P-76

SI-M-1

S. MAJUMDAR & CO.

PATENT & TRADEMARK ATTORNEYS

Undertakings : Intellectual Property Laws
Patents, Trademarks, Designs, Copyrights.
Licencing, Investigations, Litigations
DOMESTIC AND INTERNATIONAL

202, Elecon Chambers, Behind Saki Naka Tel. Ex., Off Kurla-Andheri Road, Saki Naka, Mumbai- 400 072, India
Tel. : 91-22-2852 2901/ 2902, Fax : 91-22-2852 2903, e-mail : bom@patentindia.com

30.3.09

The Controller of Patents
The Patent Office
Mumbai.

March 30, 2009

Dear Sir,

Re: Opposition under Section 25(2) against
Patent No. 215758
Patent Application No: IN/PCT/2001/799/MUM dated 05.07.2001
Applicant: BAYER CORPORATION
Opponent: Cipla Ltd.
Our Ref: PII273

In connection with the aforesaid patent we submit herewith the following documents -

I) Notice of Opposition on Form-7 (in duplicate) along with the prescribed fee of Rs.6000/-
II) Written statement of opposition (in duplicate) along with Annexure 1 and *Exhibit 1, Exhibit 2 and Exhibit 3.*

In accordance with Rule 57 of the Rules we are sending copies of the aforesaid documents to the Patentee's Agent.

It is requested that in terms of Section 25(3)(a) the Ld. Controller may kindly notify the patentee of the opposition and constitute an Opposition Board in terms of Section 25(3)(b) of the Act.

We request you to kindly take the opposition on record under intimation to us.

Yours faithfully,

Abhishek Sen

Abhishek Sen
Of S. MAJUMDAR & CO.
(Opponent's Agent)

30/03/2009

Encl : 1. Form-7
2. Full Written Statement of opposition along with the annexures.
3. Power of Attorney in our favour.
6. Prescribed fee of Rs. 6000/- (Cheque No. 106943 dated 30.03.09 drawn on Bank of India)

P-11, F-7, OPPn

30/3/09

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Head office. : 5, Harish Mukherjee Road, Kolkata - 700 025, India, Tel. : 91-33-2455 7484/85/86,
Fax : 91-33-2455 7487/88, e-mail : cal@patentindia.com

cc: REMFRY & SAGAR
79, Nariman Bhavan,
7th Floor,
227, Backbay Reclamation,
Nariman point,
Mumbai - 400.021.

Fee: Rs. 6000.00

FORM - 7

THE PATENTS ACT, 1970

(39 OF 1970)

&

THE PATENTS RULES, 2003

6000/- [illegible]
CBR [illegible]
[illegible]

NOTICE OF OPPOSITION

(See section 25 (2) and rule 55A)

We, CIPLA LTD., A COMPANY INCORPORATED UNDER THE COMPANIES ACT, 1956, HAVING ITS REGISTERED OFFICE AT 289, BELLASIS ROAD, MUMBAI CENTRAL, MUMBAI-400 008, an Indian Company hereby, give notice of opposition to patent No. **215758** granted on Application No. **IN/PCT/2001/799/MUM** dated July 05, 2001 published on March 28, 2008, made by BAYER CORPORATION, 100 BAYER ROAD, PITTSBURG, PENNSYLVANIA, 15202, U.S.A., on the grounds: -

a. that the invention so far as claimed in any claim of the complete specification is obvious and clearly does not involve any inventive step, having regard to the matter published as mentioned in clause (b) of section 25(2) or having regard to what was used in India before the priority date of the applicant's claim;
b. that the subject of any claim of the complete specification is not an invention within the meaning of this Act, or is not patentable under this Act;
c. that the patentee has failed to disclose to the Controller the information required by section 8 or has furnished the information which is in any material particular was false to his knowledge.

Our address for service in India is

S. MAJUMDAR & CO., 5, Harish Mukherjee Road, Calcutta - 700 025, State of West Bengal. Phone: 0-33-4557484/4557485/4557486; Fax: 0-33-4557487/4557488

Dated this 30th day of March 2009

Abhishek Sen
Of S. MAJUMDAR & CO.
(Opponents' Agent)

To
The Controller of Patents
The Patent Office,
At MUMBAI.

BEFORE THE CONTROLLER OF PATENTS

PATENT OFFICE,

MUMBAI.

In the matter of The Patents Act, 1970 *as amended by* The Patents (Amendment) Act 2005,

And

In the matter of The Patents Rules, 2003 as amended by the Patents Amendment Rules 2006

And

IN THE MATTER of Patent No.215758 (IN/PCT/2001/799/MUM) dated July 05, 2001 in the name of BAYER CORPORATION, 100 BAYER ROAD, PITTSBURG, PENNSYLVANIA, 15202, U.S.A.,

..... Patentee

And

IN THE MATTER of opposition thereto by Cipla Limited, 289, Bellasis Road, Mumbai Central, Mumbai– 400 008.

..... Opponent

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WRITTEN STATEMENT OF OPPOSITION U/S 25(2)

We, CIPLA Limited, 289, Bellasis Road, Mumbai Central, Mumbai– 400 008, (hereinafter called 'opponent') make the following statement in support of the grounds of opposition submitted by us in opposing the patent indicated in the cause title.

1. INTERESTED PERSON

1.1 The opponent is a Company incorporated under the Companies Act, 1956, and having its principal office CIPLA Limited, 289, Bellasis Road, Mumbai Central, Mumbai– 400 008 carrying on business, inter alia, of manufacture, research and sale of anticancer agents.

1.2 The subject matter of the patent under opposition relates to drug substances those are capable of inhibiting the enzyme raf kinase. The opponent is involved in the manufacture and marketing of API which belong to the same therapeutic category of substances claimed in the patent under opposition. The opponent has access to the latest technologies relating to the manufacture of anticancer drug substances. The opponent is involved in the area of technology to which the impugned patent specification relates and has interest in opposing the patent. The opponent therefore has both manufacturing and trading interest and is therefore a person interested and thus eligible to institute the present opposition.

2. LIMITATION

2.1 The impugned application for patent No. 215758(IN/PCT/2001/799/MUM) was made on July 05, 2001 and was accompanied by a complete specification. The grant of the patent was notified in the Official Journal of the Patent Office dated March 28, 2008. Therefore the deadline for filing an opposition under Section 25(2) is March 28, 2009 and the present opposition is within time.

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3. GROUNDS OF OPPOSITION

3.1. The application has been opposed on the following grounds:

a. that the invention so far as claimed in any claim of the complete specification is obvious and clearly does not involve any inventive step, having regard to the matter published as mentioned in clause (b) of section 25(2) or having regard to what was used in India before the priority date of the applicant's claim;

b. that the subject of any claim of the complete specification is not an invention within the meaning of this Act, or is not patentable under this Act;

c. that the patentee has failed to disclose to the Controller the information required by section 8 or has furnished the information which is in any material particular was false to his knowledge.

4. ANALYSIS OF THE PATENTEE'S SPECIFICATION NO. 215758

4.1. Patent No. 215758 entitled "Carboxyaryl substituted diphenyl ureas" in the name of BAYER CORPORATION, 100 BAYER ROAD, PITTSBURG, PENNSYLVANIA, 15202, U.S.A., was originally filed as application no. IN/PCT/2001/799/MUM in India on 05th July 2001. The application was published in the patent office journal dated 4th March 2005. The grant of patent u/s 43(2) was notified in the patent office journal dated 28th March 2008. This patent claims three US priorities dated 13th January 1999, 25th February 1999 and 22nd October 1999 of application numbers 60/115,877 , 09/257,266 and 09/425,228 respectively.

4.2. The claimed invention allegedly relates to the use of a group of carboxyaryl substituted diphenyl ureas, which fall in the therapeutic category of anticancer (raf mediated diseases) drugs. The compounds of the alleged invention are substituted diphenyls derived from urea. The specific compound covered by the patent under opposition is N-(4-chloro-3-(trifluoromethyl) phenyl)-N'-(4-(2-(-N-methylcarbamoyl)-4-pyridyloxy) phenyl)urea (INN name: sorafenib) or its pharmaceutically acceptable salts amongst others.

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4.3. Admittedly the compounds of Formula I may be prepared by the use of known chemical reactions and procedures, some from starting materials which are commercially available.

4.4. Entry 42 (Table 4) on page 66 of the complete specification pertains to sorafenib and the same is prepared by following the methods A2 and C1a.

5. ANALYSIS OF CLAIMS AND MERITS OF THE INVENTION

5.1. The patent entitled "Carboxyaryl substituted diphenyl ureas" contains a statement of 22 claims and for ready reference the said claims are annexed hereto as "Annexure A". It is stated that the claims 2 to 14 derive their dependency and patentability directly or indirectly from claim 1.

5.2. Claim 1 of the patent is a product claim directed to compound selected from the groups viz 4-chloro-3-(trifluoro methyl)phenyl urea, 4-bromo-3-(trifluoro methyl)phenyl urea and 2-methoxy-4-chloro-5-(trifluoromethyl)phenyl ureas claming 12 compounds or a pharmaceutically acceptable salt thereof inclusive of sorafenib which is N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy)phenyl)urea.

5.3. Claim 2 provides the pharmaceutically acceptable salts of the compounds claimed in claim 1 to be basic salts of organic acids and inorganic acids or acid salts of organic and inorganic bases containing cations.

5.4. Claim 3 provides a pharmaceutical composition of a compound as claimed in claim 1 or a pharmaceutically acceptable salt and a physiologically acceptable carrier which therefore is a mere admixture.

5.5. Claim 4 merely claims 06 compounds already claimed in claim 1 selected from the total 12 compounds claimed in claim 1.

5.6. Claim 5 and 6 merely repeat the same contents as claimed in claims 2 and 3 except for the difference that these two claims derive their dependency from claim 4.

5.7. Claim 7 provides all the compounds claimed in claim 1and a pharmaceutically acceptable salt thereof for the manufacture of medicines for the treatment of a cancerous cell growth mediated by raf kinase.

5.8. Claim 8 merely provides all the compounds included in claim 1 and a pharmaceutically acceptable salt thereof for the manufacture of medicines for the treatment of a carcinoma of lungs, pancreas, thyroid, bladder, colon, myeloid leukemia or villous colon adenoma.

5.9. Claim 9 merely provides all the compounds included in claim 1 and a pharmaceutically acceptable salt thereof for the manufacture of medicines for the treatment of tumors.

5.10. Claim 10 is a mere repetition of claim 7 except for the difference that this claim derives it dependency from claim 4.

5.11. Claim 11 and 12 are a mere repetition of claims 8 and 9 except for the difference that these claims derive dependency from claim 4.

5.12. Claim 13 provides a compound N-(4-chloro-3(trifluoromethyl) phenyl)-N'-(4-(2-(N-carbamoyl)-4-pyridyloxy) phenyl) urea as in claim 1.

5.13. Claim 14 provides a compound N-(4-chloro-3(trifluoromethyl) phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy) phenyl) urea which is sorafenib as in claim 1.

5.14. Claim 15 provides a pharmaceutical composition of the compound that is claimed in claim 13 or a pharmaceutically acceptable salt thereof and a physiologically acceptable carrier.

5.15. Claim 16 provides a pharmaceutical composition of the compound that is claimed in claim 14 or a pharmaceutically acceptable salt thereof and a physiologically acceptable carrier.

5.16. Claim 17 provides a compound claimed in claim 13 and a pharmaceutically acceptable salt thereof for the manufacture of medicines for the treatment of a cancerous cell growth mediated by raf kinase.

5.17. Claim 18 provides a compound claimed in claim 14 and a pharmaceutically acceptable salt thereof for the manufacture of medicines for the treatment of a cancerous cell growth mediated by raf kinase.

5.18. Claim 19 provides pharmaceutically acceptable salt of the compounds as claimed in claim 17 and 18.

5.19. Claim 20 specifies that a pharmaceutically acceptable salt of compound claimed in claim 19 is a basic salt of an organic or an inorganic acid.

5.20. Claim 21 specifies that a pharmaceutically acceptable salt of compounds claimed in claim 13 is a tosylate salt.

5.21. Claim 22 provides a pharmaceutical composition comprising a pharmaceutically acceptable salt of claim 19, 29 or 21 and pharmaceutically acceptable carrier.

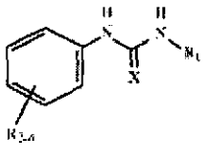
6. PRIOR ART

The opponent wishes to rely on the following prior art as evidence in support of the grounds of opposition *(published prior to the earliest priority date of 13.01.1999)*:

- US5773459 published on June 30, 1998 hereinafter referred to as Exhibit 1;
- US4904668 published on February 27, 1990 hereinafter to as Exhibit 2.

7. OBVIOUSNESS AND LACK OF INVENTIVE STEP

7.1. The opponent states that Exhibit 1 teaches urea and thiourea type compounds for the prevention and treatment of cell proliferative disorders. Particularly Exhibit 1 teaches compounds having the formulae:

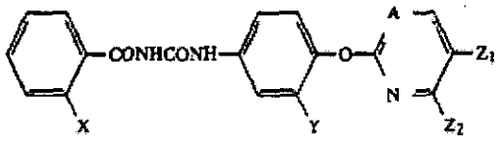


and pharmaceutically acceptable salts thereof, wherein: X is O or S;

R_1 is selected from the group consisting of optionally substituted aryl, alkylaryl, and heteroaryl

$R_{2\text{-}6}$ are independently selected from the group consisting of hydroxy, H, alkyl, alkoxy, CN, nitro, halo, trihalomethyl, amide, carboxamide, sulfonyl, and sulfoxamide.

7.2. It is stated that Exhibit 2 teaches antitumor benzoyl urea compounds having an improved absorbability through the gut. In particular Exhibit 2 teaches compounds with the generic structure



wherein X is a halogen atom, a nitro group or a trifluoromethyl group, provided that when Y is a nitro group, X is a halogen atom or a nitro group, Y is a hydrogen atom, a halogen atom, a nitro group or a trifluoromethyl group, Z_1 is a halogen atom or a trifluoromethyl group, Z_2 is a hydrogen atom or a halogen atom, and A is a =CH-- group or a nitrogen atom, or ....., characterized in that its average particle size is not larger than 1 μm.

7.3. The opponent relies upon the opinion of the expert Dr. A. A. Natu annexed hereto and referred to hereinafter as Exhibit 3.

7.4. It is stated that the definitions provided to the substituents in the exhibits 1 and 2 which ultimately teach substituted urea derivatives with antitumor activity, make it very evident that a person skilled in the art will be able to arrive at the structure of sorafenib by routine trial and error methods. It is further stated that the same is established beyond doubt by the sequential analysis of Dr. Natu in his expert evidence to arrive at the structure of sorafenib in the light of the teachings of Exhibit 1 and Exhibit 2. It is stated that the combined teachings of Exhibit 1 and Exhibit 2 lead to a compound with maximum structural resemblance to sorafenib as depicted in figure 3 of page 17 of Annexure A1.

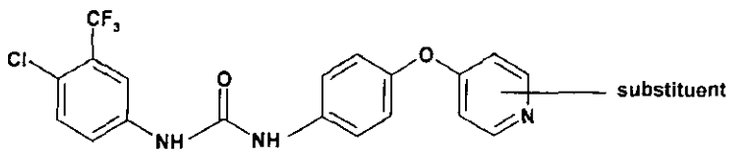


Figure 3

It is stated that a person skilled in the art will interpret that this moiety is required for antitumor activity and introduce such substituents so as to maintain the said activity. It is stated that the sorafenib allegedly claimed in the impugned patent is a mere modification of compounds which can be clearly envisaged from the prior art in specific Exhibit 1 and Exhibit 2 and thus is devoid of inventive merit.

7.5. The opponent states the combined teachings of the prior art documents viz Exhibit 1 and Exhibit 2 make it evident to a person skilled in the art that compounds with the skeletal structure of urea bearing a trifluoromethyl bearing on the N atom and a pyridyloxy phenyl group on the N' atom will possess antitumor activity. It is stated that a skilled researcher will obviously try to make the claimed invention in view of the substantial structural similarity of sorafenib to the prior art compound derived cumulatively from Exhibit 1 and Exhibit 2. It is further stated that any synthetic organic chemist interested in medicinal chemistry, will invariably work on physiologically active compounds of known structure so as to achieve compounds with superior activity or atleast equivalent activity. It is stated the inventors of the impugned patent by virtue of being skilled in the art, will try minor structural variations to known compounds by routine techniques and test such compounds obtained for their antitumor activity. It is stated that the alleged invention claimed in the instant impugned patent is obvious over the combined teachings of Exhibit 1 and Exhibit 2.

7.6. It is stated that the arguments in the aforesaid paragraphs 7.1 to 7.5 make it evident that the compound sorafenib allegedly claimed in the impugned patent is obvious and devoid of inventive merit. It is stated that a preparation of a suitable pharmaceutically acceptable salt of sorafenib is a routine job as a part of drug development and thus the tosylate salt of sorafenib also doe not involve any inventive merit.

8. <u>NOT AN INVENTION / NOT PATENTABLE</u>

8.1. The opponent states that the claimed invention falls under the mischief of Section 2(1)(ja) being devoid of inventive step as according to definition of Inventive step, the invention should be an 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. The opponent states that the invention claimed in the patent under opposition is neither a technical advancement nor it is giving any economic significance, on the face of what is already known in the prior art and cited herein above. It is stated that to claim an invention, an inventor has to show a positive

advancement of the relevant art and in the present case no technical advancement flows out of the various facets of the alleged invention claimed by the patentee.

8.2. The opponent states that the invention claimed in the patent under opposition is not an invention within the meaning of Section 2(1)(j) of the Patents Act, 1970. Therefore, the claimed invention fails to meet the definition of an invention according to Section 2(1)(j) of the Act.

8.3. The opponent states that the composition claimed in claims 3, 6, 15, 16 and 22 contravenes the provisions of Section 3(e) of the Patents Act, 1970. It is stated that these claims do not satisfy the definition of the term 'composition' since these merely refer to an admixture of an active or its salt form and a pharmaceutically acceptable carrier. These claims are therefore liable to be rejected on this ground.

9. BREACH OF SECTION 8

9.1. The opponent states that there has been deliberate breach on the part of the patentee to make bonafide disclosure of the prosecution history of the corresponding applications and thereby obtained the impugned patent by suppression of material facts. The patentee has therefore failed to comply with the provisions of Section 8 of the Act. The opponent warrants rejection on this ground also.

10. RELIEF SOUGHT

10.1. *In the circumstances aforesaid the opponent prays for the following reliefs:*

1) revocation of the patent in toto;
2) award cost in favour of the opponent;
3) such other relief or reliefs as the Controller may deem appropriate.

Dated this 30th day of March 2009.

Abhishek Sen
Of S.Majumdar & Co.
Opponents' agent

To

The Controller of Patents

The Patent Office

Mumbai.

Enclosures:

- Annexure A;
- Exhibit 1;
- Exhibit 2;
- Exhibit 3.

WE CLAIM:

1. A compound selected from the group consisting of the 4-chloro-3-(trifluoromethyl)phenyl ureas:

N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(3-(2-carbamoyl-4-pyridyloxy)phenyl)urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(3-(2-(N-methylcarbamoyl)-4-pyridyloxy)phenyl)urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-carbamoyl)-4-pyridyloxy)phenyl)urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy)phenyl)urea and
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(2-chloro-4-(2-N-methylcarbamoyl)(4-pyridyloxy)phenyl)urea,

the 4-bromo-3(trifluoromethyl)phenyl ureas:

N-(4-bromo-3-(trifluoromethyl)pheny)-N'-(3-(2-(N-methylcarbamoyl)-4-pyridyloxy)phenyl)urea,
N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy)phenyl)urea,
N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(3-(2-(N-methylcarbamoyl)-4-pyridylthio)phenyl)urea,
N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(2-chloro-4-2-(N-methylcarbamoyl)(4-pyridyloxy))phenyl)urea, and
N-(4-bromo-3-(trifluoromethyl))phenyl)-N'-(3-chloro-4-2-(N-methylcarbamoyl)(4-pyridyloxy)phenyl)urea.

the 2-methoxy-4-chloro-5-(trifluoromethyl)phenyl ureas:

N-(2-methoxy-4-chloro-5-(trifluoromethyl)phenyl)-N'-(4-2-(N-methylcarbamoyl)-4-pyridyloxy)phenyl)urea.

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N-(2-methoxy-4-chloro-5-(trifluoromethyl)phenyl)-N'-(2-chloro-4-(2-(N-methylcarbamoyl)(4-pyridyloxy))phenyl)urea.

or a pharmaceutically acceptable salt thereof.

2. A compound as claimed in claim 1, wherein a pharmaceutically acceptable salt thereof selected from the group consisting of:

a) basic salts of organic acids and inorganic acids selected from the group consisting of hydrochloric acid hydrobromic acid, sulphuric acid, phophoric acid, methanesulphonic acid, trifluorosulphonic acid, benzenesulfonic acid, p-toluene sulphonic acid (tosylate salt), 1-napthalene sulfonic acid, 2-napthalne sulfonic acid, acetic acid, trifluoroacetic acid, malic acid, tartaric acid, citric acid, lactic acid, oxalic acid, succinic acid, fumaric acid, maleic acid, benzoic acid, salicylic acid, phenylacetic acid, and mandelic acid; and

b) acid salts of organic and inorganic bases containing cations selected from the group consisting of alkaline cations, alkaline earth cations, the ammonium cation, aliphatic substituted ammonium cations and aromatic substituted ammonium cations.

3. A pharmaceutical composition comprising a compound as claimed in claim 1 or a pharmaceutically acceptable salt thereof and a physiologically acceptable carrier.

4. A compound as claimed in claim 1 selected from the group consisting of:

N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl-pyridyloxy)phenyl) urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-carbamoyl-4-pyridyloxy)phenyl) urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(2-chloro-4-(2-(N-methylcarbamoyl)(4-pyridyloxy))phenyl] urea,

N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy) phenyl) urea,
N-(2-methoxy-4-chloro-5-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy)phenyl) urea,
N-(2-methoxy-4-chloro-5-(trifluoromethyl)phenyl)-N'-(2-chloro-4-(2-(N-methylcarbamoyl) (4-pyridyloxy)) phenyl) urea,

or a pharmaceutically acceptable salt thereof.

5. A compound as claimed in claim 4 which is a pharmaceutically acceptable salt thereof selected from the group consisting of:

a) basic salts of organic adds and inorganic acids selected from the group consisting of hydrochloric acid, hydrobromic acid, sulphuric acid, phosphoric acid, methanesulphonic acid, trifluorosulphonic acid, benzenesulfonic acid, p-toluene sulphonic acid (tosylate salt), 1-napthalene sulfonic acid, 2-napthalene sulfonic acid, acetic acid, trifluoroacetic acid, malic acid, tartaric acid, citric acid, lactic acid, oxalic acid, succinic acid, fumaric acid, maleic acid, benzoic acid, salicylic acid,
phenylacetic acid, and mandelic acid; and
b) acid salts of organic and inorganic bases containing cations selected from the group consisting of alkaline cations, alkaline earth cations, the ammonium cation, aliphatic substituted ammonium cations and aromatic substituted ammonium cations.

6. A pharmaceutical composition comprising a compound as claimed in claim 4 or a pharmaceutically acceptable salt thereof and a physiologically acceptable carrier.

7. A compound as claimed in claim 1 selected from the group consisting of:
the 4-chloro-3-(trifluoromethyl)phenyl ureas:

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N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(3-(2-carbamoyl-4-pyridyloxy)phenyl) urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(3-(2-(N-methylcarbamoyl)-4-pyridyloxy) phenyl) urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-carbamoyl-4-pyridyloxy)phenyl) urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)4-pyridyloxy) phenyl) urea and
N-(4-chloro-3-(trifluoromethyl)phenyl)-N-(2-chloro-4-(2-(N-methylcarbamoyl)(4-pyridyloxy))phenyl) urea,

the 4-bromo-3(trifluoromethyl)phenyl ureas:

N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(3-(2-(N-methylcarbamoyl)-4-pyridyloxy) phenyl) urea,
N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy) phenyl urea.
N-(4-bromo-3-(trrifluoromethyl)phenyl)-N'-(3-(2-(N-methylcarbamoyl)-4-pyridylthio) phenyl) urea,
N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(2-chloro-4-(2-(N-methylcarbamoyl)(4-pyridyloxy))phenyl) urea and
N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(3-chloro-4-(2-(N-methylcarbamoyl)(4-pyridyloxy))phenyl) urea;
the 2-methoxy-4-chloro-5-(trifluoromethyl)phenyl ureas:
N-(2-methoxy-4-chloro-5-(trifluoromethylphenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy)phenyl) urea,
N-(2-methoxy- 4-chloro-5-(trifluoromethyl) phenyl)-N'-(2-chloro-4-(2-(N-methylcarbamoyl) (4-pyridyloxy)) phenyl) urea

or a pharmaceutically acceptable salt thereof for the manufacture of a medicament for the treatment of a cancerous cell growth mediated by raf kinase.

8. A compound as claimed in claim 1 selected from the group consisting of

the 4-chloro-3-(trifluoromethyl)phenyl ureas:

N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(3-(2-carbamoyl-4-pyridyloxy)phenyl) urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(3-(2-(N-methylcarbamoyl)-4-pyridyloxy) phenyl) urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-carbamoyl-4-pyridyloxy)phenyl) urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy) phenyl) urea and
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(2-chloro-4-(2-(N-methylcarbamoyl)(4-pyridyloxy))phenyl) urea,

the 4-bromo-3(trifluoromethyl)phenyl ureas:

N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(3-(2-(N-methylcarbamoyl)4-pyridyloxy) phenyl) urea,
N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy) phenyl urea,
N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(3-(2-(N-methylcarbamoyl)-4-pyridylthio) phenyl) urea,
N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(2-chloro-4-(2-(N-methylcarbamoyl)(4-pyridyloxy))phenyl) urea and
N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(3-chloro-4-(2-(N-methylcarbamoyl)(4-pyridyloxy))phenyl) urea;

the 2-methoxy-4-chloro-5-(trifluoromethyl)phenyl ureas:

N-(2-methoxy-4-chloro-5-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy)phenyl) urea,

N-(2-methoxy-4-chloro-5-(trifluoromethyl) phenyl)-N'-(2-chloro-4-(2-(N-methylcarbamoyl) (4-pyridyloxy)) phenyl) urea

or a pharmaceutically acceptable salt thereof for the manufacture of a medicament for the treatment of:

i) carcinoma of the lungs, pancreas, thyroid, bladder, colon,
ii) myeloid leukemia or
iii) villous colon adenoma.

