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New pyrazolo[3,4-*d*]pyrimidines endowed with A431 antiproliferative activity and inhibitory properties of Src phosphorylation

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Abstract—New 4-aminopyrazolo[3,4-*d*]pyrimidines bearing various substituents at the position 1 and 6, were synthesized. The new compounds showed antiproliferative activity toward A431 cells, were found to be inhibitors of Src phosphorylation, and induced apoptotic cell death. In particular, **2h** was a better inhibitor of Src phosphorylation than the reference compound PP2.

The understanding of the fundamental biology of cancer increased dramatically in recent years[1] and has strongly impacted on both experimental and gradually also on clinical tumor therapy. We now believe that the future of tumor therapy is in the development of molecularly targeted agents that specifically block key mechanisms involved in development and progression of specific types of cancer.[2] Due to their critical role in tumor development and progression, molecules capable of inhibiting protein kinases are of central interest in targeted cancer therapy. In particular, tyrosine kinases (TKs) are enzymes that catalyze the specific phosphorylation of tyrosine residues on proteins.

Protein tyrosine kinases (PTKs) participate in a wide variety of cellular activities including proliferation, secretion, adhesion, and responses to mitogens and stress.[3,4] The TK Src is the prototype member of the nonreceptor Src family of PTKs that include c-Yes, Fyn, c-Fgr, Lyn, Lck, Hck, Clk, Yrk, and c-Src.[5] In particular, the nonreceptor pp60c-Src PTK transduces signals that control the above cellular processes.[6–8] Src is activated following engagement of many different classes of cellular receptors and participates as a convergence point in different signaling pathways.[3,4,9] In this regard, Src is a critical component of the signaling cascades initiated by tyrosine kinase-linked receptors, such as the epidermal growth factor receptor (EGFR), and G-protein coupled receptors and is directly associated with, and may regulate signaling via, the EGFR and HER-2/neu receptor PTKs,[10,11] both of which are involved in cancer. Finally, Src overexpression and activation has been correlated with a large number of growth-regulatory processes in which Src participates. In particular, the Src protein is overexpressed in many tumors such as colon, breast, gastric, and prostatic tumors and plays a key role in controlling their proliferation and invasiveness.[12,13]

On the basis of these considerations, inhibitors of Src phosphorylation process may stop uncontrolled tumor cell growth and play an important role as new therapeutic agents for the treatment of cancer.

During the past decade, examples of pyrazolo[3,4-*d*]-pyrimidines active as TKs inhibitors have been reported in the literature by different authors. Many of these compounds were highly active, and some of them have been largely used as standard or reference compounds and as a tool to set up in vitro assays and to prove their functionality. However, only a few demonstrated in vivo activity. As an example, approaches aimed at identifying ATP-competitive small molecules led to the characterization of derivatives **1** (Fig. 1) as potent inhibitors of

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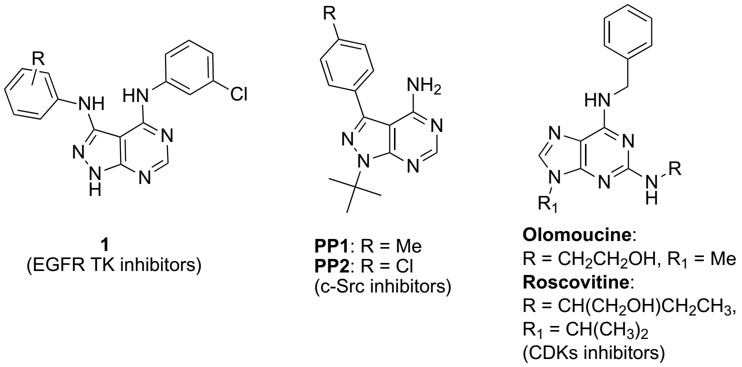


1
(EGFR TK inhibitors)

PP1: R = Me
PP2: R = Cl
(c-Src inhibitors)

Olomoucine:
R = CH_2CH_2OH, R_1 = Me
Roscovitine:
R = $CH(CH_2OH)CH_2CH_3$,
R_1 = $CH(CH_3)_2$
(CDKs inhibitors)

Figure 1.

