A shikonin derivative, β-hydroxyisovalerylshikonin, is an ATP-non-competitive inhibitor of protein tyrosine kinases

Kazuyasu Nakaya[a] and Tadashi Miyasaka[a]

Studies of the mechanism of action of a shikonin derivative, β-hydroxyisovalerylshikonin (β-HIVS), have revealed that β-HIVS inhibits the protein tyrosine kinase (PTK) activities of the receptor for epidermal growth factor and v-Src. In this review, we compare the characteristics of the inhibition of PTK activity by β-HIVS with those of other inhibitors of PTKs. The chemical structure of β-HIVS is completely different from that of ATP and it does not resemble any of the PTK inhibitors reported to date, except that it includes the benzilidene moiety. In contrast to most PTK inhibitors, the mechanism of inhibition by β-HIVS is non-competitive with respect to ATP, but competitive with respect to its peptide substrate. This feature of the mechanism of inhibition of PTK by β-HIVS suggests that it might be useful in a clinical setting with other PTK inhibitors. When Bcr–Abl-positive, human leukemia K562 cells were treated simultaneously with β-HIVS and STI571 (Gleevec), these compounds had a synergistic effect on both the induction of apoptosis in K562 cells and the inhibition of the phosphorylation activity of PTK, probably because the mechanism of interference with phosphorylation by β-HIVS and the binding site of β-HIVS are different from those of STI571. *Anti-Cancer Drugs* 14:683–693 © 2003 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2003, **14**:683–693

Keywords: β-hydroxyisovalerylshikonin, cancer, inhibitor of tyrosine kinase, protein tyrosine kinase, shikonin

[a]School of Pharmaceutical Sciences, Showa University, Tokyo, Japan.

Correspondence to K. Nakaya, Laboratory of Biological Chemistry, School of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan.
Tel: +81 3 3784 8217; fax: +81 3 3784 8219;
e-mail: nakaya@pharm.showa-u.ac.jp

Received 10 June 2003 **Revised form accepted** 9 July 2003

Introduction

Protein tyrosine kinases (PTKs) play important roles in a wide variety of cellular signal-transduction pathways and regulate a variety of cellular activities, such as cell growth, mitogenesis, development, differentiation and cell death [1,2]. Defects in the normal functioning of PTKs are closely associated with carcinogenesis [1,3]. Furthermore, the products of many oncogenes are PTKs. Thus, PTKs are attractive targets for anticancer drugs. In the last decade, a variety of low-molecular-weight inhibitors of PTKs have been developed as candidates for anticancer drugs and some are or soon will be in clinical trials [2–7]. In terms of clinical applicability, one of the most useful anticancer drugs developed to date is STI571 (Gleevec), which is used in the treatment of patients with chronic myelocytic leukemia (CML) [4,8]. In patients with CML, a reciprocal translocation between chromosomes 9 and 22, t(9:22), results in a Bcr–Abl fusion gene whose product has elevated PTK and transforming activity [9]. The PTK activity of the protein product of Bcr–Abl, p210, is essential for its transforming activity [10] and ectopic expression of p210 induces a CML-like syndrome in mice [11,12]. Furthermore, cells that express Bcr–Abl, such as K562 cells, are resistant to the apoptotic effects of antileukemic drugs [13,14]. One of the reasons for this resistance is the fact that the product of the Bcr–Abl gene inhibits apoptosis due to diverse stimuli by blocking release of cytochrome *c* from mitochondria [15]. The product of Bcr–Abl (p210) is, therefore, an attractive target for the design of drugs for treatment of CML. STI571 is a drug that was designed by reference to the crystal structure of the ATP-binding site of protein kinases [6], and it was shown to inhibit the protein kinase activity of p210 and to induce complete remission in most patients with CML [16]. In its inhibition of the PTK activity of p210, STI571 inhibits the tyrosine kinase activities of platelet-derived growth factor (PDGF) and c-kit [17,18]. Thus, STI571 appears to be effective for the clinical treatment of fibroblast-derived tumors and gastrointestinal stromal tumors, in which PDGF and c-kit, respectively, are overexpressed [19,20].

The level of expression of epidermal growth factor receptor (EGFR) is elevated in a large variety of tumors and this receptor is also a target for many low-molecular-weight inhibitors of PTK. Among the various inhibitors of the PTK activity of EGFR, ZD1839 (Iressa) has been clinically tested as an orally administrated drug for the treatment of non-small cell lung cancer [21]. Although a phase II trial of ZD1839 in patients with lung cancer resulted in significant improvement in a limited number of patients, the phase III trial in patients with lung cancer failed to prolong the lives of patients in the trial [22]. The failure of ZD1839 to cure lung cancer can be interpreted to mean that the cancer cells are not solely dependent on the activity of EGFR for their survival. It

0959-4973 © 2003 Lippincott Williams & Wilkins DOI: 10.1097/01.cad.0000092781.37568.df

seems, therefore, that treatment with drugs targeted to EGFR alone is not sufficient to inhibit the proliferation of cancer cells.

We showed previously that β-hydroxyisovalerylshikonin (β-HIVS), which can be isolated from the roots of the traditional oriental herb *Lithospermum radix*, inhibited the growth of various lines of human tumor cells at low concentrations between 10^{-8} to 10^{-6} M and induced apoptosis in leukemia HL-60 cells through a mechanism different from those of conventional inducers of apoptosis [23]. Extracts of the roots of *L. radix*, which contain shikonin and various derivatives of shikonin, were used in ancient Japan for the preparation of ointments for the treatment of cuts and burns. They were also taken internally as an antidote, and as an antipyretic and anti-inflammatory agent. More recently, shikonin was reported to exhibit antitumor activity in mice implanted with sarcoma 180 tumor cells [24]. The mechanisms responsible for these activities of shikonin and its derivatives are unknown, but we have shown that β-HIVS specifically inhibits the PTK activities of EGFR and v-Src [25]. In this review, we provide a summary of the recent development of inhibitors of PTKs and a comparison of the characteristics of β-HIVS with those of other inhibitors of PTKs.

Classification of PTK inhibitors

The inhibitors of PTKs can be divided into several groups (Fig. 1). The first group consists of compounds with a phenolic hydroxyl group, which resembles the side chain of tyrosine residues. This group includes erbstatin [26,27] and lavendastin-A [28], which are non-selective inhibitors of EGFR. Methyl 2,5-dihydroxycinnamate was synthesized as an analog of erbstatin [29]. The first synthetic inhibitor of PTK to be developed was tyrphostin. This compound is designated as a tyrosine mimetic and it interacts with the active site of PTKs [30]. TX-1123 was synthesized as a compound with lower mitochondrial toxicity than the tyrphostin derivative [31], tyrphostin AG17 [32]. AG537 is a dimeric derivative of tyrphostin and was designed to bind to the dimerized active form of EGFR [33]. Methyl 7,8-dihydroxyisoquinoline-3-carboxylate is a bicyclic phenol [34].

