Last Findings on Dual Inhibitors of Abl and Src Tyrosine-Kinases

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Abstract: Chronic myelogenous leukemia (CML) is a myeloproliferative disease characterized by the presence of the Philadelphia chromosome that expresses the constitutively activated tyrosine kinase Bcr-Abl; this enzyme causes hyperproliferation of the stem cells and the consequent pathology of the disease. Targeted inhibitors of Bcr-Abl have antiproliferative effects on the leukemic cells and induce apoptosis, favouring a regression of the CML chronic phase, but in the successive blast crisis phase cancer cells frequently develop resistance to Bcr-Abl inhibitors. Src is a family of non-receptor tyrosine kinases, fundamental for cell development, growth, replication, adhesion, motility and is overexpressed in a wide number of human cancers. Recently it was demonstrated that Src is increased in hematopoietic cells expressing Bcr-Abl and is involved in the oncogenic pathway that causes CML. For this reason and also for the development of resistance to classical Bcr-Abl inhibitors, various dual Src/Abl inhibitors have been recently synthesized and tested. This mini review will be focused on the latest finding on this matter.

Key Words: Chronic myelogenous leukemia, tyrosine kinases, Src, Bcr-Abl, dual inhibitors, Imatinib, resistance.

INTRODUCTION

Chronic myelogenous leukemia (CML) is a myeloproliferative disease and accounts for 15% of all adult leukemias, with ~4500 new cases diagnosed each year in the US and 750 in the UK [1]. The disease progresses through three phases (chronic proliferative phase, accelerated phase, blast crisis phase) becoming more resistant to treatment in each successive phase. The last phase is also characterized by the presence of genomic instability and is ultimately fatal [2,3].

CML is characterized by the presence of the Philadelphia chromosome in 90% of patients. This chromosome, carrying the Bcr-Abl (Breakpoint Cluster Region-Abelson) oncogene, derives from the fusion of a *bcr* gene segment from chromosome 9, to a region of the *c-abl* gene from chromosome 22 [1] and encodes the Bcr-Abl protein tyrosine kinase. The greater degree of this kinase activity correlates with the acuity of the disease.

C-abl gene encodes the cytoplasmatic tyrosine kinase (TK) c-Abl present in normal cells where its activity is closely regulated.

c-Abl is a protein constituted by ~1150 aminoacids [4]. The N-terminal portion includes a "cap" of 80 residues important for autoinhibition [5], followed by an SH3 domain, an SH2 domain, a tyrosine kinase domain and a C-terminal last exon region (Fig. (**1**)). Except for the "cap" region, unique to c-Abl, the N-terminal part of c-Abl is very similar to that of Src TKs, another family of cytoplasmatic kinases, more involved in different diseases (see later in this review) [6]. The C-terminal portion of c-Abl includes binding elements for SH3 domains, nuclear localization and an actin binding domain. Human cells express two variants of c-Abl, Abl 1a and Abl 1b, which differ only in the terminal region. Abl 1b is myristoylated, Abl 1a is not.

The Abl catalytic domain is similar to that of other tyrosine kinases: it shows a bilobate structure with a smaller N-terminal lobe and a larger C-terminal lobe. The cleft between the two lobes contains the amino acids critical for catalysis, including the ATP-binding loop (P-loop) and the activation loop (A-loop). In the closed and inactive conformation, the A-loop blocks the catalytic centre of the kinase, preventing the ATP and the substrate binding. In the open and active conformation, the A-loop swings away from the catalytic centre making possible the interaction of the enzyme with the substrates. The conformation of the A-loop is regulated by phosphorylation of critical tyrosine residues. Although the A-loop conformations of different active kinases are very similar, pronounced differences are present in the inactive states [7].

Bcr-Abl proteins possess constitutive catalytic activity and cause malignancies, even if the amino acid sequence of the Abl segment of Bcr-Abl is preserved without mutation to that of c-Abl, except for a small N-terminal region, upstream from the SH3 domain [8].

The fusion with Bcr disrupts the internal control that keeps c-Abl in an inactive form, even if the exact mechanism of dysregulation of Abl kinase activity upon fusion with Bcr is still not clearly understood, despite two decades of research. A mechanism of this dysregulation has been recently reported by Van Etten [9]: the Bcr-Abl protein maintains the Abl SH3 domain but lacks Abl first exon sequences and the myristoylation domain while it gains sequences from the N-terminus of Bcr.

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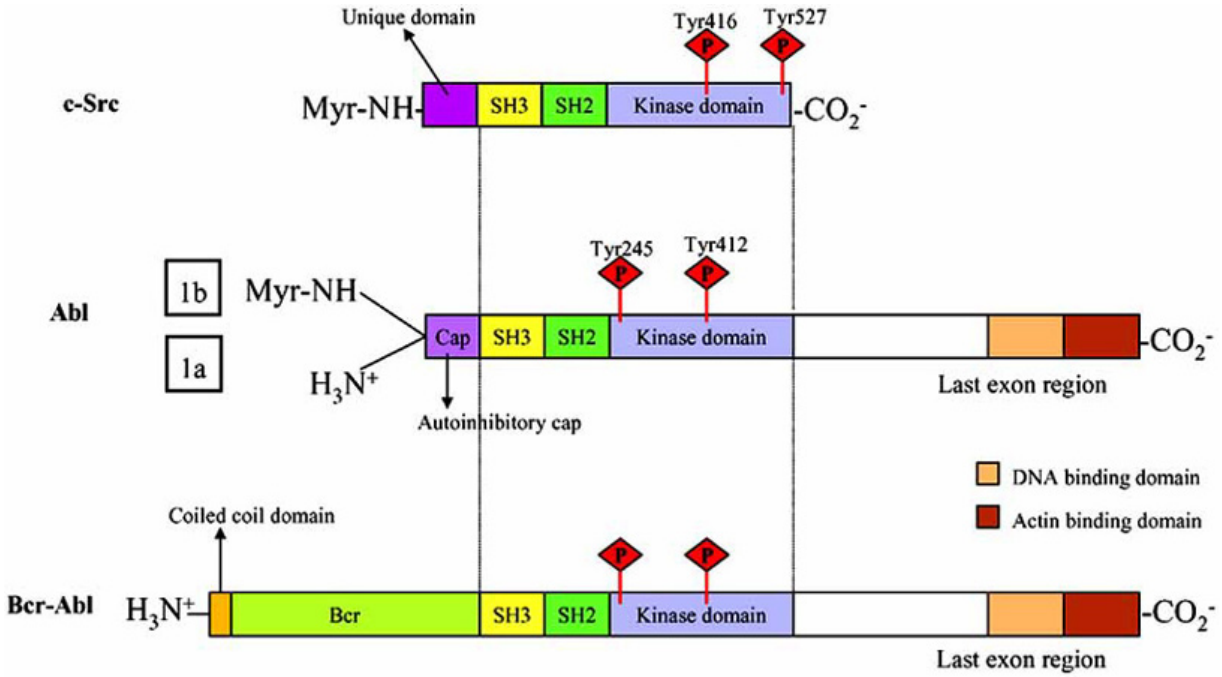