EGFR TK,[14] while PP1 and PP2 were described as very strong and selective inhibitors of the c-Src family of kinases.[15] Unfortunately, attempts to improve the biological profile of the latter compounds have so far met little success. Following these studies, some other inhibitors, possessing different chemical structures and interesting c-Src inhibitory activity, have been recently reported. In particular, pyrrolo[2,3-*d*]pyrimidines have been described as selective and potent inhibitors of c-Src with inhibition potency of about 10 nM and 20-fold selectivity toward a panel of other TKs.[16] Other efforts directed to c-Src kinase inhibitors included substituted 5,10-dihydropyrimido[4,5-*b*]quinolines, showing an in vitro c-Src inhibition activity in the nanomolar range.[17]

Finally, well known compounds as olomoucine and roscovitine (Fig. 1), representative of purine derivatives, strictly correlated with the pyrazolo-pyrimidine compounds, exhibited a moderate inhibitory activity but good selectivity for a panel of CDKs kinases,[18] that have recently raised considerable interest as the principal regulators of the cell division cycle.

To summarize, a careful observation of compounds depicted in Figure 1 reveals that small variations on the chemical structure (either the scaffold or the all around substituents) might shift the inhibitory activity of a compound from EGFR to c-Src, to CDK serine threonine kinase.

All these experimental evidences prompted us to synthesize a new family of compounds **2** (Scheme 1, Table 1), bearing a pyrazolo[3,4-*d*]pyrimidine scaffold with substituents at the position 1 (namely, a 2-chloro-2-phenylethyl, or a 2-hydroxy-2-phenylethyl, or a styryl side chain), 4 (linear and cyclic, aliphatic and aromatic amines), and 6 (a alkylthio group),[19] to be tested as possible inhibitors of c-Src and, consequently, as antiproliferative agents.

Compounds **2a**–**n** were prepared starting from the ethyl ester of 5-amino-1-(2-hydroxy-2-phenylethyl)-1*H*-pyrazole-4-carboxylic acid (**3**), in turn obtained following our reported procedure.[20] Reaction of **3** with benzoyl isothiocyanate in THF at reflux for 8 h yielded the intermediate **4**, which was cyclized to the pyrazolo[3,4-*d*]pyrimidine derivative **5** by treatment with 1 M NaOH at 100 °C for 10 min, followed by acidification with acetic acid (80% yield). Alkylation of the 6-thio group with methyl or ethyl iodide in THF at reflux afforded the 6-alkylthio derivatives **6**, that were in turn treated with the Vilsmeier complex ($POCl_3$/DMF, 10 equiv) in $CHCl_3$ to obtain the dihalogenated compounds **7**, bearing the chlorine atom in both the position 4 of the pyrimidine nucleus and on the side chain at N1. Compounds **7** were purified in good yield by chromatography on Florisil® column. Finally, regioselective substitution of the C4 chlorine atom with an excess of various amines afforded the desired compounds **2a**–**n** in yields ranging between 60% and 80%. Notably, the chlorine atom on the N1 side chain has never been substituted by the amine in spite of its benzylic position, as shown by the ^1H NMR chemical shifts of the CH_2–CH side chain, which give an ABX complex pattern, similar to that of the starting material.