9. A compound as claimed in claim 1 selected from the group consisting of:

the 4-chloro-3-(trifluoromethyl)phonyl ureas:

N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(3-(2-carbamoyl-4-pyridyloxy)phenyl) urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(3-(2-(N-methylcarbamoyl)-4-pyridyloxy) phenyl) urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-carbamoyl-4-pyridyloxy)phenyl) urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy) phenyl) urea and
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(2-chloro-4-(2-(N-methylcarbamoyl)(4-pyridyloxy))phenyl) urea,

the 4-bromo-3(trifluoromethyl)phenyl ureas:

N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(3-(2-(N-methylcarbamoyl)4-pyridyloxy) phenyl) urea,
N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy) phenyl urea,
N-(4-bromo-3-(trifluoromethyl)phenyl)-N-(3-(2-(N-methylcarbamoyl)-4-pyridylthio) phenyl) urea,
N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(2-chloro-4-(2-(N-methylcarbamoyl)(4-pyridyloxy))phenyl) urea and

N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(3-chloro-4-(2-(N-methylcarbamoyl)(4-pyridyloxy))phenyl) urea;

the 2-methoxy-4-chloro-5-(trifluoromethyl)phenyl ureas:

N-(2-methoxy-4-chloro-5-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy)phenyl) urea,

N-(2-methoxy-4-chloro-5-(trifluoromethyl)phenyl)-N'-(2-chloro-4-(2-(N-methylcarbamoyl)(4-pyridyloxy)phenyl) urea,

or a pharmaceutically acceptable salt thereof for the manufacture of a medicament for the treatment of tumors.

10. A compound as claimed in claim 4 selected from the group consisting of:

N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-N-methylcarbamoyl)-4-pyridyloxy)phenyl) urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-carbamoyl-4-pyridyloxy)phenyl) urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(2-chloro-4-(2-(N-methylcarbamoyl)(4-pyridyloxy))phenyl) urea,
N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4 pyridyloxy) phenyl) urea,
N-(2-methoxy-4-chloro-5-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy)phenyl) urea,
N-(2-methoxy-4-chloro-5-(trifluoromethyl)phenyl)-N'-(2-chloro-4-(2-(N-methylcarbamoyl) (4-pyridyloxy))phenyl) urea.

or a pharmaceutically acceptable salt hereof for the manufacture of a medicament for the treatment of a cancerous cell growth mediated by raf kinase.

11. A compound as claimed in claim 4 selected from the group consisting

N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-N-methylcarbamoyl)-4-pyridyloxy)phenyl) urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-carbamoyl-4-pyridyloxy) phenyl) urea,
N-(4-chloro-3-(trifluoromethyl)phanyl)-N'-(2-chloro-4-(2-(N-methylcarbamoyl) (4-pyridyloxy))phenyl) urea,
N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4 pyridyloxy)phenyl)urea,
N-(2-methoxy-4-chloro-5-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy)phenyl) urea,
N-(2-methoxy-4-chloro-5-(trifluoromethyl)phenyl)-N'-(2-chloro-4-(2-(N-methylcarbamoyl) (4-pyridyloxy)) phenyl) urea

or a pharmaceutically acceptable salt hereof for the manufacture of a medicament for the treatment of:

i) carcinoma of the lungs, panaeas, thyroid, bladder, colon,
ii) myeloid leukemia or
iii) villous colon adenoma.

12. A compound as claimed in claim 4 selected from the group consisting of:

N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-N-methylcarbamoyl)-4-pyridyloxy)phenyl)urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-carbamoyl-4-pyridyloxy)phenyl) urea,
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(2-chloro-4-(2-(N-methylcarbamoyl)(4-pyridyloxy))phenyl) urea,

N-(4-bromo-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4 pyridyloxy)phenyl)urea,
N-(2-methoxy-4-chloro-5-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy)phenyl)urea,
N-(2-methoxy-4-chloro-5-(trifluoromethyl)phenyl)-N'-(2-chloro-4-(2-(N-methylcarbamoyl)(4-pyridyloxy))phenyl) urea

or a pharmaceutically acceptable salt hereof for the manufacture of a medicament for the treatment of tumors.

13. A compound as claimed in claim 1 which is N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-carbamoyl-4-pyridyloxy)phenyl) urea.

14. A compound as claimed in claim 1 which is N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy)phenyl) urea

15. A pharmaceutical composition comprising N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-carbamoyl-4-pyridyloxy)phenyl) urea or a pharmaceutically acceptable salt thereof and a physiologically acceptable carrier.

16. A pharmaceutical composition comprising N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy) phenyl) urea or a pharmaceutically acceptable salt thereof and a physiologically acceptable carrier.

17. N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-carbamoyl-4-pyridyloxy) phenyl) urea,
or a pharmaceutically acceptable salt thereof for the manufacture of a medicament for the treatment of a cancerous cell growth mediated by raf kinase.

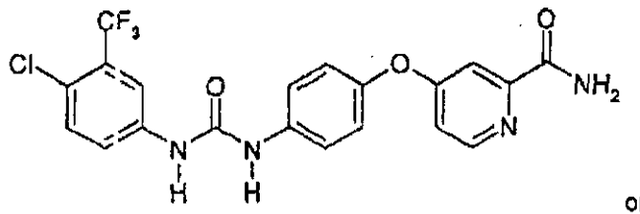
18. N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-

20

pyridyloxy)phenyl) urea or a pharmaceutical acceptable salt thereof for the manufacture of a medicament for the treatment of a cancerous cell growth mediated by raf kinase.

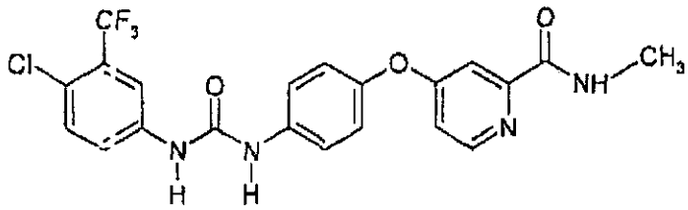
19. A pharmaceutically acceptable salt of a compound which is:

N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-carbamoyl-4-pyridyloxy)phenyl) urea of the formula:



or

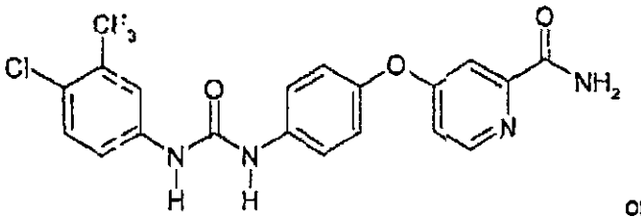
N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl-4-pyridyloxy)phenyl) urea of the formula:



20. A pharmaceutically acceptable salt as claimed in claim 19 which is a basic salt of an organic acid or an inorganic acid which is hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, methanesulfonic acid, trifluoromethanesulfonic acid, benzenesulfonic acid, p-toluene sulfonic acid (tosylate salt), 1-napthalene sulfonic acid, 2-napthalene sulfonic acid, acetic acid, trifluoroacetic acid, malic acid, tartaric acid, citric acid, lactic acid, oxalic acid, succinic acid, fumaric acid, maleic acid, benzoic acid, salicylic acid, phenylacetic acid, or mandelic acid.

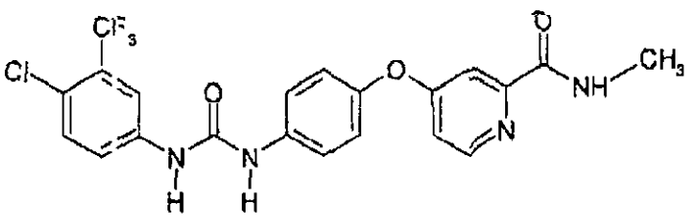
21. A pharmaceutically acceptable salt which is the tosylate salt of

N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-carbamoyl-4-pyridyloxy) phenyl) urea of the formula:



or

N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-N-methylcarbamoyl-4-pyridyloxy)phenyl) urea of the formula:



22. A pharmaceutical composition comprising a pharmaceutically acceptable salt of claim 19, 20 or 21 and pharmaceutically acceptable carrier.

Dated this 5th day of July, 2001.

[RANJNA MEHTA-DUTT]
OF REMFRY & SAGAR
ATTORNEY FOR THE APPLICANT(S)

Exhibit 1

22

US005773459A

United States Patent [19]

Tang et al.

[11] **Patent Number: 5,773,459**

[45] **Date of Patent: Jun. 30, 1998**

[54] ***UREA- AND THIOUREA-TYPE COMPOUNDS***

[75] Inventors: **Peng Cho Tang**, Moraga; **Gerald McMahon**, Kenwood, both of Calif.

[73] Assignee: **Sugen, Inc.**, Redwood City, Calif.

[21] Appl. No.: **486,816**

[22] Filed: **Jun. 7, 1995**

[51] **Int. Cl.**[6] **A61K 31/38**; C07D 333/10

[52] **U.S. Cl.** **514/445**; 514/326; 514/327; 514/347; 514/371; 514/426; 514/585; 514/596; 514/597; 546/23; 546/208; 546/212; 546/216; 546/306; 548/196; 548/557; 548/559; 549/63

[58] **Field of Search** 514/585, 596, 514/597, 445, 347, 327, 326, 371, 426; *564/26, 48, 49; 546/306, 216, 23, 212,* 208; 548/196, 557, 559; 549/63

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Primary Examiner—Shailendra Kumar

Attorney, Agent, or Firm—Lyon & Lyon LLP

[57] **ABSTRACT**

The present invention relates to molecules capable of modulating tyrosine signal transduction to prevent and treat cell proliferative disorders or cell differentiation disorders associated with particular tyrosine kinases by inhibiting one or more abnormal tyrosine kinase activities.

18 Claims, No Drawings

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Walchshofer et al., "Recherche des paramètres structuraux influencant l'activiteè anthelminthque anthelminthique d'urèes dèrivès thiadiazoles" *Eur. J. Med. Chem.–Chim. Ther.* 22:467–471 (1987).

UREA- AND THIOUREA-TYPE COMPOUNDS

FIELD OF THE INVENTION

The present invention relates generally to the field of tyrosine kinase inhibition. More specifically, the present invention relates to the use of small organic molecules to prevent and treat cell proliferative disorders or cell differentiation disorders associated with particular tyrosine kinases by inhibiting one or more abnormal tyrosine kinase activities.

BACKGROUND OF THE INVENTION

Cellular signal transduction is a fundamental mechanism whereby external stimuli that regulate diverse cellular processes are relayed to the interior of cells. Reviews describing intracellular signal transduction include Aaronson, *Science*, 254:1146–1153, 1991; Schlessinger, *Trends Biochem. Sci.*, 13:443–447, 1988; and Ullrich and Schlessinger, *Cell*, 61:203–212, 1990. One of the key biochemical mechanisms of signal transduction involves the reversible phosphorylation of tyrosine residues on proteins. The phosphorylation state of a protein is modified through the reciprocal actions of tyrosine kinases (TKs) and tyrosine phosphatases (Tps).

Tyrosine kinases can be of the receptor type (having extracellular, transmembrane and intracellular domains) or the non-receptor type (being wholly intracellular). There are 19 known families of receptor tyrosine kinases including the Her family (EGFR, Her 2, Her 3, Her 4), the insulin receptor family (insulin receptor, IGF-1R, insulin-related receptor), the PDGF receptor family (PDGF-Rα and β, CSF-1R, kit, Flk2), the Flk family (Flk-1, Flt-1, Flk-4), the FGF-receptor family (FGF-Rs 1 through 4), the Met family (Met, Ron), etc. There are 11 known famiolies of non-receptor type tyrosine kinases including the Src family (src, yes, fyn, lyn, lck, blk, Hck, Fgr, yrk), Abl family (Abl, Arg), Zap 70 family (Zap 70, Syk) and Jak family (Jak 1, Jak 2, Tyk 2, Jak 3). Many of these tyrosine kinases have been found to be involved in cellular signalling pathways leading to pathogenic conditions such as cancer, psoriasis, hyperimmune response, etc.

Protein tyrosine kinases play an important role in cellular signaling pathways that regulate the control of cell growth and differentiation (for review, see Schlessinger & Ullrich, 1992, *Neuron*, 9:383–391). Aberrant expression or mutations in receptor tyrosine kinases (RTKs) have been shown to lead to either uncontrolled cell proliferation (e.g. malignant tumor growth) or to defects in key developmental processes. In some instances, a single tyrosine kinase can inhibit, or stimulate, cell proliferation depending on the cellular environment in which it is expressed. Consequently, the biomedical community has expended significant resources to discover the specific biological role of members of the RTK family, their function in differentiation processes, their involvement in tumorigenesis and in other diseases, the biochemical mechanisms underlying their signal transduction pathways activated upon ligand stimulation and the development of novel antineoplastic drugs.

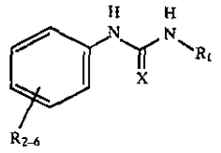
Attempts have been made to identify RTK "inhibitors" using a variety of approaches, including the use of mutant ligands (U.S. Pat. No. 4,966,849), soluble receptors and antibodies (Application No. WO 94/10202; Kendall & Thomas, 1994, *Proc. Nat'l Acad. Sci* 90:10705–09; Kim, et al., 1993, *Nature* 362:841–844), RNA ligands (Jellinek, et al., 19 *Biochemistry* 33:10450–56), protein kinase C inhibitors (Schuchter, et al., 1991, *Cancer Res.* 51:682–687); Takano, et al., 1993, *Mol. Bio. Cell* 4:358A; Kinsella, 20 et al., 1992, *Exp. Cell Res.* 199:56–62; Wright, et al., 1992, *J. Cellular Phys.* 152:448–57) and tyrosine kinase inhibitors (WO 94/03427; WO 92/21660; WO 91/15495; WO 94/14808; U.S. Pat. No. 5,330,992; Mariani, et al., 1994, *Proc. Am. Assoc. Cancer Res.* 25 35:2268).

Attempts have also been made to identify small molecules which act as tyrosine kinase inhibitors. For example, bis monocyclic, bicyclic or heterocyclic aryl compounds (PCT WO 92/20642), vinylene-azaindole derivatives (PCT WO 94/14808) and 1 -cycloproppyl-4-pyridyl-quinolones (U.S. Pat. No. 5,330,992) have been described generally as tyrosine kinase inhibitors. Styryl compounds (U.S. Pat. No. 5,217,999), styryl-substituted pyridyl compounds (U.S. Pat. No. 5,302,606), certain quinazoline derivatives (EP Application No. 0 566 266 Al), seleoindoles and selenides (PCT WO 94/03427), tricyclic polyhydroxylic compounds (PCT WO 92/21660) and benzylphosphonic acid compounds (PCT WO 91/15495) have been described as compounds for use as tyrosine kinase inhibitors for use in the treatment of cancer.

SUMMARY OF THE INVENTION

The present invention relates to molecules capable of modulating tyrosine signal transduction to prevent and treat cell proliferative disorders or cell differentiation disorders associated with particular tyrosine kinases by inhibiting one or more abnormal tyrosine kinase activities.

More specifically, the invention is generally directed to compounds having the formulae:



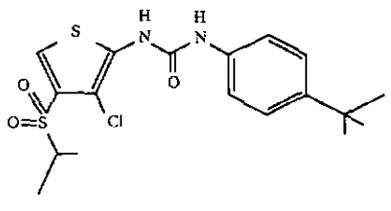
and pharmaceutically acceptable salts thereof, wherein:

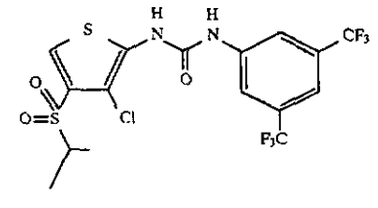
X is O or S;

R_1 is selected from the group consisting of optionally substituted aryl, alkylaryl, and heteroaryl

$R_{2-6}$are independently selected from the group consisting of hydroxy, H, alkyl, alkoxy, CN, nitro, halo, trihalomethyl, amide, carboxamide, sulfonyl, and sulfoxamide.

Example of preferred compounds include:





The present invention also provides pharmaceutical compositions and methods for inhibiting cell proliferation or

differentiation and related disorders. Examples of such disorders include cancers, blood vessel proliferative disorders, psoriasis, hyperimmune response and fibrotic disorders. Examples of other disorders include the HER2 disorders, EGF disorders, IGFR disorders, PDGFR disorders, met disorders, SVC disorders, and KDR/FLK-1 disorders described herein. It is to be understood that compounds which are effective for diseases related to one RTK will also likely be effective for diseases related to other RTK's, especially those from the same family. Thus, for example, compounds shown to have good effect against Her2 are likely to also have good effect against other members of the Her family, i.e., EGFR, Her3, and Her4.

Chemical Definitions

The following is a list of some of the definitions used in the present disclosure. An "alkyl" group refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably, it is a lower alkyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, *cyano, alkoxy,* =O, =S, NO_2, $N(CH_3)_2$, *amino, or SH.*

An "alkenyl" group refers to an unsaturated hydrocarbon group containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has 2 to 12 carbons. More preferably it is a lower alkenyl of from 2 to 7 carbons, more preferably 2 to 4 carbons. The alkenyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2, halogen, $N(CH_3)_2$, amino, or SH. An "alkynyl" group refers to an unsaturated hydrocarbon group containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkynyl group has 2 to 12 carbons. More preferably, it is a lower alkynyl of from 2 to 7 carbons, more preferably 2 to 4 carbons. The alkynyl group may be substituted or unsubstituted. When substituted, the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2, $N(CH_3)_2$, amino or SH.

An "alkoxy" group refers to an "—O-alkyl" group, where "alkyl" is defined as described above.

An "aryl" group refers to an aromatic group which has at least one ring having a conjugated pi electron system and includes carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which may be optionally substituted. Preferably, the aryl is a substituted or unsubstituted phenyl or pyridyl. Preferred aryl substituent(s) are halogen, *trihalomethyl, hydroxyl, SH, OH, NO_2, amine, thioether,* cyano, alkoxy, alkyl, and amino groups.

An "alkylaryl" group refers to an alkyl (as described above), covalently joined to an aryl group (as described above). Preferably, the alkyl is a lower alkyl.

"Carbocyclic aryl" groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted.

"Heterocyclic aryl" groups are groups having from 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen, and include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, imidazolyl and the like, all optionally substituted.

An "amide" refers to an —C(O)—NH—R, where R is either alkyl, aryl, alkylaryl or hydrogen.

A "thioamide" refers to —C(S)—NH—R, where R is either alkyl, aryl, alkylaryl or hydrogen. An "ester" refers to an —C(O)—OR', where R' is either alkyl, aryl, or alkylaryl.

An "amine" refers to a —N(R")R"', where R" and R"', is independently either hydrogen, alkyl, aryl, or alkylaryl, provided that R" and R"' are not both hydrogen.

A "thioether" refers to —S—R, where R is either alkyl, aryl, or alkylaryl.

A "sulfonyl" refers to —$S(O)_2$—R, where R is aryl, C(CN)=C-aryl, CH_2—CN, alkylaryl, NH-alkyl, NH-alkylaryl, sulfonamide, or NH-aryl.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Cell Proliferative and Cell Differentiation Disorders

Cell proliferative and cell differentiation disorders which can be treated or further studied by the present invention include any disorder associated with a tyrosine kinase signalling pathway, for example cancers, blood vessel proliferative disorders, psoriasis, hyperimmune response and fibrotic disorders. These disorders are not necessarily independent. For example, fibrotic disorders may be related to, or overlap, with blood vessel proliferative disorders. For example, atherosclerosis (which is characterized herein as a blood vessel disorder) results, in part, in the abnormal formation of fibrous tissue.

Blood vessel proliferation disorders refer to angiogenic and vasculogenic disorders generally resulting in abnormal proliferation of blood vessels. The formation and spreading of blood vessels, or vasculogenesis and angiogenesis respectively, play important roles in a variety of physiological processes such as embryonic development, wound healing and organ regeneration. They also play a role in cancer development. Examples of blood vessels disorders include restenosis, retinopathies, and atherosclerosis.

Fibrotic disorders refer to the abnormal formation of extracellular matrix. Examples of fibrotic disorders include *hepatic cirrhosis and mesangial cell proliferative disorders.* Hepatic cirrhosis is characterized by the increase in extracellular matrix constituents resulting in the formation of a hepatic scar. Hepatic cirrhosis can cause diseases such as cirrhosis of the liver. An increased extracellular matrix resulting in a hepatic scar can also be caused by viral infection such as hepatitis. Lipocytes appear to play a major role in hepatic cirrhosis.

Mesangial cell proliferative disorders refer to disorders brought about by abnormal proliferation of mesangial cells. Mesangial proliferative disorders include various human renal diseases, such as glomerulonephritis, diabetic nephropathy, malignant nephrosclerosis, thrombotic microangiopathy syndromes, transplant rejection, and glomerulopathies. PDGFR has been implicated in the maintenance of mesangial cell proliferation. (Floege, J. et al., Kidney International 43S:47–54 (1993))

HER2, EGFR, IGFR, PDGFR, met, src and KDR/FLK-1 driven cancers and disorders are described in detail below and are a preferred subset of the disorders to be treated. A cancer cell refers to various types of malignant neoplasms, most of which can invade surrounding tissues, and may metastasize to different sites, as defined by Stedman's Medical Dictionary 25th edition (Hensyl ed. 1990).

A. HER2 Cell Proliferation Disorders

The HER-2 protein is a member of the class I receptor tyrosine kinase (RTK) family. Yarden and Ullrich, *Annu. Rev. Biochem.* 57:443, 1988; Ullrich and Schiessinger, *Cell* 61:203, 1990. HER-2 protein is structurally related to EGF-R, p180(HER-3), and p180(HER-4). Carraway, et al., *Cell* 78:5, 1994; Carraway, et al., *J. Biol. Chem.* 269:14303, 1994. These receptors share a common molecular architecture and contain two cysteine-rich regions within their cytoplasmic domains and structurally related enzymatic regions within their cytoplasmic domains.

Activation of HER-2 protein can be caused by different events such as ligand-stimulated homodimerization, ligand-stimulated hetero-dimerization and ligand-independent homo-dimerization. Ligand-stimulated hetero-dimerization appears to be induced by EGF-R to form EGF-R/HER-2 complexes and by neu differentiation factor/heregulin (NDF/HRG) to form HER-2/HER-3 and/or HER2/HER-4 complexes. Wada et al., *Cell* 61:1339, 1990; Slikowski et al., *J. Biol. Chem.* 269:14661, 1994; Plowman et al., *Nature* 266:473, 1993. Ligand-dependent activation of HER-2 protein is thought to be mediated by neuactivating factor (NAF) which can directly bind to p165(HER-2) and stimulate enzymatic activity. Dougall et al., *Oncogene* 9:2109, 1994; Samata et al., *Proc. Natl. Acad. Sci. USA* 91:1711, 1994. Ligand-independent homodimerization of HER-2 protein and resulting receptor activation is facilitated by over-expression of HER-2 protein.

HER-2 protein substrates are acted upon by activated HER-2 complexes such as HER-2/EGF-R, HER-2/HER-2, HER2/HER-3, and HER-2/HER-4 activated complexes. An activated HER-2 complex acts as a phosphokinase and phosphorylates different cytoplasmic proteins. Examples of HER-2 substrates include IP_3 kinase and PI4-kinase. Scott et al., *Journal of Biological Cheinistry* 22:14300, 1991. Proteins bind to an activated HER-2 complex and then another protein. For example, GRB-7 binding to a HER-2 complex may be sufficient to initiate the GRB-7 signaling pathway without phosphorylation. Stein et al., *EMBO Journal* 13:1331, 1993.

Thus, HER-2 protein activities include: (1) phosphorylation of HER-2 protein, HER-3 protein or HER-4 protein; (2) phosphorylation of a HER-2 protein substrate; (3) interaction with a HER-2 adapter protein; and/or (4) HER-2 protein *surface expression. Additional HER-2 protein activities can* be identified using standard techniques. For example, a partial agonistic monoclonal antibody recognizing HER-2 protein can be used to activate HER-2 protein and examine *signal transduction of HER-2 protein. Scott et al., Journal of Biological Chemistry* 22:14300, 1991. HER2 activity can be assayed by measuring one or more of the following activities: (1) phosphorylation of HER2; (2) phosphorylation of a *HER2* substrate; (3) activation of an *HER2* adapter molecule; and (4) increased cell division. These activities can be measured using techniques described below and known in the art.

HER2 driven disorders are characterized by inappropriate or over-activity of HER2. Inappropriate HER-2 activity refers to either: (1) HER2 expression in cells which normally do not express HER2; (2) increased HER-2 expression leading to unwanted cell proliferation such as cancer; (3) increased HER-2 activity leading to unwanted cell proliferation, such as cancer; and/or overactivity of HER-2. Over-activity of HER2 refers to either an amplification of the gene encoding HER2 or the production of a level of HER2 activity which can be correlated with a cell proliferative disorder (i.e., as the level of HER2 increases the severity of one or more of the symptoms of the cell proliferative disorder increases). HER2 driven disorders are typically cell proliferative or differentiation disorders such as cancers. HER2 driven disorders appear to be responsible for a sub-population of different types of cancers. For example, as noted above, Slamon et al., found about 30% of breast cancer cells to have increased HER2 gene expression. Slamon et al., also found a correlation between her2 (c-erbB-2) amplification and poor patient prognosis.

Treatment of patients suffering from a HER2 disorder is facilitated by first determining whether the cell proliferative disorder is characterized by an overactivity of HER2. After *the disorder is identified, patients suffering from such a* disorder can be identified by analysis of their symptoms using procedures well known to medical doctors. Such identified patients can then be treated as described herein. *The use of the present invention to treat breast cancer is* preferred because of the prevalence and severity of breast cancer. Carcinoma of the breast is the most common cancer among women and their second leading cause of cancer death (Marshall, E., *Science* 259:618–621, 1993) . The incidence of breast cancer has been increasing over the past several decades (Marshall, supra, and Harris, JR., et al, *New Engl. J. Med.*, 327(5):319–328, 1992). In addition to breast cancers, increased HER2 activity or gene expression has been associated with certain types of blood cancers, stomach adenocarcinomas, salivary gland adenocarcinomas, endometrial cancers, ovarian adenocarcinomas, gastric cancers, colorectal cancers, non-small cell lung cancer, and glioblastomas. The methods described herein can be used to identify the sub-populations of these different cancers which are characterized by over-activity of HER2.

B. EGFR Disorders

Some of the featured compounds can be used to treat cell proliferative and/or cell differentiation disorders characterized by inappropriate EGFR activity. "Inappropriate EGFR" activity refers to either: (1) EGF-receptor (EGFR) expression in cells which normally do not express EGFR; (2) EGF *expression by cells which normally do not express EGF;* (3) increased EGF-receptor (EGFR) expression leading to unwanted cell proliferation; (4) increased EGF expression leading to unwanted cell proliferation; and/or (5) mutations leading to constitutive activation of EGF-receptor (EGFR) The existence of inappropriate or abnormal EGF and EGFR levels or activities is determined by procedures well known in the art.