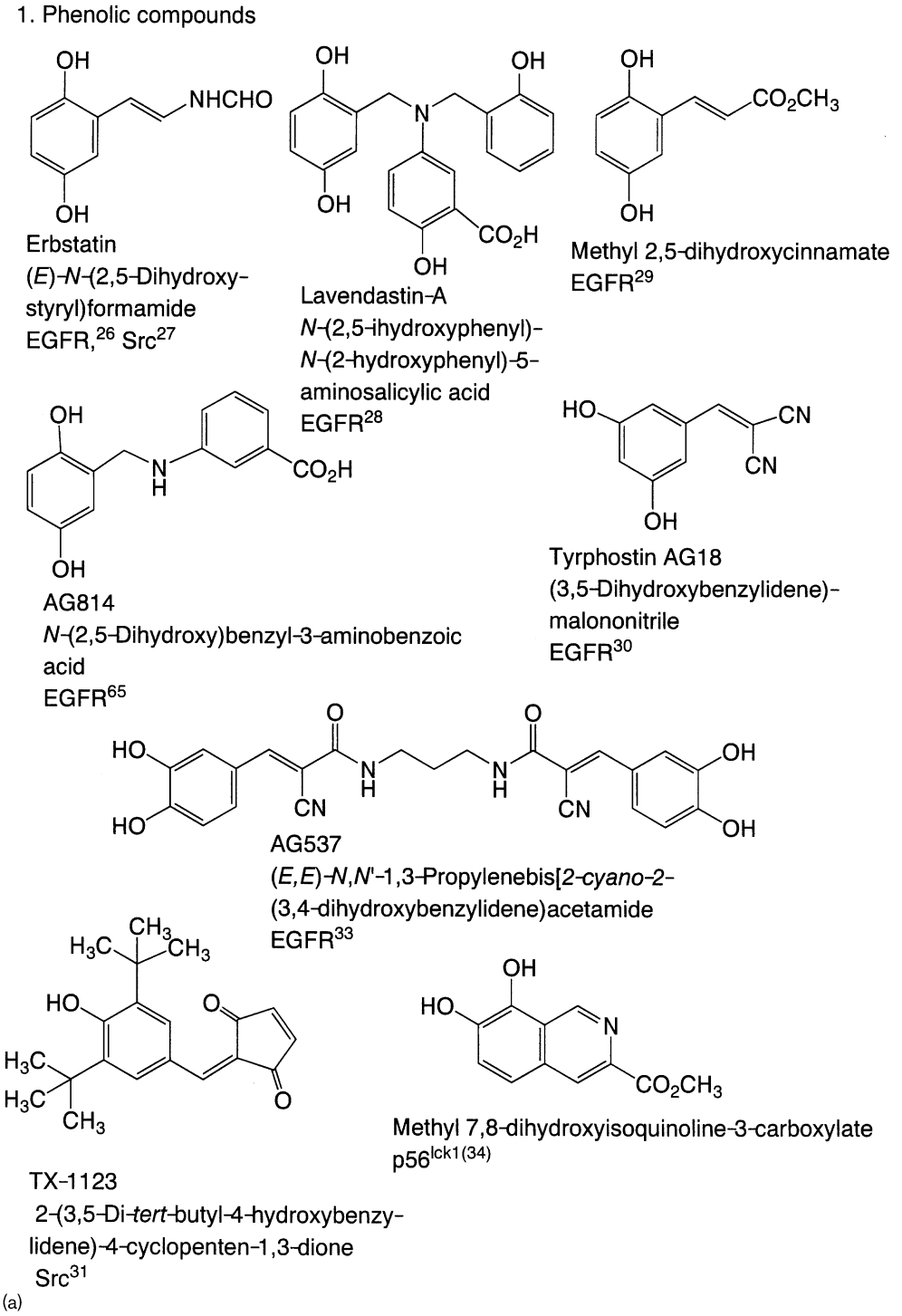
The second group consists of flavones, such as quercetin [35–37], and isoflavones, such as genistein [38]. The third group, pyrrolopyrimidines, was identified by random screening as lead structures for inhibitors of PTKs [7,39]. This group includes PP1 and PP2 [40], PKI166 [41,42], and 4-(phenylamino)-7*H*-pyrrolo[2,3-*d*]pyrimidine [43]. The identification of useful derivatives of quinazoline, the fourth group, by large-scale screening, combined with molecular modeling of the catalytic sites of PTKs and of compounds that might bind to such sites, markedly improved the potency and specificity of inhibitors of PTKs. Quinazoline derivatives that are being tested for efficacy include PD153035 [44], PD0165557 [45], ZD1839 (Iressa) [21,46], OSI774 [47], GW572016 [48,49] and CI1033 [50]. Further investigations of quinazoline in terms of structure–activity relationships on quinazolines resulted in the synthesis of the fifth group, the pyridopyrimidines [39], such as PD158780 [45], PD173074 [51], PD173955 [52] and PD180970 [53,54]. Group six, consisting of phenylaminopyrimidines such as STI571, was also identified by large-scale screening combined with investigations of structure–activity relationships of pyridopyrimidines [39]. Most of the PTK inhibitors described above inhibit EGFR or members of the Src family, as indicated in Figure 1. In contrast to these PTK inhibitors that inhibit EGFR and members of the Src family, oxindoles (group seven), such as PD146568, SU5416 and SU6668 [55–58], have selective inhibitory activity against receptors for vascular endothelial growth factor (VEGFR). The inhibition of EGFR by PD146568 is irreversible and non-competitive with respect to ATP, suggesting that this drug might bind covalently to a sulfhydryl residue near the catalytic site [56]. PD173074 inhibits the tyrosine kinase activity of fibroblast growth factor receptor (FGFR) [51]. Oxindole inhibitors, such as SU5416 and PD173074, suppress angiogenesis, and induce the destruction of the vasculature that is needed for the growth and proliferation of tumor cells *in vivo* [51,57].

There are other inhibitors of PTK that cannot be classified into the groups mentioned above. For example, PTK787/ZK222584 is a phthalazine derivative [59,60]. Herbimycin A, a specific inhibitor of intracellular v-Src whose effects are irreversible, is a benzoquinoid ansamycin antibiotic [61]. Clavilactones, isolated from fungal metabolites, are benzoquinoid 10-membered-ring macrolides that include a 2,3-epoxy-γ-lactone [62]. We were the first to demonstrate that shikonin and its derivative β-HIVS inhibit the activities of PTKs such as EFGR and v-Src [25]. The inhibitory activity of β-HIVS was the strongest among the shikonin derivatives that we tested [25].

Characteristics of the mechanism of action of PTK inhibitors

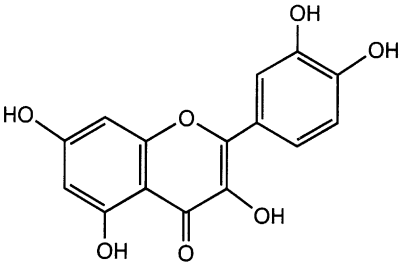
The active center of a protein kinase (PK) consists of an ATP-binding site and a binding site for the peptide substrate [4,7,63,64]. Most of the inhibitors of PTKs reported to date are competitive inhibitors with respect to ATP, as shown in Table 1. This observation suggests that the inhibitors bind to the ATP-binding site in the catalytic domain of the enzymes. The inhibition of the PTK activity of EGFR by genistein [38], erbstatin [26], lavendastin-A [28], AG814 [65], PD158780 and PD0165557 [44] appears to be competitive with respect to ATP, as does inhibition of v-Src by quercetin [35].

