Available online at www.sciencedirect.com
ScienceDirect
European Journal of Medicinal Chemistry 43 (2008) 2665–2676
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://www.elsevier.com/locate/ejmech

Original article

# Synthesis, biological evaluation and docking studies of 4-amino substituted 1*H*-pyrazolo[3,4-*d*]pyrimidines

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Received 26 November 2007; received in revised form 22 January 2008; accepted 23 January 2008
Available online 6 February 2008

**Abstract**

The synthesis of new 4-amino substituted pyrazolo[3,4-*d*]pyrimidines along with their activity in cell-free enzymatic assays on Src and Abl tyrosine kinases is reported. Some compounds emerged as good dual inhibitors of the two enzymes, showed antiproliferative effects on two Bcr–Abl positive leukemia cell lines K-562 and KU-812, and induced apoptosis, as demonstrated by the PARP assay. Docking studies have been also performed to analyze the binding mode of compounds under study and to identify the structural determinants of their interaction with both Src and Abl.


*Keywords:* Abl; Src; Pyrazolo-pyrimidines; Chronic myeloid leukemia; Docking

## 1. Introduction

c-Src is a non-receptor tyrosine kinase that plays important roles in several signal transduction pathways and is involved in development and progression of human solid malignancies, including colon, breast, pancreatic and lung cancers [1–3]. Src kinase activity is also linked with tumor metastatic growth, being involved in the disruption of focal adhesions that ensure the interaction between the cell and the extracellular matrix, and of adherens junctions that permit the adhesion of the cells to each other and are critical for cell migration [4,5]. Transfection of cancer cells with activated c-Src leads to disorganized and less effective focal adhesion structures, with reduced cell clustering, promoting the release of cells from the matrix [6]. High level or overexpression of Src in tumors is correlated with poor prognosis.

Besides having a role in solid tumors, Src is also involved in the progression of haematological malignancies, in particular chronic myeloid leukemia (CML) and acute myeloid leukemia (AML). These diseases are characterized by the presence of the Philadelphia chromosome, derived from a reciprocal translocation between chromosomes 9 and 22 [7]; the translocation fuses the breakpoint cluster region (Bcr) and the Abl genes, forming the Bcr–Abl oncogene which encodes a constitutively active cytoplasmatic tyrosine kinase (TK) Bcr–Abl. This deregulated enzyme causes hyperproliferation of the leukemic cells and the consequent pathology of the disease. The finding that Bcr–Abl is the cause of the leukemic phenotype and that the tyrosine kinase activity of Abl is fundamental for Bcr–Abl-mediated transformation made this kinase an important target for the development of specific therapies. In the recent past, advances in the selective inhibition of Bcr–Abl kinase activity led to the

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doi:10.1016/j.ejmech.2008.01.034

development of Imatinib mesylate (Gleevec) that now represents the first line treatment for CML. Unfortunately, either overexpression or mutations of Bcr–Abl confer resistance to Gleevec. As a consequence, there is a growing interest in the development of second generation small molecule inhibitors, able to treat Gleevec resistant CML [8–11].

It has been recently demonstrated that Src kinase activity remains active following Imatinib inhibition of Bcr–Abl in leukemic cells [12], and that simultaneous inhibition of both Bcr–Abl and c-Src kinases results in long-term survival of mice with AML [13].

These reasons, together with the striking similarity between the catalytically active states of the c-Src and Abl kinases, prompted medicinal chemists, helped by computational methods, to synthesize dual Src/Abl inhibitors that might be active against Philadelphia positive forms of leukemia [14]. Actually, structurally different compounds identified as c-Src inhibitors were subsequently also described as Abl inhibitors [15]. The most recent and important dual Src/Abl inhibitors include the pyrido-pyrimidine PD166326 **1** [16], the purine AP23846 **2**, by Ariad Pharmaceuticals [17], the anilinoquinazoline AZD0530 **3**, by AstraZeneca [18], the quinolinecarbonitrile SKI-606 (bosutinib) **4** by Wyeth [19–21] and its recent furyl derivatives [22], the benzotriazine **5** [23]; finally the thiazole-carboxamide BMS-354825 **6** (dasatinib), by Bristol-Myers Squibb [24–26], was approved by the FDA in 2006 for the treatment of CML [27] (Fig. 1).

In this context, in continuing our efforts to find new anticancer agents, we have recently synthesized several pyrazolo[3,4-*d*]pyrimidines that showed inhibition properties toward Src in a cell-free assay, as well as antiproliferative activity toward the epidermoid (A431) and breast cancer (BC-8701) cell lines, blocking Src phosphorylation and inducing apoptosis. In particular, compound **7a** (Table 1) showed a submicromolar activity toward isolated Src and an inhibitory activity about two-fold higher than that of the

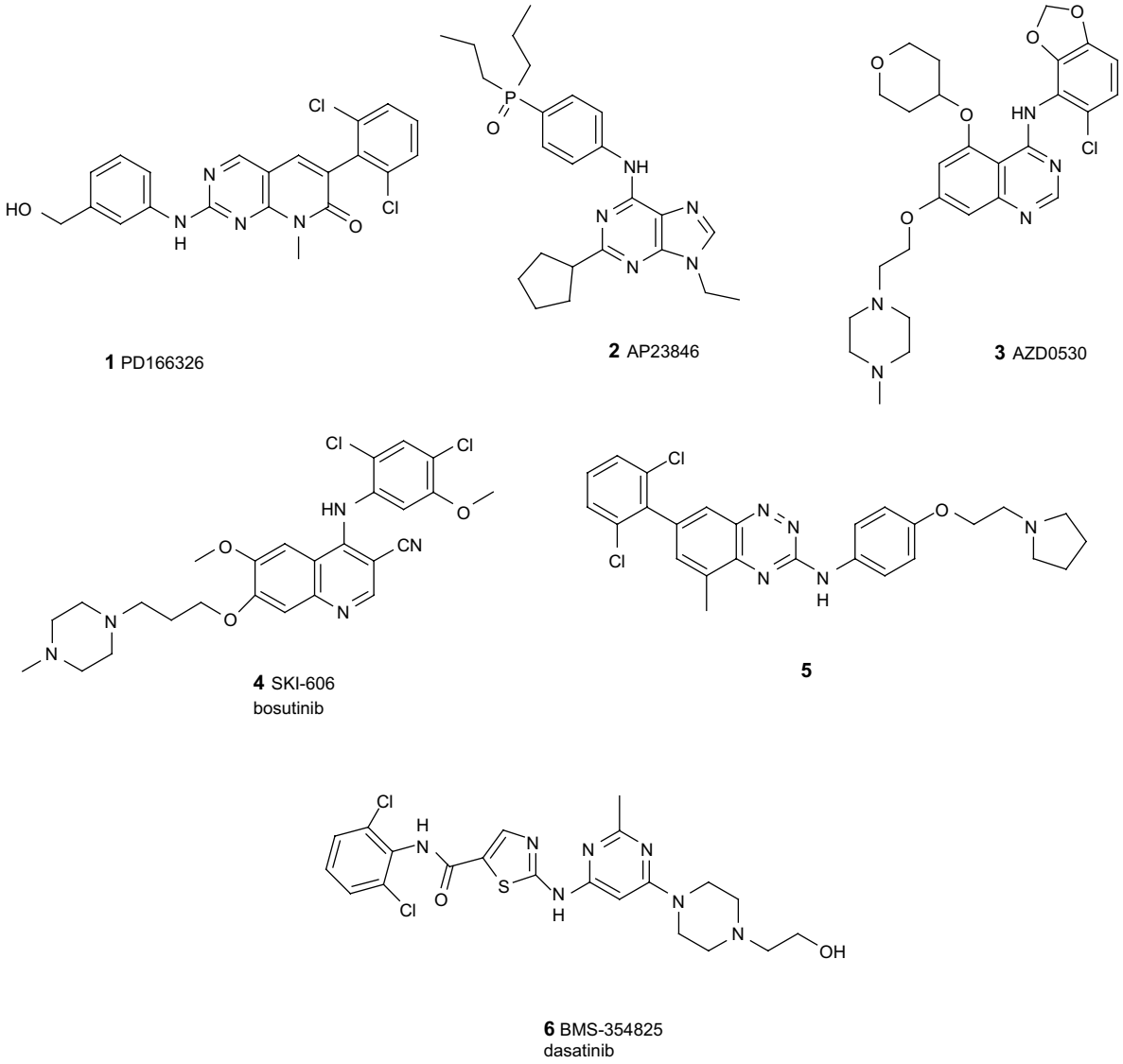
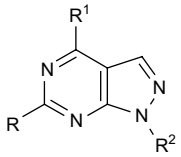



Fig. 1. Structures of Abl/Src dual inhibitors.

Table 1
Structure and inhibitory activity of compounds **7a–m**, **8a–q**, **9a–h** and **10a–c** toward isolated Abl and Src kinases