An increase in EGF activity or expression is characterized by an increase in one or more of the activities which can occur upon EGF ligand binding such as: (1) EGF-R dimerization; (2) auto-phosphorylation of EGFR, (3) phosphorylation of an EGFR substrate (e.g., PLC, see Fry supra) , (4) activation of an adapter molecule, and/or (5) increased cell division. These activities can be measured using techniques described below and known in the art. For example auto-phosphorylation of EGFR can be measured as described in the examples below using an anti-phosphotyrosine antibody, and increased cell division can be performed by measuring ^{3}H-thymidine incorporation into DNA. Preferably, the increase in EGFR activity is characterized by an increased amount of phosphorylated EGFR and/or DNA synthesis.

Unwanted cell proliferation and/or differentiation can result from inappropriate EGFR activity occurring in different types of cells including cancer cells, cells surrounding a cancer cell, and endothelial cells. Examples of disorders characterized by inappropriate EGF activity include cancers

identify compounds which regulate the signal transduction pathway are directly applicable to the identification of compounds which may be used to regulate the human signal transduction pathway, and more specifically, activity related to the KDR receptor. Chemical compounds identified as *inhibitors of KDR/FLK-1 in vitro, will be confirmed in* suitable in vivo models. Both in vivo mouse and rat animal models have been demonstrated to be of excellent value for the examination of the clinical potential of agents acting on the KDR/FLK-1 induced signal transduction pathway.

This invention is therefore directed to compounds which regulate, modulate and/or inhibit vasculogenesis and/or angiogenesis by affecting the enzymatic activity of the KDR/FLK-1 receptor and interfering with the signal transduced by KDR/FLK-1. *More particularly, the present invention* is directed to compounds which regulate, modulate and/or inhibit the KDR/FLK-1 mediated signal transduction pathway as a therapeutic approach to cure many kinds of solid tumors, including but not limited to glioblastoma, melanoma and Kaposi's sarcoma, and ovarian, lung, mammary, prostate, pancreatic, colon and epidermoid carcinoma. In addition, data suggest the administration of compounds which inhibit the KDR/FLK1 mediated signal transduction pathway may be used for the treatment of hemangioma and diabetic retinopathy.

The invention also relates to the inhibition of vasculogenesis and angiogenesis via other receptor-mediated pathways, including the pathway comprising the highly related flt-1 receptor. Receptor tyrosine kinase mediated signal transduction is initiated by extracellular interaction with a specific growth factor (ligand), followed by receptor dimerization, transient stimulation of the intrinsic protein tyrosine kinase activity and autophosphorylation. Binding sites are thereby created for intracellular signal transduction molecules and lead to the formation of complexes with a spectrum of cytoplasmic signalling molecules that facilitate the appropriate cellular response. (E.g., cell division, metabolic effects to the extracellular microenvironment) See, Schlessinger and Ullrich, 1992, *Neuron* 9:1–20.

The close homology of the intracellular regions of KDR/FLK-1 with that of the PDGF-β-Receptor (50.3% homology) and/or the highly related flt-1 receptor indicates the induction of overlapping signal transduction pathways. For example, for the PDGF-βReceptor, members of the src family (Twamley et al., 1993, *Proc. Natl. Acad. Sci. USA* 90:7696–7700), phosphatidylinositol-3'-kinase (Hu et al., 1992, *Mol. Cell. Biol.* 12:981–990), phospholipase c-γ (Kashishian & Cooper, 1993, *Mol. Cell. Biol.* 4:49–51), ras-GTPase-activating protein, (Kashishian et al., 1992, *EMBO J.* 11:1373–1382), PTP-ID/syp (Kazlauskas et al., 1993, *Proc. Natl. Acad. Sci. USA* 90:6939–6943), Grb2 (Arvidsson et al., 1994, *Mol. Cell. Biol.* 14:6715–6726), and the adapter molecules Shc and Nck (Nishimura et al., 1993, *Mol. Cell. Biol.* 13:6889–6896), have been shown to bind to regions involving different autophosphorylation sites. See generally, Claesson-Welsh, 1994, prog. Growth Factor Res. 5:37–54. Thus, it is likely that signal transduction pathways activated by KDR/FLK-1 include the ras pathway (Rozakis et al., 1992, *Nature* 360:689–692), the PI-3'-kinase pathway and the src-mediated and plcγ-mediated pathways. Each of these pathways may play a critical role in the angiogenic and/or vasculogenic effect of KDR/FLK-1 in endothelial cells. Consequently, the present invention is also directed to the use of the organic compounds discussed herein to modulate angiogenesis and vasculogenesis as such processes are controlled by these pathways.

E. C-MET Related Disorders

The c-met protooncogene is a growth factor receptor with tyrosine kinase activity and a suspected involvement in hepatocarcinogenesis. C-met protein expression has been correlated with poor to moderate differentiation of cancer cells whereas in one study all cases have increased proliferative activity, thus suggesting an important role in the development of hepatocellular-carcinoma see Suzuki et al., *Hepatology* 20:1231–1236, 1994.

The met gene is selectively expressed in several epithelial tissues and high levels of met mRNA have been found in liver, gastrointestinal tract, thyroid and kidney. Normal or increased levels of met mRNA and met protein were consistently found in fresh samples of carcinomas as well as epithelial tumor cell lines and in thyroid carcinomas of a specific histiotype. The amount of met protein was found to be increased more than 100 fold suggesting a role in growth control of epithelial cells other than hepatocytes and suggesting the increase in expression may convert growth advantage to neoplasm cells. Renzo et al., Oncogene 6:1997–2003, 1991.

The c-met oncogene is expressed not only in hepatocytes but also in a variety of tissues and over expression of c-met is found in some cell lines and tumors. It is amplified and overexpressed in a gastric carcinoma cell line, gtl-16 and it has been reported that the expression of c-met is enhanced in colorectal, gastric and thyroid cancer. The met gene is overexpressed in some cases of human leukemia and lymphoma. See Jucker et al. *Leukemia Res., 18:7–16, 1994.* Expression of the met gene was detected in patients with Hodgkins disease, Burkitt's, lymphoma cell line and acute myeloid leukemia. Expression of c-met encoded HGFR in human melonocytic neoplasms has been used to demonstrate the relationship to malignant tumor progressions. Natali, *Br. J. Cancer* 68:746–750, 1993.

The role of c-met in human tumors is reviewed in Giordano et al., *European Jrnl. Cancer Prevention*, 1:45–49, 1992. Examples of human tumors believed to be associated with c-met include colon cancer tumor, epithelial tumors, gastrointestinal tumors, thyroid tumors, and others. The expression of HGFR in human pancreatic cancer is described in Renzo et al., *Cancer Res.*, 55:1129–1138, 1995. The TPR/MET oncogenic rearrangement is present and expressed in human gastric carcinoma and precursor legion, see Soman et al., *Proc. Natl. Acad. Sci. USA*, 88:4892–4896, 1991. It has been reported that HGF gene deletion leads to death knockout mice see *Bioworld Today* Feb. 24, 1995. The molecular characteristics of HGF-SF and its role in cell motility and invasion is reviewed in Widner et al., *Hepatocyte Growth Factor Scatter Factor (HGSF)* and the C MET Receptor Editors Goldberg and Rosen, 1993.

F. PDGFR Driven Disorders

PDGFR driven disorders are described in U.S. patent applications Ser. Nos. 08/370,574 and 08/426,789, filed Jan. 6, 1995 and Apr. 21, 1995, both of which are incorporated herein by reference in their entirety including any drawings.

II. Diagnostic Uses

Another use of the compounds described herein is to help diagnose whether a disorder is driven, to some extent, by a particular receptor tyrosine kinase. Some cancers may be driven by more than one receptor tyrosine kinases. For example, Wada et al, *Oncogene* 5.489–495, 1990, describes co-expression of EGFR and HER2.

A diagnostic assay to determine whether a particular cancer is driven by a specific receptor can be carried out

using the following steps: (1) culturing test cells or tissues; (2) administering a compound which can inhibit one or more receptor tyrosine kinase; and (3) measuring the degree of growth inhibition of the test cells.

These steps can be carried out using standard techniques in light of the present disclosure. For example, standard techniques can be used to isolate cells or tissues and culturing or in vivo. An example of an in vitro assay is a cellular kinase assay as described below. An example of an in vivo assay is a xenograft experiment where the cells or tissues are implanted into another host such as a mouse.

Compounds of varying degree of selectivity are useful for diagnosing the role of a receptor tyrosine kinase. For example, compounds which inhibit more than one type of receptor tyrosine kinase can be used as an initial test compound to determine if one of several receptor tyrosine kinases drive the disorder. More selective compounds can then be used to further eliminate the possible role of different receptor tyrosine kinases in driving the disorder. Test compounds should be more potent in inhibiting receptor tyrosine kinase activity than in exerting a cytotoxic effect (e.g., an IC_{50}/LD_{50} of greater than one). IC_{50} and LD_{50} can be measured by standard techniques, such as described in the present application and using an MTT assay as described by Mossman supra, or by measuring the amount of LDH released (Korzeniewski and Callewaert, J. supra; Decker and Lohmann-Matthes, supra). The degree of IC_{50}/LD_{50} of a compound should be taken into account in evaluating the diagnostic assay. Generally, the larger the ratio the more reliable the information. Appropriate controls to take into account the possible cytotoxic effect of a compound, such as treating cells not associated with a cell proliferative disorder (e.g., control cells) with a test compound, can also be used as part of the diagnostic assay.

III. Pharmaceutical Formulations and Modes of Administration

The particular compound that affects the protein complexes and the disorder of interest can be administered to a patient either by themselves, or in pharmaceutical compositions where it is mixed with suitable carriers or excipient (s). In treating a patient exhibiting a disorder of interest, a therapeutically effective amount of a agent or agents such as these is administered. A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms or a prolongation of survival in a patient.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds which exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} as determined in cell culture (i.e., the concentration of the test compound which achieves a half-maximal disruption of the protein complex, or a half-maximal inhibition of the cellular level and/or activity of a complex component). Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by HPLC.

The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g. Fingl et al., in *The Pharmacological Basis of Therapeutics*, 1975, Ch. 1 p. 1). It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administrated dose in the management of the oncogenic disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

Depending on the specific conditions being treated, such agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in *Remington's Pharmaceutical Sciences*, 18th ed., Mack Publishing Co., Easton, Pa. (1990). Suitable routes may include oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, just to name a few. For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice of the invention into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier and suitable manufacturing practice, the compositions of the present invention, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes, then administered as described above. Liposomes are spherical lipid bilayers with aqueous interiors. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external microenvironment and, because liposomes fuse

with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intracellularly.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions. The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levitating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with fillers such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the kinase modulating effects, or *minimal effective concentration* (MEC). The MEC will vary for each compound but can be estimated from in vitro data; eg the concentration necessary to achieve a 50–90% inhibition of the kinase using the assays described herein. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, HPLC assays or bioassays can be used to determine plasma concentrations.

Dosage intervals can also be determined using the MEC value. Compounds should be administered using a regimen which maintains plasma levels above the MEC for 10–90% of the time, preferably between 30–90% and most preferably between 50–90%.

EXAMPLES

Examples are provided below to illustrate different aspects and embodiments of the present invention. These examples are not intended in any way to limit the disclosed invention. Rather, they illustrate methodology by which drugs having the disclosed formulas can be readily identified by routine procedure to ensure that they have the desired activity, and the synthesis of different compounds described herein. Compounds within a formula claimed herein can be screened to determine those with the most appropriate activity prior to administration to an animal or human. Other compounds can also be screened to determine suitability for use in methods of this invention.

Example	X	R1	R2
1.	O	4-t-Bu	3-chloro-4-isopropylsulfonylthien-2-yl
2.	O	3,5-di-trifluoro methyl	3-chloro-4-isopropylsulfonyl thien-2-yl
3.	O	4-trifludro 5-yl	2-(2,4-dichlorophenoxy)pyrid-methylmercapto
4.	S	4-cyano	4-(piperid-1-ylsulfonyl)phenyl
5.	O	4-t-butyl	3-chloro-4-isopropylsulfonyl thien-2-yl
6.	O	4-trifluoro methyl	3-chloro-4-isopropylsulfonyl thien-2-yl
7.	S	4-trifluoro methyl	3-chloro-4-isopropylsulfonyl thien-2-yl
8.	S	4-(piperid-1-yl sulfonyl)	3-chloro-4-isopropylsulfonyl thien-2-yl
9.	S	4-phenylamino sulfonyl	3-chloro-4-isopropylsulfonyl thien-2-yl
10.	S	4-(piperid-1-yl sulfonyl)	4-(4-chlorophenylsulfonyl) thien-3-yl
11.	S	4-trifluoro methyl	4-(4-chlorophenylsulfonyl) thien-3-yl
12.	O	4-trifluoro methyl	5-nitrothiazol-2-yl
13.	S	4-trifluoro methyl	5-nitrothiazol-2-yl
14.	S	4-phenylamino sulfonyl	5-nitrothiazol-2-yl
15.	O	4-t-butyl thien-2-yl	3-ethoxycarbonyl-4-methyl
16.	S	4-trifluoro methyl	3-ethoxycarbonyl-4-methyl thien-2-yl
17.	S	4-(piperid-1-yl sulfonyl)	3-ethoxycarbonyl-4-methyl thien-2-yl
18.	O	4-t-butyl	2,4-dinitrothien-5-yl
19.	O	4-t butyl	3-cyano-5-methoxycarbonyl-4-

-continued

Example	X	R1	R2
			methylthien-2-yl
20.	O	4-trifluoro methyl	3-cyano-5-methoxycarbonyl-4-methylthien-2-yl
21.	S	4-phenylamino sulfonyl	3-cyano-5-methoxycarbonyl-4-methylthien-2-yl
22.	S	4-trifluoro methyl	3-methylthien-2-yl
23.	S	4-(piperid-1-yl sulfonyl)	3-methylthien-2-yl
24.	O	4-t-butyl	4-(4-chlorophenyl)-3-cyano pyrrol-2-yl
25.	S	4-trifluoro methyl	4-(4-chlorophenyl)-3-cyano pyrrol-2-yl
26.	S	4-(piperid-1-yl sulfonyl)	4-(4-chlorophenyl)-3-cyano pyrrol-2-yl
27.	O	4-t-butyl	4-isopropylsulfonylthien-2-yl
28.	S	4-trifluoro methyl	4-isopropylsulfonylthien-2-yl
29.	S	4-(piperid-1-yl sulfonyl)	4-isopropylsulfonylthien-2-yl
30.	O	4-t-butyl	4-[4-(trifluoromethyl)phenylamino sulfonyl)thien-2-yl
31.	S	4-(piperid-1-yl sulfonyl	4-[4-(trifluoromethyl)phenylamino sulfonyl)thien-2-yl
32.	S	4-trifluoro methyl	3-aminocarbonyl-4-methylthien-2-yl
33.	S	4-(piperid-1-yl sulfonyl)	4-trifluoromethylphenyl
34.	S	4-(piperid-1-yl sulfonyl)	4-trifluoromethylbenzyl
35.	S	4-trifluoro methyl	2-(2,4-dichlorophenoxy)pyrid-5-yl

EXAMPLES

Example 1 (Z1)

N-[3-Chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-(4-t-butylphenyl)urea

2-Amino-3-chloro-4-isopropylsulfonylthiophene (260 mg) was stirred for 4 hours with 4-t-butylphenylisocyanate (119 mg) in 1 mL of pyridine at 60 ∞C. The pyridine was evaporated to dryness and the residue taken up in a mixture of ethyl acetate and 1N hydrochloric acid. The ethyl acetate layer was washed with water, saturated sodium bicarbonate and brine, and dried over anhydrous magnesium sulfate. Concentration and crystallization of the residue from ethanol and water gave 100 mg of N-[3-chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-(4-t-butyl-phenyl)urea, an off-white solid.

Example 2 (Z2)

N-[3-Chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-(3,5-di-trifluoromethylphenyl)urea

3,5-Di-trifluoromethylphenylisocyanate was treated with 2-amino-3-chloro-4-isopropylsulfonylthiophene as in Example 1 to give N-[3-chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-(3,5-di-trifluoromethylphenyl) urea, an off-white solid.

Example 3 (Z3)

N-[2-(2,4-Dichlorophenoxy)pyrid-5-yl]-N'-[4-trifluoromethylmercapto)phenyl]urea

5-Amino-2-chloropyridine(643 mg)in 3 mL of chloroform containing 505 mg of triethylamine was stirred with 853 mg of benzyl chloroformate for 4 hours at 60 degrees C. The mixture was then diluted with 20 mL of chloroform and washed with water, saturated sodium bicarbonate and brine, dried over anhydrous magnesium sulfate and evaporated to give 700 mg of 5-benzyloxycarbonylamino-2-chloropyridine. 5-Benzyloxy carbonylamino-2-chloropyridine (272 mg) and 163 mg of 2,4-dichlorophenol were stirred overnight at room temperature in 1 mL of dimethylformamide containing 138 mg of potassium carbonate. The reaction mixture was evaporated to dryness *under high vacuum and partitioned between ethyl acetate* and water. The ethyl acetate layer was washed with water, saturated sodium bicarbonate and brine, dried over anhydrous magnesium sulfate and evaporated. Hydrogenolysis in methanol over palladium on carbon gave 5-amino-2-(2,4-dichlorophenoxy) pyridine. The 5-amino-2-(2,4-dichlorophenoxy)pyridine was treated with 4-(trifluoromethylmercapto)phenylisocyanate under conditions similar to those in Example 1 to give N-[2-(2,4-*dichlorophenoxy)pyrid-5-yl*]-N'-[4-*trifluoromethyl* mercapto)phenyl]urea.

Example 4 (Z4)

N-(4-Cyanophenyl)-N'-[4-[(piperid-1-yl)sulfonyl]phenyl] *thiourea*

4-Chlorosulfonylphenylisothiocyanate (233 mg) was stirred with 85 mg of piperidine and 115 mg of N-ethylmorpholine in 1 mL of chloroform at 0 degrees C for two hours. The mixture was diluted into 10 mL of ice cold chloroform and washed with ice cold water, saturated sodium bicarbonate, 1N hydrochloric acid and brine, dried over anhydrous magnesium sulfate and concentrated to dryness. The residue was treated with 118 mg of 4-aminobenzonitrile as in Example 1 to give N-(4-cyanophenyl)-N'-[4-[(piperid-1-yl)sulfonyl]phenyl] thiourea, an off-white solid.

The following examples were prepared in a manner similar to that of Example 1.

Example 5

N-(4-t-Butylphenyl)-N'-[3-chloro-4-(isopropylsulfonyl) thien-2-yl]urea.

Example 6

N-[3-chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-(4-*trifluoromethylphenyl)urea.*

Example 7

N-[3-Chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-4-trifluoromethylphenyl)thiourea.

Example 8

N-[3-Chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-[4-(piperid-1-yl)sulfonylphenyl]thiourea.

Example 9

N-[3-Chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-[4-phenylaminosulfonylphenyl]thiourea.

Example 10

N-[4-(4-Chlorophenylsulfonyl)thien-3-yl]-N'-[4-(piperid-1-yl)sulfonylphenyl]thiourea.

Example 11

N-[4-(4-Chlorophenylsulfonyl)thien-3-yl}-N'-(4-trifluoromethylphenyl)thiourea.

Example 12

N-(5-Nitrothiazol-2-yl)-N'-(4-trifluoromethylphenyl)urea.

Example 13

N-(5-Nitrothiazol-2-yl)-N'-(4-trifluoromethylphenyl) thiourea.

Example 14

N-(5-Nitrothiazol-2-yl)-N'-[4-(phenylaminosulfonyl) phenyl]thiourea.

Example 15

N-(4-t-Butylphenyl)-N'-(3-ethoxycarbonyl-4-methylthien-2-yl)urea.

Example 16

N-(3-Ethoxycarbonyl-4-methylthien-2-yl)-N'-(4-trifluoromethylphenyl)thiourea.

Example 17

N-(3-Ethoxycarbonyl-4-methylthien-2-yl)-N'-[4-[(piperid-1-yl)sulfonyl]phenyl]thiourea.

Example 18

N-(4-t-Butylphenyl)-N'-(2,4-dinitrothien-5-yl)urea

Example 19

N-(4-t-Butylphenyl)-N'-(3-cyano-5-methoxycarbonyl-4-methylthien-2-yl)urea.

Example 20

N-(3-Cyano-5-methoxycarbonyl-4-methylthien-2-yl)-N'-(4-trifluoromethylphenyl)urea.

Example 21

N-(3-Cyano-5-methoxycarbonyl-4-methylthien-2-yl)N'-[4-(phenylaminosulfonyl)phenyl]thiourea.

Example 22

N-(3-Methylthien-2-yl)-N'-(4-trifluoromethylphenyl) thiourea.

Example 23

N-(3-Methylthien-2-yl)-N'-[4-[(piperid-1-yl)sulfonyl] phenyl]thiourea.

Example 24

N-(4-t-Butylphenyl)-N'-[4-(4-chlorophenyl)-3-cyanopyrrol-2-yl]urea.

Example 25

N-[4-(4-Chlorophenyl)-3-cyanotpyrrol-2-yl]-N'-(4-trifluoromethylphenyl)urea.

Example 26

N-[4-(4-Chlorophenyl)-3-cyanopyrrol-2-yl]-N'-[4-[(piperid-1-yl)sulfonyl]phenyl]thiourea.

Example 27

N-(4-t-Butylphenyl)-N'-[4-(isopropylsulfonyl)thien-2-yl] urea.

Example 28

N-[4-(Isopropylsulfonyl)thien-2-yl]-N'-4-trifluoromethylphenyl]thiourea.

Example 29

N-[4-(Isopropylsulfonyl)thien-2-yl]-N'-[4-[(piperid-1 -yl) sulfonyl]phenyl]thiourea.

Example 30

N-(4-t-Butylphenyl)-N'-[4-[4-(trifluoromethyl) phenylaminosulfonyl]thien-2-yl]urea.

Example 31

N-[4-[(Piperid-1-yl)sulfonyl]phenyl]-N'-[4-[4-(trifluoromethyl)phenylaminosulfonyl]thien-2-yl] thiourea.

Example 32

N-(3-Aminocarbonyl-4-methylthien-2-yl)-N'-(4-trifluoromethylphenyl)thiourea.

Example 33

N-[4-[(Piperid-1-yl)sulfonyl]phenyl]-N'-4-trifluoromethylphenyl)thiourea.

Example 34

N-[4-[(Piperid-1-yl)sulfonyl]phenyl]-N'-[4-(4-trifluoromethylbenzyl]thiourea.

Example 35

N-[2-(2,4-dichlorophenoxy)pyrid-5-yl]-N'-(4-trifluoromethylphenyl)thiourea.

Receptor tyrosine kinases can be used as initial test compounds to determine if one of several receptor tyrosine kinases drive the disorder. More selective compounds can then be used to further eliminate the possible role of different receptor tyrosine kinases in driving the disorder. Test compounds should be more potent in inhibiting receptor tyrosine kinase activity than in exerting a cytotoxic effect (e.g., an IC_{50}/LD_{50} of greater than one). As noted above, IC_{50} and LD_{50} can be measured by standard techniques, such as described in the present application and using an MTT assay as described by Mossman supra, or by measuring the amount of LDH released (Korzeniewski and Callewaert, J. supra; Decker and Lohmann-Matthes, supra). The degree of IC_{50}/LD_{50} of a compound should be taken into account in evaluating the diagnostic assay. Generally, the larger the ratio the more reliable the information. Appropriate controls to take into account the possible cytotoxic effect of a compound, such as treating cells not associated with a cell proliferative disorder (e.g., control cells) with a test compound, can also be used as part of the diagnostic assay.

The following examples illustrate the ability of the exemplary compounds to inhibit receptor tyrosine kinases, such as HER2 and/or EGFR. The following target cells were used for cellular kinase assays: NIH3T3 clone C7 (Honegger et al., supra) engineered to over-express human EGF receptor; NIH3T3 cells engineered to over-express a chimeric receptor containing the EGFR extracellular domain and the HER2 intracellular kinase domain; the human mammary carcinoma line BT474 (ATCC HTB2) expressing HER2; and the human glioblastoma line U1242 that expresses PDGFR-beta. Growth assays were carried out using human mammary epithelial SKBR3 (ATCC HTB30) cells (SKBR3 cells

over-express HER2), SKOV3 (ATCC HTB77) human ovarian cancer cell line (SKOV3 cells also over-express HER2), *A431 cells (A431 cells over-express EGFR) MCF7 human* breast carcinoma cells, MCF7 cells overexpressing the HER2 kinase (MCF7-HER2), NIH3T3 cells, and NIH3T3 cells overexpressing the HER2 kinase (3T3-HER2.

The assay procedures described below were used to generate the data in the tables showing the effectiveness of the compounds of the present invention.

GROUP II ELISA TYPE ASSAYS

EXAMPLE 1

EGFR Whole Cell Kinase Assay

EGFR kinase activity (EGFR-3T3 assay) in whole cells was measured as described below:

Materials & Reagents

1) EGF Ligand: stock concentration=16.5 μM; EGF 201, TOYOBO, Co., Ltd. Japan.

2) 05–101 (UBI) (a monoclonal antibody recognizing an EGFR extracellular domain).

3) Anti-Phosphotyosine antibody (polyclonal) (made according to Fendley et al., *Cancer Research* 50: 1550–1558, 1990).

4) TAGO antibody: Goat anti-rabbit IgG horse radish peroxidase conjugate, TAGO, Inc., Burlingame, Calif.

5) TBST buffer:

Tris-HCl, pH 7.2,	50 nM
NaCl,	150 mM,
Triton X-100	0.1%

6) HNTG 5X stock:

HEPES	0.1 M
NaCl	0.75 M
Glycerol	50%
Triton X-100	1.0%

7) ABTS stock:

Citric Acid	100 mM
Na_2HPO_4	250 mM
HCl, conc.	4.0 pH
ABTS*	0.5 mg/ml

*(2,2'-azinobis(3-ethylbenzthiazotinesulfonic acid). Keep solution in dark at 4° C. until use.

8) Stock reagents of:

EDTA	100 mM; pH 7.0
Na_3VO_4	*0.5 M*
Na_4PQ	0.2 M

Procedure

I. Pre-coat ELISA Plate

A. Coat ELISA plates (Corning, 96 well, Cat. #25805-96) with 05–101 antibody at 0.5 μg per well in PBS, 150 μl final volume/well, and store overnight at 4¤C. Coated plates are good for up to 10 days when stored at 4¤C.

B. On day of use, remove coating buffer and replace with blocking buffer (5% Carnation Instant NonFat Dry Milk in PBS). Incubate the plate, shaking, at room temperature (about 23¤C to 25¤C) for 30 minutes. Just *prior to use, remove blocking buffer and wash plate 4* times with TBST buffer.