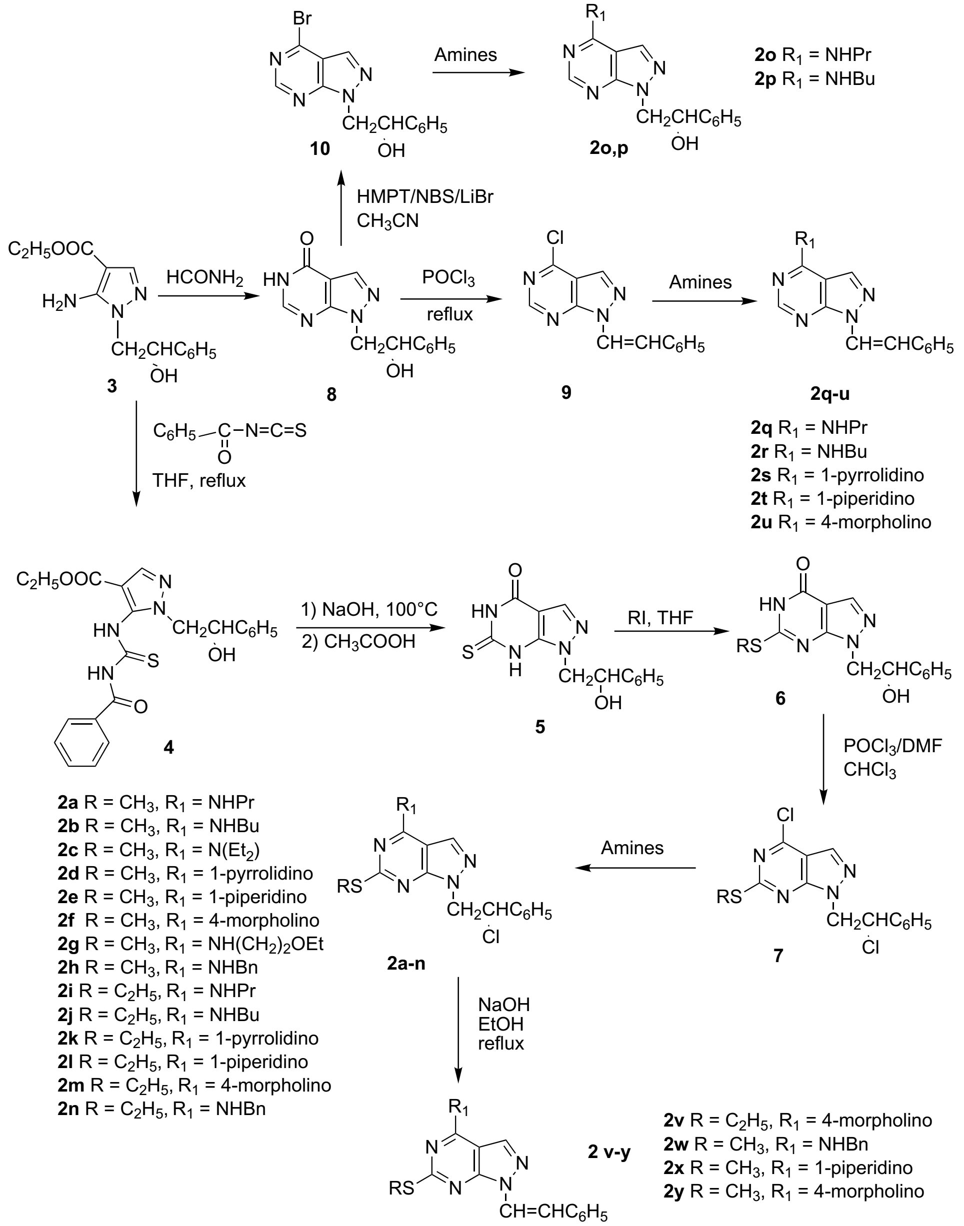
Compounds **2v**–**y** have been obtained by treatment of the corresponding chlorophenylethyl derivatives **2m**, **2h**, **2e**, and **2f**, respectively, with a 4 M solution of NaOH in EtOH/water (1:8) at reflux for 4 h.

Reaction of **3** with formamide in excess at 190 °C for 8 h afforded the pyrazolo[3,4-*d*]pyrimidinone **8**, which was purified by dissolving the crude in 2 M NaOH, boiling with charcoal, followed by precipitation with acetic acid.

Compound **10** was prepared in a yield of 44% following the Beal and Véliz procedure[21] by treatment of **8** with a mixture of HMPT/NBS in acetonitrile at −20 °C followed by addition of LiBr and refluxing. It is interesting to point out that the secondary OH on the side chain remained unaltered by this procedure, as shown by its ^1H NMR spectrum. Treatment of **10** with propylamine or butylamine gave the desired compounds **2o** and **2p**.

Reaction of **8** at reflux with $POCl_3$ afforded the 1-styryl derivative **9**, which was reacted with an excess of various amines in toluene to give **2q**–**u** in good yield.

The new pyrazolo[3,4-*d*]pyrimidine derivatives **2a**–**y** were studied in an assay system with the aim of evaluating their antiproliferative properties on the human epidermoid carcinoma A431 cells,[22] known to overexpress both EGFR and Src. Results are reported in



Scheme 1.

Table 1. A431 cells appeared to be sensitive to the activity of these new compounds, some of them showing an inhibition of cell growth similar or higher to that of PP2 (Fig. 2), known as a very potent and selective inhibitor of Src and chosen as the reference compound. Analysis of the antiproliferative activity toward A431 cells led us to rule out general considerations on the relationships between biological data and structural properties of such compounds.