| Compd. | R | $R^1$ | $R^2$ | Src enzyme $K_i$[a] (μM) | Abl enzyme $K_i$[b] (μM) |
|---|---|---|---|---|---|
| **7a**[c] | $SCH_3$ | $NHCH_2CH_2C_6H_5$ | $CH_2{-}CHCl{-}C_6H_5$ | 0.7 ± 0.2 | 7.3 ± 1.2 |
| **7b**[c] | $SCH_3$ | $NHCH_2C_6H_5$ | $CH_2{-}CHCl{-}C_6H_5$ | 3.7 ± 0.9 | 0.3 ± 0.1 |
| **7c**[c] | $SCH_3$ | $NHC_3H_7$ | $CH_2{-}CHCl{-}C_6H_5$ | 2.9 ± 0.8 | 4.8 ± 0.6 |
| **7d**[c] | $SCH_3$ | $NHC_4H_9$ | $CH_2{-}CHCl{-}C_6H_5$ | 1.7 ± 0.4 | 1.2 ± 0.3 |
| **7e**[c] | $SCH_3$ | $NH(CH_2)_2OC_2H_5$ | $CH_2{-}CHCl{-}C_6H_5$ | NA | 1.5 ± 0.5 |
| **7f**[c] | $SCH_3$ | 1-Pyrrolidinyl | $CH_2{-}CHCl{-}C_6H_5$ | ND | NA |
| **7g**[c] | $SCH_3$ | 1-Piperidinyl | $CH_2{-}CHCl{-}C_6H_5$ | 2.4 ± 0.7 | NA |
| **7h**[c] | $SCH_3$ | 4-Morpholinyl | $CH_2{-}CHCl{-}C_6H_5$ | 6.5 ± 1.0 | NA |
| **7i**[c] | $SCH_3$ | $N(C_2H_5)_2$ | $CH_2{-}CHCl{-}C_6H_5$ | 0.5 ± 0.1 | 0.40 ± 0.05 |
| **7l**[c] | $SCH_3$ | $NHC_6H_5$ | $CH_2{-}CHCl{-}C_6H_5$ | 1.2 ± 0.3 | 0.40 ± 0.07 |
| **7m** | $SCH_3$ | $NHC_6H_4$-*m*F | $CH_2{-}CHCl{-}C_6H_5$ | 1.4 ± 0.3 | 0.40 ± 0.1 |
| **8a** | $SCH_3$ | $NHCH_2CH_2C_6H_4$-*o*F | $CH_2{-}CHCl{-}C_6H_5$ | 10.2 ± 1.8 | 0.40 ± 0.1 |
| **8b** | $SCH_3$ | $NHC_3H_7$ | $CH_2{-}CHCl{-}C_6H_4$-*p*F | 2.61 ± 1.2 | 0.57 ± 0.1 |
| **8c** | $SCH_3$ | $NHC_4H_9$ | $CH_2{-}CHCl{-}C_6H_4$-*p*F | 5.02 ± 0.9 | 0.11 ± 0.02 |
| **8d** | $SCH_3$ | 4-Morpholinyl | $CH_2{-}CHCl{-}C_6H_4$-*p*F | 3.32 ± 1.0 | 0.97 ± 0.3 |
| **8e** | $SCH_3$ | $NHCH_2C_6H_4$-*p*F | $CH_2{-}CHCl{-}C_6H_4$-*p*F | 0.31 ± 0.07 | 0.22 ± 0.03 |
| **8f** | $SCH_3$ | $NHCH_2C_6H_4$-*o*F | $CH_2{-}CHCl{-}C_6H_4$-*p*F | 0.30 ± 0.06 | 0.34 ± 0.02 |
| **8g** | $SCH_3$ | $NHCH_2CH_2C_6H_5$ | $CH_2{-}CHCl{-}C_6H_4$-*p*F | 3.25 ± 0.8 | 0.20 ± 0.04 |
| **8h** | $SCH_3$ | $NHC_6H_4$-*m*F | $CH_2{-}CHCl{-}C_6H_4$-*p*F | 0.21 ± 0.02 | 0.22 ± 0.04 |
| **8i** | $SCH_3$ | $NHC_3H_7$ | $CH_2{-}CHCl{-}C_6H_4$-*p*Cl | 1.32 ± 0.4 | 0.40 ± 0.03 |
| **8j** | $SCH_3$ | $NHC_4H_9$ | $CH_2{-}CHCl{-}C_6H_4$-*p*Cl | 1.53 ± 0.6 | 0.39 ± 0.06 |
| **8k** | $SCH_3$ | $N(C_2H_5)_2$ | $CH_2{-}CHCl{-}C_6H_4$-*p*Cl | 1.62 ± 0.5 | 0.30 ± 0.1 |
| **8l** | $SCH_3$ | 4-Morpholinyl | $CH_2{-}CHCl{-}C_6H_4$-*p*Cl | 3.21 ± 0.7 | 0.50 ± 0.05 |
| **8m** | $SC_2H_5$ | $N(C_2H_5)_2$ | $CH_2{-}CHCl{-}C_6H_5$ | 5.28 ± 0.5 | 0.33 ± 0.1 |
| **8n** | $SC_3H_7$ | $NHC_3H_7$ | $CH_2{-}CHCl{-}C_6H_5$ | 3.60 ± 0.4 | 0.25 ± 0.04 |
| **8o** | $SC_3H_7$ | $NHC_4H_9$ | $CH_2{-}CHCl{-}C_6H_5$ | 2.56 ± 0.7 | 0.52 ± 0.08 |
| **8p** | $SC_3H_7$ | $N(C_2H_5)_2$ | $CH_2{-}CHCl{-}C_6H_5$ | 5.37 ± 0.9 | 0.28 ± 0.01 |
| **8q** | $SC_3H_7$ | $NHCH_2C_6H_5$ | $CH_2{-}CHCl{-}C_6H_5$ | 0.51 ± 0.1 | 0.10 ± 0.01 |
| **9a** | $SCH_3$ | $NHC_3H_7$ | $CH_2{-}CHBr{-}C_6H_5$ | 2.55 ± 0.5 | 0.88 ± 0.2 |
| **9b** | $SCH_3$ | $NHC_4H_9$ | $CH_2{-}CHBr{-}C_6H_5$ | 0.81 ± 0.3 | 0.32 ± 0.04 |
| **9c** | $SCH_3$ | $NH(CH_2)_2OC_2H_5$ | $CH_2{-}CHBr{-}C_6H_5$ | 5.66 ± 0.9 | 0.55 ± 0.04 |
| **9d** | $SCH_3$ | 1-Pyrrolidinyl | $CH_2{-}CHBr{-}C_6H_5$ | 1.63 ± 0.4 | 1.40 ± 0.3 |
| **9e** | $SCH_3$ | 1-Piperidinyl | $CH_2{-}CHBr{-}C_6H_5$ | 0.32 ± 0.03 | 0.88 ± 0.4 |
| **9f** | $SCH_3$ | 4-Morpholinyl | $CH_2{-}CHBr{-}C_6H_5$ | 2.57 ± 0.7 | 0.97 ± 0.3 |
| **9g** | $SCH_3$ | $NHCH_2C_6H_5$ | $CH_2{-}CHBr{-}C_6H_5$ | 0.22 ± 0.01 | 0.19 ± 0.03 |
| **9h** | $SCH_3$ | $NHCH_2CH_2C_6H_5$ | $CH_2{-}CHBr{-}C_6H_5$ | 2.80 ± 0.4 | 0.27 ± 0.03 |
| **10a** | $N(CH_3)_2$ | $NHC_3H_7$ | $CH_2{-}CHCl{-}C_6H_5$ | 4.10 ± 0.5 | 0.32 ± 0.04 |
| **10b** | $N(CH_3)_2$ | 4-Morpholinyl | $CH_2{-}CHCl{-}C_6H_5$ | 3.63 ± 0.4 | 0.28 ± 0.02 |
| **10c** | $N(CH_3)_2$ | $NHCH_2C_6H_5$ | $CH_2{-}CHCl{-}C_6H_5$ | 4.22 ± 0.5 | 0.16 ± 0.01 |
| PP2 | | | | 0.50 ± 0.1 | 0.52 ± 0.2 |
| Imatinib | | | | 31.0 ± 2.2 | 0.013 ± 0.005 |

[a] $K_i$ values toward isolated Src calculated according to the following equation: $K_i = (ID_{50} - E_0/2)/\{E_0 - [S_0/K_{m(ATP)} - 1]/E_0\}$, where $E_0$ and $S_0$ are the enzyme and the ATP concentrations (0.0125 and 0.0160 μM, respectively). NA = not active ($ID_{50}$ >2 mM). ND = not determined.

[b] $K_i$ values toward isolated Abl calculated according to the following equation: $K_i = ID_{50}/\{E_0+[E_0(K_{m(ATP)}/S_0)]\}/E_0$, where $E_0$ and $S_0$ are the enzyme and the ATP concentrations (0.005 and 0.012 μM, respectively). NA = not active ($ID_{50}$ >2 mM). ND = not determined.

[c] Structures and inhibitory activity of previously published compounds [29,30].

reference compound 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)-pyrazolo[3,4-*d*]pyrimidine (PP2) [28,29]. Successively, based on the fact that compounds acting as c-Src inhibitors often also showed activity toward Bcr–Abl, we tested a set of our pyrazolo[3,4-*d*]pyrimidine derivatives on Abl isolated enzyme and on a panel of leukemia cell lines: the studied compounds were able to inhibit Bcr–Abl and c-Src phosphorylation, induced apoptosis and, as the activation of Src and Abl is an important step in the progression of leukemia cells (in particular, CML) [30], reduced cell proliferation.

We are reporting here the synthesis of a new series of 4-amino substituted pyrazolo[3,4-*d*]pyrimidines bearing a 2-chloro-2-phenylethylamino N1 side chain, present in the

most active compounds previously reported by us. We decided to insert a halogen atom (F or Cl) on the N1 phenyl ring side chain in a number of compounds (**8b–l**), to verify the possible influence of such substitution on the activity. Moreover in derivatives **9a–h** the chlorine atom on the C2 of side chain was replaced with a bromine. Thio-methyl, -ethyl and -propyl groups at C6 have been maintained, the only exception being derivatives **10a–c**, where a dimethylamino group has been inserted at C6, using a different chemical approach. The newly synthesized compounds have been tested in cell-free enzymatic assays against c-Src and Abl and the most active derivatives of the series have been also tested for their antiproliferative effects on Bcr–Abl-expressing cells, namely the CML K-562 cells and the basophilic leukemia KU-812 cells.

## 2. Chemistry

The synthesis of derivatives **8a–q**, **9a–h** and **10a–c** are depicted in Schemes 1, 2 and 3, respectively.

Compounds **8a–q** were prepared starting from 1-substituted-4-chloro-6-(alkylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidines **11a–e**, obtained in four steps from the appropriate ethyl 5-amino-1-[2-phenyl or 2-(4-halophenyl)-2-hydroxyethyl]-1*H*-pyrazole-4-carboxylates, following our reported procedure [31,32]. Regioselective substitution of the C4 chlorine atom with an excess of various amines in anhydrous toluene at room temperature afforded the desired compounds **8a–q** in high yields (Scheme 1).

Treatment of intermediate **12** [31] with phosphorous tribromide and pyridine in anhydrous toluene and anhydrous DMF afforded the 1-(2-bromo-2-phenylethyl)-6-(methylthio)-1,5-dihydro-4*H*-pyrazolo[3,4–*d*]pyrimidin-4-one **13**, that was in turn treated with the Vilsmeier complex ($POCl_3$:DMF 1:1, 15 equiv) in $CHCl_3$ to obtain the C4 chlorinated derivative **14**. This compound was purified in good yield by chromatography on Florisil column. Finally, reaction of **14** with an excess of various amines in anhydrous toluene at room temperature afforded compounds **9a–h** (Scheme 2).