II. Seeding Cells

A. EGFR/C7 cell line (Honegger, et al., supra) can be used for this assay.

B. Choose dishes having 80–90% confluence for the *experiment.*

Trypsinize cells and stop reaction by adding 10% CS DMEM medium. Suspend cells in DMEM medium (10% CS DMEM medium) and centrifuge once at 1000 rpm, and once at room temperature for 5 minutes.

C. Resuspend cells in seeding medium (DMEM, 0.5% bovine serum), and count the cells using trypan blue. Viability above 90% is acceptable. Seed cells in DMEM medium (0.5% bovine serum) at a density of 10,000 cells per well, 100 μl per well, in a 96 well microtiter plate. Incubate seeded cells in 5% CO_2 at 37¤C for about 40 hours.

III. Assay Procedures.

A. Check seeded cells for contamination using an inverted *microscope. Dilute drug stock (10 mg/ml in DMSO)* 1:10 in DMEM medium, then transfer 5 μl to a test well for a final drug dilution of 1:200 and a final DMSO concentration of 1%.

Control wells receive DMSO alone. Incubate in 5% CO_2 at 37° C. for one hour.

B. *Prepare EGF ligand: dilute stock EGF in DMEM so* that upon transfer of 10 μl dilute EGF (1:12 dilution), 25 nM final concentration is attained.

C. Prepare fresh HNTG sufficient for 100 μl per well; and place on ice.

HNTG:*	*10 ml*
HNTG stock (5×)	*2.0 ml*
milli-Q H_2O	7.3 ml
EDTA, (100 mM, pH 7.0)	0.5 ml
Na_3VO_4, (0.5 M)	0.1 ml
Na_4PO_3, (0.2 M)	0.1 ml

D. *After two hours incubation with drug, add prepared* EGF ligand to cells, 10 μl per well, to yield a final concentration of 25 nM. Control wells receive DMEM alone. Incubate, shaking, at room temperature, for 5 minutes.

E. Remove drug, EGF, and DMEM. Wash cells twice with PBS. Transfer HNTG* to cells, 100 μl per well. Place *on ice for 5 minutes. Meanwhile, remove blocking* buffer from other ELISA plate and wash with TBST as described above.

F. With a pipette tip securely fitted to a micropipettor, scrape cells from plate and homogenize cell material by repeatedly aspirating and dispensing the HNTG* lysis buffer. Transfer lysate to a coated, blocked, and washed *ELISA plate. Incubate shaking at room temperature for* one hour.

G. Remove lysate and wash 4 times with TBST. Transfer freshly diluted anti-Ptyr antibody to ELISA plate at 100 μl per well. Incubate shaking at room temperature for 30 minutes in the presence of the anti-Ptyr antiserum (1:3000 dilution in TBST).

H. Remove the anti-Ptyr antibody and wash 4 times with TBST. Transfer the freshly diluted TAGO 30 anti-rabbit IgG antibody (anti-rabbit IgG antibody: 1:3000 dilution in TBST) to the ELISA plate at 100 μl per well. Incubate shaking at room temperature for 30 minutes.

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I. Remove detection antibody and wash 4 times with TBST. Transfer freshly prepared ABTS/H_2O_2 solution to ELISA plate, 100 μl per well. Incubate at room temperature for 20 minutes. ABTS/H_2O_2 solution: 1.2 μl 30% H_2O_2 in 10 ml ABTS stock.

J. Stop reaction by adding 50 μl 5N H_2SO_4 (optional), and determine O.D. at 410 nm.

K. The maximal phosphotyrosine signal is determined by subtracting the value of the negative controls from the positive controls. The percent inhibition of phosphotyrosine content for extract-containing wells is then calculated, after subtraction of the negative controls.

EXAMPLE 2

EGFR-HER2 Chimeric Recetor

HER2 kinase activity (EGFR-3T3) in whole cells was measured as described below:

Materials & Reagents

The materials and regeants are identical to these used in example 1, the EGFR whole cell kinase assay.

Procedure

I. Pre-coat ELISA Plate

A. Coat ELISA plates (Corning, 96 well, Cat. #25805-96) with 05–101 antibody at 0.5 μg per well in PBS, 100 μl final volume/well, and store overnight at 4°C. Coated plates are good for up to 10 days when stored at 4° C.

B. On day of use, remove coating buffer and replace with 100 μl blocking buffer (5% Carnation Instant Non-Fat Dry Milk in PBS). Incubate the plate, shaking, at room temperature (about 23°C to 25°C) for 30 minutes. Just prior to use, remove blocking buffer and wash plate 4 times with TBST buffer.

II. Seeding Cells

A. An NIH3T3 cell line overexpressing a chimeric receptor containing the EGFR extracellular domain and extracellular HER2 kinase domain can be used for this assay.

B. Choose dishes having 80–90% confluence for the experiment. Trypsinize cells and stop reaction by adding 10% fetal bovine serum. Suspend cells in DMEM medium (10% CS DMEM medium) and centrifuge once at 1500 rpm, at room temperature for 5 minutes.

C. Resuspend cells in seeding medium (DMEM, 0.5% bovine serum) , and count the cells using trypan blue. Viability above 90% is acceptable. Seed cells in DMEM medium (0.5% bovine serum) at a density of 10,000 cells per well, 100 μl per well, in a 96 well microtiter plate. Incubate seeded cells in 5% CO_2 at 37°C for about 40 hours.

III. Assay Procedures

A. Check seeded cells for contamination using an inverted microscope. Dilute drug stock (10 mg/ml in DMSO) 1:10 in DMEM medium, then transfer 5 l to a TBST well for a final drug dilution of 1:200 and a final DMSO concentration of 1%. Control wells receive DMSO alone. Incubate in 5% CO_2 at 37° C. for two hours.

B. Prepare EGF ligand: dilute stock EGF in DMEM so that upon transfer of 10 μl dilute EGF (1:12 dilution), 100 nM final concentration is attained.

C. Prepare fresh HNTG* sufficient for 100 μl per well; and place on ice.

HNTG*:	10 ml
HNTG stock	2.0 ml

-continued

milli-Q H_2O	7.3 ml
EDTA, 100 mM, pH 7.0	0.5 ml
Na_3VO_4, 0.5 M	0.1 ml
Na_4PO_7, 0.2 M	0.1 ml

D. After 120 minutes incubation with drug, add prepared SGF ligand to cells, 10 μl per well, to a final concentration of 100 nM. Control wells receive DMEM alone. Incubate, shaking, at room temperature, for 5 minutes.

E. Remove drug, EGF, and DMEM. Wash cells twice with PBS. Transfer HNTG* to cells, 100 μl per well. Place on ice for 5 minutes. Meanwhile, remove blocking buffer from other ELISA plate and wash with TBST as described above.

F. With a pipette tip securely fitted to a micropipettor, scrape cells from plate and homogenize cell material by repeatedly aspirating and dispensing the HNTG* lysis buffer. Transfer lysate to a coated, blocked, and washed ELISA plate. Incubate shaking at room temperature for one hour.

G. Remove lysate and wash 4 times with TBST. Transfer freshly diluted anti-Ptyr antibody to ELISA plate at 100 μl per well. Incubate shaking at room temperature for 30 minutes in the presence of the anti-Ptyr antiserum (1:3000 dilution in TBST).

H. Remove the anti-Ptyr antibody and wash 4 times with TBST. Transfer the freshly diluted TAGO anti-rabbit IgG antibody (anti-rabbit IgG antibody: 1:3000 dilution in TBST) to the ELISA plate at 100 μl per well. Incubate shaking at room temperature for 30 minutes.

I. Remove detection antibody and wash 4 times with TBST. Transfer freshly prepared ABTS/H_2O_2 solution (ABTS/H_2O_2 solution: 1.0 μl 30% H_2O_2 in 10 ml ABTS stock) to ELISA plate, 100 μl per well. Incubate shaking at room temperature for 20 minutes.

J. Stop reaction by adding 50 μl 5N H_2SO_4 (optional), and determine O.D. at 410 nm.

K. The maximal phosphotyrosine signal is determined by subtracting the value of the negative controls from the positive controls. The percent inhibition of phosphotyrosine content for extract-containing wells is then calculated, after subtraction of the negative controls.

EXAMPLE 3

HER2-ELISA

HER2-BT474 assays measuring whole cell HER2 activity was carried out as described below:

Materials & Reagents

1. The cell line used in this assay is BT-474 (ATCC HBT20), a human breast tumor cell line which expresses high levels of HER2 kinase.

2. BT-474 is grown in an incubator with 5% CO_2 at 37° C. The growth media is RPMI+10% FBS+GMS-G (Gibco supplement)+Glutamine.

3. A monoclonal anti-HER2 antibody is used in ELISA.

4. D-PBS:

KH_2HPO_4	0.20 g/l 10 (GIECO, 310-4190AJ)
K_2HPO_4	2.16 g/l:

-continued

KCl	0.20 g/l
NaCl	8.00 g/l pH 7.2

5. Blocking Buffer: TBST plus 5% Milk (Carnation Instant Non-Fat Dry Milk).

6. TBST buffer:

Tris-HCl	50 mM pH 7.2 (HCl, 10 N)
NaCl	150 mM
Triton X-100	0.1%

*Stock solution of TES (10X) is prepared, and Triton X-100 is added to the buffer during dilution.

7. HNTG buffer:

HEPES	20 mM; pH 7.2 (HCl, 1 N)
NaCl	150 mM
Glycerol	10%
Triton X-100	0.2%

*Stock solution (5x) is prepared and kept in 4° C.

8. EDTA-HCl: 0.5M pH 7.0 (10N HCl) as 500X stock.

9. Na_3VO_4: 0.5M as 100X stock is kept at −80° C. as aliquots.

10. $Na_4P_2O_7$: 0.2M as 100X stock.

11. Polyclonal antiserum anti-phosphotyrosine.

12. Goat anti-rabbit IgG, horse raddish peroxidase (POD) conjugate, Tago (Cat. No. 4520; Lot No. 1802): Tago, Inc., Burlingame, Calif.

13. ABTS solution:

Citric acid	100 mM,
Na_2HPO_4	250 mM; pH 4.0 (1 N HCl)
ABTS	0.5 mg/ml

* ABTS: 2.2'-azinobis(3-ethylbenzthiazolinesulfonic acid)

*ABTS solution should be kept in the dark at 40C. The solution should be discarded when it turns green.

14. Hydrogen Peroxide: 30% solution is kept in dark and 4° C.

Procedure

All the following steps are at room temperature and aseptically performed, unless stated otherwise. All ELISA plate washing is by rinsing with distilled water three times and once with TBST.

1. Cell Seeding

(a) Grow BT474 cells in tissue culture dishes (10 cm, Corning 25020-100) to 80–90% confluence and collect using Trypsin-EDTA (0.25%, GIBCO).

(b) Resuspend the cells in fresh medium and transfer to 96-well tissue culture plates (Corning, 25806-96) at about 25,000–50,000 cells/well (100 μl/well). Incubate the cells in 5% CO_2 at 37° C. overnight.

2. ELISA Plate Coating and Blocking

(a) Coat the ELISA plate (Corning 25805-96) with anti HER2 antibody at 0.5 μg/well in 150 μl PBS overnight at 4°C, and seal with parafilm. The antibody coated plates can be used up to 2 weeks, when stored at 4° C.

(b) On the day of use, remove the coating solution, replace with 200 μl of Blocking Buffer, shake the plate, and then remove the blocking buffer and wash the plate just before adding lysate.

3. Assay Procedures

(a) TBST the drugs in serum-free condition. Before adding drugs, the old media

(b) Dilute drug stock (in 100% DMSO) 1:10 with RPMI, and transfer 10 μl/well of this solution to the cells to achieve a final drug DMSO concentration at 1%. Incubate the cells in 5% CO_2 at 37° C.

(c) Prepare fresh cell lysis buffer (HNTG*)

HNTG	2 ml
EDTA	0.2 ml
Na_3VO_4	0.1 ml
$Na_4P_2O_7$	0.1 ml
H_2O	7.3 ml
HNTG*	10 ml

(d) After drug preincubation for two hours remove all the solution from the plate, transfer HNTG* 100 μl/well to the cells, and shake for 10 minutes.

(e) Use a 12-channel pipette to scrape the cells from the plate, and homogenize the lysate by repeat aspiration and dispensing. Transfer all the lysate to the ELISA plate and shake for 1 hour.

(f) Remove the lysate, wash the plate, add anti-pTyr (1:3,000 with TBST) 100 μl/well, and shake for 30 minutes.

(g) Remove anti-pTyr, wash the plate, add goat anti-rabbit IgG conjugated antibody (1:5,000 with TBST) 100 μl/well, and shake for 30 minutes.

(h) Remove anti-rabbit IgG antibody, wash the plate, and add fresh ABTS/H_2O_2 (1.2 μl 30% H_2O_2 in 10 ml ABTS) 100 μl/well to the plate to start color development, which usually takes 20 minutes.

(l) Measure OD 410 nM, Dynatec MR5000.

EXAMPLE 4

PDGF-R Cellular Assay

The PDGF cellular kinase assay was carried out as follows: cells are lysed in 0.2M Hepes, 0.15M NaCl, 10% V/V glycerol, 0.04% Triton X-100, 5 mM EDTA, 5 mM Na+ vanadate and 2 mM Na+ pyrophosphate; cell lysates are then added to an ELISA plate coated with an anti-PDGF receptor antibody (Genzyme); ELISA plates are coated at 0.5 μg of antibody/well in 150 μl of PBS for 18 hours at 4° C. prior to the addition of the lysate; the lysate is incubated in the coated plates for 1 hour and then washed four times in TBST (35 mM Tris-HCl pH 7.0, 0.15M NaCl, 0.1% Triton X100); anti-phosphotyrosine antibody (100 μl in PBS) is added and the mixture is incubated for 30 minutes at room temperature; the wells were then washed four times in TBST, a secondary antibody conjugated to POD (TAGO) is added to each well, and the treated well are incubated for 30 minutes at room temperature; the wells are then washed four times in TBST, ABTS/H_2O_2 solution is added to each well and the wells are incubated for two minutes; absorbance is then measured at 410 nm.

EXAMPLE 5

Cellular IGF-1 Receptor ELISA (Version I)

U1242 MG cells were plated in 96-well plates at a concentration of 5×10^4 cells/well in cultured media contain-

ing 0.5% FBS. The cells were incubated for 24 hours. The cells were then treated with a particular compound for 2 hours followed by the addition of 100 ng/ml PDGF-BB and incubation for 10 minutes.

Cells were lysed in 0.2M Hepes, 0.15M NaCl, 10% V/V glycerol, 0.04% Triton X-100, 5 mM EDTA, 5 mM Na+ vanadate and 2 mM Na+ pyrophosphate. Cell lysates were then added to an ELISA plate coated with an anti-PDGF receptor antibody (Genzyme). ELISA plates were coated at 0.5 μg of antibody/well in 150 μl of PBS for 18 hours at 4° C. prior to the addition of the lysate.

The lysate was incubated in the coated plates for 1 hour and then washed four times in TBST (35 mM Tris-HCl pH 7.0, 0.15M NaCl, 0.1% Triton X-100). Anti-phosphotyrosine antibody (100 μl in PBS) was added and the mixture was incubated for 30 minutes at room temperature. The wells were then washed four times in TBST, a secondary antibody conjugated to POD (TAGO) was added to each well, and the treated well were incubated for 30 minutes at room temperature. The wells were then washed four times in TBST, ABTS/H_2O_2 solution was added to each well and the wells were incubated for two minutes. Absorbance was then measured at 410 nm.

MATERIALS AND REAGENTS

(1). The cell line used in this assay is 3T3/IGF-1R, a cell line which overexpresses IGF-1 Receptor.

(2). 3T3/IGF-1 R is grown in an incubator with 5% CO2 at 37° C. The growth media is DMEM+10% FBS (heat inactivated)+2 mM L-Glutamine.

(3). For ELISA plate coating, the anti-IGF-1 R antibody named 17–69 is used. Antibodies are purified by the Enzymology Lab, SUGEN, Inc.

(4). D-PBS:

KH2PO4	0.20 g/l (GIBCO, 310-4190AJ)
K2HPO4	2.16 g/l
KCl	0.20 g/l
NaCl	8.00 g/l; pH 7.2

(5). Blocking Buffer: TBST plus 5% Milk (Carnation Instant Non-Fat Dry Milk)

(6). TBST buffer: Tris-HCl 50 mM NaCl 150 mM pH 7.2 (HCl, 10N) Triton X-100 0.1% *. Stock solution of TBS (10X) is prepared, and Triton X-100 is added to the buffer during dilution.

(6). HNTG buffer: HEPES 20 mM NaCl 150 mM pH 7.2 (HCl, 1N) Glycerol 10% Triton X-100 0.2 *. Stock solution (5X) is prepared and kept at 4° C.

(7). EDTA.HCl: 0.5M pH 7.0 (NaOH) as 100X stock.

(8). Na3VO4: 0.5M as 100X stock and aliquots are kept in −80° C.

(9). Na4P207: 0.2M as 100X stock.

(10). Insulin-like growth factor-1 from Promega (Cat# G5111).

(11). Polyclonal antiserum Anti-phosphotyrosine:

(12). Goat anti-rabbit IgG, POD conjugate (detection antibody), Tago (Cat. No. 4520; Lot No. 1802): Tago, Inc., Burlingame, Calif.

(13). ABTS solution: Citric acid 100 mM Na2HPO4 250 mM pH 4.0 (1N HCl) ABTS 0.5 mg/ml * ABTS: 2.2'-azinobis(3-ethylbenzthiazolinesulfonic acid) *. ABTS solution should be kept in dark and 4° C. The solution should be discarded when it turns green.

(14). Hydrogen Peroxide: 30% solution is kept in the dark and at 4° C.

V. PROCEDURE

All the following steps are conducted at room temperature unless it is specifically indicated. All ELISA plate washings are performed by rinsing the plate with tap water three times, followed by one TBST rinse. Pat plate dry with paper towels.

1. Cell Seeding

(1). The cells, grown in tissue culture dish (10 cm, Corning 25020-100) to 80–90% confluence, are harvested with Trypsin-EDTA (0.25%, 0.5 ml/D-100, GIBCO).

(2). Resuspend the cells in fresh DMEM+10% FBS+2 mM L-Glutamine, and transfer to 96-well tissue culture plate (Corning, 25806-96) at 20,000 cells/well (100 ul/well). Incubate for 1 day then replace medium to serum-free medium (90/ul) and incubate in 5% CO2 and 37° C. overnight.

2. ELISA Plate Coating and Blocking

(1). Coat the ELISA plate (Corning 25805-96) with Anti-IGF-1 R Antibody at 0.5 ug/well in 100 ul PBS at least 2 hours.

(2). Remove the coating solution, and replace with 100 ul Blocking Buffer, and shake for 30 minutes. Remove the blocking buffer and wash the plate just before adding lysate.

3. Assay Procedures

(1). The drugs are tested in serum-free condition.

(2). Dilute drug stock (in 100% DMSO) 1:10 with DMEM in 96-well poly-propylene plate, and transfer 10 ul/well of this solution to the cells to achieve final drug dilution 1:100, and final DMSO concentration of 1.0%. Incubate the cells in 5% CO2 at 37° C for 2 hours.

(3). Prepare fresh cell lysis buffer (HNTG HNTG 2 ml EDTA 0.1 ml Na3VO4 0.1 ml Na4P207 0.1 ml H20 7.3 ml HNTG* 10 ml.

(4). After drug incubation for two hours, transfer 10 ul/well of 200 nM IGF-1 Ligand in PBS to the cells (Final Conc=20 nM), and incubate at 5% CO2 at 37° C. for 10 minutes.

(5). Remove media and add 100 ul/well HNTG* and shake for 10 minutes. Look at cells under microscope to see if they are adequately lysed.

(6). Use a 12-channel pipette to scrape the cells from the plate, and homogenize the lysate by repeat aspiration and dispense. Transfer all the lysate to the antibody coated ELISA plate [V.2.(2)], and shake for 1 hour.

(7). Remove the lysate, wash the plate, transfer anti-pTyr (1:3,000 with TBST) 100 ul/well, and shake for 30 minutes.

(7). Remove anti-pTyr, wash the plate, transfer detection antibody (1:3,000 with TBST) 100 ul/well, and shake for 30 minutes.

(8). Remove detection antibody, wash the plate, and transfer fresh ABTS/H2O2 (1.2 ul H2O2 to 10 ml ABTS) 100 ul/well to the plate to start color development.

(9). Measure OD (410 nm) in Dynatec MR5000, which is connected to Ingres.

EXAMPLE 6

Cellular Insulin Receptor ELISA (Version I)

The following protocol describes the cell line, reagents and procedures used to measure phosphotyrosine level on

Insulin Receptor, which indicates Insulin Receptor tyrosine kinase activity.

MATERIALS AND REAGENTS

(1). The cell line used in this assay is H25 (ATCC #CRL 8017), an NIH3T3 cell line which overexpresses Insulin Receptor.

(2). H25 cells are grown in an incubator with 5% CO2 at 37° C. The growth media is DMEM+10% FBS (heat inactivated)+2 mM L-Glutamine.

(3). For ELISA plate coating, the monoclonal anti-IR antibody named BBE is used. Antibodies are purified by the Enzymology Lab, SUGEN, Inc.

(4). D-PBS: KH2PO4 0.20 g/l (GIBCO, 310-4190AJ) K2HPO4 2.16 g/l KCl 0.20 g/l NaCl 8.00 g/l pH 7.2.

(5). Blocking Buffer: TBST plus 5% Milk (Carnation Instant Non-Fat Dry Milk)

(6). TBST buffer: Tris-HCl 50 mM NaCl 150 mM pH 7.2 (HCl, 10N) Triton X-100 0.1%. *. Stock solution of TBS (10X) is prepared, and Triton X-100 is added to the buffer during dilution.

(6). HNTG buffer: HEPES 20 mM NaCl 150 mM pH 7.2 (HCl, 1N) Glycerol 10% Triton X-100 0.2% *. Stock solution (5X) is prepared and kept at 4° C.

(7). EDTA.HCl: 0.5M pH 7.0 (NaOH) as 100X stock.

(8). Na3VO4: 0.5M as 100X stock and aliquots are kept in −80° C.

(9). Na4P207: 0.2M as 100X stock.

(10). Insulin from GIBCO BRL (Cat# 18125039).

(11). Polyclonal antiserum Anti-phosphotyrosine: rabbit sera generated by Enzymology Lab., SUGEN Inc.

(12). Goat anti-rabbit IgG, POD conjugate (detection antibody), Tago (Cat. No. 4520; Lot No. 1802): Tago, Inc., *Burlingame, Calif.*

(13). ABTS solution: Citric acid 100 mM Na2HPO4 250 mM pH 4.0 (1N HCl) ABTS 0.5 mg/ml *. ABTS: 2.2'-azinobis(3-ethylbenzthiazolinesulfonic acid) *. ABTS solution should be kept in dark and 4° C. The solution should be discarded when it turns green.

(14). Hydrogen Peroxide: 30% solution is kept in the dark and at 4° C.

IV. PROCEDURE

All the following steps are conducted at room temperature unless it is specifically indicated. All ELISA plate washings are performed by rinsing the plate with tap water three times, followed by one TBST rinse. Pat plate dry with paper towels.

1. Cell Seeding

(1). The cells, grown in tissue culture dish (10 cm, Corning 25020-100) to 80–90% confluence, are harvested with Trypsin-EDTA (0.25%, 0.5 ml/D-100, GIBCO).

(2). Resuspend the cells in fresh DMEM+10% FBS+2 mM L-Glutamine, and transfer to 96-well tissue culture plate (Corning, 25806-96) at 20,000 cells/well (100 ul/well). Incubate for 1 day then replace medium to 0.01% serum medium (90/ul) and incubate in 5% CO2 and 37° C. overnight.

2. ELISA Plate Coating and Blocking

(1). Coat the ELISA plate (Corning 25805-96) with Anti-IR Antibody at 0.5 ug/well in 100 ul PBS at least 2 hours.

(2). Remove the coating solution, and replace with 100 ul Blocking Buffer, and shake for 30 minutes. Remove the blocking buffer and wash the plate just before adding lysate.

3. Assay Procedures

(1). The drugs are tested in serum-free condition.

(2). Dilute drug stock (in 100% DMSO) 1:10 with DMEM in 96-well poly-propylene plate, and transfer 10 ul/well of this solution to the cells to achieve final drug dilution 1:100, and final DMSO concentration of 1.0%. Incubate the cells in 5% CO2 at 37° C. for 2 hours.

(3). Prepare fresh cell lysis buffer (HNTG*) HNTG 2 ml EDTA 0.1 ml Na3VO4 0.1 ml Na4P2O7 0.1 ml H20 7.3 ml HNTG* 10 ml.

(4). After drug incubation for two hours, transfer 10 ul/well of 1 μM Insulin in PBS to the cells (Final Conc=100 nM), and incubate at 5% CO2 at 37° C. for 10 minutes.

(5). Remove media and add 100ul/well HNTG* and shake for 10 minutes. Look at cells under microscope to see if they are adequately lysed.

(6). Use a 12-channel pipette to scrape the cells from the plate, and homogenize the lysate by repeat aspiration and dispense. Transfer all the lysate to the antibody coated ELISA plate [V.2.(2)], and shake for 1 hour.

(7). Remove the lysate, wash the plate, transfer anti-pTyr (1:3,000 with TBST) 100 ul/well, and shake for 30 minutes.

(8). Remove anti-pTyr, wash the plate, transfer detection antibody (1:3,000 with TBST) 100 ul/well, and shake for 30 minutes.

(9). Remove detection antibody, wash the plate, and transfer fresh ABTS/H2O2 (1.2 ul H2O2 to 10 ml ABTS) 100 ul/well to the plate to start color development.

(10). Measure OD (410 nM) in Dynatec MR5000.

EXAMPLE 7

ELISA Assay to Measure Kinase Activity of FLK-1 Receptor in FLK-1/NIH Cells

An ELISA assay was conducted to measure the kinase activity of the FLK-1 receptor and more specifically, the inhibition or activiation of protein tyrosine kinase activity on the FLK-1 receptor.

