. BMS-354825 (*N*-(2-chloro-6-methylphenyl)-2-[[6-[4-(2-hydroxyethyl)-1-piperazinyl]-2-methyl-4-pyrimidinyl]amino]-5-thiazolecarboxamide) was synthesized by Bristol-Myers Squibb (New York, NY; ref. 14). AMN107 (NVP-AMN107-AA; 4-methyl-*N*-[3-(4-methyl-1*H*-imidazol)-5-(trifluoromethyl)phenyl]-3-(3-pyridinyl)-2pyridinyl]amino] benzamide, hydrochloride) was synthesized by Novartis Pharmaceuticals (12). Imatinib was purchased from the Oregon Health and Science University pharmacy. Each compound was prepared as a 10 mmol/L stock in DMSO and experiments were done with dilutions of the stock solutions.

Cell lines. Ba/F3 transfectants (expressing full-length wild-type Bcr-Abl or Bcr-Abl with kinase domain point mutations) were generated, selected, and maintained as previously described (10). Parental Ba/F3 cells were supplemented with interleukin-3 (IL-3).

Kinase autophosphorylation assays with glutathione *S*-transferase–Abl kinase domains. Kinase assays using wild-type and mutant glutathione *S*-transferase (GST)–Abl fusion proteins (c-Abl amino acids 220-498) were done as described, with minor alterations (15). GST-Abl fusion proteins were released from glutathione-Sepharose beads before use; the concentration of ATP was 5 μmol/L. Immediately before use in kinase autophosphorylation and *in vitro* peptide substrate phosphorylation assays, GST-Abl kinase domain fusion proteins were treated with LAR tyrosine phosphatase according to the manufacturer's instructions (New England Biolabs, Beverly, MA). After 1-hour incubation at 30°C, LAR phosphatase was inactivated by addition of sodium vanadate (1 mmol/L). Immunoblot analysis comparing untreated GST-Abl kinase to dephosphorylated GST-Abl kinase was routinely done using phosphotyrosine-specific antibody 4G10 to confirm complete (>95%) dephosphorylation of tyrosine residues and c-Abl antibody CST 2862 to confirm equal loading of GST-Abl kinase. The inhibitor concentration ranges for IC_{50} determinations were 0 to 5,000 nmol/L (imatinib and AMN107) or 0 to 32 nmol/L (BMS-354825). The BMS-354825 concentration range was extended to 1,000 nmol/L for mutant T315I. These same inhibitor concentrations were used for the *in vitro* peptide substrate phosphorylation assays. The three inhibitors were tested over these same concentration ranges against GST-Src kinase (Cell Signaling Technology, Boulder, CO) and GST-Lyn kinase (Stressgen, Victoria, BC, Canada).

***In vitro* peptide substrate phosphorylation assays with glutathione *S*-transferase–Abl kinase domains.** The effects of imatinib (0-5,000 nmol/L), AMN107 (0-5,000 nmol/L), and BMS-354825 (0-32 nmol/L) on the catalytic activity of unphosphorylated GST-Abl kinase were assessed using a synthetic, NH_2-terminal biotin-linked peptide substrate (biotin-EAIYAAPFAKKK-amide; ref. 16). Assays were carried out at 30°C for 5 minutes in 25 μL of reaction mixture consisting of kinase buffer [25 mmol/L Tris-HCl (pH 7.5), 5 mmol/L β-glycerophosphate, 2 mmol/L DTT, 0.1 mmol/L Na_3VO_4, 10 mmol/L $MgCl_2$; Cell Signaling Technology], 50 μmol/L peptide substrate, 10 nmol/L wild-type or mutant GST-Abl kinase, and 50 μmol/L ATP/[γ-^{32}P]ATP (5,000 cpm/pmol). Reactions were terminated by addition of guanidine hydrochloride to a final concentration of 2.5 mol/L.

A portion of each terminated reaction mixture was transferred to a streptavidin-coated membrane (SAM^2 biotin capture membrane; Promega, Madison, WI), washed, and dried according to the manufacturer's instructions; phosphate incorporation was determined by scintillation counting. Results were corrected for background binding to the membranes as determined by omitting peptide substrate from the kinase reaction. Time course experiments to establish the linear range of enzymatic activity preceded kinase assays. Similar *in vitro* peptide substrate phosphorylation assays were conducted with two Src family kinases: GST-Src kinase (Cell Signaling Technology) and GST-Lyn kinase (Stressgen). For Src family kinases, SignaTECT PTK biotinylated peptide substrate 2 (Promega) was the peptide substrate; all other conditions were as described for the GST-Abl kinase assays.

Cellular proliferation assays. Ba/F3 cell lines were plated in triplicate and incubated with escalating concentrations of imatinib, AMN107, or BMS-354825 for 72 hours. Proliferation was measured using a methanethiosulfonate-based viability assay (CellTiter96 Aqueous One Solution Reagent; Promega). IC_{50} and IC_{90} values are reported as the mean of three independent experiments done in quadruplicate. The inhibitor concentration ranges for IC_{50} and IC_{90} determinations were 0 to 2,000 nmol/L (imatinib and AMN107) or 0 to 32 nmol/L (BMS-354825). The imatinib concentration range was extended to 6,400 nmol/L for mutants with IC_{50} >2,000 nmol/L. The BMS-354825 concentration range was extended to 200 nmol/L for mutant T315I.

Immunoblotting. Ba/F3 cell lines (1×10^6 cells) were incubated for 3 hours in media containing escalating doses of either imatinib or AMN107. Cells were collected by centrifugation and lysed in SDS sample buffer. Following SDS-PAGE, proteins were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) for immunoblotting. Tyrosine-phosphorylated Bcr-Abl was detected with mouse monoclonal phosphotyrosine antibody 4G10 (Supplementary Fig. S1). Bcr-Abl expression was detected using rabbit c-Abl antibody CST 2862 (Cell Signaling Technology).

Apoptosis assays. Ba/F3 cell lines (6×10^4 cells/well) were incubated in 1 mL media containing vehicle, imatinib (300 nmol/L, 1,500 nmol/L), or AMN107 (30, 300, and 1,500 nmol/L) for 72 hours. Detection of apoptosis was done using a Guava Nexin apoptosis kit (Annexin V-phycoerythrin and 7-amino-actinomycin D; Guava Technologies, Hayward, CA) and a Guava Technologies PCA instrument. Results based on three independent experiments are reported as the mean ± SE (Supplementary Fig. S2).

Results and Discussion

In this study, we compared the activity of imatinib and two promising Bcr-Abl inhibitors, AMN107 and BMS-354825, against wild-type Bcr-Abl and imatinib-resistant Bcr-Abl mutants. Biochemical assays with purified, dephosphorylated wild-type GST-Abl kinase showed that AMN107 inhibited Abl-catalyzed peptide substrate phosphorylation with ~20-fold higher potency than imatinib (IC_{50}: 15 versus 280 nmol/L), whereas BMS-354825 had a two-log (~325-fold) increased potency relative to imatinib (IC_{50}: 0.6 versus 280 nmol/L). Corresponding experiments with mutant Abl kinase domains revealed that the ~20-fold improved potency of AMN107 compared with imatinib is also seen with the imatinib-resistant mutants (Table 1). The one exception was the Abl mutant

Table 1. Imatinib, AMN107, and BMS-354825 IC_{50} values (nmol/L) for purified GST-Abl kinase and purified GST-Src kinase autophosphorylation and peptide substrate phosphorylation assays

	Purified GST-Abl kinase and GST-Src kinase assays											
	Peptide substrate phosphorylation						Autophosphorylation					
	Imatinib		AMN107		BMS-354825		Imatinib		AMN107		BMS-354825	
	IC_{50} (nmol/L)	Fold change	IC_{50} (nmol/L)	Fold change	IC_{50} (nmol/L)	Fold change	IC_{50} (nmol/L)	Fold change	IC_{50} (nmol/L)	Fold change	IC_{50} (nmol/L)	Fold change
WT Abl	280	1	15	1	0.6	1	300	1	7	1	0.8	1
M244V	220	0.8	19	1.3	0.8	1.3	380	1.3	13	2	0.9	1.1
G250E	1,650	6	36	2	0.3	0.5	1,000	3	35	5	2.0	3
Q252H	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Y253F	4,300	15	28	2	0.4	0.6	>5,000	>17	55	8	3.1	4
Y253H	>5,000	>18	400	27	1.8	3	>5,000	>17	190	27	1.4	2
E255K	>5,000	>18	59	4	0.2	0.3	2,800	9	110	16	1.5	2
E255V	3,700	13	160	11	0.6	1	>5,000	>17	275	39	1.7	2
F311L	800	3	57	4	0.9	1.5	775	3	17	2	0.5	0.6
T315l	>5,000	>18	>5,000	>333	>10,000	>16,667	>5,000	>17	>5,000	>714	>1,000	>1,250
F317L	800	3	29	2	0.9	1.5	900	3	20	3	1.15	1.4
M351T	440	2	12	0.8	0.1	0.2	820	3	9	1.3	1.6	2
F359V	900	3	170	11	0.5	0.8	4,700	16	35	5	1.7	2
V379I	950	3	21	1.4	0.7	1.2	800	3	50	7	2.25	3
L387M	825	3	18	1.2	0.5	0.8	1,500	5	50	7	3.7	5
H396P	2,000	7	9	0.6	0.5	0.8	800	3	55	8	3.0	4
H396R	1,400	5	28	2	0.8	1.3	1,950	7	31	4	2.0	3
Src	>5,000	na	>5,000	na	0.8	1.3	>5,000	na	>5,000	na	6	8
Lyn	>5,000	na	>5,000	na	2.8	4.67	>5,000	na	>5,000	na	4.4	5.5

NOTE: Fold change refers to the fold difference in the IC_{50}, relative to wild type, which is set to 1.
Abbreviations: WT, wild type; na, not applicable; nd, not determined.

T315I, which was completely insensitive to AMN107 (highest concentration tested: 5,000 nmol/L). To facilitate comparisons within Table 1, the IC_{50} data are also expressed as fold change relative to a baseline of one for wild-type Abl kinase. By viewing the data in this way, it is apparent that the overall pattern of mutant sensitivity to AMN107 closely parallels that of imatinib. Because imatinib and AMN107 are predicted to share an absolute requirement for a specific inactive Bcr-Abl conformation and to bind in very similar ways, it is logical to expect largely the same pattern of effectiveness for AMN107 as for imatinib, but with the range of effectiveness shifted to a value more than an order of magnitude lower than that of imatinib. In contrast, BMS-354825 potently inhibited wild-type Abl kinase and all mutants except T315I over a narrow range ($IC_{50} \leq 1.7$ nmol/L). We also did a complete set of Abl kinase autophosphorylation assays with each of the inhibitors and obtained similar results to those from peptide substrate assays (Table 1). Thus, biochemical assays establish that AMN107 and BMS-354825 directly target wild-type and mutant Abl kinase domains and inhibit autophosphorylation and substrate phosphorylation in a concentration-dependent manner. Similar assays with the Src family kinases Src and Lyn (17, 18), which is expressed at high levels in primary CML blast crisis cells, showed that BMS-354825 is a potent inhibitor of these Src family kinases, whereas imatinib and AMN107 are inactive against these kinases (Table 1).

To extend the results with isolated kinase domains into a cellular context, we carried out cellular proliferation assays using Ba/F3 cells expressing wild-type or kinase domain mutants of Bcr-Abl. Similar to the data using isolated kinase domains, AMN107 inhibited the growth of cells expressing wild-type Bcr-Abl with 20-fold higher potency than imatinib (IC_{50}: 13 versus 260 nmol/L); BMS-354825 (IC_{50}: 0.8 nmol/L) displayed 325-fold greater potency compared with imatinib against cells expressing wild-type Bcr-Abl (Fig. 1; Table 2). For both inhibitors, similar improvements were maintained for all imatinib-resistant mutants tested, with the notable exception of T315I. We have previously reported that inhibition of Bcr-Abl by imatinib or the Src/Abl inhibitor AP23464 results in the accumulation of cells expressing WT Bcr-Abl in the G_0-G_1 cell cycle phase (19). We confirmed that this is also the case for AMN107 in the current study (data not shown).

The data for AMN107 is summarized in Fig. 2, which shows a model of AMN107 in complex with Abl kinase mutant M351T, and shows that the sensitivity of Bcr-Abl mutants to AMN107 segregates into four categories: high ($IC_{50} \leq 70$ nmol/L: M244V, G250E, Q252H, F3llL, F317L, M351T, V379I, L387M, H396P, H396R), medium ($IC_{50} \leq 200$ nmol/L: Y253F, E255K, F359V), low ($IC_{50} \leq 450$ nmol/L: Y253H, E255V), and insensitive ($IC_{50} > 2$ μmol/L: T315I). This pattern is highly reminiscent of the corresponding ranking of imatinib sensitivities (Table 2) and expected given the highly related structures of these two compounds and the binding

Table 2. Imatinib, AMN107, and BMS-354825 IC_{50} values (nmol/L) for cellular proliferation and cellular Bcr-Abl tyrosine phosphorylation assays

	Ba/F3 cellular assays								
	Cellular proliferation						Bcr-Abl tyrosine phosphorylation		
	Imatinib		AMN107		BMS-354825		Imatinib	AMN107	BMS-354825*
	IC_{50} (nmol/L)	Fold change	IC_{50} (nmol/L)	Fold change	IC_{50} (nmol/L)	Fold change	IC_{50} (nmol/L)	IC_{50} (nmol/L)	IC_{50} (nmol/L)
WT Bcr-Abl	260	1	13 (28)†	1	0.8 (2.6)†	1	280	10	<10
M244V	2,000	8	38 (56)	3	1.3 (1.8)	2	500	8	<10
G250E	1,350	5	48 (152)	4	1.8 (3)	2	1,000	7	10-100
Q252H	1,325	5	70 (156)	5	3.4 (11.2)	4	1,500	15	<10
Y253F	3,475	13	125 (215)	10	1.4 (3)	2	4,200	55	<10
Y253H	>6,400	>25	450 (1,024)	35	1.3 (3)	2	>5,000	155	<10
E255K	5,200	20	200 (415)	15	5.6 (12.8)	7	5,000	70	10-100
E255V	>6,400	>25	430 (2,000)	33	11 (27)	14	>5,000	250	<10
F311L	480	2	23 (48)	2	1.3 (2.8)	2	600	44	nd
T315l	>6,400	>25	>2,000	>154	>200	>250	>5,000	>5,000	>1,000
F317L	1,050	4	50 (116)	4	7.4 (15)	9	400	47	<10
M351T	880	3	15 (31)	1.2	1.1 (3)	1.4	500	8	<10
F359V	1,825	7	175 (385)	13	2.2 (4.8)	3	3,100	43	10-100
V379l	1,630	6	51 (115)	4	0.8 (1.6)	1	800	15	nd
L387M	1,000	4	49 (115)	4	2 (4)	3	2,700	33	nd
H396P	850	3	41 (69)	3	0.6 (1.2)	0.8	2,700	70	nd
H396R	1,750	7	41 (73)	3	1.3 (2.7)	2	1,000	22	<10
Parental Ba/F3	>6,400	>25	>2,000	>154	>200	>250	ns	ns	nd

NOTE: Fold change refers to the fold difference in the IC_{50}, relative to wild type, which is set to 1.
Abbreviation: ns, no signal detected, Bcr-Abl not expressed.
*Estimated from ref. (14).
† Values in parentheses are IC_{90} values, given for AMN107 and BMS-354825 only.

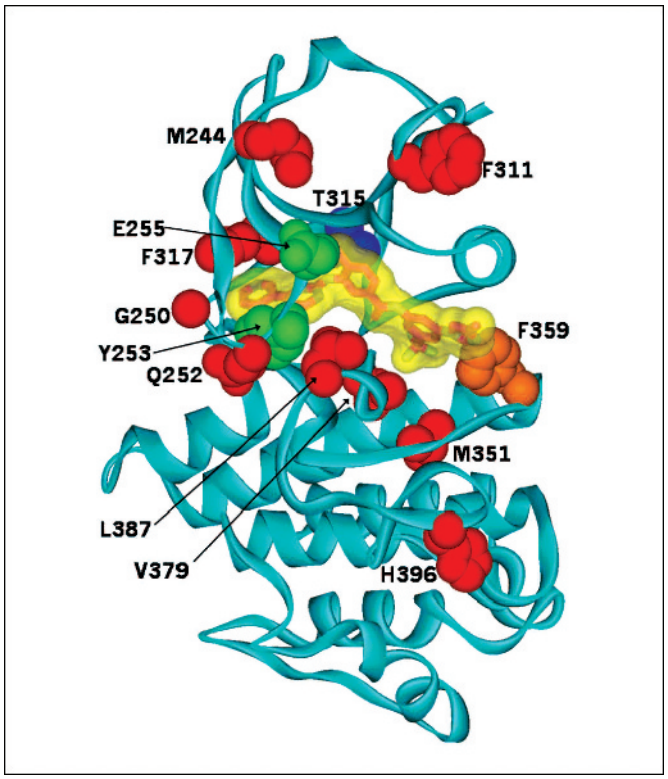

Figure 2. AMN107 in complex with kinase domain of Abl mutant M351T. Schematic diagram showing the locations of residues on Abl kinase corresponding to imatinib-resistant mutant forms of Bcr-Abl detected in patients. The residues are color coded according to their sensitivity to inhibition by AMN107, which is shown as a tube representation with a transparent yellow surface, as bound in a crystal structure of M351T Abl kinase (*light blue*). Mutations of residues shaded in red are highly sensitive to AMN107 ($IC_{50} \leq 70$ nmol/L: M244V, G250E, Q252H, F3llL, F317L, M351T, V379I, L387M, H396P, H396R), residues in orange show medium sensitivity ($IC_{50} \leq 200$ nmol/L: Y253F, E255K, F359V), residues in green show low sensitivity ($IC_{50} \leq 450$ nmol/L: Y253H, E255V), and the blue residue (T315I) is insensitive to AMN107 ($IC_{50} > 2$ μmol/L). Note that the level of AMN107 sensitivity at positions 253 and 255 (*green*) is dependent on the specific amino acid substitution. Thus, mutants Y253F and E255K fall in the medium (*orange*) classification, whereas Y253H and E255V comprise the low (*green*) category.

constraints they share. However, there are a few differences in the fold differences in sensitivities of the mutants to imatinib and AMN107. This is particularly noteworthy for M351T. Due to the structural differences between AMN107 and imatinib, this residue comes in close proximity to imatinib, but is less critical for coordinating binding of AMN107. BMS-354825, on the other hand, was found to be an extremely potent inhibitor of proliferation in cells expressing all mutants except T315I. As in the biochemical assays, the range of IC_{50} values for cells treated with BMS-354825 was narrow (0.8-11 nmol/L), in line with the proposal that the Bcr-Abl structural requirements for binding BMS-354825 are much less stringent than for imatinib family members.

Cellular Bcr-Abl tyrosine phosphorylation immunoblot analysis confirmed that AMN107 is much more potent than imatinib in reducing Bcr-Abl tyrosine phosphorylation levels in cells expressing wild-type Bcr-Abl and all imatinib-resistant mutants except T315I (Table 2; Supplementary Fig. S1). As a basis of comparison, published values for BMS-354825 are included in Table 2 (14). With respect to AMN107, the highly imatinib-resistant mutants Y253H (AMN107 IC_{50}: 155 nmol/L) and E255V (AMN107 IC_{50}: 250 nmol/L) were the least sensitive mutants. For all other mutants, treatment with 250 nmol/L AMN107 resulted in complete (>95%) inhibition of the phosphorylated Bcr-Abl tyrosine kinase signal (Supplementary Fig. S1). As expected, T315I was completely insensitive to AMN107 treatment ($IC_{50} > 5{,}000$ nmol/L) and no Bcr-Abl signal was detected in lysates from untransfected, parental Ba/F3 cells (data not shown).

In agreement with cellular proliferation and tyrosine phosphorylation assay results, AMN107 induced apoptosis at significantly lower concentrations than imatinib in cells expressing wild-type Bcr-Abl or any of the kinase domain mutants except T315I (Supplementary Fig. S2). At the highest AMN107 concentration tested (1.5 μmol/L), >90% of the cells were Annexin positive except in the cases of Y253H (65%), E255V (65%), and T315I (3%). Parental Ba/F3 cells and Ba/F3 cells expressing Bcr-Abl mutant T315I did not undergo apoptosis above vehicle-treated control levels in response to either inhibitor.

A conservative estimate for imatinib steady-state trough levels in patients treated with 400 mg imatinib per day is 1.5 μmol/L (2, 20). The pharmacokinetic profile and maximum tolerated dose for AMN107 have not been reported, and its effectiveness as a therapy for imatinib-resistant CML will depend on the concentration that can be reached in humans. Preliminary findings from the phase I/II study indicate that orally administered AMN107 at 200 mg per day is well tolerated with biological and marrow effects in some patients (21). Taking inhibition at or above the IC_{90} value (Table 2) as a benchmark for clinical benefit, AMN107 at a trough level of 1.5 μmol/L would be predicted to be an effective single agent therapeutic for cells expressing wild-type Bcr-Abl and all mutants tested except T315I. If a trough level of only 500 nmol/L is achievable, three mutants (Y253H, E255V, and T315I) are predicted to be substantially resistant.

BMS-354825 is ~325-fold more potent than imatinib and 16-fold more potent than AMN107 against wild-type Bcr-Abl. Again, invoking inhibition at or above the IC_{90} value (Table 2) as an indicator of clinical benefit, BMS-354825 would be predicted to be an effective single agent therapeutic for cells expressing wild-type Bcr-Abl and all mutants tested except T315I at a trough level of 50 nmol/L. Establishing and utilizing the minimum effective concentration may be especially important in the case of dual Src/Abl inhibitors, such as BMS-354825 due to concerns pertaining to off-target effects.

In summary, the cellular and biochemical experiments directly comparing AMN107 and BMS-354825 to imatinib show that both inhibitors are more potent than imatinib against all cell lines and purified Abl kinase domains tested except T315I. The mutants, other than T315I, that were least responsive to AMN107 in all three cellular assays were Y253H and E255V (Fig. 2). Analogous to imatinib, the extent of sensitivity to AMN107 depended on the specific substitution at a given position (e.g., E255V less sensitive than E255K to AMN107).

Both AMN107 and BMS-354825 hold promise for treating patients with imatinib-resistant CML except when the disease is driven by Bcr-Abl mutant T315I. Although both inhibitors efficiently block Bcr-Abl tyrosine kinase catalytic activity, they do so by binding to distinct, partially overlapping sites in the kinase domain and by placing different conformational requirements on the Abl kinase domain. If results of clinical trials and pharmacokinetic studies indicate that AMN107 and BMS-354825 are safe and effective, the feasibility of using these drugs in combination should be evaluated. In support of this approach, we recently investigated the use of imatinib in combination with BMS-354825 as a strategy for confronting drug resistance in

CML.[6] Specifically, treating Ba/F3 cells expressing wild-type or imatinib-resistant Bcr-Abl kinase domain mutants with various combinations of imatinib and BMS-354825 produced additive inhibitory effects. Even at imatinib concentrations above clinically achievable levels, no antagonism of the Src/Abl inhibitor was observed. A particularly appealing therapeutic option is to use a Bcr-Abl inhibitor cocktail containing these inhibitors as well as an as yet undiscovered T315I inhibitor. Advantages of combinatorial therapy are that clones resistant to one of the Bcr-Abl inhibitors may be vulnerable to another component of the cocktail (22), the potential to eliminate a wider spectrum of mutants, including those that predate therapeutic intervention (23), and eradication of a higher proportion of residual leukemic cells (8). Therefore, using Bcr-Abl inhibitor combinations to treat newly diagnosed, chronic-phase CML patients may represent the best strategy to prevent or significantly delay the onset of acquired drug resistance. In addition, these Bcr-Abl kinase inhibitors could also increase response rates and duration of response due to their increased potency.

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AMN107, a Novel Aminopyrimidine Inhibitor of Bcr-Abl, Has *In vitro* Activity against Imatinib-Resistant Chronic Myeloid Leukemia

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Abstract Resistance to or intolerance of imatinib in patients with Philadelphia chromosome–positive chronic myelogenous leukemia (CML) has encouraged the development of more potent Bcr-Abl inhibitors. AMN107 is a novel, orally bioavailable ATP-competitive inhibitor of Bcr-Abl. The effects of AMN107 were compared with those of imatinib on imatinib-sensitive (KBM5 and KBM7) and imatinib-resistant CML cell lines (KBM5-STI571$^{R1.0}$ and KBM7-STI571$^{R1.0}$). Compared with the antiproliferative activity of imatinib, AMN107 was 43 times more potent in KBM5 (IC_{50} of 11.3 versus 480.5 nmol/L) and 60 times more potent in KBM7 (IC_{50} of 4.3 versus 259.0 nmol/L) cells. IC_{50} for AMN107 and imatinib were 2,418.3 and 6,361.4 nmol/L, respectively, in KBM5-STI571$^{R1.0}$, and 97.2 and 2,497.3 nmol/L, respectively, in KBM7-STI571$^{R1.0}$ cells. AMN107 inhibited autophosphorylation of Bcr-Abl kinase more effectively than imatinib in all cell lines. They had similar effects on cell cycle progression and apoptotic response in these cell lines. Among severe combined immunodeficient mice bearing KBM5 cells, mean survival times of groups treated with 10, 20, and 30 mg/kg/d of AMN107, starting day 20 after leukemic cell grafting and continuing for 20 days, were 144%, 159%, and 182%, respectively, compared with controls. These results strongly support investigation of the clinical efficacy of AMN107 in patients with CML.

Identification of the central role of the *BCR-ABL* gene and its product, a chimeric protein having a tyrosine kinase domain rendered permanently active through autophosphorylation of its ATP-binding site (1), has stimulated the development of drugs targeting this pathway. The most effective of these drugs developed to date, imatinib (Gleevec; refs. 2–4), is now thought to conduct much of its activity by stabilizing an inactive, non–ATP-binding tyrosine kinase conformation (5, 6). By preventing activation of the kinase through autophosphorylation, imatinib critically impacts transmission of downstream regulatory signals resulting in target-specific inhibition of cell proliferation (2–4).

Imatinib has significant activity in all three phases of Philadelphia chromosome–positive chronic myelogenous leukemia (CML)—chronic, accelerated, and blastic (7–14). However, responses to imatinib in patients with more advanced disease are generally transient (7–10). In early chronic CML, high cytogenetic and molecular response rates are obtained (11–15), particularly with higher-dose imatinib regimens (15). If patients are unable to tolerate an adequate imatinib dose, response rates are lower (15). Resistance to imatinib may manifest at the hematologic, cytogenetic, or molecular level particularly in patients with blastic phase disease (7–9) and may be evidenced by the development of more advanced CML (8, 10). There is a wealth of information documenting heterogeneity of cellular mechanisms associated with inherent or acquired resistance to imatinib *in vitro* (16–24) and *in vivo* (16–18, 25–28). Imatinib resistance in CML can be caused by several mechanisms: increased expression or mutation of the kinase, resulting in reduction or loss of imatinib effectiveness; changes in the ability of the cells to maintain an effective intracellular imatinib concentrations at the cellular level; or emergence of additional mutations decreasing the dependence of CML on Bcr-Abl (16–18). *In vitro*, increasing drug concentrations can overcome some mechanisms of imatinib resistance (19), and clinical studies have replicated this finding in patients with CML (15, 29). These data have encouraged the search for more potent Bcr-Abl inhibitors for treatment of CML (30).

AMN107 is a novel inhibitor of the protein tyrosine kinase activity of Bcr-Abl. Structural biology studies have confirmed that the molecule binds to an inactive conformation of the protein, occupying a region of space analogous to that which would be occupied by ATP in the active conformation of the enzyme (Fig. 1). In animals, AMN107 is well absorbed following oral administration, has a good pharmacokinetic profile, and is well tolerated. Therefore, AMN107 may prove to be an effective treatment for Bcr-Abl–associated diseases, including CML. An important preclinical issue in the development of AMN107 is its

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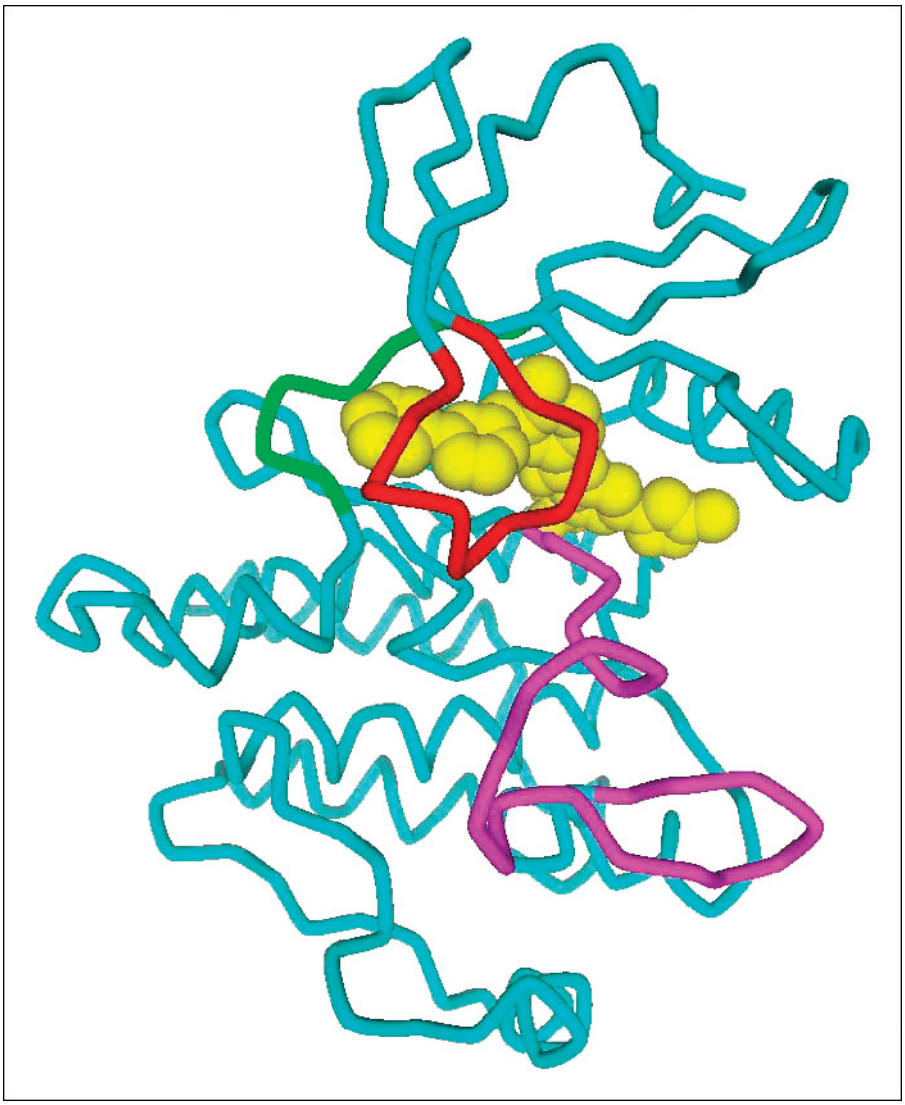

Fig. 1. Schematic diagram, based on an X-ray crystal structure, of the Abl kinase domain (*cyan*) in complex with AMN107 (*yellow*). The hinge region is shown in green, the glycine-rich loop is shown in red, and the activation loop is shown in magenta. In this structure, the activation loop adopts an inactive "DFG out" conformation similar to that observed in the imatinib-Abl complex. The picture was kindly supplied by S. Cowan-Jacob. Novartis Institutes of Biomedical Research, Basel, Switzerland.

activity relative to that of imatinib in both imatinib-sensitive and imatinib-resistant *BCR-ABL*–positive leukemic cells. We investigated the activity of AMN107, relative to imatinib, in a series of human Philadelphia chromosome–positive cell lines. These cell lines differ by degree of sensitivity to imatinib and by mechanisms of resistance to imatinib. We then assessed the efficacy of AMN107 in a severe combined immunodeficient (SCID) mouse model of CML.

Materials and Methods

Cell lines. Human cell lines used in this study included the Philadelphia chromosome–positive KBM5 (31) and KBM7 (32) cell lines, and the imatinib-resistant counterparts to these cell lines (19), KBM5-STI571$^{R1.0}$ and KBM7-STI571$^{R1.0}$. KBM5 cells contain multiple copies of the Philadelphia chromosome, express p210 Bcr-Abl protein, but lack normal c-Abl (33). KBM5-STI571$^{R1.0}$ cells have a T315I mutation in the ATP-binding site of Bcr-Abl (34). KBM7 cells have a near-haploid karyotype and express p210 Bcr-Abl. In KBM7-STI571$^{R1.0}$ cells, prominent features associated with resistance to imatinib are amplification of the *BCR-ABL* fusion gene and a corresponding increase in the Bcr-Abl protein (19). KBM5 and KBM7 cells differ in their response to imatinib: In KBM5 cells, imatinib does not elicit an apoptotic response but does cause G_0-G_1 cell cycle arrest; in KBM7 cells, imatinib causes increased apoptosis with no significant alterations in cell cycle progression (19).