The starting product for the synthesis of dimethylamino derivatives **10a–c** is 5-amino-1-(2-hydroxy-2-phenylethyl)-1*H*-pyrazole-4-carbonitrile **15**, obtained in good yield by reaction of 2-hydrazino-1-phenylethanol and ethoxymethylenemalononitrile in ethanol at reflux as previously reported [33]. Intermediate **15** was reacted with *N*,*N*-dimethylphosgeniminium chloride in dichloroethane at reflux for 2 h to give the corresponding dimethylcarbamimidic chloride **16**, then cyclized with anhydrous hydrochloric acid to the 4-chloro-1-(2-chloro-2-phenylethyl)-*N*,*N*-dimethyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-6-amine **17** in satisfactory yield (65%). C4 chlorine atom was substituted by reaction with the appropriate amines in anhydrous toluene at room temperature for 24 h to give the desired compounds **10a–c** (Scheme 3).

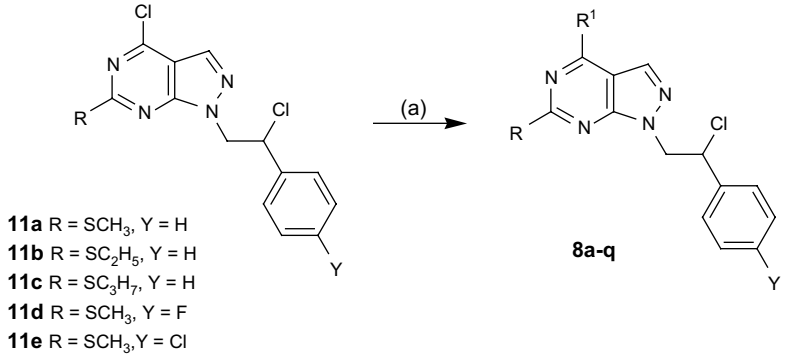
## 3. Biology

The affinity toward c-Src and Abl TKs of the newly synthesized compounds was evaluated in cell-free assays. Enzymatic data are reported in Table 1 along with those of the reference compounds PP2 and Imatinib. Three of the most interesting molecules were then tested to assess their effects on the proliferation of two Bcr–Abl positive leukemia cell lines, namely K-562 and KU-812, and their activity was compared to that of PP2, chosen as reference compound. In fact, PP2 inhibits either Src [34] or Abl TK [35] with similar potency, thus resulting an appropriate reference when the activity toward both kinases is to be compared. Moreover, a huge amount of literature has been recently published on PP2, giving us exhaustive information on its biological profile toward both TKs; finally, PP2 is an easily available commercial compound. Assays on proapoptotic activity of selected compounds have been performed as well.

## 4. Results and discussion

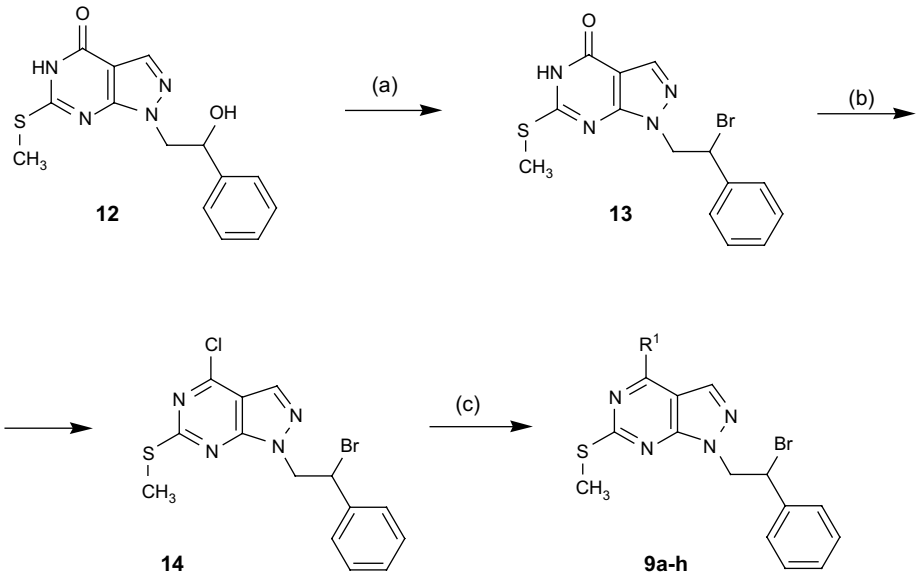
### 4.1. Enzymatic assays

Compounds **8a–q, 9a–h**, and **10a–c** showed interesting dual Src/Abl activity, with a general improvement in the affinity toward both the enzymes as compared with that previously reported by us [29,30], even if generally resulted more active toward Abl than c-Src when tested in cell-free assays (Table 1). In more detail, they possessed $K_i$ values lower than 0.5 μM for Abl, the only exceptions being **8d**, **9a**, **9d**, **9e** and **9f**, whose $K_i$




**11a** R = $SCH_3$, Y = H
**11b** R = $SC_2H_5$, Y = H
**11c** R = $SC_3H_7$, Y = H
**11d** R = $SCH_3$, Y = F
**11e** R = $SCH_3$,Y = Cl

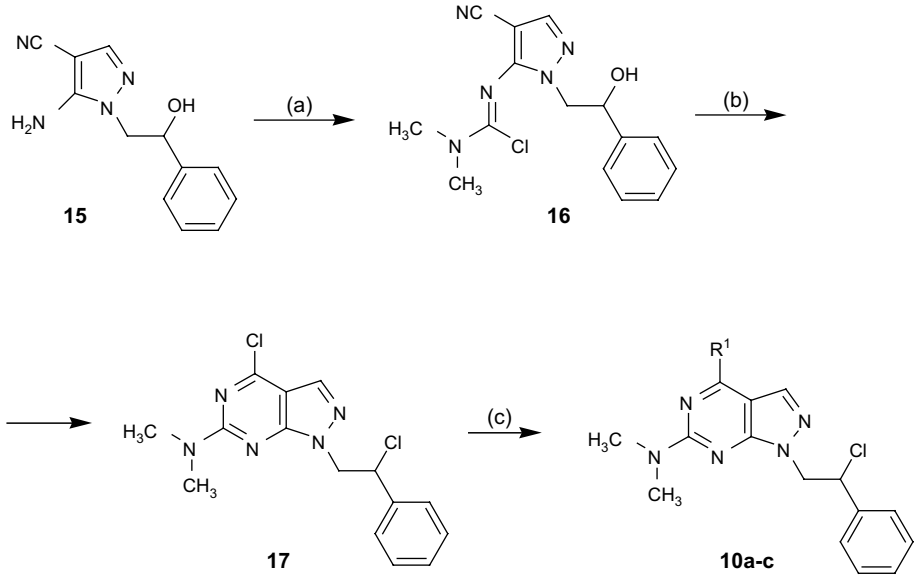
Scheme 1. a) Amines, toluene, r.t.




Scheme 2. (a) $PBr_3$, Py, toluene, DMF, r.t.; (b) $POCl_3$/DMF, $CHCl_3$, reflux; (c) amines, toluene, r.t.

values ranged between 0.88 and 1.40 μM. The most active derivatives were **8c**, **8e**, **8g**, **8h, 8n**, **8q**, **9g** and **10c**, with $K_i$ values of 0.11, 0.22, 0.20, 0.22, 0.25, 0.10, 0.19 and 0.16 μM, respectively, but also **8f**, **8k**, **8m**, **8p**, **9b**, **9h**, **10a** and **10b** showed interesting potency with $K_i$ values lower than 0.35 μM.

Regarding Abl inhibition, the most important structural feature was represented by the amino-substituent on C4; in fact, a benzylamino group in such position conferred a higher activity (see **8e**, **8q**, **9g** and **10c**), independently from the C6 and the N1 side chain. The *meta*-fluoro anilino derivative **8h** and the phenylethylamino derivatives **8g** and **9h** ($K_i$ values of 0.22, 0.20 and 0.27 μM, respectively) showed a good potency, pointing out the importance of a phenyl-substituted side chain at C4. Short aliphatic amino or cyclo-amino groups generally induced a drop in the activity, with the notable exception of the butylamino-derivative **8c**, possessing a $K_i$ value of 0.11 μM; this compound was one of the most potent compounds of this series, together with **10b** (0.28 μM), bearing a morpholino substituent group at C4. As a general trend, the 2-bromine atom of the 2-halo-2-phenylethyl chain at N1 is preferable to chlorine; moreover, among the 2-chloro-2-phenylethyl derivatives, a *para*-fluoro or *para*-chloro phenyl substitution at the N-1 side chain was present in a number of active compounds, namely **8c**, **8e**, **8g**, **8h** and **8k**. Overall, the new derivatives bearing a halogen substitution on the phenyl ring of the N1 side chain were more active on Abl when compared to those unsubstituted and previously reported by us [30]. As an example, the C4 butylamino-1-(2-chloro-2-phenylethyl)-6-methylthio derivative **7d** possessed a $K_i$ of 1.2 μM, while




Scheme 3. a) *N*,*N*-Dimethylphosgeniminium chloride, $ClCH_2CH_2Cl$, reflux; (b) HCl (g), $ClCH_2CH_2Cl$, r.t.; (c) amines, toluene, r.t.

the corresponding *para*-fluoro **8c** and *para*-chloro **8j** showed $K_i$ values of 0.11 and 0.39 μM, respectively; the same behaviour was detected in the *n*-propyl derivatives, for which the activity increased by an order of magnitude (4.8 μM for the unsubstituted derivative **7c** and 0.57 and 0.40 μM for the *para*-fluoro derivative **8b** and for the *para*-chloro **8i** derivative, respectively).

As regards the activity toward c-Src, **8e**, **8f**, **8h**, **8q**, **9e** and **9g** emerged as the most potent compounds of the series, with $K_i$ values of 0.31, 0.30, 0.21, 0.51, 0.32 and 0.22 μM, respectively; noteworthy, also in this case the importance of a benzylamino or anilino-group on C4 (the only exception being the piperidino-derivative **9e**) and of the 2-chloro-2-(4-fluorophenyl)ethyl chain at N1 was confirmed.

Also the C-6 substitution seemed to play a fundamental role for Src activity: in fact, the activity decreased with the lengthening of the *S*-alkyl group from methyl to ethyl or propyl, with the only exception of the benzylamino derivative **8q**, endowed with a $K_i$ value of 0.51 μM toward c-Src.

A similar trend was observed for derivatives **10a–c**, bearing at C6 a bulkier dimethylamino group, which showed a drop in activity when compared with the corresponding thio-methyl compounds.

