In-vitro antiproliferative activities and kinase inhibitory potencies of glycosyl-isoindigo derivatives

Mathieu Sassatelli[a], Fadoua Bouchikhi[a], Bettina Aboab[a], Fabrice Anizon[a], Doriano Fabbro[b], Michelle Prudhomme[a] and Pascale Moreau[a]

In the course of studies on the preparation of potential kinase inhibitors, we were interested in the synthesis of diversely substituted glycosyl-isoindigo derivatives. To get an insight into the effect of the substitution pattern of the isoindigo aromatic and carbohydrate moieties on the biological activities and to identify the cellular target(s) involved in the in-vitro antiproliferative activity of these derivatives, their inhibitory activities toward a panel of 10 different kinases were examined. The best inhibitory activities were found toward cyclin-dependent kinase 2/cyclin A. Molecular modelling experiments were carried out to investigate the binding interactions between the active site of cyclin-dependent kinase 2 and the lead compound of this series.

***Anti-Cancer Drugs* 18:1069–1074 © 2007 Lippincott Williams & Wilkins.**

Anti-Cancer Drugs 2007, **18:**1069–1074

Keywords: antiproliferative activities, isoindigos, kinase inhibitory activities

[a]Laboratoire SEESIB, Université Blaise Pascal, UMR 6504 du CNRS, Aubière, France and [b]Novartis Institutes for Biomedical Research, Basel, Switzerland

Correspondence to Dr Pascale Moreau, PhD, Laboratoire SEESIB, Université Blaise Pascal, UMR 6504 du CNRS, 24 avenue des Landais, 63177 Aubière Cedex, France
Tel: +33 4 73 40 79 63; fax: +33 4 73 40 77 17;
e-mail: Pascale.MOREAU@univ-bpclermont.fr

Received 19 January 2007 **Revised form accepted** 19 March 2007

Introduction

Protein kinases play important roles in regulating most cellular functions such as proliferation/cell cycle, cell metabolism, survival/apoptosis, DNA damage repair, cell motility and response to the microenvironment. Recent investigations reveal that there are over 500 human kinases. Many kinase inhibitors are currently in different phases of clinical development. Moreover, some kinase inhibitors are already used for the treatment of different cancers: gefitinib and erlotinib are epidermal growth factor receptor inhibitors used for the treatment of nonsmall cell lung cancer, and imatinib mesylate is a c-Abl inhibitor used for treatment of chronic myeloid leukaemia (Fig. 1).

In the course of studies on the preparation of potential kinase inhibitors, we were interested in the synthesis of indolinones [1–4], more particularly indigoid derivatives. Indigo, indirubin and isoindigo, containing a bis-indole framework, have been isolated from various natural sources (Fig. 2).

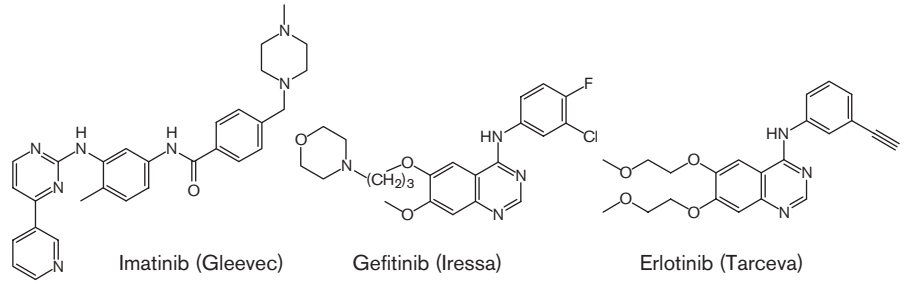
The bis-indole indirubin is the active ingredient of Danggui Longhui Wan, a mixture of plants used in traditional Chinese medicine to treat chronic diseases. Indirubins are potent inhibitors of several kinases such as glycogen synthase kinase (GSK)-3β and cyclin-dependent kinases (CDKs) [5]. Indirubin is known to interact with the ATP-binding site of CDK2, CDK5 and GSK-3 [6]. Moreover, structure–activity relationship studies have shown that 5-nitro and 5-bromoindirubin are more potent kinase inhibitors (GSK-3β, CDK1, CDK5) than their parent indirubin [5]. Indirubin and indigo are also potent aryl hydrocarbon receptor ligands [7,8].