The two imatinib-resistant cell lines were grown in the presence of 1 μmol/L concentration of imatinib at a proliferation rate similar to the proliferation rate of untreated parental cells. Acute myelomonocytic leukemic cell lines HL60 and U937 were used as *BCR-ABL*–negative controls. All cell lines were maintained in Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin solution (Gemini Bio-Products, Woodland, CA).

Drugs. Imatinib and AMN107 monohydrochloride were provided by (Novartis, East Hanover, NJ). Stock dilutions were prepared in DMSO and stored as 10 mmol/L solutions at −20°C. Only freshly thawed aliquots were used in experiments.

Proliferation inhibition assay. The 3-(4,5-dimethylthiazol-2-yl)-5-(carboxymethoxyphenyl)-2(4-sulophenyl)-2*H*-tetrazolium (MTS) assay (CellTiter 96 Aqueous One Solution Reagent, Promega Corporation, Madison, WI) was used to measure the effect of imatinib and AMN107 on proliferation of human leukemic cells *in vitro*. The assay was done according to the manufacturer's recommendations. Cells were seeded in triplicate in 96-well microtiter plates (Falcon, Franklin Lakes, NJ), incubated in the presence of different drug concentrations for 72 hours and the proliferation was measured as a percentage of the proliferation of untreated cells. Each experiment was done at least thrice. The drug

concentration resulting in 50% inhibition of cell proliferation (IC_{50}) was determined. The resistance index was calculated by dividing the IC_{50} for resistant cells by the IC_{50} for parental cells.

Detection of caspase-3 activity. The fluorogenic substrate PhiPhiLux G1D2 (Oncoimmunin, Gaithersburg, MD) was used to monitor caspase-3 activity by means of flow cytometry. Following treatment with imatinib or AMN107, cells were washed in Ca^{2+}-free PBS, resuspended in 25 μL of substrate solution, and incubated for 1 hour in a humidified chamber at 37°C in the dark. After incubation, cells were washed and resuspended in Ca^{2+}-free PBS. Propidium iodide was added to permit identification and exclusion of necrotic cells during analysis. Prepared cell samples were run on a flow cytometer (FACScan, Becton Dickinson, Franklin Lakes, NJ), and the resulting data were analyzed using the program CellQuest (Becton Dickinson).

Cell cycle analysis by flow cytometry. To investigate the impact of drug exposure on cell cycle distribution and apoptotic response, cells were exposed to imatinib or AMN107 for 24, 48, or 72 hours. Equipotent concentrations of the drugs, ranging between IC_{60} and IC_{90}, were chosen on the basis of the MTS-determined cell sensitivities. After drug treatment, cells were collected, washed in Ca^{2+}-free PBS, and fixed overnight in 70% ethanol at −20°C. Cells were then washed twice in cold PBS, resuspended in hypotonic propidium iodide solution [25 μg of propidium iodide per milliliter, 0.1% Triton X-100, 30 mg of polyethylene glycol per milliliter, and 3,600 units of RNase per milliliter, dissolved in 4 mmol/L sodium citrate buffer (pH 7.8); Sigma], and incubated for at least 1 hour at 4°C in the dark. Cellular DNA contents were determined by flow cytometry (FACScan). Cell cycle distribution was analyzed using ModFit LT software (Becton Dickinson). Cells with hypodiploid DNA content were considered apoptotic.

Immunoprecipitation of BCR-ABL. Following treatment of cells with AMN107 or imatinib for 3 hours, Bcr-Abl was immunoprecipitated as previously reported (4, 19). Aliquots of 20×10^6 cells per cell line were treated with different concentrations of imatinib or AMN107 for 3 hours. Cells were then collected, washed thrice with cold PBS, resuspended in 250 μL of lysis buffer, and incubated on ice for 1 hour. Cell lysate was then centrifuged at 14,000 rpm at 4°C for 40 minutes. Supernatant was removed and mixed with 25 μL of anti–Bcr-Abl P6D monoclonal antibody (kindly provided by Dr. R.B. Arlinghaus, University of Texas M. D. Anderson Cancer Center, Houston, TX) for 1 hour on ice. Fifty microliters of protein-A/G agarose slurry (sc-2003, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added; the solution was incubated overnight at 4°C with constant rotation. The antibody-protein complex was subjected to Western blotting.

Western blotting. The immunoprecipitated complex was eluted from the agarose with 2× loading buffer and run on a 9.5% SDS-PAGE gel. Western blotting was done overnight at 4°C after incubation of membranes with mouse antiphosphotyrosine (py99, Santa Cruz Biotechnology) diluted in 2.5% bovine serum albumin and 2.5% nonfat milk (dilution 1:8,000). The active band for phosphorylated Bcr-Abl was detected using conjugated HOURP-sheep anti-mouse antibody (NA931V, Amersham). Detection was done by enhanced

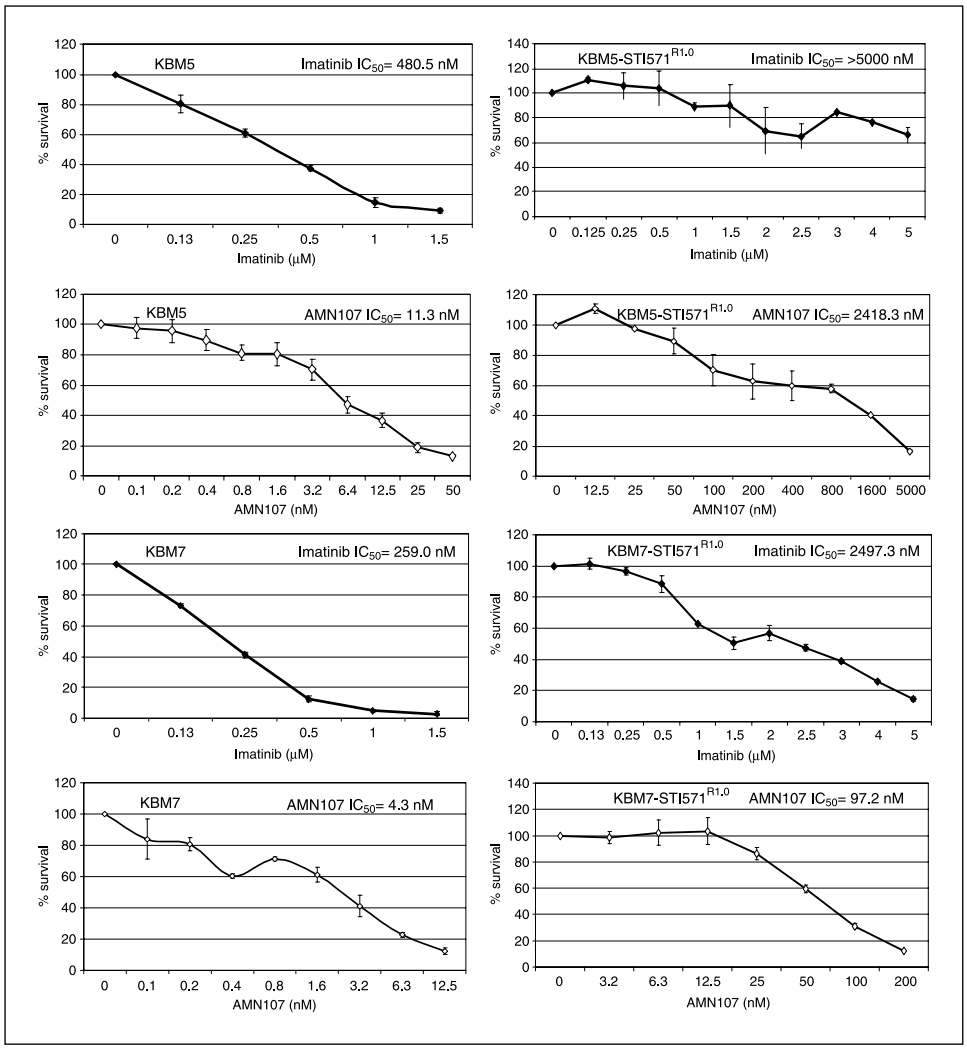

Fig. 2. Effect of increasing concentrations of imatinib and AMN107 on imatinib-sensitive (KBM5, KBM7) and imatinib-resistant (KBM5-STI571$^{R1.0}$, KBM7-STI571$^{R1.0}$) cell lines *in vitro*. *Points,* means of three MTS assays.

chemiluminescence as specified by the manufacturer (ECL, Amersham, Piscataway, NJ).

Stripped membranes were reprobed with mouse anti Bcr-Abl antibody (8E9; dilution 1:4,000) overnight at 4°C. The active band for Bcr-Abl was detected with HOURP-sheep anti-mouse antibody.

In vivo *experiments.* To investigate the efficacy of AMN107 *in vivo*, the SCID mouse model of clinically advanced blastic phase CML was selected (31). The experiments were done according to a research protocol approved by the Animal Care and Use Committee at M. D. Anderson Cancer Center. After acclimatization and 1 day after whole-body irradiation (250 cGy), SCID mice (5-6 weeks old, female, Taconic, Germantown, NY) were injected i.p. with 2.4×10^7 KBM5 cells (31) and randomly assigned to treatment groups. Starting 20 days after KBM5 cell inoculation, at a time when most mice were visibly ill and some had evident tumor masses, mice were treated with AMN107 at doses of 10, 20, or 30 mg/kg body weight i.p. daily for 20 days. AMN107 was dissolved in DL-lactic acid (L-6661, Sigma) and then diluted 80-fold with 5% glucose water (G8644, Sigma) containing 1% pluronic F68 solution (v/v; P5556, Sigma) to 0.14 mol/L, the final molarity of DL-lactic acid. Mice in the control group received vehicle only. Animals displaying signs of pain and suffering were euthanized by CO asphyxiation. Survival was measured to the time of spontaneous death or CO asphyxiation.

We expressed the median survival time of treated animals as a percentage of the median survival time of control animals. By National Cancer Institute criteria, if the mean survival time of treated animals exceeds 125% of that of control animals, the treatment has significant antitumor activity.

Results

Effect of imatinib on cell proliferation. The proliferation rates, viability, and saturation density of the resistant sublines were similar to those of the corresponding parental cell lines cultured concomitantly without imatinib. The IC_{50} values for imatinib in KBM5 and KBM7 cells were 0.48 and 0.24 μmol/L, respectively. The IC_{50} values for imatinib in KBM5-STI571$^{R1.0}$ and KBM7-STI571$^{R1.0}$ cells were 6.40 and 3.30 μmol/L, respectively. The calculated resistance indexes of 13.3 for KBM5-STI571$^{R1.0}$ cells and 13.8 for KBM7-STI571$^{R1.0}$ confirmed comparable degrees of imatinib resistance (Fig. 2).

Effect of AMN107 on cell proliferation and potency of AMN107 compared with that of imatinib. AMN107 was a more potent inhibitor of proliferation than imatinib in both KBM5 and KBM7 cells. The mean IC_{50} values for imatinib and AMN107 were 480.5 and 11.3 nmol/L, respectively, for KBM5 cells and 259.0 and 4.3 nmol/L, respectively, for KBM7 cells. Thus, AMN107 was 43 times as potent as imatinib in KBM5 cells and 60 times as potent as imatinib in KBM7 cells (Fig. 2). AMN107 was also a more potent proliferation inhibitor than imatinib in KBM5-STI571$^{R1.0}$ and KBM7-STI571$^{R1.0}$ cells. In KBM5-STI571$^{R1.0}$ cells, characterized by the presence of a T315I mutation (34), the mean IC_{50} values for imatinib and AMN107 were 6,361 and 2,418 nmol/L, respectively, indicating that AMN107 was thrice as potent as imatinib (Fig. 2). In KBM7-STI571$^{R1.0}$ cells, which have amplification of the *BCR-ABL* fusion gene and a corresponding increase in Bcr-Abl protein (19), mean IC_{50} values for imatinib and AMN107 were 2,497 and 97 nmol/L, respectively, indicating that AMN107 was 26 times as potent as imatinib (Fig. 2). The differences in the relative potency of AMN107 in these two imatinib-resistant cell lines suggested that different mechanisms of resistance to imatinib might determine the activity of AMN107. Sensitivity to AMN107 was Bcr-Abl selective. Philadelphia chromosome–negative acute myelomonocytic leukemia (U937, HL-60) cell lines lacked sensitivity to AMN107 or imatinib at concentrations of up to 10 μmol/L (data not shown).

Cell cycle analysis and caspase-3 activity. Exposure of KBM5 cells to AMN107 or imatinib resulted in an accumulation of cells in the G_0-G_1 phase of the cell cycle (Table 1). Similar but less pronounced perturbation of cell cycle progression was observed in KBM5-STI571$^{R1.0}$ cells exposed to imatinib or AMN107 (Table 1). AMN107 was more effective than imatinib in inducing accumulation of cells in G_0-G_1. In KBM7 and in KBM7-STI571$^{R1.0}$ cells, the effect of AMN107 or imatinib on cell cycle distribution was much less pronounced. Overall, at equipotent drug doses, the effect of AMN107 on cell cycle progression was similar to that of imatinib and was comparable in imatinib-sensitive and imatinib-resistant cells.

In KBM7 cells, intermediate to high concentrations of imatinib induced a time-dependent activation of caspase-3 and an increase in apoptosis. In KBM5 cells, these effects were not seen except at the longest (72 hours) imatinib exposure

Table 1. Percentage of cells in G_0-G_1 phase of cell cycle (A) and percentage of apoptotic cells (B) in sensitive and resistant sublines after treatment with AMN107 and imatinib at equipotent doses

Exposure time (h)	KBM5 (~IC_{90})			KBM5-STI571R1.0 (~IC_{60})			KBM7 (~IC_{90})			KBM7-STI571R1.0 (~IC_{80})		
	0	Imatinib (1,000 nmol/L)	AMN107 (25 nmol/L)	0	Imatinib (8,000 nmol/L)	AMN107 (3,000 nmol/L)	0	Imatinib (1,000 nmol/L)	AMN107 (25 nmol/L)	0	Imatinib (4,000 nmol/L)	AMN107 (160 nmol/L)
A. Percentage of cells in G_0-G_1												
24	32.0	73.9	75.7	25.9	43.0	63.9	30.3	25.7	32.6	34.9	54.8	59.7
48	32.1	87.4	88.4	27.5	41.0	55.7	29.0	35.5	41.5	29.5	48.0	49.5
72	24.5	76.3	83.3	29.6	52.0	60.8	26.5	36.8	36.6	34.3	45.1	48.9
B. Percentage of apoptotic cells												
24	4.8	5.3	5.6	9.0	11.4	17.2	11.5	24.8	24.7	28.7	37.9	36.6
48	3.6	8.3	7.9	8.3	13.7	29.7	9.2	44.7	40.6	28.8	49.0	61.1
72	3.8	14.7	17.6	10.3	15.1	30.0	15.0	59.4	53.5	25.9	53.8	67.4

NOTE: Propidium iodide staining and flow cytometry were used to determine cell cycle contents. PhiPhiLux G1D2 kit was used to detect caspase-3 activity by flow cytometry.

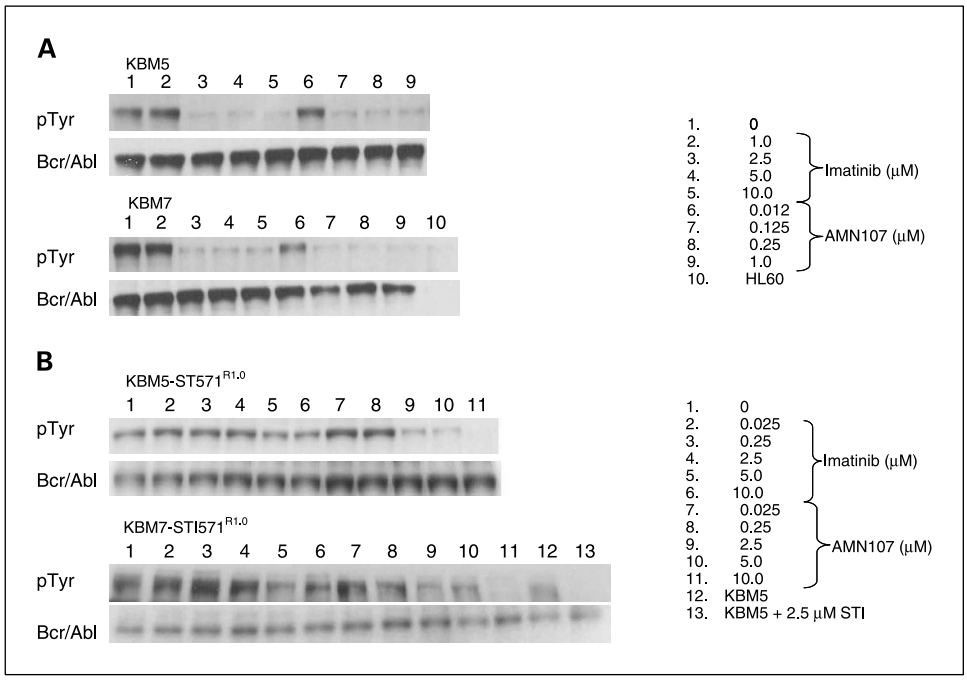

Fig. 3. Bcr-Abl expression and phosphorylation after imatinib or AMN107 exposure in imatinib-sensitive (*A*) and imatinib-resistant (*B*) leukemic cell lines. Cells (2×10^7) were exposed to different concentrations of imatinib or AMN107 for 3 hours, lysed, and whole cell lysates immunoprecipitated with Bcr-Abl antibody. Western blot analysis using antiphosphotyrosine was done on immunoprecipitated complex. After stripping, the membranes were reprobed with antibody to Bcr-Abl to assess the total amount of Bcr-Abl protein. HL60 cells were used as a Bcr-Abl – negative control. *STI*, imatinib.

(Table 1). A slight time-dependent increase in the apoptotic response was noted in KBM5-STI571$^{R1.0}$ cells exposed to AMN107 but not in KBM5-STI571$^{R1.0}$ cells exposed to imatinib. These findings indicate that AMN107 is either more potent than imatinib in inducing apoptosis by inhibiting mutated Bcr-Abl or that AMN107 and imatinib affect different pathways in these imatinib-resistant cells. In KBM7 and KBM7-STI571$^{R1.0}$ cells, imatinib and AMN107 induced similar degrees of apoptotic response, although the percentage of apoptotic cells seemed to be higher in cells treated with AMN107 for >24 hours (Table 1). Thus, at equipotent concentrations, imatinib and AMN107 induced a similar degree of apoptotic response in all but KBM5-STI571$^{R1.0}$ cells (Table 1).

Bcr-Abl expression and phosphorylation in sensitive and resistant cells. In KBM5 and KBM7 cells, a short exposure to imatinib completely inhibited Bcr-Abl phosphorylation at a concentration of 2.5 μmol/L; AMN107 achieved the same effect at 125.0 nmol/L, indicating ~20 times higher potency (Fig. 3A). In KBM5-STI571$^{R1.0}$ and KBM7-STI571$^{R1.0}$ cells, imatinib failed to completely inhibit Bcr-Abl phosphorylation even when cells were exposed to concentrations up to 10 μmol/L; AMN107 inhibited Bcr-Abl phosphorylation at 2.5 μmol/L (KBM5-STI571$^{R1.0}$) or 10 μmol/L (KBM7-STI571$^{R1.0}$; Fig. 3B), again indicating higher potency of AMN107 compared with imatinib.

***Effect of AMN107 on human chronic myelogenous leukemia cells* in vivo.** As previously established for the SCID mouse model of advanced blastic phase CML (31), mice developed ascites, macroscopically visible tumors in the peritoneum or at the site of inoculation or both, and dissemination of leukemia into organs. Mice tolerated administration of AMN107 well, without overt evidence of toxicity. Animals in the control group died between 23 and 32 days after tumor inoculation (median, 27 days). Median survival times for groups treated with AMN107 ranged from 39 to 49 days (Fig. 4). AMN107 was effective at all doses tested and the increases in survival times were dose related (Fig. 4; Table 2).

Discussion

In the study reported here, we evaluated AMN107, a novel aminopyrimidine inhibitor of Bcr-Abl, in imatinib-sensitive and imatinib-resistant CML cell lines and CML-derived cell lines growing as xenografts in nude mice. The resistant cell lines we chose manifested two of the best appreciated *BCR-ABL* – dependent mechanisms of imatinib resistance: (*a*) increased amounts of Bcr-Abl tyrosine kinase associated with amplification of the *BCR-ABL* gene (KBM7-STI571$^{R1.0}$ cells; refs. 19, 20 – 22) 22) and (*b*) a point mutation in the *BCR-ABL* domain coding for the ATP-binding site of the Bcr-Abl tyrosine kinase (KBM5-STI571$^{R1.0}$ cells; refs. 16, 19, 34).

In MTS assays, AMN107 was more effective in KBM7 cells, which express a low number of *BCR-ABL* gene copies and transcripts, than in KBM5 cells, which have a high degree of *BCR-ABL* gene amplification. AMN107 was 60 times more potent than imatinib in KBM7 cells and 43 times more potent in KBM5 cells. Given the large difference in the number of gene copies in these two cell lines, the sensitivity of the cells to AMN107 does not seem to be directly related to the extent of *BCR-ABL* gene amplification. In both these imatinib-naïve cell lines, AMN107 completely inhibited the autophosphorylation of the Bcr-Abl tyrosine kinase, with a potency ~20 times that of imatinib. Although resistance to imatinib was associated with a decrease in the effectiveness of AMN107, AMN107 remained more potent than imatinib in both resistant cell lines: It was 26 times more potent than imatinib in KBM7-STI571$^{R1.0}$ cells and thrice as potent in KBM5-STI571$^{R1.0}$ cells. AMN107 was also more effective than imatinib in inhibiting autophosphorylation of Bcr-Abl kinase in imatinib-resistant cells.

The resistant cell lines used in our study had similar degrees of resistance to imatinib (resistance index ~13). AMN107 was more effective in KBM7-STI571$^{R1.0}$ cells, with resistance presumed due to *BCR-ABL* gene amplification (19), than in

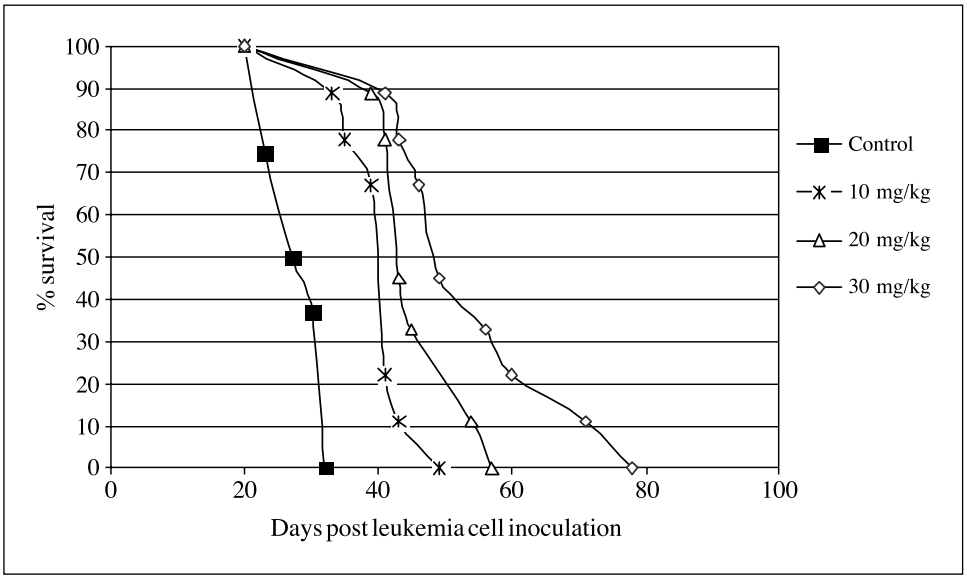

Fig. 4. Survival of SCID mice bearing KBM5 cells treated with AMN107. Irradiated female 5-week-old SCID mice were injected i.p. with 2.4×10^7 KBM5 cells on day 0. Starting on day 20, AMN107 was delivered i.p. daily for 20 days at a dose of 10, 20, or 30 mg/kg. Groups consisted of eight mice (control group) or nine mice (all experimental groups).

KBM5-STI571$^{R1.0}$ cells, characterized by T315I mutation (19, 34). This finding suggests that AMN107 has superior activity against cells with imatinib resistance due to quantitative rather than qualitative changes in the Bcr-Abl protein, perhaps because AMN107 interferes more effectively with activation of the native Bcr-Abl kinase than with activation of the kinase modified by T315I mutation in the activation site(s). The T315I mutation involves the ATP-binding pocket of the Abl kinase and confers a high degree of clinical resistance to imatinib. Of 15 *BCR-ABL* isoforms transfected into Ba/F3 cells, T315I was the only mutation that conferred complete resistance to a new inhibitor of SRC-family kinases, BMS-354825 (30). BMS-354825 treatment of animals grafted with Ba/F3 cells harboring the T315I mutant *BCR-ABL* failed to improve survival (30). These and our findings document the unique impact of this mutation on the interaction of Bcr-Abl with a variety of small-molecular inhibitors. Therefore, even the modest activity of AMN107 in this highly imatinib-resistant human CML model is of interest for future clinical studies and for elucidating the impact of subtle structural changes in Bcr-Abl on the binding and inhibitory activity of new agents.

In experiments focused on cell cycle analysis and caspase-3 activity, the cell cycle distribution and apoptotic responses induced by imatinib and AMN107 seemed generally similar, although in KBM5-STI571$^{R1.0}$ cells, treatment with AMN107 was associated with a more discernible increase in apoptosis than was treatment with imatinib.

In addition to a higher potency *in vitro*, AMN107 had significant antileukemia activity at a range of doses in the KBM5 xenograft model (31). The survival time of SCID mice bearing KBM5 cells treated with AMN107 was extended over that of controls. SCID mouse models of leukemia have been an effective aid in the development of subsequent clinical studies (35–38). We were less successful, however, in evaluating activity of AMN107 in a similar xenograft model using imatinib-resistant KBM5 STI571$^{R1.0}$ cells, the main difficulty being an inconsistent engraftment of resistant cells in the strain of SCID mice and experimental conditions used. Therefore, the weight of our report is with the *in vitro* data, which now seems to be supported by experience in the phase I clinical trial.

In conclusion, the currently reported data provided a rationale for clinical studies of AMN107 in patients with CML who are resistant to or intolerant of imatinib. The preliminary results of phase I studies of AMN107 in advanced stages of chronic myelogenous leukemia support the prediction of our *in vitro* studies by showing documented activity of AMN107 in imatinib-resistant patients (39).

Table 2. *In vivo* antitumor activity of AMN107 in SCID mice bearing KBM5 CML cells

Treatment	No. mice per group	Survival time, range (d)	Median survival time (d)	Treated survival as percentage of control survival*
Control	8	23-32	27	—
AMN107, 10 mg/kg	9	33-49	39	144%
AMN107, 20 mg/kg	9	39-57	43	159%
AMN107, 30 mg/kg	9	41-78	49	182%

*Median survival time of treated mice as percentage of median survival time of control mice.

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Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl

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Summary

The Bcr-Abl tyrosine kinase oncogene causes chronic myelogenous leukemia (CML) and Philadelphia chromosome-positive (Ph+) acute lymphoblastic leukemia (ALL). We describe a novel selective inhibitor of Bcr-Abl, AMN107 (IC_{50} < 30 nM), which is significantly more potent than imatinib, and active against a number of imatinib-resistant Bcr-Abl mutants. Crystallographic analysis of Abl-AMN107 complexes provides a structural explanation for the differential activity of AMN107 and imatinib against imatinib-resistant Bcr-Abl. Consistent with its in vitro and pharmacokinetic profile, AMN107 prolonged survival of mice injected with Bcr-Abl-transformed hematopoietic cell lines or primary marrow cells, and prolonged survival in imatinib-resistant CML mouse models. AMN107 is a promising new inhibitor for the therapy of CML and Ph+ ALL.

Introduction

Chronic myelogenous leukemia (CML) constitutes about 15% of adult leukemias and annually affects 1–2 people per 100,000. The disease progresses in three phases (O'Dwyer et al., 2002): the initial chronic phase, which has a median duration of 4–6 years, is a clonal myeloproliferative disorder characterized by a massive accumulation of functional granulocytes and immature myeloid cells in blood, marrow, and spleen. In untreated patients, the disease may progress via an accelerated phase, characterized by the appearance of undifferentiated blast cells in blood and bone marrow, to a terminal blast crisis phase of the disease. In the blastic phase, for which median survival is 18 weeks, more than 30% of the blood and bone marrow cells are blasts, and myeloid precursors may also form tumors in the lymph nodes, skin, and bone (Kantarjian and Talpaz, 1988).

The underlying cause of CML is the *BCR-ABL* oncogene, which results from a reciprocal t(9;22) chromosome translocation in a hematopoietic stem cell (Deininger et al., 2000). This fusion gene encodes a chimeric Bcr-Abl protein, in which the tyrosine kinase activity of Abl is constitutively activated. CML patients express the 210 kDa Bcr-Abl, whereas patients with Ph+ ALL usually express a p190 kDa Bcr-Abl protein arising from a different breakpoint in the *BCR* gene (Melo et al., 1994; Ravandi et al., 1999).

Expression of either p210 or p190 Bcr-Abl in hematopoietic cell lines abrogates the growth factor requirements for cell proliferation and survival by three major mechanisms: constitutive activation and enhancement of mitogenic signaling (Puil et al., 1994), reduced responsiveness to apoptotic stimuli (Bedi et al., 1994), and altered adhesion to stroma cells and extracellular matrix (Gordon et al., 1987). The constitutively activated tyrosine kinase of Bcr-Abl is essential for the transforming activity (Lugo et al., 1990).

Imatinib mesylate (Gleevec, STI571; Novartis Pharma AG) is a drug targeting the tyrosine kinase activity of Bcr-Abl (Buchdunger et al., 2001) and is an effective therapy for CML. After a median 19 months of treatment, newly diagnosed patients show an estimated 97% complete hematological response (CHR) and 76% complete cytogenetic response (CCR; no detectable Ph+ cells) (O'Brien et al., 2003). However, in ALL or in CML patients who have progressed to either the accelerated or blastic phases of the disease, response rates to imatinib

SIGNIFICANCE

Imatinib is an effective therapy for chronic phase CML, but advanced stage CML and Ph+ ALL patients frequently relapse due to the development of resistance caused by point mutations within the kinase domain of Bcr-Abl. New Abl kinase inhibitors with higher potency against native and imatinib-resistant mutants of Bcr-Abl could have substantial clinical utility. AMN107 is a high-affinity inhibitor that targets many imatinib-resistant mutants of Bcr-Abl, and which may therefore be useful in CML, reducing the incidence of resistant mutants, and in the treatment of imatinib-resistant disease.

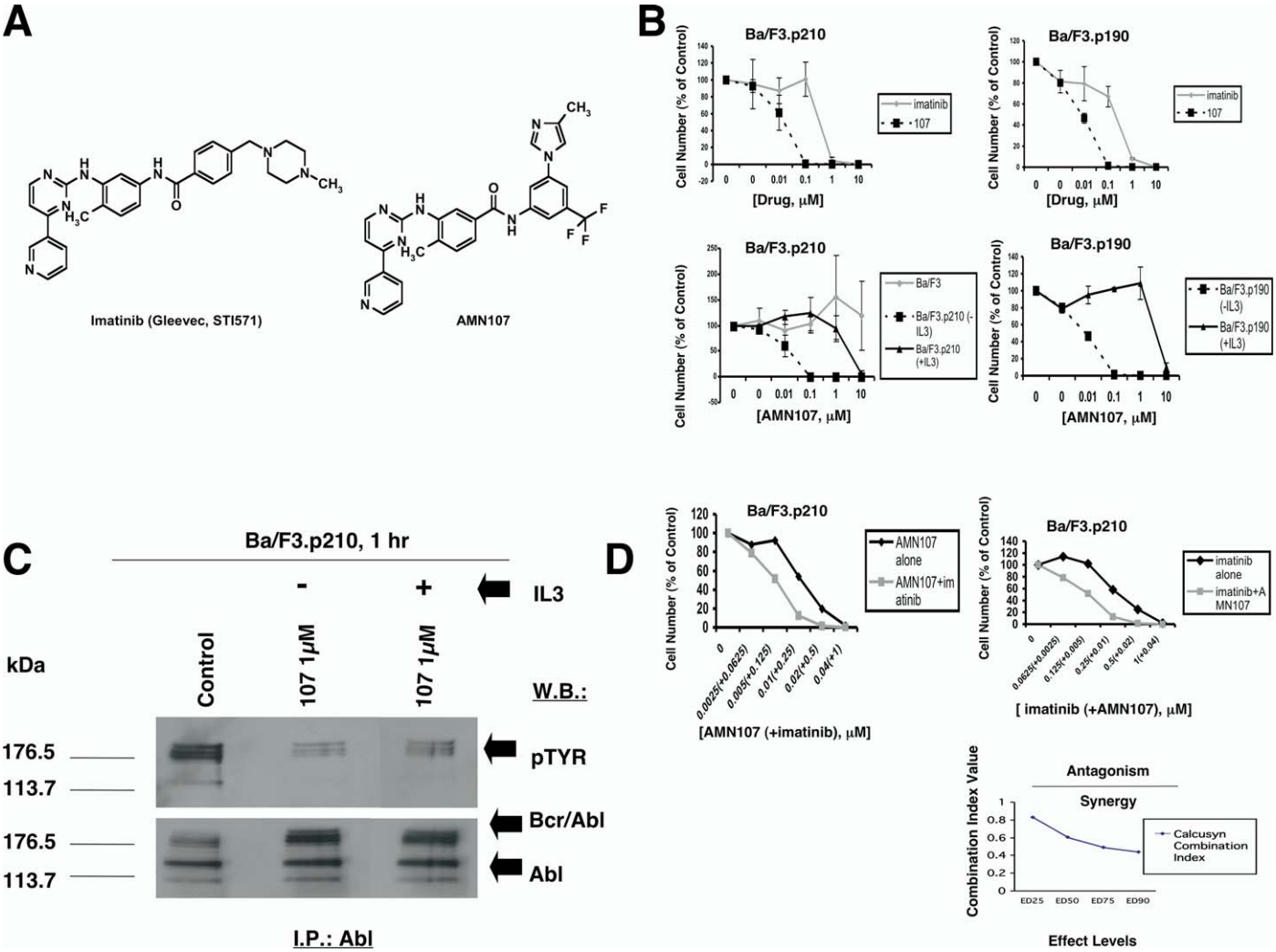

Figure 1. Effects of AMN107 on Bcr-Abl-expressing cell lines in vitro

A: The chemical structures of imatinib and AMN107.