Derivative **9g** has been also tested on a panel of TKs showing selectivity for Src and Abl [36].

### 4.2. Inhibition of proliferation

Compounds that showed lower $K_i$ values on Abl isolated enzyme, namely **8c**, **8q** and **9g**, were also evaluated for their inhibitory activity on two leukemia cell lines, using PP2 as a reference compound. Results of antiproliferative assays (Table 2) showed that the selected compounds also possessed an interesting cellular activity, in particular toward the K-562 cell line, with $IC_{50}$ values comparable with those of the reference compound. However, it is important to note that the improved enzymatic potency of pyrazolo-pyrimidine derivatives with respect to PP2 had no marked effect on the cellular activity. Experimental and theoretical pharmacokinetic data regarding pyrazolo-pyrimidines similar to those reported herein suggested that for some of the studied compounds the scarce activity in cellular assay may be due to their metabolic instability or, alternatively, to their low solubility in the assay conditions, which in part would prevent compounds from entering cells [32].

Table 2
Inhibitory activity of compounds **8c**, **8q** and **9g** on human leukemia K-562 and KU-812 cell lines

| | $IC_{50}$[a] (μM) | |
|---|---|---|
| | K-562 | KU-812 |
| PP2 | 24.8 ± 0.9 | 45.2 ± 2.9 |
| **8c** | 29.2 ± 1.5 | 37.5 ± 4.1 |
| **8q** | 27.3 ± 1.0 | 67.1 ± 1.8 |
| **9g** | 25.0 ± 1.3 | 35.9 ± 2.0 |

[a] $IC_{50}$ values are means ± SD of five experiments, each performed in triplicate.

The same compounds, besides showing interesting antiproliferative activity, were also able to inhibit Bcr–Abl phosphorylation with an activity comparable to PP2 on both cell lines (data not shown).

### 4.3. Proapoptotic activity

Given the antiproliferative activity showed by compounds **8c**, **8q** and **9g** on both K-562 and KU-812 cell lines, the same agents were subsequently tested for their proapoptotic activity on a poly-ADP-Ribose-Polymerase (PARP) assay [29]. Compounds **8c**, **8q** and **9g** potently induced apoptosis in KU-812 cell line, with cleaved/uncleaved PARP ratio higher than that of PP2 (39, 33, 35 and 20% for **8c**, **8q**, **9g** and PP2, respectively). Also on K-562 cell line **8c**, **8q** and **9g** showed significant proapoptotic activity (24, 22, 25 and 23% for **8c**, **8q**, **9g** and PP2, respectively), though lower in comparison to that showed on KU-812 cell line. On the basis of the ability of **9g** to induce apoptosis in both cell lines, we also investigated the expression of Bax/Bcl-xL mRNA in KU-812 cells treated with this compound.

It is known that molecular mechanisms of apoptosis involve change in expression of distinct genes. As an example, the ratio between proapoptotic Bax and antiapoptotic Bcl-2 or Bcl-xL genes is a critical determinant to induce cells toward apoptosis [37]. Death signals induce an increase in expression of the Bax mRNA message, leading Bax itself to gain proapoptotic activity [38]. On the other hand, the proapoptotic action of Bax is antagonized by Bcl-2 and BcL-xL, which are both able to inhibit the release of cytochrome C from mitochondria. In this context, Bcr–Abl participates in maintenance of an antiapoptotic environment through the regulation of Bcl-2 antiapoptotic members [39]. For the above reasons, the ratio between Bax mRNA and Bcl-xL mRNA expression may be used as a direct index of the induction of the apoptotic process and could be useful in explaining the molecular mechanism of apoptosis induction. Incubation of KU-812 cells for 72 h in the presence of the inhibitor **9g** led to an increase in the Bax/Bcl-xL ratio (that was 100 for the medium control and about 280 in the presence of 50 μM of **9g**).

### 4.4. Modeling studies

Docking studies have been performed on the novel pyrazolo-pyrimidines to explore the structural features responsible for their activity toward both c-Src and Abl kinases. Likewise previous molecules of the same series, compounds **8a–q**, **9a–h** and **10a–c** were shown to act as ATP competitive inhibitors [29,30]; they were therefore subjected to focused docking experiments within the ATP binding site of the two target enzymes. Computational analysis was performed by using a recently deposited crystallographic structure of Src (PDB entry: 1YOL) [40]; it involved the same procedures previously applied by our research group to address an analogous task [29,30,41] and provided very similar results, as expected. In fact, for all derivatives reported herein we identified the binding mode that we have already described for other

6-substituted pyrazolo-pyrimidines of the same family. In detail, in both enzymes compounds **8a–q**, **9a–h** and **10a–c** located their pyrazolo-pyrimidine nucleus within the adenine region of the ATP binding site, directing the side chains at C4 and N1 toward two hydrophobic regions, labeled as hydrophobic regions I and II, and the alkylthio substituent toward the external region of the binding pocket, though still involved in hydrophobic contacts. However, whereas the hydrophobic region I in Src was occupied by C4 substituents, the same region in Abl hosted the N1 side chain. Conversely, the hydrophobic pockets II of c-Src and Abl were contacted by substituents at N1 and C4, respectively. Finally, some differences also involved the location of the alkylthio groups, which occupied an additional small hydrophobic region in c-Src while sharing the hydrophobic region II with the side chain at N1 in the case of Abl. Concerning the hydrogen bonds, two interactions were identified between the studied molecules and the Src binding site (the second hydrogen bond was observed only for compounds bearing a secondary amino group, which could act as hydrogen bond donor, at C4) whereas only one interaction characterized the binding with Abl. As an example, Fig. 2 reports a schematic representation of the binding models proposed for **8h**, one of the most interesting compounds as dual inhibitor, for both c-Src and Abl.

## 5. Conclusion

The inhibition properties of some new pyrazolo[3,4-*d*]pyrimidines toward Abl and c-Src in cell-free assays along with their antiproliferative activity toward two human leukemia cell lines are reported, demonstrating that some compounds (**8e**, **8f**, **8h**, **8q** and **9g**) are interesting dual inhibitors of Abl/Src and could be important hits in the field of antileukemic agents, as they probably affect a pathway that is pivotal for the growth of transformed cells. In particular, such compounds showed good enzymatic activity toward Abl, suggesting that some of them could be effective agents in the chemotherapy armamentarium against CML. However, at the moment we cannot exclude that their antiproliferative activity could in part derive from targeting other tyrosine kinases, and further experiments are therefore ongoing to better investigate the mechanism of action of such compounds.

## 6. Experimental protocols

### 6.1. Chemistry

Starting materials were purchased from Aldrich-Italia (Milan, Italy). Melting points were determined with a Büchi 530 apparatus and are uncorrected. IR spectra were measured in KBr with a Perkin–Elmer 398 spectrophotometer. $^1$H NMR spectra were recorded in a $(CD_3)_2SO$ solution on a Varian Gemini 200 (200 MHz) instrument. Chemical shifts are reported as $\delta$ (ppm) relative to TMS as internal standard, $J$ in hertz. $^1$H patterns are described using the following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, sx = sextet, m = multiplet, br = broad.

All compounds were tested for purity by TLC (Merck, Silica gel 60 $F_{254}$, $CHCl_3$ as the eluant).

Analyses for C, H, N and S were within ±0.3% of the theoretical value.

#### 6.1.1. 1-(2-Bromo-2-phenylethyl)-6-(methylthio)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one **13**

To a mixture of 1-(2-hydroxy-2-phenylethyl)-6-(methylthio)-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one **12** (3.02 g, 10 mmol) [31] in anhydrous dimethylformamide (DMF) (20 mL) was added dropwise at 0 °C a solution of $PBr_3$ (3.27 g, 12.1 mmol), pyridine (0.5 mL) and anhydrous toluene

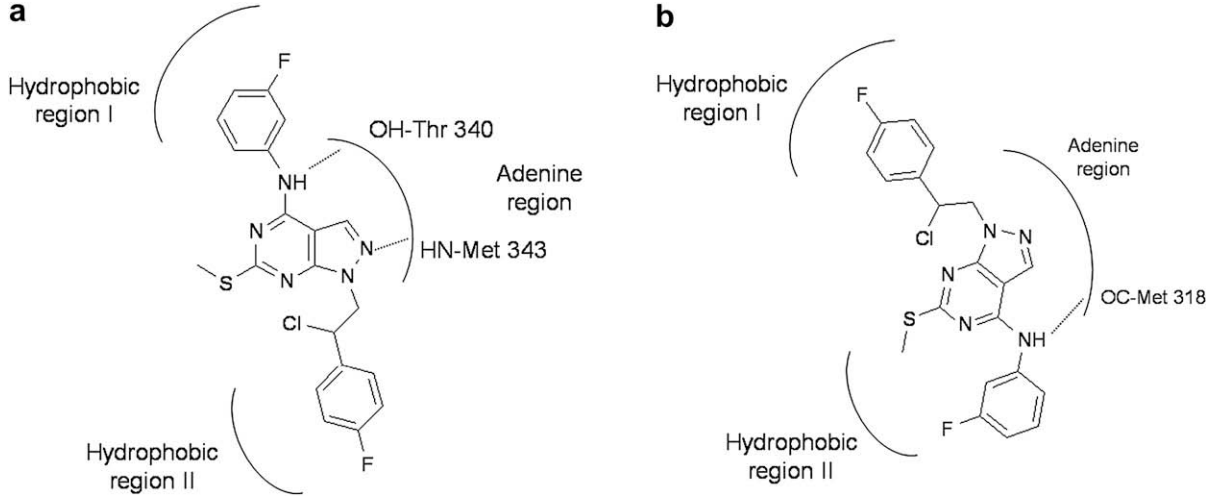



Fig. 2. Schematic representation of the binding mode of compound **8h** into the ATP binding site of (a) Src: **8h** establishes two hydrogen bonds (black dashed lines) with Src, the first between the amino group at C4 and the side chain of Thr340, and the second between the N2 and the NH backbone of Met343 and (b) Abl: **8h** is engaged in a single hydrogen bond involving the C4 amino group and the carbonyl backbone of Met318. For the sake of clarity, only a few residues among those interacting with the compound are displayed. The figure shows that the location of side chains at N1 and C4 is inverted in the two enzymes, and that the alkylthio moiety occupies a slightly different position within the two binding pockets.