Fig. 1

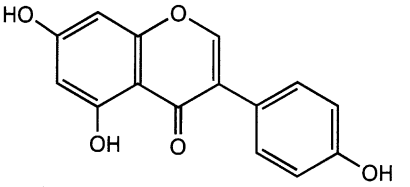


Examples of low-molecular-weight inhibitors of PTKs. References as superscripts.

2. Flavones and isoflavones

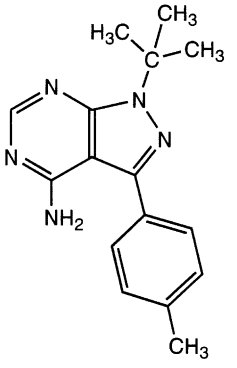


Quercetin
Src[35], PKC,[36] PI3 kinase[37]

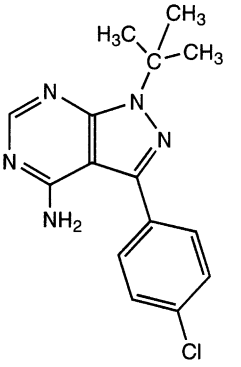


Genistein
EGFR[38]

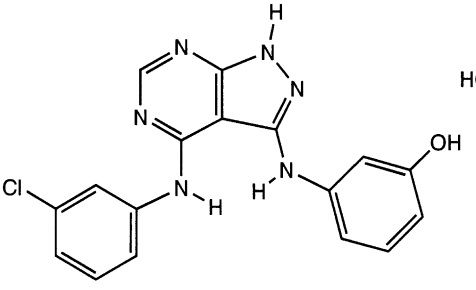
3. Pyrazolopyrimidines and pyrrolopyrimidines



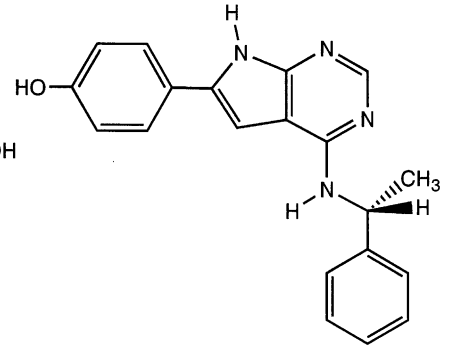
PP1
1-*tert*-Butyl-3-(4-methylphenyl)-
1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine
Lck,[40] Fyn T[40]



PP2
1-*tert*-Butyl-3-(4-chlorophenyl)-
1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine
Lck,[40] Fyn T[40]



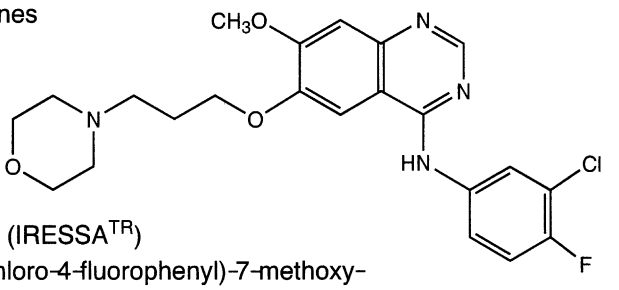
N-[4-(3-Chloroanilino)-1*H*-
pyrazolo[3,4-*d*]pyrimidin-3-yl]-
3-amino]phenol
EGFR[43]



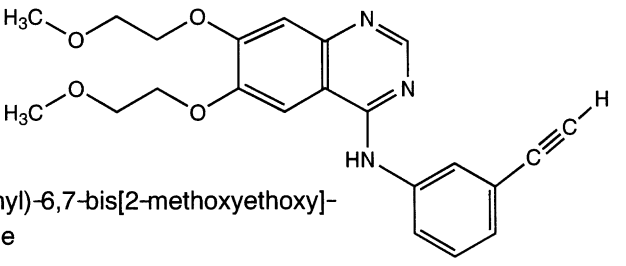
PKI166
(*R*)-4-[4-(1-Phenylethylamino)-7*H*-pyrrolo-
[2,3-*d*]pyrimidin-6-yl]phenol
EGFR[41,42]

(b)

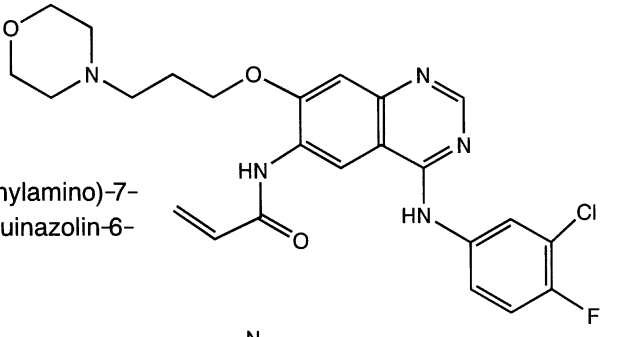
4. Quinazolines



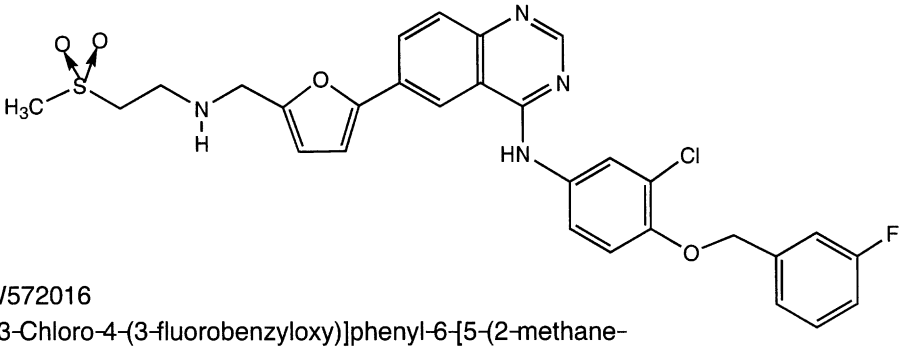
ZD1839 (IRESSA^{TR})
N -(3-Chloro-4-fluorophenyl)-7-methoxy-6-[3-(morpholino)propoxy]quinazolin-4-amine (INN: Gefitinib)
EGFR[21]



OSI774
N -(3-Ethynylphenyl)-6,7-bis[2-methoxyethoxy]-quinazolin-4-amine
EGFR[47]