Fig. (1). Schematic structural organization of Src family, Abl and Bcr-Abl kinases.

The end of the Bcr protein has a tetramerization domain, called coiled coin, and through it the Bcr-Abl oncoprotein associates in tetramers. In detail Zhao and coworkers determined the crystal structure of the N-terminal oligomerization domain (residues 1-72) and found the mode of oligomer formation. Two monomers dimerize by swapping N-terminal helices and by forming an antiparallel coiled coin between C-terminal helices. Two dimers then stack onto each other to form a tetramer [10].

He and coworkers reported that the coiled coin domain and the Grb2 (Growth factor Receptor Bound protein 2) binding site at Tyr177 play an important role in myeloproliferative disease induction by Bcr/Abl in a murine model similar to the human CML [11].

Smith, Yacobi and Van Etten proposed a model for Bcr-Abl activation. In the autoinhibited state, Bcr-Abl is monomeric, unphosphorylated and the Abl SH3 domain interacts with a proline residue present in the linker region between the SH2 and the catalytic domain. After, an oligomerization occurs, followed by a primary phosphorylation at Tyr1294 and a rapid secondary phosphorylation at Tyr1127, which disrupts the SH3 linker interaction and results in full catalytic activity [12]. However, there was no direct evidence that oligomerization is critical for Bcr-Abl activity [9].

Extensive work has been performed to identify Bcr-Abl substrates and the possible mechanism that leads to a myeloid expansion. Many authors have characterized different protein substrates phosphorylated by this enzyme in different cell lines. Moreover key signal transduction pathways involving phosphoinositol 3-kinase, Ras, Myc, c-Jun kinase and STAT5 (signal transducer and activator of transcription 5) are activated in a Bcr-Abl kinase-dependent manner [13, 14]. Protein associated with cytoskeletal functions, including paxillin, FAK (Focal Adhesion Kinase) and talin are phosphorylated by Bcr-Abl. [15].

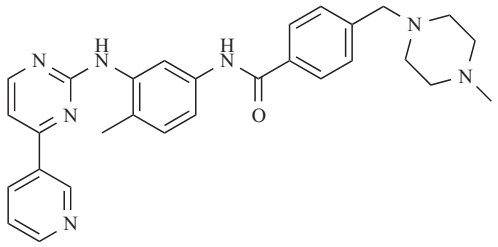
Bcr-Abl also activates other non receptor TKs, including the Src family kinases, in particular Hck and Lyn. Bcr-Abl is directly associated with these enzymes and results in their increased activities [16,17].

Bcr-Abl stimulates different signalling pathways responsible for protection from apoptosis and induction of growth factor independent proliferation and transformation [18, 19].

However, despite new knowledge on the downstream factor activated by Bcr-Abl, the exact mechanism by which the leukemic state is achieved is not completely understood.

The genetic events that cause the progression of chronic phase to blast crisis are poorly understood, being the disease characterized by genetic instability. Recently Radich and coworkers performed a study aimed at determining the changes in gene expression that occur in the evolution of CML, with the hope of identifying gene pathways useful as prognostic marker or target for therapeutics. Authors identified a "progression specific" gene expression and the genes that are aberrantly expressed as the disease progresses [20].

In 1996 Novartis, during a program aimed at identifying selective TK inhibitors, reported the first potent Abl kinase inhibitor CGP57148 or STI571, Imatinib mesylate, Gleevec™ or Glivec® (**1**) (Fig. (**2**)) [21], approved by FDA in 2001, that became within a few years of its discovery the first line therapy generally well tolerated for the treatment of CML. In the chronic phase, Imatinib is highly effective, with excellent and durable haematological and cytological responses in the majority of patients [22].



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IMATINIB
PDGFr IC_{50} 0.05 μM
PKC IC_{50} > 100 μM
v-Abl-K IC_{50} 0.038 μM
Src IC_{50} > 100 μM
c-Kit IC_{50} 0.1 μM

Fig. (2).

From a chemical point of view Imatinib was derived from a non-selective inhibitor with activity at PDGF, PKCα and Src [23]. The observation of Abl activity for this series was made later and the PKCα and Src activities were eliminated *via* the introduction of a "flag methyl" group on the phenylaminopyrimidine scaffold [24] (Fig. (**2**)).