B: Comparison of effects of 3-day treatment of Bcr-Abl-expressing cell lines with imatinib and AMN107 (upper panels), in the presence and the absence of WEHI conditioned medium as a source of IL-3 (lower panels). Bars are SEM, n = 2. In all dose-response curves, viable cell counts are represented as percent of control cells for each drug dose, and were determined by trypan blue exclusion assay.

C: Inhibition of Bcr-Abl autophosphorylation by AMN107 in Bcr-Abl-expressing cells. Autophosphorylation was determined by Bcr-Abl immunoprecipitation, followed by a pTyr immunoblot. Treatment with AMN107 was carried out in both the absence and the presence of IL-3.

D: Dose-response curves generated for combinations of AMN107 and imatinib against Ba/F3.p210 (upper panels), demonstrating synergy (lower panel). Combination studies using Ba/F3.p210 were performed in duplicate; shown are the data for one representative experiment.

therapy are significantly decreased and, of those who initially respond to treatment, many relapse within 12 months. Relapse is often associated with point mutations in Bcr-Abl that reduce the binding affinity of imatinib, or occasionally with amplification of the *BCR-ABL* gene (Gorre et al., 2001; Cowan-Jacob et al., 2004; le Coutre et al., 2000; Weisberg and Griffin, 2000; Mahon et al., 2000; Campbell et al., 2002; Hochhaus et al., 2002; Morel et al., 2003). Thus, there is a need for additional Bcr-Abl tyrosine kinase inhibitors that are more potent and active against imatinib-resistant Bcr-Abl mutants.

The molecular details of the interaction of imatinib with the Abl kinase domain have been revealed from crystal structures of complexes (Schindler et al., 2000; Nagar et al., 2003; Cowan-Jacob et al., 2004), and further supported by an analysis of the effects on binding of point mutations in the protein (Cowan-Jacob et al., 2004). Based upon this structural data, we hypothesized that more potent and selective compounds could be designed by incorporating alternative binding groups for the *N*-methylpiperazine group, while retaining an amide pharmacophore to maintain the H-bond interactions to Glu286 and Asp381 (Manley et al., 2004). This approach resulted in the discovery of AMN107 (Figure 1A), and here we detail the characterization of this molecule in vitro and in experimental Bcr-Abl-driven models of leukemia in mice. AMN107 has superior potency to imatinib as an inhibitor of Bcr-Abl in vitro and in vivo. Furthermore, we report on the efficacy of AMN107 in inhibiting some imatinib-resistant mutant forms of the kinase both in vitro and in vivo.

Table 1. Comparison of imatinib and AMN107 for effects on autophosphorylation and proliferation in cells

	Imatinib (IC_{50} in nM)		AMN107 (IC_{50} in nM)	
Kinase (cell type)	Autophosphorylation	Proliferation	Autophosphorylation	Proliferation
wt-32D + IL-3	NA	6140 ± 449, n = 14	NA	6134 ± 228, n = 3
wt-Ba/F3 + IL-3	NA	7384 ± 766, n = 5	NA	>10000, n = 15
p210 Bcr-Abl (32D)	194 ± 7, n = 94	334 ± 37, n = 23	20 ± 1, n = 53	9.2 ± 0.3, n = 3
Bcr-Abl (K562)	470 ± 59, n = 15	272 ± 27, n = 21	43 ± 15, n = 3	12 ± 2, n = 3
Bcr-Abl (Ku-812F)	466 ± 59, n = 7	80 ± 35, n = 13	60 ± 19, n = 5	8 ± 2, n = 6
p210 Bcr-Abl (Ba/F3)	220 ± 36, n = 12	649 ± 52, n = 18	21 ± 2, n = 5	25 ± 2, n = 49
p190 Bcr-Abl (Ba/F3)	122 ± 15, n = 3		33 ± 4, n = 3	
E255K Bcr-Abl (Ba/F3)	2108 ± 367, n = 3	>6000, n = 3	150 ± 12, n = 3	566 ± 107, n = 3
E255V Bcr-Abl (Ba/F3)	6499 ± 666, n = 13	6368 ± 892, n = 11	246 ± 36, n = 8	681 ± 72, n = 12
T315I Bcr-Abl (Ba/F3)	>10000, n = 20	7393 ± 157, n = 3	>10000, n = 12	>10000, n = 15
F317L Bcr-Abl (Ba/F3)	818 ± 99, n = 10	1583 ± 236, n = 14	41 ± 5, n = 8	80 ± 6, n = 11
M351T Bcr-Abl (Ba/F3)	595 ± 63, n = 10	1285 ± 180, n = 13	31 ± 4, n = 8	33 ± 3, n = 10
F486S Bcr-Abl (Ba/F3)	1230 ± 121, n = 10	2728 ± 676, n = 7	43 ± 6, n = 4	87 ± 4, n = 4
M244V Bcr-Abl (Ba/F3)	NA	3100^{*wst}, n = 1	NA	39^{wst}, n = 1
L248R Bcr-Abl (Ba/F3)	NA	$>20000^{*wst}$, n = 1	NA	919^{wst}, n = 1
Q252H Bcr-Abl (Ba/F3)	NA	2900^{*wst} , n = 1	NA	16^{wst}, n = 1
Y253H Bcr-Abl (Ba/F3)	NA	17700^{*wst} , n = 1	NA	751^{wst}, n = 1
E279K Bcr-Abl (Ba/F3)	NA	9900^{*wst} , n = 1	NA	75^{wst}, n = 1
E282D Bcr-Abl (Ba/F3)	NA	1300^{*wst} , n = 1	NA	39^{wst}, n = 1
V289S Bcr-Abl (Ba/F3)	NA	1400^{*wst} , n = 1	NA	7^{wst}, n = 1
L384M Bcr-Abl (Ba/F3)	NA	2800^{*wst} , n = 1	NA	39^{wst}, n = 1
G250E Bcr-Abl (Ba/F3)	NA	4800^{*wst} , n = 1	NA	219^{wst}, n = 1
PDGFR-α + PDGFR-β (A31)	74 ± 11, n = 11		71 ± 7, n = 20	
PDGFR-β (Tel Ba/F3	NA	39 ± 4, n = 8	NA	57 ± 7, n = 18
c-Kit exon13 mutant (GIST882)	96 ± 12, n = 7	120 ± 6, n = 15	200 ± 13, n = 19	160 ± 12, n = 17
c-Kit del 560-561 (Ba/F3)	27, n = 1	27 ± 2, n = 2	27 ± 3, n = 3	26 ± 1, n = 4
VEGFR-2 (CHO-VEGFR2)	>10000, n = 6		3720 ± 920, n = 5	NA
c-erbB-2 (BT-474)	>10000, n = 4		>10000, n = 2	>10000, n = 6
c-erbB-2 (Ba/F3-erbB-2)		$>3000^{AB}$, n = 2		$>3000^{AB}$, n = 2
Flt3-ITD (Ba/F3-NPOS)		$>3000^{AB}$, n = 2		$>3000^{AB}$, n = 2
Ret (Ba/F3-PTC-3)		$>3000^{AB}$, n = 2		$>3000^{AB}$, n = 2
Met (Ba/F3-Tpr-Met)		$>3000^{AB}$, n = 2		$>3000^{AB}$, n = 2
IGF-1R (NWT-21)	>10000, n = 1		>10000, n = 4	
Ins-R (A14)	>10000, n = 1		>10000, n = 4	
FGFR-1 (Ba/F3-Bcr-FGFR1)		$>3000^{AB}$, n = 3		$>3000^{AB}$, n = 2
JAK-2 (Ba/F3-JAK-2)		$>3000^{AB}$, n = 2		$>3000^{AB}$, n = 2
Ras (Ba/F3-H-Ras-G12V)		$>3000^{AB}$, n = 2		$>3000^{AB}$, n = 2
NPM-Alk (Ba/F3-NPM-Alk cl. 1)		$>3000^{AB}$, n = 2		$>3000^{AB}$, n = 2
Akt (Ba/F3-MyrAkt, cl. 21)		>3000, n = 1		>3000, n = 2

The influence of compounds on kinase autophosphorylation or cell viability was calculated as percentage inhibition. Dose-response curves were used to calculate IC_{50} values, expressed as mean ± SEM, n = number of experiments. The antiproliferative activity was assessed using either the ATPlite assay kit (Perkin-Elmer) or, where indicated (^{AB}), the Alamar Blue assay kit (Biosource International Inc.). ^{wst}, WST-1 reagent (Roche), as previously described (Azam et al., 2003), was used to generate IC50 values. *, values as reported previously for imatinib (Azam et al., 2003)

Results

AMN107 selectively inhibits proliferation of Bcr-Abl-expressing cells and inhibits Bcr-Abl autophosphorylation

AMN107 inhibited the proliferation of Ba/F3 cells expressing p210- and p190-Bcr-Abl, or K562 and Ku-812F cells with IC_{50} values $\leq$12 nM (Figure 1B and Table 1). AMN107 was at least 10-fold more potent than imatinib against Bcr-Abl expressing cell lines, but like imatinib, did not inhibit untransformed Ba/F3 cells growing in IL-3 at concentrations $\leq$6 μM. Ba/F3 cells expressing p210 and p190 Bcr-Abl could also be partially rescued from the inhibitory effects of up to 1 μM AMN107 when cultured in the presence of IL-3 (Figure 1B). At concentrations of AMN107 higher than 1 μM, cells died even in the presence of IL-3 (Figure 1B and Supplemental Data). Inhibition of cell growth by AMN107 was associated with induction of apoptosis (Supplemental Data). AMN107 did not reduce the formation of normal human myeloid and erythroid progenitor cells (assayed as CFU-GM [colony-forming unit, granulocyte/macrophage] and BFU-E [burst-forming unit, erythroid]) at concentrations $\leq$100 nM, but resulted in ~50% reduction in colony numbers at 1 μM (Supplemental Data). In preliminary mouse tolerability studies, AMN107 (50 or 150 mg/kg p.o. b.i.d. for 14 days) was well tolerated, and there were no significant reductions in blood erythrocytes, reticulocytes, leukocytes, or platelets.

AMN107 potently inhibited proliferation of cells transformed by activated mutants of Arg, Kit, PDGFRα, and PDGFRβ (Table 1 and Supplemental Data), but had no significant effect on the viability or proliferation of Ba/F3 cells rendered factor-independent through expression of activated forms of erbB2, Flt3, Met, Ret, IGF-1R, or NPM-ALK, and several other tyrosine kinases at concentrations $\leq$3 μM (Table 1). Taken together, these data suggest that AMN107 selectively inhibits Bcr-Abl, Kit, and PDGFR tyrosine kinases, but does not significantly affect any of the kinases required for IL-3 signaling, such as JAK2, or a

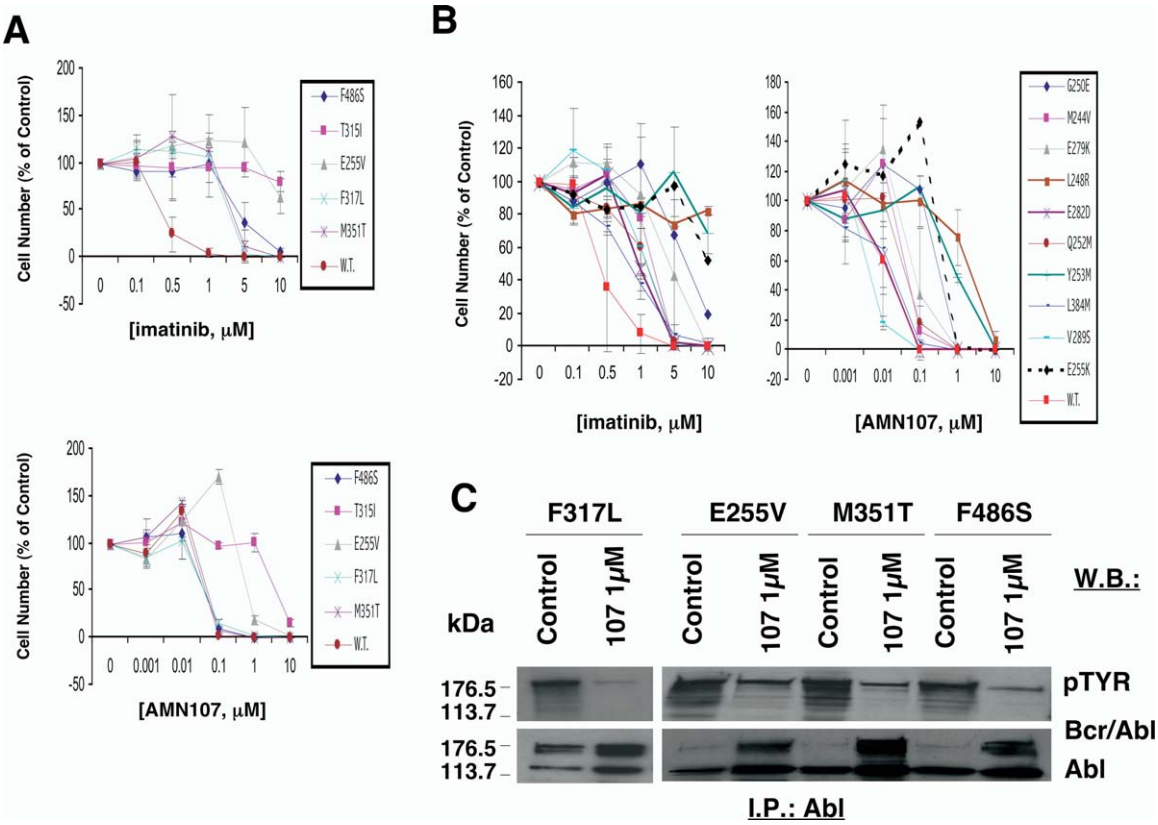

Figure 2. Effects of AMN107 on imatinib-resistant and wild-type Bcr-Abl-expressing cells in vitro

A and B: Treatment of Bcr-Abl mutant-expressing and wild-type Bcr-Abl-expressing Ba/F3 cells with imatinib versus AMN107. **A:** Upper panel: Imatinib treatment; bars are SEM, n = 3. Lower panel: AMN107 treatment, n = 3. **B:** Left panel: Imatinib treatment. Right panel: AMN107 treatment, n = 2.
C: Inhibition of Bcr-Abl autophosphorylation by AMN107 in imatinib-resistant Bcr-Abl-expressing cells. Bcr-Abl autophosphorylation was determined as in Figure 1C.

broad variety of other receptor tyrosine kinases or tyrosine kinase oncogenes. Furthermore, in cell-free assays, AMN107 at concentrations <3 μM had no significant effect on transphosphorylation catalyzed by the GST-fusion kinase domains of CDK-1, FGFR-1, Flt-3, HER-1, IGF-1R, InsR, c-Met, PKA, PKB, c-Src, Tie-2, or VEGFR-2 (data not shown).

Imatinib and AMN107 were compared quantitatively via capture ELISA for their effects on cellular Bcr-Abl autophosphorylation activity and on Bcr-Abl-dependent cell proliferation (Table 1). AMN107 (IC_{50} 20–60 nM) was consistently more potent than imatinib (IC_{50} 120–470 nM) in inhibiting Bcr-Abl tyrosine kinase activity in cell lines. In the presence of IL-3, AMN107 inhibited autophosphorylation but no longer inhibited cell viability (Figures 1B and 1C).

Exposure of Ba/F3.p210 (Figure 1D) or Ba/F3.p190 (data not shown) cells to both AMN107 and imatinib simultaneously across a range of concentrations resulted in synergistic cytotoxicity.

AMN107 selectively inhibits the proliferation of imatinib-resistant Bcr-Abl-expressing cells and autophosphorylation of imatinib-resistant Bcr-Abl mutants

Imatinib and AMN107 were compared for effects on cells expressing point mutants of Bcr-Abl associated with imatinib resistance in patients (Hofmann et al., 2002; Shah et al., 2002; von Bubnoff et al., 2002; Branford et al., 2002; Roche-Lestienne et al., 2002). A series of Ba/F3 cell lines stably expressing E255V, T315I, F317L, M351T, F486S, G250E, M244V, L248R, Q252H, Y253H, E255K, E279K, E282D, V289S, and L384M Bcr-Abl mutants were generated after transfection with expression plasmids containing each mutant (Azam et al., 2003).

As reported, imatinib effectively inhibited proliferation of Ba/F3 cells expressing nonmutated Bcr-Abl, but was substantially less active against cells expressing any of these Bcr-Abl point mutants (IC_{50} values $\geq$1 μM; Figure 2A, 2B, and Table 1) (Cowan-Jacob et al., 2004). In contrast, AMN107 inhibited proliferation of Ba/F3 cells expressing G250E, E255K(V), F317L, M351T, F486S, M244V, L248R, Q252H, Y253H, E255K, E279K, E282D, V289S, and L348M Bcr-Abl mutants at <1 μM concentrations (Table 1). However, the T315I mutant remained resistant to AMN107 below 10 μM. Also, four of these mutants had IC_{50} values >500 nM (E255K[V], L248R, Y253H), indicating intermediate sensitivity. AMN107-induced cytotoxicity of Ba/F3 cells expressing E255V, F317L, M351T, and F486S mutants could be rescued by the addition of IL-3, suggesting that the critical target was probably Bcr-Abl itself (Supplemental Data).

Similarly, AMN107, in contrast to imatinib, potently inhibited the tyrosine autophosphorylation of the E255K, E255V, F317L, M351T, and F486S Bcr-Abl mutants with mean IC_{50} values of

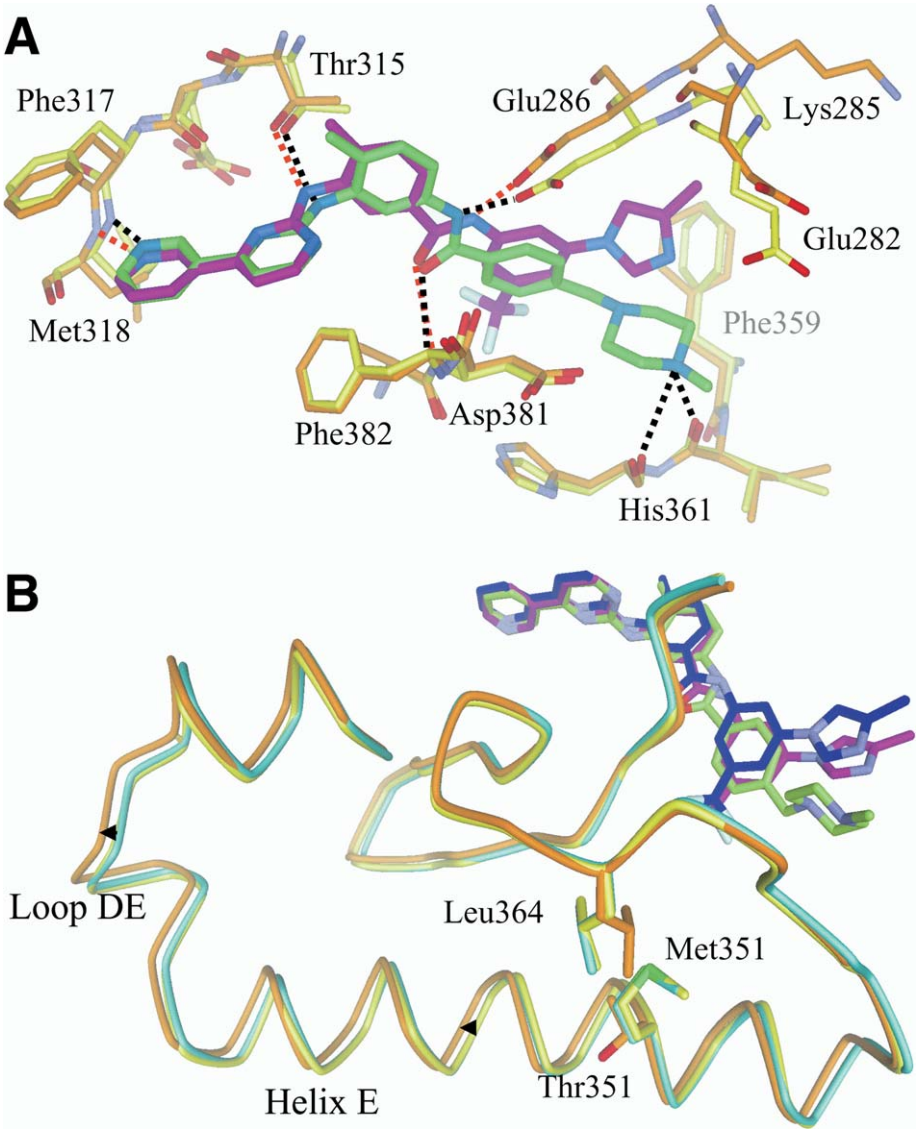

Figure 3. Abl-AMN107 complex

A: Superposition of AMN107 (magenta) bound to Abl^{M351T} (orange), and imatinib (green) bound to Abl (yellow). H bonds within the AMN107-Abl^{M351T} complex are depicted as dashed red lines, whereas those in the imatinib complex are shown in black. The variability in the positions of side chains from the C-helix (top right corner) is due to crystal contacts that influence the position of the N-terminal lobe of the kinase. The methyl-imidazole group of AMN107 packs in a hydrophobic pocket formed by these residues with the nitrogen exposed to solvent.

B: Superposition of parts of the backbone structures of imatinib-Abl (yellow), AMN107-Abl^{M351T} (orange), and AMN107-Abl (cyan). The inhibitors are shown in green, magenta and blue, respectively. The small black arrows show the shifts within helix E and the preceding loop, DE.

150, 246, 41, 31, and 43 nM, respectively (Figure 2C and Table 1). These effects were not associated with a decrease in Abl or Bcr-Abl protein levels (Figure 2C and Supplemental Data). Autophosphorylation of the T315I mutant was unaffected by AMN107. Overall, these results indicate that many imatinib-resistant Bcr-Abl mutants are relatively or absolutely more sensitive to AMN107.

AMN107 also inhibited ligand-induced cellular PDGFR kinase activity and the growth of cells whose proliferation is dependent on activated forms of PDGFR with mean IC_{50} values of 71 and 57 nM (Table 1). In addition, AMN107 inhibited constitutively activated (autophosphorylated) c-Kit, harboring gain-of-function mutations in exon-13 (GIST882 cells) or exon-11 (juxtamembrane domain deletion 560–561, expressed in Ba/F3 cells), with mean IC_{50} values of 200 nM and 27 nM, respectively (Table 1). The inhibition of these cellular kinase activities was well correlated with the effects on cellular viability and cell proliferation, and the results are comparable with those obtained with imatinib (Table 1 and Supplemental Data).

Crystallographic structural analysis of AMN107-Abl complexes

Structural analysis of the binding of AMN107 and imatinib to Abl can explain the differential sensitivity of Abl point mutations to these two compounds. The binding modes of AMN107 to the tyrosine kinase domains of both wild-type Abl and the Abl^{M351T} mutant were elucidated from the crystal structures of the complexes (Figure 3). Additional details of the structure of Abl in complex with AMN107 are described elsewhere (P.W.M., unpublished data). Here we report two structures of AMN107 in complex with Abl^{M351T} solved in two different space groups, such that common differences between the structures and the wild-type Abl-AMN107 complex probably result from the mutation and not from artifacts such as crystal contacts. In all of the structures, AMN107 was found to bind to the inactive conformation of Abl as observed for imatinib (Nagar et al., 2003). In the Abl-AMN107 structure, the helix containing Met351 (residues 338–358) is locked into position by the methionine side-chain hooking into a cavity adjacent to Leu364. However,

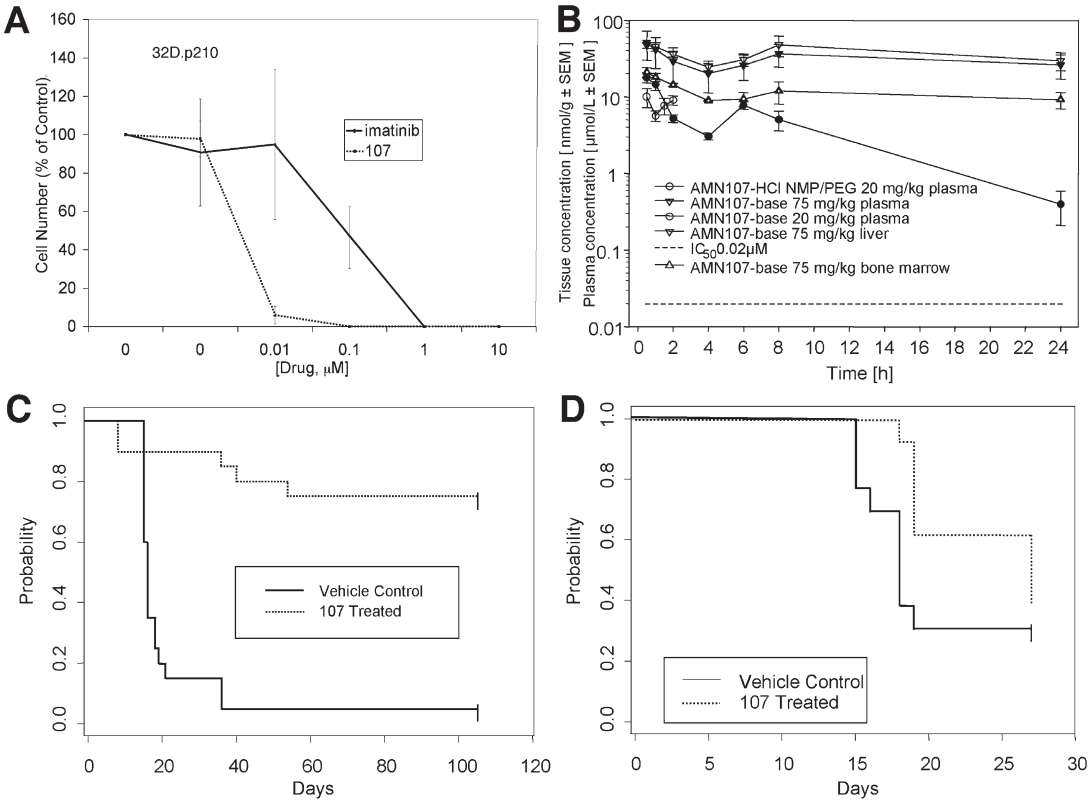

Figure 4. In vivo investigation of AMN107 against 32D.p210 and 32D-E255V cells

A: 32D.p210 cells following 3 days of treatment with increasing concentrations of imatinib or AMN107. Bars are SEM, n = 4.

B: Mean plasma concentrations of AMN107-base following a single oral dose of either 20 mg/kg or 75 mg/kg to naïve mice. Different groups (n = 4) of female OF1 mice received a single oral dose of 20 mg/kg in the respective formulation (based on the free base). The AMN107-base was formulated in 10% N-methyl pyrrolidone/90% PEG200 (v/v). At the allotted times, mice were sacrificed, blood and tissue removed, and the concentration of compound determined by reversed-phase HPLC/MS-MS analysis. Bars are SEM, n = 4. The limit of quantitation (LOQ) was set to 5 ng/ml. Antiproliferative IC_{50} against Ba/F3 Bcr-Abl p210 cells (0.022 μM) is inserted.

C: Kaplan-Meier plot of survival for 32D.p210-injected Balb/c mice treated with either vehicle or AMN107.

D: Kaplan-Meier plot of survival for 32D-E255V-injected Balb/c mice treated with either vehicle or AMN107.

within Abl^{M351T}, this hook is absent, and the whole helix can translate up to 1 Å along its axis. This translation slightly shifts the residues in the loop preceding helix E, which are involved in contacting the SH2 domain in the assembled inactive state (Nagar et al., 2003), and also form part of the myristate binding site.

AMN107 prolongs survival of mice with Bcr-Abl+ leukemias

The pharmacokinetic properties of AMN107 were evaluated following single administration of a solution of either 20 or 75 mg/kg in 10% NMP/90% PEG300 by gavage to naïve female Balb/c mice. Sample analysis was based on an HPLC-MS method with LOQ 0.01 μM, and pharmacokinetic parameters were calculated from the concentration versus time profiles. AMN107 was orally bioavailable and well absorbed, with mean plasma levels of 5.6, 5.4, and 0.4 μM, or 29, 30, and 25 μM, at 2, 8, and 24 hr following administration of either 20 mg/kg (AUC_{0-24h} 82 hr/μmol/l) or 75 mg/kg (AUC_{0-24h} 641 hr/μmol/l), respectively (details are presented in Figure 4B and Table 2).

To directly assess the in vivo antitumor efficacy of AMN107, we developed mouse models of CML in which tumor burden was quantified by noninvasive imaging of the luminescent tumor cells. Murine 32D.p210 cells were engineered to stably express firefly luciferase and evaluated for their responsiveness to imatinib and AMN107 in vitro: AMN107 inhibited the proliferation of 32D.p210 cells in vitro with a mean IC_{50} of 9 nM (imatinib IC_{50} 300 nM; Table 1 and Figure 4A).

Sublethally irradiated NOD-SCID mice were then inoculated with these cells and noninvasive imaging was used to serially assess tumor burden. Mice with established leukemia were divided into cohorts with equivalent tumor burden, and oral administration was initiated with AMN107 or vehicle (Figure 5). Mice with 32D.p210-Luc+ leukemia treated with AMN107 (100 mg/kg/day) showed a profound and rapid reduction in tumor burden (Figure 5). After 4 doses, there was a one-log reduction in overall leukemia burden in AMN107-treated mice, in comparison to a 1.5 log increase in tumor burden in vehicle-treated mice. The results suggested that AMN107 could reduce the accumulation of leukemic cells in marrow, spleen, lymph node areas, and liver.

To determine if this ability to suppress leukemic cell growth

Table 2. Pharmacokinetic parameters of AMN107/AA-salt following single oral administration of either 20 mg/kg or 75 mg/kg to naïve mice

PK parameters	20 mg/kg plasma	75 mg/kg plasma	75 mg/kg bone marrow	75 mg/kg liver
C_{max} (µmol/l; nmol/g)	17.92 ± 2.71	47.90 ± 14.34	21.05 ± 3.14	51.11 ± 2.85
C_{last} (µmol/l; nmol/g)	0.39 ± 0.18	29.69 ± 7.80	9.20 ± 2.22	26.18 ± 10.57
t_{max} (h)	0.5	0.5	0.5	0.5
t_{last} (h)	24	24	24	24
AUC_{0-24h} (h·µmol/l)	81.8	879	261	722
$AUC_{0-24h, dose}$ ([h·µmol/l] [mg/kg])	4.0	11.7	3.48	9.6

Area under the plasma concentration versus time curve (AUC) was calculated from the mean concentrations by linear/log trapezoidal rule using noncompartmental analysis (WinNonlin, Pharsight). The pharmacokinetic parameters Cmax, Clast, tmax, and tlast were determined by inspection of the data.

would prolong survival, AMN107 was administered to a larger cohort of Balb/c mice at an oral dose of 75 mg/kg/day over a 16-day period, commencing three days after injection of 32D.p210 cells. Vehicle-treated animals (19/20) developed a lethal disease (median survival 16 days, range 15–36 days), characterized by splenomegaly (Figure 4C). One control mouse failed to develop signs of leukemia, but was included in the survival analysis. Treatment with AMN107 resulted in the survival of 15/20 animals over 105 days of observation.