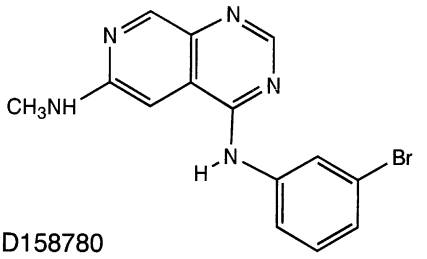
CI1033
N-{4-(3-Chloro-4-fluorophenylamino)-7-[3-(4-morpholino)propoxy]quinazolin-6-yl}-acrylamide
EGFR[50]



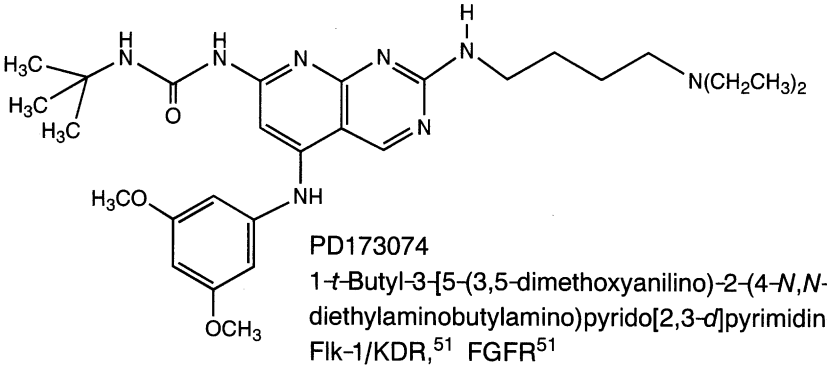
GW572016
N-[3-Chloro-4-(3-fluorobenzyloxy)]phenyl-6-[5-(2-methanesulfonylethylaminomethyl)furan-2-yl]quinazolin-4-amine
EGFR[49]

(c)

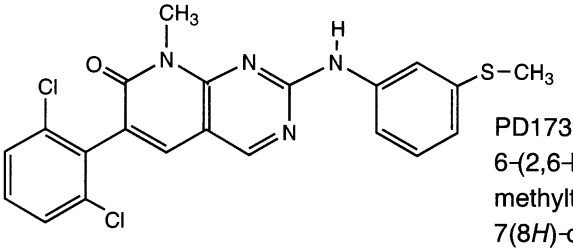
5. Pyridopyrimidines



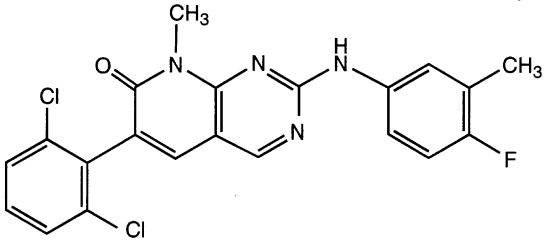
PD158780
4-(3-Bromoanilino-6- (methylamino)-
pyrido[3,4-*d*]pyrimidine
EGFR[45]



PD173074
1-*t*-Butyl-3-[5-(3,5-dimethoxyanilino)-2-(4-*N*,*N*-
diethylaminobutylamino)pyrido[2,3-*d*]pyrimidin-7-yl]urea
Flk-1/KDR,[51] FGFR[51]



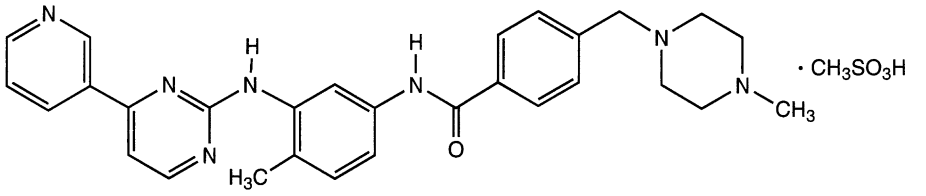
PD173955
6-(2,6-Dichlorophenyl)-8-methyl-2-(3-
methylthioanilinopyrido[2,3-*d*]pyrimidin-
7(8*H*)-one
Lck,[52] Fyn T[52]



PD180970
6-(2,6-Dichlorophenyl)-2-(4-fluoro-3-methylanilino)-
8-methylpyrido[2,3-*d*]pyrimidin-7(8*H*)-one
c-Src,[53] Bcr-Abl[54]

(d)

6. Phenylaminopyrimidines

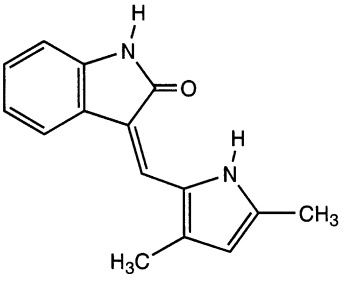


STI571 (Gleevec^{TR})

4-(4-Methyl-1-piperazinomethyl)-*N*-[4-methyl-3-[4-(pyridin-3-yl)pyrimidin-2-ylamino]phenyl]benzamide monomethanesulfonate (INN: Imatinib Mesylate)

Bcr-Abl,[7,8,66] PDGFR,[7,8] c-Kit[7]

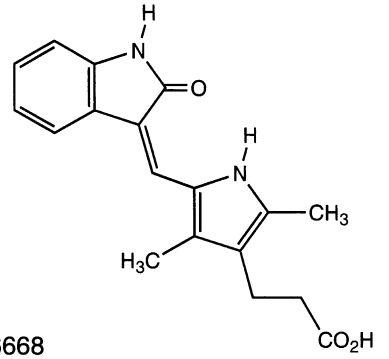
7. Oxindoles and Indoles



SU5416

(*Z*)-3-(3,5-Dimethylpyrrol-2-yl)methylidene-2,3-dihydroindol-2(1*H*)one

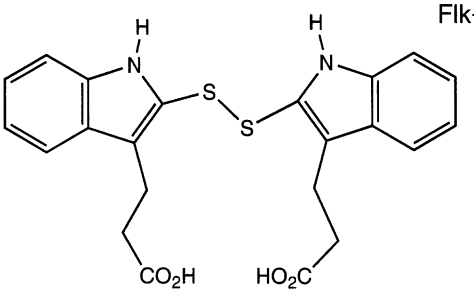
Flk-1/KDR,[57] c-Kit[58]



SU6668

(Z)-3-[2-(2,3-Dihydro-2(1*H*)-oxoindol-3-ylidene)methyl-3,5-dimethylpyrrol-4-yl]-propionic acid

Flk-1/KDR,[55] PDGFR,[55,58] FGFR,[55,58] cKit[58]



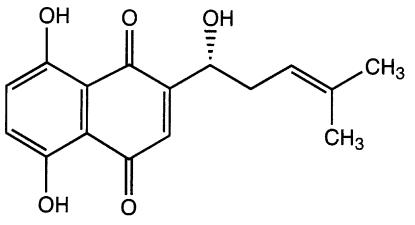
PD146568

2,2'-Dithiobis[3-(1*H*-indol-3-yl)propionic acid)