Imatinib is an ATP-competitive inhibitor of Abl, with K_i values of 85 nM [25], c-Kit, PDGFr, devoid of significant activity against other TKs [26]. The crystal structure of the complex between Imatinib and Abl showed the reasons of this specificity [25, 27, 28]. Imatinib binds the inactive form of Abl; in more detail the compound is bound in the ATP-binding cleft of the unphosphorylated activation loop of Abl and forms extensive contacts with residues lining the cleft or just outside it. The nucleotide binding loop changes considerably upon binding of Imatinib, blocking Abl and also Bcr-Abl in their inactive, autoinhibited conformations. The selectivity of Imatinib for Abl kinase *vs* Src is achieved in part through "conformational specificity". In fact, although the Abl amino acids interacting with Imatinib are identical to those of Src, with the exclusion of Abl Tyr253 (corresponding to Phe in Src) it has been found that Imatinib is a weak inhibitor of Src. The activation loop of unphosphorylated, inactive kinases shows preferential conformations different among kinases, some of which are not sterically compatible with the binding mode of Imatinib [29]. In cellular assays this compound inhibits the autophosphorylation of Abl, c-Kit and PDGFr and cell proliferation. Very interestingly the antiproliferative and apoptotic activity is seen only in cells expressing activated forms of these TKs, for example Bcr-Abl [30]. It has been shown that Imatinib blocks the proliferation of KU812 cells, a Bcr-Abl dependent human CML line, with an IC_{50} between 100-300 nM, and also other human CML lines, including K562 and MC3 [31]. Several studies performed in animal models of CML demonstrated the *in vivo* antitumour activity of this compound.

In patients, Imatinib shows remarkable efficacy and high tolerability with single oral daily dosing giving mean plasma concentration 10-fold above the IC_{50} for the inhibition of *in vitro* activity [32].

Nevertheless primary refractoriness and acquired resistance to Imatinib are observed in patients in the accelerated phase or blast crisis.

Even if the reasons for Imatinib resistance are multiple and well described elsewhere [33], the two main mechanisms are the overexpression of Bcr-Abl, mainly due to gene amplification [34], and more frequently mutations in the kinase domain of Bcr-Abl [35,36].

Other reasons for resistance include drug efflux mechanisms or binding to plasmatic protein [37].

Mutations in Bcr-Abl that confer resistance to Imatinib have been identified as the major cause of relapse during Imatinib therapy [38,39]. Point mutations result in changes of critical amino acids in contact with the inhibitor or ensure access of the inhibitor to the ATP binding site so provoking the resistance of the kinase to Imatinib. In particular, point mutations in the kinase domain of Bcr-Abl were detected in 50% to 90% of patients with acquired resistance, with more than 100 different described amino acid substitutions and 20 clinically relevant ones [40,41]. The emergence of clones expressing mutants of Bcr-Abl might derive from a leukemic population of non-cycling G_o quiescent stem cells, which have not been eliminated by Imatinib [42].

The most relevant mutations can be divided into four clusters based on their position in the kinase domain. The first group of mutations includes amino acids in the phosphate-binding loop for ATP (P-loop) and represents 40% of the mutations. The second group is in the T315 region, which is the Imatinib binding site and interacts directly with the inhibitor *via* hydrogen bonds or Van der Waals' interactions (25% of the mutations). The third group is in the M351 region in closer proximity of the catalytic domain (25% of the mutations). Finally 5% of amino acid mutations are located in the activation loop, whose conformation controls kinase activation or inactivation [40, 43, 44].

The amino acid Thr315 is of particular relevance in determining sensitivity to Imatinib and other inhibitors. In fact mutation of Thr315 to Ile confers high resistance to Abl-Bcr [34]. This replacement would prevent formation of a hydrogen bond between the enzyme and inhibitor and would also cause a steric conflict due to the increased size of the side chain [44].

Cools and coworkers defined Thr315 as a kind of "gatekeeper" that controls access of small molecule inhibitors [45].

Studies on the crystal structure of the human Abl kinase domain in complex with Imatinib [25,28] and an Imatinib variant showed the reasons for the resistance [27]. The inhibitor binds to an inactive conformation of the enzyme, where the glycine rich P-loop folds over the ATP binding site and the activation loop adopts a conformation in which it occludes the substrate binding site and disrupts the ATP phosphate binding site, blocking the catalytic activity of the enzyme. This binding mode induces changes in the orientation of the side chain of the N-terminal part of the activation

loop (AspPheGly, DGF motif), that results in a conformation unable to bind ATP. In addition Imatinib binds into a hydrophobic pocket and an allosteric inhibitor binding site adjacent to the ATP binding pocket [46].

A series of hydrogen bonds are formed between the Abl and Imatinib: the pyridine-N with the Met318 backbone NH, the anilino NH with the Tyr315 side chain, the amide-NH to the side chain of Glu286, the amide-carbonyl with the Ala380 backbone-NH and the protonated N-methylpiperazine to the backbone carbonyl of Ile360. Moreover the pyrimidine ring of Imatinib is placed between two hydrophobic side chains of Val299 and Phe382, while the pyridine is surrounded by apolar side chains of other amino acids. This unusual binding and inhibition mode explains the selectivity profile of Imatinib [25].

Recently Levinson and coworkers presented a structure of the Abl kinase domain in which the protein adopts an inactive conformation that closely resembles that of Src kinase and reported that probably one class of mutations in Bcr-Abl that confers resistance to Imatinib appears to destabilize the inactive Src-like conformation of Bcr-Abl [47].

A second mechanism of resistance is Bcr-Abl gene amplification, that results in overproduction of the kinase [35,48]. While amino acid mutations alter the affinity to the kinase for the inhibitor, amplification has no effect on the sensitivity of the kinase to the inhibitor [46]. It is presumed that the intracellular concentration of Imatinib is insufficient to inhibit the increased level of Bcr-Abl. In this situation the increase of the inhibitor dose could be helpful [49], even if amplification of the target kinase is the main mechanism of resistance observed in cell lines cultured in gradually increasing concentrations of inhibitors [50].