The indolin-2-one skeleton is found in the structure of indirubin and isoindigo (Fig. 2). Indolin-2-one derivatives are usually known as ATP competitive inhibitors of receptor tyrosine kinase such as vascular endothelial growth factor receptor, fibroblast growth factor receptor and platelet-derived growth factor receptor [9,10] (e.g. SU6668, SU11248). Moreover, some imidazo[2,1-*b*]-thiazolylmethylene-2-indolinones such as compounds **A** or indolylmethylene-2-indolinones such as compounds **B** have been described as CDK1/cyclin B inhibitors [11] (Fig. 3). Recently, SU9516, possessing an indolin-2-one framework was described as an ATP competitive inhibitor of CDKs (Fig. 3) [12].

We have reported in previous papers the synthesis and in-vitro antiproliferative activities of 13 isoindigo derivatives bearing a sugar residue attached to one of the oxindole nitrogens and diversely substituted on the aromatic rings in the 5, 6 or 5′ positions (Fig. 4) [2,3]. Some of these glycosyl-isoindigo derivatives have shown significant cytotoxicities. To get an insight into the best substitution pattern required to get relevant biological activities and to identify the cellular target(s) involved in the in-vitro antiproliferative activity of these isoindigo derivatives, their inhibitory potencies toward a panel of 10 different kinases were examined. The kinases tested were either receptor tyrosine kinase, nonreceptor tyrosine kinases or

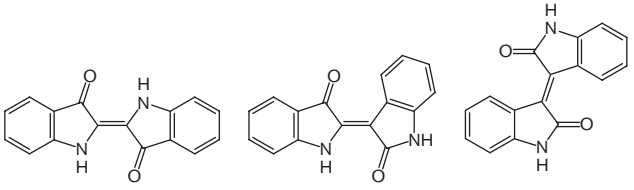
0959-4973

Fig. 1



Structures of imatinib, gefitinib and erlotinib.

Fig. 2



Structures of indigo, indirubin and isoindigo.

serine/threonine kinases. The receptor tyrosine kinases tested were KDR, Flt-3, IGF-1R, Tek, c-Met and RET. The nonreceptor tyrosine kinases tested were Src and Abl. The serine/threonine kinase tested were protein kinase A and CDK2. Molecular modelling experiments were also performed to investigate the binding interactions between the active site of CDK2 and the lead compound of this series.

Materials and methods

In-vitro kinase inhibition assays

The in-vitro kinase assays were performed in 96-well plates (30 ml) at ambient temperature for 15–45 min using the recombinant glutathione S-transferase-fused kinase domains (4–100 ng, depending on specific activity) prepared previously [13,14]. [γ^{33}P]ATP was used as phosphate donor and polyGluTyr-(4 : 1) peptide as acceptor. With the exception of PKA for which the heptapeptide Leu–Arg–Arg–Ala–Ser–Leu–Gly (known as Kemptide; Bachem, Bubendorf, Switzerland) was used as peptide substrate. Assays were optimized for each kinase using the following ATP concentrations: 1.0 µmol/l (c-Met and RET), 5.0 µmol/l (c-Abl), 8.0 µmol/l (Flt-3, KDR, IGF-1R, Tek) and 20.0 mmol/l (c-Src and PKA). The reaction was terminated by the addition of 20 µl of 125 mmol/l EDTA. Either 30 (c-Abl, c-Src, IGF-1R, RET) or 40 µl (all other kinases) of the reaction mixture was transferred onto Immobilon-polyvinylidene difluoride membrane (Millipore, Bedford, Massachusetts, USA), pre-soaked with 0.5% H_3PO_4 and mounted on a vacuum manifold. Vacuum was then applied and each well rinsed with 200 µl of 0.5% H_3PO_4. Membranes were removed and washed four times with 1.0% H_3PO_4 and once with ethanol. Dried membranes were counted after mounting in a Packard TopCount 96-well frame and with the addition of 10 µl/well of Microscint. For RET kinase assay, either glutathione S-transferase–wild-type RET (15 ng) or glutathione S-transferase–RET-Men2B protein (15 ng) was used. The CDK2/cyclin A assay was performed as described previously [15]. The IC_{50} values were calculated by linear regression analysis of the percentage inhibition.