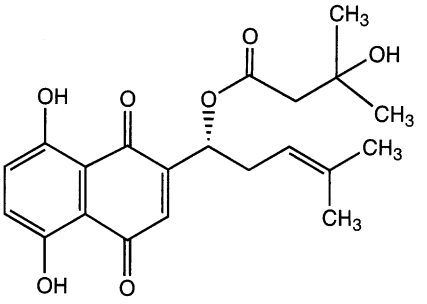
EGFR,[2,56] v-Src[2,56]

(e)

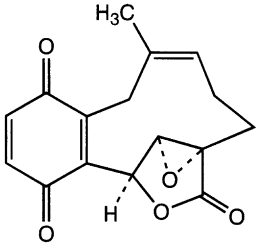
8. Others (Quinone Derivatives)



Shikonin
EGFR,[25] v-Src[25]



β-Hydroxyisovalerylshikonin
(β-HIVS)
EGFR,[25] v-Src[25]



Clarrilactone CB
EGFR,[62] PDGFR,[62] Flt-1,[62] v-Src[62]

(f)

Table 1 Competition by inhibitors of PTKs with respect to ATP and the substrate protein

ATP competitive	Erbstatin [65], Tyrphostin [65], AG814 [66], Quercetin [35], Genistein [38], Lavendastin-A [6], 4-(3-bromoanilino)-quinazoline [44], OSI774 [47], STI571 [4,8], PD158780 [45], PD0166326 [53], PD180970 [53,54], 4-(phenylamino)-7*H*-pyrrolo[2,3-*d*]pyrimidine [43], SU6668 [55]
ATP non-competitive	
substrate competitive	β-HIVS [25], 5-*S*-glutathionyl-*N*-β-alanyl-L-dopa [69], Tyrphostins [30], AG537 [65]
substrate-non competitive	Clavilactone CB [62]

STI571 and SU6668 compete with ATP for the ATP-binding site of the catalytic domain of the product of the Bcr–Abl gene and of FGFR, respectively [4,55]. The crystal structure of the kinase domain of the c-Abl protein in a complex with STI-571 indicates that STI-571 binds specifically to an inactive and unphosphorylated form of the protein, thereby freezing the kinase in an inactive conformation [66]. Inhibition by ZD1839 is competitive with respect to ATP and seems to be non-competitive with respective to the protein substrate, as deduced from an analysis with an analog, 4-(3-chloroanilino)quinazoline [67]. In contrast to these ATP-competitive inhibitors of PTKs, β-HIVS does not compete with a binding site with ATP, but competes with the substrate peptide, an observation that suggests β-HIVS binds to the peptide-binding site in the catalytic domain [25]. Derivatives of hydroxynaphthalene, such as 2-carbonyl-3,6-dihydroxy-naphthalene, have already been developed as ATP-non-competitive inhibitors of PTK [68]. Moreover, the proposal that 5-*S*-glutathionyl-*N*-β-alanyl-L-dopa inhibits PTK in an ATP-non-competitive and protein substrate-competitive manner is reasonable, since the structure of the inhibitor is similar to that of a tyrosine residue, thereby allowing it to bind to the protein substrate-binding site [69].

Characteristics of inhibition by β-HIVS

In contrast to most of the PTK inhibitors reported to date, β-HIVS has a chemical structure that bears no resemblance to ATP. However, it includes a benzilidene moiety similar to those in erbstatin, tryphostin, flavones and isoflavones. A very restricted number of PKs is inhibited by β-HIVS. When a lysate of SR-3Y1 cells that express v-Src was treated with β-HIVS, only the autophosphorylating activity of v-Src was inhibited [25]. The activities of PKA and PKC were almost completely

unaffected by β-HIVS [25]. In addition to that of v-Src, the PTK activity of EGFR was inhibited by β-HIVS with an IC_{50} (concentration for 50% inhibition) of approximately 700 nM [25]. The PTK activities of VEGFRs, such as KDR/Flk-1 and Flt-1, are also inhibited by β-HIVS, but to a lesser extent than those of EGFR and v-Src [25]. The fact that the inhibitory activity of β-HIVS against EGFR and v-Src was much stronger that of shikonin indicates that the side chain of β-HIVS contributes to an increase in the inhibitory activity against PTKs. These results also strongly suggest that much more potent and more specific inhibitors of PTK will be produced when changes are made in the side chain of shikonin.

In contrast to most PTK inhibitors, β-HIVS does not compete with ATP [25]. This feature of β-HIVS is very useful, because it means that β-HIVS does not need to be present at millimolar levels, to compete with ATP, in the intracellular environment. Although the IC_{50} values of β-HIVS for EGFR and v-Src are substantially higher than those of ST-571, the inhibitory effect of β-HIVS on the autophosphorylating activity of EGFR can be enhanced considerably if it is used in combination [70]. The fact that the mechanism of inhibition of PTKs by β-HIVS is different from that of most known inhibitors of PTK also allows us to use β-HIVS effectively with other PTK inhibitors. Thus, for example, simultaneous treatment of Bcr–Abl-positive, human leukemia K562 cells with β-HIVS and STI571 revealed that these drugs had a synergistic effect on the induction of apoptosis in human leukemia cells [71]. Furthermore, the inhibition of PTK activity in K562 cells by STI571 was enhanced by simultaneous treatment of the cells with β-HIVS [71]. The most advantageous feature of β-HIVS is that an extract of *L. radix* containing β-HIVS as a major constituent has been administered orally to Asian patients for centuries as an antidote, antipyretic and anti-inflammatory agent without any serious side effects. It is now necessary to synthesize various other derivatives of shikonin, and to examine their PTK-inhibitory activities and side effects on animals.

Future prospects

Although STI-571 has been used successfully for the clinical treatment of patients with CML, clinical resistance to STI-571 has emerged as a serious problem [72]. Resistance to STI-571 has been associated with the reactivation of Bcr–Abl signal transduction that is caused by genetic mutation or amplification of the Bcr–Abl gene in the patients examined to date [72]. To overcome the resistance to STI-571, we need additional new inhibitors of PTKs with different chemical structures, which can be expected to inhibit PTK activity by different mechanisms. While STI-571 binds to the inactive and unphosphorylated form of the kinase domain of the c-Abl protein and inhibits kinase activity, PD173955 binds to the active conformation of the kinase [66]. Moreover, PD180970, which is structurally related to PD173955, induces apoptosis in STI571-resistant Bcr–Abl-positive CML cells [73].

Since most of the PTK inhibitors developed to date are ATP-competitive inhibitors, many possibilities remain for the development of ATP-non-competitive inhibitors. When more novel inhibitors of PTKs have been developed, it will become possible to select effective combinations that will attack different molecular targets simultaneously. When STI571 was combined with antileukemic agents, such as interferon-α, daunorubicin, and cytosine arabinoside, or with As_2O_3 for the treatment of Bcr-positive K562 cells, the respective drugs had additive or synergistic effects on cell growth [74,75], even though the latter agents are not PTK inhibitors. As described above, STI571 and β-HIVS had a synergistic effect with respect to the induction of apoptosis, when applied together to K562 cells [71].