Recently Shah and coworkers found a four-compartment model, developed by a mathematical approach and based on the known biology of haematopoietic differentiation, that might explain the kinetics of the molecular response to Imatinib in a patient data set. The model suggests that Imatinib is a potent inhibitor of the production of differentiated leukemic cells, but does not eliminate the leukemic stem cells. Importantly authors calculated the probability of developing Imatinib resistance thus providing the first quantitative insight into the *in vivo* kinetics of a human cancer [51].

The Src family kinases (SFKs) is the first characterized and the most studied among the non-receptor tyrosine kinases. In 1911, the pathologist Peyton Rous isolated a chicken sarcoma virus [52], but only in the late 70s, the studies of Brugge and colleagues demonstrated that this avian tumour is caused by a viral TK, named v-Src [53]. Further experiments demonstrated that v-Src has a highly conserved and ubiquitous cellular homologue, called c-Src, present in normal cells [54]. This gene was then identified in the viral genome and subsequently sequenced by Bishop and Varmus, who won the Nobel Prize in 1989.

SFKs include Src, Fyn, Yes and Fgr, widely present in different tissues, and Lck, Blk, Lyn and Hck restricted to hematopoietic cells [55, 56].

Src kinases consist of six different regions, including Src-homology (SH) domains that are highly conserved in the different members of this family. The Src structure has been extensively studied and characterized [for recent reviews, see 57, 58]. Between the N- and the C- termini, several structural elements are present: the SH4 domain, which is myristoylated or palmitoylated for the membrane localization, the unique region, poorly conserved in the different Src family members, the SH3 domain, the SH2 domain, the catalytic or SH1 domain, and the C-terminal tail, containing the negative regulatory Tyr527 in mice and Tyr530 in humans (see Fig. (**1**)). The catalytic kinase domain is constituted by a bilobate structure [59]: a small N-terminal lobe, that binds ATP, and a larger C-terminal lobe, where an activation loop is present, containing a conserved tyrosine residue (Tyr416 in mice and chickens and 419 in humans), phosphorylated in the active form of Src. The C-terminal lobe binds the protein substrate to be phosphorylated. Kinase domain structures of different members of the Src family have been solved by X-ray crystallography [60,61]. Tyr527 and Tyr416 are fundamental for the enzyme activation. These two amino acids have opposite effects on Src activity, in fact, Tyr416 autophosphorylation promotes activation, while the phosphorylation of Tyr527 causes inactivation of the enzyme.

Src activity is regulated through a complex series of intramolecular interactions that restrict the accessibility of ATP and substrates to the kinase domain active site. Crystallographic studies have demonstrated that interactions of the C-terminus with the SH2 domain and that of the kinase domain with the SH3 domain lead to a less active or closed conformation [57].

Very recently, the crystal structure of an unphosphorylated form of c-Src has been determined, characterized by the SH2 domain not bound to the C-terminal tail, resulting in an open and active conformation of the kinase domain [62]. Other Authors have reported crystal structures showing how the kinase domain can adopt a more open and flexible conformation, in the absence of rigidifying interdomain interactions [63].

Src and its family play key roles in cell morphology, replication, growth, adhesion and motility. All these cellular functions regulated by Src have been extensively studied and reported [64,65].

SFKs act at the point of integration, relaying signals from cell surface receptors to the nucleus [66] and have a key role in promoting a variety of diseases, first of all human malignancies, both solid and haematological [67]. Overexpression and/or hyperactivation of Src appears to be correlated with the tumour grade and with poor prognosis and has been described for many different cancers, including colorectal [68], breast [69,70], lung [71], hepatocellular [72], pancreatic [73], prostate [74,75], brain and neuronal [76], and ovarian [77] carcinomas. C-Src mutations have been described in colon and endometrial cancers, but are relatively rare [78-79].

Src is involved in different pathologic situations such as bone diseases, rheumatoid arthritis, cardiovascular diseases and immune system disorders.

Regarding bone diseases, Src plays an important role in osteoporosis, Paget' disease, hypercalcemia, cancer bone

metastasis [80,81] and, recently, it has been also demonstrated that enhanced Src activity contributes to the proliferation of human osteosarcoma [82].

New studies have reported the involvement of Src pathways in different diseases, including prion diseases [83] and viral infections such as hepatitis B virus [84] and Japanese encephalitis [85].

Moreover Src is involved in angiogenesis by association with different factors, as described in a number of literature reports [see for example 86, 87].

Several Authors have reported that the Src family might be involved either directly or indirectly in signal transduction by Bcr-Abl. The first evidence for a proleukemogenic potential of Src kinases came from the observation that v-Src, the viral homologue of c-Src, characterized by the lack of the C-terminal negative regulation site, is able to induce interleukin-3 (IL-3) independent growth in different hematopoietic cell lines [88] and to inhibit the granulocytic differentiation of myeloid progenitor cell lines [89]. Not all members of the Src family show the same involvement in the leukemogenic activity as that possessed by v-Src; in particular the two members of the Src family, Lyn and Hck, are upregulated in both Imatinib resistant cell lines and in CML blast crisis. K562-R cell lines have shown higher levels of Lyn and a more activated form of this kinase than was seen in the parent line [90]. Studies with knockout mice and bone marrow transplantation techniques demonstrated the different activities of Src members family and are well reported by Warmuth and coworkers [67]. The same Authors also demonstrated that the interaction of Src and Abl-Bcr *in vivo* might be mediated by multiple domains, in addition to SH2 and SH3 domains as previously reported, and that inactivation of the Bcr-Abl by a targeted mutation did neither inhibit the complex formation with Src nor their activation by Bcr-Abl. Active Src kinases may promote Bcr-Abl phosphorylation and interaction with Grb2 [67, 91]. Studies with dominant-negative mutants and Src inhibitors suggest that Src kinases may contribute to the proliferation of myeloid cell lines expressing Bcr-Abl *in vitro* [92].