6.1. Materials And Methods

Materials. The following reagents and supplies were used:

a. Corning 96-well ELISA plates (Corning Catalog No. 25805-96);

b. Cappel Goat anti-rabbit IgG (catalog no. 55641);

c. PBS (Gibco Catalog No. 450-1300EB);

d. TBSW Buffer (50 mM Tris (pH 7.2)m 150 mM NaCl and 0.1% Tween-20);

e. Ethanolamine stock (10% ethanolamine (pH 7.0), stored at 4° C.);

f. HNTG buffer (20 mM HEPES buffer (pH 7.5), 15 OmM NaCl, 0.2% Triton X-IOO, and 10% Glycerol);

g. EDTA (0.5M (pH 7.0) as a IOOX stock);

h. Sodium Ortho Vanadate (0.5M as a IOOX stock)

i. Sodium pyro phosphate (0.2M as a IOOX stock);

j. NUNC 96 well V bottom polypropylene plates (Applied Scientific Catalog No. AS-72092);

k. N1H3T3C7#3 Cells (FLK-1 infected cells);

l. DMEM with IX high glucose L Gulatamine (catalog No. 11965-050);

m. FBS, Gibco (catalog no. 16000-028);

n. L-glutamine, Gibco (catalog no. 25030-016);

o. VEGF, PeproTech, Inc. (catalog no.100-20) (kept as 1 ug/100 ul stock in Milli-Q dH_2O and stored at −20° C.;

p. Affinity purified anti-flk-I antiserum, Enzymology Lab, Sugen, Inc.;

q. UB40 monoclonal antibody specific for phophotyrosine, Enzymology Lab, Sugen, Inc.;

r. EIA grade Goat anti-mouse IgG-POD (BioRad catalog no. 172-1011)

S. 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) solution (100 mM citric acid (anhydrous), 250 mM Na_2HPO_4 (pH 4.0), 0.5 mg/ml ABTS (Sigma catalog no. A-1888)), solution should be stored in dark at 4° C. until ready for use;

t . H_2O_2 (30% solution) (Fisher catalog no. 11325);

u. ABTS/H_2O_2 (15 ml ABTS solution, 2 ul H_2O_2) prepared 5 minutes before use and left at room temperature;

v. 0.2M HCl stock in H_2O;

w. dimethylsulfoxide (100%) (Sigma Catalog No. D-8418) ; and

y. Trypsin-EDTA (Gibco BRL Catalog No. 25200-049)

Protocol. The following protocol was used to conduct the ELISA Assay:

1. Coat Corning 96-well elisa plates with 1.0 ug per well Cappel Anti-rabbit IgG antibody in 0.1M Na2CO3 pH 9.6. Bring final volume to 150 ul per well. Coat plates overnight at 4° C. Plates can be kept up to two weeks when stored at 4° C.

2. Grow cells in 30 ml of Growth media (DMEM. 2.0 mM L-Glutamine, 10% FBS) until confluent in 150cm tissue culture dishes at 37ºC, 5% CO_2.

3. Harvest cells by tyrpsination and seed in Corning 25850 polystyrene 96-well roundbottom cell plates, 25.000 cells/well in 200 uL of growth media.

4. Grow cells at least one day at 37° C., 5% CO_2.

5. Wash cells with D-PBS IX.

6. Add 200 ul/well of starvation media (DMEM, 2.0 mM 1-Glutamine, 0.1% FBS). Incubate overnight at 37° C., 5% CO_2.

7. Dilute Compound 1:20 in polyproplyene 96 well plates using starvation media. Dilute dimethylsulfoxide 1:20 for use in control wells.

8. Remove starvation media from 96 well cell culture plates and add 162 ul of fresh starvation media to each well.

9. Add 18 ul of 1:20 diluted Compound dilution (from step #7) to each well plus the 1:20 dimethylsulfoxide dilution to the control wells (+/- VEGF), for a final dilution of 1:200 after cell stimulation. Final dimethylsulfoxide is 0.5%. Incubate the plate at 37° C., 5% CO_2 for two hours.

10. Remove unbound antibody from Elisa plates by inverting plate to remove liquid. Wash 3 times with TBSW+ 0.5% Ethanolamine, pH 7.0. Pat the plate on a paper towel to remove excess liquid and bubbles.

11. Block plates with TBSW+0.5% Ethanolamine, pH 7.0. 150 ul per well. Incubate plate thirty minutes while shaking on a microtiter plate shaker.

12. Wash plate 3 times as described in step 10.

13. Add 0.5 ug/well affinity purified anti-flk-I polyclonal rabbit antiserum. Bring final volume to 150 μl/well with TBSW+0.5% Ethanolamine pH 7.0. Incubate plate for thirty minutes while shaking.

14. Add 180 ml starvation medium to the cells and stimulate cells with 20 ul/well 10.0 mM Sodium Ortho Vanadate and 500 ng/ml VEGF (resulting in a final concentration of 1.0 mM Sodium Ortho Vanadate and 50 ng/ml VEGF per well) for eight minutes at 37° C., 5% CO_2. Negative control wells receive only starvation medium.

15. After eight minutes, media are removed from the cells and washed one time with 200 ul/well PBS.

16. Lyse cells in 150 ul/well HNTG while shaking at room temperature for five minutes. HNTG formulation includes sodium ortho vanadate, sodium pyro phosphate and EDTA.

17. Wash Elisa plate three times as described in step 10.

18. Transfer cell lysates from the cell plate to elisa plate and incubate while shaking for two hours. To transfer cell lysate pipette up and down while scrapping the wells.

19. Wash plate three times as described in step 10.

20. Incubate Elisa plate with 0.02 ug/well UB40 in TBSW+05% ethanolamine. Bring final volume to 150 ul/well. Incubate while shaking for 30 minutes.

21. Wash plate three times as described in step 10.

22. Incubate elisa plate with 1:10,000 diluted EIA grade Goat anti-mouse IgG conjugated horseradish peroxidase in TBSW+0.5% ethanolamine, pH 7.0. Bring final volume to 150 ul/well. Incubate while shaking for thirty minutes.

23. Wash plate as described in step 10.

24. Add 100 ul of ABTS/H202 solution to well. Incubate ten minutes while shaking.

25. Add 100 ul of 0.2M MCTh for 0.1M MCL final to stop the color development reaction. Shake 1 minute at room temperature. Remove bubbles with slow stream of air and read the ELISA plate in an ELISA plate reader at 410 nm.

GROUP III—IN VITRO CELL GROWTH ASSAYS

EXAMPLE 8

Sulforhodamine B (SRB) Assay for Adherent Cells

Sulforhodamine B assays for measuring effects of TBST compounds on cell growth were based on procedures described by Skehan et al. J. Natl. Cancer Inst. 82:1107, 1990 incorporated herein by reference in its entirety, including any drawings. Unless otherwise stated the assays were carried out aseptically as follows:

Material & Methods

(1) Sulforhodamine B Sigma Catalog S-9012 Working solution: 0.4% Sulforhodamine B=4 gram/liter 1% Acetic Acid.

(2) Trichloroacetic Acid (TCA)—Fisher Catalog #A322 Working solution: 10% TCA=100 gram TCA+1 liter H_2O.

(3) Acetic Acid, Glacial- Fisher Catalog A38 Working solution: 1 Acetic Acid=10 ml Acetic Acid+990 ml H_2O.

(4) Tris, crystallized free base—Fisher Catalog 5BP152 Working solution: 10 mM tris=1.211 gram Trizma base/liter H_2O.

Procedure

(1) Aspirate growth media from 96 well plate containing control cells or cell treated with compounds, rinse wells 2 or 3 times with PBS and layer 200 μl cold 100 TCA onto each well. Fix cells for 60 minutes at 4° C.

(2) Discard TCA and rinse wells 5 times with distilled H_2O. Dry plate upside down on paper towel.

(3) Stain fixed cells for 10 minutes with 100 μl 0.4% SRB per well.

(4) Pour off SRB solution and rinse wells 5 times with 1% acetic acid.

(5) Dry plate upside down on paper towel.

(6) After wells are completely dry, solubilize dye with 100 μl 10 mM Tris base per well for 5–10 minutes on titer plate shaker.

(7) Read optical density at dual wavelength mode 570 nm and 630 nm on Dynatech ELISA plate reader, Model MR 5000.

EXAMPLE 9

Soft Aqar Assay Protocol

The soft agar assay is well known in the art as a method for measuring the effects of substances on cell growth. Unless otherwise stated the soft agar assays were carried out as follows:

Material & Reagents

(1) A Water bath set at 39° C. and another water bath at 37° C.

(2) 2X assay medium is comprised of 2X Dulbecco's 5Modified Eagle's Medium (DMEM) (Gibco Cat. # CA400-4ANO3) supplemented by the following:
20% Fetal Bovine Serum (FBS) 2 mM Sodium Pyruvate 4 mM Glut amine
20 mM HEPES
Non-essential Amino Acids (1:50 from lOOx stock)

(3) 1X assay medium made of IX DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM glutamine, 10 mM HEPES, non-essential amino acid (1:100 from lOOx stock)

(4) 1.6% SeaPlaque Agarose in autoclave bottle

(5) Sterile 35 mm Corning plates (FMC Bioproducts Cat. #50102)

(6) Sterile 5 ml glass pipets (individually wrapped)

(7) Sterile 15 ml and 50 ml conical centrifuge tubes

(8) Pipets and sterile tips

(9) Sterile microcentrifuge tubes

(10) Cells in T75 flasks: SKOV-3 (ACTT HTB77)

(11) 0.25% Trypsin solution (Gibco #25200-015)

Procedure for making the base layer:

(a) Have all the media warmed up in the 37° C. water bath.

(b) To make 1X of assay medium +0.8% agar: make a 1:2 (vol:vol) dilution of melted agar (cooled to 39° C.), with 2X assay medium.

(c) Keep all media with agar warm in the 39° C. water bath when not in use.

(d) Dispense 1 ml of 1X assay medium +0.8% agar into dishes and gently swirl plate to form a uniform base layer. Bubbles should be avoided.

(e) Refrigerate base layers to solidify (about 20 minutes). Base layers can be stored overnight in the refrigerator.

Procedure for collecting cells:

(a) Take out one flask per cell line from the incubator; aspirate off medium; wash once with PBS and aspirate off; add 3 ml of trypsin solution.

(b) After all cells dissociate from the flask, add 3 ml of 1X assay media to inhibit trypsin activity. Pipet the cells up and down, then transfer the suspension into a 15 ml tube.

(c) Determine the concentration of cells using a Coulter counter, and the viability by trypan blue exclusion.

(d) Take out the appropriate volume needed to seed 3300 viable cells per plate and dilute it to 1.5 ml with 1X assay medium.

Procedure for making the upper 0.4% agarose layer:

(a) Add TBST compounds at twice the desired final assay concentration; +1.5 ml of cell suspension in 1X assay medium 10% FBS; +1.5 ml of 1X assay medium +0.8% agarose: *Total=3.0 ml 1X media 10% FBS +0.4% agarose with 3300 viable cells/ml, with and without TBST compounds.

*(Made by 1:2 dilution of 2X media with 1.6% agar for the base layer procedure above.)

(b) Plate 1 ml of the Assay Mix onto the 1 ml base layer. The duplicates are plated from the 3 ml volume.

(c) Incubate the dishes for 2–3 weeks in a 100% humidified, 10% CO_2 incubator.

(d) Colonies that are 60 microns and larger are scored positive.

EXAMPLE 10

MCF-7 SRB Growth Assay

MCF-7 cells are seeded at 2000 cells/ well in a 96-well flat bottom plate in normal growth media, which was 10% FBS/RPMI supplemented with 2 mM Glutamine. The plate of cells is incubated for about 24 hours at 37° C. after which it receives an equal volume of compound dilution per well making the total volume per well 200 μl. The compound is prepared at 2 times the desired highest final concentration and serially diluted in the normal growth media in a 96-well round bottom plate and then transferred to plate of cells. DMSO serves as the vector control up to 0.2% as final concentration. The cells are then incubated at 37° C. in a humidified 5% CO_2 incubator.

Four days following dosing of compound, the media is discarded and 200 μl/well of ice-cold 10% TCA (Trichloroacetic Acid) is added to fix cells. After 60 minutes at 4° C., the TCA is discarded and the plate is rinsed 5 times with water. The plate is then air-dried and 100 μl/well of 0.4% SRB (Sulforhodamine B from Sigma) 20 in 1% Acetic Acid is added to stain cells for 10 minutes at room temperature. The SRB is discarded and the plate is rinsed 5 times with 1% Acetic Acid. After the plate is completely dried, 100 μl/well of 10 mM Tris-base is added to solubilize the dye. After 5 to 10 minutes, the plate is read on a Dynatech ELISA Plate Reader at dual wavelengths at 570 nm and 630 nm.

EXAMPLE 11

MCF-7/HER-2 SRB Growth Assay

The protocol is basically the same as that above (for the MCF-7 Growth Assay) except that immediately before the 30 compound is added, the normal growth media is removed and 0.5% FBS/RPMI supplemented with 2 mM Glutamine is added onto the cells. The compound is also prepared in this 0.5% serum media. The plate of cells is incubated for four days and developed as usual.

EXAMPLE 12

3T3 Growth Assay

The 3T3 growth assay was carried out as follows:

Materials and Reagents

(1) Dulbecco's Modified Eagle Medium (D-MEM), Gibco 511965-050;

(2) Calf serum, Gibco 16170-029;

(3) Trypsin-EDTA, Gibco 25200-056;

(4) Fetal Bovine Serum Certified, Gibco 16000-028;

(5) Dulbecco' 5 Phosphate-Buffered Saline (D-PBS), 10 Gibco 14190-029;

(6) Sulforhodamine B (SRB), Sigma 5-9012 0.4% SRB in 1% acetic acid;

(7) 10 mM Tris-base, Fisher BP152-5;

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(8) 10% TCA, Trichroloacetic acid, Fisher A322-500;

(9) 96-well flat bottom plate (sterile), Corning 08-757-155;

(10) 100 ml reagent reservoir 9 (sterile), Matrix Technologies Corporation, 8086;

(11) Sterile pipet tips, Fisher 21-197-8E;

(12) 50 ml sterile TBST tubes, Fisher 05-539-6.

Cell Lines

NIH3T3C7 cells in 10% CS+2 mM GLN DMEM
HER2C7 cells in 2% FBS+2 mM GLN DMEM

Procedures

(1) HER2C7 (engineered to express HER2) and NIH3T3C7 (as the control) cells are used for this assay. NIH3T3C7 cells are seeded at 2500 cells/well, 10 μl/well in 10% CS+2 mM GLN DMEM, in a 96 well plate; HER2C7 cells are seeded at 6000 cells/well, 100 μl/well in 2% FBS+2 mM GLN DMEM, in a 96 well plate. Cells are incubated at 37° C., 5% CO_2 overnight to allow for cell attachment to the plate;

(2) The TBST compound is added to the cells at day 2. The compounds are prepared in the appropriate growth medium (10% CS+2 mM) GLN DMEM for NIH3T3C7 cells; 2% FBS+2 mM GLN DMEM for HER2C7 cells) in a 96 well plate, and serially diluted. A total of 100 μl/well medium of the diluted compounds is added into the cells. The total volume of each well is 200 μl. Quadruplicates (wells) and 11 concentration points are applied to each compound tested.

(3) After the cells are treated with the compound for 4 days, the cells are washed with PBS and fixed with 200 μl/well ice-cold 10% TCA for one hour at 0°–5° C. condition.

(4) Remove TCA and rinse wells 5 times with deionized water. Dry plates upside down with paper towels. Stain cells with 0.4% SRB at 100 μl well for 10 minutes.

(5) Pour off SRB and rinse plate 5 times with 1% acetic acid. Dry plate completely.

(6) Solubilize the dye with 10 mM Tris-base at 100 μl/well for 10 minutes on a shaker.

(7) Read the plate at dual wavelengths at 570 nm and 630 nm on Dynatech Elisa plate reader.

EXAMPLE 13

HUV-EC-C Flk-1 assay

The HUV-EC-C Flk-1 assay can be performed as follows:

DAY 0

1. Wash and trypsinize HUV-EC-C cells (human umbilical vein endothelial cells, American Type Culture Collection; catalogue no. 1730-CRL). Wash with Dulbecco's phosphate-buffered saline (D-PBS; obtained from Gibco BRL; catalogue no. 14190-029) 2 times at about 1 ml/10 cm^2 of tissue culture flask. Trypsinize with 0.05% trypsin-EDTA in non-enzymatic cell dissociation solution (Sigma Chemical Company; catalogue no. C-1544). The 0.05% trypsin was made by diluting 0.25% trypsin/1 mM EDTA (Gibco; catalogue no. 25200-049) in the cell dissociation solution. Trypsinize with about 1 ml/25–30 cm^2 of tissue culture flask for about 5 minutes at 37° C. After cells have detached from the flask, add an equal volume of D-PBS and transfer to a 50 ml sterile centrifuge tube (Fisher Scientific; catalogue no. 05-539-6).

2. Wash the cells with about 35 ml D-PBS in the 50 ml sterile centrifuge tube by adding the D-PBS, centrifuge for 10 minutes at approximately 200×g, aspirate the supernatant, and resuspend with 35 ml D-PBS. Repeat the wash two more times, resuspend the cells in about 1 ml assay medium/15 cm^2 of tissue culture flask. Assay medium consists of F12K medium (Gibco BRL; catalogue no. 21127-014)+0.5% heat-inactivated fetal bovine serum. Count the cells with a Coulter Counter$^{(R)}$(Coulter Electronics, Inc.) and add assay medium to the cells to obtain a concentration of 0.8–1.0×10^5 cells/ml.

3. Add cells to 96-well flat-bottom plates at 100 ul/well or 0.8–10.×10^4 cells/well; incubate ~24 h at 37° C., 5% CO_2.

DAY 1

1. Make up two-fold drug titrations in separate 96-well plates, generally 50 uM on down to 0 uM. Use the same assay medium as mentioned in day 0, step 2 above. Titrations are made by adding 120 ul/well of drug at 200 uM (4X the final well concentration) to the top well of a particular plate column. Since the stock drug concentration is 10 mM and in 100% DMSO, the 200 uM drug concentration is 0.5% DMSO. Therefore, diluent made up of 0.5% DMSO in assay medium (F12K+0.5% fetal bovine serum) is used as diluent for the drug titrations in order to dilute the drug but keep the DMSO concentration constant. Add this diluent to the remaining wells in the column at 60 ul/well. take 60 ul from the 120 ul of 200 uM drug dilution in the top well of the column and mix with the 60 ul in the second well of the column. Take 60 ul from this well and mix with the 60 ul in the third well of the column, and so on until two-fold titrations are completed. When the next-to-the-last well is mixed, take 60 ul of the 120 ul in this well and discard it. Leave the last well with 60 ul of DMSO/media diluent as a non-drug-containing control. Make 9 columns of titrated drug, enough for triplicate wells each for 1) vascular endothelial growth factor (VEGF; obtained from Pepro Tech Inc., catalogue no. 100-20), 2) endothelial cell growth factor (ECGF; also known as acidic fibroblast growth factor, or aFGF; obtained from Boehringer Mannheim Biochemica, catalogue no. 1439 600), and assay media control. ECGF comes as a preparation with sodium heparin.

2. Transfer 50 ul/well of the drug dilutions to the 96-well assay plates containing the 0.8–1.0×10^4 cells/100 ul/well of the HUV-EC-C cells from day 0 and incubate ~2 h at 37° C., 5% CO_2.

3. In triplicate, add 50 ul/well of 80 ug/ml VEGF, 20 ng/ml ECGF, or media control to each drug condition. As with the drugs, the growth factor concentrations are 4X the desired final concentration. Use the assay media from day 0 step 2 to make the concentrations of growth factors. Incubate ~24 h at 37° C., 5% CO_2. Each well will have 50 ul drug dilution, 50 ul growth factor or media, and 100 ul cells, =200 ul/well total. Thus the 4X concentrations of drugs and growth factors become 1X once everything has been added to the wells.

DAY 2

1. Add ^{3}H-thymidine (Amersham; catalogue no. TRK-686) at 1 uCi/well (10 ul/well of 100 uCi/ml solution made up in RPMI media+10% heat-inactivated fetal bovine serum) and incubate ~24 h at 37° C., 5% CO_2. Note: ^{3}H-thymidine is made up in RPMI media because all of the other applications for which we use the ^{3}H-thymidine involve experiments done in RPMI. The media difference at this step is probably not significant. RPMI was obtained from Gibco BRL, catalogue no. 11875-051.

DAY 3

1. Freeze plates overnight at 20° C.

DAY 4

1. Thaw plates and harvest with a 96-well plate harvester (Tomtec Harvester 96(R)) onto filter mats (Wallac; catalogue no. 1205-401); read counts on a Wallac Betaplate(TM) liquid scintillation counter.

EXAMPLE 14

IGF-1 Receptor Growth Assay

Screen III

Cell lines: 3T3/IGF-1 R (10% FBS/2 mM glutamine/DMEM) NIH 3T3 c7 (10% calf serum/2 mM glutamine/DMEM) NOTE: NIH 3T3 cells (and cells derived from them) should never be allowed to become confluent because this increases the chance of spontaneous transformation. If they show signs of being transformed (morphological changes, piling up, moving into clumps), throw them away and thaw a fresh batch.

Materials: 10% FBS/2 mM glutamine/DMEM 0.5% FBS/2 mM glutamine/DMEM 10% calf serum/2 mM glutamine/DMEM IGF-1, 5 μM in sterile PBS (Promega/Fisher cat. #G5111) DMSO, tissue culture grade (Sigma cat. #D 2650) Hits from screen 11, 100 mM in DMSO 96-well plates, flat and round bottom 8 channel pipettor and sterile tips sterile reagent reservoirs sterile tubes (1.5 or 15 ml)

Methods (carry all steps out under asceptic conditions *until fixing the cells for the SRB assay*):

Day 0: Cell Plating—Trypsinize and count 3T3/IGF-1R and NIH 3T3 c7 cells. Dilute in growth media to 2000 cells/200 μl and seed flat bottom 96-well plates with 200 μl/well, one plate for two compounds for each cell line.

Day 1: Compound preparation—Add DMSO to each compound to make 100 mM stock solutions. If a compound *does not go into solution with vortexing, add extra DMSO* to make 50 mM or less, as required.

Aliquot each compound to 3–4 sterile screw cap tubes and store at −20° C. After thawing, make sure the compound has gone completely back into solution. Throw away after 3 freeze/thaws.

3T3/IGF-1R cells—For each 96-well plate, make 15 ml of 10 nM IGF-1/0.5% FBS/2 mM glutamine/DMEM (30 μl of 5 μM IGF-1/15 ml).

Aliquot 1.5 ml 10 nM IGF-1/0.5% FBS to a sterile tube for each compound to be tested (the first time a compound is tested, use a 15 ml tube in case it is necessary to add extra medium to get it into solution).

Add 3 μl of 100 mM stock of each compound to a tube so 200 μM final. Shake or vortex until it goes into solution. If it is necessary to add additional medium, note the final concentration.

For the DMSO control, prepare 0.5 ml/plate of 0.2% DMSO/10 nM IGF-1/0.5% FBS (2 μl DMSO/ml).

For every two compounds, aliquot 130 μl 10 nM IGF-1/0.5% FBS to wells in columns 2–11 of a 96-well round bottom plate.

Add 260 μl of each compound to four wells in column 12.

Do 2-fold dilutions (130 μl) from columns 12 to 3 on each plate (column 2 will be for the untreated control), mixing thoroughly.

Remove medium from 3T3/IGF-1 R cells, one plate at a time.

Transfer 120 μl from each well on a compound dilution *plate to the corresponding well of cells.*

Add 120 μl 0.2% DMSO/10 nM IGF-1/0.5% FBS to four wells in column 1.

Add 120 μl 0.5% FBS (without IGF-1) to other four wells in column 1 for negative control.

NIH 3T3 c7 cells—Carry out the same steps as for 3T3/IGF-1 R cells except use 10% calf serum instead of 0.5% FBS and do not include IGF-1.

Day 4: Refeed—Repeat steps above, adding exactly the same IGF-1 and compound concentration to each well as before.

Day 6: Analysis of cells—Look at wells with the highest concentrations for each compound to make sure it has not precipitated. If so, mark wells and do not use for data calculations.

Also scan plates to make sure none of the wells are contaminated. If so, mark wells and do not use for data calculations.

Detection—Follow the steps for fixing and staining described in the SOP for SRB Assays.

Whenever: Data analysis—Find averages and standard deviations for each set of four OD's.

Using wells in column 2 (treated with IGF-1 but not compound) as 100%, calculate percent of control for each concentration of compound.

Determine the fold difference between the IGF-1-treated and untreated cells. It should be 2–3-fold.

Determine the percent of control for 0.2% DMSO. If it is less than 95%, do not use the highest compound concentration to calculate the IC_{50} value.

Use a curve fit method to graph percent of control vs. log(molar concentration) and determine the IC_{50}.

GROUP IV—IN VIVO

EXAMPLE 15

VEGF pellet model

Basic Procedures

Theory—VEGF packaged into a time-release pellet and implanted subcutaneously on the abdomen of nude mice. This implant induces a 'reddening' response and subsequent swelling around the pellet. The objective of these studies is to implant Flk-1 inhibitors in methylcellulose near the VEGF pellet in an attempt to inhibit the 'reddening' response and subsequent swelling.

Materials—

VEGF—human., recombinant, lyophilized (Peprotech, Inc., Princeton Business Park, G2; P.O. box 275, Rocky Hill, N.J. 08553)

VEGF Packaged into 21 day release pellets by Innovative Research of America, using patented matrix driven delivery system. Pellets packaged at 0.20, 0.21, or2.1 ug VEGF/pellet. These doses approximate 10 and 100 ng/day release of VEGF. (Innovative Research of America, 3361 Executive Parkway, P.O. box 2746, Toledo, Ohio 43606)

Methylcellulose

Water (sterile)

Methanol

Appropriate drugs/inhibitors

10 cm culture plates

parafilm

Methods—

VEGF purchased from Peprotech and sent to Innovative Research for Custom Pellet preparation.

Methylcellulose prepared at 1.5% (w/v) in sterile water

Drugs solublized in methanol (usual concentration range= 10 to 20 mg/ml)

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Place sterile parafilm in sterile 10 cm plates

150 ul of drug in methanol added to 1.35 ml of 1.5% methylcellulose and mixed/vortexed thoroughly.

25 ul aliquots of homogenate placed on parafilm and dried into discs.

Mice (6–10 wk. Balb/C athymic nu/nu, female) anesthetized via isoflurane inhalation. VEGF pellets and methylcellulose discs implanted subcutaneously on the abdomen.

Mice scored at 24 hours and 48 hours for reddening and swelling response.

Experimental Design

N=4 animals/group

Controls—VEGF pellet+drug placebo VEGF placebo+drug pellet

The examples provided herein describe experiments that indicate the compounds of the present invention are useful in inhibiting certain in vitro activities of receptors and other signalling molecules associated with cell proliferative and cell differentiation disorders. Animal model systems can also be used to further measure the therapeutic effect of a compound. Examples of suitable animal models include subcutaneous xenograft model and in situ mammary fat pad model. Another suitable animal model described herein is the VEGF pellet model.