(5 mL). The mixture was stirred at room temperature for 60 h. Toluene was removed under reduced pressure, then the mixture was poured into cold water; standing in a refrigerator for 12 h, a white solid was obtained, then filtered, washed with $H_2O$ and purified by column chromatography (silicagel, 100–200 mesh) using as the eluant a mixture of $CHCl_3$/$CH_3OH$ (9:1), to afford the pure product **13** (2.37 g, 65%) as a white solid, mp 216–217 °C. $^1$H NMR: $\delta$ 2.61 (s, 3H, $SCH_3$), 4.86–5.00 (m, 2H, $CH_2N$), 5.64–5.73 (m, 1H, CHBr), 7.25–7.56 (m, 5H Ar), 7.99 (s, 1H, H-3), 12.35 (br s, 1H, NH disappears with $D_2O$). IR cm$^{-1}$: 3100–2800 (NH), 1671 (CO). Anal. $C_{14}H_{13}N_4OBrS$ (C, H, N, S).

### 6.1.2. 1-(2-Bromo-2-phenylethyl)-4-chloro-6-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine **14**

The Vilsmeier complex, previously prepared from $POCl_3$ (23 g, 150 mmol) and anhydrous DMF (10.97 g, 150 mmol) was added to a suspension of 1-(2-bromo-2-phenylethyl)-6-(methylthio)-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one **13** (3.65 g, 10 mmol) in $CHCl_3$ (50 mL). The mixture was refluxed for 12 h. After cooling, the solution was washed with $H_2O$ (2 × 20 mL), dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The crude oil was purified by column chromatography (Florisil, 100–200 mesh) using diethyl ether as the eluant, and afforded the pure product **14**, which crystallized on standing in a refrigerator by adding a mixture of diethyl ether/petroleum ether (bp 40–60 °C) (1:1) as a light yellow solid (3.57 g, 93%), mp 89–90 °C. $^1$H NMR: $\delta$ 2.66 (s, 3H, $SCH_3$), 4.78–5.16 (m, 2H, $CH_2N$), 5.47–5.62 (m, 1H, CHBr), 7.25–7.47 (m, 5H Ar), 8.02 (s, 1H, H-3). Anal. $C_{14}H_{12}N_4ClBrS$ (C, H, N, S).

### 6.1.3. N′-[4-Cyano-1-(2-hydroxy-2-phenylethyl)-1H-pyrazol-5-yl]-N,N-dimethylcarbamimidic chloride **16**

To a solution of 5-amino-1-(2-hydroxy-2-phenylethyl)-1*H*-pyrazole-4-carbonitrile **15** (2.28 g, 10 mmol) in 1,2-dichloroethane (50 mL), *N*,*N*-dimethylphosgeniminium chloride (6.5 g, 40 mmol) was added and the solution was refluxed for 2 h. The solvent was removed by distillation under reduced pressure, the oily residue was crystallized by adding diethyl ether (10 mL). The yellow solid obtained was purified by recrystallization with absolute ethanol to afford the pure product **16** (2.22 g, 70%) as a pale yellow solid, mp 123–124 °C. $^1$H NMR: $\delta$ 3.26 (s, 6H, $2CH_3$), 4.36–4.62 (m, 2H, $CH_2N$), 5.26–5.37 (m, 1H, CHO), 7.24–7.38 (m, 5H Ar), 7.67 (s, 1H, H-3). IR cm$^{-1}$: 3443 (OH), 2224 (C≡N). Anal. $C_{15}H_{16}N_5ClO$ (C, H, N).

### 6.1.4. 4-Chloro-1-(2-chloro-2-phenylethyl)-N,N-dimethyl-1H-pyrazolo[3,4-d]pyrimidin-6-amine **17**

In a solution of *N*′-[4-cyano-1-(2-hydroxy-2-phenylethyl)-1*H*-pyrazol-5-yl]-*N*,*N*-dimethylcarbamimidic chloride (3.17 g, 10 mmol) in 1,2-dichloroethane (50 mL), anhydrous hydrochloric acid was bubbled for 4 h and the solution was stirred at room temperature for 2 days. The solvent was removed by distillation under reduced pressure to afford a crude that was purified by column chromatography (silica gel, 100 mesh), using a mixture of diethyl ether/petroleum ether (bp 40–60 °C) (1:1) as the eluant, to afford the pure product **17** (2.18 g, 65%) as a white solid, mp 118–119 °C. $^1$H NMR: $\delta$ 3.25 (s, 6H, $2CH_3$), 4.61–4.84 (m, 2H, $CH_2N$), 5.38–5.67 (m, 1H, CHCl), 7.17–7.56 (m, 5H Ar), 7.82 (s, 1H, H-3). Anal. $C_{15}H_{15}N_5Cl_2$ (C, H, N).

### 6.1.5. General procedure for 4-amino-1H-pyrazolo[3,4-d]pyrimidines **8a–q, 9a–h, 10a–c**

To a solution of **11a–e**, **14**, **17** (5 mmol) in anhydrous toluene (20 mL), the proper amine (20 mmol) was added. The reaction mixture was stirred at room temperature for 24–36 h and then extracted with $H_2O$ (2 × 20 mL). The organic phase was dried ($MgSO_4$), filtered and concentrated under reduced pressure. The oily residue was crystallized by adding diethyl ether (10 mL) to give the final products as white solids.

*Compound* ***8a***. White solid, yield 81%, mp 111–112 °C. $^1$H NMR: $\delta$ 2.49 (s, 3H, $SCH_3$), 2.93 (t, $J = 6.8$, 2H, $CH_2Ar$), 3.74 (q, $J = 6.8$, 2H, $CH_2NH$), 4.58–4.86 (m, 2H, $CH_2N$), 5.38–5.52 (m, 1H, CHCl), 6.86–7.43 (m, 9H Ar), 7.63 (s, 1H, H-3). IR cm$^{-1}$: 3371 (NH). Anal. $C_{22}H_{21}N_5ClFS$ (C, H, N, S).

*Compound* ***8b***. White solid, yield 60%, mp 108–109 °C. $^1$H NMR: $\delta$ 1.03 (t, $J = 7.2$, 3H, $CH_3$), 1.74 (sx, $J = 7.2$, 2H, $CH_2CH_3$), 2.59 (s, 3H, $SCH_3$), 3.56 (q, $J = 7.2$, 2H, $CH_2NH$), 4.72–4.96 (m, 2H, $CH_2N$), 5.26 (br s, 1H, NH, disappears with $D_2O$), 5.48–5.59 (m, 1H, CHCl), 6.94–7.08 and 7.26–7.46 (2m, 4H Ar), 7.78 (s, 1H, H-3). IR cm$^{-1}$: 3241 (NH). Anal. $C_{17}H_{19}N_5ClFS$ (C, H, N, S).

*Compound* ***8c***. White solid, yield 65%, mp 108–109 °C. $^1$H NMR: $\delta$ 0.99 (t, $J = 7.2$, 3H, $CH_3$), 1.47 (sx, $J = 7.2$, 2H, $CH_2CH_3$), 1.67 (quint, $J = 7.2$, 2H, $CH_2CH_2CH_3$), 2.60 (s, 3H, $SCH_3$), 3.60 (q, $J = 7.2$, 2H, $CH_2NH$), 4.70–4.94 (m, 2H, $CH_2N$), 5.47–5.58 (m, 1H, CHCl), 6.94–7.06 and 7.36–7.44 (2m, 4H Ar), 7.77 (s, 1H, H-3). IR cm$^{-1}$: 3239 (NH). Anal. $C_{18}H_{21}N_5ClFS$ (C, H, N, S).

*Compound* ***8d***. White solid, yield 70%, mp 147–148 °C. $^1$H NMR: $\delta$ 2.58 (s, 3H, $SCH_3$), 3.81–3.88 and 3.90–4.00 (2m, 8H, $4CH_2$ morph), 4.72–4.96 (m, 2H, $CH_2N$), 5.46–5.59 (m, 1H, CHCl), 6.95–7.09 and 7.34–7.46 (2m, 4H Ar), 7.82 (s, 1H, H-3). Anal. $C_{18}H_{19}N_5OClFS$ (C, H, N, S).

*Compound* ***8e***. White solid, yield 85%, mp 151–152 °C. $^1$H NMR: $\delta$ 2.51 (s, 3H, $SCH_3$), 4.60–4.86 (m, 4H, $CH_2N + CH_2Ar$), 5.35–5.50 (m, 1H, CHCl), 5.55 (br s, 1H, NH, disappears with $D_2O$), 6.82–7.04 and 7.11–7.57 (2m, 8H Ar), 7.63 (s, 1H, H-3). IR cm$^{-1}$: 3294 (NH). Anal. $C_{21}H_{18}N_5ClF_2S$ (C, H, N, S).

*Compound* ***8f***. White solid, yield 80%, mp 151–152 °C. $^1$H NMR: $\delta$ 2.55 (s, 3H, $SCH_3$), 4.60–4.86 (m, 4H, $CH_2N + CH_2Ar$), 5.32–5.47 (m, 1H, CHCl), 6.86–7.36 (m, 8H Ar), 7.74 (s, 1H, H-3). IR cm$^{-1}$: 3278 (NH). Anal. $C_{21}H_{18}N_5ClF_2S$ (C, H, N, S).

*Compound* ***8g***. White solid, yield 61%, mp 121–122 °C. $^1$H NMR: $\delta$ 2.61 (s, 3H, $SCH_3$), 3.01 (t, $J = 7.2$, 2H, $CH_2Ar$), 3.87 (q, $J = 7.2$, 2H, $CH_2NH$), 4.70–4.89 (m, 2H, $CH_2N$), 5.36 (br s, 1H, NH disappears with $D_2O$), 5.46–5.59 (m, 1H, CHCl), 6.92–7.05 and 7.18–7.45 (2m, 9H Ar), 7.72 (s, 1H, H-3). IR cm$^{-1}$: 3252 (NH). Anal. $C_{22}H_{21}N_5ClFS$ (C, H, N, S).

*Compound* ***8h***. White solid, yield 55%, mp 250–251 °C. $^1$H NMR: δ 2.56 (s, 3H, $SCH_3$), 4.64–4.87 (m, 2H, $CH_2N$), 5.36–5.50 (m, 1H, CHCl), 6.82–7.53 (m, 9H, 8Ar + H-3). IR cm$^{-1}$: 3098 (NH). Anal. $C_{20}H_{16}N_5ClF_2S$ (C, H, N, S).