Aside from these Bcr-Abl-dependent mechanisms of resistance, activation of Src kinases has been described in rare patients. Cell lines established from these patients are sensitive to Src kinase inhibitors, implying that a Src kinase has replaced Bcr-Abl [90].

Keeping in mind the incidence of Imatinib resistance to Abl and the involvement of Src family members in the Bcr-Abl signal pathway it seems reasonable to develop dual Abl/Src inhibitors that could in principle act on mutated forms of Abl and may reduce disease progression into the blast phase.

Excluding the unique domain of Src, Abl and Src share a similar domain organization containing SH2 and SH3 domains followed by the catalytic domain (Fig. **1**); also the latter possesses a high degree of homology in Src and Abl kinases [93].

In spite of this analogy Imatinib showed, as already reported, an IC_{50} of 38 nM in an Abl kinase assay, and an IC_{50} greater than 100 μM in a Src kinase assay [24]. In fact the catalytically active states of the two enzymes are very similar, but their inactive states have a unique conformation, consistent with the inability of Imatinib to inhibit Src kinases [28,94].

On the other hand, the similarity of the catalytically active states of Src and Abl explains the inhibition of Abl by ATP-competitive molecules originally developed as Src inhibitors [95].

In addition many Src/Abl inhibitors are smaller than Imatinib, contact fewer residues, and require fewer Abl conformational changes for their binding [94]. Moreover dual inhibitors Abl/Src appear to bind both active and inactive conformations of Abl [7].

A large number of reviews of dual Abl/Src inhibitors is present in the literature (see for example 22, 44, 45, 95, 96).

We shall report in this article the historical development from the first dual agents to the most recent findings in this field, with major focus on chemical structures and on SAR data.