EXAMPLE 16

Xenoraft Model

The ability of human tumors to grow as xenografts in athymic mice (e.g., Balb/c, nu/nu) provides a useful in vivo model for studying the biological response to therapies for human tumors. Since the first successful xenotransplantation of human tumors into athymic mice by Rygaard and Povisen (Rygaard, J. and Povisen, C.O., *Acta Pathol. Microbial. Scand.,* 77:758–760, 1969.), many different human tumor cell lines (e.g., mammary, lung, genitourinary, gastrointestinal, head and neck, glioblastoma, bone, and malignant melanomas) have been transplanted and successfully grown in nude mice. Human mammary tumor cell lines, including MCF-7, ZR75-1, and MDA-MB-231, have been established as subcutaneous xenografts in nude mice (Warri, A.M., et al, *Int. J. Cancer,* 49:616–623,1991; Ozzello, L. and Sordat, M., *Eur. J. Cancer,* 16:553–559, 1980; Osborne, C. K., et al, *Cancer* 25 Res., 45:584–590, 1985; Seibert, K., et al, *Cancer Res.,* 43:2223–2239, 1983).

To study the effect of anti-tumor drug candidates on HER2 expressing tumors, the tumor cells should be able to grow in the absence of supplemental estrogen. Many mammary cell lines are dependent on estrogen for in vivo growth in nude mice (Osborne et al., supra), however, exogenous estrogen suppresses her2 expression in nude mice (Warri et al., supra, Dati, C., et al, *Oncogene,* 5:1001–1006, 1990) . For example, in the presence of estrogen, MCF-7, ZR-75-1, and T47D cells grow well in vivo, but express very low levels of HER2 (Warri et al., supra, Dati, C., et al, *Oncogene,* 5:1001–1006).

The following type of xenograft protocol can be used: (1) implant tumor cells (subcutaneously) into the hindflank of five- to six-week-old female Balb/c nu/nu athymic mice; (2) administer the anti-tumor compound; (3) measure tumor growth by measuring tumor volume. The tumors can also be analyzed for the presence of a receptor, such as HER2, EGF or PDGF, by Western and immunohistochemical analyses. Using techniques known in the art, one skilled in the art can vary the above procedures, for example through the use of different treatment regimes.

EXAMPLE 17

Mammary Fat Pad Model

The mammary fat pad model is particularly useful for measuring the efficacy of compounds which inhibit HER2, because of the role HER2 plays in breast cancer. By implanting tumor cells directly into the location of interest, in situ models more accurately reflect the biology of tumor development than do subcutaneous models. Human mammary cell lines, including MCF-7, have been grown in the mammary fat pad of athymic mice (Shafie, S. M. and Grantham, F. H., J. Natl. Cancer Instit., 67:51–56, 1981; Gottardis, M. M., et al, J. *Steroid Biochem.,* 30:311–314, (1988). For example the following procedure can be used: (1) MDA-MB-231 and MOF-7 cells transfected with her2 are implanted at various concentrations into the axillary mammary fat pads of female athymic mice; (2) the compound is administered; and (3) tumor growth is measured at various time points. The tumors can also be analyzed for the presence of a receptor such as HER2, by Western and immunohistochemical analyses. Using techniques known in the art, one skilled in the art can vary the above procedures, for example through the use of different treatment regimes.

EXAMPLE 18

Toxicity

Therapeutic compounds should be more potent in inhibiting receptor tyrosine kinase activity than in exerting a cytotoxic effect. A measure of the effectiveness and cell toxicity of a compound can be obtained by determining the therapeutic index: IC_{50}/LD_{50}. IC_{50}, the dose required to achieve 50% inhibition, can be measured using standard techniques such as those described herein. LD_{50}, the dosage which results in 50% toxicity, can also be measured by standard techniques, such as using an MTT assay as described by Mossman J. Immunol. Methods 65:55–63 (1983), by measuring the amount of LDH released (Korzeniewski and Callewaert, *J. inimunol. Methods* 64:313 (1983); Decker and Lohmann-Matthes, *J. Immunol. Methods* 115:61 (1988), or by measuring the lethal dose in animal models. Compounds with a large therapeutic index are preferred. The therapeutic index should be greater than 2, preferably at least 10, more preferably at least 50.

In addition to measuring tumor growth to achieve a compound range which can safely be administered to a patient in the animal models, plasma half-life and biodistribution of the drug and metabolites in plasma, tumors, and major organs can be determined to facilitate the selection of drugs most appropriate for the inhibition of a disorder. Such measurements can be carried out, for example, using HPLC analysis. Compounds that show potent inhibitory activity in the screening assays, but have poor pharmacokinetic characteristics, can be optimized by altering the chemical structure and retesting. In this regard, compounds displaying good pharmacokinetic characteristics can be used as model.

Toxicity studies can also be carried out by measuring the blood cell composition. For example, toxicity studies can be carried out as follows: (1) the compound is administered to mice (an untreated control mouse should also be used); (2) blood samples are periodically obtained via the tail vein from one mouse in each treatment group; and 3) the samples are analyzed for red and white blood

TABLE 1

ELISA DATA

Compound #	IGF-1R	IR	EGFR	PDGFR	HER2 - (BT474)	HER2 - (3T3)	FLK-1 - (Cell.)	FLK-1
Z3	16.5	>100	69.5	>100	9.9	>100		
Z2	>100	>100		>50	3.2	31.7	>100	
Z1	>100	>100	>100	>50	11.8	10.3	1	>100
Z4	>100		>100	>100	9.3	>100		

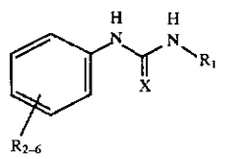
TABLE 2

GROWTH DATA

Compound#	3T3/IGF -1R-0.5% FBS	3T3/IGF -1R-10% FBS	3T3/HE R2 2% FBS	3T3/HE R2 10% FBS	MCF7-HER2 0.5% FBS	MCF7 10% FBS	HUV-EC-C/VEGF	HUV-EC-C/aFGF
Z3	0.6	18	0.35	18	0.2	2	nt	nt
Z2	0.7	20	0.6	18	0.3	1	nt	nt
Z1	nt	nt	1.0	15	0.9	5	2.9	30
Z4	nt	nt			1	3	nt	nt

What is claimed:

1. A method for treating a cell proliferative or cell differentiation disorder by inhibiting a tyrosine kinase associated with said disorder, wherein said disorder is selected from the group consisting of a cancer, restenosis, retinopathy, psoriasis, hyperimmune response, hepatic cirrhosis, a mesangial cell proliferative disorder, a HER2 disorder, an EGFR disorder, an IGFR disorder, a PDGFR disorder, a met disorder, a Src disorder, and a KDR/Flk-1 disorder, comprising the step of administering a therapeutically effective amount of a compound of the formula



or a pharmaceutically acceptable salt thereof to a patient in need of such treatment, wherein;

X is oxygen or sulfur

R_1 is selected from the group consisting of optionally substituted aryl, alkylaryl, and heteroaryl and

R_{2-6} are independently selected rom the group consisting of hydroxy, hydrogen, alkyl, alkoxy, cyano, nitro, halo trihalomethyl, amide, carboxamide, sulfonyl, and sulfoxamide.

2. A compound selected from the group consisting of

N-[3-Chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-(4-t-butylphenyl)urea,

N-[3-Chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-(3,5-ditrifluoromethylphenyl)urea,

N-[2-(2,4-Dichlorophenoxy)pyrid-5-yl]-N'-[4-trifluoromethylmercapto)phenyl]urea,

N-(4-Cyanophenyl)-N'-[4-[(piperid-1-yl)sulfonyl]phenyl] thiourea,

N-(4-t-Butylphenyl)-N'-[3-chloro-4-(isopropylsulfonyl) thien-2-yl]urea,

N-[3-chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-(4-trifluoromethylphenyl)urea,

N-[3-Chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-4-trifluoromethylphenyl)thiourea,

N-[3-Chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-[4-(piperid-1-yl)sulfonylphenyl]thiourea,

N-[3-Chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-[4-phenylaminosulfonylphenyl]thiourea,

N-[4-(4-Chlorophenylsulfonyl)thien-3-yl]-N'-[4-(piperid-1-yl)sulfonylphenyl]thiourea,

N-[4-(4-Chlorophenylsulfonyl)thien-3-yl}-N'-(4-trifluoromethylphenyl)thiourea,

N-(5-Nitrothiazol-2-yl)-N'-(4-trifluoromethylphenyl)urea,

N- (5-Nitrothiazol-2-yl)-N'-(4-trifluoromethylphenyl) thiourea,

N-(5-Nitrothiazol-2-yl)-N'-[4-(phenylaminosulfonyl) phenyl]thiourea,

N-(4-t-Butylphenyl)-N'-(3-ethoxycarbonyl-4-methylthien-2-yl)urea,

N-(3-Ethoxycarbonyl-4-methylthien-2-yl)-N'-(4-trifluoromethylphenyl)thiourea,

N-(3-Ethoxycarbonyl-4-methylthien-2-yl)-N'-[4-[(piperid-1-yl)sulfonyl]phenyl]thiourea,

N-(4-t-Butylphenyl)-N'-(2,4-dinitrothien-5-yl)urea,

N-(4-t-Butylphenyl)-N'-(3-cyano-5-methoxycarbonyl-4-methylthien-2-yl)urea,

N-(3-Cyano-5-methoxycarbonyl-4-methylthien-2-yl)-N'-(4-trifluoromethylphenyl)urea,

N-(3-Cyano-5-methoxycarbonyl-4-methylthien-2-yl)N'-[4-(phenylaminosulfonyl)phenyl]thiourea,

N-(3-Methylthien-2-yl)-N'-(4-trifluoromethylphenyl) thiourea,

N-(3-Methylthien-2-yl)-N'-[4-[(piperid-1-yl)sulfonyl] phenyl]thiourea,

N-(4-t-Butylphenyl)-N'-[4-(4-chlorophenyl)-3-cyanopyrrol-2-yl]urea,

N-[4-(4-Chlorophenyl)-3-cyanotpyrrol-2-yl]-N'-(4-trifluoromethylphenyl)urea,

N-[4-(4-Chlorophenyl)-3-cyanopyrrol-2-yl]-N'-[4-[(piperid-1-yl)sulfonyl]phenyl]thiourea,

N-(4-t-Butylphenyl)-N'-[4-(isopropylsulfonyl)thien-2-yl] urea,

N-[4-(Isopropylsulfonyl)thien-2-yl]-N'-4-trifluoromethylphenyl]thiourea,

N-[4-(Isopropylsulfonyl)thien-2-yl]-N'-[4-[(piperid-1-yl) sulfonyl]phenyl]thiourea,

N-(4-t-Butylphenyl)-N'-[4-[4-(trifluoromethyl) phenylaminosulfonyl]thien-2-yl]urea,

N-[4-[(Piperid-1-yl)sulfonyl]phenyl]-N'-[4-[4-(trifluoromethyl)phenylaminosulfonyl]thien-2-yl] thiourea,

N-(3-Aminocarbonyl-4-methylthien-2-yl)-N'-(4-trifluoromethylphenyl)thiourea,

N-[4-[(Piperid-1-yl)sulfonyl]phenyl]-N'-4-trifluoromethylphenyl)thiourea,

N-[4-[(Piperid-1-yl)sulfonyl]phenyl]-N'-[4-(4-trifluoromethylbenzyl]thiourea,

N-[2-(2,4-dichlorophenoxy)pyrid-5-yl]-N'-(4-trifluoromethylphenyl)thiourea.

3. A pharmaceutical composition comprising a compound of claim **2** and a pharmaceutically acceptable carrier.

4. The method of claim **1**, the compound of claim **2**, or the composition of claim **3**, wherein said compound is N-[3-Chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-(4-t-butylphenyl)urea.

5. The method of claim **1**, the compound of claim **2**, or the composition of claim **3**, wherein said compound is N-[3-Chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-(3,5-di-trifluoromethylphenyl)urea.

6. The method of claim **1**, the compound of claim **2**, or the composition of claim **3**, wherein said compound is N-[2-(2,4-Dichlorophenoxy)pyrid-5-yl]-N'-[4-trifluoromethylmercapto)phenyl]urea.

7. The method of claim **1**, the compound of claim **2**, or the composition of claim **3**, wherein said compound is N-(4-Cyanophenyl)-N'-[4-[(piperid-1-yl)sulfonyl]phenyl] thiourea.

8. The method of claim **1**, the compound of claim **2**, or the composition of claim **3**, wherein said compound is selected from the group consisting of:

N-(4-t-Butylphenyl)-N'-[3-chloro-4-(isopropylsulfonyl) thien-2-yl]urea;

N-[3-chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-(4-trifluoromethylphenyl)urea;

N-[3-Chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-4-trifluoromethylphenyl)thiourea;

N-[3-Chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-[4-(piperid-1-yl)sulfonylphenyl]thiourea.

9. The method of claim **1**, the compound of claim **2**, or the composition of claim **3**, wherein said compound is selected from the group consisting of:

N-[3-Chloro-4-(isopropylsulfonyl)thien-2-yl]-N'-[4-phenylaminosulfonylphenyl]thiourea;

N-[4-(4-Chlorophenylsulfonyl)thien-3-yl]-N'-[4-(piperid-1-yl)sulfonylphenyl]thiourea;

N-[4-(4-Chlorophenylsulfonyl)thien-3-yl}-N'-(4-trifluoromethylphenyl)thiourea;

N-(5-Nitrothiazol-2-yl)-N'-(4-trifluoromethylphenyl)urea;

N-(5-Nitrothiazol-2-yl)-N'-(4-trifluoromethylphenyl) thiourea; and

N-(5-Nitrothiazol-2-yl)-N'-[4-(phenylaminosulfonyl) phenyl]thiourea.

10. The method of claim **1**, the compound of claim **2**, or the composition of claim **3**, wherein said compound is selected from the group consisting of:

N-(4-t-Butylphenyl)-N'-(3-ethoxycarbonyl-4-methylthien-2-yl)urea;

N-(3-Ethoxycarbonyl-4-methylthien-2-yl)-N'-(4-trifluoromethylphenyl)thiourea;

N-(3-Ethoxycarbonyl-4-methylthien-2-yl)-N'-[4-[(piperid-1-yl)sulfonyl]phenyl]thiourea;

N-(4-t-Butylphenyl)-N'-(2,4-dinitrothien-5-yl)urea;

N-(4-t-Butylphenyl)-N'-(3-cyano-5-methoxycarbonyl-4-methylthien-2-yl)urea;

N-(3-Cyano-5-methoxycarbonyl-4-methylthien-2-yl)-N'-(4-trifluoromethylphenyl)urea;

N-(3-Cyano-5-methoxycarbonyl-4-methylthien-2-yl)N'-[4-(phenylaminosulfonyl)phenyl]thiourea; and

N-(3-Methylthien-2-yl)-N'-(4-trifluoromethylphenyl) thiourea.

11. The method of claim **1**, the compound of claim **2**, or the composition of claim **3**, wherein said compound is selected from the group consisting of:

N-(3-Methylthien-2-yl)-N'-[4-[(piperid-1-yl)sulfonyl] phenyl]thiourea;

N-(4-t-Butylphenyl)-N'-[4-(4-chlorophenyl)-3-cyanopyrrol-2-yl]urea;

N-[4-(4-Chlorophenyl)-3-cyanotpyrrol-2-yl]-N'-(4-trifluoromethylphenyl)urea;

N-[4-(4-Chlorophenyl)-3-cyanopyrrol-2-yl]-N'-[4-[(piperid-1-yl)sulfonyl]phenyl]thiourea;

N-(4-t-Butylphenyl)-N'-[4-(isopropylsulfonyl)thien-2-yl] urea; and

N-[4-(Isopropylsulfonyl)thien-2-yl]-N'-4-trifluoromethylphenyl]thiourea.

12. The method of claim **1**, the compound of claim **2**, or the composition of claim **3**, wherein said compound is selected from the group consisting of:

N-[4-(Isopropylsulfonyl)thien-2-yl]-N'-[4-[(piperid-1-yl) sulfonyl]phenyl]thiourea;

N-(4-t-Butylphenyl)-N'-[4-[4-(trifluoromethyl) phenylaminosulfonyl]thien-2-yl]urea;

N-[4-[(Piperid-1-yl)sulfonyl]phenyl]-N'-[4-[4-(trifluoromethyl)phenylaminosulfonyl]thien-2-yl] thiourea;

N-(3-Aminocarbonyl-4-methylthien-2-yl)-N'-(4-trifluoromethylphenyl)thiourea;

N-[4-[(Piperid-1-yl)sulfonyl]phenyl]-N'-4-trifluoromethylphenyl)thiourea;

N-[4-[(Piperid-1-yl)sulfonyl]phenyl]-N'-[4-(4-trifluoromethylbenzyl]thiourea; and

N-[2-(2,4-dichlorophenoxy)pyrid-5-yl]-N'-(4-trifluoromethylphenyl)thiourea.

13. The method of claim **1**, wherein said disorder is a cancer.

14. The method of claim **1**, wherein said disorder is a HER2 disorder.

15. The method of claim **1**, wherein said disorder is an EGFR disorder.

16. The method of claim **1**, wherein said disorder is an IGFR disorder.

17. The method of claim **1**, wherein said disorder is a PDGFR disorder.

18. The method of claim **1**, wherein said disorder is a KDR/Flk-1 disorder.

* * * * *

Exhibit 2

46

United States Patent [19]

Kondo et al.

[11] **Patent Number: 4,904,668**

[45] **Date of Patent: Feb. 27, 1990**

[54] **BENZOYL UREA COMPOUND**

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[73] Assignees: **Ishihara Sangyo Kaisha Ltd.**; **The Green Cross Corporation**, both of Osaka, Japan

[21] Appl. No.: **101,005**

[22] Filed: **Sep. 25, 1987**

[30] **Foreign Application Priority Data**

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Jun. 30, 1987 [JP] Japan 62-164496

[51] **Int. Cl.**[4] **C07D 239/34**; A61K 31/505

[52] **U.S. Cl.** **514/274**; 544/316

[58] **Field of Search** 544/316; 514/274

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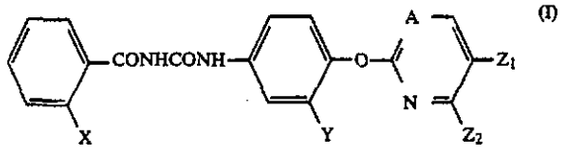
Primary Examiner—John M. Ford

Attorney, Agent, or Firm—Oblon, Spivak, McClelland, Maier & Neustadt

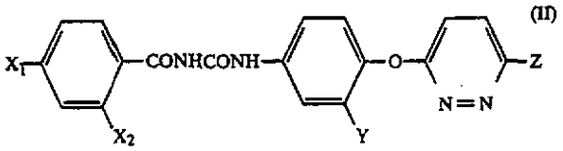
[57] **ABSTRACT**

A benzoyl urea compound having the formula:



(I)

wherein X is a halogen atom, a nitro group or a trifluoromethyl group, provided that when Y is a nitro group, X is a halogen atom or a nitro group, Y is a hydrogen atom, a halogen atom, a nitro group or a trifluoromethyl group, Z_1 is a halogen atom or a trifluoromethyl group, Z_2 is a hydrogen atom or a halogen atom, and A is a =CH— group or a nitrogen atom, or the formula:



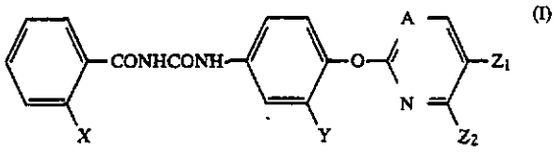
(II)

wherein each of X_1 is X_2 is a hydrogen atom, a halogen atom or a nitro group, provided that when Y is a nitro group, X_1 is a hydrogen atom, Y is a hydrogen atom, a halogen atom, a nitro group or a trifluoromethyl group, and Z is a hydrogen atom, a halogen atom or a trifluoromethyl group, characterized in that its average particle size is not larger than 1 μm.

9 Claims, No Drawings

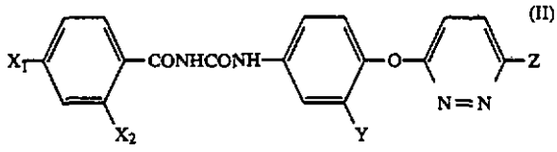
BENZOYL UREA COMPOUND

The present invention relates to a benzoyl urea compound represented by the following formula I or II (hereinafter referred to as a benzoyl urea compound (I) or (II)) with its average particle size being not larger than 1 μm. More particularly, the present invention relates to an antitumour benzoyl urea compound having the formula:



(I)

wherein X is a halogen atom, a nitro group or a trifluoromethyl group, provided that when Y is a nitro group, X is a halogen atom or a nitro group, Y is a hydrogen atom, a halogen atom, a nitro group or a trifluoromethyl group, Z_1 is a halogen atom or a trifluoromethyl group, Z_2 is a hydrogen atom or a halogen atom, and A is a =CH— group or a nitrogen atom, or the formula:



(II)

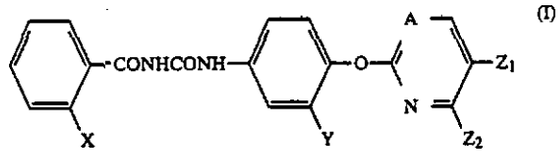
wherein each of X_1 and X_2 is a hydrogen atom, a halogen atom or a nitro group, provided that when Y is a nitro group, X_1 is a hydrogen atom, Y is a hydrogen atom, a halogen atom, a nitro group or a trifluoromethyl group, and Z is a hydrogen atom, a halogen atom or a trifluoromethyl group, having an improved absorbability through the gut.

The benzoyl urea compound (I) or (II) is substantially known to have excellent antitumour activities (Japanese Unexamined Patent Publications No. 109721/1982, No. 1670/1986, No. 33176/1986, No. 93163/1986, No. 5959/1987 and No. 116566/1987). However, this compound is hardly soluble in water, and its absorbablity through e.g. the gut is poor. Therefore, in order to obtain adequate antitumour activities, it is necessary to increase the dose, whereby there is a possible danger of adverse effects due to the excessive admistration.

It is an object of the present invention to provide a benzoyl urea compound (I) or (II) having an improved absorbability through the gut.

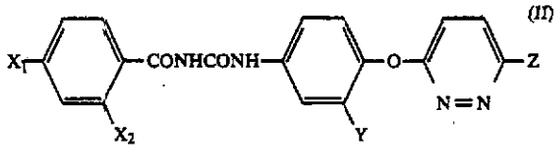
The present inventors have conducted extensive research with an aim to improve the absorbability of the benzoyl urea compound (I) or (II) through the gut, and as a result, have found it possible to increase the absorbability of the benzoyl urea compound (I) or (II) through the gut by adjusting the average particle size of the benzoyl urea compound (I) or (II) to a level of not larger than 1 μm. The present invention has been accomplished on the basis of this discovery.

Namely, the present invention provides a benzoyl urea compound having the formula:



(I)

wherein X is a halogen atom, a nitro group or a trifluoromethyl group, provided that when Y is a nitro group, X is a halogen atom or a nitro group, Y is a hydrogen atom, a halogen atom, a nitro group or a trifluoromethyl group, Z_1 is a halogen atom or a trifluoromethyl group, Z_2 is a hydrogen atom or a halogen atom, and A is a =CH— group or a nitrogen atom, or the formula:



(II)

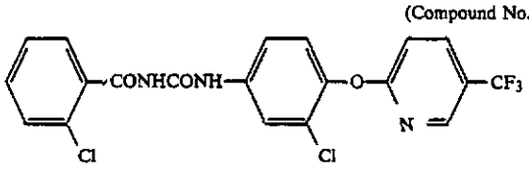
wherein each of X_1 and X_2 is a hydrogen atom, a halogen atom or a nitro group, provided that when Y is a nitro group, X_1 is a hydrogen atom, Y is a hydrogen atom, a halogen atom, a nitro group or a trifluoromethyl group, and Z is a hydrogen atom, a halogen atom or a trifluoromethyl group, characterized in that its average particle size is not larger than 1 μm.

Now, the present invention will be described in detail with reference to the preferred embodiments.

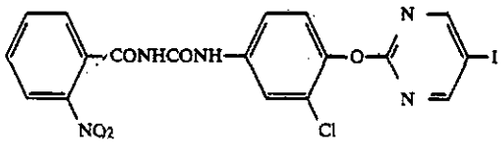
In this specification, the halogen atom is preferably a chlorine atom or a bromine atom.

The following compounds may be mentioned as typical examples of the benzoyl urea compound (I) or (II).

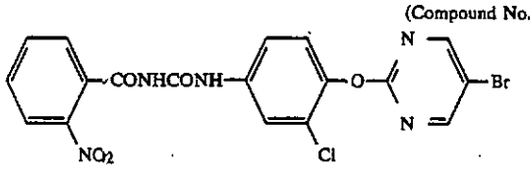
(Compound No. 1)



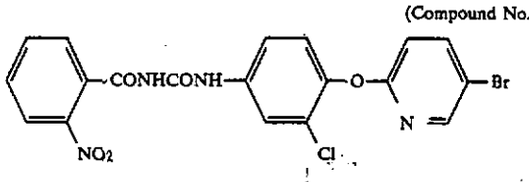
(Compound No. 2)



(Compound No. 3)

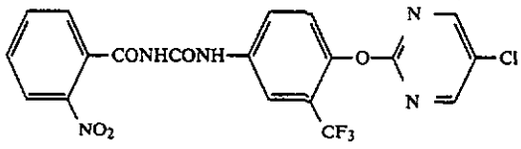


(Compound No. 4)

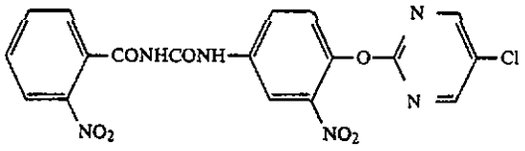


-continued

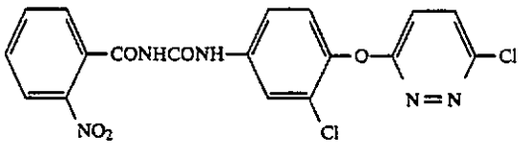
(Compound No. 5)



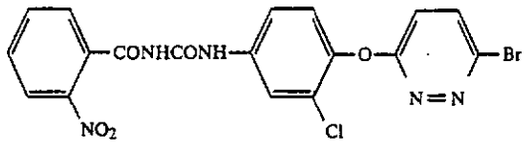
(Compound No. 6)



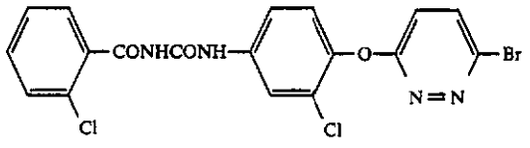
(Compound No. 7)



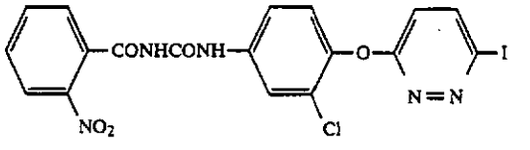
(Compound No. 8)



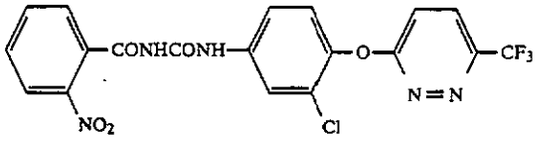
(Compound No. 9)



(Compound No. 10)



(Compound No. 11)



The benzoyl urea compound (I) or (II) is substantially a known compound, and it may be prepared by a method disclosed in e.g. Japanese Unexamined Patent Publication No. 109721/1982, No. 1670/1986, No. 33176/1986, No. 93163/1986, No. 227572/1986, No. 5959/1987, No. 116566/1987 or No. 135463/1987 or by a similar method.