Five of the AMN107-treated mice either died or were sacrificed; median survival was not reached. All remaining mice were sacrificed at the planned end of the study (day 105), and were censored in the survival analysis. Body weights and spleen weights were available for 17/20 AMN107-treated mice, and were found to be within the normal range (median body weight 21.9 g, median spleen weight 0.085 g; spleen as a % of body weight: 0.4%). In comparison, vehicle-treated mice had a median body weight of 16.6 g and a median spleen weight of 0.51 g (spleen as % of body weight: 3.2%). Using the Wilcoxon rank sum test, these parameters differed significantly between the vehicle control and the AMN107-treated mice, with p values < 0.0001 for body weight, = 0.0001 for spleen weight, and <0.0001 for spleen as a percentage of body weight. p values were two-sided, and were obtained from the normal approximation to the Wilcoxon. Treatment with AMN107 was associated with a significant prolongation of survival, $p < 0.00001$. The log rank test was used to assess differences in survival.

The effects of AMN107 were also evaluated in a bone marrow transplant model. Bone marrow cells from normal Balb-c mice were transduced with Bcr-Abl and transferred to sublethally irradiated hosts. Such mice develop a reproducible myeloproliferative disease similar to human CML. Groups of mice (n = 12) were treated with vehicle (control), imatinib (125 mg/kg/day in two divided doses), or AMN107 (75 mg/kg daily) by oral gavage, which was started on day 8 following transplant. Mice were sacrificed when moribund as assessed by consistent standard criteria.

The animals in the control group developed splenomegaly and marked leukocytosis as observed previously (Mohi et al., 2004), and all were sacrificed by day 18 posttransplantation (Figure 6). There was a significantly prolonged survival in mice treated with either imatinib or AMN107, all of which were alive at the study end point 20 days after transplantation ($p < 0.001$, Figure 6A). Disease burden, as evidenced by spleen weights at the time of sacrifice, was compared for mice treated with imatinib and AMN107. There was a significant reduction in tumor bulk in mice treated with AMN107 ($p < 0.001$, Figure 6D). In contrast, imatinib failed to control spleen weights despite prolonged survival at 20 days.

AMN107 prolongs survival of mice with leukemia due to imatinib-resistant mutants of Bcr-Abl

The results shown in Figures 4–6 demonstrate that AMN107 is effective in prolonging survival in mice with Bcr-Abl+ leukemias. To determine if AMN107 would also prolong survival in mice with imatinib-resistant Bcr-Abl+ leukemias, we replaced native Bcr-Abl with E255V Bcr-Abl in both the 32D.p210 cell line and BMT models. The E255V mutant is known to be resistant to imatinib (Hofmann et al., 2002; Shah et al., 2002; von Bubnoff et al., 2002; Table 1 and Figure 2). An oral dose of 75 mg/kg/day AMN107 administered over a 16-day period, commencing three days after injection of parental 32D.p210-E255V

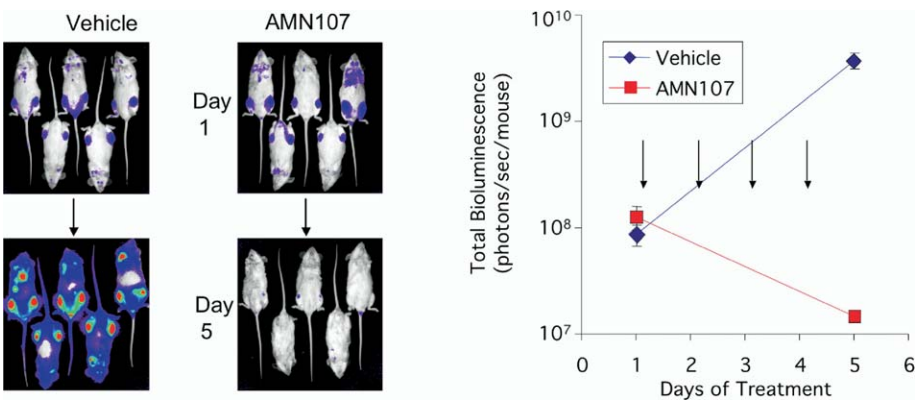

Figure 5. Efficacy of AMN107 against 32D.p210- and 32D-E255V-Luc+ cells in vivo

Left panel: Bioluminescence of vehicle- or AMN107-treated mice. Right panel: Quantitation of AMN107 effects against 32D.p210-Luc+ cells in vivo. Bars are SEM, n = 5.

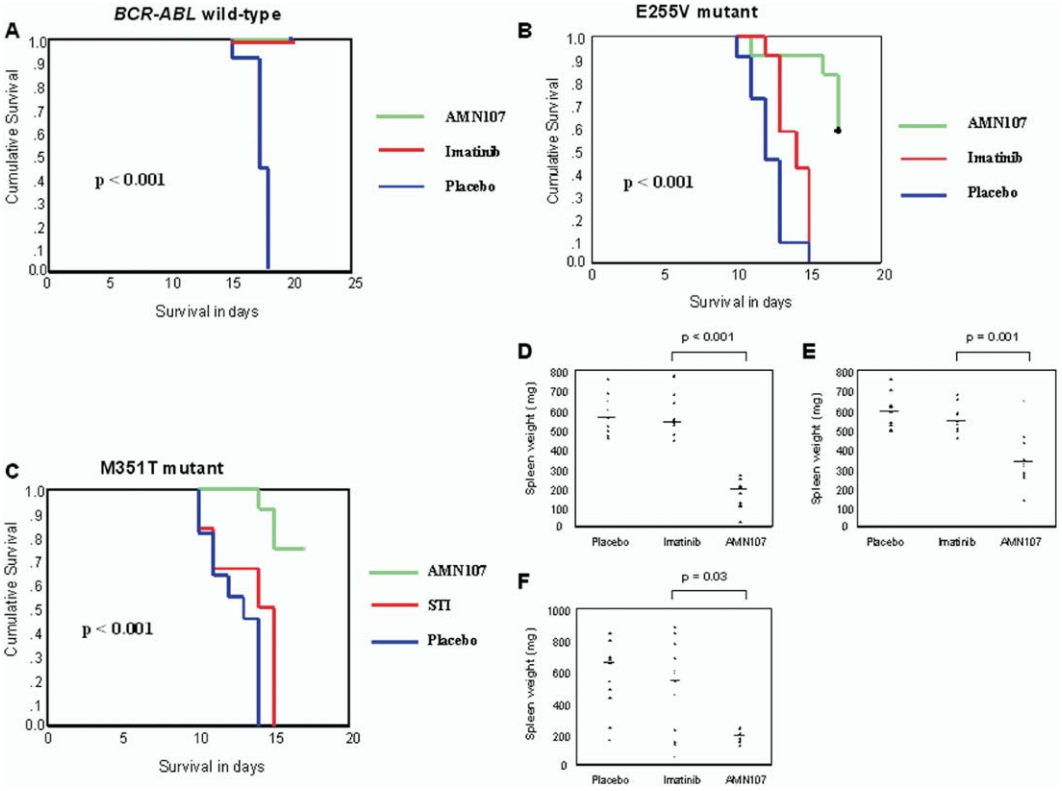

Figure 6. AMN107 treatment prolongs survival and decreases tumor burden in an imatinib-resistant Bcr-Abl mutant BMT model

A–C: Kaplan-Meier plots demonstrating survival for mice transplanted with marrow transduced with wild-type Bcr-Abl (**A**), the imatinib-resistant Bcr-Abl mutant E255V (**B**), and the imatinib-resistant Bcr-Abl mutant M351T (**C**). A significant difference in survival was demonstrated between mice treated with AMN107 and imatinib (log-rank test, $p < 0.001$ for the E255V and M351T mutants).

D–F: Scatter diagrams demonstrating spleen weights for mice treated with either placebo, imatinib, or AMN107 for mice transplanted with marrow transduced with wild-type Bcr-Abl (**D**), the imatinib-resistant Bcr-Abl mutant E255V (**E**), or the imatinib-resistant Bcr-Abl mutant M351T (**F**). Horizontal bars represent median values. Comparisons between values for mice treated with imatinib and AMN107 demonstrate a significant reduction in spleen weights for AMN107-treated animals (p values shown).

cells, resulted in a delayed onset of leukemia in drug-treated mice versus vehicle-treated controls (Figure 4D). The Wilcoxin p value for a difference in time of onset of tumor development was 0.04.

A similar trial was performed for mice transplanted with bone marrow transduced with the imatinib-resistant E255V mutant of *BCR-ABL*, and treated with vehicle, imatinib, or AMN107. Animals in the vehicle-treated control group (12 mice) again developed myeloproliferative disease, as did the mice treated with imatinib, and all were sacrificed by day 15 posttransplantation (Figure 6B). In contrast, treatment with AMN107 significantly prolonged survival in mice transplanted with the E255V mutant, with 7/12 mice still alive at the study endpoint, 17 days posttransplant ($p < 0.001$, Figure 6B). Tumor burden was again compared between mice treated with imatinib or AMN107, and a significant reduction in spleen weight was demonstrated for those animals treated with AMN107 (Figure 6E). Similar results were obtained when the M351T mutant was tested in mice treated with imatinib or AMN107, with significantly prolonged survival in AMN107-treated mice ($p < 0.001$, Figure 6C), and a significant reduction in spleen weight in mice treated with AMN107 (Figure 6F). Liver weights and white cell counts (wcc) were also used as a determinant of tumor burden in mice transplanted with native, E255V, and M351T-Bcr-Abl, and, similar to spleen, a significant reduction was observed in AMN107-treated mice (Supplemental Data). Taken together, these results show that AMN107 can prolong survival of mice with two different imatinib-resistant mutants of Bcr-Abl. However, in both murine models, survival of AMN107-treated mice with native Bcr-Abl appeared to be superior to that of mice with either E255V or M351T mutants, consistent with the fact that these mutants are 27- and 2-fold less sensitive to AMN107 than is native Bcr-Abl (Table 1).

Discussion

Imatinib, an orally administered drug that inhibits the tyrosine kinase activity of Bcr-Abl and of the c-Kit and PDGF receptors, has proven to be an effective treatment for CML- and c-Kit-positive gastrointestinal stromal tumors (GISTs) (Buchdunger et al., 1996; Druker et al., 1996; Carroll et al., 1996; Buchdunger et al., 2000; Demetri et al., 2002). In chronic phase CML, imatinib induces complete hematologic remissions in almost all patients, and in a substantial number produces cytogenetic responses (O'Brien et al., 2003). However, resistance to imatinib occurs in a small number of chronic phase patients, with some

patients relapsing after months or years of treatment. Chronic phase CML patients who achieve a 1000-fold reduction in *BCR-ABL* transcript levels have a negligible risk of disease progression over the subsequent 12 months; this level of cytogenetic response has been achieved in 39% of patients receiving a standard dose regimen of imatinib (O'Brien et al., 2003). In contrast, patients diagnosed with Ph+ ALL, as well as many patients with more advanced stage CML (accelerated phase and blast crisis), fail to achieve a complete cytogenetic response and frequently develop resistance to therapy and relapse (Sawyers et al., 2002; Ottmann et al., 2002).

Resistance to imatinib often results from the emergence of clones expressing mutant forms of Bcr-Abl that exhibit a decreased sensitivity toward inhibition by imatinib. These include G250E, Y253H, E255K(V), T315I, F317L, and M351T (Branford et al., 1999; Gorre et al., 2001; Barthe et al., 2001; Hochhaus et al., 2001; Barthe et al., 2002; Ricci et al., 2002), and more than 30 such mutants have now been isolated from patients (Hochhaus and La Rosee, 2004). Crystallographic studies revealed that imatinib binds to Bcr-Abl by filling a pocket created in the ATP binding site by the DFG motif of the activation loop being displaced from the position that it occupies in the catalytically active conformation of the enzyme (Schindler et al., 2000; Nagar et al., 2003). Point mutations of Bcr-Abl have been characterized as either those that destabilize this inactive protein conformation, or those that sterically impede direct contact between the protein and imatinib (Shah et al., 2002; Corbin et al., 2003; Cowan-Jacob et al., 2004). In general, point mutations affecting residues in close contact with imatinib confer a greater degree of resistance than those affecting the stability of the protein conformation.

A number of strategies to prevent the emergence of resistant clones have been proposed, including combination of imatinib with other agents (Krystal, 2001). Additive/synergistic effects have been achieved when imatinib was combined with standard chemotherapeutic agents such as interferon α, daunorubicin, cytosine arabinoside, and homoharringtonine (Thiesing et al., 2000; Tipping et al., 2002). Agents that disrupt signaling pathways associated with Bcr-Abl or that lead to accelerated catabolism of the Bcr-Abl protein have also been tested for synergy with imatinib (Sun et al., 2001; Gorre et al., 2002; Hoover et al., 2002; Nimmanapalli et al., 2002; Klejman et al., 2002; Nakajima et al., 2003; Warmuth et al., 2003). These alternative strategies are being clinically evaluated alone and in combination with imatinib.

There has also been increasing interest in identifying new Bcr-Abl inhibitors with greater potency than imatinib, or that retain the ability to inhibit imatinib-resistant point mutants to Bcr-Abl (Mow et al., 2002; La Rosee et al., 2002; Shah et al., 2004; O'Hare et al., 2004). Dual Abl and Src kinase inhibitors have the potential attraction that Src may be involved in signaling by Bcr-Abl (Danhauser-Riedl et al., 1996). However, it does not appear that Lyn, hck, or Fgr are important for myeloid disease in mice (Hu et al., 2004), and preliminary studies with agents such as BMS354825 suggest that inhibition of Src kinases in the setting of the highly drug-resistant T315I mutant of Bcr-Abl results in no inhibition at all. The added value of such agents, and potential for added toxicity, will need to be studied in clinical trials.

AMN107 is a new and highly potent inhibitor of Abl that has certain advantages over imatinib. First, AMN107 is 10- to 50-fold more potent as an inhibitor of Bcr-Abl than imatinib, as assessed by its ability to block proliferation of Bcr-Abl dependent cell lines derived from CML patients (K562, Ku-812F) and cell lines (32D or Ba/F3) transfected to express the Bcr-Abl protein. Similarly, AMN107 is 10- to 20-fold more active than imatinib in reducing Bcr-Abl autophosphorylation (IC_{50} values ranging from 20–60 nM). Proliferation of the parental 32D and Ba/F3 cell lines was unaffected at 100-fold greater concentrations, indicating a lack of general toxicity. Similarly, normal myeloid progenitor cells are not inhibited at concentrations of AMN107 <100 nM, and mice show no evidence of hematopoietic toxicity after exposure to high concentrations of drug for 14 days.

AMN107 also inhibited the tyrosine kinase activity of the PDGF and c-Kit receptors, displaying similar efficacy to imatinib, and therefore possessing greater selectivity toward Bcr-Abl. AMN107 showed no activity against a wide panel of other protein kinases at concentrations below 3 μM, including c-Src.

A key feature of AMN107 is the ability to inhibit some Bcr-Abl point mutants resistant to imatinib. The majority of the 15 mutants tested were sensitive to AMN107 to a variable degree, with IC_{50} values ranging from <10 nM to approximately 1000 nM (when assessed in proliferation assays in vitro), with 10 mutants <100 nM (Table 1). Mutants G250E, Q252H, Y253H, E255K/V, T315I, and M351T are the most common mutants in patients with imatinib resistance (>5% incidence each), while the others tested are detected in 1% to 5% of patients (Hochhaus and La Rosee, 2004). Overall, L248R, Y253H, E255K/V, and L248R were the least sensitive to AMN107, with IC_{50} values 100–1000 nM, while T315I was resistant at an IC_{50} value of >10,000 nM. Since plasma levels of AMN107 in excess of 10,000 nM can be readily achieved in mice, these results suggest that many imatinib-resistant Bcr-Abl mutants might be effectively targeted by AMN107.

In an effort to explain the differences between imatinib and AMN107 as inhibitors of Abl, crystallographic structural analysis of an Abl-AMN107 complex was performed. Like imatinib, AMN107 binds to the inactive conformation of Abl kinase (Cowan-Jacob et al., 2004). From analysis of crystal structures, the greater affinity of AMN107 compared to imatinib results from a better topological fit of AMN107 to the protein, contrasting with the need to desolvate/deprotonate the highly basic *N*-methylpiperazine and the slightly larger constraints on the binding surface for this group in imatinib. The binding affinity contributed by the pyridinyl and pyrimidinyl groups is therefore relatively large for imatinib, and small compared to the total energy for AMN107, where the trifluoromethyl/imidazole substituted phenyl group (Figure 1A) contributes greatly to the potency. Hence, mutations such as F317L from the hinge region and E255K/V in the P loop, which contact the pyridinyl and pyrimidinyl groups, have less effect on the overall affinity of AMN107 than imatinib. The crystal structure of Abl^{M351T} shows that residues of the C-terminal lobe lining the imatinib binding site are only marginally affected by the M351T mutation, and could explain the small reduction in affinity to Abl^{M351T} compared to wt-Abl, although energetic differences between the two states might be of greater importance. If imatinib must induce Abl to adopt a specific conformation for binding, then the affinity will be greater if that conformational state is of lower energy. The M351T mutation facilitates other positions of helix E, increasing the entropy and thus increasing the energy re-

quired to adopt the imatinib binding mode. This mutation has little effect on AMN107 affinity, since there is less stringent induced-fit binding. In contrast to imatinib, which makes directional H bonds to Ile360 and His361, the imidazole moiety has less critical interactions with the C-terminal lobe. Similar energetic effects would be expected for other mutants, e.g. M244V and F486S, which are distant from the imatinib binding site and cause mild resistance to imatinib. The T315I mutant remains insensitive to binding of AMN107 due to the loss of a hydrogen bond and introduction of a steric clash, as in the case of imatinib (Schindler et al., 2000). A different inhibitor scaffold would be required to overcome resistance to this mutant. The reduced sensitivity of the G250E mutant toward AMN107 is probably because the glutamate stabilizes the active conformation of Abl. This is in contrast to other mutations in this region, such as Y253F/H and E255K/V, which destabilize the inactive conformation of the P loop (Cowan-Jacob et al., 2004).

Having demonstrated the potent in vitro efficacy of AMN107, we evaluated the compound against Bcr-Abl-induced leukemia in animal models. AMN107 was well absorbed and displayed good bioavailability in mice; oral administration of 20 mg/kg of AMN107 yielded a mean plasma level in the range of 6.0–12.1 μM after 2 hr, which is >100-fold greater than the concentrations required to inhibit either Bcr-Abl autophosphorylation or hematopoietic cell proliferation in vitro. Following a single 75 mg/kg dose, high plasma and bone marrow concentrations were maintained out to 24 hr, and the compound was well tolerated at oral doses up to 150 mg/kg/day.

The potential of AMN107 for in vivo activity was tested in a short-term model where nude mice were injected with 32Dp210Bcr/Abl cells additionally expressing the luciferase gene. Serial imaging indicated that compared to vehicle, AMN107 dramatically reduced the accumulation of leukemic cells in the bone marrow, spleen, liver, and lymph node areas, indicating effective distribution into multiple tissues in vivo. To determine whether AMN107 could also extend the survival of mice injected with 32Dp210Bcr-Abl cells, Balb/c mice were treated with an oral dose of 75 mg/kg q.d. over a 16-day period, commencing three days after the injection of 32D.p210 Bcr-Abl cells, or vehicle control. Treatment with AMN107 resulted in the survival of 15/20 animals over the 105 days of planned observation, whereas 19/20 vehicle-treated mice had progressive disease. Spleen weights of the animals receiving AMN107 were within the normal range at the end of the experiment. Taken together, these two studies with 32Dp210Bcr-Abl cell lines indicate the potential of AMN107 to rapidly and profoundly suppress disease development.

To determine if the therapeutic effects of AMN107 on Bcr-Abl+ cell lines would extend to Bcr-Abl+ primary hematopoietic cells, mice were transplanted with marrow infected with a Bcr-Abl retrovirus, followed 8 days later by treatment with AMN107, imatinib, or vehicle control. In this model, mice develop a highly reproducible CML-like myeloproliferative syndrome characterized by granulocytosis and splenomegaly. Treatment with AMN107 reduced morbidity and, at the end of the study, yielded spleen weights within the normal range, as observed for the long-term survival experiments.

As noted above, the availability of agents that could be used to treat imatinib-resistant clones of Bcr-Abl+ leukemias would have significant therapeutic value. This possibility was assessed using a highly imatinib-resistant mutant, E255V Bcr-Abl, both in mice injected with 32D.p210-E255V cells and in mice receiving bone marrow transplants after infection with the Bcr-Abl mutant, E255V. In both of these models, AMN107, but not imatinib, increased survival and decreased disease volume. These results were extended by testing a second imatinib-resistant mutant, M351T. AMN107 significantly prolonged survival compared to imatinib ($p < 0.001$, Figure 6E), and also resulted in a significant reduction in spleen weight and other measures of disease burden (Figure 6F).

Overall, the data presented here suggest that AMN107 is highly cytotoxic to both cell lines and primary hematopoietic cells expressing Bcr-Abl, and that it could have certain advantages over imatinib in terms of higher potency and the ability to inhibit some imatinib-resistant mutants. However, since the IC_{50} value of AMN107 for some imatinib-resistant mutants is higher than for wild-type Bcr-Abl, it may be necessary to achieve substantially higher plasma concentrations of AMN107 in such patients to achieve responses.

If human clinical trials validate the effectiveness of AMN107 demonstrated in the preclinical studies reported here, it may be possible to either use AMN107 in selected patients with imatinib resistance, or to use both agents together, simultaneously or sequentially. Highly potent inhibitors of Bcr-Abl should reduce the number of residual Bcr-Abl+ cells capable of undergoing mutation. Consequently, monotherapy with highly potent Abl inhibitors, or combinations of Abl inhibitors with different mechanisms of action, might prevent or delay the emergence of some types of drug-resistant mutants of Bcr-Abl. In support of simultaneous administration, AMN107 was shown to be synergistic when combined with imatinib against cells expressing p210Bcr-Abl or p190Bcr-Abl, despite the fact that both inhibitors bind to the same site.

While we anticipate that new Bcr-Abl point mutations could eventually emerge to confer resistance to AMN107, it may be possible to cycle or combine agents to suppress or delay the emergence of resistant clones. Thus, the availability of novel, high-potency, Abl tyrosine kinase inhibitors will usher in a new generation of clinical studies that will hopefully result in additional major advances in the therapy of CML and Ph+ ALL.

Experimental procedures

Systemic 32D Bcr-Abl leukemia model

32D.p210 and 32D-E255V cells free of *Mycoplasma* and viral contamination were washed once with Hank's Balanced Salt Solution (HBSS; Mediatech, Inc.,VA), and resuspended in HBSS prior to administration to mice. Solutions of AMN107 were prepared just prior to administration, by dissolving 75 mg in 1.0 ml of NMP to give a clear solution and diluting with 9.0 ml PEG300. Female BALB/c mice (weighing 15–18 g and 6–7 weeks of age at delivery; Taconic, NY) were administered suspensions containing 32D.p210 or 32D-E255V cells by tail vein injection (1×10^5 32D.p210 cells/mouse; 7×10^5 32D-E255V cells/mouse; day 0). 32D.p210-injected mice were treated via gavage with either vehicle (10% NMP-90% PEG300) or AMN107 (75 mg/kg/day) on days 3, 4, 7, 8, 9, 10, 11, 15, 16, 17, and 18 (eleven doses total), and monitored for signs of leukemia. 32D-E255V-injected mice were treated via gavage with either vehicle or AMN107 (100 mg/kg/day) once daily for 21 days. Mice were sacrificed if they became morbid, according to institute protocols. At the planned end of each study, any remaining mice were sacrificed, body and spleen weights were recorded, and tissues preserved in 10% formalin for histopathological analysis.

Survival was measured as time from cell injection to death or sacrifice. All starting animals were included in the statistical analysis. Survival analysis was performed using the method of Kaplan and Meier with statistical significance assessed using the log rank test.

Bioluminescent Bcr-Abl model of CML

Cells were transduced with a retrovirus encoding firefly luciferase (MSCV-Luc), and selected with puromycin at 2 μg/ml to produce 32D.p210-Luc+ and 32D-E255V-Luc+ cell lines. Female NOD-SCID mice (8–10 weeks of age; Jackson Laboratory) were sublethally irradiated with a single fraction of 300 rads, and 3–6 hr later, a total of 1×10^6 cells were administered by tail vein injection. Mice were imaged and total body luminescence quantified as previously described (Armstrong et al., 2003). Baseline imaging 5–7 days after tumor cell inoculation was used to establish treatment cohorts with matched tumor burden. Cohorts of mice were treated with oral administration of vehicle (formulated as above), 75 mg/kg imatinib (twice daily), or 100 mg/kg/day AMN107 (formulated as above). Repeat imaging was performed every 3–4 days.

Bone marrow transplant Bcr-Abl model of CML

The murine BMT assays and drug treatment were performed as described previously (Liu et al., 2000; Cools et al., 2003; Kelly et al., 2002). In brief, 1×10^6 bone marrow cells transduced with distinct retroviral constructs (either native p210-BCR-ABL or BCR-ABL E255V mutant both in MSCV-IRES-GFP) were injected into the lateral tail veins of lethally irradiated (450cGy X2) syngeneic Balb/c recipient mice. After 8 days, mice were administered either vehicle (10% NMP-90% PEG300), 75 mg/kg/day AMN107 (formulated in 10% NMP-90% PEG300), or 125 mg/kg/day imatinib (two divided doses formulated in 0.05% methylcellulose) via oral gavage. Diseased animals, identified by splenomegaly or moribund appearance, were sacrificed. Spleen and liver weights were recorded and histopathological analyses were performed for each animal; single-cell suspensions of bone marrow, spleen, and peripheral blood were analyzed by flow cytometry as described previously (Schwaller et al., 1998). Survival analysis was performed using the method of Kaplan and Meier with statistical significance assessed using the log rank test. Tumor burden as assessed by white cell counts (WCC) and spleen and liver weights were compared by the Mann-Whitney U test for each treatment category.

Supplemental data

Information on cell lines and cell culture, chemical compounds and biologic reagents, antibodies, cell proliferation studies, apoptosis assays, immunoprecipitation and immunoblotting, effects of imatinib and AMN107 on phosphorylation status of target kinases in cells, determination of AMN107 binding sites in Abl (including preparation of the c-Abl^{M351T}-AMN107 complex, crystal structure determination of the c-Abl^{M351T}-AMN107 complex, and structure determination and refinement), and synergy studies can be found in the Supplemental Data at http://www.cancercell.org/cgi/content/full/7/2/129/DC1/.

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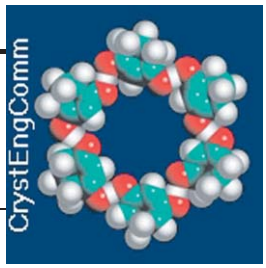
Hydrate formation in NH^+-containing salts of pharmaceutically acceptable anions: A CSD survey†



Paper

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The CSD has been systematically searched for salts of N-based cations and pharmaceutically acceptable groups of anions. The tendency of particular anions to crystallise with particular cations has been assessed, and the occurrence of hydrates within particular groups of salts has been analysed. It was found that pyridinium carboxylate salts show a reduced tendency to form hydrates when compared to other salts.

1. Introduction

Salts of pharmaceutical candidate molecules are often prepared in order to improve the physical properties of the molecule, such as solubility, hygroscopicity and crystallinity.[1] The process of salt preparation and selection of salt forms of pharmaceutical candidates can be long and expensive, and the process of choosing a counterion is not selective. We are therefore carrying out an analysis of the effect of specific counterions on crystal structure, and hence on crystal properties, with the eventual objective of enabling specific selection of a counterion to yield crystalline material with the desired properties.

Our general approach has been to begin by searching the Cambridge Structural Database (CSD)[2] to establish the existence of possible trends, such as the frequent occurrence of particular hydrogen bonding motifs within subsets of salts.[3] On the basis of these observations experiments were then designed to examine the general validity of the apparent trend.

The basic group on a drug molecule is frequently an amine, a cyclic nitrogen or an aromatic nitrogen. Stahl and Wermuth[1] have listed salt-forming acids that are pharmaceutically acceptable, generally taken to be acids which are biologically non-toxic and are unlikely to interfere with the action of the drug. The anions of these acids can be subdivided into carboxylates and carbonate, halides, sulfate and sulfonates, inorganic phosphate, nitrate and thiocyanate. These functional groups are all either pharmaceutically acceptable anions themselves (PO_4^{3-}, Cl^-, SO_4^{2-}), or are the charge-carrying group on several pharmaceutically acceptable anions ($MeCOO^-$, $PhSO_3^-$).[1] The CSD has been systematically searched for combinations of these N-bearing cations and pharmaceutically acceptable anions, and trends in hydration across the groups have been investigated.

2. Methods

Searches were done on CSD version 5.25 (November 2003) using ConQuest version 1.6[4] (unless stated otherwise). The search criteria used were drawings of the respective ions (Fig. 1.), with two filters in place: hits must have their 3-D coordinates determined, and have no transition metals present. Hydrate in all cases is defined as a structure containing H_2O or H_3O^+.

The anion groups were chosen on the basis of the list of pharmaceutically acceptable acids in *Handbook of Pharmaceutical Salts* (see supplementary material†).[1] We have previously analysed the distribution of pharmaceutically acceptable anions in the CSD, and investigated the occurrence of hydrates of particular anions.[5] We have observed a slightly higher than average occurrence of hydrates in zwitterions in the CSD (22.50% hydrates in salts with zwitterions excluded, and 25.53% hydrates in zwitterions).[6] This effect is much more pronounced in the various anion groups under investigation in this study (Fig. 2.). We have therefore excluded zwitterions from our analysis. This gives a sample of 7439 non-zwitterionic NH^+-containing salts, which is the sample used in this study.

3. Results and discussion

The results of CSD searches for combinations of N-based cations and pharmaceutically acceptable anions are given in Table 1. Several general observations can be made based on these data.

3.1 Anion profiles

Firstly we note that 2085 (28%) of the N-based organic salts in the CSD have counterions that are not in one of the pharmaceutically acceptable groups used in this study. Fig. 3 shows the anion profiles of each cation group, with the occurrence of each anion expressed as a percentage of the total number of hits for all pharmaceutically acceptable anion groups. There is a high occurrence of halide and carboxylate salts in all the cation subgroups, and a much lower occurrence of phosphates and sulfates. There is increased occurrence of halide salts in the tertiary amines and cyclic amines at the expense of the other anion groups, and a slightly higher occurrence of nitrate salts when the cation is pyridinium.

3.2 Hydrate formation

Analysis of the data on hydration reveals some interesting trends. Overall, there is a decrease in hydration in the halide series that correlates with the increase in ion size, *i.e.* F^- shows the highest hydrate occurrence of the halides and I^- shows the lowest (Table 2). There is a higher percentage of hydrate

† Electronic supplementary information (ESI) available: List of pharmaceutically acceptable acids, based on the *Handbook of Pharmaceutical Salts*, excluding polymers. See http://www.rsc.org/suppdata/ce/b5/b503309h/

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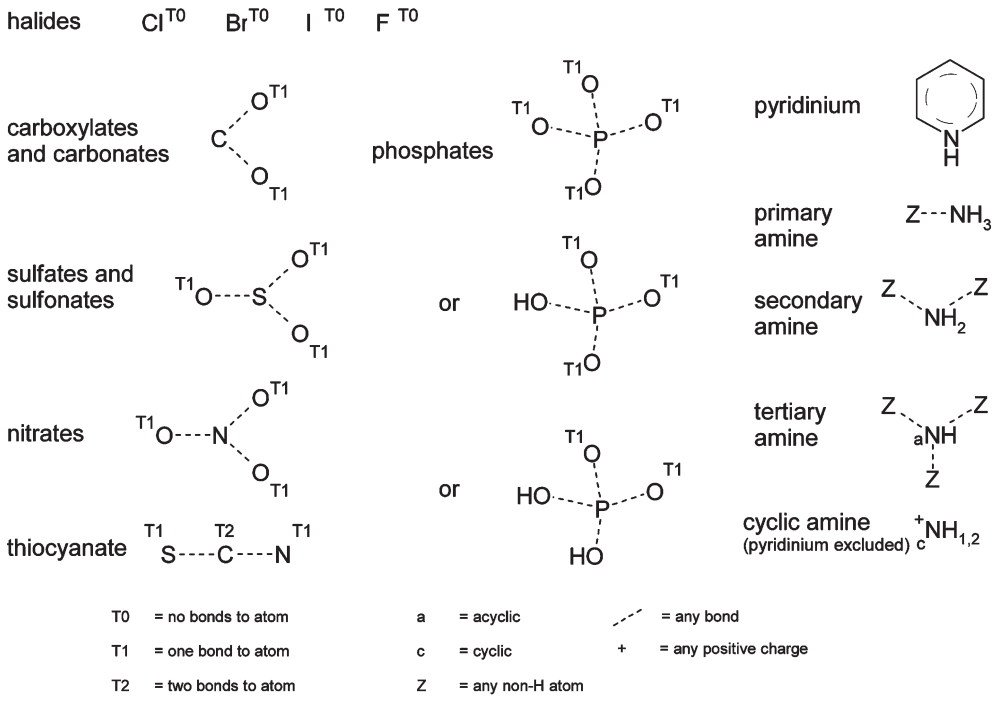

Fig. 1 Sketches used to define ion groups in ConQuest

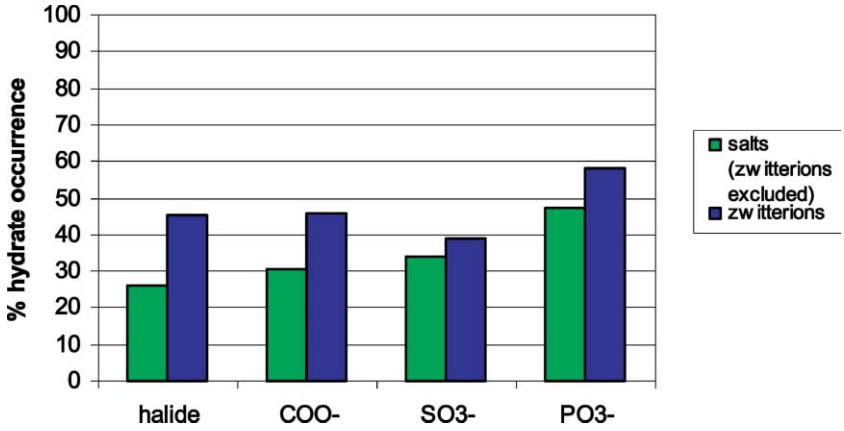

Fig. 2 Percentage occurrence of hydrates in salts with zwitterions excluded (18 003 hits) and zwitterions (4191 hits) in the CSD.