*Compound* ***8i***. White solid, yield 65%, mp 124–125 °C. $^1$H NMR: δ 1.04 (t, $J = 7.4$, 3H, $CH_3$), 1.73 (sx, $J = 7.4$, 2H, $CH_2CH_3$), 2.59 (s, 3H, $SCH_3$), 3.56 (q, $J = 7.4$, 2H, $CH_2NH$), 4.74–4.90 (m, 2H, $CH_2N$), 5.46–5.58 (m, 1H, CHCl), 7.21–7.43 (m, 4H Ar), 7.77 (s, 1H, H-3). IR cm$^{-1}$: 3269 (NH). Anal. $C_{17}H_{19}N_5Cl_2S$ (C, H, N, S).

*Compound* ***8j***. White solid, yield 77%, mp 119–120 °C. $^1$H NMR: δ 0.97 (t, $J = 7.4$, 3H, $CH_3$), 1.47 (sx, $J = 7.4$, 2H, $CH_2CH_3$), 1.65 (quint, $J = 7.4$, 2H, $CH_2CH_2CH_3$), 2.59 (s, 3H, $SCH_3$), 3.59 (q, $J = 7.4$, 2H, $CH_2NH$), 4.64–4.93 (m, 2H, $CH_2N$), 5.31 (br s, 1H, NH disappears with $D_2O$), 5.42–5.58 (m, 1H, CHCl), 7.11–7.54 (m, 4H Ar), 7.77 (s, 1H, H-3). IR cm$^{-1}$: 3246 (NH). Anal. $C_{18}H_{21}N_5Cl_2S$ (C, H, N, S).

*Compound* ***8k***. White solid, yield 66%, mp 67–68 °C. $^1$H NMR: δ 1.22 (t, $J = 7.0$, 6H, $2CH_3$ diethylam.), 2.50 (s, 3H, $SCH_3$), 3.65 (q, $J = 7.0$, 4H, $2CH_2$ diethylam.), 4.60–4.87 (m, 2H, $CH_2N$), 5.43–5.54 (m, 1H, CHCl), 6.86–7.02 and 7.22–7.41 (2m, 4H Ar), 7.64 (s, 1H, H-3). Anal. $C_{18}H_{21}N_5Cl_2S$ (C, H, N, S).

*Compound* ***8l***. White solid, yield 79%, mp 109–110 °C. $^1$H NMR: δ 2.59 (s, 3H, $SCH_3$), 3.80–3.88 and 3.90–4.00 (2m, 8H, $4CH_2$ morph), 4.72–4.96 (m, 2H, $CH_2N$), 5.47–5.58 (m, 1H, CHCl), 7.24–7.44 (m, 4H Ar), 7.82 (s, 1H, H-3). Anal. $C_{18}H_{19}N_5OCl_2S$ (C, H, N, S).

*Compound* ***8m***. White solid, yield 77%, mp 83–84 °C. $^1$H NMR: δ 1.22 (t, $J = 7.0$, 6H, $2CH_3$ diethylam.), 1.38 (t, $J = 7.4$, 3H, $CH_3CH_2S$), 3.10 (q, $J = 7.4$, 2H, $SCH_2$), 3.64 (q, $J = 7.0$, 4H, $2CH_2$ diethylam.), 4.57–4.92 (m, 2H, $CH_2N$), 5.43–5.57 (m, 1H, CHCl), 7.14–7.46 (m, 5H Ar), 7.66 (s, 1H, H-3). Anal. $C_{19}H_{24}N_5ClS$ (C, H, N, S).

*Compound* ***8n***. White solid, yield 34%, mp 97–98 °C. $^1$H NMR: δ 0.88–1.09 (m, 6H, $2CH_3$), 1.54–1.84 (m, 4H, $2CH_2$), 3.08 (t, $J = 7.0$, 2H, $SCH_2$), 3.48 (q, $J = 6.4$, 2H, $CH_2NH$), 4.60–4.88 (m, 2H, $CH_2N$), 5.16 (br s, 1H, NH disappears with $D_2O$), 5.40–5.52 (m, 1H, CHCl), 7.16–7.40 (m, 5H Ar), 7.70 (s, 1H, H-3). IR cm$^{-1}$: 3371 (NH). Anal. $C_{19}H_{24}N_5ClS$ (C, H, N, S).

*Compound* ***8o***. White solid, yield 49%, mp 77–78 °C. $^1$H NMR: δ 0.90 (t, $J = 7.4$, 3H, $CH_3$), 1.02 (t, $J = 7.4$, 3H, $CH_3$), 1.26–1.43 and 1.49–1.63 and 1.65–1.83 (3m, 6H, $3CH_2$), 3.07 (t, $J = 7.4$, 2H, $SCH_2$), 3.48 (q, $J = 7.4$, 2H, $CH_2NH$), 4.61–4.88 (m, 2H, $CH_2N$), 5.38–5.53 (m, 1H, CHCl), 7.14–7.41 (m, 5H Ar), 7.70 (s, 1H, H-3). IR cm$^{-1}$: 3275 (NH). Anal. $C_{20}H_{26}N_5ClS$ (C, H, N, S).

*Compound* ***8p***. White solid, yield 81%, mp 80–81 °C. $^1$H NMR: δ 0.94–1.06 and 1.10–1.30 (2m, 9H, $3CH_3$), 1.75 (sx, $J = 7.2$, 2H, $SCH_2CH_2CH_3$), 3.06 (t, $J = 7.2$, 2H, $SCH_2$), 3.52–3.73 (m, 4H, $2CH_2N$ diethylam.), 4.56–4.91 (m, 2H, $CH_2N$), 5.42–5.56 (m, 1H, CHCl), 7.16–7.43 (m, 5H Ar), 7.65 (s, 1H, H-3). Anal. $C_{20}H_{26}N_5ClS$ (C, H, N, S).

*Compound* ***8q***. White solid, yield 68%, mp 136–137 °C. $^1$H NMR: δ 1.01 (t, $J = 7.0$, 3H, $CH_3$), 1.73 (sx, $J = 7.0$, 2H, $CH_2CH_3$), 3.08 (t, $J = 7.0$, 2H, $SCH_2$), 4.56–4.87 (m, 4H, $CH_2N + CH_2Ar$), 5.38–5.51 (m, 1H, CHCl), 7.06–7.38 (m, 10H Ar), 7.65 (s, 1H, H-3). IR cm$^{-1}$: 3250 (NH). Anal. $C_{23}H_{24}N_5ClS$ (C, H, N, S).

*Compound* ***9a***. White solid, yield 50%, mp 123–124 °C. $^1$H NMR: δ 1.02 (t, $J = 7.2$, 3H, $CH_3$), 1.71 (sx, $J = 7.2$, 2H, $CH_2CH_3$), 2.61 (s, 3H, $SCH_3$), 3.54 (q, $J = 7.2$, 2H, $CH_2NH$), 4.84–5.09 (m, 2H, $CH_2N$), 5.40 (br s, 1H, NH disappears with $D_2O$), 5.57–5.67 (m, 1H, CHBr), 7.20–7.54 (m, 5H Ar), 7.77 (s, 1H, H-3). IR cm$^{-1}$: 3239 (NH). Anal. $C_{17}H_{20}N_5BrS$ (C, H, N, S).

*Compound* ***9b***. White solid, yield 66%, mp 97–98 °C. $^1$H NMR: δ 0.98 (t, $J = 7.4$, 3H, $CH_3$), 1.37–1.45 and 1.61–1.82 (2m, 4H, $2CH_2$ butylam.), 2.61 (s, 3H, $SCH_3$), 3.59 (q, $J = 7.4$, 2H, $CH_2NH$), 4.87–5.09 (m, 2H, $CH_2N$), 5.30 (br s, 1H, NH disappears with $D_2O$), 5.57–5.68 (m, 1H, CHBr), 7.20–7.55 (m, 5H Ar), 7.77 (s, 1H, H-3). IR cm$^{-1}$: 3240 (NH). Anal. $C_{18}H_{22}N_5BrS$ (C, H, N, S).

*Compound* ***9c***. White solid, yield 61%, mp 126–127 °C. $^1$H NMR: δ 1.24 (t, $J = 7.0$, 3H, $CH_3$), 2.61 (s, 3H, $SCH_3$), 3.55 (q, $J = 7.0$, 2H, $CH_2CH_3$), 3.67 (t, $J = 4.6$, 2H, $CH_2CH_2O$), 3.79 (q, $J = 4.6$, 2H, $CH_2NH$), 4.81–5.08 (m, 2H, $CH_2N$), 5.56–5.68 (m, 1H, CHBr), 5.76 (br s, 1H, NH disappears with $D_2O$), 7.24–7.53 (m, 5H Ar), 7.77 (s, 1H, H-3). IR cm$^{-1}$: 3311 (NH). Anal. $C_{18}H_{22}N_5OBrS$ (C, H, N, S).

*Compound* ***9d***. White solid, yield 60%, mp 149–150 °C. $^1$H NMR: δ 1.93–2.25 (m, 4H, $2CH_2$ pyrr.), 2.62 (s, 3H, $SCH_3$), 3.67–3.87 (m, 4H, $2CH_2N$ pyrr.), 4.85–5.08 (m, 2H, $CH_2N$), 5.60–5.70 (m, 1H, CHBr), 7.15–7.57 (m, 5H Ar), 7.79 (s, 1H, H-3). Anal. $C_{18}H_{20}N_5BrS$ (C, H, N, S).

*Compound* ***9e***. White solid, yield 90%, mp 109–110 °C. $^1$H NMR: δ 1.47–1.79 (m, 6H, $3CH_2$ pip.), 2.60 (s, 3H, $SCH_3$), 3.81–3.95 (m, 4H, $2CH_2N$ pip.), 4.85–5.09 (m, 2H, $CH_2N$), 5.61–5.72 (m, 1H, CHBr), 7.25–7.53 (m, 5H Ar), 7.82 (s, 1H, H-3). Anal. $C_{19}H_{22}N_5BrS$ (C, H, N, S).

*Compound* ***9f***. White solid, yield 90%, mp 98–99 °C. $^1$H NMR: δ 2.61 (s, 3H, $SCH_3$), 3.80–3.89 and 3.90–3.99 (2m, 8H, $4CH_2$ morph), 4.80–5.10 (m, 2H, $CH_2N$), 5.55–5.70 (m, 1H, CHBr), 7.26–7.52 (m, 5H Ar), 7.82 (s, 1H, H-3). Anal. $C_{18}H_{20}N_5OBrS$ (C, H, N, S).