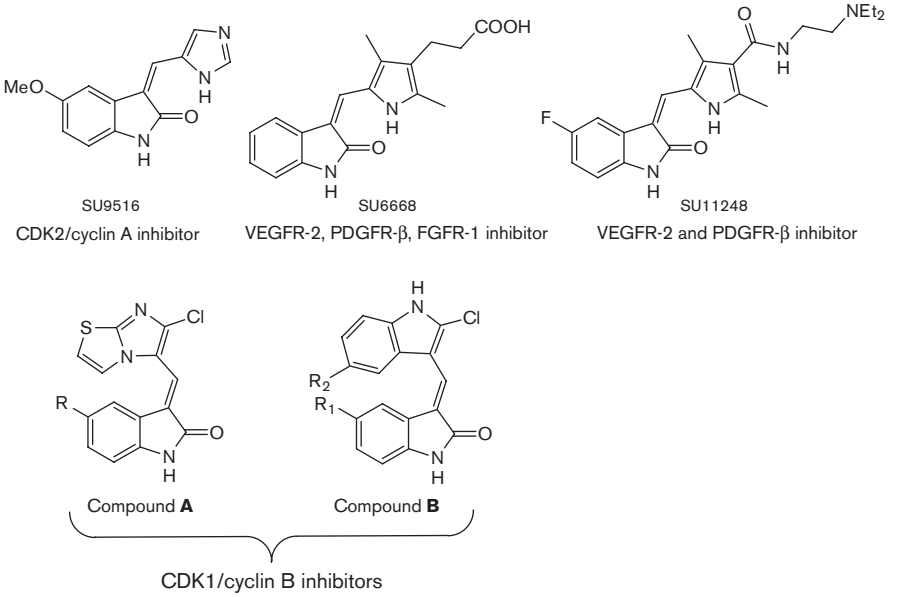
Molecular modelling

To investigate the binding interactions involved between CDK2 and compound **1** and **13**, we used the crystal structure 1KE7 [16] from the Protein Data Bank. All molecular mechanics calculations and molecular surface were performed by the Macromodel (Shroedinger, Portland, Oregon, USA) molecular modelling software. Energy minimization was done with AMBER force field [17,18] using the Truncated Newton Conjugate Gradient method.

Results and discussion

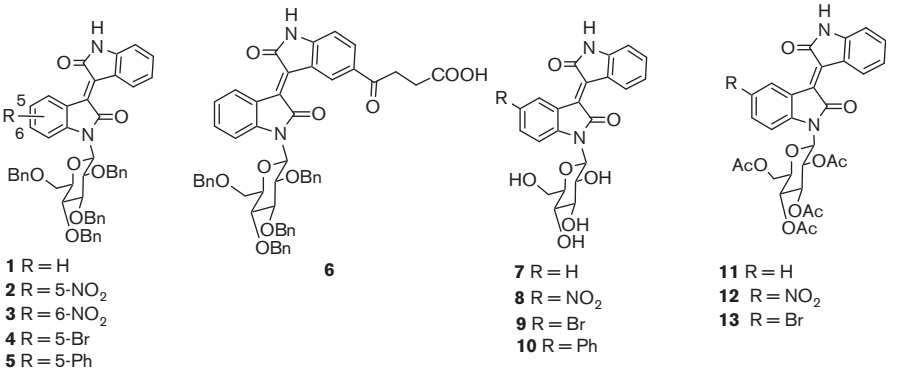
The kinases inhibition percentages, at a drug concentration of 10 µmol/l, of isoindigo and compounds **1–13** were determined (Table 1). The IC_{50} values were calculated for compounds **1–6** and **13** toward KDR, IGF-1R, c-Met, Ret, c-Src and CDK2/A (Table 1). Isoindigo was almost inactive toward all the tested kinases. On the contrary, some protected glycosyl-isoindigos (**1–6** and **13**) were efficient toward different kinases indicating that the presence of a protected sugar moiety is favorable to the interaction with the ATP-binding site of the target enzyme(s). Four categories of glycosyl-isoindigos can be distinguished according to their structure: the first one with the aromatic moiety substituted or not in the 5 or 6

Fig. 3



Indolin-2-one derivatives. CDK, cyclin-dependent kinase; VEGFR, vascular epithelial growth factor receptor; PDGFR, platelet-derived growth factor receptor; FGFR, fibroblast growth factor receptor.