It is also important to clarify the signal transduction pathways involved in the inhibition by PTK inhibitors. When the signal transduction pathways inhibited by PTK inhibitors have been characterized, we shall be able to select molecular targets for development of new PTK inhibitors. Recently, activation of the tyrosine kinase activity of the Flt3 receptor was found in about 30% of patients with acute myelogenous leukemia (AML) and specific inhibitors of the PTK activity of the Flt3 receptor, such as PKC412 [76] and CT53518 [77], have been identified and are being evaluated in clinical trials. In these cases too, the specificity of each PTK inhibitor must be defined. Since the total number of protein kinases encoded by the human genome is estimated to be greater than 2000 and since approximately 100 of them are likely to be PTKs [2,78], it will be difficult to find inhibitors of PTKs that inhibit only a single PTK selectively. For example, even STI571 inhibits c-kit and PDGF in addition to the product of Bcr–Abl gene [17,18]. Elucidation of the specificity of PTK inhibitors will help us also to predict the side effects of these anticancer agents. When the molecular targets for the induction of apoptosis in tumor cells are identified and when specific inhibitors of PTKs with limited side effects have been selected, it will be possible to develop much better and more specific anticancer therapies.

References

1 Hunter T. Protein kinases and phosphatases: the Yin and Yang of protein phosphorylation and signaling. *Cell* 1995; **80**:225–236.
2 Al-Obeidi FA, Lam KS. Development of inhibitors for protein tyrosine kinases. *Oncogene* 2000; **19**:5690–5701.
3 Kolibaba KS, Druker BJ. Protein tyrosine kinases and cancer. *Biochim Biophys Acta* 1997; **1333**:F217–F248.
4 Druker BJ, Lydon NB. Lessons learned from the development of an Abl tyrosine kinase inhibitor for chronic myelogenous leukemia. *J Clin Invest* 2000; **105**:3–7.

5 Noonberg SB, Benz CC. Tyrosine kinase inhibitors targeted to the epidermal growth factor receptor subfamily. Role as anticancer agents. *Drugs* 2000; **59**:753–767.

6 Levitzki A, Gazit A. Tyrosine kinase inhibition: an approach to drug development. *Science* 1995; **267**:1782–1788.

7 Fabbro D, Ruetz S, Buchdunger E, Cowan-Jacob SW, Fendrich G, Liebetanz J, *et al.* Protein kinases as targets for anticancer agents: from inhibitors to useful drugs. *Pharamacol Ther* 2002; **93**:79–98.

8 Druker BJ, Tamura S, Buchunger E, Ohno S, Segal GM, Fanning S, *et al.* Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr–Abl-positive cells. *Nat Med* 1996; **2**:561–566.

9 McWhirter JR, Galasso DL, Wang JYJ. A coiled-coil oligomerization domain of Bcr is essential for the transforming function of Bcr–Abl oncoproteins. *Mol Cell Biol* 1993; **13**:7587–7595.

10 Lugo TG, Pendergast A-M, Muller AJ, Witte ON. Tyrosine kinase activity and transformation potency of *bcr–abl* oncogene products. *Science* 1990; **247**:1079–1082.

11 Daley GQ, Van Etten RA, Baltimore D. Induction of chronic myelogenous leukemia in mice by the $p210^{bcr/abl}$ gene of the Philadelphia chromosome. *Science* 1990; **247**:824–830.

12 Kelliher MA, McLaughlin J, Witte ON, Rosenberg N. Induction of a chronic myelogenous leukemia-like syndrome in mice with v-*abl* and *BCR/ABL*. *Proc Natl Acad Sci USA* 1990; **87**:6649–6653.

13 Lozzio CB, Lozzio BB. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. *Blood* 1975; **45**:321–334.

14 Ray S, Bullock G, Nunez G, Tang C, Ibrado AM, Huang Y, *et al.* Enforced expression of $Bcl\text{-}x_s$ induces differentiation and sensitizes chronic myelogenous leukemia-blast crisis K562 cells to 1-β-D-arabinofuranosylcytosine-mediated differentiation and apoptosis. *Cell Growth Differ* 1996; **7**:1617–1623.

15 Amarante-Mendes GP, Kim CN, Liu L, Huang Y, Perkins CL, Green DR, *et al.* Bcr–Abl exerts its antiapoptotic effect against diverse apoptotic stimuli through blockage of mitochondrial release of cytochrome *c* and activation of caspase-3. *Blood* 1998; **91**:1700–1705.

16 O'Dwyer ME, Druker BJ. Chronic myelogenous leukemia—new therapeutic principles. *J Intern Med* 2001; **250**:3–9.

17 Buchdunger E, Cioffi CL, Law N, Stover D, Ohno-Jones S, Druker BJ, *et al.* Abl protein-tyrosine kinase inhibitor STI571 inhibits *in vitro* signal transduction mediated by c-kit and platelet-derived growth factor receptors. *J Pharmacol Exp Ther* 2000; **295**:139–145.

18 Heinrich MC, Griffith DJ, Druker BJ, Wait CL, Ott KA, Zigler AJ. Inhibition of c-kit receptor tyrosine kinase activity by STI571, a selective tyrosine kinase inhibitor. *Blood* 2000; **96**:925–932.

19 Sjoblom T, Shimizu A, OBrien KP, Pietras K, Cin PD, Buchdunger E, *et al.* Growth inhibition of dermatofibrosarcoma protuberans tumors by the platelet-derived growth factor receptor antagonist STI571 through induction of apoptosis. *Cancer Res* 2001; **61**:5778–5783.

20 Tuveson DA, Willis NA, Jacks T, Griffin JD, Singer S, Fletcher CDM, *et al.* STI571 inactivation of the gastrointestinal stromal tumor c-KIT oncoprotein: biological and clinical implications. *Oncogene* 2001; **20**:5054–5058.

21 Wakeling AE, Guy SP, Woodburn JR, Ashton SE, Curry BJ, Barker AJ, *et al.* ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. *Cancer Res* 2002; **62**: 5749–5754.

22 Couzin J. Smart weapons prove tough to design. *Science* 2002; **298**: 522–525.

23 Hashimoto S, Xu M, Masuda Y, Aiuchi T, Nakajo S, Cao J, *et al.* β-Hydroxyisovaleryl-shikonin inhibits cell growth of various cancer cell lines and induces apoptosis in leukemia HL-60 cells by a mechanism different from those of Fas and etoposide. *J Biochem* 1999; **125**:17–23.

24 Sankawa U, Ebizuka Y, Miyazaki T, Isomura Y, Otsuka H, Shibata S, *et al.* Antitumor activity of shikonin and its derivatives. *Chem Pharm Bull* 1977; **25**:2392–2395.

25 Hashimoto S, Xu Y, Masuda Y, Aiuchi T, Nakajo S, Uehara Y, *et al.* β-Hydroxyisovalerylshikonin is a novel and potent inhibitor of protein tyrosine kinases. *Jpn J Cancer Res* 2002; **93**:944–951.

26 Umezawa H, Imoto M, Sawa T, Isshiki K, Matsuda N, Uchida T, *et al.* Studies on a new epidermal growth factor-receptor kinase inhibitor, erbstatin, produced by MH435-hF3. *J Antibiot (Tokyo)* 1986; **39**:170–173.