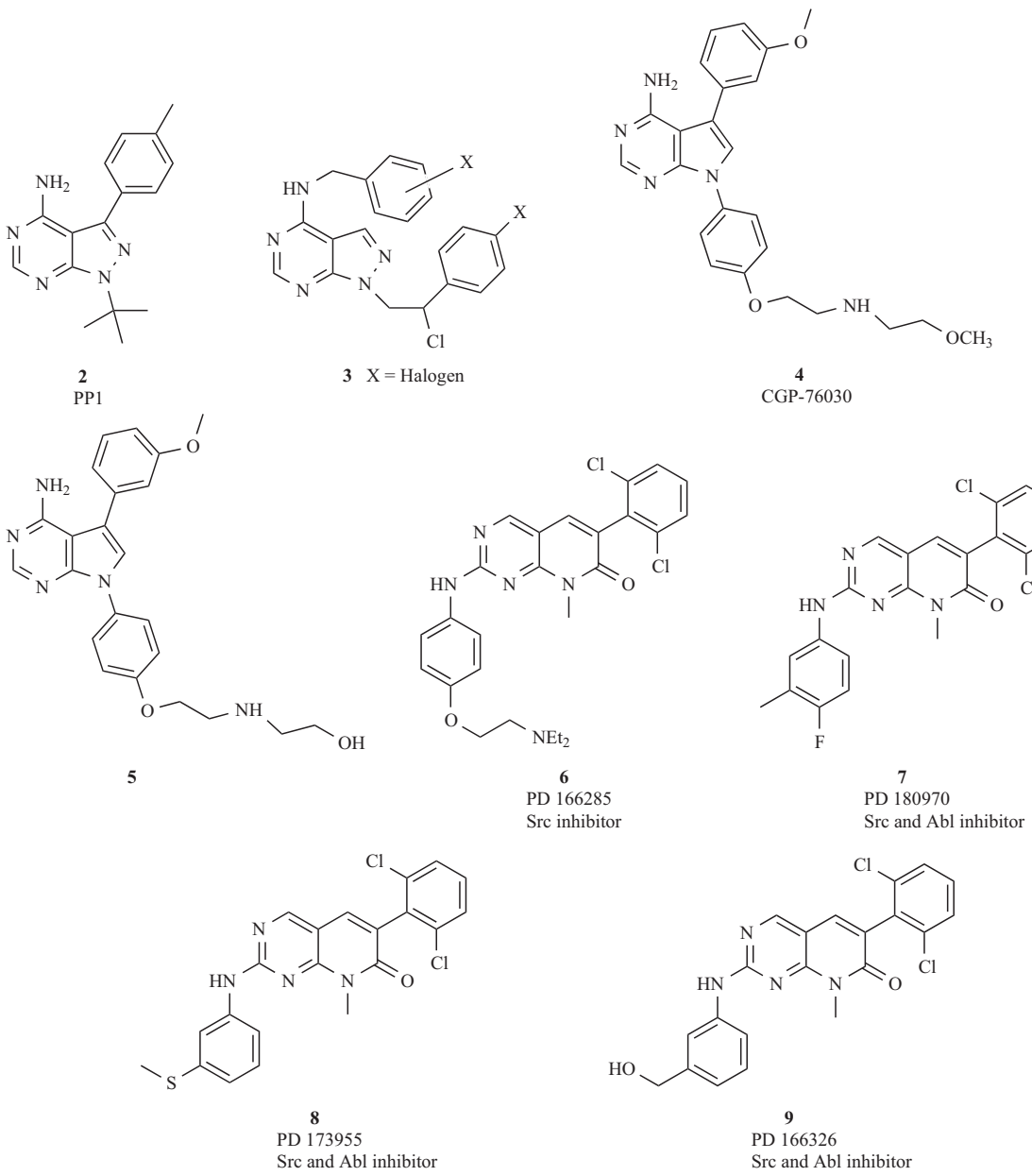
PYRAZOLO[3,4-*d*]PYRIMIDINES

During a program to find specific Src members inhibitors, Pfizer researchers synthesized in 1996 a series of 4-aminopyrazolo[2,3-*d*]pyrimidines [97]. Particularly, PP1 (**2**) (Fig. (**3**)) is the first potent SFK inhibitor reported in the literature, even if with poor selectivity toward the different members of SFK enzymes. A very large amount of biological and computational data has been reported for PP1 (reviewed for example in 98). More recently it has been found that PP1 inhibits also Abl with an IC_{50} value of 300 nM, making this compound the first reported Src/Abl dual inhibitor [44]. Both PP1 and CGP76030 (a pyrrolo-pyrimidine derivative cited above) block growth and survival of cells expressing Imatinib resistant Brc-Abl kinases containing several mutated forms of the key threonine 315 residue; in contrast to Imatinib, the two compounds bind and inhibit Bcr-Abl independently of the activation loop conformation [99].

Interestingly, some new results from our laboratory have shown that a family of 4-aminopyrazolo[3,4-*d*]pyrimidines (**3**) possess interesting antiproliferative activity on KU812, K562 and MEG-01 leukemic cell lines, inhibiting Bcr-Abl and Src phosphorylation, and inducing apoptosis. In enzymatic assays compounds showed IC_{50} values in the range of 0.5-1 μM and 25-100 nM for Src and Abl respectively (unpublished data).

PYRROLO[2,3-*d*]PYRIMIDINES

In 1999, Novartis researchers reported pyrrolo[2,3-*d*]pyrimidine derivatives as Src selective inhibitors with activity in animal models of osteoporosis [100] and closely related to PP1, but bearing a carbon atom at position 6 of the 5 membered ring instead of a nitrogen atom and a substituted phenyl group at C-7 instead of a *t*-butyl group. Successively a new derivative of this series, CGP76030, (**4**) was found to possess dual activity showing IC_{50} values of 27 and 180 nM for the inhibition of Src and Abl respectively; substitution of



2 PP1

3 X = Halogen

4 CGP-76030

5

6 PD 166285 Src inhibitor

7 PD 180970 Src and Abl inhibitor

8 PD 173955 Src and Abl inhibitor

9 PD 166326 Src and Abl inhibitor

Fig. (3). pyrazolo and pyrrolo-pyrimidines, pyrido-pyrimidones.

the methoxy group with a hydroxy one on the alkoxy side chain led to derivative (**5**), that showed better IC_{50} values for Abl (10 nM), but scarce oral bioavailability in respect of CGP76030 [101].

PYRIDO[2,3-*d*]PYRIMIDIN-7(*8H*)-ONES

In 1998 Parke Davis researchers disclosed a class of pyrido[2,3-*d*]pyrimidines, bearing on C-6 a 2,6-dichlorophenyl ring, as ATP-competitive inhibitors of several tyrosine kinases [102], for this reason later called by Palmer "pan-kinase" inhibitors [103]. One of these molecules, PD166285 (**6**) was quite Src selective showing an IC_{50} value of 9 nM and activity in several tumour xenograft models [104]. Other molecules in this series such as PD180970 (**7**), PD173955 (**8**) and PD166326 (**9**) [105], bearing different substitutions (4-fluoro-3-methyl, 3-thiomethyl and 3-hydroxymethyl respectively) on the anilino moiety on C-2, showed IC_{50} values of 17, 25 and 5.7 nM respectively for c-Src and less than 5 nM for Lck, another member of Src kinase family and remarkable selectivity over other kinases, such as PDGFR, FGFR and EGFR. With further investigation it was also found that (**7**), (**8**), and (**9**) inhibited also Abl with IC_{50} in the range 1-5 nM in cell free assays [17, 102, 105-107].

Other biological data regarding the activity of pyrido-pyrimidines on Bcr/Abl or Src depending cell lines and in

animal models are extensively reviewed elsewhere [44, 108], demonstrating that these molecules are more potent than Imatinib and act on the Imatinib resistant Bcr/Abl mutants, with the only exception of the mutation Tyr315Ile in the ATP-binding pocket.

In particular PD166326, the most active compound in this family, inhibits the growth of the Bcr-Abl K562 cell line with an IC_{50} in the picomolar range (300 pM), leading to apoptotic G1 arrest, whereas the non Bcr-Abl cell type requires a concentration 1000 times higher. Even if the Tyr315Ile mutant Bcr-Abl protein is resistant to PD166326, the growth of Bcr-AblT315I-driven cells is partially sensitive to this compound, like the inhibition of the Bcr-Abl pathway [17]. Recently Wolff and coworkers reported that PD166326 has greater antileukemic activity than Imatinib in a murine model of chronic myeloid leukemia at a dose of 50 mg/kg twice a day [109]. Mutations conferring resistance to this compound have been also reported by von Bubnoff. However, the frequency of resistant colonies emerging from the use of PD166326 was significantly lower than those reported with Imatinib. In addition, PD166326 produced a distinct pattern of Bcr-Abl mutations. The majority of mutations occurring with both Imatinib and PD166326 could be suppressed by increasing the dose of PD166326, while a few mutations could be suppressed by increasing the Imatinib dose [110].

Very recently the Azam group in collaboration with Ariad researchers reported that PD166326 inhibited the proliferation of BaF3 cells transformed with Bcr/Abl showing an IC_{50} value of 13.4 nM, but did not inhibit the untransformed Il-3-dependent BaF3 cells at a concentration of 10 μM in a study comparing the activity of PD166326 and Ariad molecule AP23464 cited below. Both compounds potently inhibit most mutant variants, and *in vitro* drug selection demonstrates that the two compounds are less susceptible to resistance than Imatinib. Combinations of inhibitors essentially suppressed all the resistance mutations, with the notable exception of T315I [111].