In the present invention, the benzoyl urea compound is in the form of fine particles having an average particle size of not larger than 1 μm, preferably from 0.2 to 1 μm, more preferably from 0.3 to 0.8 μm. If the average particle size exceeds 1 μm, the absorbability through the gut tends to deteriorate.

The benzoyl urea compound having the specified particle size of the present invention can be prepared, for instance, by pulverizing the benzoyl urea compound (I) or (II) in an aqueous solution containing a dispersant such as a nonionic surfactant. There is no particular restriction as to the nonionic surfactant to be used for this purpose. Any nonionic surfactant may be employed so long as it is useful as an additive for pharmaceuticals. Its HLB value (Hydrophile-Lipophile Balance) is preferably at least 3. Specific examples of such nonionic surfactants include polyoxyethylene hardened caster oil 20, polyoxyethylene hardened caster oil 40, polyoxyethylene hardened caster oil 60, polyoxyethylene hardened caster oil 100, polysorbate 60, polysorbate 65, polysorbate 80, polyoxyethylene polyoxypropylene glycol, a sucrose fatty acid ester, a glycerol fatty acid ester, a sorbitan fatty acid ester, a propylene glycol fatty acid ester, a polyglycerol fatty acid ester, a polyoxyethylene sorbitan fatty acid ester, a polyoxyethylene sorbitol fatty acid ester, a polyoxyethylene glycerol fatty acid ester, a polyethylene glycol fatty acid ester and a polyoxyethylene castor oil.

The pulverization is preferably conducted by wet pulverization. The wet pulverization is a method wherein the material to be pulverized is rotated or shaken together with beads (particularly glass beads) in a solution containing the dispersant. A machine such as Dyno-mill (KDL-model, manufactured by Willy A. Bachofen Company) may be employed for this purpose. The concentration of the benzoyl urea compound in the aqueous solution during the pulverization, is from 1 to 70 w/v%, preferably from 20 to 50 w/v%. Particularly when the pulverization is conducted in a wet pulverization system by using Dyno-mill, the concentration of the benzoyl urea compound in the aqueous solution is preferably within the above range. The concentration of the nonionic surfactant as a dispersant is usually from 1 to 30 w/v%, preferably from 2 to 20 w/v%. The glass beads employed usually have a size of from 0.1 to 1.5 mm in diameter, preferably from 0.25 to 0.5 mm in diameter. The pulverization time is usually from 5 minutes to 1 hour, preferably from 30 minutes to 1 hour. After the completion of the wet pulverization, glass beads will be removed by sieving, and if necessary additives such as a sweetening agent or a perfume may be added thereto. If necessary, the composition is then subjected to autoclave sterilization or to filtration for the removal of bacteria, to obtain the benzoyl urea compound dispersed in water (which will be referred to as a liquid composition).

Further, the benzoyl urea compound of the present invention may be formed into a dry formulation, if necessary. The dry formulation may be prepared by freeze-drying the above liquid composition by a usual method, preferably after an addition of a suitable amount of a disintegrator. As such a disintegrator, saccharides, saccharide alcohols, silicic anhydride or a nonionic surfactant (the same as mentioned above) may be employed. Particularly preferred is a nonionic surfactant. The saccharides as a disintegrator include a monosaccharide such as glucose or fructose, a disaccharide such as sucrose, maltose or lactose and a polysaccharide such as starch, dextrin or cellulose. The saccharide alcohols include, for example, xylitol, mannitol and sorbitol.

The silicic anhydride as the disintegrator is preferably a light anhydrous silicic acid.

The nonionic surfactant as the disintegrator is preferably those exemplified above as a dispersant, more preferably a sucrose fatty acid ester or a polyoxyethylene polyoxypropylene glycol. The nonionic surfactant can be used both as the dispersant and as the disintegrator,

preferably used differently as each of them. For instance, in the case of using polyglycerol fatty acid ester (e.g. decaglycerol monolaurate) or polyoxyethylene hardened caster oil (e.g. polyoxyethylene hardened caster oil 60) as the dispersant, a sucrose fatty acid ester is preferably used as the disintegrator.

The amount of disintegrator is added preferably in an amount of from 1 to 90 w/v%, more preferably from 20 to 70 w/v%.

Thus, it is possible to obtain a formulation having excellent granularity as a dry formulation and having excellent dispersibility in water.

In the preparation of the present invention, the ratio of the benzoyl urea compound to be dispersant and the disintegrator is, for instance, the benzoyl urea compound:the dispersant:the disintegrator=1-70:1-30:1-90 by weight, preferably 20-50:2-20:10-70 by weight.

The benzoyl urea compound of the present invention can be formulated into other pharmaceutical formulations by conventional methods. As such pharmaceutical formulations, oral formulations such as powders, fine particles, granules, capsules, tablets and liquid drugs may be mentioned.

Such formulations may be prepared by removing water from the above-mentioned liquid composition by heat drying, freeze drying, centrifugal separation, membrane filtration, etc., and then following a conventional method for formulation by using or without using conventional pharmaceutical additives.

The benzoyl urea compound having the specified particle size of the present invention may usually orally be administered to mammals (e.g. human beings, horses, cattles, dogs, mice, rats, etc.). The dose varies depending upon the diseases condition, the sex, the body weight, the formulation, etc. However, for instance, when the benzoyl urea compound of the present invention is orally administered against human malignant lymphoma or lung cancer, the benzoyl urea compound is administered in a daily dose of from 5 to 100 mg/kg to an adult in one to three times per week.

With the benzoyl urea compound having the specified particle size of the invention, the absorption through the gut is remarkably improved, and the stability of the particles in a liquid state is excellent.

By using the benzoyl urea compound having the specified particle size according to the present invention, it is possible to reduce the dose of the benzoyl urea compound and thus to reduce the side effects or the pain to the the patient when it is administered to the patient.

TEST EXAMPLE 1 (Formulation Conditions)

(1) Type of the benzoyl urea compound

Compound No. 3, No. 5 or No. 6 (4 w/v%) as the benzoyl urea compound and polyoxyethylene hardened caster oil 60 (HCO-60, manufactured by Nikko Chemical K.K., 10 w/v%) as a dispersant were suspended in water, and after an addition of glass beads (from 1 to 1.4 mm in diameter) in an amount of the same volume, subjected to rotary pulverization by Dyno-mill for 20 minutes. The average particle size of the benzoyl urea compound thus obtained was measured and shown in Table 1.

TABLE 1

Sample*1	Particle size (μm)
Compound No. 3	0.43
Compound No. 5	1.13
Compound No. 6	0.72

*1Benzoyl urea compound

The average particle size was measured in such a manner that the benzoyl urea compound was diluted to a concentration of 20 μg/ml and the average particle size was measured by a light scattering method (Autosizer, Model 700, manufactured by Maruburn Company) (the same applies hereinafter).

(2) Type of the dispersant

Compound No. 3 (4 w/v%) and the following dispersant (10 w/v%) were suspended in water, and after an addition of glass beads (from 1 to 1.4 mm in diameter) in an amount of the same volume, subjected to rotary pulverization by Dyno-mill for 45 minutes. As the dispersant, polyoxyethylene hardened caster oil 60 (HCO-60, manufactured by Nikko Chemical K.K.), polyoxyethylene (160) polyoxypropylene (30) glycol (F68, manufactured by Asahi Denka Kogyo K.K.), a decaglycerol fatty acid ester (Decagly.ester, Nikko Chemical K.K.) or polysolbate 80 (Tween 80, manufactured by Nakarai Kagaku K.K.) was employed.

TABLE 2

Dispersant	Particle size (μm)
HCO-60	0.9
F68	0.9
Decagly.ester	0.6
Tween 80	0.6

(3) Concentration of the benzoyl urea compound

Compound No. 3 (from 10 to 40 w/v%), HCO-60 (5 w/v%) as a dispersant and glass beads (from 1 to 1.5 mm in diameter) were subjected to rotary pulverization by Dyno-mill for pulverization time of 30 minutes. Then, the average particle size of the benzoyl urea compound was measured.

TABLE 3

Sample (w/v %)	Particle size (μm)
10	0.56
20	0.50
40	0.46

(4) Concentration of the dispersant

Compound No. 3 (20 w/v%), HCO-60 (from 2.5 to 10 w/v%) as the dispersant and glass beads (from 1 to 1.5 mm in diameter) were subjected to rotary pulverization by Dyno-mill for a pulverization time of 30 minutes. Then, the average particle size of the benzoyl urea compound was measured.

TABLE 4

Dispersant (w/v %)	Particle size (μm)
2.5	0.54
5	0.50
10	0.45

(5) Size of beads

Compound No. 3 (40 w/v%), HCO-60 (5 w/v%) as the dispersant and glass beads (three types having a diameter of from 0.25 to 0.5, from 0.5 to 0.75 and from 1 to 1.5 mm, respectively) were subjected to rotary pulverization by Dyno-mill for a pulverization time of

30 minutes. Then, the average particle size of the benzoyl urea compound was measured.

TABLE 5

Diameter of beads (mm in diameter)	Particle size (μm)
0.25–0.5	0.30
0.5–0.75	0.33
1–1.5	0.46

(6) Pulverization time

Compound No. 3 (40 w/v%), HCO-60 (5 w/v%) as the dispersant and glass beads (from 0.25 to 0.5 mm in diameter), were subjected to rotary pulverization by Dyno-mill for a pulverization time of from 10 to 60 minutes. Then, the average particle size of the benzoyl urea compound was measured.

TABLE 6

Pulverization time (minutes)	Particle size (μm)
10	0.38
20	0.33
30	0.29
40	0.27
50	0.26
60	0.26

TEST EXAMPLE 2

The stability of particles in the liquid composition of the benzoyl urea compound was investigated.

Namely, a wet pulverization formulation comprising compound No. 3 (40 w/v%) and HCO-60 (10 w/v%) was stored at room temperature, whereby the stability of the particles was investigated. The results are shown in Table 7.

TABLE 7

Storage duration (months)	Particle size (μm)
0	0.7
1	0.8
3	1.3

TEST EXAMPLE 3

Each of four wet pulverized formulations having different particle sizes [composition: Compound No. 3 (40 w/v%) and HCO-60 (10 w/v%)] was forcibly orally administered by an oral sonde to a group of 5 Wister male rats (body weight: 200 g) starved for 18 hours (dose: 50 mg/5 ml/kg). Then, blood (0.3 ml) was periodically sampled with heparin from the jugular vein.

The blood thus obtained was subjected to separation of the plasma and removal of proteins by using acetonitrile, and then Compound No. 3 was quantitatively analyzed by a high speed liquid chromatography using a reversed phase column (Nova Pak C_{18}, 5μ, 3.9 mm in diameter×150 mm, Nihon Waters), and the curve of the concentration in blood was prepared.

From the curve of the concentration in blood, the area below the curve was obtained by using a trapezoid formula and presented as AUC (Area Under the Curve).

TABLE 8

Particle size (μm)	AUC (0–24 hr) (μg/ml. hr.)
0.31	11.6 ± 2.3
0.66	10.0 ± 1.6
1.5	8.1 ± 1.2
2.5	6.9 ± 1.4

EXAMPLE 1

Compound No. 3 (20 g) was suspended in 50 ml of a 5 w/v% HCO-60 aqueous solution, and the suspension was wet pulverized (3000 rpm for 45 minutes) by Dyno-mill by using 50 g of glass beads (from 0.25 to 0.5 mm in diameter). After the completion of the pulverization, glass beads were removed by sieving, to obtain a wet pulverized formulation of Compound No. 3.

The wet pulverized formulation thus obtained was sterilized in an autoclave to obtain a liquid formulation of a final form. The average particle size of Compound No. 3 in this liquid formulation was 0.68 μm. Instead of the sterilization in an autoclave, it is possible to employ filtration to remove bacteria. If necessary, a sweetening agent, a perfume, etc. may be added.

EXAMPLE 2

To 50 ml of the liquid formulation obtained in Example 1, 20 g of a sucrose fatty acid ester (P1670, manufactured by Mitsubishi Chemical Industries, Ltd.) was added. The mixture was freezed with dry ice-methanol and then subjected to vacuum drying for 24 hours to remove water. The solid thus obtained was filled in capsules to obtain capsule drugs.

EXAMPLE 3

Compound No. 3 (15 g) was suspended in 50 ml of a 5 w/v% decaglycerol monolaurate (Decagline 1 L, manufactured by Nikko Chemical K.K.) aqueous solution, and the suspension was wet pulverized (3000 rpm for 45 minutes) by Dyno-mill by using 50 g of glass beads (from 0.25 to 0.5 mm in diameter). After the completion of the pulverization, glass beads were removed by sieving, to obtain a wet pulverized formulation of Compound No. 3.

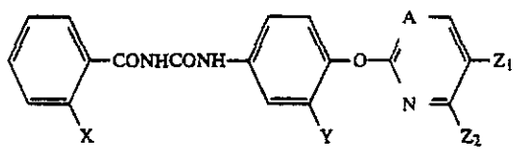
The wet pulverized formulation thus obtained was sterilized in an autoclave to obtain a liquid formulation of a final form. The average particle size of Compound No. 3 in this liquid formulation was 0.75 μm. Instead of the sterilization in an autoclave, it is possible to employ filtration to remove bacteria. If necessary, a sweetening agent, a perfume, etc. may be added.

EXAMPLE 4

To 50 ml of the liquid formulation obtained in Example 3, 30 g of a sucrose fatty acid ester (P1670, manufactured by Mitsubishi Chemical Industries, Ltd.) was added. The mixture was freezed with dry ice-methanol and then subjected to vacuum drying for 24 hours to remove water. The solid thus obtained was filled in capsules to obtain capsule drugs.

We claim:

1. A benzoyl urea compound have the formula:



(I)

wherein X is a halogen atom, a nitro group or a trifluoromethyl group, provided that when Y is a nitro group, X is a halogen atom or a nitro group, Y is a hydrogen atom, a halogen atom, a nitro group or a trifluoromethyl group, Z_1 is a halogen atom or a trifluoromethyl group, Z_2 is a hydrogen atom or a halogen atom, and A is a nitrogen atom, characterized in that its average particle size is not larger than 1 μm.

2. The benzoyl urea compound according to claim 1, which is N-(2-nitrobenzoyl)-N'-[3-chloro-4-(5-bromo-2-pyrimidinyloxy)phenyl]urea.

3. The benzoyl urea compound according to claim 1, of which an average particle size is within a range of from 0.2 to 1 μm.

4. An antitumor composition comprising an effective amount of the benzoyl urea compound according to claim 1, which is suspended in an aqueous solution containing a dispersant.

5. The benzoyl urea compound according to claim 1, which is prepared by pulverization in an aqueous solution containing a nonionic surfactant as a dispersant.

6. The benzoyl urea compound according to claim 5, wherein the nonionic surfactant as a dispersant has at least 3 HLB value.

7. The benzoyl urea compound according to claim 5, wherein the nonionic surfactant as a dispersant is at least one selected from the group consisting of polyoxyethylene hardened caster oil 20, polyoxyethylene hardened caster oil 40, polyoxyethylene hardened caster oil 60, polyoxyethylene hardened caster oil 100, polysorbate 60, polysorbate 65, polysorbate 80, polyoxyethylene polyoxypropylene glycol, a sucrose fatty acid ester, a glycerol fatty acid ester, a sorbitan fatty acid ester, a propylene glycol fatty acid ester, a polyglycerol fatty acid ester, a polyoxyethylene sorbitan fatty acid ester, a polyoxyethylene sorbitol fatty acid ester, a polyoxyethylene glycerol fatty acid ester, a polyethylene glycol fatty acid ester and a polyoxyethylene caster oil.

8. An antitumor composition comprising an effective amount of the benzoyl urea compound according to claim 1, which is in the form of a freeze-dried formulation.

9. An antitumor composition comprising an effective amount of the benzoyl urea compound according to claim 1, which is in the form of a freeze-dried formulation after an addition of a suitable amount of a disintegrator.

* * * * *

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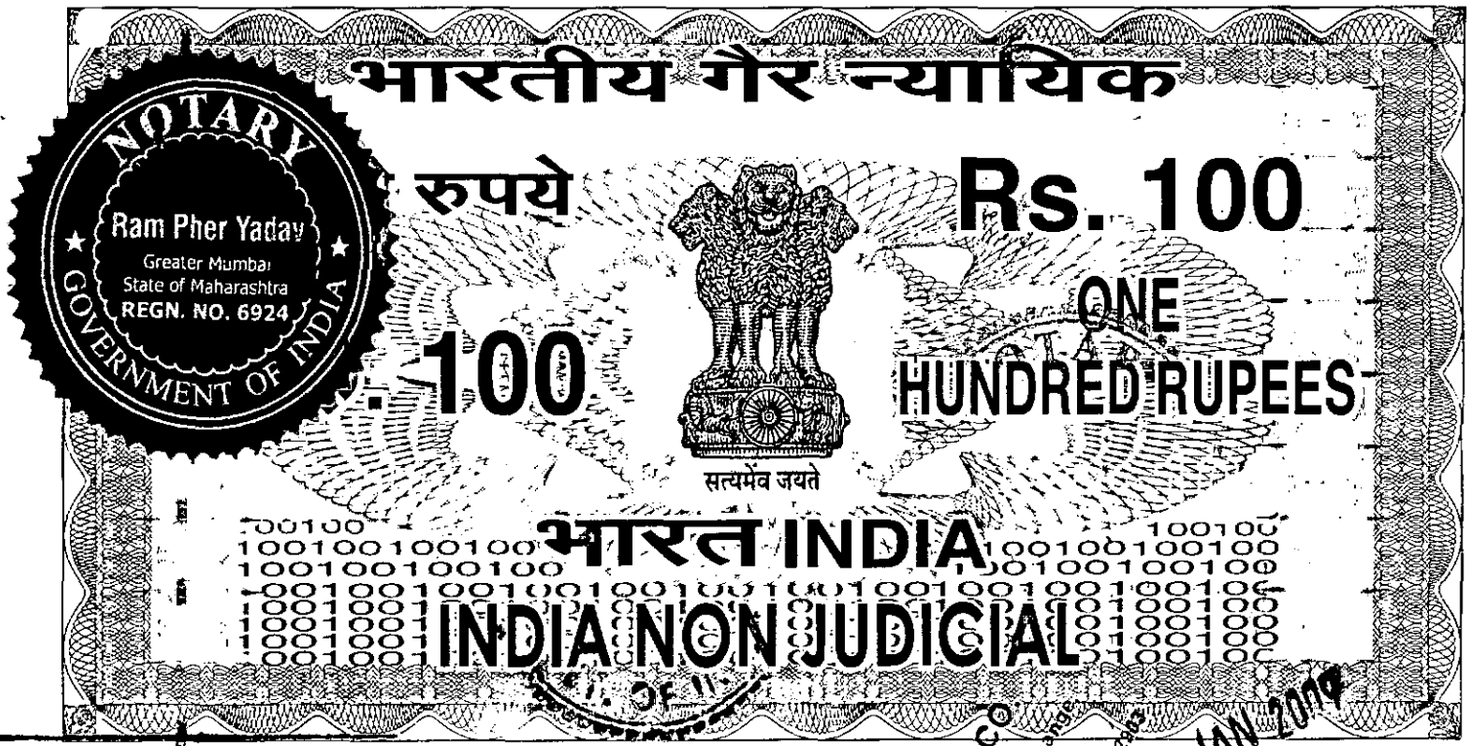
Exhibit 3



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Shri. R. S. Sawant

BEFORE THE CONTROLLER OF PATENTS

PATENT OFFICE,

MUMBAI

NOTARY
Ram Pher Yadav
Greater Mumbai
Maharashtra
Regn. No. 6924
GOVT. OF INDIA

In the matter of The Patents Act, 1970 as amended by The Patents (Amendment) Act 2005,

And

In the matter of The Patents Rules, 2003 as amended by the Patents Amendment Rules 2006

And

Natureau
26/3/09

IN THE MATTER of Patent No.215758 (In/PCT/2001/799/MUM) dated July 05, 2001 in the name of BAYER CORPORATION, 100 BAYER ROAD, PITTSBURG, PENNSYLVANIA, 15202, U.S.A.,

..... Patentee

And

IN THE MATTER of opposition thereto by Cipla Limited, 289, Bellasis Road, Mumbai Central, Mumbai-- 400 008.

..... Opponent

NOTARY Ram Pher Yadav Greater Mumbai Maharashtra Reg. No. 6924 GOVT. OF INDIA

AFFIDAVIT

I, ARVIND ANANT NATU working for gain at National Chemical Laboratory, Pune solemnly affirm and declare as under:

1. I have been working as a Scientist in NCL since 1978 after finishing my Ph.D. degree from the University of Pune and post-doctoral fellowship at the Technical University of Berlin, Germany, for two years, where I worked on natural products and characterized more than 90 new compounds.

2. After rejoining NCL, I was mainly engaged in the asymmetric induction using alkaloids as catalyst, where besides finding out new catalysts for various reactions, I developed new NMR methods for determination of optical yields. Application of phase transfer catalyst in various organic reactions for better selectivity and yields was another field explored by me. Several industrial

chemicals are now produced using these procedures. Besides that, I developed process for drugs and drug intermediates which are practiced in industry today.

Later, I switched to Bioorganic Chemistry. I have established state-of-art facility for the synthesis of modified oligonucleotides and peptides for the first time in the country, which now caters to the needs of most of leading hospitals. I was involved in the development of several DNA based diagnostic methods for genetic disorders, viruses, etc. Use of fluorescent oligonucleotides for diagnosis was a major breakthrough in this field. This method is now in practice in two hospitals for the diagnosis of Thalessimia. Recently, I have used combinatorial chemistry for the synthesis of an anti-inflammatory drug, besides the synthesis of natural products and new heterocycles.

3. I am a faculty member in the Department of Biotechnology, University of Pune, and am responsible for designing and teaching a course of macromolecular biopolymers. I am a member of several professional bodies and delivered lectures in number of Symposia. I am a technical consultant to four American/Indian companies. I have visited Germany, Switzerland, Poland, Russia, UK and several other countries and delivered invited lectures in the various Universities abroad. Details of my qualifications and various experiences in the field of medicinal chemistry are outlined in the below table:

1.	Name	:	Dr. Arvind Anant Natu
2.	Designation & Institution	:	Professor Emeritus Indian Institute of Science ,Education and research 900 ,NCL Innovation Park Homi Bhabha road Pune 411008 Telephone: +91-20-25898023 Ext 119 +91-20-25898021

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			Fax No. +91-20-25898022 www.iiserpune.ac.in
4.	Academic Qualifications	:	PROVIDED BELOW

	Degree	University	Year	Class
(A)	B.Sc.	Poona	1967	I[st] (Hons.)
	M.Sc.	Poona	1969	I[st]
	Ph.D.	Poona	1976	--
(B)	Diploma in German Language	Poona	1981	3[rd] in the University

4. My research experience includes research conducted by me at the following universities, which are NCL **Pune**, Technical University **Berlin**, Bielefeld University, Institute of Biomedical Science/ Pharmacia **Upsala,** Shemyakin Institute of Bioorganic Chemistry **Moscow** and Institute of Molecular Biology, **Poland**. The detailed chronologies of the research carried out by me are as hereunder:

1969-74	Synthesis of number of terpenoid and steroidal derivatives using modern synthetic reactions and reagents. Elucidation of stereochemistry using mainly PMR as a tool.
1974-76	Modification of waxes, extraction of Colchicine on industrial scale. Project identification. Project planning, costing and standardization of a veterinary drug 'Quinapyramine' which involves ten step syntheses. This is the first injectable vaccine developed at NCL.
1976-78	Isolation and structure determination of biologically important compounds and their synthesis. Use of special techniques in spectroscopy such as Lanthenide

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	shifts, NOE, double resonance ^{13}C-NMR, WH-90, PMR chemical ionisation, mass spectrometry, etc. I have isolated and determined the structures of about 60-70 compounds, out of which 30 were new (at the end – Technical University, Berlin).
1979-82	Synthesis and structure determination (using modern techniques such as ESCA) of various types of homogeneous catalysts derived from alkaloids and metal complexes. Their use in various reactions for optical induction. Synthesis of enantiomerically pure sulphur compounds and amino alcohols. Application of these catalysts to the synthesis of drugs. Determination of plausible reaction mechanism for optical induction. Determination of enantiomeric excess using PMR techniques.
1982-87	Standardization of the new process for the preparation of chloramphenicol, involving mainly asymmetric induction. Use of PTC technique for optical induction in various reactions. Enzymatic and swing resolution of the amino acids or amino diols.
1987 onwards	Synthesis of bio active compounds ,Asymmetric synthesis, Synthesis and structural studies of oligonucleotides /peptides (solid phase/solution phase synthesis), PCR based diagnostics; Combinatorial techniques in Drug discovery; Natural Product Processing; High Throughput Screening; Synthesis of Novel antifungals; In charge of Chem bio resource center

5. A brief summary of my post-doctoral research experience carried out in foreign countries is brought out in the below table:

1976-78	Institute of Organic Chemistry Technical University, Berlin **W.Germany** (Prof.F.Bohlmann)	Isolation and synthesis of natural products

1987	Institute of Organic Chemistry Bielefeld University, Bielefeld **W.Germany** (Prof.E.V.Dehlmon)	Phase transfer catalysed organic reaction
1990	Institute of Biomedical Science/ Pharmacia, Upsala, **Sweden**	Solid phase automatic DNA synthesis
1993	Shemyakin Institute of Bioorganic Chemistry, Moscow, **USSR** (Prof.V.A.Effimov)	Synthesis of natural and modified monomers for oligonucleotides and their incorporation Synthesis of peptides; use of DNA diagnostics oligonucleotides, combinatorial chemistry for building of Libraries for new drugs.
1997	Institute of Molecular Biology, **Poland** (Prof.G.Stec)	Large scale synthesis of modified DNA for therapeutics

Awards/Fellowships:

- Hon. Advisor to German Academic Exchange Service
- Labdhi RDE Life time achievement award (Rs. 1,00 , 000)
- Junior & Senior Research Fellowship of CSIR, New Delhi
- Visiting Scientist at Bielefeld University, Germany
- Shemyakin Institute of Bioorganic Chemistry, USSR
- Institute for Macromolecular Chemistry, Poland

Teaching Experience:

All teaching experience at post graduate and graduate levels. I am involved in faculty positions at various institutes for last 10-15 years, besides my NCL research activities.