Derivatives bearing a methylthio group at the position 6 were found more active than the corresponding ethylthio counterparts, suggesting that the size of the alkyl group at this position was an important element to influence antiproliferative activity.

Among the methylthio compounds, both linear primary alkylamino groups (such as propyl- and butylamino chains), cyclic amino moieties (such as 1-pyrrolidino, 1-piperidino, and 4-morpholino rings), and aromatic amines (such as a benzylamino substituent) at the position 4 led to compounds with antiproliferative activity comparable or higher to that of the reference compound PP2, found to be 32 μM. In particular, elongation of the propyl chain of **2a** (32 μM) to a butyl group, afforded compound **2b** with slightly enhanced activity (27 μM). On the other hand, when the bulkiness of the amino side chain was increased as for compound **2c**, antiproliferative activity underwent a significant decrease (70 μM). Moreover, compounds **2d–f** bearing a 1-pyrrolidino, a 1-piperidino, and a 4-morpholino substituent at the position 4, were found to have an activity of 32, 38, and 24 μM, respectively. The morpholino derivative was also found to be the most active compound among all the pyrazolo-pyrimidines presented in this paper. Transformation of the morpholino ring into

Table 1. Physicochemical and antiproliferative activity of compounds **2a**–**y** toward A431 cells

Compound	R	R_1	Mp (°C)	Yield (%)	IC_{50} (μM)[a]
2a	Me	NHPr	126–127	76	32 ± 0.5
2b	Me	NHBu	106–107	60	27 ± 0.5
2c	Me	$N(Et_2)$	91–92	80	70 ± 1.4
2d	Me	1-Pyrrolidino	151–152	60	32 ± 0.8
2e	Me	1-Piperidino	94–95	65	38 ± 0.8
2f	Me	4-Morpholino	116–117	75	24 ± 0.4
2g	Me	$NH(CH_2)_2OEt$	115–116	78	32 ± 0.6
2h	Me	NHBn	142–143	81	25 ± 0.7
2i	Et	NHPr	72–73	71	79 ± 1.8
2j	Et	NHBu	102–103	61	40 ± 0.4
2k	Et	1-Pyrrolidino	131–132	70	89 ± 1.7
2l	Et	1-Piperidino	92–93	81	176 ± 1.9
2m	Et	4-Morpholino	130–131	80	42 ± 0.7
2n	Et	NHBn	165–166	76	124 ± 2.5
2o		NHPr	104–105	65	91 ± 1.4
2p		NHBu	125–126	70	83 ± 1.8
2q		NHPr	132–133	75	61 ± 1.0
2r		NHBu	106–107	45	91 ± 2.0
2s		1-Pyrrolidino	151–152	53	63 ± 1.0
2t		1-Piperidino	94–95	60	69 ± 1.2
2u		4-Morpholino	116–117	75	59 ± 1.0
2v	Et	4-Morpholino	155–156	65	50 ± 0.8
2w	Me	NHBn	185–186	72	40 ± 0.6
2x	Me	1-Piperidino	155–156	90	54 ± 0.7
2y	Me	4-Morpholino	161–162	88	36 ± 0.6
AG1478[b]					20 ± 0.4
PP2[b]					32 ± 0.7

[a] IC_{50} values are means ± SEM of series separate assays, each performed in quadruplicate.

[b] To test our system, we have used the well known antiproliferative agent AG1478. As a result, the IC_{50} of such compound toward A431 cells (20 μM), was comparable with that reported in the literature.[31] However, since the studied compounds were found to inhibit Src phosphorylation, we discussed their structure–activity relationships in comparison to the activity of PP2, known as a very potent and selective inhibitor of Src phosphorylation.

the corresponding opened counterpart yielded the ethoxyethylamino derivative **2g** with an antiproliferative activity of 32 μM. Finally, introduction of an arylamino moiety at the same position led to **2h** characterized by an activity of 25 μM, comparable to that of the corresponding morpholino derivative **2f**.