Table 1 Percentage hydrate occurrence in NH^+ salts in the CSD. Ncompounds is the number of crystal structures found in each case, Nhydrates is the number of these crystals which are hydrates

Cation		Halide	Carboxylate or carbonate	Sulfate or sulfonate	Phosphate	Nitrate	SCN^-	Other[a]	Total
Pyridinium	Ncompounds	210	143	44	11	35	3	321	729
	Nhydrates	73	13	15	4	6	1	58	163
	% hydrates	34.8	9.1	34.1	36.4	17.1	33.3	18.1	22.4
1° Amine	Ncompounds	553	514	131	90	42	3	382	1710
	Nhydrates	146	114	58	32	3	0	100	453
	% hydrates	26.4	22.2	44.3	35.6	7.1	0.0	26.2	26.5
2° Amine	Ncompounds	272	172	41	25	8	2	207	725
	Nhydrates	36	30	12	17	1	0	21	117
	% hydrates	13.2	17.4	29.3	68.0	12.5	0.0	10.1	16.1
3° Amine	Ncompounds	272	72	20	6	10	1	268	649
	Nhydrates	51	12	5	6	1	0	19	94
	% hydrates	18.75	16.7	25.0	100.0	10.0	0.0	7.1	14.5
Cyclic amine (excluding pyridinium)	Ncompounds	1374	513	183	24	76	10	703	2871
	Nhydrates	412	126	80	10	15	3	133	771
	% hydrates	30	24.6	43.7	41.7	19.7	30.0	18.9	26.9
Total	Ncompounds	2809	1590	610	754	198	25	2085	7439
	Nhydrates	742	353	185	236	29	4	367	1723
	% hydrates	26.4	22.2	30.3	31.3	14.7	16.0	17.6	23.2

[a] These are non-pharmaceutically acceptable anions.

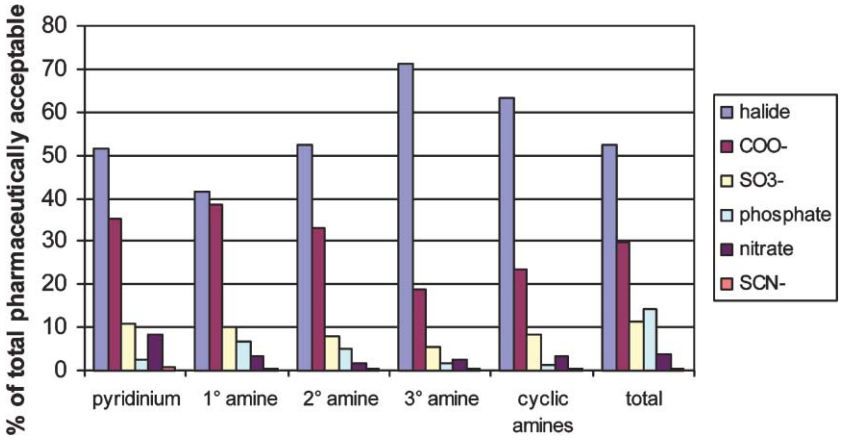

Fig. 3 Percentage occurrence of salts of N-based cations with pharmaceutically acceptable anions in the CSD

Table 2 Hydrate occurrence in NH^+-containing halide salts in the CSD. Ncompounds is the number of crystal structures found in each case, Nhydrates is the number of these crystals which are hydrates

Cation		I^-	Br^-	Cl^-	F^-
Pyridinium	Ncompounds	8	42	156	4
	Nhydrates	1	10	62	0
	% hydrates	12.5	23.8	39.7	0.0
1° Amine	Ncompounds	28	103	420	2
	Nhydrates	4	26	115	1
	% hydrates	14.3	25.2	27.4	50.0
2° Amine	Ncompounds	8	37	225	2
	Nhydrates	2	4	29	1
	% hydrates	25.0	10.8	12.9	50.0
3° Amine	Ncompounds	9	56	202	5
	Nhydrates	1	9	41	0
	% hydrates	11.1	16.1	20.3	0.0
Cyclic amine (excluding pyridinium)	Ncompounds	56	356	958	9
	Nhydrates	12	96	300	7
	% hydrates	21.4	27.0	31.3	77.8
Total	Ncompounds	127	635	2030	22
	Nhydrates	24	151	561	9
	% hydrates	18.9	23.8	27.7	40.9

occurrence in sulfates and phosphates within all cation groups, and a lower hydrate occurrence when the anion is 'other'. The higher than average occurrence of hydrates for pharmaceutically acceptable ions has been reported by us elsewhere.[5] The higher occurrence of hydrates in sulfates and phosphates is not surprising, as hydrate occurrence in a total survey of the CSD increases with increasing number of polar groups on a molecule, and with increasing charge.[7]

Generally, within the groups of anions studied, halides tend to be more highly hydrated than carboxylates (Fig. 4). The secondary amines are an exception to this. Also, secondary and tertiary amines show generally lower levels of hydrate occurrence than the other cation groups. However, the most pronounced trend in this data is the low occurrence of hydrate formation in pyridinium carboxylate salts: only 9.1% (13 of 143 hits) of pyridinium carboxylate salts are hydrates, as compared to between 16.7 and 24.6% of carboxylate hydrates in the other cation groups. This is particularly interesting when compared to the occurrence of hydrates in pyridinium salts in general: the next lowest occurrence of hydrates is for the pyridinium nitrates at 17.1%, pyridinium halides have a hydrate occurrence of 34.8% and the overall percentage of hydrate occurrence for pyridinium salts is 22.4%.

This effect is restricted to pyridinium salts: in carboxylate salts of other aromatic NH^+-containing rings 20.6% are hydrates, 24.6% of other cyclic amine carboxylate salts are hydrates (Table 1), and in the carboxylate salts of 3-coordinate NH^+ (excluding pyridinium) in general, 23.7% are hydrates. It should be noted that the sample of 143 pyridinium carboxylate salts analysed here includes a wide range of different types of molecules, from simple pyridinium trifluroacetate (CSD reference code RUVYIQ), to complex larger molecules (*e.g.* RIZWIG). It is therefore surprising that there should be such a definite trend towards reduced hydration for carboxylates in the presence of the pyridinium cation. Inspection of the 13 hydrated pyridinium carboxylate salts in the CSD does not reveal any explanation as to why these salts are hydrated. In fact, at least one salt exists both as a hydrate (WADPEX) and in a non-hydrated form (DUVLUB).

Current experimental work is focussed on the synthesis and structural characterisation of a systematic series of pyridinium

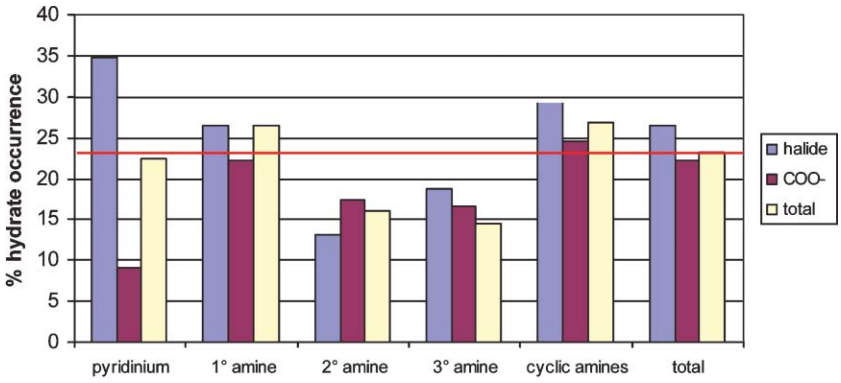

Fig. 4 Percentage of hydrates in cation groups in the CSD. The red line indicates the percentage of hydrates in non-zwitterionic salts in the CSD as a whole (22.5%).

carboxylate salts. This work aims to confirm the reduced occurrence of hydrates in pyridinium carboxylate salts, and to ensure that the results discussed here are not a result of bias in the CSD. This systematic study may also shed some light on why this trend is observed.

4. Conclusions

CSD studies have revealed several potentially useful trends in NH^+-containing salts in the database. Tertiary amine and cyclic amine cations show a slight preference for crystallising with halide anions, and secondary and tertiary amine salts generally have a lower number of hydrates. However, the most pronounced trend observed was a trend towards reduced hydration in pyridinium carboxylate salts. This result implies that in choosing a counterion for a pyridinium based pharmaceutical, the choice of a carboxylate anion would give a good chance of crystal formation (approximately 35% of the pyridinium salts in the CSD are carboxylates), and a diminished chance of hydrate formation.

Acknowledgements

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7 Lourdes Infantes, James Chisholm and Sam Motherwell, *CrystEngComm*, 2003, **5**, 480–486.

Chapter 11

Selected Procedures for the Preparation of Pharmaceutically Acceptable Salts

by **Camille G. Wermuth*** and **P. Heinrich Stahl**

Contents

1. Introduction

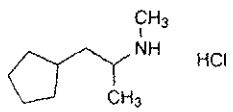
The preparation of pharmaceutical salts is usually not a matter of university teaching, and most of the organic chemists are not trained to prepare salts. For this reason, it appeared useful to the authors to collect some preparation methods from the literature and to present them in this chapter as possible models. Most of the 'recipes' stem from the patent literature and, therefore, are not optimized. Nevertheless they illustrate the state of the art and sometimes propose several variants for the preparation of a given salt form.

2. Preparation of Salts of Basic Drug Substances

2.1. Hydrochlorides

Hydrochlorides do sometimes require strictly anhydrous conditions to be prepared (*e.g.*, HCl gas in anhydrous diethyl ether (Et_2O)), at other times they require stoichiometric amounts of H_2O to be present in order to yield hydrated crystals. A typical procedure is to dissolve the organic base in the minimal amount of hot isopropanol (i-PrOH) and to add the calculated volume of concentrated HCl (85 ml ≈ 1 mol). If, after cooling, crystallization does not occur, it can be induced by progressive additions of Et_2O. Only if both procedures fail, salt formers other than HCl should be envisaged.

Cyclopentamine Hydrochloride
Clopane® (*Lilly*)
Procedure [1]



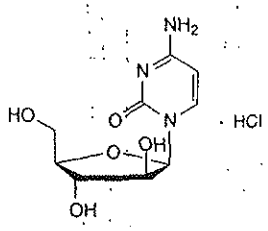
Dissolve 141 g (1 mol) of 3-cyclopentyl-*N*-methylpropan-2-amine in 500 ml of dry Et_2O, and pass dry HCl into the solution, until the weight of the mixture and container has increased by 36 g. During the addition of the HCl, the HCl salt of 3-cyclopentyl-*N*-methylpropan-2-amine precipitates as a white powder. The salt is filtered off and washed with dry Et_2O. 3-cyclopentyl-*N*-methylpropan-2-amine hydrochloride thus prepared melts at *ca.* 113 – 115 °C. The yield is practically quantitative.

Caution!

When this procedure is performed on a small scale, it is often observed that the initially formed hydrochloride re-dissolves. This is due to the fact that an excess of gaseous HCl in anhydrous Et_2O produces the very polar di-

ethyloxonium chloride, which solubilizes the initially precipitated hydrochloride.

Cytarabine Hydrochloride
Cytosar® (*Upjohn*)

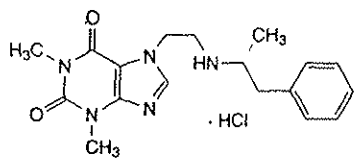


Procedure [2]

A solution of 250 mg D-1-β-arabinofuranosylcytosine in absolute methanol (MeOH) was warmed and stirred with decolorizing charcoal. The mixture was filtered through a bed of filter aid, and the filter bed was washed repeatedly with absolute MeOH. The combined filtrate and washings were pale yellow. The solution was diluted to faint cloudiness with anhydrous Et_2O, and an excess of anhydrous HCl was introduced. Crystallization began at *ca.* 25 °C and further crystallization was induced by chilling at 0 °C for 14 hours. The crystalline product was collected on a filter, washed with anhydrous Et_2O, and dried in air. There was thus obtained 180 mg of pale yellow D-1-β-arabinofuranosylcytosine hydrochloride melting at 186 to 189 °C.

The pale yellow product was dissolved in warm, absolute MeOH, and after treatment with decolorizing charcoal the solution was filtered through a bed of filter aid. The filter bed was washed with warm absolute MeOH, and the combined methanolic filtrate and washings were warmed and diluted with anhydrous Et_2O to incipient crystallization. The MeOH/Et_2O mixture was kept at *ca.* 25 °C for *ca.* 1 hour and then chilled, first at 0 °C, and then at −20 °C. The resulting colorless needles were collected on a filter, washed with anhydrous Et_2O, and dried at 85 °C, yielding 100 mg of D-1-β-arabinofuranosylcytosine hydrochloride with a melting point of 186 – 188 °C.

Fendiline Hydrochloride
Sensit® (*Thiemann*)

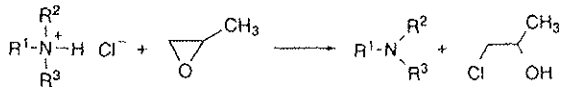


Procedure [3]

The freshly distilled base, 25.38 g of 1,7-dihydro-1,3-dimethyl-7-{2-[(1-methyl-2-phenylethyl)amino]ethyl}-2*H*,6*H*-purine-2,6-dione is dissolved in 134 ml of 96% EtOH, whereupon 26.8 ml of concentrated HCl and 201 ml of hot H_2O are added while cooling with ice-water. The precipitate is filtered off and dried *in vacuo* at 100 °C: 22.98 g of hydrochloride salt are obtained. M.p. 200 – 201 °C. On recrystallization from 285 ml of a 2:1 mixture of H_2O and 96% EtOH the melting point remains unchanged.

Conversion of a Hydrochloride to the Free Base

The usual way to prepare free bases from hydrochlorides and, more generally from salts, consists in adding an inorganic base (NaOH, K_2CO_3) and extracting the free base by means of a water-immiscible organic solvent. However, under some circumstances it may be desirable to work under anhydrous conditions. This can be achieved for example in bringing the hydrochloride to react with an HCl scavenger such as propylene oxide, according to the reaction below:



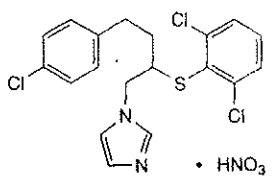
The 1-chloropropan-2-ol formed can be easily removed under reduced pressure. One of the interesting applications of this procedure is the transition from hydrobromides, the usual product of demethylation reactions, to the corresponding hydrochlorides, *via* the free base.

Procedure [4]

To a solution of 3.3 g (13.3 mmol) of L-3-hydroxy-4-methoxyphenylalanine hydrochloride in 150 ml of EtOH are added portionwise over 3 hours 12,5 ml propylene oxide. The mixture is allowed to stand 20 hours at ambient temperature (*ca.* 25 °C). The precipitated crystals are collected and purified by dissolution in 40 ml of absolute EtOH and inducing the crystallization by adding some drops of H_2O. The yield is 2.15 g of colorless crystals of L-3-hydroxy-4-methoxyphenylalanine melting at 251 – 252 °C.

2.2. Nitrates

Butoconazole Nitrate
Femstat® (Syntex)



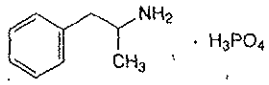
Procedure [5]

To a solution of 10 g (*ca.* 0.025 mol) of butoconazole (= 1-{4-(4-chlorophenyl)-2-[(2,6-dichlorophenyl)sulfanyl]butyl}-1*H*-imidazole) base in 300 ml of dry Et_2O, 70% HNO_3 ($d = 1.42$) is added dropwise until the precipitation is complete (*ca.* 2.2 ml). The resulting nitrate salt is recrystallized from ace-

tone/AcOEt to give butoconazole nitrate as colorless blades; yield: 9.6 g corresponding to *ca.* 80%.

2.3. Phosphates

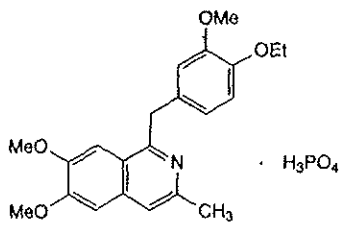
Amphetamine Phosphate



Procedure [6]

Amphetamine (= α-methylbenzeneethanamine; 135 g, 1 mol) is stirred into 300 ml of acetone in a stainless-steel vessel. To the resultant solution, 115.3 g of 85% H_3PO_4 (containing 1 mol of H_3PO_4) are added under constant agitation, care being taken to avoid any sudden rise in temperature or local overheating due to the considerable amount of heat that is evolved. During the addition of the H_3PO_4, a fine, white, flocculent precipitate appears, which becomes more and more dense and abundant, as the quantity of added acid increases. When the entire quantity of the H_3PO_4 has been added, agitation of the mixture is continued for *ca.* ½ hour or more to insure complete conversion. The precipitate is then allowed to settle, the supernatant liquid is discarded, and the residue is filtered. The precipitate thus separated may, if desired, be washed with acetone and is then dried by evaporation to constant weight, providing a fine, white, impalpable powder, which consists of pure monobasic amphetamine phosphate.

Dioxyline Phosphate
Paveril® (*Lilly*)

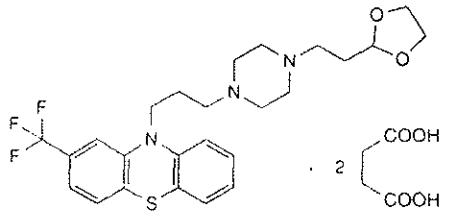


Procedure [7]

A solution of 5 g of 6,7-dimethoxy-3-methyl-1-(4-ethoxy-3-methoxybenzyl)isoquinoline in 100 ml of EtOH is treated with a solution of 1.5 g of H_3PO_4 in 10 ml of EtOH. Thereafter, 10 ml of H_2O are added to effect complete solution, and the reaction mixture is then cooled, and Et_2O is added, until precipitation of the salt is complete. The precipitate of 6,7-dimethoxy-3-methyl-1-(4-ethoxy-3-methoxybenzyl)isoquinoline phosphate is filtered off and recrystallized from 85% EtOH by the addition of 2 volumes of Et_2O.

2.4. Succinates

Oxaflumazine Succinate
Oxaflumine® (*Diamant*)

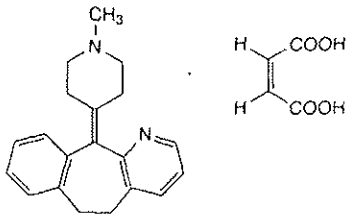


Procedure [8]

A solution of 257 g oxaflumazine (= 10-(3-{4-[2-(1,3-dioxan-2-yl)ethyl]-piperazin-1-yl}propyl)-2-(trifluoromethyl)-10*H*-phenothiazine) base in 500 ml of acetone is added with stirring to a solution of 118 g of succinic acid in 4000 ml of acetone. The agitation is continued at room temperature until the desired salt crystallizes. The mixture is allowed to stand overnight at 0 °C. The weight of the collected crystals is 256 g (yield, 70%). After recrystallization in 1000 ml of acetonitrile (MeCN) the acid disuccinate melts at 136–138 °C.

2.5. Maleates

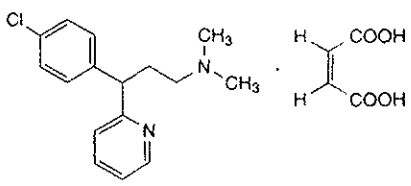
Azatadine Maleate
Optimine® (*Schering*)



Procedure [9]

To a solution containing 4.3 g of 6,11-dihydro-11-(1-methylpiperidin-4-ylidene)-5*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridine in 55 ml of AcOEt, add a solution of 3.45 g of maleic acid dissolved in AcOEt. Filter the resulting precipitate and recrystallize the desired product from an AcOEt/MeOH mixture to yield the dimaleate salt. M.p. 152–154 °C.

Dexchlorpheniramine Maleate
Polaramine® (*Schering*)

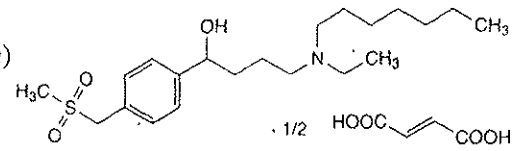


Procedure [10]

(+)-Dexchlorpheniramine (= γ-(4-chlorophenyl)-*N*,*N*-dimethylpyridine-2-propanamine) (4.3 g) and maleic acid (1.8 g) are dissolved in 20 ml of isopropyl acetate (AcO(i-Pr)) and kept at room temperature until crystallization

is complete. The crystals are filtered, washed with AcOEt and recrystallized from 15 ml of this solvent in the same manner. The crystalline maleate salt so formed is filtered off and dried. M.p. 113 – 115 °C.

Ibutilide Fumarate
Corvert® (*Pharmacia & Upjohn*)

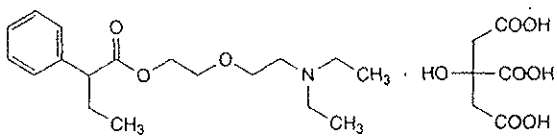


Procedure [11]

Ibutilide (= {3-[(ethyl)(heptyl)amino]propyl}{4-[(methylsulfonyl)methyl]-phenyl}methanol; 31.9 g, 0.0829 mol) is dissolved in 135 ml of absolute EtOH and mixed with a solution of fumaric acid (4.3 g, 0.037 mol) in absolute EtOH (203 ml). The solution was concentrated under reduced pressure, and the residue was dissolved in hot acetone and allowed to crystallize. Recrystallization from acetone gave a solid that was dried under reduced pressure for 4 days at 60 °C to give 31.4 g of the (*E*)-but-2-enoate salt of ibutilide (2:1). M.p. 117 – 119 °C.

2.6. Citrates

Butamirate Citrate
Sinecod® (*Hommel*)

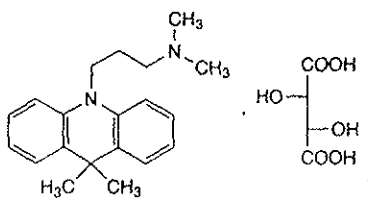


Procedure [12]

To 10 g of high-vacuum distilled 2-[2-(diethylamino)ethoxy]ethyl α-ethylbenzeneacetate is added a solution of 7 g of citric acid in 30 ml of warm acetone. After standing for some time, the citrate crystallizes out. After suction filtration and washing with acetone, the citrate is recrystallized from acetone. M.p. 75 °C.

2.7. Tartrates

Dimetacrine Tartrate
Isotonil® (*Siegfried*)

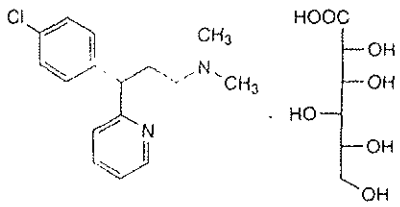


Procedure [13]

N,N,9,9-Tetramethyl-9*H*-acridine-10-propanamine (43 g) is dissolved in 229 ml of 1N aqueous (+)-tartaric acid, and the clear solution obtained is evaporated to dryness under reduced pressure. The residue is dissolved in 150 ml of 90% EtOH. The solution gives after cooling, the corresponding tartrate as white needles. The salt contains 1 mol of tartaric acid per mol of the base and is easily soluble in cold H_2O. M.p. 155 – 156 °C.

2.8. Gluconates

Dexchlorpheniramine Gluconate
Polaramine® [Gluconate] (Schering)

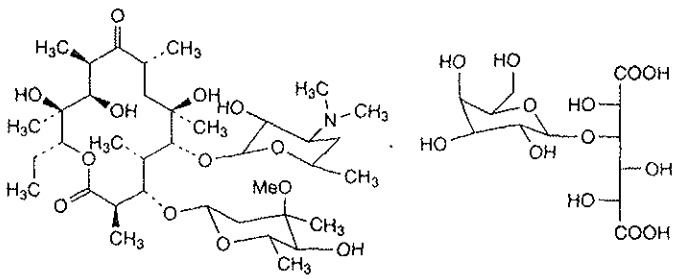


Procedure [10]

D-γ-(4-Chlorophenyl)-*N,N*-dimethylpyridine-2-propanamine (10 g) and 6.5 g of D-gluconolactone are mixed in 50 ml of 50% aqueous EtOH and kept at 50 °C for 2 hours. The solvent is then removed under reduced pressure to leave the desired salt as viscous colorless oil.

2.9. Lactobionates

Erythromycin Lactobionate
Erythrocin lactobionate® (Abbott)



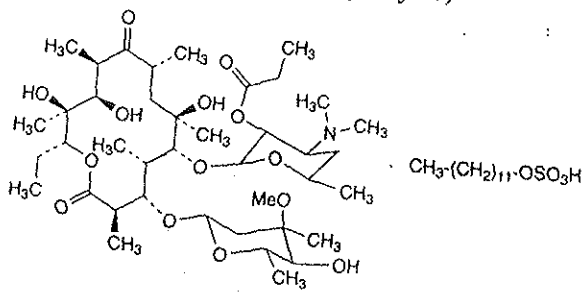
Procedure [14]

A solution of erythromycin base is prepared by dissolving 8.0 g of erythromycin in 25 ml of acetone. One the other hand, a solution of 4.0 g of δ-lactobionolactone is dissolved in 25 ml of H_2O, providing the free lactobionic acid (= 4-*O*-β-D-galactopyranosyl-D-gluconic acid). The two solutions are mixed and evaporated to a gummy residue, which is dissolved in 60 ml of H_2O, and

the resulting solution is frozen and dried *in vacuo* by lyophilization. The dried residue of erythromycin lactobionate (11.7 g) is a white amorphous powder with solubility in H_2O of *ca.* 200 mg/ml. M.p. 145 – 150 °C.

2.10. Lauryl Sulfate Salts

Erythromycin Estolate (Erythromycin Propionate Laurylsulfate)
Ilosone® (*Dista*)

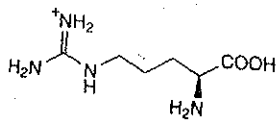


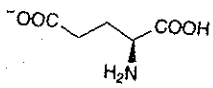
Procedure [15]

Monopropionylerythromycin (16.7 g) is dissolved in 50 ml of warm acetone. To the solution is added sodium lauryl sulfate (6.4 g), dissolved in 50 ml of distilled H_2O containing 2 ml of glacial AcOH. The white crystalline precipitate of monopropionylerythromycin lauryl sulfate separated is filtered off and dried. M.p. *ca.* 135 – 137 °C.

2.11. Glutamates

Arginine Glutamate
Modamate® (*Abbott*)





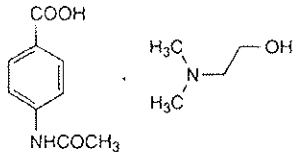
Procedure [16]

This water-soluble salt may be prepared by mixing L-arginine with L-glutamic acid in H_2O and crystallizing the resulting salt by the addition of a polar water-miscible organic solvent. For instance, when 17.2 g of L-arginine and 14.5 g of L-glutamic acid are dissolved in 155 g of H_2O, a clear homogeneous solution results, which has a pH of 5.3. After filtration, this solution is concentrated at 50 °C under reduced pressure to a solid content of *ca.* 45%. Absolute MeOH (220 g) is added, and this mixture is cooled to 5 °C for 1 hour. The resulting solid salt is removed by filtration and washed with absolute MeOH. The salt is left to become air-dry and is then further dried in a vacuum oven at 60 °C for 3 hours. Thus, 30 g (94.6% of the theoretical yield based on the amounts of

L-arginine and L-glutamic acid employed) of the L-arginine L-glutamate salt are obtained. M.p. 193 – 194.5 °C with decomposition.

2.12. Acetamidobenzoates

Deanol Acetamidobenzoate
Deaner® (Riker)



Procedure [17]

4-(Acetylamino)benzoic acid (*ca.* 40 g, 0.223 mol) is dissolved in 600 ml of absolute MeOH, and the solution is heated to reflux temperature. Heating is discontinued, and, under mechanical stirring, 2-(dimethylamino)ethanol (19.9 g, 0.223 mol) is added through a dropping funnel as fast as the exothermic nature of the reaction permits. The reaction mixture is allowed to cool to room temperature (2.5 – 3 hours) under mechanical agitation, and the solution is suction-filtered through *Celite* filter aid. The filtrate is poured into 500 ml of anhydrous Et_2O and seeded with a few crystals of 2-(dimethylamino)ethanol 4-(acetylamino)benzoate. The seeding crystals are previously obtained by introducing 3 to 6 drops of the filtered reaction mixture into a test tube containing 10 ml of anhydrous Et_2O. The contents of the test tube are thoroughly shaken and allowed to stand at room temperature. The salt crystallizes within not more than 10 – 15 minutes.

The crude product (48.4 g, 80.9% yield) is recrystallized from an absolute EtOH/AcOEt mixture by suspending the salt in boiling anhydrous AcOEt. Just enough absolute EtOH is gradually added to effect solution, then the solution is concentrated to *ca.* ⅔ of the original volume on the steam bath, treated with charcoal, and suction-filtered through *Celite* filter aid. The white crystals of 2-(dimethylamino)ethanol 4-(acetylamino)benzoate obtained are dried at room temperature at a pressure of 0.08 mm Hg for 15 hours. M.p. 159.0 – 161.5 °C.

3. Preparation of Salts of Acidic Drug Substances

3.1. Potassium and Sodium Salts

These salts can be prepared by means of sodium or potassium hydroxide, carbonate, or alkoxide. The preferred preparations make use of rather anhydrous sodium or potassium carboxylates, which are well soluble in polar organic solvents.

the resulting solution is frozen and dried *in vacuo* by lyophilization. The dried residue of erythromycin lactobionate (11.7 g) is a white amorphous powder with solubility in H_2O of *ca.* 200 mg/ml. M.p. 145 – 150 °C.

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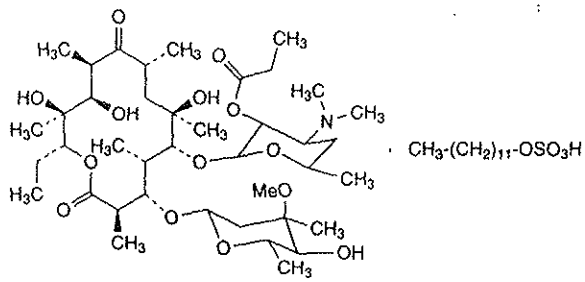
Erythromycin Estolate (Erythromycin Propionate Laurylsulfate)
Ilosone® (*Dista*)



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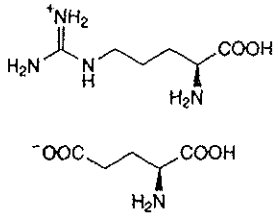
Modamate® (*Abbott*)



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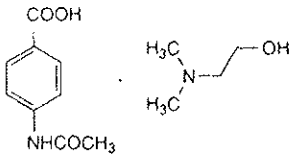
Deanol Acetamidobenzoate
Deaner® (Riker)



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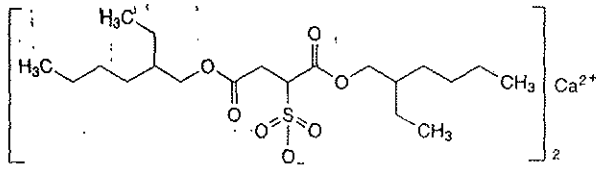
3. Preparation of Salts of Acidic Drug Substances

3.1. Potassium and Sodium Salts

These salts can be prepared by means of sodium or potassium hydroxide, carbonate, or alkoxide. The preferred preparations make use of rather anhydrous sodium or potassium carboxylates, which are well soluble in polar organic solvents.