Regarding the binding mode, the crystal structure of Abl in complex with PD173955 was reported, in comparison with the Abl-Imatinib complex [28]. The pyrido[2,3-*d*]pyrimidine binds either the active or the inactive conformations of Abl, due to its smaller size as compared with Imatinib. Although Imatinib and PD173955 bind to the same site in the Abl kinase, 21 protein residues interact with the first compound and only 11 with PD173955, but in spite of the minor binding interface, PD173955 is more active than Imatinib [28].

4-ANILINO-3-QUINOLINECARBONITRILES

SKI-606 (**10**) (Fig. (**4**)) is a potent Src and Abl inhibitor from Wyeth researchers acting with an ATP-competitive mechanism included in this class of derivatives. This compound bearing a (2,4-dichloro-5-methoxy)anilino group on C4, showed an IC_{50} of 1.2 nM in an enzymatic assay and an IC_{50} of 100 nM on Src cell proliferation [112].

SKI-606 was found to be also an Abl kinase inhibitor with an IC_{50} of 1 nM in an Abl enzymatic assay. Moreover this compound inhibits the growth of three Brc-Abl positive leukemia cell-lines K562, KU812 and MEG-01 with IC_{50} values of 20, 5, 20 nM, respectively. Once daily oral administration of SKI-606 at 100 mg/kg for 5 days caused complete regression of large K562 xenografts in nude mice [113]. SKI-606 was also docked into the active site of c-Src (Brookhaven protein data bank accession code: 2src, inactive form), showing strong hydrogen bonding interactions between the backbone NH of Met341 and N1 of quinoline, the side chain hydroxyl group of Thr338 and the nitrogen atom of the CN group and between the quinoline C2 and the carbonyl oxygen of Glu339. The anilino group is positioned in the kinase-specific pocket, while the 4-methylpiperazine is oriented toward the solvent region. Recently computational studies on SKI-606 have been performed, docking the compound inside the active sites of both c-Src and Abl, demonstrating that the orientation and hydrogen bonding interaction of SKI-606 are similar in both kinases [114].

An important Src/Abl dual inhibitor recently reported by the same company is compound (**11**) [115] that showed an IC_{50} of 2.7 nM in a LANCE format Src enzyme assay and an IC_{50} of 0.78 nM in an Abl enzyme assay while in a cell growth test, showed an IC_{50} of 100 nM on Src cells and IC_{50} values of 5.8 and 18 nM on KU812 and K562 leukemia cell lines, respectively. Among the high number of anilinoquinolinecarbonitriles or isosthere carbonitriles synthesized by Wyeth and showing dual Abl/Src action [44], here we report compound (**12**), bearing a phenylaminothieno[3,2-*b*]pyridine ring and the 2,4-dichloro-5-methoxy substitution on the anilino moiety, always present in the most active compounds of this series. Compound (**12**) showed an IC_{50} value of 13 and 720 nM in an enzymatic assay and in an Src cell assay respectively. Moreover this compound showed an IC_{50} of 2.3 nM toward Abl kinase and IC_{50} of 32 nM in a proliferation assay on K562 cells [116, 117].

THIAZOLE-CARBOXAMIDES (BMS-354825)

In 2004 Bristol-Myers Squibb researchers presented a new family of dual Scr/Abl inhibitor, including BMS-354825, Dasatinib, (**13**) a potent inhibitors with IC_{50} values of 1 pM and 30 pM for Src and Bcr-Abl respectively, orally active in a K562 xenograft model of CML in mice.

Dasatinib is a competitive inhibitor 100 to 300-fold more potent than Imatinib. It binds both the inactive and active configuration of Abl, and makes fewer contacts with Abl protein than that of Imatinib, as shown by crystallographic structure [118-120].

Very recently Bristol-Myers Squibb researchers reported a crystallography structure of Desatinib bound to Abl kinase suggesting that the increased binding affinity of Dasatinib over Imatinib is probably due to its ability to recognize multiple states of Bcr-Abl. The structure also provides an explanation for the activity of Dasatinib against Imatinib-resistant Bcr-Abl mutants [121].

Dasatinib, a substituted thiazole carboxamide, presents chemical features quite different from the byciclic Abl/Src inhibitors previously reported in this review, that mime in some way the purinic structure of ATP. Dasatinib is active against most Abl kinase mutants, except Thr315Ile, prolongs survival of mice with Bcr-Abl driven disease [122] and when