Regarding the ethylthio compounds, antiproliferative activity followed a trend similar to that found for the corresponding methylthio derivatives, with IC_{50} values significantly higher. In fact, while activity of the methylthio compounds ranged from 24 to 70 μM, the corresponding ethylthio derivatives showed activity spanning from 40 to 176 μM. In particular, in agreement with what found for **2a** and **2b**, the butyl chain of **2j** was preferred to the propyl group of **2i**, being 40 and 79 μM the activity values of these compounds toward A431 cells. It is also important to note that **2j** showed the best activity among the ethylthio derivatives, similarly to **2b** that was one of the most active compounds among the methylthio counterparts. Moreover, activity of **2k**–**m** (89, 176, and 42 μM, respectively) followed the same trend of the corresponding methylthio compounds, with the antiproliferative activity enhanced in the order 4-morpholino > 1-pyrrolidino > 1-piperidino. Finally, an unexpected low activity (124 μM) was measured for **2n**, about 5-fold decreased in comparison to the methylthio counterpart **2h** (25 μM). This loss of antiproliferative activity could be rationalized by the fact that the presence, at the same time, of a benzylamino chain at C4 and an ethylthio group at C6 (both of them being the bulkiest substituents added to these positions) could led to steric clashes with the corresponding receptor counterparts, in turn avoiding profitable interactions.

The substituent at N1 has been varied to evaluate its influence on the ability of the compounds to inhibit the proliferation of the A431 cells. In particular, when the 2-chloro-2-phenylethyl chain was replaced by a styryl group, keeping fixed the methylthio group at C6, a slight decrease of activity was observed. In fact, compounds **2w**–**y** showed an activity (40, 54, and 36 μM, respectively) lower with respect to the corresponding chlorophenylethyl derivatives **2h**, **2e**, and **2f**. Similarly, the ethylthio analogue of **2y** (**2v**), bearing a morpholino ring at C4 and a styryl group at N1, was characterized by an activity of 50 μM, lower than that of the corresponding chlorophenylethyl derivative **2m** (42 μM). These experimental results evidenced that the optimal combination for substituents at N1 and C6 is represented by a 2-chloro-2-phenylethyl chain and a methylthio group, respectively.

To further investigate the structure–activity relationships of these pyrazolo-pyrimidines, two additional subclasses were designed and synthesized, both of them lacking the C6 substituent, and bearing a 2-hydroxy-2-phenylethyl or styryl side chain at N1. Regarding the

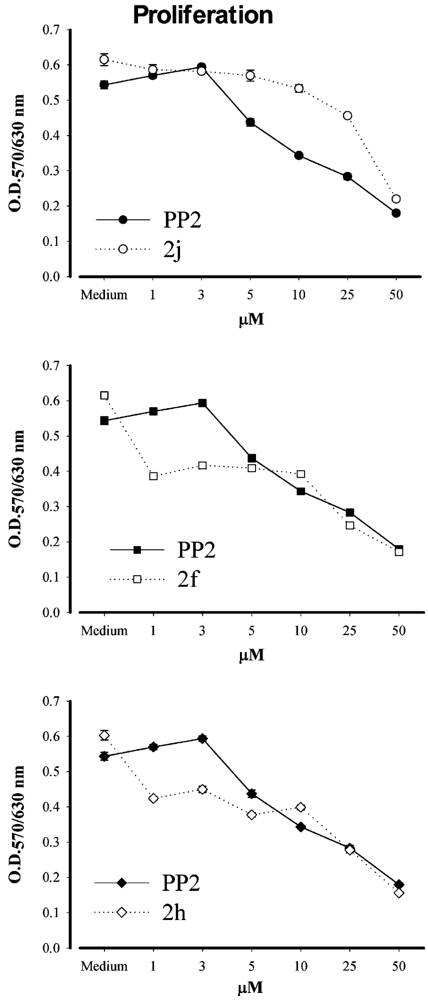


Figure 2. Inhibitory effects of compounds **2f**, **2h**, and **2j** on A431 cells measured by the MTT assay.[30] Values are means ± SEM of three independent experiments performed in duplicates.

hydroxy derivatives, they showed the lowest antiproliferative activity in comparison to the corresponding congeneric compounds (i.e., propylamino and butylamino analogues) belonging to the other subclasses, with the exception of **2p** that was found to have an activity of 83 μM, slightly higher than the corresponding styryl derivative **2r** (91 μM). Moreover, among the styryl compounds lacking a C6 substitution, the activity values of the alkylamino derivatives depended from the length of the alkyl chain in the order propyl > butyl, differently to that found in the other three subclasses. In fact, activity of **2q** and **2r** was found to be 61 and 91 μM, respectively. Finally, insertion of a heterocyclic amino moiety at C4, such as 1-pyrrolidino, 1-piperidino, and 4-morpholino, led to **2s–u**, respectively, whose activity values followed the same trend found for the corresponding 2-chloro-2-phenylethyl derivatives **2d–f** and **2k–m** with an increase in activity in the order 4-morpholino > 1-pyrrolidino > 1-piperidino.