*Compound* ***9g***. White solid, yield 70%, mp 137–138 °C. $^1$H NMR: δ 2.61 (s, 3H, $SCH_3$), 4.73–5.07 (m, 4H, $CH_2N + CH_2Ar$), 5.53–5.66 (m, 1H, CHBr), 7.20–7.53 (m, 10H Ar), 7.72 (s, 1H, H-3). IR cm$^{-1}$: 3243 (NH). Anal. $C_{21}H_{20}N_5BrS$ (C, H, N, S).

*Compound* ***9h***. White solid, yield 73%, mp 112–113 °C. $^1$H NMR: δ 2.63 (s, 3H, $SCH_3$), 3.00 (t, $J = 7.0$, 2H, $CH_2Ar$), 3.87 (q, $J = 7.0$, 2H, $CH_2NH$), 4.86–5.10 (m, 2H, $CH_2N$), 5.30 (br s, 1H, NH disappears with $D_2O$), 5.57–5.68 (m, 1H, CHBr), 7.21–7.52 (m, 10H Ar), 7.73 (s, 1H, H-3). IR cm$^{-1}$: 3240 (NH). Anal. $C_{22}H_{22}N_5BrS$ (C, H, N, S).

*Compound* ***10a***. White solid, yield 51%, mp 112–113 °C. $^1$H NMR: δ 1.03 (t, $J = 7.4$, 3H, $CH_3$ prop.), 1.70 (sx, $J = 7.4$, 2H, $CH_2CH_3$), 3.22 (s, 6H, $2NCH_3$), 3.52 (q, $J = 7.4$, 2H, $CH_2NH$), 4.62–4.88 (m, 2H, $CH_2N$), 5.06 (br s, 1H, NH, disappears with $D_2O$), 5.54–5.66 (m, 1H, CHCl), 7.21–7.51 (m, 5H Ar), 7.64 (s, 1H, H-3). IR cm$^{-1}$: 3365 (NH). Anal. $C_{18}H_{23}N_6Cl$ (C, H, N).

*Compound* ***10b***. White solid, yield 79%, mp 104–105 °C. $^1$H NMR: $\delta$ 3.21 (s, 6H, $2NCH_3$), 3.80–3.93 (m, 8H, $4CH_2$ morph), 4.66–4.88 (m, 2H, $CH_2N$), 5.57–5.68 (m, 1H, CHCl), 7.26–7.52 (m, 5H Ar), 7.70 (s, 1H, H-3). Anal. $C_{19}H_{23}N_6OCl$ (C, H, N).

*Compound* ***10c***. White solid, yield 79%, mp 70–71 °C. $^1$H NMR: $\delta$ 3.24 (s, 6H, $2CH_3$), 4.62–4.87 (m, 4H, $CH_2N + CH_2Ar$), 5.24 (br s, 1H, NH, disappears with $D_2O$), 5.54–5.66 (m, 1H, CHCl), 7.25–7.50 (m, 10H Ar), 7.61 (s, 1H, H-3). IR cm$^{-1}$: 3359 (NH). Anal. $C_{22}H_{23}N_6Cl$ (C, H, N).

## 6.2. Biology

PP2 (AG 1879, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine) and Imatinib mesylate, used as the reference compounds, were obtained from Calbiochem (San Diego, CA) and Novartis Pharma AG (Basel, Switzerland), respectively.

### 6.2.1. Enzymatic assays with recombinant Src

Recombinant human Src was purchased from Upstate (Lake Placid, NY). Activity was measured in a filter-binding assay using a commercial kit (Src Assay kit, Upstate), according to the manufacturer's protocol. $K_i$ (expressed as a μM concentration) toward recombinant human Src was calculated according to the following equation: $K_i = (ID_{50} - E_0/2)/\{E_0 - [S_0/K_m - 1]/E_0\}$, where $S_0$ is the concentration of the competing substrate (ATP), and $E_0$ is the concentration of the enzyme. Each experiment was done in triplicate and mean values were used for the interpolation. Curve fitting was performed with the program GraphPad Prism.

*Cell-free assay with recombinant Abl*. Recombinant human Abl was purchased from Upstate Biotechnology (Waltham, MA) and used to investigate the mechanism of kinase inhibition, as previously reported [30]. In detail, activity was measured in a filter-binding assay using an Abl specific peptide substrate (Abltide, Upstate Biotechnology) in the presence of 0.012 μM [γ-$^{32}$P]ATP, 50 μM peptide, and 0.005 μM c-Abl. The apparent affinity ($K_m$) of Abl for the peptide and ATP substrates was determined separately (1.5 and 10 μM, respectively). Each experiment was done in triplicate and mean values were used for the interpolation. Curve fitting was performed with the program GraphPad Prism. $K_i$ values toward isolated Abl were calculated according to the following equation: $K_i = ID_{50}/\{E_0+[E_0(K_{m(ATP)}/S_0)]\}/E_0$, where $E_0$ and $S_0$ are the enzyme and the ATP concentrations (0.005 and 0.012 μM, respectively).

### 6.2.2. Cell culture

Human CML K-562 cells in blast crisis [42] and human CML KU-812 [43] cell line in myeloid blast crisis were obtained from the American Type Culture Collection and were grown in RPMI 1640 medium (BioWhittaker, Vallensbaek, DK) containing 10% fetal calf serum (FCS) (BioWhittaker). The cultures were free of mycoplasma. For the proliferation assay, cell lines ($2 \times 10^4$ cells mL$^{-1}$) were incubated overnight in 100 μL RPMI 640 culture medium, supplemented with 0.5% FCS and antibiotics (100 U mL$^{-1}$ penicillin and 100 μg mL$^{-1}$ streptomycin) at 37 °C in 5% $CO_2$. Later the spent medium was removed and the culture was refreshed with new medium or medium containing different concentrations (0–100 μM) of the studied compounds. After 3 days (control cultures did not reach confluence), the antiproliferative effect of the compounds was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) proliferation assay. Briefly, cells were treated with an MTT solution (10 μL, 5 mg mL$^{-1}$) and, 4 h later, acid propan-2-ol (100 μL, 0.04 M HCl in propan-2-ol) was added to dissolve the formazan product. The microplates were read using an ELISA plate reader at 570 nm with a reference wavelength of 630 nm. Data analysis for $IC_{50}$ calculations was performed with the LSW Data Analysis Package plug-in for Excel (Microsoft). Results are reported as mean ± SD of five experiments, each performed in triplicate.

### 6.2.3. Proapoptotic activity

Proapoptotic effect of selected pyrazolo[3,4-*d*]pyrimidines was tested using a poly-ADP-Ribose-Polymerase (PARP) assay (Roche Dignostic, Milan, Italy). KU-812 cell line was cultured at a concentration of $2 \times 10^5$ cells mL$^{-1}$ and challenged with the compound (50 μM). After 72 h, cells were harvested and lysed in an appropriate buffer containing 1% Triton X-100. Immunoblot analysis was performed using PARP-specific antibodies to both the uncleaved (113 kDa) and cleaved (89 kDa) forms of PARP. Quantification of cleaved PARP over uncleaved PARP expression was achieved with Sigma Gel analysis software and the results were expressed as the mean of three independent experiments. Statistical analyses were performed using Student's *t*-test and the Bonferroni's correction.

### 6.2.4. mRNA expression of apoptotic genes

The mRNA expression of apoptotic genes was performed using qRT-PCR. KU-812 cells were treated as reported above, and lysed in an appropriate buffer (OMNI-zol for RNA–DNA–Protein extraction Kit, Eurocione, Devon, UK). The extract was processed for the extraction of mRNA. For qRT-PCR analysis, Bcl-xL (antiapoptotic gene) and Bax (proapoptotic gene) expression in KU-812 cells was determined using an MJ MiniOpticon Cycler (Bio-Rad Laboratories, Hercules, CA). First strand cDNA synthesis was performed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories). qRT-PCR was performed using iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories) and specific primers from GenBank. Data were quantitatively analyzed on an MJ OpticonMonitor detection system (Bio-Rad Laboratories). The values were expressed as fold increase relative to the expression of β-actin.

## 6.3. Docking studies

Consistent with the previous works [29,30,41], docking studies were performed on the newly synthesized compounds within the ATP binding site of c-Src and Abl kinases using

the software packages Autodock 3.0.5 [44] and Gold 3.0.1 [45–47], respectively. Structures of inhibitors were represented using MacroModel 8.5 [48] and minimized with the Amber force field using the Polak–Ribiere conjugated gradient method (0.001 kJ/mol Å convergence or 10 000 iterations). To remove unfavourable contacts, a preliminary structure optimization was performed with MacroModel 8.5 on the X-ray crystallographic structures of c-Src (PDB entry: 1YOL) [40] and c-Abl (PDB entry: 1M52) [49] through the all-atom Amber* force field and Polak-Ribiere conjugate gradient method. A continuum solvation method, with water as the solvent, was also applied. Extended cutoffs were used and convergence was set to 0.01 kJ/mol Å.

With respect to Autodock simulations, the Lamarckian genetic algorithm (LGA) was employed to explore the possible orientations/conformations of the inhibitors into the binding site. For each of the 100 independent runs, a maximum number of 1 500 000 operations were performed with a population size of 200 individuals. The binding pocket was inserted into a grid box centred on the bound ligand CGP77675 and enclosing residues lying within about 8 Å from the ligand itself.

As regards Gold calculations, the ChemScore was chosen as fitness function. For each of the 50 independent genetic algorithm runs, a maximum number of 100 000 GA operations were performed on a set of five groups with a population size of 200 individuals. The remaining GA parameters were kept to their default values. Hydrophobic fitting points were calculated on the target for a 10 Å radius around the co-crystallized ligand PD173955.

Finally, results differing less than 1.5 Å in ligand–all atom RMSD were clustered together.

For each inhibitor, the first ranked solution as well as the lowest energy conformation of the most populated cluster, were selected for further analysis.

## Acknowledgements

Financial support provided by the Italian MIUR (PRIN 2006030948) and by the Fondazione Monte dei Paschi di Siena is gratefully acknowledged. We thank Dr. Giovanni Gaviraghi (Sienabiotech S.p.A.) for helpful discussion. We are indebted to Dr. Doriano Fabbro (Novartis Institutes for Biomedical Research, Basel, Switzerland) for assays on the tyrosine kinase panel.