Fig. 4



Iso-indigo derivatives.

Table 1 **Percentage of kinase inhibition at a drug concentration of 10 μmol/l**

Kinases	Isoindigo	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
KDR	18	43 (0.91)	60 (0.79)	67 (1.1)	59 (0.85)	58 (0.36)	49 (4.5)	1 (–)	4 (–)	7 (–)	0 (–)	17 (–)	0 (–)	87 (2.2)
Flt-3	14	0	0	1	0	0	11	10	12	11	0	15	0	45
IGF-1R	30	42 (1.0)	70 (3.2)	64 (3.0)	76 (2.7)	60 (1.3)	13 (>10)	0 (–)	0 (–)	3 (–)	0 (–)	26 (–)	0 (–)	77 (1.6)
Tek	3	22	41	48	38	34	34	6	10	8	0	16	4	38
c-Met	28	90 (0.43)	89 (0.51)	88 (0.79)	92 (0.34)	87 (0.48)	81 (2.8)	6 (–)	16 (–)	18 (–)	0 (–)	22 (–)	0 (–)	69 (9.5)
Ret	33	86 (1.7)	86 (2.0)	85 (3.3)	91 (1.5)	88 (2.1)	89 (6.9)	0 (–)	19 (–)	17 (–)	0 (–)	38 (–)	0 (–)	79 (9.6)
c-Src	5	97 (4.0)	98 (3.8)	98 (3.7)	97 (2.6)	98 (3.4)	96 (5.4)	0 (–)	0 (–)	4 (–)	0 (–)	33 (–)	0 (–)	85 (>10)
c-Abl	11	33	33	32	38	43	24	0	0	15	0	16	0	48
PKA	0	2	0	0	29	5	0	0	0	3	0	0	0	12
CDK2/A	21	86 (0.14)	73 (0.76)	72 (0.13)	87 (0.20)	81 (0.21)	57 (4.2)	0 (–)	0 (–)	2 (–)	0 (–)	25 (–)	0 (–)	99 (1.2)

IC_{50} values (μmol/l) are given in parantheses. PKA, protein kinase A; CDK, cyclin-dependent kinase.

positions and with the sugar residue substituted with benzyl groups (**1–5**), the second one with the aromatic moiety substituted or not in the 5 position and with a nonprotected sugar residue (**7–10**), the third one with the aromatic moiety substituted or not in the 5 position and with the sugar residue substituted with acetyl groups (**11–13**) and the last one substituted with a carboxylic acid side chain on the upper aromatic moiety (**6**).

Compounds **1–5** have a similar inhibition profile. They were particularly active toward CDK2 with IC_{50} values in the 0.1 – 0.2 μmol/l range, except compound **2** for which the IC_{50} value was higher. They were all efficient toward KDR and c-Met with IC_{50} values ≤ 1 μmol/l. Moreover, these compounds were active toward IGF-1R, RET and c-src with IC_{50} values in the micromolar range. These results indicated that in the benzylated series, the substitution pattern on the lower aromatic moiety does not deeply affect the kinase inhibition profiles.