27 Hori T, Kondo T, Tsuji T, Imoto M, Umezawa K. Inhibition of tyrosine kinase and *src* oncogene functions by stable erbstatin analogues. *J Antibiot (Tokyo)* 1992; **45**:280–282.

28 Onoda T, Inuma H, Sasaki Y, Hamada M, Isshiki K, Naganawa H, *et al.* Isolation of a novel tyrosine kinase inhibitor, lavendastin-A from *Streptomyces griseolavendus*. *J Nat Prod* 1989; **52**:1252–1257.

29 Umezawa K, Imoto M. Use of erbstatin as protein-tyrosine kinase inhibitor. *Methods Enzymol* 1991; **201**:379–385.

30 Yaish P, Gazit A, Gilon C, Levitzki A. Blocking of EGR-dependent cell proliferation by EGF receptor kinase inhibitors. *Science* 1988; **242**: 933–935.

31 Hori H, Nagasawa H, Ishibashi M, Uto Y, Hirata A, Saijo K, *et al.* TX-1123: an antitumor 2-hydroxyarylidene-4-cyclopentene-1,3-dione as a protein tyrosine kinase inhibitor having low mitochondrial toxicity. *Bioorg Med Chem* 2002; **10**:3257–3265.

32 Burger AM, Kaur G, Alley MC, Supko JG, Malspeis L, Grever MR, *et al.* Tyrphostin AG17, [(3,5-di-*tert*-butyl-4-hydroxybenzylidene)-malononitrile], inhibits cell growth by disrupting mitochondria. *Cancer Res* 1995; **55**:2794–2799.

33 Gazit A, Osherov N, Gilon C, Levitzki A. Tyrphostins. 6. Dimeric benzylidenemalononitrile tyrphostins: potent inhibitors of EGF receptor tyrosine kinase *in vitro*. *J Med Chem* 1996; **39**:4905–4911.

34 Burke TR, Lim Jr B, Marquez VE, Li Z-H, Bolen JB, Stefanova I, *et al.* Bicyclic compounds as ring-constrained inhibitors of protein-tyrosine kinase $p56^{lck1}$. *J Med Chem* 1993; **36**:425–432.

35 Graziani Y, Erikson E, Erikson RL. The effects of quercetin on the phosphorylation activity of the Rous sarcoma virus transforming gene product *in vitro* and *in vivo*. *Eur J Biochem* 1983; **135**:583–589.

36 Gschwendt M, Horn F, Kittstein W, Marks F. Inhibition of the calcium- and phospholipid-dependent protein kinase activity from mouse brain cytosol by quercetin. *Biochem Biophys Res Commun* 1983; **117**:444–447.

37 Matter WF, Brown RF, Vlahos CJ. The inhibition of phosphatidylinositol 3-kinase by quercetin and analogs. *Biochem Biophys Res Commun* 1992; **186**:624–631.

38 Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, *et al.* Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem* 1987; **262**:5592–5595.

39 Bridges AJ. The rationale and strategy used to develop a series of highly potent, irreversible inhibitors of the epidermal growth factor receptor family of tyrosine kinases. *Curr Med Chem* 1999; **6**:825–843.

40 Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, *et al.* Discovery of a novel, potent, and Src family-selective kinase inhibitor. *J Biol Chem* 1996; **271**:695–701.

41 Bruns CJ, Solorzano CC, Harbison MT, Ozawa S, Tsan R, Fan D, *et al.* Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. *Cancer Res* 2000; **60**:2926–2935.

42 Solorzano CC, Baker CH, Tsan R, Traxler P, Cohen P, Buchdunger E, *et al.* Optimization for the blockade of epidermal growth factor receptor signaling for therapy of human pancreatic carcinoma. *Clin Cancer Res* 2001; **7**:2563–2572.

43 Traxler P, Bold G, Frei J, Lang M, Lydon N, Mett H, *et al.* Use of a pharmacophore model for the design of EGFR-tyrosine kinase inhibitors: 4-(phenylamino)pyrazolo[3,4-*d*]pyrimidines. *J Med Chem* 1997; **40**: 3601–3616.

44 Bridges AJ, Zhou H, Cody DR, Rewcastle GW, McMichael A, Showalter HDH, *et al.* Tyrosine kinase inhibitors. 8. An unusually steep structure–activity relationship for analogues of 4-(3-bromoanilino)-6,7-dimethoxyquinazoline (PD 153035), a potent inhibitor of the epidermal growth factor receptor. *J Med Chem* 1996; **39**:267–276.

45 Rewcastle GW, Murray DK, Elliot WL, Fry DW, Howard CT, Nelson JM, *et al.* Tyrosine kinase inhibitors. 14. Structure–activity relationships for methylamino-substituted derivatives of 4-[(3-bromophenyl)amino]-6-(methylamino)-pyrido[3,4-*d*]pyrimidine (PD158780), a potent and specific inhibitor of the tyrosine kinase activity of receptors for the EGF family of growth factors. *J Med Chem* 1998; **41**:742–751.

46 Ciardiello F, Caputo R, Bianco R, Damiano V, Pomatico G, De Placido S, *et al.* Antitumor effect of potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res* 2000; **6**:2053–2063.

47 Moyer JD, Barbacci EG, Iwata KK, Arnold L, Boman B, Cunningham A, *et al.* Induction of apoptosis and cell cycle arrest by CP-358,774, an inhibitor of epidermal growth factor receptor tyrosine kinase. *Cancer Res* 1997; **57**:4838–4848.

48 Xia W, Mullin RJ, Keith BR, Liu L-H, Ma H, Rusnak DW, *et al.* Anti-tumor activity of GW572016: a dual tyrosine kinase inhibtor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene* 2002; **21**:6255–6263.

49 Rusnak DW, Lackey K, Affleck K, Wood ER, Alligood KJ, Rhodes N, *et al.* The effects of the novel, reversible epidermal growth factor receptor/ErbB-2 tyrosine kinase inhibitor, GW2016, on the growth of human normal and