3.2. Calcium Salts

Docusate Calcium
Surfak® (Hoechst)

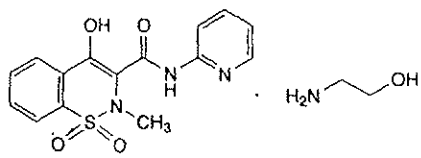


Procedure [22]

1,4-Bis(2-ethylhexyl)sulfobutanedioate sodium (88 g) is dissolved in i-PrOH (100 ml), and another solution of $CaCl_2$ (25 g) in MeOH (50 ml) is prepared. Both solutions are mixed and stirred for *ca.* 3 hours and then cooled with ice. The NaCl, which precipitates from the cold mixture, is removed by filtration, and most of the alcohol is evaporated from the resulting filtrate with heat. The remaining liquor is poured into 88 ml of H_2O, and the resulting precipitate is washed with H_2O to free from chloride ion. The calcium salt is then dried.

3.3. 2-Aminoethanol Salts

Benzothiazine Dioxide 2-Aminoethanol

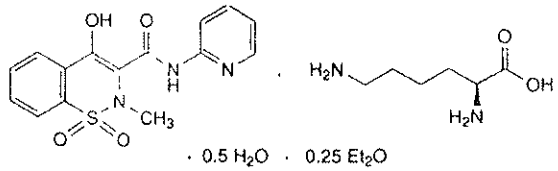


Procedure [23]

In a 2-l, three-necked round bottomed reaction flask equipped with magnetic stirrer, dropping funnel (250 ml), and thermometer, a filtered solution of 55.0 g (0.166 mol) 4-hydroxy-2-methyl-*N*-(pyridin-2-yl)-1,2-2*H*-benzothiazine-3-carboxamide 1,1-dioxide dissolved in 660 ml of CH_2Cl_2 is placed. The solution, which also contained 0.1 g of the mono(2-aminoethanol) salt of 4-hydroxy-2-methyl-*N*-(pyridin-2-yl)-1,2-2*H*-benzothiazine-3-carboxamide 1,1-dioxide as seed, is initially prepared by first dissolving the solid material in 610 ml of CH_2Cl_2 in an *Erlenmeyer* flask at 25 °C under gentle magnetic stirring. The remaining 50 ml of CH_2Cl_2 is then used for washing during the transfer of the solution to the aforementioned reaction flask. At this point, the latter flask and its contents are heated to 27 °C with the aid of a steam bath, and the entire system was subjected to constant and vigorous agitation, while a solution consisting of 10.7 g (0.175 mole) of 2-aminoethanol dissolved in 110 ml of fresh CH_2Cl_2 is slowly added during 50 minutes. Upon completion of this step, the reaction mixture was stirred at 27 °C for 1 hour and then filtered on a *Büchner* funnel to afford the crystalline salt, which is dried in a vacuum oven at 35 °C. Yield: 63 g of the pure mono(2-aminoethanol) salt. M.p. 171 – 174 °C.

3.4. Lysine Salts

Benzothiazine Dioxide Lysine Salt



· 0.5 H_2O · 0.25 Et_2O

Procedure [24]

In a 1000-ml *Erlenmeyer* flask equipped with magnetic stirrer and reflux condenser, a filtered solution of 3.5 g (0.0106 mol) of 4-hydroxy-2-methyl-*N*-(pyridin-2-yl)-1,2-2*H*-benzothiazine-3-carboxamide 1,1-dioxide, 1,54 g (0.0106 mol) of L-lysine, and 700 ml of EtOH is placed to form a yellow suspension. This suspension is then heated to reflux and treated with 10 ml of H_2O added slowly at the reflux point. The resulting yellow solution was then cooled to room temperature (*ca.* 25 °C) and evaporated to near dryness under reduced pressure to afford a yellow foam. This foam is subsequently treated with 400 ml of Et_2O by slurrying overnight and then filtered to give a fine yellow solid. Yield: 4,5 g (89%) of the pure amorphous L-lysine salt as the hemihydrate with 0.25 mol of Et_2O.

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Review

Pharmaceutical Solids: A Strategic Approach to Regulatory Considerations

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Purpose. This review describes a conceptual approach to the characterization of pharmaceutical solids. ***Methods.*** Four flow charts are presented: (1) polymorphs, (2) hydrates, (3) desolvated solvates, and (4) amorphous forms. ***Results.*** These flow charts (decision trees) are suggested as tools to develop information on pharmaceutical solids for both scientific and regulatory purposes. ***Conclusions.*** It is hoped that this review will lead to a more direct approach to the characterization of pharmaceutical solids and ultimately to faster approval of regulatory documents containing information on pharmaceutical solids.

KEY WORDS: polymorph; hydrate; amorphous form; desolvated solvate.

Interest in the subject of pharmaceutical solids stems in part from the Food and Drug Administration's (FDA's) drug substance guideline that states "appropriate" analytical procedures should be used to detect polymorphic, hydrated, or amorphous forms of the drug substance. These guidelines suggest the importance of controlling the crystal form of the drug substance. The guideline also states that it is the applicant's responsibility to control the crystal form of the drug substance and, if bioavailability is affected, to demonstrate the suitability of the control methods.

Thus, while it is clear that the New Drug Application (NDA) should contain information on solid state properties, particularly when bioavailability is an issue, the applicant may be unsure about how to scientifically approach the gathering of information and perhaps what kind of information is needed. This review is intended to provide a strategic approach to remove much of this uncertainty by presenting concepts and ideas in the form of flow charts rather than a set of guidelines or regulations. This is especially important because each individual compound has its own peculiarities which require flexibility in approach. The studies proposed herein are part of the Investigational New Drug (IND) process.

Solid drug substances display a wide and largely unpredictable variety of solid state properties. Nevertheless, application of basic physicochemical principles combined with appropriate analytical methodology can provide a strategy for scientific and regulatory decisions related to solid state behavior in the majority of cases. By addressing *fundamental* questions about solid state behavior at an early stage of drug development, both the applicant and the FDA are in a better position to assess the possible effects of any variations in the solid state properties of the drug substance. The resulting early interaction of the parties with regard to this area would not only tend to ensure uniformity of the materials used throughout the clinical trials but also fully resolve solid state issues before the critical stages of drug development. A further benefit of these scientific studies is the development of a meaningful set of solid state specifications which critically describe the solid form of the drug substance. These specifications would thus also facilitate the approval of a change in supplier or chemical process.

Our approach in this review is to suggest a sequence for collecting data on a drug substance that will efficiently answer specific questions about solid state behavior in a logical order. In "difficult" cases, perhaps where mixtures of forms must be dealt with, or other unusual properties are encountered, the suggested sequences would still have to be followed as a first stage in this investigation.

We have chosen to present this approach in the form of a series of decision trees, or flow charts (algorithms), one for each of the most common solid state forms. The charts are accompanied by examples from the literature representing the kind of data that would be useful in supporting the various decisions.

Decision trees provide conceptual frameworks for understanding how the justification for different crystal forms might be presented in the drug application. Industry may wish to use these decision trees as a strategic tool to organize the gathering of information early in the drug development process. Put another way, these decision trees provide a thought process that will lead to development of the most

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appropriate analytical controls. One should also note that it is the responsibility of the industry to select the appropriate test or tests to identify the phase of the solid and determine its relevant pharmaceutical properties. This approach is superior to simply performing a broad range of tests without regard to their relevance.

We should point out that, from a regulatory standpoint, if a company can establish a specification/test to ensure production of a well defined solid form of the drug substance, then it is not necessary to do all of the physical/chemical testing outlined in the decision trees. From a scientific standpoint, however, such an approach is risky since new forms may appear unpredictably during various stages of the development process. The appearance of these new forms usually slows the drug approval process and makes planning difficult.

Four decision trees are described in the sections that follow: Polymorphs; Hydrates (Solvates); Desolvated Solvates; and Amorphous Forms. Polymorphs exist when the drug substance crystallizes in different crystal packing arrangements all of which have the same elemental composition (Note that hydrates can exist in polymorphs). Hydrates exist when the drug substance incorporates water in the crystal lattice in either stoichiometric or non-stoichiometric amounts. Desolvated solvates are produced when a solvate is desolvated (either knowingly or unknowingly) and the crystal retains the structure of the solvate. Amorphous forms exist when a solid with no long range order and thus no crystallinity is produced. It is apparent that the appropriate flow chart can only be determined after the solid has been characterized using some of the tests described in the first decision point of the decision trees/flow charts (i.e. X-ray powder diffraction, elemental analysis, etc.). If there is no interest in marketing or producing an amorphous form or desolvated solvate at any stage in the process, then the corresponding flow charts do not need to be addressed. As already mentioned, it is advisable to investigate the drug substance for the existence of polymorphs and hydrates since these may be encountered at any stage of the drug manufacturing process or upon storage of the drug substance or dosage form.

All of the flow charts end (see for example Figure 1) with an indication of the types of controls which will be required based on whether a single morphic form or a mixture will be produced as the drug substance. Although this ending provides a simplistic view of a very complicated process of selecting appropriate controls, it is included to illustrate the consequence of the decisions made with regard to the drug substance. The reader should realize that the actual selection of the appropriate control could be the subject of another review which might contain another set of flow charts or decision trees.

POLYMORPHS

The flow chart/decision tree for polymorphs is shown in Figure 1. It outlines investigations of the formation of polymorphs, the analytical tests available for identifying polymorphs, studies of the physical properties of polymorphs and the controls needed to ensure the integrity of drug substance containing either a single morphic form or a mixture.

A. Formation of Polymorphs—Have Polymorphs Been Discovered?

The first step in the polymorphs decision tree is to crystallize the substance from a number of different solvents in order to attempt to answer the question: Are polymorphs possible? Solvents should include those used in the final crystallization steps and those used during formulation and processing and may also include water, methanol, ethanol, propanol, isopropanol, acetone, acetonitrile, ethyl acetate, hexane and mixtures if appropriate. New crystal forms can often be obtained by cooling hot saturated solutions or partly evaporating clear saturated solutions. The solids produced are analyzed using X-ray diffraction and at least one of the other methods. In these analyses, care must be taken to show that the method of sample preparation (i.e. drying, grinding) has not affected the solid form. If the analyses show that the solids obtained are identical (e.g. have the same X-ray diffraction patterns and IR spectra) then the answer to the question "Are polymorphs possible?" is "No",

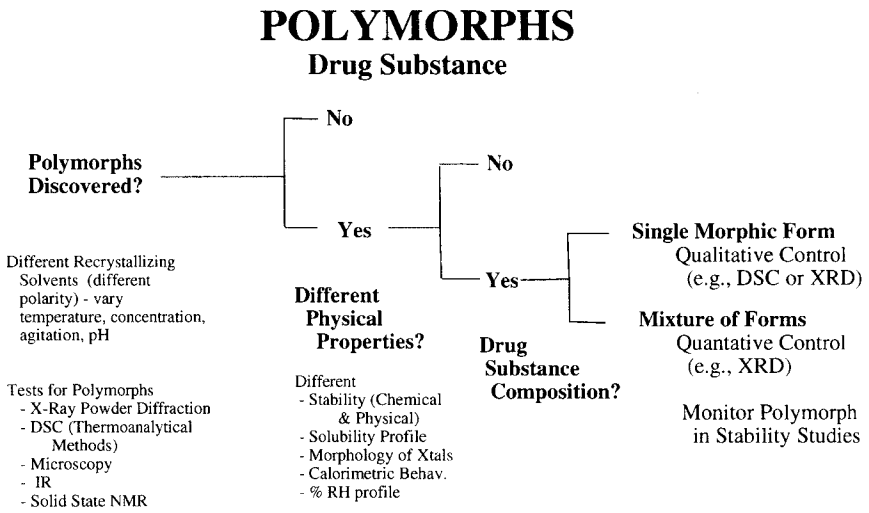

Figure 1. Flow chart/decision tree for polymorphs.

and further research is not needed. The work of Miyamae *et al.* serves as a good example of solid state studies of a drug substance which exists as polymorphs (1). Powder diffraction showed that there were two crystal forms (see Figure 2).

These workers also carried out single crystal analysis of the two crystal forms of the compound. The structures are shown in Figure 3. While such studies are not required, and indeed sometimes not possible, they provide an unequivocal confirmation of the existence of polymorphs. Moreover, once the single crystal structure of a phase has been determined, it is possible to calculate the corresponding X-ray powder pattern. This provides an irrefutable standard for identifying the phase by that method.

The DSC thermal curves of the two forms are slightly different, as shown in Figure 4 and thus may not be the preferred way of differentiating these polymorphs.

The IR spectra of the two polymorphs are quite similar(1), and IR does not appear to be a powerful method for differentiating the crystal forms in this case. Thus, for 8-(2-methoxycarbonylamino-6-methylbenzyloxy)-2-methyl-3-(2-propynyl)-imidazo{1,2-a}pyridine, powder diffraction appears to be the best method for differentiating the two forms.

Solid-state NMR is another powerful technique for analyzing different crystal forms (2,3). Figure 5 shows the solid-state C-13 NMR spectra of Forms I and II of prednisolone. Differences in the positions of the two resonances in the 120 ppm range clearly differentiate the two forms. In principle, solid state NMR is an absolute technique in which the signal intensity is proportional to the number of nuclei provided appropriate conditions are met. In addition, solid state NMR is a bulk technique which is not very sensitive to surface changes. This method appears to be very sensitive and will undoubtedly be used more often in the future as a tool to detect different crystal forms. However, with present technology, errors in solid-state quantitative studies may be rather large.

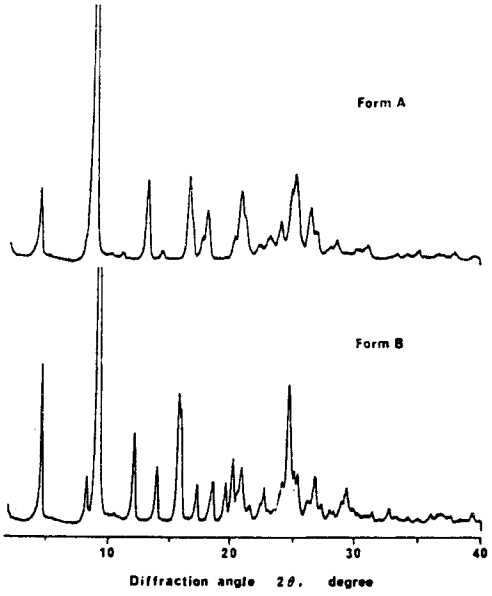

Figure 2. Powder X-ray diffraction patterns of the polymorphs of 8-(2-methoxycarbonylamino-6-methylbenzyloxy)-2-methyl-3-(2-propynyl)-imidazo{1,2-a}pyridine (1).

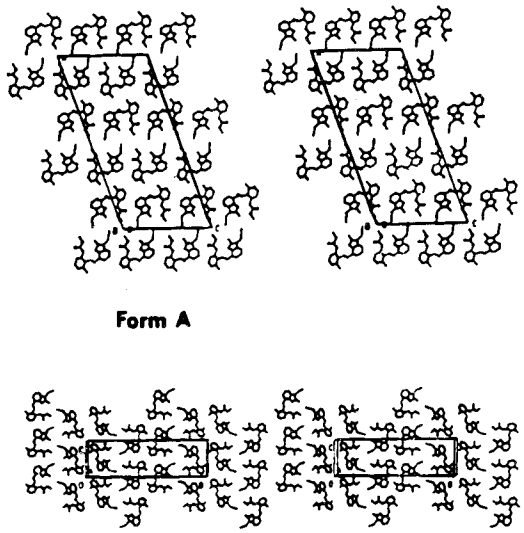

Figure 3. Stereoscopic drawings of the crystal packing of both polymorphs of 8-(2-methoxycarbonylamino-6-methylbenzyloxy)-2-methyl-3-(2-propynyl)-imidazo{1,2-a}pyridine viewed along the shortest axis (Form A, b-axis; Form B, a-axis) (1).

B. Do the Polymorphs Have Different Physical Properties?

If polymorphs exist then it is necessary to examine the physical properties of the different polymorphs that can affect dosage form performance (bioavailability and stability) or manufacturing reproducibility. The properties of interest are solubility profile (intrinsic dissolution rate, equilibrium solubility), stability (chemical and physical), and crystal morphology (including both shape and particle size), calorimetric behavior, and %RH profile. If there are no discernible differences between these physico-chemical properties, then the answer to the second question in the decision tree, "Different physical properties?" is "No."

The variable physical properties of several drugs with different polymorphs are reported in the literature. For example, the dissolution profiles of the polymorphs of chloramphenicol are significantly different (4). In addition, van't Hoff solubility analysis has been used to elucidate the dif-

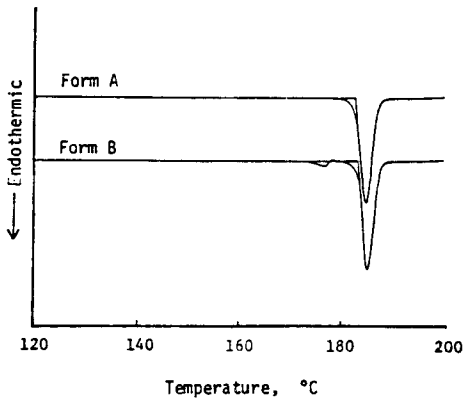

Figure 4. DSC thermal curves of the polymorphs of 8-(2-methoxycarbonylamino-6-methylbenzyloxy)-2-methyl-3-(2-propynyl)-imidazo{1,2-a}pyridine (1). These curves show that Form A melts whereas Form B undergoes a small endothermic transition and then melts at the same temperature as Form A.

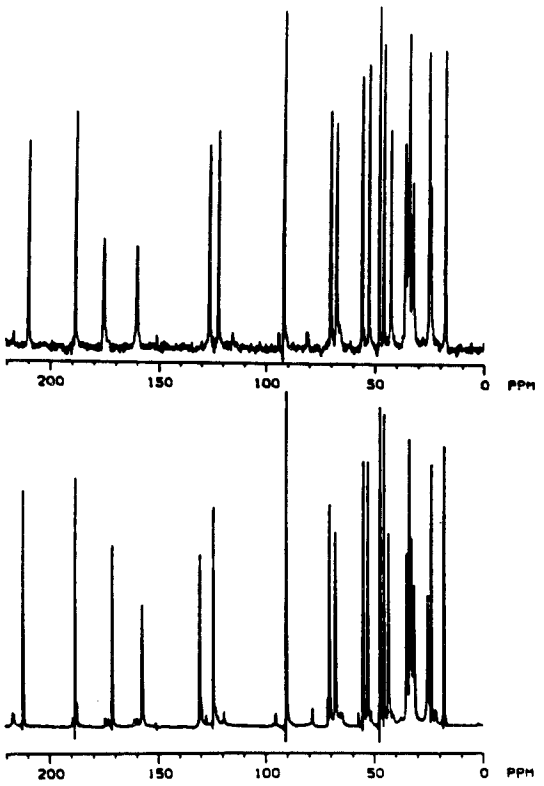

Figure 5. Solid state NMR of the two Crystal Forms of Prednisolone (2).

ferent solubilities of two polymorphs of methyl prednisolone(5). This method involves determining the equilibrium solubility of each polymorph at various temperatures. The log of the equilibrium solubility is then plotted vs 1/T. This should give straight lines for each polymorph and the temperature at which the curves intersect is the transition temperature. This technique does not work if the polymorphs interconvert.

For balance, it is important to point out that there are also cases where polymorphs exist but they have virtually identical dissolution properties(6).

C. Drug Substance Control

The important question lies in the properties that differ among polymorphs and whether those properties affect the dosage form performance (i.e., quality or bioavailability). If they do then from a regulatory standpoint it is appropriate to establish a specification/test (e.g. powder X-ray diffraction or IR) to ensure the proper form is produced. From a production standpoint, it is important to develop a process that reproducibly produces the desired polymorph.

If mixtures of forms cannot be avoided, then quantitative control is needed to ensure that a fixed proportion of forms is obtained. Furthermore, the method of analyzing for the proportion of forms would have to be validated. Also, the proportion of forms would have to remain within stated limits through the retest date of the drug substance and potentially throughout the shelf life of the product; a difficult requirement if the forms interconvert. Thus, the way to avoid a substantial amount of work in this area is to select a single solid form for production. Usually, this would be the most physically stable form when their bioavailabilities are not significantly different. Selection of the most stable from would, of course, insure that it there would be no conversion into other forms.

Powder diffraction is often a useful method to determine the percentages of polymorphs in a mixture; however, the detection limit is variable from case to case and can be as high as 15%. Matsuda (7) carried out a mixture analysis of phenylbutazone polymorphs. Diffraction lines disappear and appear as the ratio of the crystal forms change. Some of these calibration curves developed from this analysis are almost horizontal, meaning that any given mixture gives the same line intensity in this mixture range. However, other calibration curves are sloped and would appear to allow a reasonable analysis. It is fair (although Matsuda did not carry out an estimate) to estimate the errors in this analysis as ±15%.

Tanninen and Ylirussi (8) used computer curve fitting to carry out a mixture analysis of prazosin. In this particular case, they reported a highly accurate analysis, and, in fact, showed a calibration curve that could detect 0.5% of one form in another. This is obviously a highly accurate mixture analysis by powder diffraction and shows the power of this method for some applications. However, this analysis required extreme care in sample preparation and may be more difficult to carry out in a production setting where particle size may not be controlled. Similar comments apply to the analysis of mixtures by IR, where the accuracy and precision may also vary considerably from case to case. Given the analytical problems in dealing with mixtures of forms, it may generally be simpler to develop a method to prepare only one crystal form.

In summary, it is important to determine whether polymorphs are present and to solve any problems before pivotal clinical studies are initiated.

D. Determination of the Polymorph Present in the Drug Product

In cases where stability or bioavailability issues exist, the solid form present in the drug product should be investigated, if possible.

For bulk drug substances, X-ray powder diffraction and other techniques can identify the polymorph; however, solid state NMR appears to be the best method for the study of the drug substance in the dosage form (2, 3). Solid-state NMR study of three commercial products containing prednisolone showed that the products A and B contain Form I, whereas product C contains Form II.. This analysis was possible even though these tablets contain approximately 95 mg of excipients and 5 mg of drug. There are numerous cases, often involving complex mixtures or low dose products, where solid state NMR (and, in fact, any technique) will not be sensitive enough to identify the polymorph present in the drug product. However, the safety and efficacy is, of course, controlled by the potency assays and by the physical tests (e.g., dissolution).

HYDRATES (SOLVATES)

The flow chart/decision tree for hydrates (solvates) is shown in Figure 6. It outlines investigations of the formation

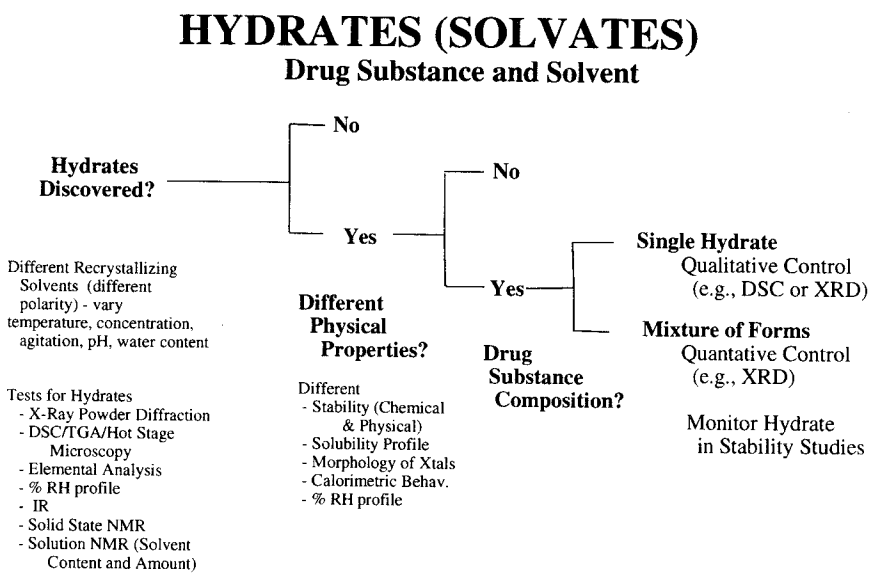

Figure 6. Flow chart for solvates or hydrates.

of hydrates (solvates), the analytical tests available for hydrates (solvates), studies of the physical properties of hydrates (solvates) and the controls needed to ensure the integrity of drug substance containing either a single morphic form or a mixture.

A. Have Hydrates (Solvates) Been Discovered?

The flow chart for hydrates (solvates) (Figure 7) is applied after the preliminary crystallizations have been completed. These are essentially the same as in the polymorph decision tree but, in addition, should include solvent-water mixtures in order to maximize the chance for hydrate formation. These experiments can be guided by the moisture uptake (% RH) studies. Any solids that indicate a significant change in water content as indicated by the % RH-moisture profile should also be examined. The resulting solid phases are preferably characterized by a combination of methods—two for phase identity and two to reveal composition and stoichiometry.

With a very few exceptions, the structural solvent contained in marketed crystalline drug products is water. It is nevertheless often desirable to characterize other solvated crystalline forms of a drug for several reasons: they may be the penultimate form used to crystallize the final product and thus require controlled characterization; they may form if the final crystallization from solvents, especially mixed solvents, is not well controlled; they may be the actual crystallized form of a final product that is desolvated during a final drying step; they may be the form used in recovery for subsequent rework. The relevance of these points will vary from case to case, but for the present discussion we shall treat the subject of solvates in its broadest form.

Examples taken from the literature serve to illustrate the kind of data that proves useful in characterizing solvated crystal forms. For example, a recent report from our laboratory showed that IR and solid state NMR was useful for the identification of the different crystal forms of dirithromycin(9). TGA is another powerful method for the analysis of solvates. For example, one early study showed that TGA could differentiate three different hydrated salts of fenoprofen(10). Combined with IR or other methods, TGA is an unequivocal method for the verification of the existence of solvates. In addition, TGA is a good method for looking at mixtures of solvated and unsolvated crystal forms, and probably can be developed into an analytical method for determining the ratios of solvated and unsolvated forms.

DSC is also a good method for detecting solvates since there is usually heat change involved in desolvation, especially for hydrates(11). Specifically, DSC by itself does not prove the existence of a solvate, but once other analytical

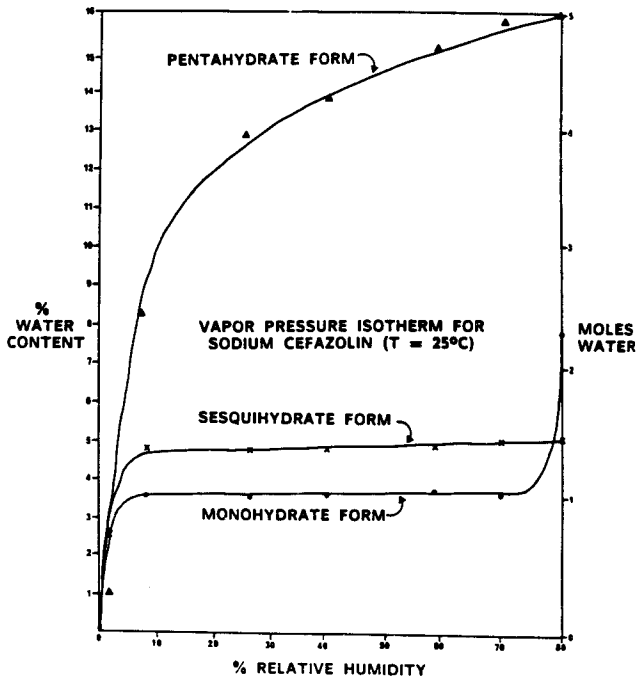

Figure 7. Water uptake vs percent relative humidity for sodium cefazolin.

data from TGA, NMR, etc. are available, DSC becomes a good method for analyzing solvates and determining a percentage of solvates present.

The three solvates of ethynylestradiol (0.5 acetonitrile, 1.0 methanol, 0.5 water) provides another interesting example (12). These solvates have different cell parameters and are crystallographically completely distinct materials. The hemihydrate was obtained from an organic solvent which is not completely miscible with water but was saturated with water. In fact, it is known that crystallization from water-immiscible solvents containing small but slightly different proportions of water can produce different hydrates of a substance.

The DSC/TGA of the three ethynylestradiol solvates(12) are unique and in this case it may be possible to develop DSC/TGA into an analytical procedure for determining the proportions of each solvate. The DSC in some of these traces appears to show a melt and recrystallization corresponding to the loss of solvent of crystallization. However, the exact interpretation of this is not possible without either a DSC microscope or interrupting the tracing to analyze the sample at various temperatures. The methanolate appears to lose solvent in two equal steps, indicating that there may also be a hemimethanolate of this compound. Again, confirmation of this would require interrupting the heating and analyzing the substance after the first solvent loss has occurred. In addition, the DSC/TGA traces suggest that all of the forms are converted to an anhydrous form which then melts at a higher temperature. Thus, interrupting any one of these thermal curves just prior to the final melt could reveal a new form that gives the powder pattern for the anhydrate. Unfortunately, no data of this type is provided in the case cited.

DSC analysis of solvates should be carried out using either an open pan or a pan with a pin-prick; otherwise, unusual and variable results will be obtained because the solvent is not provided a way of escape from the pan. One advantage of using an open pan for DSC is that it reproduces the conditions under which the TGA is performed.

Comparison of the ethinylestradiol powder diffraction patterns clearly establishes that these solvates are different crystal forms as would be expected from the single crystal data(12). In summary, DSC, TGA, and powder diffraction are all good methods for analysis of the different crystal forms of ethinylestradiol.

Figure 7 shows a percent relative humidity versus water uptake study of the type recommended by the USP committee on water(13) In this case, there are two hydrates which are relatively well behaved insofar as they are completely hydrated at about 10% relative humidity and remain uniformly hydrated throughout a wide humidity range. On the other hand, the so-called pentahydrate, which really is only a pentahydrate at very high humidity, changes water content considerably as the relative humidity is changed. The USP committee on moisture specifications recommended that moisture uptake vs relative humidity studies should be routinely performed on all drug substances and excipients (13).

B. Do the Hydrates (Solvates) Have Different Physical Properties?

The physical properties of hydrates are often quite different from the anhydrate form. Figure 8 shows the dissolution profile of theophylline hydrate and anhydrate. This figure shows that the anhydrate reaches a much higher solubility in water, and on extended exposure recrystallizes to the less soluble hydrate. Such differences must be further examined for possible effects on bioavailability.

In our laboratory we have described the different crystal forms of hydrocortisone-21-tertiary butylacetate(14). Our studies showed that the nonstoichiometric ethanolate is oxygen-sensitive and, of course, would show different physical properties from the stoichiometric ethanolate and the other solvates. Prednisolone tertiary-butylacetate also exists as a nonstoichiometric hydrate which is oxygen sensitive(15). Thus, these are cases where different crystal forms have different chemical stability, although there may be no significant differences in solubility.

C. Mixtures of Polymorphs and Hydrates

Other drug substances exist as both polymorphs and solvates. For example, furosemide exists in two polymorphs, two solvates, and an amorphous form (16, 17). The polymorphs are enantiotropically related, which means that at one temperature one polymorph is more stable, but at a different temperature the other polymorph is physically more stable. That is, plots of solubility versus temperature cross for the two polymorphs. In addition, the different crystal forms have different photostability (chemical stability in light) and moreover have different dissolution rates. Thus, there are significant differences in both chemical and physical properties.

The five different forms, or modifications of furosemide, give clearly different powder patterns. Thus, powder diffraction is a good method for analysis of these different forms. There are similarities between the IR spectra of the five different forms but there are also some significant differences, and expansion and careful analysis could lead to an FT/IR method for analysis of these different forms. IR would probably be a useful method for analysis at least for pairs of these compounds. However, it is not clear whether IR could be used to determine the percentages of several different forms in a more complex mixture. The DSC and TGA of the different forms are significantly different. As expected, the solvates show weight loss in the TGA.

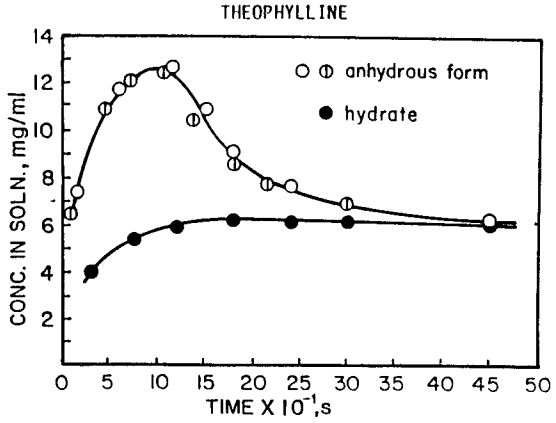

Figure 8. The dissolution-time curves for anhydrous and hydrated theophylline in water at 25°. The two types of open circles represent successive experiments (18).