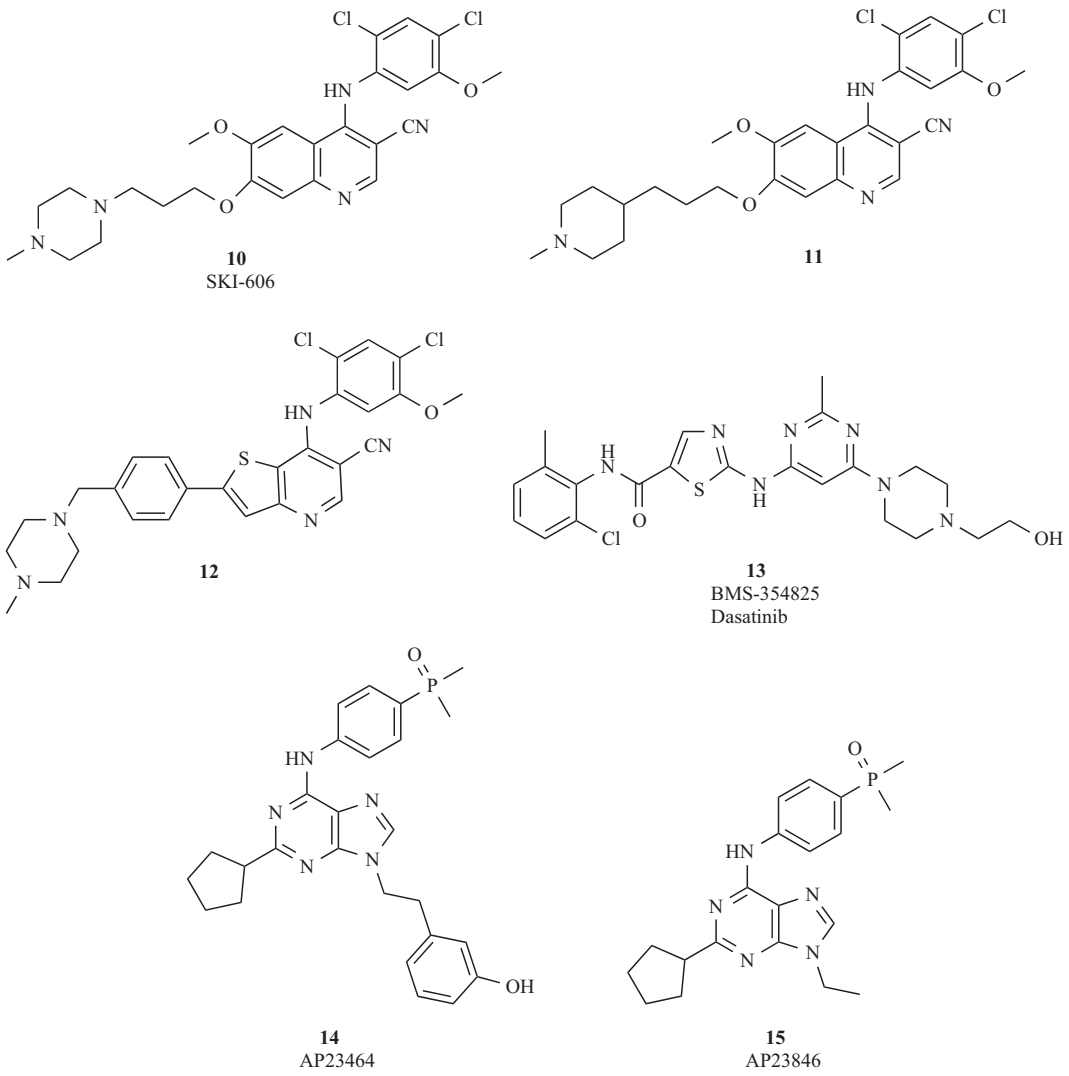


Fig. (4). Quinoline carbonitriles, BMS-354825, purine derivatives.

combined with Imatinib shows an additive effect, targeting a wider range of resistant clones than either single agent and delaying the onset of acquired drug resistance [123, 94].

In a mutagenesis-based screening with combinations of Imatinib, Dasatinib and Nilotinib (a recent Abl inhibitor) maximal suppression of resistant clone outgrowth was achieved at lower concentrations compared to single agents, suggesting that such combinations may be equipotent to higher doses of the single agents. Mutation Thr315Ile is insensitive also to this drug combination [124]

Copland and coworkers reported that Dasatinib targets an earlier progenitor population of CML cells than Imatinib, but does not eliminate the most primitive quiescent fraction, that appears to be resistant to both drugs [125].

Results from the phase I clinical trial demonstrated that Dasatinib induces hematologic and cytogenetic responses in patients with CML who cannot tolerate or are resistant to Imatinib, with the exception of the T315I mutation [126]; Dasatinib is now in phase II studies.

PURINES

AP23464, (**14**), from Ariad Pharmaceutical, is a potent ATP competitive inhibitor of Src and Abl kinases and shows antiproliferative activity against a human CML cell line and Bcr-Abl-transduced Ba/F3 cells with an IC_{50} value of 14 nM. AP23464 inhibits Bcr-Abl tyrosine phosphorylation, blocks cell cycle progression, and promotes apoptosis of Bcr-Abl-expressing cells. Importantly, AP23464 is active in the low nanomolar range on the frequently observed Imatinib mesylate-resistant Bcr-Abl mutants, including nucleotide binding P-loop mutants Q252H, Y253F, E255K, C-terminal loop mutant M351T, and activation loop mutant H396P, with the only exception of T315I [127]. Authors reported a 3D molecular model of AP23464 complexed to the Abl kinase domain and predict the binding of the compounds only to the active conformation of Abl. The bulky Ile of the T315I mu-

tant blocks access of the compound to the ATP binding site, thus explaining the resistance of this mutant to AP23464.

Very recently the same Authors guided by mutagenesis studies and molecular modelling designed a series of AP23464 analogues to target T315I. AP23846 (**15**), bearing an ethyl group on N9, instead of the bulkier ethyl-phenol substituent of AP23464, can dock within the Abl active site and does not clash with the T315I mutation. In fact, very importantly, compound AP23846 inhibited both native and T315I variants of Bcr-Abl in the submicromolar range, so becoming the first compound active on this mutant. Unfortunately (**15**) showed nonspecific cellular toxicity, inhibiting the BaF3 cell line transformed by Bcr-Abl, but also the proliferation of the parental BAF3 cell line [111].

As shown by the recent results obtained by different Groups it seems possible to obtain new potent dual Src-Abl inhibitors for the treatment of CML, but much work need to be done to develop less toxic drugs and more active against all forms of mutations.

ACKNOWLEDGEMENTS

Financial support from Italian MIUR (PRIN 2004059221_004) is gratefully acknowledged.

Financial support provided by the Fondazione Monte dei Paschi di Siena is gratefully acknowledged. We also thank Dr. Giovanni Gaviraghi (Sienabiotech S.p.A.) for helpful discussion.

S.S. thanks Dr. Chiara Brullo for library work and assistance.

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Received: 08 September, 2006 Revised: 21 September, 2006 Accepted: 05 October, 2006