tumor-derived cell lines *in vitro* and *in vivo*. *Mol Cancer Ther* 2001; **1**: 85–94.
50 Erlichman C, Boerner SA, Hallgren CG, Spieker R, Wang X-Y, James CD, *et al.* The Her tyrosine kinase inhibitor CI1033 enhances cytotoxicity of 7-ethyl-10-hydroxycamptothecin and topotecan by inhibiting breast cancer resistance protein-mediated drug efflux. *Cancer Res* 2001; **61**:739–748.
51 Mohammadi M, Froum S, Hamby JH, Schroeder MC, Panek RL, Lu GH, *et al.* Crystal structure of an angiogenesis inhibitor bound to the FGF receptor tyrosine kinase domain. *EMBO J* 1998; **17**:5896–5904.
52 Moasser MM, Srethapakdi M, Sachar KS, Kraker AJ, Rosen N. Inhibition of Src kinases by a selective tyrosine kinase inhibitor causes mitotic arrest. *Cancer Res* 1999; **59**:6145–6152.
53 Kraker AJ, Hartl BG, Amar AM, Barvian MR, Schwalter HDH, Moore CW. Biochemical and cellular effects of c-Src kinase-selective pyrido[2,3-*d*]pyrimidine tyrosine kinase inhibitors. *Biochem Pharmacol* 2000; **60**: 885–898.
54 Dorsey JF, Jove R, Kraker AJ, Wu J. The pyrido[2,3-*d*]pyrimidine derivative PD180970 inhibits $p210^{Bcr-Abl}$ tyrosine kinase and induces apoptosis of K562 leukemic cells. *Cancer Res* 2000; **60**:3127–3131.
55 Laird AD, Vajkoczy P, Shawver LK, Thurnher A, Liang C, Mohammadi M, *et al.* SU6668 is a potent antiangiogenic and antitumor agent that induces regression of established tumors. *Cancer Res* 2000; **60**:4152–4160.
56 Showalter HDH, Sercel AD, Leja BM, Wolfangel CD, Ambroso LA, Elliott WL, *et al.* Tyrosine kinase inhibitors. 6. Structure–activity relationships among *N*- and 3-substituted 2,2′-diselenobis(1*H*-indoles) for inhibition of protein tyrosine kinases and comparative *in vitro* and *in vivo* studies against selected sulfur congeners. *J Med Chem* 1997; **40**:413–426.
57 Fong TAT, Shawver LK, Sun L, Tang C, App H, Powell TJ, *et al.* SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. *Cancer Res* 1999; **59**:99–106.
58 Krystal GW, Honsawek S, Kiewlich D, Liang C, Vasile S, Sun Li, *et al.* Indolinone tyrosine kinase inhibitors block kit activation and growth of small cell lung cancer cells. *Cancer Res* 2001; **61**:3660–3668.
59 Bold G, Altman K-H, Frei J, Lang M, Manley PW, Traxler P, *et al.* New anilinophthalazines as potent and orally well absorbed inhibitors of the VEGF receptor kinases useful as antagonists of tumor-driven angiogenesis. *J Med Chem* 2000; **43**:2310–2323.
60 Lin B, Podar K, Gupta D, Tai Y-T, Li S, Weller E, *et al.* The vascular endothelial growth factor receptor tyrosine kinase inhibitor PTK767/ZK222584 inhibits growth and migration of multiple myeloma cells in the bone marrow microenvironment. *Cancer Res* 2002; **62**:5019–5026.
61 Uehara Y, Murakami Y, Suzukake-Tsuchiya K, Moriya Y, Sano H, Shibata K, *et al.* Effects of herbimycin derivatives on *src* oncogene function in relation to antitumor activity. *J Antibiot (Tokyo)* 1988; **41**:831–834.
62 Cassinelli G, Lanzi C, Pensa T, Gambetta RA, Nasini G, Cuccuru G, *et al.* Clavilactones, a novel class of tyrosine kinase inhibitors of fungal origin. *Biochem Pharmcol* 2000; **59**:1539–1547.
63 Xu W, Harrison SC, Eck MJ. Three-dimensional structure of the tyrosine kinase c-Src. *Nature* 1997; **385**:595–602.
64 Sicher F, Moarefi I, Kuriyan J. Crystal structure of the Src family tyrosine kinase Hck. *Nature* 1997; **385**:602–609.
65 Posner I, Engel M, Gazit A, Levitzki A. Kinetics of inhibition by tyrphostins of the tyrosine kinase activity of the epidermal growth factor receptor and analysis by a new computer program. *Mol Pharmacol* 1994; **45**: 673–683.
66 Nagar B, Bornmann WG, Pellicena P, Schindler T, Veach DR, Miller WT, *et al.* Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571). *Cancer Res* 2002; **62**:4236–4243.
67 Ward WHJ, Cook PN, Slater AM, Davies DH, Holdgate GA, Green LR. Epidermal growth factor receptor tyrosine kinase. Investigation of catalytic mechanism, structure-based searching and discovery of a potent inhibitor. *Biochem Pharmacol* 1994; **48**:659–666.
68 Marsilje TH, Milkiewicz KL, Hangauer DG. The design, synthesis and activity of non-ATP competitive inhibitors of $pp60^{c-src}$ tyrosine kinase, Part 1: hydroxynaphthalene derivatives. *Bioorg Med Chem Lett* 2000; **10**:477–481.
69 Zheng Z-B, Nagai S, Imanami N, Kobayashi A, Natori S, Sankawa U. Inhibition effects of 5-*S*-glutathionyl-*N*-β-alanyl-L-dopa analogues against Src protein tyrosine kinase. *Chem Pharm Bull* 1999; **47**:777–782.
70 Xu Y, Kajimoto S, Nakajo S, Nakajo S, Nakaya K. β-Hydroxyisovalerylshikonin and cisplatin act synergistically to inhibit growth and to induce apoptosis of human lung cancer DMS114 cells via a tyrosine kinase-dependent pathway. *Oncology* 2003; in press.
71 Masuda Y, Nishida A, Hori K, Hirabayashi T, Kajimoto S, Nakajo S, *et al.* β-Hydroxyisovalerylshikonin induces apoptosis in human leukemia cells by inhibition the activity of a polo-like kinase 1. *Oncogene* 2003; **22**: 1012–1023.
72 Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN, *et al.* Clinical resistance to STI-571 cancer therapy caused by BCR–ABL gene mutation or amplification. *Science* 2001; **293**:876–880.
73 Huang M, Dorsey JF, Epling-Burnette PK, Nimmanapalli R, Landowski TH, Mora LB, *et al.* Inhibition of Bcr–Abl kinase activity by PD180970 blocks constitutive activation of Stat5 and growth of CML cells. *Oncogene* 2002; **21**:8804–8816.
74 Thiesing JT, Ohno-Jones S, Kolibaba KS, Druker BJ. Efficacy of STI571, an Abl tyrosine kinase inhibitor, in conjunction with other antileukemic agents against Bcr–Abl-positive cells. *Blood* 2000; **96**:3195–3199.
75 Porosnicu M, Nimmanapalli R, Nguyen D, Worthington E, Perkins C, Bhalla KN. Co-treatment with As_2O_3 enhances selective cytotoxic effects of STI-571 against Brc–Abl-positive acute leukemia cells. *Leukemia* 2001; **15**:772–778.
76 Kelly LM, Yu J-C, Boulton CL, Apatira M, Li J, Sullivan CM, *et al.* CT53518, a novel selective FLT3 antagonist for the treatment of acute myelogenous leukemia (AML). *Cancer Cell* 2002; **1**:421–432.
77 Weisberg E, Boulton C, Kelly LM, Manley P, Fabbro D, Meyer T, *et al.* Inhibition of mutant FLT3 receptors in leukemia cells by the small molecule tyrosine kinase inhibitor PKC412. *Cancer Cell* 2002; **1**:433–443.
78 Hunter T. 1001 protein kinases redux—towards 2000. *Semin Cell Biol* 1994; **6**:367–376.