The interconversion of the different forms of furosemide have been analyzed and a diagram constructed. Such a diagram can be experimentally difficult when so many pairs of crystal forms must be studied for possible interconversions and under different conditions. It is clear from this diagram that many of the forms of furosemide can be converted to form I. This study is one of the most complete reports of solvates and polymorphs available in the literature and serves as a model for studies of such systems for regulatory submissions.

D. Determination of the Hydrate Present in the Drug Product

Another important area is the analysis of the material which is produced after wet granulation of a substance which can form hydrates. We are aware of cases where the bulk drug substance is manufactured and stored as the anhydrate. However, upon wet granulation, there is a conversion (either partial or complete) to a hydrate. Subsequent drying is sometimes not adequate to convert the substance back to the anhydrate, and a hydrate or a mixture of hydrate and anhydrate remain. The formation of a hydrate and its subsequent drying can result in a change in particle size of the drug substance (19). It may also be possible to cause transformations during other processing steps. It is thus recommended that if wet granulation or processing that subjects the drug to even brief changes in temperature or pressure (e.g. milling or compression) is contemplated, then extensive studies of the ability to convert the drug substance to a new crystal form be carried out by mimicking the processing step in the laboratory.

DESOLVATED SOLVATES

The term "desolvated solvates" refers to compounds that are crystallized as solvates but undergo desolvation prior to analysis. Often these "desolvated solvates" retain the structure of the solvate with relatively small changes in the lattice parameters and atomic coordinates, but no longer contain the solvent. In addition, desolvated solvates are apt to be less ordered that their crystalline counterparts. These forms are particularly difficult to characterize properly since analytical studies indicate that they are unsolvated materials (anhydrous crystal forms) when, in fact, they have the structure of the solvated crystal form from which they were derived. Several observations may give clues that one is dealing with a desolvated form: (1) The form can be obtained from only one solvent; (2) On heating, the form converts to a structure known to be unsolvated; and (3) The form has a particularly low density compared to other forms of the same substance. Experiments that help to clarify whether an apparently solvent free modification is a desolvated form or a true anhydrate include: (1) Single crystal X-ray structure determination in the presence of mother liquor from the crystallization; (2) comparison of the X-ray powder diffraction patterns and solid state NMR spectra of the solvated and desolvated crystal forms; and (3) determination of the vapor pressure isotherm by varying the vapor pressure of the specific solvent involved. A desolvated form will often take up stoichiometric amounts of the relevant solvent. In addition, crystals of the form directly isolated from the crystallizing medium will show a plateau in their isotherm as the vapor pressure of the solvent is reduced.

Figure 9 shows the flow chart used to address regulatory issues involving desolvated solvates. It is similar to the polymorphs flow chart except that the first question involves determining whether a solvate was formed initially and then desolvated, perhaps by "air drying." The remaining questions are identical to the polymorphs flow chart.

Figure 10 shows the behavior of three different crystal forms of the same antibiotic. One crystal form takes up about two waters of hydration and further analysis indicated that it was the dimethanolate which had been desolvated by drying. The second crystal form takes up approximately one water and was originally the monomethanolate which had been desolvated by drying. The third crystal form also takes up

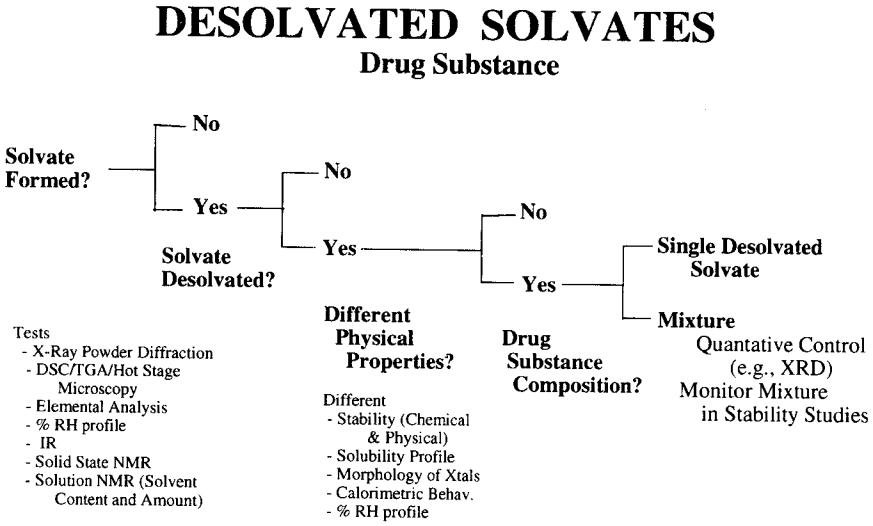

Figure 9. Flow chart for desolvated solvates.

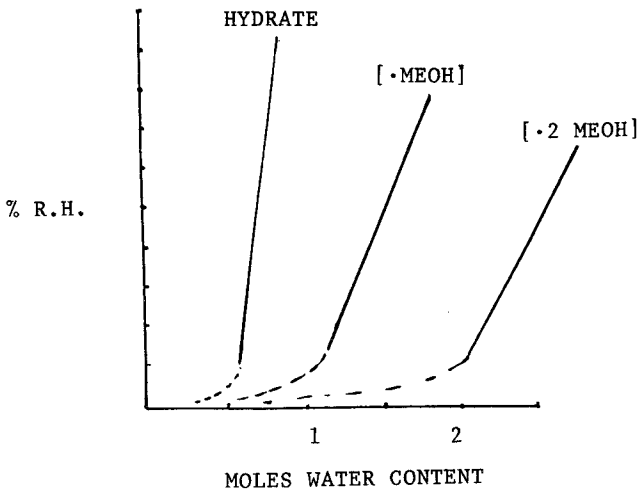

Figure 10. Water sorption by three crystal forms of cephaloridine. The brackets indicate the crystal form produced by desolvating the designated methanolate.

about one molecule of water and is the 0.75 hydrate typically obtained from water solution.

AMORPHOUS FORMS

Amorphous forms are of substantial interest because they usually are much more soluble than their crystalline counterparts. Indeed, there are cases where the amorphous forms is the only solid form that has adequate bioavailability. The initial question with this flow chart (Figure 11) is similar to the previous ones: "Are amorphous forms possible?" Amorphous forms can be prepared in different ways, for example, by spray drying or by freeze drying. One can test whether an amorphous form has been produced by using one of the methods listed. X-ray powder diffraction and microscopy are the two primary methods for determining whether an amorphous form has been produced. Powder diffraction is an excellent method for determining the existence of an amorphous form since they usually exhibit a broad hump between 2 and 20° 2θ. An amorphous form is expected to have no peaks in the powder diffraction pattern. The USP test for the presence of an amorphous form involves determining, by microscopy, whether the material lacks birefringence. IR and solid-state NMR may be useful for detecting amorphous forms since the amorphous nature of the solid sometimes results in broad lines, or in NMR, altered relaxation times. The next question on the flow chart is: "Do the amorphous forms have different physical properties?" The answer to this question will almost certainly be "Yes." Three differences from crystalline forms may generally be expected: 1) Amorphous forms would have greater solubility, 2) Amorphous forms take up water more extensively, and 3) Amorphous forms are sometimes less chemically stable. Another key question for an amorphous form is: "Does it crystallize, and how and when?" This question is very important since inadvertent crystallization can greatly affect the solubility and dissolution rate, and lead to other failures in formulation. Attempts to purposely cause amorphous forms to crystallize can provide information on the parameters involved in crystallization of amorphous forms. Specific questions include: (1) "Does the amorphous form crystallize upon exposure to heat and/or humidity?," and (2) "What other factors (e.g. mechanical pressure and seeding) can lead to the crystallization of the amorphous forms?"

The amorphous form of any substance can be partly characterized by the glass transition temperature, T_g (11). When heated to a temperature above T_g, the solid transforms from a glassy state to a more fluid-like rubbery state. The corresponding increased molecular mobility greatly raises the likelihood of two adverse events: (1) Crystallization and subsequent decreased solubility; and (2) Reduced chemical stability in the more reactive amorphous solid. Amorphous solids are also often prone to absorb moisture and this water sorption reduces the glass transition temperature further. The weight of water required to reduce the glass transition

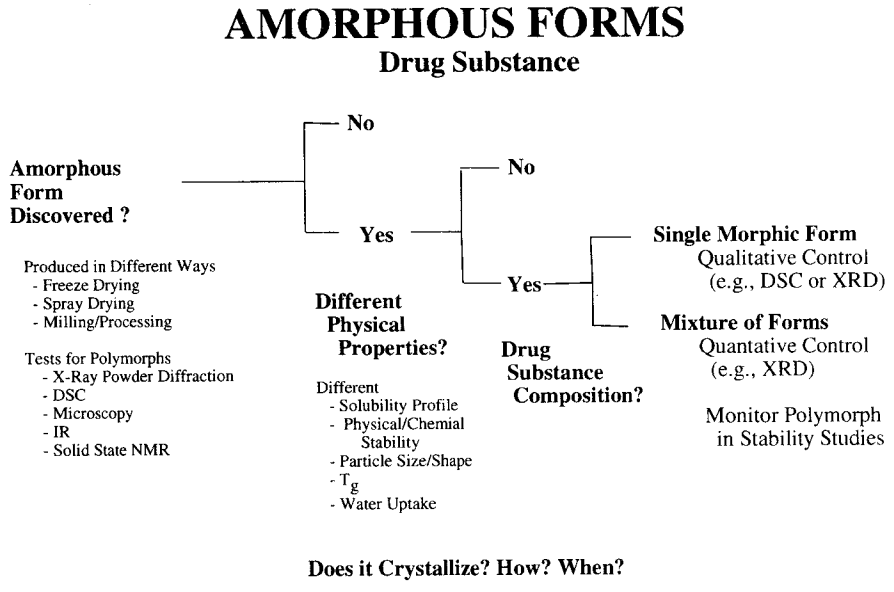

Figure 11. Flow chart for amorphous solids.

temperature to room temperature is of obvious interest and is termed W_g. Table I shows a series of interesting studies on amorphous forms of some common pharmaceuticals.

The table compares the glass transition temperatures (T_g) of a number of pharmaceutical solids with the melting temperatures (T_m). It is interesting that the average ratio of the glass transition temperature to the melting temperature is about 0.70. This table provides a simple rule of thumb which allows the prediction of the glass transition temperature of pharmaceuticals from the known melting point. Crystallization and other solid-state phenomena, such as degradation reactions, as we have said, would be more likely to occur at temperatures above the glass transition temperature. For stability, one might, therefore, wish to prepare amorphous forms only for drugs which have a T_g well above room temperature.

Amorphous indomethacin crystallizes upon standing at room temperature(Figure 12). Obviously, formulations containing amorphous indomethacin are at significant risk to crystallize and thus become less soluble. This has to some extent hampered preparing more bioavailable indomethacin dosage forms.

Quantitative analysis of mixtures of amorphous and crystalline forms provides some challenges. Cefixamine trihydrate is the subject of some early research in this area. This antibiotic, upon grinding, became a mixture of crystalline and amorphous forms. A calibration curve based upon analyzing the height of a selected powder X-ray peak was constructed and used to determine the crystallinity versus grinding time for this system. It is clear that powder diffraction provides a way to estimate the amount of amorphous cefiximine. These studies show that milling and other similar processing steps can create amorphous material and that this process may be detectable. As with wet granulation where transitions to hydrated forms can occur, processing of the drug substance can promote the formation of amorphous drug.

Pikal has compared the analysis of mixtures of crystalline and amorphous forms of several antibiotics by powder diffraction and calorimetry (20). His studies indicate that calorimetry can be a more accurate method for analysis of percent crystallinity but are complicated by water sorption. Zografi and co-workers (unpublished results) have developed a powerful method for the determination of low percentages of amorphous material based on the general propensity of amorphous materials to sorb moisture.

Table I. Pharmaceuticals Forming Glasses Above Room Temperature (21)

Pharmaceutical	T_g (K)	T_m (K)	T_g/T_m
Cholecalciferol	296	352	0.84
Sulfisoxazole	306	460	0.67
Stilbestrol	308	439	0.70
Phenobarbital	321	443	0.72
Quinidine	326	445	0.73
Salicin	333	466	0.71
Sulfathiazole	334	471	0.71
Sulfadimethoxine	339	465	0.73
Dehydrocholic acid	348	502	0.69
17β-Estradiol	351	445	0.80

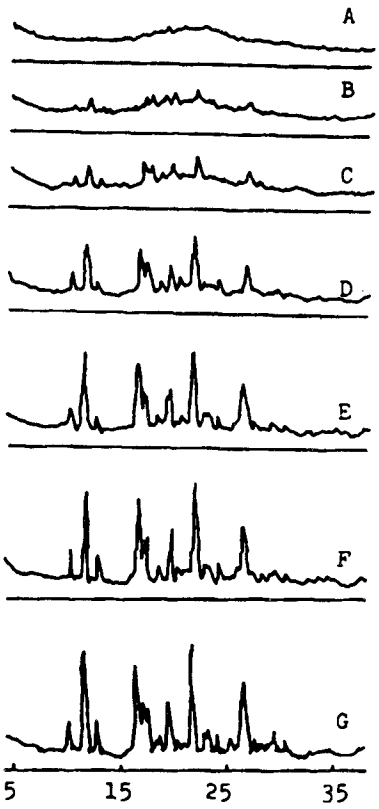

Figure 12. Behavior of amorphous indomethacin upon standing: A, at start; B 24 h; C, 48 h; D, 7d; E, 14d; F, 30d; G, 67d (22).

SUMMARY

Four flow charts which describe approaches to regulatory issues involving pharmaceutical solids have been developed. These flow charts are for the different types of solids generally encountered (polymorphs, solvates, desolvated solvates, and amorphous forms). It is hoped that these flow charts will guide the solid-state research needed to prepare a comprehensive regulatory submission on the physicochemical properties of a pharmaceutical. It is also hoped that this review has provided enough information to allow the generation of results and information necessary to prepare a drug substance submission that will be quickly approved.

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(54) Title: PROCESS FOR THE PREPARING ZIPRASIDONE MONOHYDROCHLORIDE HYDRATE

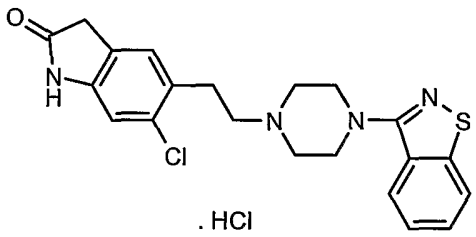
(57) Abstract: The present invention describes a process for preparing 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one (Ziprasidone) monohydrochloride hydrate or a pharmaceutically acceptable acid addition salt thereof, which is well known agent for treating various disorders including schizophrenia, migraine pain and anxiety. The manufacturing process of Ziprasidone comprises the reaction of hydrochloride salt of 1,2-benzisothiazole-3-piprazinyl with 2-Chloroethyl-6-chlorooxindole in aqueous sodium carbonate solution. Its hydrochloride salt is prepared in aqueous methanol using hydrogen chloride.

PROCESS FOR THE PREPARING ZIPRASIDONE MONOHYDROCHLORIDE HYDRATE

FIELD OF THE INVENTION

The present invention relates to a process for the preparation of 5-(2-(4-1,2-Benziso-thiazol-3-yl) piperazinyl) ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one (Ziprasidone) monohydrochloride hydrate having Formula I

Formula I



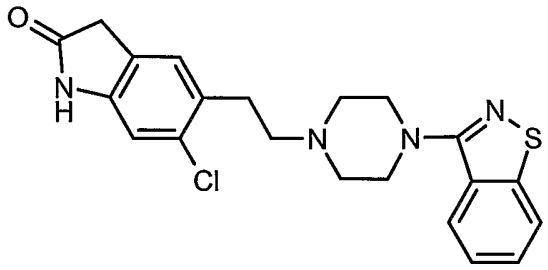
. HCl

: hydrate

The Formula I product is prepared by condensing 1,2-benzisothiazole-3-piprazinyl hydro-chloride with 2-chloroethyl-6-chloro-oxindole in presence of sodium iodide. The crude product is purified by re-crystallization in a mixture of tetrahydrofuran and dimethyl forma-mide followed by their transformation into hydrochloride hydrate in aqueous methanol having characteristic IR, TGA and DSC properties.

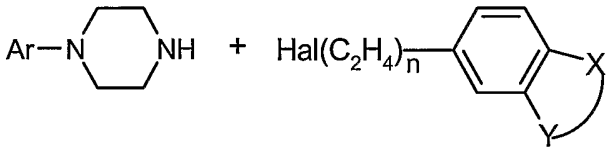
BACKGROUND OF THE INVENTION

The neuroleptic activity of 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one (Ziprasidone) and its pharmaceutically acceptable salts are useful for treating psychotic disorders in human subject. For example Ziprasidone is useful for treating psychotic disorders of the schizophrenic types and in particular it is useful for removing or ameliorating such symptoms as anxiety, agitation, excessive aggression, tension and social or emotional withdrawal in psychotic patients. Ziprasidone is a well-known compound having the chemical structure:



Arylpiperazinylethyl heterocyclic compounds and their use in the treatment of psychiatric disorders are disclosed in the US Patent No. 4,558,060. The aryl group in the prior art is pyrimidinyl or an optionally substituted phenyl. Compounds with a butyl between the piperazinyl and heterocyclic group are not disclosed, and heterocyclic groups other than benzoxazolones are not disclosed.

US Patent 4,831,031 discloses that arylpiperazinyl ethyl (or butyl) heterocyclic compounds may be prepared by reacting an arylpiperazine of the formula 1 with a fused bicyclic compound of the formula 2 as follows:

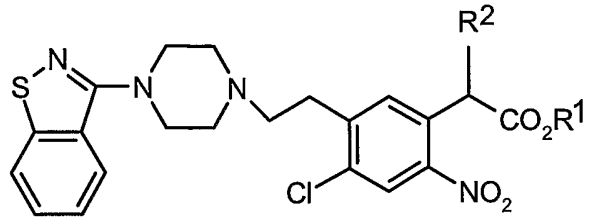


1 2

the coupling reaction is generally conducted in a polar solvent (such as a lower alcohol, dimethylformamide or methylisobutyl ketone) and in the presence of a weak base and preferably the reaction is carried out in presence of a catalytic amount of sodium iodide and a neutralizing agent for hydrochloride such as sodium carbonate.

Yevich, et al, J. Med. Chem, 29, No.3, page 359 to 369 (1986) relates to a method of producing 1-(1,2-benzisothiazol-3-yl) and (1,2-Benzisoxazole-3-yl)piperazine derivatives. Several reaction schemes are disclosed including reaction schemes wherein coupling occurs in a free base.

On the other hand US Patent No. 5,359,068 describes the synthesis of Ziprasidone by treating a compound having formula:



wherein R^2 is hydrogen, CN, or CO_2R^1 and R1 is hydrogen or (C_{1-6}) alkyl with a reducing agent selected from the group consisting of sodium hydrosulfite, hydrogen in the presence of a hydrogenation catalyst, iron in acetic acid, zinc and calcium chloride in acetic acid and NH_2-PO_2 in the presence of Pd/C with proviso that when R_2 is CN or CO_2R^1 or (C_{1-6}) alkyl the product of the reduction is heated with an 6 or 3N hydrochloric acid or acetic acid.

US Patent No. 6,150,366 , discloses a manufacturing process of ziprasidone hydrochloride monohydrate ,which states:

1) dissolving ziprasidone free base in a solvent comprising THF and water, in a volume ratio of about 22-35 unit volumes of THF to about 1.5-8 volumes of water;
2) heating the solution resulting from step (1);
3) adding HCl to the solution resulting from step (2); and
4) cooling the solution resulting from step (3) and crystals were collected by filtration and drying.

Apart from these, US Patent 5,312,925 describes a process for the synthesis of monohydrate of 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one hydrochloride and its characterization based on IR, XRD and moisture content. US Patent 5,312,925 also discloses that the hemihydrate may be obtained by the process described in Example 16 of US Patent No. 4,831,031 and their characterization by IR, XRD and moisture content. It also discloses the IR, XRD and moisture content of anhydrous Ziprasidone hydrochloride. According to the invention in "925" patent water content having 3.97, 2.55 and 0.37 % were used for the IR and XRD study of Ziprasidone hydrochloride monohydrate, hemihydrate and anhydrous. In this invention, the monohydrate of Ziprasidone hydrochloride was prepared by reacting anhydrous 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one with aqueous hydrochloric acid. The temperature range of the reaction was maintained between 60 to 65 °C and aqueous HCl used for salt formation was around 0.7 M. Depending on the reaction temperature and other

conditions, the reaction times were set around 3 to 24 hours. The final product thus obtained was dried carefully monitored conditions to make certain that water content was from about 3.8 % to about 4.5 % to obtain the stable monohydrate.

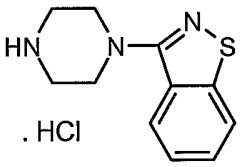
SUMMARY OF THE INVENTION

The present invention is directed to a process for the manufacture of 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one monohydrochloride hydrate which has utility as a neuroleptic, and is thus useful as an antipsychotic.

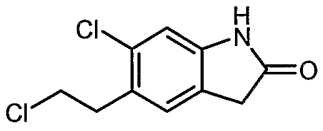
In a first embodiment , the invention is directed to crystalline Ziprasidone hydrochloride hydrate.

In a second embodiment, the invention is directed a process for the preparation of the compound of Formula I, comprising reactions of piperazine hydrochloride salt derivatives of Formula II with alkyl halide derivatives of oxindole of Formula III:

Formula II



Formula III



in aqueous medium using NaI as catalyst. The yield of the desired product is quite high and purity is more than 99. 5 % (based on HPLC assessment). The moisture content in the Formula I product is around 5.0 to 6.0 % by weight. This is in contrast to water content having 3.97, 2.55 and 0.37 % disclosed by the US Patent 5,312,925. Ziprasidone salt of the present invention is having the water content as surface instead of water of crystallization and this is supported by IR Spectrum, Thermal Gravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC). The hydrated form of Ziprasidone hydrochloride in the present invention is neither monohydrate nor hemihydrate as evident from their IR spectrum and TGA analysis. TGA clearly demonstrates that water content (up to 6 %) which is associated with the sample of this invention is not the water of crystallization, since water loss is at 85 to 90 °C.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a Infra Red Spectrum of Ziprasidone hydrochloride hydrate.

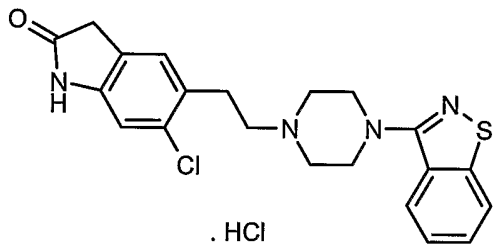
Figure 2 is a Thermal Gravimetric Analysis of Ziprasidone hydrochloride hydrate; and

Figure 3 is a Differential Scanning Calorimetry (DSC) of Ziprasidone hydrochloride hydrate

DETAILED DESCRIPTION OF THE INVENTION

This invention is directed to an anti-psychiatric disorder compound having the therapeutic value and a process of its manufacture. In particular, the present invention is directed to monhydrochloride salt of 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one hydrate having the chemical formula:

Formula I



. HCl

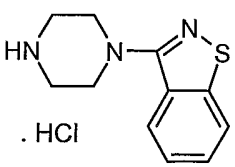
: hydrate

According to one embodiment the present invention, 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one and its hydrated hydrchloride salt is prepared according to the following synthetic reaction scheme:

(a) Reaction of 1,2-benzisothiazole-3-piperazinyl HCl with 2-Chloroethyl-6-chloro oxindole

1,2-Benzisothiazole-3-piperazinyl Hydrochloride (Formula II):

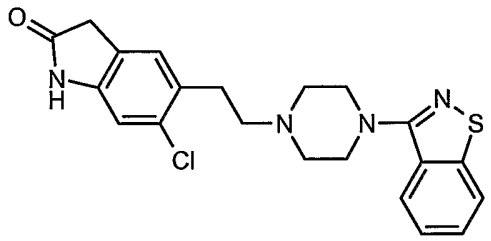
Formula II



. HCl

is reacted with a molar excess of 2-Chloroethyl-6-chloro oxindole (Formula III) in a solvent in the presence of a mild base to form 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)- ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one (Formula IV).

Formula IV



The molar excess of 2-Chloroethyl-6-chloro oxindole used in this reaction stage is typically between about 1 and about 3 fold preferably about 2 fold. Suitable solvents for this synthetic stage include water. 1,-Benzisothiazole-3-piprazinyl hydrochloride is added to the aqueous sodium carbonate solution at ambient temperature. Sodium carbonate dissolved in water is around 2-6 % by its weight. The mixture is stirred for one hour to get the suspension. 2-Chloroethyl-6-chloro-oxindole followed by sodium iodide is added to the suspension and heated the combined mixture between about 50 to 100 °C for 20 to 30 hours. After completion of the reaction, it was cooled to ambient temperature and filtered. The crude Ziprasidone thus obtained is again heated with water and cooled to room temperature, filtered and dried to get in greater than 95 % HPLC purity.

In present invention the coupling reaction is conducted in water. This in-situ aqueous based coupling process is not only more efficient but has a much lower environmental burden since the handling and disposal of organic solvents are eliminated. This process has not shown formation of any side products and does not require special isolation procedures process, e.g. extraction or distillation.

(b) Purification of Crude Ziprasidone

Ziprasidone has poor solubility in most of the common organic solvents. Therefore, the further objective of the present invention is to find out a suitable solvent or solvent mixture to purify crude Ziprasidone Base to get the required quality without loss of material. In the same pursuance, crude Ziprasidone obtained from step (a) is dissolved in a mixture of Tetrahydrofuran and N,N-Dimethylformamide (7:3, v/v) along with activated charcoal and

heated to make clear solution. The solution thus obtained is filtered, washed with cold solvent mixture of THF and DMF. Finally dried stuff is obtained in 99 % HPLC purity.

(c) Reaction of 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one with hydrogen chloride

5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one is reacted with hydrogen chloride in aqueous organic solvent, to generate 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one hydrochloride hydrate (Formula I).

Suitable aqueous organic solvent for this reaction includes, but not limited to, organic solvents such as aliphatic alcohols, e.g., methanol, ethanol, 2-propanol or n-butanol etc. The volume ratio of alcohol and water is typically from about 1:1 to about 8:2 more particularly around 7:3. The hydrochloric acid is added to the solution drop-wise and stirred for about 20 hours at about 65 °C. Concentration of the said hydrochloric acid solution in the said reaction is from about 5.0 to about 7.0 % by weight or around 6.0 moles of Conc. HCl. After washing with the cold solvent mixture of THF and DMF solvents, Ziprasidone is dried at 60 °C for 10 to 12 hours to get the 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one hydrochloride hydrate (Formula I). The final product thus obtained is 99.5 % pure based on HPLC and has the characteristic IR, TGA and DSC values which confirms it as Ziprasidone monohydrochloride hydrate. The so obtained Ziprasidone monohydrochloride is having moisture content in the range of 5.0% to 6.0%. Ziprasidone salt of the present invention is having the water content as surface water instead of water of crystallization which has been confirmed by IR Spectrum, Thermal Gravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC).

Ziprasidone monohydrochloride anhydrous is mentioned in US Patent 5,312,925. The product of the present invention i.e. Ziprasidone monohydrochloride hydrate can be made anhydrous having moisture content up to 0.7% by weight, on prolonged drying. But it has been observed that it re-absorbs moisture up to 6% when it is exposed at 60% relative humidity.

IR spectrum of Zonisamide of the present invention is characterized by the following peaks at about 3424, 3197, 2931, 2669, 2604, 2458, 1715, 1632, 1494, 1382, 1289, 1262, 1243, 1179, 1085, 991, 973, 775, 744 and 651 cm^{-1} (Figure 1). The infrared spectrum of

Ziprasidone monohydrochloride hydrate shows sharp bands at **3424 cm^{-1}** and **2458 cm^{-1}** in contrast to the IR spectrum of the Ziprasidone Monohydrochloride monohydrate disclosed in the US Patent 5,312,925.

Thermal Gravimetric Analysis of the present invention is characterized by the weight loss at about 85 to 90 °C (Figure 2).

The Differential Scanning Calorimetry (DSC) theromgram of 5-(2-(4-1,2-benziso-thiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one hydrochloride hydrate of the invention shows a peak endotherm at 104.40 °C (Figure 3).

The TGA data of Ziprasidone monohydrochloride hydrate clearly showed a weight loss at 85 to 90 °C indicating that the moisture lost is surface water and not the water of crystallization. Based on the above data of IR and TGA, it shows that the product of the present invention, i.e., Ziprasidone Monohydrochloride is having only surface moisture and not as water of crystallization.

The following examples illustrate the invention, but is not limiting thereof.

EXAMPLE 1

5-(2-(4-(1,2-benzisothiazol-3-yl)-1-piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indole-2-one (Ziprasidone)

In a 10 liter three-necked round bottom flask sodium carbonate (742 g) and pure water (3.06 liter) is charged. To the above stirred solution 1,2-benzisothiazole-3-piperazinyl HCl (611 g, 2 moles) is added at ambient temperature and stirred the reaction mixture additionally for 1 hour. To the obtained suspension 2-Chloroethyl-6-chloro oxindole (430 g, 2 moles) and sodium iodide (20 g) is added. The combined reaction mixture is heated at 60 to 90 °C for 20 to 30 hours. After completion of the reaction, reaction mass is cooled to ambient temperature and the slurry thus obtained is stirred for 1 hour more and then filtered. The wet tan colored crude free base is suspended in water (7.50 liter) , heated to 75 to 80 °C under stirring for 1 hour and filtered at 45 to 50 °C temperature. Wet compound is dried at 65 to 70 °C for 10 to 12 hours to get desired product in 752 gm yield (HPLC Purity 95 to 96%).

EXAMPLE 2

Crystallization of Crude Ziprasidone Base

Into a mixture of Tetrahydrofuran : Dimethylformamide (30 liter, 7:3, v/v), crude Ziprasidone (750 gm) is charged with activated charcoal (15 gm) and stirred the mixture for 1 hour at 65 to 67 °C. The clear solution is filtered under suction and reaction mass is concentrated up to its 3.0 to 5.0 liter volume. The slurry thus obtained is cooled to 0 to 5 °C temperature and stirred further for 1 hour. Crystallized product is filtered and washed with a mixture of Tetrahydrofuran : Dimethylformamide (250 ml), dried at 60 to 70 °C under high vacuum for 10 to 12 hours to obtain 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one free base (603 g) in more than 99% HPLC purity.

EXAMPLE 3

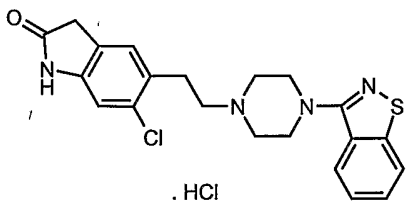
5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one monohydrochloride hydrate

Into a 10 liter three necked round bottom flask 5-(2-(4-(1,2-benzisothiazol-3-yl)-1-piperazinyl)-ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one free base (600 g, 6.452 mole) is charged in a mixture of methanol : water (6 liter, 7 : 3 v/v). To the stirred slurry concentrated hydrochloride acid (900 g) is added through a dropping funnel within 30 minutes and the slurry is kept for standing at 65 °C for 20 hours. The slurry is cooled to room temperature; filtered and washed the cake with 500 ml of the cold methanol : water mixture and then dried at 60 °C for 10 to 12 hours that yields 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one monohydrochloride hydrate (665 g) in 99.5 % HPLC purity.

We Claim:

1 A compound of Formula I

Formula I

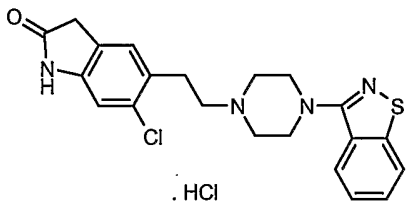


. HCl

: hydrate

2 A process for the manufacturing a compound of Formula I

Formula I



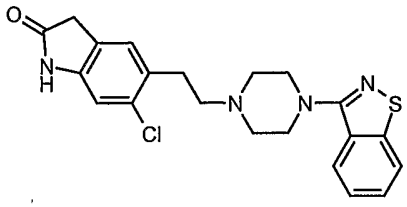
. HCl

: hydrate

the said method comprising:

(a) subjecting a compound of the Formula IV

Formula IV



with hydrochloric acid in the mixture of solvents for a sufficient time to form 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one monohydrochloride hydrate; and

(b) isolating the 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one monohydrochloride hydrate.