- Course on Biopolymers (design, teaching and evaluation) M.Sc. Biotechnology, University of Pune.

- Staff Development Courses for Post-graduate Teachers at Mumbai, Pune, Shivaji, Nanded Universities, National Defence Academy, Pharmacy Colleges & Hospitals.

Facilities-in-Charge:

- Established a total drug discovery unique facility comprising of combinatorial chemistry, processing of high throughput screening (Combinatorial Chemistry Bio-Resource Center).
- Established and running a full-fledged facility for the synthesis of normal and modified oligonucleotides and peptides catering to the needs of major hospitals/labs in the country.
- In-charge of analytical facility (Organic), responsible for NMR, GC, LC, FPLC, UV, Fluorescence Spectrophotometer, MPLC, Amino Acid Analyser, etc.

Memberships:

1. Founder Member of Indian Society of Bioorganic Chemists
2. Founder member Of Indian peptide society
3. Society of Biological Chemistry
4. Associated Referee of ICUMSA
5. Referee for two international journals
6. Members of the National Committee on GLP
7. Member of the Research Society of H.N. Hospital, Mumbai.
8. Convener of Bureau of Indian standards (DIN in Germany)

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Ram Pher Yadav
Greater Mumbai
Maharashtra
Reg. No. 6924
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Other Achievements:

- 25 years of research experience in synthetic Organic chemistry, bioorganic chemistry, combinatorial chemistry and drug discovery in NCL;
- Consultant to Indian and International pharmaceutical companies (mainly in strategic planning, project monitoring, vendor /site audits/ qualification, synthesis, IPR, regulatory affairs, impurity profiles, polymorphs etc.);
- Last five years mainly involved in setting up unique "state of the art "Combi Chem – Bio Center in NCL;
- Trained in India, Germany and other European countries;
- Teaching experience of 15 years mostly at post graduate and graduate levels (Organic and bio organic chemistry);
- Can speak, read, and write German;
- Recently (1st May 2007 onwards) joined Indian Institute of Science, Education and research.

List of Publications:

1. Partial synthesis of protoprimulogenin; A.A.Natu and C.R.Narayanan, Current Science, 42, 403 (**1973**);

2. Synthesis of some bridged triterpene ethers, A.A.Natu and C.R.Narayanan, J.Org.Chem. 30, 2689 (**1974**);

3. Triterpene acids from Indian clove buds, A.A.Natu and C.R.Narayanan, Phytochemistry, 13, 1999 (**1974**);

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4. Terpen-derivative aus Senocio Arten, F.Bohlmann, K.H.Knoll, C.Zdero, P.K.Mahanta, M.Grenz, A.Suwita, D.Ehlers, Le Van Ngo, W.R.Abraham and A.A.Natu, Phytochemistry, 16, 965 (**1977**);

5. Neue sesquiterpenlakton und andere Inhalstoffe aus, Vertretern der Euoatorium gruppe, F.Bohlmann, P.K.Mahanta, A.Suwita, A.A.Natu, Zdero, W.Dorner, D.Ehlers and M.Grenz, Phytochemistry, 16, 1973 (**1977**);

6. Uber weiters-longipinen-derivative aus composition, F.Bohlmann, A.Suwita, A.A.Natu, H.Czerson and A.Suwita Chemische Berichte, 110, 3572 (**1977**);

7. New germacronolides from isocarpha species, F.Bohlmann, P.K.Mahanta, A.A.Natu, R.M.King and H.Robinson, Phytochemistry, 17, 471 (**1978**);

8. Neue diterpene und germacronolide aus mikania arten, F.Bohlmann, A.A.Natu and P.K.Mahanta Phytochemistry, 17, 483 (**1978**);

9. New sesquiterpene lactones from inula species F.Bohlmann, A.A.Natu, R.C.Rastogi, J.Jackupovic and P.K.Mahanta, Phytochemistry, 17, 1165 (**1978**);

10. Weitere Bisabolen derivative und andero inhaltstoffe aus Sudafrikanischen senecio arten, F.Bohlmann, C.Zdero and A.A.Natu, Phytochemistry, 17, 1757 (**1978**);

11. Thymolderivative aus neurolaena arten, F.Bohlmann, A.A.Natu and K.Kerr, Phytochemistry, 18, 489 (**1979**);

12. Optical Induction Part-IV. Optical induction by using homogeneous catalyst, R.R.Ahuja, A.A.Natu and V.N.Gogte, Tet. Lett. 21, 4743 (**1980**);

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13. Optical Induction Part-III, Some mechanistic studies on the reaction between thiophenol and unsaturated ketone using quinine/quinidine catalyst, R.R.Ahuja, S.I.Bhole, N.N.Bhongle, V.N.Gogte and A.A.Natu, Ind.J.Chem. 21B, 299 (**1982**);

14. Optical Induction Part-V. Michael addition of thiophenol to benzalacetone catalysed by quinine and quinidine derivatives, N.N.Bhongle, V.N.Gogte and A.A.Natu, Ind.J.Chem. 21B, 304 (**1982**);

15. Prenylated flavonoids and Teprosia purpurea seeds, B.Sinha, A.A.Natu and D.D.Nanavati, Phytochemistry, 21, 1468 (**1982**);

16. Oxidative coupling reactions under PTC conditions, V.N.Gogte, A.A.Natu and V.S.Pandit, Tetrahedron Letters, 21, 4131 (**1983**);

17. Determination of enantiomeric excess using ^{13}C-NMR spectroscopy, V.N.Gogte, A.A.Natu and V.S.Pandit, Org.Mag.Resonance, 22, 624 (**1984**);

18. Alkylation of ethyl-nitroacetate under PRC conditions, V.S.Pore and A.A.Natu, Synthetic Communications, 17, 1421 (**1987**);

19. Enantioselective synthesis of R & S phenyl alanine, V.N.Gogte, J.Kenya and A.A.Natu, Chemistry & Industry, London, 243 (**1986**);

20. Optical Induction Part-VIII, Addition of htiophenol to Mannich bases using cinchona alkaloids, Synthetic Communications (1987)/1988;

21. Optical Induction Part-VIII, Opening of phenyl oxiranes with different amino nucleophiles, V.N.Gogte, A.A.Natu and V.S.Pandit, Ind.J.Chem. 25B, 603 (**1986**);

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22. Structure of 4R(+)-1-benzyl-N-dichloroacetyl 1,3-oxazolidine, V.G.Puranik, S.S.Tawale, T.N.Guru Row, V.A.Kumar and A.A.Natu, Acta Crystallographica (**1986**);

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24. Chlorocarboxylation of polyethylene, Part-I, S.G.Joshi and A.A.Natu, Angewandte Makromolecular Chemie, 143, 99 (**1986**);

25. Chlorocarboxylation of polymers, IUPAC International Conference on Modified Polymer Short Lectures, 91 (**1988**);

26. A non-phosgene route for the synthesis of sym. N,N'-diethyldiphenyl-urea, N.R.Ayyangar, U.R.Kalkote, A.R.Choudhary and A.A.Natu, Chemistry & Industry, 599 (**1988**);

27. A simple and economical method for the alkylation of benzanilide, N.R.Ayyangar, U.R.Kalkote, A.R.Choudhary and A.A.Natu, Synthetic Communications, 18, 2011 (**1988**);

28. Facile synthesis of substituted N-monoalkylaromatic amines using PTC conditions, U.R.Kalkote, A.R.Choudhary, A.A.Natu, R.J.Lahoti and N.R.Ayyangar, Synthetic Communications, 21, 1889-1900 (**1991**);

29. Phase transfer catalyzed N-monoalkylation of aminoanthraquinones, U.R.Kalkote, A.R.Choudhary, A.A.Natu and N.R.Ayyangar, Synthetic Communications, 21, 1129-1135 (**1991**);

30. Detection of Bovine herpes virus by polymerase chain reaction, Indian Veterinary Congress, Bangalore (**1992**);

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31. Synthesis and application of fluorescent nucleotides, V[th] National Bioorganic Symposium, Kolhapur (**1995**);

32. Detection of genetic diseases by PCR, Indo-German Symposium in Organic Chemistry, Hyderabad (**1996**);

33. PCR based test for the detection of virus in India, Indian Scientific & Research Letters, 2(2), 148-150 (**1994**);

34. 5-Aminocarboxyfluorescein-2'-dU oligonucleotides Nucleosides/ Nucleotides, 00 (**1997**);

35. Psoriasia a retrovial link, Australian Journal of ..., R.B.Gadgil, M.H.Gharpure and A.A.Natu, Presentation at 19[th] World Congress of Dermatology, Sydney, 2002, (1997);

36. Dental plaque: an unlikely reservoir of Helicobacter pylori, A.H.Kamat, P.R.Mehta and A.A.Natu, Int.J. of Gastroentrology, 17, 138 (**1998**);

37. Chemoselective carbon-carbon coupling of organocuprates with (Bromo-methyl) methyl maleic anhydride: Synthesis of chaetomellic acid A, A.M.Deshpande, A.A.Natu and N.P.Argade, J.Org.Chem. 63, 9557 (**1998**);

38. Synthesis and screening of a combinatorial library of naphthalene substituted chalcones: Inhibitors of leukotriene B_4, A.M.Deshpande, N.P.Argade and A.A.Natu, Bioorganic & Medicinal Chemistry, 7(6), 1237 (**1999**);

39. Chemo- and regioselective nucleophilic reactions of (Bromomethyl) methyl maleic anhydride. Synthesis of α-quinoxalinyl- and α-benzothiazinyl acrylic acid, A.M.Deshpande, A.A.Natu and N.P.Argade, Heterocycles, 51, 0000 (**1999**);

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40. Biocontrol of root infecting pathogenic fungus, chitin metabolism inhibitors, M. v. Deshpande and A. A. Natu, Journal of Biocontrols ,15(2) ,157 **(2001)**;

41. Facile synthesis of (±) 2,3-disubstituted maleic anhydride segment .A. M Deshpande, N. P. Argade, A. A. Natu , Synthesis, 5, 702, **(2001);**

42. A new strategy for the synthesis of furan-3,4 dicarboxylic acid Deshpande, N. P. Argade, A. A. Natu , Synthesis **(2002)**;

43. Expedient synthesis of 1,2,3-triazole-fused tetracyclic compounds by intramolecular Huisgen ('click') reactions on carbohydrate-derived azido-alkynes, Srinivas Hotha,* Ramakrishna I. Anegundi and Arvind A. Natu, Tetrahedron Letters 46 **(2005)** 4585–4588;

44. "Diversity oriented synthesis of 1,2,3-triazole and 1,2,3,4- tetrazole-fused glycosides and nucleosides by an intramolecular 1,3- dipolar cycloaddition reaction", J. Comb. Chem **(000 2007)**.

To be communicated:

1. Enzymatic resolution of amino acids using subtisiline;
2. Backbone rearrangement of 3β-4β-diacetoxy-5α-6α-epoxy-cholestane;
3. On the configuration of Echinocystic acid diacetate lactone;
4. Reversal of backbond rearrangements in steroids;
5. Detection of unusual mutation in thallesimia; and
6. PCR detection of Psorasis.

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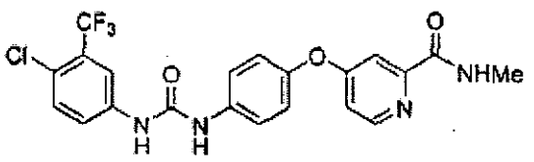
Major scientific areas of interest:

- Bioorganic Chemistry
- Synthesis of Oligonucleotides/Peptides
- Synthetic Organic Chemistry
- Drug Discovery & Process Development

6. I have been given a copy of Patent specification of granted Indian Patent No. 215758 and copies of US Patent No.5773459 published on June 30, 1998 referred to as Exhibit 1 and US Patent No.4904668 published on February 27, 1990 referred to as Exhibit 2. I have been particularly asked to study and appreciate the invention disclosed and claimed in the Patent specification of granted Indian Patent No. 215758 on the face of the state of art namely Exhibits 1 and 2 and any other document known to me which is published prior to January 13, 1999 and to thereafter assess whether the invention claimed in the Indian Patent No. 215758 presents any technical advancement which is unobvious in nature. More particularly I have asked to give my opinion with reference to the compound with the INN name sorafenib or pharmaceutically acceptable salts thereof in particular the tosylate salt of sorafenib.

7. I say that sorafenib is a compound with the IUPAC name N-(4-chloro-3-(trifluoromethyl)phenyl)-N'-(4-(2-(N-methylcarbamoyl)-4-pyridyloxy)phenyl)urea and structurally represented as shown below.

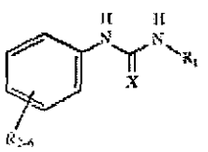


8. While sorafenib or a pharmaceutically acceptable salt thereof is claimed in claim 1 of the patent, the compound specifically in the form of its tosylate is claimed in claim 20. I have carefully gone through the patent specification of Patent No. 215758 as also exhibits 1and 2. I have understood the invention claimed in Patent

NOTARY Ram Pher Yadav Greater Mumbai Maharashtra Regd. No. 6924 GOVT. OF INDIA

No. 215758 as also the teachings contained in Exhibits 1 and 2. I say that in view of my qualifications, experience and knowledge in the field of Organic and Medicinal Chemistry I am competent to carry out the assessment as to whether the invention claimed in Patent No. 215758, in particular sorafenib or sorafenib tosylate present any technical advancement, which is unobvious in nature having regard to the teachings contained in Exhibits 1 and 2 and other documents known to me. Accordingly I am competent to depose this affidavit as an expert.

9. I say that that Exhibit 1 relates to urea and thiourea type compounds for the prevention and treatment of cell proliferative disorders. Particularly Exhibit 1 discloses compounds having the formulae:



and pharmaceutically acceptable salts thereof, wherein: X is O or S;

R_1 is selected from the group consisting of optionally substituted aryl, alkylaryl, and heteroaryl

$R_{2\text{-}6}$ are independently selected from the group consisting of hydroxy, H, alkyl, alkoxy, CN, nitro, halo, trihalomethyl, amide, carboxamide, sulfonyl, and sulfoxamide.

I say that that column 2 (lines 43 to 46) clearly discloses that the phenyl ring can contain independently $R_{2\text{-}6}$ including halo and trihalomethyl groups. This has a clear similarity to the structure of sorafenib in the phenyl ring fragment of the same. I say that the above thus leads us to compound with the partial structure as shown:

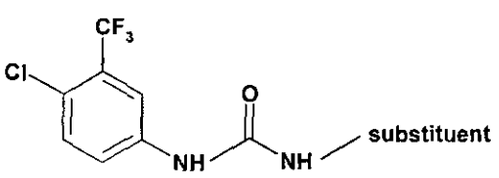


Figure 1

10. Similarly regarding the substitution on the N' atom, I say that Exhibit 1 on column 2 lines 41, 42 and column 3 lines 44 to 52 disclose that R1 is an optionally substituted aryl group which is preferably a phenyl group. I say that this drives to a compound with the partial structure:

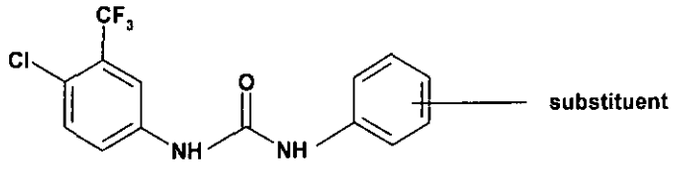
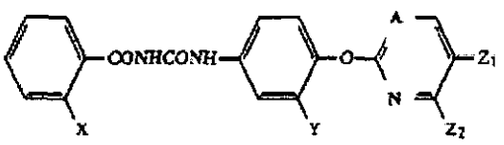


Figure 2

11. I say that that Figure 2 depicts a compound with the maximum structural resemblance to sorafenib that is allegedly claimed in the patent under opposition. I say that this comparison makes it very clear that Exhibit 1 discloses an essential structural feature that is required for a molecule to function as a kinase inhibitor i.e. the preferable presence of the trifluoromethyl phenyl ring on the N-atom of urea and the presence of the substituted phenyl group on the N' atom of the urea. I say that a urea derivative the substitutions as defined above on the N atom and N' atom of urea is capable of functioning as an anticancer agent.

12. I say that the specific pyridyloxy phenyl substitution on the N' atom of urea is motivated by the teachings of Exhibit 2. Exhibit 2, which refers to US4904668, discloses antitumor benzoyl urea compounds having an improved absorbability through the gut. In particular Exhibit 2 discloses compounds with the generic structure



wherein X is a halogen atom, a nitro group or a trifluoromethyl group, provided that when Y is a nitro group, X is a halogen atom or a nitro group, Y is a hydrogen atom, a halogen atom, a nitro group or a trifluoromethyl group, Z_1 is a halogen atom or a trifluoromethyl group, Z_2 is a hydrogen atom or a halogen

atom, and A is a =CH-- group or a nitrogen atom, or ....., characterized in that its average particle size is not larger than 1 μm.

13. I say that specific compounds that satisfy the above definition of formula I are disclosed in columns 2 and 3 of Exhibit 2. I say that compound no 1, 4 bear a substituted pyridyloxy phenyl group on the N' atom of the parent urea. I say that Exhibit 2 further discloses in column 5 lines 42 to 50 *"With the benzoyl urea compound having the specified particle size of the invention, the absorption through the gut is remarkably improved, and the stability of the particles in a liquid state is excellent.*

By using the benzoyl urea compound having the specified particle size according to the present invention, it is possible to reduce the dose of the benzoyl urea compound and thus to reduce the side effects or the pain to the patient when it is administered to the patient."

I say that the teachings of Exhibit 2 make it evident that urea compounds bearing a pyridyloxy phenyl group on one of the N atoms exhibit excellent stability and also marked antitumor effects.

14. I say that from the combined disclosure of Exhibit 1 and Exhibit 2, a compound with the following partial structure may be readily arrived at:

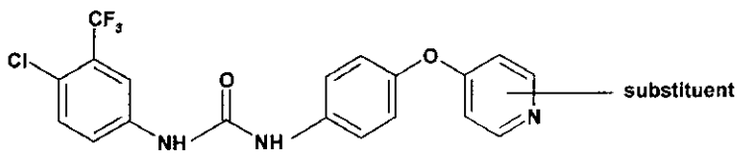


Figure 3.

I say that a person skilled in the art reading Exhibit 1 in conjunction with Exhibit 2 will clearly predict that urea derivatives wherein one of the N atom bears a trifluoromethyl phenyl substituent and the other N atom bears a pyridyloxy phenyl substituent (as shown in Figure 3) will have the potential to exhibit antitumor properties. I say that the inventors of the patent under opposition being persons skilled in the art have merely combined the teachings of Exhibit 1 and Exhibit 2 to prepare the desired molecule i.e. sorafenib.

NOTARY
Ram Pher Yadav
Greater Mumbai
Maharashtra
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GOVT. OF INDIA

15. I say that while none of Exhibit 1 and Exhibit 2 do not explicitly disclose sorafenib, nonetheless provide more than sufficient motivation and expectation of success since the combined teachings of these documents are directed towards compounds which bear maximum structural proximity to sorafenib. I say that these documents viz Exhibit 1 and Exhibit 2 disclose urea derivatives and suitable substitutions on either of the N atoms of urea, the various combinations of which will lead to new compounds, which will also possess potent antitumor activity. I say that both Exhibit 1 and Exhibit 2 bring to light the fact that substituted urea compounds are capable of exhibiting antitumor activity. Also these documents individually teach that presence of certain substituents on the N and N' atom of urea is preferred for pronounced activity. I say that Exhibit 1 discloses that a trifluoromethyl group is preferred whereas Exhibit 2 discloses that a pyridyloxy phenyl group is preferred. I say that sorafenib which is also a urea derivative bearing a trifluoromethyl group on the N atom and a pyridyloxy phenyl group on the N' atom of urea, claimed in the impugned patent in my opinion is obvious and devoid of inventive merit since the same flows from the combined teachings of Exhibit 1 and Exhibit 2. I say that every new substitution cannot be rewarded with a patent unless there is a genuine and enforceable development. In this case it was always worthwhile to study the efficacy of the different substituents in the present case and the inventors arrived at the claimed compound by substituting trifluoromethyl group for the N atom and a pyridyloxy phenyl group for the N' atom of urea which the patentee clearly has done and the compound of the impugned patent arrived at by trial and error method. I say that there was no guarantee flowing from Exhibit 1 and Exhibit 2 as to the specific attributes of the compound of the patent but the substitution was clearly motivated and thus obvious to try.

16. Turning to the tosylate salt of sorafenib I say that preparation of a suitable pharmaceutically acceptable salt of sorafenib is a routine job as a part of drug development thus the tosylate salt of sorafenib also cannot involve any inventive merit.

NOTARY Ram Pher Yadav Greater Mumbai Maharashtra Reg. No. 6924 GOVT. OF INDIA

17. *The statements made herein are true to my knowledge.*

Declared at Mumbai this 26th day of March 2009

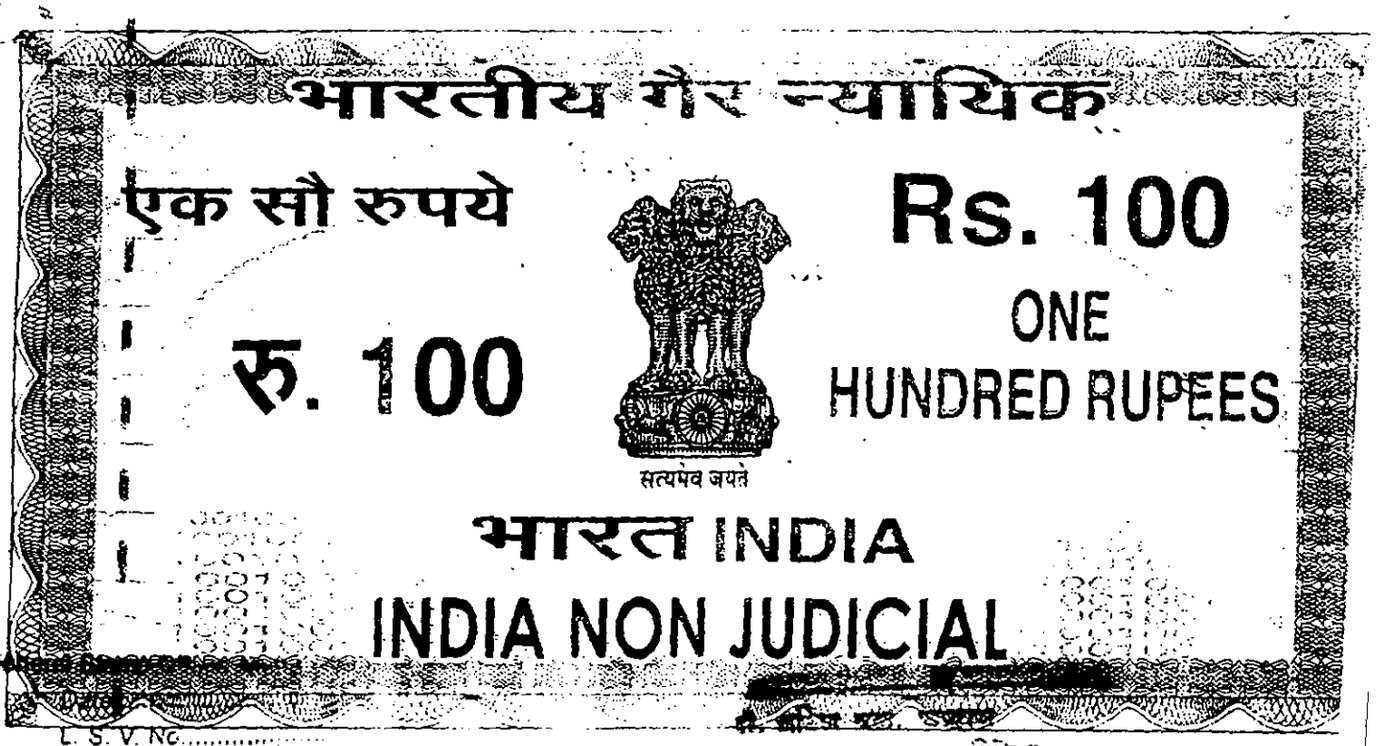
Dr. ARVIND ANANT NATU

Identified by me.
26/03/2009.

ASHOK KUMAR TIWARI
B.A. LL.B
Advocate High Court
Mumbai.

26/03/09

RAM PHER YADAV
NOTARY GR. MUMBAI (GOVT. OF INDIA)
Bandrekarwadi, Mahadev Hande Chawl,
Jogeshwari (E), Mumbai - 400 060.



Proper Officer

28 APR 2006

GENERAL POWER OF ATTORNEY

In the matter of The Patents Act, 1970 *as amended by* The Patents (Amendment) Act of 1999 and 2002, and The Patents Amendment Act, 2005,

And

In the matter of The Patents Rules, 2003 *as amended by* The Patents (Amendment) Rules of 2005,

And

In the matter of CIPLA LIMITED, Mumbai Central, Mumbai 400 008, India

Original has been filed with Application No. 994/mum/2006

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TRUE COPY.

true copy 30/03/09

Authorised Sign

We, the abovenamed CIPLA LIMITED do hereby retain, constitute and appoint **S. MAJUMDAR, M. MAJUMDAR, DR. SANCHITA GANGULI, RAJU KUMAR, ABHISHEK SEN, ASHWINI SANDU, AMIT CHAKRABORTY, A. MUKHERJEE, SONALI BHOKARIKAR, POONAM KOLHE** representatives of the Firm of S. MAJUMDAR & CO., 5, Harish Mukherjee Road, Calcutta – 700 025, India, all of Indian nationality, jointly and severally to be our Agents and Attorneys for the purpose of all acts under the Patents Act, 1970 (as amended by the Patents (Amendment) Act, 2005 or as may be amended hereafter) for all matters in which the name of the said firm of S MAJUMDAR & CO., appears in the address for service in the respective matters and we authorize any of them to sign our name and on our behalf on all applications and other papers and writings and do such acts, as may be necessary or expedient and lastly we request that all official communications now or hereafter relating to the same may be addressed to them at their office in Calcutta and that they be recognized as our authorized Agents in all proceedings incidental thereto. Cipla Ltd. retains the power to revoke this Power of Attorney at any time at its own discretion.

We authorize them to appoint agents, advocates and attorneys on Cipla's expressed consent. We hereby confirm all actions, if any, already taken by them in this matter. This Power of Attorney supercedes all previous Powers of Attorney given in favour of said firm of S. MAJUMDAR & CO.

Dated this 23rd day of June 2006.

CIPLA LIMITED