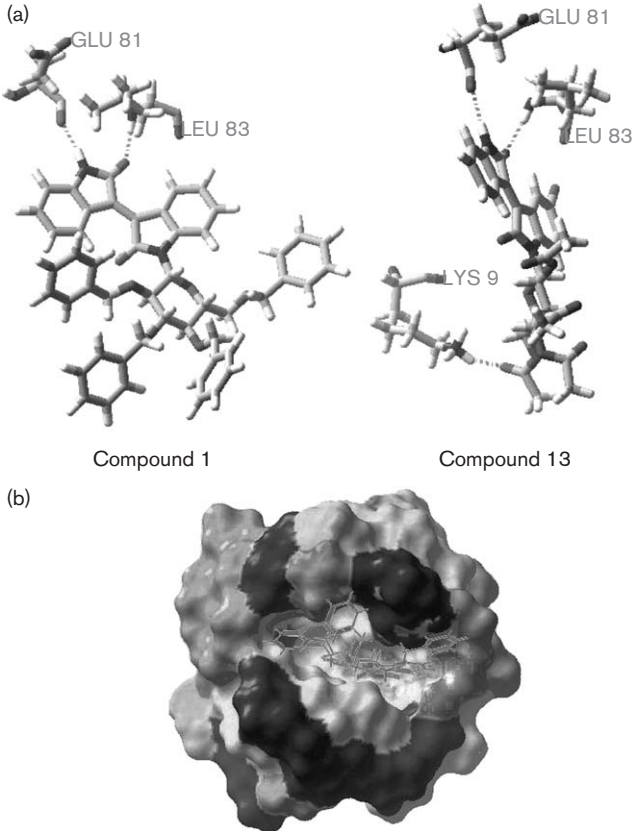
Compound **6** was in general a weaker kinase inhibitor than compounds **1–5**, indicating that the substitution of the upper aromatic moiety with a carboxylic acid side chain is detrimental to kinase inhibition.

Compounds **7–10** were almost inefficient toward all the tested kinases indicating that the presence of a nonprotected sugar residue is damaging for kinase inhibition.

Compounds **11–13**, acetylated on the sugar residue, exhibited different inhibition profiles. Compound **11**, unsubstituted on the aromatic moiety has no significant inhibitory activities toward the kinases tested. Compound **12** bearing a nitro group in the 5 position was completely inactive toward the kinases tested, whereas compound **13** bearing a bromine atom in the 5 position clearly inhibited kinases such as KDR, IGF-1R and CDK2 with IC_{50} values in the micromolar range. These results indicated that in the acetylated series, the inhibition of the kinases tested strongly depends upon the nature of the substituent in the 5 position.

In summary, compounds **1–5** with the aromatic moiety substituted or not in the 5 or 6 positions and with the sugar residue substituted with benzyl groups are potent inhibitors of CDK2. Therefore, molecular modelling experiments were carried out to investigate the binding interactions between the active site of CDK2 and compound **1**. Moreover, the binding interactions between compound **13** and CDK2 were also studied to demonstrate the differences between both categories of compounds. For these studies, the 1KE7 structure for CDK2 [16] was used.

Fig. 5



(a) Binding interactions between compounds **1** and **13** and the active site of CDK2, (b) hydrophobic surface representation of CDK2 in complex with compound **1** (hydrophobic residues are colored in light grey, polar residues in grey and charged residue in black).

For compound **1**, two hydrogen bonds were observed (Fig. 5a). The oxindole ring system interacted with CDK2 in a similar manner to that observed for indolin-2-one derivatives. Two hydrogen bonds were formed between the upper lactam moiety and CDK2. Specifically, the amide NH was H-bonded to the backbone carbonyl of Glu-81 and the amide carbonyl oxygen was H-bonded with the backbone NH of Leu-83. Two hydrogen bonds were also observed between ATP and the same residues of CDK2.

For compound **13**, three hydrogen bonds were observed (Fig. 5a). Two hydrogen bonds as already observed with the lactam moiety of compound **1**, and an extra hydrogen bond which was established between the carbonyl oxygen of 6′-acetyl group and the side chain NH of Lys-9.