## References

[1] S.J. Parsons, J.T. Parsons, Oncogene 23 (2004) 7906–7909.
[2] J.M. Summy, G.E. Gallick, Clin. Cancer Res. 12 (2006) 1398–1401.
[3] S. Schenone, F. Manetti, M. Botta, Anticancer Agents Med. Chem. 7 (2007) 660–680.
[4] M.C. Frame, J. Cell. Sci. 117 (2004) 989–999.
[5] A. Angelucci, S. Schenone, G.L. Gravina, P. Muzi, C. Festuccia, C. Vicentini, M. Botta, M. Bologna, Eur. J. Cancer 42 (2006) 2838–2845.
[6] E. Avizienyte, A.W. Wyke, R.J. Jones, G.W. McLean, M.A. Westhoff, V.G. Brunton, M.C. Frame, Nat. Cell. Biol. 4 (2002) 632–638.
[7] J.D. Rowley, Nature 243 (1973) 290–300.
[8] T. Tauchi, K. Ohyashi, Int. J. Hematol. 83 (2006) 293–300.
[9] J. Cortes, Curr. Opin. Hematol. 13 (2006) 79–86.
[10] T. O'Hare, A.S. Corbin, B.J. Druker, Curr. Opin. Genet. Dev. 16 (2006) 92–99.
[11] C. Walz, M. Sattler, Crit. Rev. Oncol. Hematol. 57 (2006) 145–164.
[12] Y. Hu, S. Swerdolw, T.M. Duffy, R. Weinmann, F.Y. Lee, S. Li, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 16870–16875.
[13] S. Li, Int. J. Biochem. Cell Biol. 39 (2007) 1483–1488.
[14] F. Manetti, G.A. Locatelli, G. Maga, S. Schenone, M. Modugno, S. Forli, F. Corelli, M. Botta, J. Med. Chem. 49 (2006) 3278–3286.
[15] S. Schenone, F. Manetti, M. Botta, Mini Rev. Med. Chem. 7 (2007) 191–201.
[16] D.R. Huron, M.E. Gorre, A.J. Kraker, C.L. Sawyers, N. Rosen, M.M. Moasser, Clin. Cancer Res. 9 (2003) 1267–1273.
[17] M. Azam, V. Nardi, W.C. Shakespeare, C.A. Metcalf III, R.S. Bohacek, Y. Wang, R. Sundaramoorthi, P. Sliz, D.R. Veach, W.G. Bornmann, B. Clarkson, D.C. Dalgarno, T.K. Sawyer, G.Q. Daley, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 9244–9249.
[18] L.F. Hennequin, J. Allen, J. Breed, J. Curwen, M. Fennel, T.P. Green, C. Lambert-van der Brempt, R. Morgentin, R.A. Norman, A. Olivier, L. Otterbein, P.A. Plé, N. Warin, G. Costello, J. Med. Chem. 49 (2006) 6465–6488.
[19] D.H. Boschelli, F. Ye, D.Y. Wang, M. Dutia, S.L. Johnson, B. Wu, K. Miller, D.W. Powell, D. Yaczko, M. Young, M. Tischler, K. Arndt, C. Discafani, C. Etienne, J. Gibbons, J. Grod, J. Lucas, J.M. Weber, F. Boschelli, J. Med. Chem. 44 (2001) 3965–3977.
[20] J.M. Golas, K. Arndt, C. Etienne, J. Lucas, D. Nardin, J. Gibbons, P. Frost, F. Ye, D.H. Boschelli, F. Boschelli, Cancer Res. 63 (2003) 375–381.
[21] M. Puttini, A.M. Coluccia, F. Boschelli, L. Cleris, E. Marchesi, A. Donella-Deana, S. Ahmed, S. Radaelli, R. Piazza, V. Magistroni, F. Andreoni, L. Scapozza, F. Formelli, C. Gambacorti-Passerini, Cancer Res. 66 (2006) 11314–11322.
[22] D.H. Boschelli, B. Wu, F. Ye, Y. Wang, J.M. Golas, J. Lucas, F. Boschelli, J. Med. Chem. 49 (2006) 7868–7876.
[23] G. Noronha, K. Barrett, A. Boccia, T. Brodhag, J. Cao, C.P. Chow, E. Dneprovskaia, J. Doukas, R. Fine, X. Gong, C. Gritzen, H. Gu, E. Hanna, J.D. Hood, S. Hu, X. Kang, J. Key, B. Klebansky, A. Kousba, G. Li, D. Lohse, C.C. Mak, A. McPherson, M.S.S. Palanki, V.P. Pathak, J. Renick, F. Shi, R. Soll, U. Splittgerber, S. Stoughton, S. Tang, S. Yee, B. Zeng, N. Zhao, H. Zhu, Bioorg. Med. Chem. Lett. 17 (2007) 602–608.
[24] N.P. Shah, C. Tran, F.Y. Lee, P. Chen, D. Norris, C.L. Sawyers, Science 305 (2004) 399–402.
[25] L.J. Lombardo, F.Y. Lee, P. Chen, D. Norris, J.C. Barrish, K. Behnia, S. Castaneda, L.A. Cornelius, J. Das, A.M. Doweyko, C. Fairchild, J.T. Hunt, I. Inigo, K. Johnston, A. Kamath, D. Kan, H. Klei, P. Marathe, S. Pang, R. Peterson, S. Pitt, G.L. Schieven, R.J. Schmidt, J. Tokarski, M.L. Wen, J. Wityak, R.M. Borzilleri, J. Med. Chem. 47 (2004) 6658–6661.
[26] A. Quintas-Cardama, H. Kantarjian, D. Jones, C. Nicaise, S. O'Brien, F. Giles, M. Talpaz, J. Cortes, Blood 109 (2007) 497–499.
[27] U.S. Food and Drug Administration, FDA gives rapid approval for a new treatment for a rare type of leukemia, http://www.fda.gov/bbs/topics/NEWS/2006/NEW01400.htlm (accessed 2006).
[28] F. Carraro, A. Pucci, A. Naldini, S. Schenone, O. Bruno, A. Ranise, F. Bondavalli, C. Brullo, P. Fossa, G. Menozzi, L. Mosti, F. Manetti, M. Botta, J. Med. Chem. 47 (2004) 1595–1598.
[29] F. Carraro, A. Naldini, A. Pucci, G.A. Locatelli, G. Maga, S. Schenone, O. Bruno, A. Ranise, F. Bondavalli, C. Brullo, P. Fossa, G. Menozzi, L. Mosti, M. Modugno, C. Tintori, F. Manetti, M. Botta, J. Med. Chem. 49 (2006) 1549–1561.
[30] F. Manetti, A. Pucci, M. Magnani, G.A. Locatelli, C. Brullo, A. Naldini, S. Schenone, G. Maga, F. Carraro, M. Botta, Chem. Med. Chem. 2 (2007) 343–353.
[31] F. Bondavalli, M. Botta, O. Bruno, A. Ciacci, F. Corelli, P. Fossa, A. Lucacchini, F. Manetti, G. Menozzi, L. Mosti, A. Ranise, S. Schenone, A. Tafi, M.L. Trincavelli, J. Med. Chem. 45 (2002) 4875–4887.

[32] F. Manetti, A. Santucci, G.A. Locatelli, G. Maga, A. Spreafico, T. Serchi, M. Orlandini, G. Bernardini, N.P. Caradonna, A. Spallarossa, C. Brullo, S. Schenone, O. Bruno, A. Ranise, F. Bondavalli, O. Hoffmann, M. Bologna, A. Angelucci, M. Botta, J. Med. Chem. 50 (2007) 5579–5588.
[33] O. Bruno, C. Brullo, F. Bondavalli, S. Schenone, A. Ranise, N. Arduino, M.B. Bertolotto, F. Montecucco, L. Ottonello, F. Dallegri, M. Tognolini, V. Ballabeni, S. Bretoni, E. Barocelli, J. Med. Chem. 50 (2007) 3618–3626.
[34] J.H. Hanke, J.P. Gardner, R.L. Dow, P.S. Changelian, W.H. Brissette, E.J. Weringer, B.A. Pollok, P.A. Connelly, J. Biol. Chem. 271 (1996) 695–701.
[35] L. Tatton, G.M. Morley, R. Chopra, A. Khwaja, J. Biol. Chem. 278 (2003) 4847–4853.
[36] The activity of compound **9g** was preliminarily assessed toward the following panel of kinases: HER-1, KDR, Flt-3, IGF-IR, Tek, c-Met, Ret, JAK-2, EphB4, FGFR-3-K560E, Axl, FAK, PKA, CDK2/A, Akt, PDK1, and B-Raf-V599E.
[37] T. Chodhuri, S. Paul, M.L. Agwarwal, T. Das, G. Sa, FEBS Lett. 512 (2002) 334–340.
[38] A. Letai, M.C. Bassik, L.D. Walensky, M.D. Sorcinelli, S. Weiler, S.J. Korsmeyer, Cancer Cell. 2 (2002) 183–192.
[39] F. Gesbert, J.D. Griffin, Blood 20 (2000) 1179–1186.
[40] C.B. Breitenlechner, A.K. Norman, K.H.S. Scheiblich, H. Koll, E. Greiter, S. Koch, W. Schafer, R. Huber, R.A. Engh, J. Mol. Biol. 353 (2005) 222–231.
[41] C. Tintori, M. Magnani, S. Schenone, M. Botta, submitted for publication.
[42] D. Hudig, M. Djobadze, D. Redelman, J. Mendelsohn, Cancer Res. 41 (1981) 2803–2808.
[43] K. Kishi, Leuk. Res. 9 (1985) 381–390.
[44] G.M. Morris, D.S. Goodsell, R.S. Halliday, R. Huey, W.E. Hart, R.K. Belew, A.J. Olson, J. Comput. Chem. 19 (1998) 1639–1662.
[45] G. Jones, P. Willett, R.C. Glen, J. Mol. Biol. 245 (1995) 43–53.
[46] G. Jones, P. Willett, R.C. Glen, A.R. Leach, R. Taylor, J. Mol. Biol. 267 (1997) 727–748.
[47] M.L. Verdonk, J.C. Cole, M.J. Hartshorn, C.W. Murray, R.D. Taylor, Proteins 52 (2003) 609–623.
[48] F. Mohamadi, N.G.J. Richards, W.C. Guida, R. Liskamp, M. Lipton, C. Caufield, G. Chang, T. Hendrickson, W.C. Still, J. Comput. Chem. 11 (1990) 440–467.
[49] B. Nagar, W.G. Bornmann, P. Pellicena, T. Schindler, D.R. Veach, W.T. Miller, B. Clarkson, J. Kuriyan, Cancer Res. 62 (2002) 4236–4243.