3 The process of claim 2, wherein step (a) is carried out in aqueous organic solvent.

4 The process of claim 3, wherein the organic solvent is an aliphatic alcohol.

5 The process of claim 4, wherein the aliphatic alcohol is methanol, ethanol, 2-propanol or n-butanol.

6 The process of claim 5, wherein more particularly aliphatic alcohol is methanol.

7 The process of claim 3, wherein the volume ratio of methanol and water is between about 1:1 to about 8: 2.

8 The process of claim 7, wherein more particularly methanol and water ratio is 7:3.

9 The process of claim 2, wherein 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one is charged to methanol-water mixture prior to addition of hydrochloic acid.

10 The process of claim 9, wherein hydrochloric acid is concentrated.

11 The process of claim 2, wherein contact ratio of 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)-ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one and hydrochloric acid is between about 1:1 to about 1:2 mol/g.

12 The process of claim 11, wherein more particularly molar ratio is about 1:1.5 mol/g.

13 The process of claim 2, wherein contacting time is conducted from about 15 to about 25 hours.

14 The process of claim 2, wherein contacting step is conducted between about 60 to about 70 °C.

15 The process of claim 2, wherein after contact time is over, mixture is cooled down to room temperature and filtered.

16 The process of claim 15, wherein isolated product is dried from about 55 to 65 °C.

17 The process of claim 16, wherein drying time is between about 10 to about 12 hours.

18 The process of claim 15-17, wherein isolated 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one monohydrochloride hydrate is isolated in more than about 99.4 % purity.

19 The compound of claim 1, wherein 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one monohydrochloride hydrate, characterized by an Infra Red Spectroscopy (IR) spectrum having the following peaks at about 3424, 3197,

2931, 2669, 2604, 2458, 1715, 1632, 1494, 1382, 1289, 1262, 1243, 1179, 1085, 991, 973, 775, 744 and 651 cm^{-1}.

20 The compound of claim 1, wherein TGA of the 5-(2-(4-1,2-benzisothiazol-3-yl)piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one monohydrochloride hydrate is characterized by the weight loss from about 85 to 90 °C.

21 The process of claim 20, wherein weight loss in TGA at about 85 to 90 °C indicates the moisture content is surface moisture.

22 The compound of claim 1, where in DSC of the 5-(2-(4-1,2-benzisothiazol-3-yl)-piperazinyl)ethyl)-6-chloro-1,3-dihydro-2H-indol-2-one monohydrochloride hydrate is characterized by a peak endotherm at about 104.40 °C.

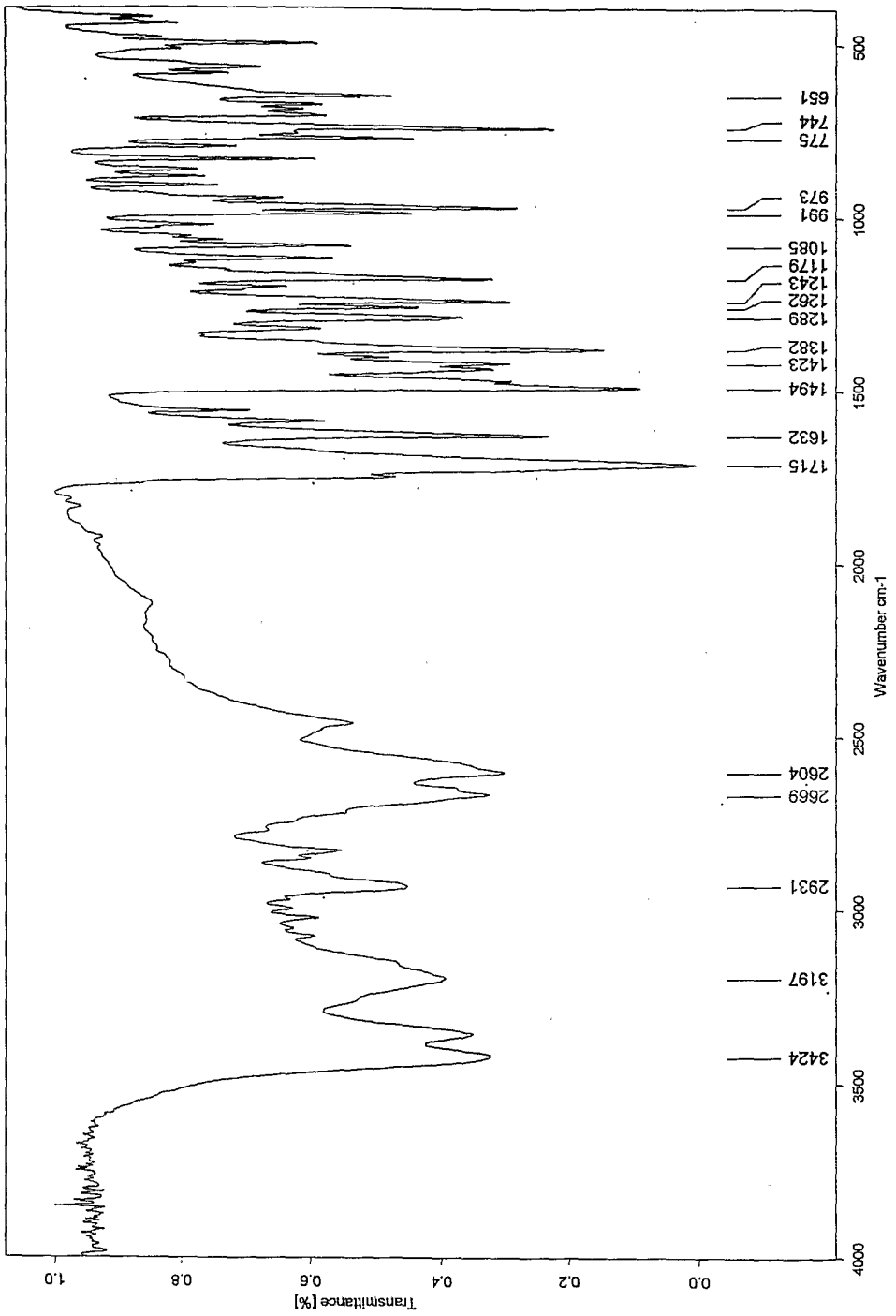

Transmittance [%]
0.0
0.2
0.4
0.6
0.8
1.0
Wavenumber cm-1
4000
3500
3000
2500
2000
1500
1000
500
3424
3197
2931
2669
2604
1715
1632
1494
1423
1382
1289
1262
1243
1179
1085
991
973
775
744
651

Figure 1

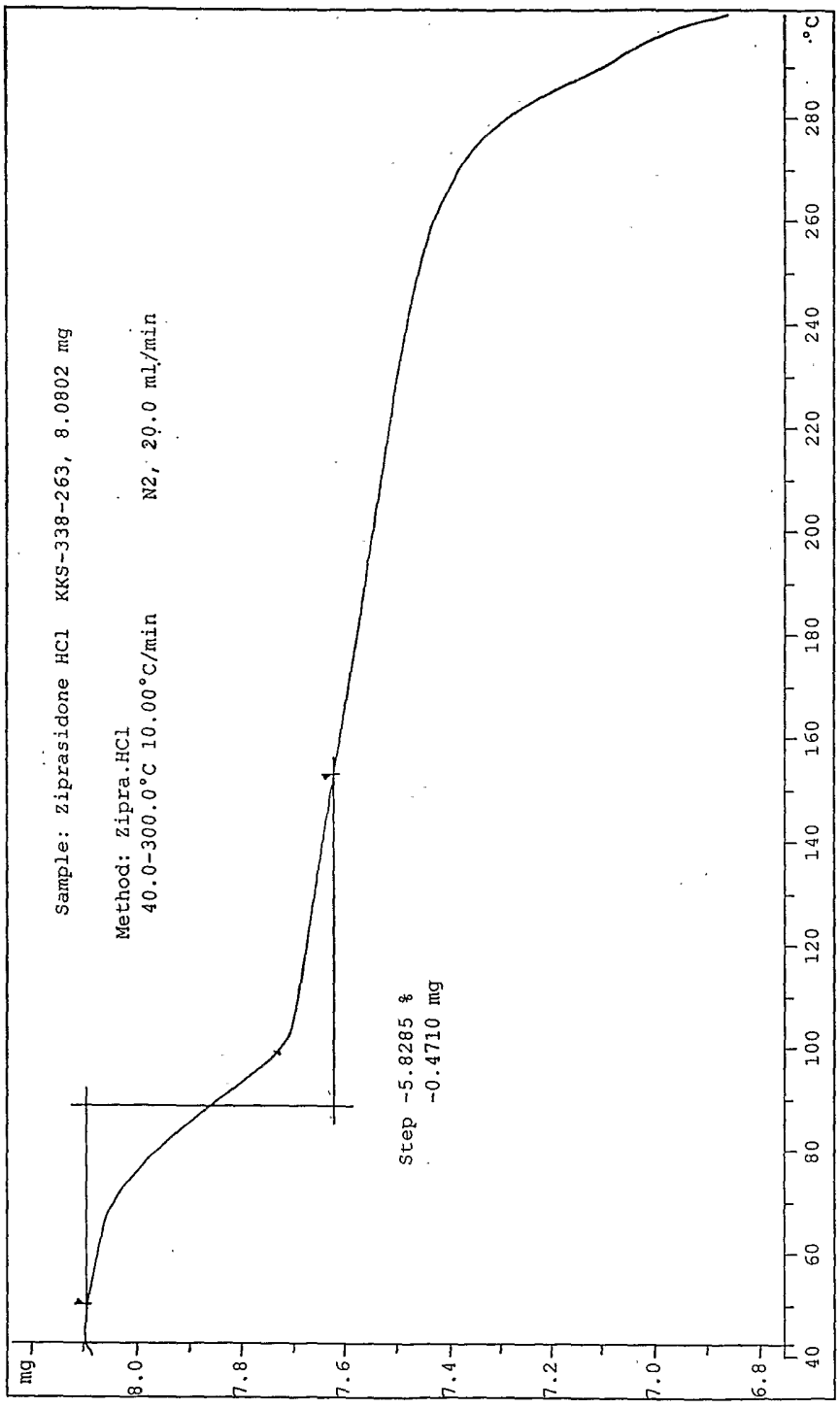

Sample: Ziprasidone HCl KKS-338-263, 8.0802 mg
Method: Zipra.HCl
40.0-300.0°C 10.00°C/min N2, 20.0 ml/min
Step -5.8285 %
-0.4710 mg
mg
°C
8.0
7.8
7.6
7.4
7.2
7.0
6.8
40
60
80
100
120
140
160
180
200
220
240
260
280

Figure 2

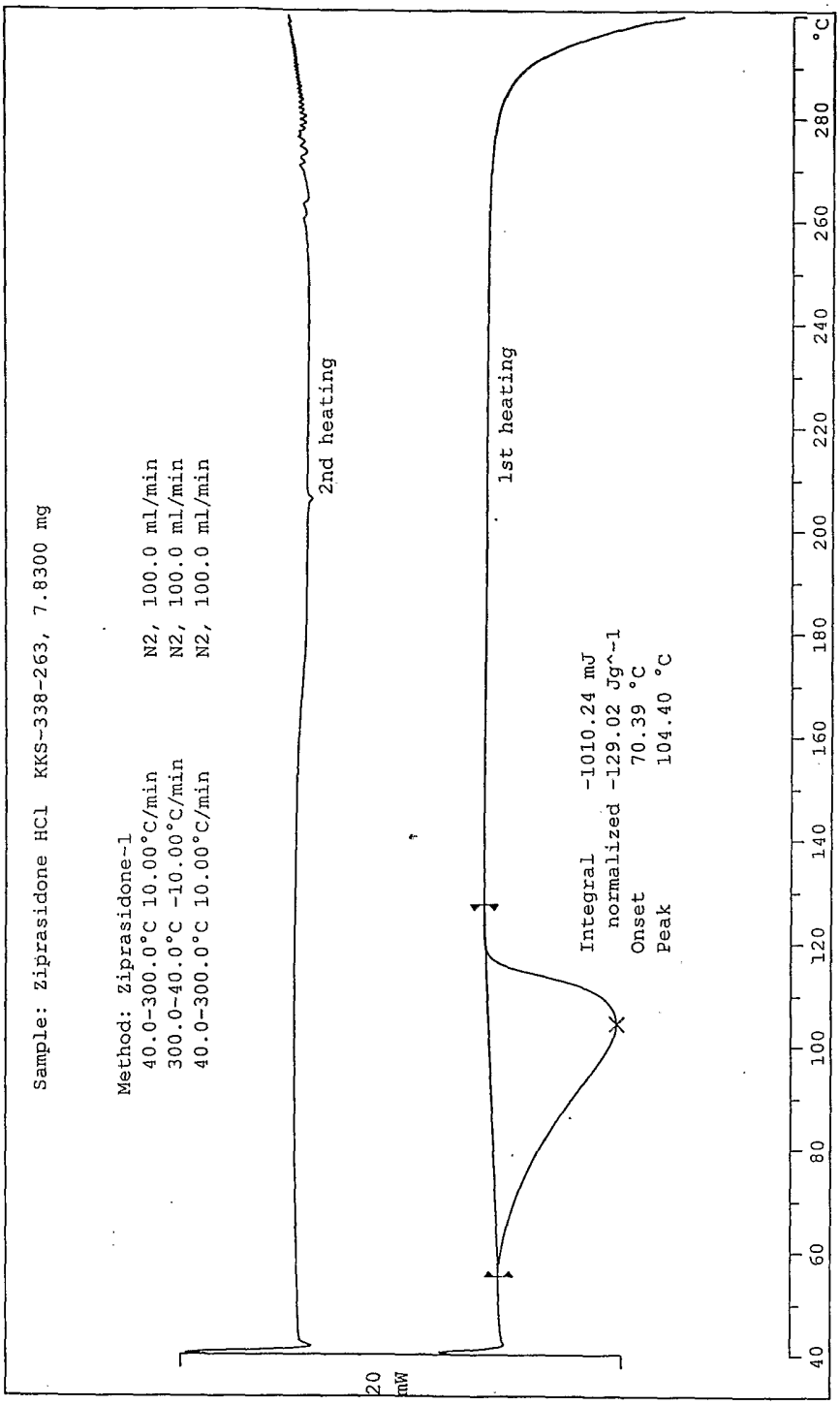

Sample: Ziprasidone HCl KKS-338-263, 7.8300 mg
Method: Ziprasidone-1
40.0-300.0°C 10.00°C/min N2, 100.0 ml/min
300.0-40.0°C -10.00°C/min N2, 100.0 ml/min
40.0-300.0°C 10.00°C/min N2, 100.0 ml/min
2nd heating
1st heating
Integral -1010.24 mJ
normalized -129.02 Jg^-1
Onset 70.39 °C
Peak 104.40 °C
20 mW
40 60 80 100 120 140 160 180 200 220 240 260 280 °C

Figure 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB03/05479

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07D 417/04
US CL : 544/368.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 544/368.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAS ONLINE STRUCTURE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,312,925 A (ALLEN et al.) 17 May 1994 (17.05.1994), see entire document especially example 1, lines 25-40 and example 2.	1,2,11-17,19-22
X	US 6,150,366 A (ARENSON et al.) 21 November 2000 (21.11.2000), see entire document especially example 10 in columns 9-10.	1-3,11-17,19-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
13 May 2004 (13.05.2004)

Date of mailing of the international search report
15 JUN 2004

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
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Alexandria, Virginia 22313-1450
Facsimile No. (703) 305-3230

Authorized officer
Emily Bernhardt

Telephone No. (571) 272-2717

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB03/05479

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claim Nos.: 18
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Exhibit 11

CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER:

22-068

PHARMACOLOGY REVIEW

Memorandum

October 24, 2007

From: David Jacobson-Kram, Ph.D., DABT Office of New Drugs
To: Robert Justice, MD
Thru: John Leighton, Ph.D.

Subject: Review of pharmacology/toxicology section of NDA 22-068

I have reviewed the pharmacology/toxicology section of NDA 22-068 and the nonclinical toxicology/pharmacology section of the proposed drug label. I agree with the primary reviewer's conclusions and with the wording of the non clinical section of the package insert.

This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature.

/s/

David Jacobson-Kram
10/24/2007 12:39:38 PM
PHARMACOLOGIST

MEMORANDUM

Date: October 23, 2007
From: John K. Leighton, PhD, DABT
Supervisory Pharmacologist
Division of Drug Oncology Products
To: File for NDA #22-068
TASIGNA (nilotinib)
Re: Approvability for Pharmacology and Toxicology

Nonclinical pharmacology and toxicology studies for nilotinib were provided in support of the NDA for Tasigna and were reviewed in detail by Dr. Shwu-Luan Lee. The studies consist of original research conducted by the applicant. Tasigna is indicated for the treatment chronic phase and accelerated phase Philadelphia chromosome positive CML in adult patients resistant or intolerant to prior therapy, including imatinib. The supporting studies included information of nilotinib's pharmacology; pharmacokinetic and ADME; safety pharmacology; general toxicology (rat, dog and monkey); genetic toxicity (complete ICH battery) of nilotinib and potential impurities; a fertility study in rats; embryo-fetal developmental toxicity in rats and rabbits; and phototoxicity.

Nilotinib is an inhibitor of Bcr-Abl and several other kinases. The pharmacology studies submitted to the NDA suggest that nilotinib may target wild-type and many, but not all, of the mutant forms of the Bcr-Abl kinase. Activity against some of the mutant forms of this kinase has been assessed clinically. In safety pharmacology studies, nilotinib showed some potential of cardiotoxicity, particularly activity in the hERG assay, suggesting potential inhibition of this ion channel. Other *in vitro* assays also showed some liability, but no acute cardiovascular toxicity was noted in the *in vivo* studies. In general toxicology studies, nilotinib showed greatest effect to the hepatobiliary system, a toxicity observed in clinical trials.

Nilotinib was negative for evidence of genetic toxicity in the standard battery of tests describe by ICH. In screening bacterial assays, several possible impurities were positive for mutagenesis. However, the CMC reviewer did not note any drug product impurities of concern [redacted] Carcinogenicity studies were not conducted for nilotinib, nor are they needed for use in this patient population. Nilotinib was negative for significant effects on fertility and teratogenesis. However, in the rat fertility study and the rat and rabbit embryo-fetal developmental toxicity studies, numerous drug-related effects were observed in the absence and presence of maternal toxicity. Pregnancy Category D is recommended based on embyro-fetal toxicity. The nonclinical findings are detailed in Dr. Lee's review and Executive Summary and reflected in the product label.

Recommendation: I concur with Dr. Lee's conclusion that pharmacology and toxicology data support the approval of NDA 22-068 for Tasigna. There are no outstanding nonclinical issues related to the approval of Tasigna.

This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature.

/s/

John Leighton
10/23/2007 11:21:45 AM
PHARMACOLOGIST

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION
CENTER FOR DRUG EVALUATION AND RESEARCH

PHARMACOLOGY/TOXICOLOGY REVIEW AND EVALUATION

NDA NUMBER:	**22-068**
SERIAL NUMBER:	**000**
DATE RECEIVED BY CENTER:	**9/28/2006**
PRODUCT:	**Tasigna® (nilotinib) capsules**
INTENDED CLINICAL POPULATION:	**Imatinib-intolrant chronic myelogenous leukemia (CML)**
SPONSOR:	**Novartis Pharmaceuticals Corporation** **One Health plaza, East Hanover, NJ 07936**
DOCUMENTS REVIEWED:	**Electronic submission**
REVIEW DIVISION:	**Division of Drug Oncology Products (HFD-150)**
PHARM/TOX REVIEWER:	**Shwu-Luan Lee, Ph.D.**
PHARM/TOX SUPERVISOR:	**John Leighton, Ph.D.**
DIVISION DIRECTOR:	**Robert Justice, M.D., M.S.**
PROJECT MANAGER:	**Janet Jamison**

Date of review submission to Division File System (DFS): **August 13, 2007**

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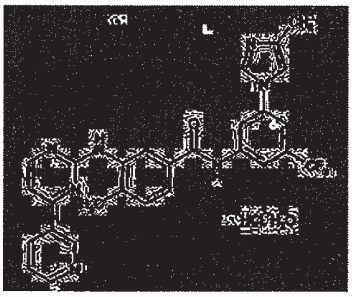
2.6 PHARMACOLOGY/TOXICOLOGY REVIEW

2.6.1 INTRODUCTION AND DRUG HISTORY

NDA number:	22-068
Review number:	1
Sequence number/date/type of submission:	000/September 28, 2006/NDA
Information to sponsor:	Yes ( ) No (x)
Sponsor and/or agent:	Norvatis Pharmaceuticals Corporation One Health Plaza East Hanover, NJ 07936-1080
Manufacturer for drug substance:	Novartis Pharma Stein AG Schaffhauserstrasse CH-4332 Stein Switzerland

Reviewer name:	Shwu-Luan Lee, Ph.D.
Division name:	Division of Drug Oncology Products
HFD #:	HFD-150
Review completion date:	8/13/2007

Drug:

Trade name:	TASIGNA®
Generic name:	Nilotinib
Code name:	AMN 107-AAA.001, NVP-AMN107-NX (free base), NVP-AMN107-AA (monohydrate salt)
Chemical name:	4-Methyl-N-[3-(4-methyl-1H-imidazol-l-yl)-5-(trifluoromethyl)phenyl]-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-benzamide, monohydrochloride, monohydrate
CAS registry number:	641571-10-0 (free base)
Molecular formula:	$C_{28}H_{22}N_7OF_3 \cdot HCl, H_2O$
Molecular weight:	583.99 gm/mole (salt form monohydrate), 565.98 gm/mole (salt form anhydrous)
Structure:	

Relevant INDs/NDAs/DMFs: IND: 69764

Study summary and discussion:
See Section 2.6.7 "Toxicology tabulated summary".

- The treatment of AMN107 in rats, dosed up to 60 mg/kg/day, reduced body weight/food consumption, affected hematological parameters (decreased erythroid parameters, increased erythrocytes and white cell counts) and clinical chemistry parameters (increased total cholesterol and triglycerides), and increased organ weights (liver, kidney, heart, brain, ovary and adrenal). The main target organ was uterus (distention/dilation).

- The moribund sacrifice of three high dose females and one control female was due to dosing trauma, and not due to drug effect.
- It was noted that females showed more reduction in food consumption, but weight loss in males persisted even in the recovery period despite the observation of recovering food intake.
- Clinical signs and clinical chemical evidences may be due to indirect drug effects. The former were potentially due to an unpleasant taste. The clinical chemistry findings, including decreased A/G ratio and chloride, were probably secondary to decrease in food consumption.
- Toxicokinetics:
  - ✧ Orally administered AMN107 was rapidly absorbed. Detectable serum levels of AMN107 were found 0.5 hr after administration (serum concentration-time profile not shown). The T_{max} was 2-6 hours.
  - ✧ The dose normalized C_{max} and AUC on Days 1 and 28 was in general proportional, but C_{max}/dose decreased at 60 mg/kg, a suggestion of saturated absorption, or an induction in metabolism.
  - ✧ Dose normalized AUC on Day 154, especially males and females at 6 mg/kg and females at 20 mg/kg, were generally higher. This may indicate accumulation of AMN107 after repeat administration.
  - ✧ Females demonstrated a higher exposure than the males (approximately 2 fold, but 4-5 fold at 20 mg/kg on Day 154), and also showed more susceptibility to the treatment, as evidenced in food consumption, hematological and clinical chemistry findings.

Study title: 4-Week oral (gavage) toxicity study in dogs with a 4-week recovery period.

Key study findings:

- AMN107-related findings included weight loss in female dogs at 45 mg/kg/day.
- The target organs/tissues of toxicity were liver (Kupffer cells and bile duct), gall bladder, and kidney, and minor changes in lung and spleen.

Note: This study was reviewed in IND 69764 (Review #1) . The IND review is reformatted and incorporated in this NDA review.

Study no.: 0370147
Volume#, and Page number: Volume #5 and page #8-1104 to 8-1299
(Note: in the NDA submission: Electronic module (pharmtox\tox\0370147.pdf))

Conducting laboratory and location: Novartis Pharmaceuticals Corporation, One Health PlazaEast, Hanover, NJ 07936-1080.
Date of study initiation: November 26, 2003
GLP compliance: yes.
QA report: yes (x) no ( ).
Drug, lot #, and % purity: AMN107 hydrochloride, lot #0351002, purity: [redacted]

Methods:
Species: Beagle dog
n: 3/sex/group, plus 2 in the control and 45 mg/kg group as recovery animals.
Age/Weight: 13-14 months/8.7-14.3 kg
Doses: 0 (control), 5, 15, and 45 mg/kg (groups 1, 2, 3 and 4, respectively).
Schedule: Once daily for 28 consecutive days. The recovery animals (Groups 1 and 4) were dosed for 28 days followed by a 28-day recovery phase (no treatment).
Route: Oral by gavage.
Formulation/vehicle: A solution in vehicle: 0.5% w/v hydroxypropyl methylcellulose (HPMC). The concentration was calculated to give a constant dose volume of 2.5 mL per kg body weight for each dose level (see table below).

Table 3-1 Study design, animal allocation and test article doses

Group	Number/sex	Animal Numbers: males	Animal Numbers: females	Dose* (mg/kg/day) Base/Salt	Concentration (mg/mL) Salt
1 Control	3 +2 recovery	1001-1003 1004-1005	1501-1502 1504** 1503**, 1505	0	0
2 Low	3	2001-2003	2501-2503	5/5.3	2.1
3 Mid	3	3001-3003	3501-3503	15/16.0	6.4
4 High	3 +2 recovery	4001-4003 4004-4005	4501-4503 4504-4505	45/48.1	19.2

*Doses are not corrected for active moiety. Salt/Base ratio is 1.069.
**Animal no. 1504 was reassigned from the recovery group to the non recovery group due to a wound on it's tail and was sacrificed with the rest of the animals at scheduled necropsy. Animal no. 1503, a non recovery animal, was reassigned as a recovery animal.

Dose justification:
Dose levels were selected based upon previously conducted 2-week oral (gavage) dose range-finding toxicity study [redacted]). For dogs treated with 6, 20 and 60 mg/kg/day (n=1/sex for the first two doses and n=2/sex for the high dose), weight loss was seen at 60 mg/kg/day (reduction compared to control: 13% in one male and 10-26% in two females). Findings in clinical chemistry, including increased ALP, ALT and total bilirubin (3 fold, 2 fold and 12 fold increase compared to the control, respectively), occurred in 1/2 females at 60 mg/kg/day. Histopathological findings were also observed at 60 mg/kg/day (3/4 animals), and mainly involved liver: periportal/sinusoidal inflammatory cell infiltration, Kupffer cell hypertrophy/hyperplasia and centrilobular bile stasis. The sponsor thus selected 45 mg/kg/day as the high dose in the 4 week study.

Observations and times:

Exhibit 12

CASE PAT034385-US-PCT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE PCT NATIONAL STAGE APPLICATION OF
Manley, Paul W. et al.

Art Unit: 1624
Examiner: Balasubramanian, V
Conf. No.:

INTERNATIONAL APPLICATION NO: PCT/US2006/027878

FILED: July 18, 2006

U.S. APPLICATION NO: 11/995898

35 USC §371 DATE: January 16, 2008

FOR: Salts of 4-Methyl-N-[3-(4-methyl-imidazol-1-yl)-5-trifluoromethyl-phenyl]-3-(4-pyridin-3-yl-pyrimidin-2-ylamino)-benzamide

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132

Sir:

I, Tsu-Han Lin, declare as follows:

1. I received a Ph.D. in Pharmaceutical Science from University of Tokyo, Japan in 1986.

2. I have worked at Novartis Pharmaceuticals Corporation in the area of Drug Metabolism & Pharmacokinetics (DMPK) / Absorption, Distribution, Metabolism and Excretion (ADME) Section since 2001.

3. Prior to working at Novartis Pharmaceuticals Corporation, I worked in this field at Merck for three years and then Southern Research Institute for 11 years.

4. Based on my education and experience, I consider myself to be an expert in DMPK, especially the ADME area.

5. The following is a summary of the results obtained from experiments that were conducted by me or under my supervision relating to the relative bioavailability in dogs of the free base and monohydrochloride salt of AMN107 (also known as nilotinib):

Animal species/strain/gender	Dog/Beagle/male	
Animal number	3	
Route	Oral gavage	
Formulation	AMN107 free base Suspension in 0.5% HPMC aqueous solution with 0.05% Tween 80	AMN107•HCl monohydrate Suspension in 0.5% HPMC aqueous solution with 0.05% Tween 80
Dose (mg/kg, free base equivalent)	20	18
Samples collected	Serial blood at designated timepoints up to 48 h postdose	
Samples analyzed	Serial plasma samples were analyzed for the parent by LC/MS/MS	
C_{max} (ng/mL)	132 ± 72	1530 ± 410
t_{max} (h)	2.0 ± 0.0	1.3 ± 0.6
AUC_{0-48h}(ng•h/mL)	790 ± 247	9300 ± 2780
$AUC_{0-\infty}$ (ng•h/mL)	897 ± -[a]	9270 ± 2800
Relative bioavailability (%)[b]	7.6	100

[a] Only two dogs since one of three dogs had an unreliable terminal slope

[b] HCl salt value was normalized to 100%

The dog bioavailability of AMN107 as free base or HCl salt was tested at the dose of 20 mg/kg in the dog. The test articles were prepared as suspension form in 0.5% HPMC aqueous solution containing 0.05% Tween 80. The relative bioavailability (%) of the AMN107 free base to the HCl salt form (normalized to 100%) was calculated based on the plasma AMN107 data according to the following equation assuming a proportional relationship between AUC0-48h and dose:

$$Relative\ bioavailability\ (\%) = \frac{AUC_{0-48h,\ free\ base} \times Dose_{HCl\ salt}}{AUC_{0-48h,\ HCl\ salt} \times Dose_{free\ base}}$$

An absorption prediction was performed using GastroPlus™ (ver. 3.2.05, Simulations Plus, Lancaster, CA) using the data of the Caco-2 permeability (7.1×10^{-5} cm/min) and physicochemical properties (solubility 0.002 mg/mL at pH6.8 for the free base and 0.14 mg/mL in water for HCl salt). The bioavailability for the HCl salt was approximately 13-fold higher than the free base as indicated by its relative bioavailability calculated from their AUC values. The GastroPlus predicted absorption was in a good agreement with that for the relative bioavailability observed in the dog. However, the later studies with other salts demonstrated that the *in vivo* system is much more complicated and cannot be simply predicted based on only CaCo-2 permeability and the compound solubility.

6. The 13-fold higher oral bioavailability of the hydrochloride salt is an advantage which permits patients to take less drug to achieve comparable plasma levels and could not have been reliably predicted.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Tsu-Han Lin Ph.D.

Date: 06-Feb-2012

Exhibit 13

PRODUCT MONOGRAPH

PrTASIGNA®

(Nilotinib Capsules)

150 mg and 200 mg nilotinib (as nilotinib hydrochloride monohydrate)

Protein-tyrosine kinase inhibitor

Novartis Pharmaceuticals Canada Inc.
385, Bouchard Blvd.
Dorval, Quebec, H9S 1A9

Date of Revision:
August 25, 2016

Control No: **195243**

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TASIGNA demonstrated efficacy in patients harboring a variety of BCR-ABL mutations associated with imatinib resistance, except T315I.

DETAILED PHARMACOLOGY

Animal Pharmacodynamics

Nilotinib has been evaluated in preclinical studies as either the free-base (AMN107-NX) or as a mono-hydrochloride salt (AMN107-AA), and has been developed as an oral formulation of the mono-hydrochloride salt. Both AMN107-NX and AMN107-AA are absorbed following oral administration to animals, and the compound is tolerated at doses showing efficacy in murine myeloproliferative disease models.

In vitro and *in vivo* pharmacology studies have been carried out to characterize and define the activity and selectivity of nilotinib (AMN107-NX). For *in vitro* studies both human CML cell lines and murine hematopoietic cells lines have been employed to characterize the antileukemic properties of the compound, and the latter cells have been employed for *in vivo* efficacy studies with nilotinib (both AMN107-NX and AMN107-AA) in mice. To assess selectivity, nilotinib (AMN107-NX) was evaluated for effects on kinase autophosphorylation and cell viability, using either engineered murine Ba/F3 cells, whose survival is dependent on the expression of constitutively activated (oncogenic) kinases, or cancer cell lines expressing the appropriate kinase.

Animal Safety pharmacology

Safety pharmacology studies were conducted to assess the safety of nilotinib in particular organ systems.

CNS safety pharmacology

The interactions of nilotinib has been evaluated in a panel of 79 *in vitro* binding assays for potential effects on G-protein coupled receptors, cell transporters, ion channels, nuclear receptors and enzymes. No significant effects on ligand-binding were seen at concentrations < 4.0 μM, other than for the human adenosine 3 receptor (IC_{50} values 2.4 and 4.2 μM) and the human adenosine transporter (IC_{50} values 0.9 and 3.5 μM).

Oral administration of nilotinib at doses up to 300 mg/kg to rats demonstrated no effects on CNS.

Respiratory effects

Oral administration of nilotinib at doses up to 300 mg/kg to rats demonstrated no effect on respiratory rate, tidal volume or minute volume.