Despite less hydrogen-binding interactions involved, compared with compound **13**, compound **1** was a more efficient CDK2 inhibitor. Indeed, the complex compound **1**–CDK2 was stabilized by hydrophobic interactions

Table 2 Antiproliferative activities of compounds 6, 11 and 13 (IC_{50} in μmol/l)

Compounds	Fibroblast	L929	A549	DLD-1	M4Beu	MCF-7	PA 1	PC-3
6	4.7 (5.1)	6.5 (6.1)	12.4 (12.4)	6.0 (5.3)	7.0 (7.1)	8.0 (5.1)	2.5 (2.7)	4.7 (5.0)
11	Inactive	8.7 (8.3)	Inactive	17.3 (11.7)	Inactive	25.1 (17.9)	6.2 (5.5)	25 (21.8)
13	Inactive	8.1 (7.3)	Inactive	17.0 (13.5)	Inactive	24.0 (12.0)	6.1 (5.5)	3.7 (3.1)

Inactive means an IC_{50} value >30 μmol/l (Rezazurin test results are given first and Hoechst test results are given in parantheses).

between the benzyl groups of the sugar moiety and the hydrophobic binding pocket of the enzyme (Fig. 5b).

In previous studies, the in-vitro antiproliferative activities of these compounds (**1–13**) were determined toward a panel of human solid cancer cell lines (PC-3, DLD-1, MCF-7, M4Beu, A549, PA1), a murine cell line (L929) and a human fibroblast primary culture [3] (Table 2). Compounds **1–5**, which exhibited potent inhibitory activities toward various kinases, have not shown any antiproliferative activity toward the cell lines tested. This could be due to a poor cellular penetration. In contrast, compound **6**, which weakly inhibited various kinases and is substituted with a carboxylic acid side chain that counterbalanced the lipophilicity and should increase its cellular penetration, was cytotoxic toward some of the cell lines tested. Compounds **7–10** and **12** that exhibited no cytotoxicity toward the cell lines tested were also inactive toward the kinases tested. Compound **11**, which was slightly, cytotoxic did not show any inhibitory activity toward the kinases tested indicating that other cellular target(s) could be involved in the cytotoxicity of this compound. In contrast, compound **13**, which inhibited significantly various kinases, was cytotoxic toward the cell lines tested indicating that these kinases could be involved in its cytotoxicity.

Conclusion

The kinases inhibition potencies and in-vitro antiproliferative activities of diversely substituted glycosyl-isoindigo derivatives were examined. The results of these structure–activity relationship studies have shown that all the benzylated compounds substituted or not on the lower aromatic ring exhibited a similar inhibition profile toward the kinases tested. None of them, however, were cytotoxic toward the cell lines tested. The presence of the benzyl groups on the sugar part improved kinase inhibition. Concerning CDK2 inhibition, the benzyl groups allowed hydrophobic interactions with the binding pocket (Fig. 5b). Nevertheless, the high lipophilicity of these compounds was detrimental to cellular penetration. In contrast, the presence of the carboxylic acid side chain on the aromatic moiety provided antiproliferative potencies to compound **6** despite lower kinase inhibitory activities compare with those observed for compounds **1–5**.

The acetylated compounds have shown different inhibition profiles. The substitution of the aromatic moiety by a nitro group (compound **12**) was detrimental to kinase inhibition, whereas the presence of a bromine atom on the heterocyclic part (compound **13**) strongly enhanced kinase inhibition. The presence of acetyl groups on the sugar residue seemed to be favorable to the cellular penetration as compound **13** has shown significant in-vitro antiproliferative activities.

References

1 Messaoudi S, Sancelme M, Polard-Housset V, Aboab B, Moreau P, Prudhomme M. Synthesis and biological evaluation of oxindoles and benzimidazolinones derivatives. *Eur J Med Chem* 2004; **39**: 453–458.

2 Sassatelli M, Saab E, Anizon F, Prudhomme M, Moreau P. Synthesis of glycosyl-isoindigo derivatives. *Tetrahedron Lett* 2004; **45**: 4827–4830.

3 Sassatelli M, Bouchikhi F, Messaoudi S, Anizon F, Debiton E, Barthomeuf C, *et al.* Synthesis and antiproliferative activities of diversely substituted glycosyl-isoindigo derivatives. *Eur J Med Chem* 2006; **41**: 88–100.

4 Sassatelli M, Debiton E, Aboab B, Prudhomme M, Moreau P. Synthesis and antiproliferative activities of indolin-2-one derivatives bearing amino acid moieties. *Eur J Med Chem* 2006; **41**:709–716.

5 Leclerc S, Garnier M, Hoessel R, Marko D, Bibb JA, Snyder GL, *et al.* Indirubins inhibit glycogen synthase kinase-3β and CDK5/P25, two protein kinases involved in abnormal Tau phosphorylation in Alzheimer's disease. *J Biol Chem* 2001; **276**:251–260.

6 Hoessel R, Leclerc S, Endicott JA, Nobel MEM, Lawrie A, Tunnah P *et al.* Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases. *Nat Cell Biol* 1999; **1**:60–67.

7 Adachi J, Mori Y, Takigami H, Fujino J, Kitagawa H, Miller CA III, *et al.* Indirubin and indigo are potent aryl hydrocarbon receptor ligands present in human urine. *J Biol Chem* 2001; **276**:31475–31478.

8 Knockaert M, Blondel M, Bach S, Leost M, Elbi C, Hager G, *et al.* Independent actions on cyclin-dependent kinases and aryl hydrocarbon receptor mediate the antiproliferative effects of indirubins. *Oncogene* 2004; **23**:4400–4412.

9 Laird AD, Cherrington JM. Small molecule tyrosine kinase inhibitors: clinical development of anticancer agents. *Exp Opin Invest Drugs* 2003; **12**: 51–64.

10 Sun L, Liang C, Shirazian S, Zhou Y, Miller T, Cui J, *et al.* Discovery of 5-[5-fluoro-2-oxo-1,2-dihydroindol-(3*Z*)-ylidenemethyl]-2,4dimethyl-1*H*-pyrrole-3-carboxylic acid (2-diethylaminoethyl)amide, a novel tyrosine kinase inhibitor targeting vascular endothelial and platelet-derived growth factor receptor tyrosine kinase. *J Med Chem* 2003; **46**: 1116–1119.

11 Andreani A, Cavalli A, Granaiola M, Leoni A, Locatelli A, Morigi R, *et al.* Imadazo[2,1-*b*]thiazolylmethylene and indolylmethylene-2-indolinones: a new class of cyclin-dependent kinase inhibitors. Design, synthesis, and CDK1/cyclin B inhibition. *Anticancer drug Des* 2000; **15**: 447–452.

12 Moshinsky DJ, Bellamacina CR, Boisvert DC, Huang P, Hui T, Jancarik J, *et al.* SU9516: biochemical analysis of cdk inhibition and crystal structure in complex with cdk2. *Biochem Biophys Res Commun* 2003; **310**: 1026–1031.

13 Wood JM, Bold G, Buchdunger E, Cozens R, Ferrari S, Frei J, *et al.* PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. *Cancer Res* 2000; **60**:2178–2189.

14 Bold G, Altmann K-H, Frei J, Lang M, Manley PW, Traxler P, *et al*. New anilinophtalazines as potent and orally well absorbed inhibitors of the VEGF receptor tyrosine kinases useful as antagonists of tumor-driven angiogenesis. *J Med Chem* 2000; **43**: 2310–2323. Erratum in: *J Med Chem* 2000; 43:3200.
15 Soni R, Fretz H, Muller L, Schoepfer J, Chaudhuri B. Novel CDK inhibitors restore TGF-β sensitivity in CDK4 overexpressing epithelial cells. *Biochem Biophys Res Commun* 2000; **272**:794–800.
16 Neal Bramson H, Corona J, Davis ST, Dickerson SH, Edelstein M, Frye SV, *et al*. Oxindole-based inhibitors of cyclin-dependent kinase 2 (CDK2): design, synthesis, enzymatic activities and X-ray crystallographic analysis. *J Med Chem* 2001; **44**:4339–4358.
17 Weiner SJ, Kollman PA, Case DA, Singh UC, Ghio C, Alagona G, *et al*. A new force field for molecular mechanical simulation of nucleic acids and proteins. *J Am Chem Soc* 1984; **106**:765–784.
18 Cornell WD, Cieplak P, Bayly CI, Gould IR, Merz KM, Ferguson DM, *et al*. A second generation force field for the simulation of proteins, nucleic acids, and organic molecules. *J Am Chem Soc* 1995; **117**: 5179–5197.