With the aim of summarizing these experimental findings, one can say that (1) the length and the bulkiness of the alkylamino side chain at the position 4 influenced activity, being a butylamino group preferred to a propylamino chain (with the only exception of compounds **2q** and **2r**), while the diethylamino substituent led to a more pronounced lowering of activity. Moreover, cyclic amino substituents were also profitable for activity, with a 4-morpholino ring preferred to pyrrolidino and piperidino groups. Finally, when at the same time a C4 benzylamino chain and a C6 methylthio group were added to the pyrazolo-pyrimidine nucleus, compounds with interesting biological profiles were obtained (namely, **2h** and **2w**). On the contrary, increasing the size of the alkylthio group of **2h** to a ethylthio substituent yielded **2n** with a very low antiproliferative activity. (2) Regarding the influence of N1 and C6 substitutions, biological data showed that a methylthio group was preferred if the 2-chloro-2-phenylethyl side chain was attached to N1. When, at the same time, the 2-chloro-2-phenylethyl group was transformed into a 2-hydroxy-2-phenylethyl chain and the alkylthio substituent was removed, the compounds obtained (**2o** and **2p**) showed antiproliferative activity significantly lower (91 and 83 μM) to the corresponding parent compounds (**2a**, **2b**, and **2i**, **2j**). Similarly, compounds without any C6 substitution and bearing both a styryl chain at N1 and a heterocycle at C4 (**2s** and **2t**) showed a higher activity with respect to the C6 ethylthio analogues **2k** and **2l** (with the exception of **2u** that was found less active than **2m**), but a significantly lower antiproliferative activity in comparison to the corresponding C6 methylthio compounds **2d–f**. Moreover, **2q** and **2r** were less active than the corresponding C6 alkylthio parents, with the exception of compound **2i** (79 μM) found to be less active than **2q**. (3) A comparison between the 2-hydroxy-2-phenylethyl derivatives **2o**, **2p** and the styryl compounds **2q** and **2r**, suggested that the antiproliferative activity of the latter increased in the order propyl > butyl, differently from that found in all the other subclasses.

To better understand the reason of the reported antiproliferative activity of these new compounds, their inhibitory property to human EGF TK receptor has been evaluated by measuring the EGF receptor and the phospho-EGF Receptor (Tyr1045) using a specific antibody (Cell Signaling Technology, MA, USA) that detects EGF receptor only when phosphorylated at Tyr1045 (a Src-independent phosphorylation site). Compounds **2a–y** demonstrated no effect on the phosphorylation (data not shown), consequently the antiproliferative activity was not mediated by inhibition of the EGF receptor. However, considering that A431 cells are known to overexpress Src, we studied the behavior of **2f**, **2h**, and **2j** (found to possess the best A431 antiproliferative activity in the two subseries of methylthio and ethylthio derivatives, respectively) on the Src phosphorylation.

Phosphorylation of Src Tyr416 is a crucial step in the mechanism of the enzyme activation.[23] A431 cells have been well characterized in respect to EGF-dependent signal transduction. In more details, these cells express high levels of EGF receptor on their surface and proliferate in response to autocrine secretion of transforming growth factor-α,[24] but they also require phosphorylation of Src to proliferate. To determine

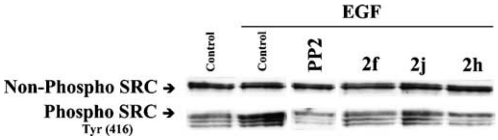


Figure 3. Phospho-Src inhibition by compounds **2f**, **2h**, and **2j** toward A431 cells. Cells were cultured at a concentration of 20^3 cells/mL in the presence of 10 μM of the specific compound for 3 h at 37 °C. EGF (0.1 μM, Cell Signaling Technology, MA, USA) was added 10 min before lysis. Cell lysates (40 μg protein) were subjected to SDS-PAGE and electrophoretically transferred to a polyvinylidene fluoride membrane. Immunoblotting was performed using the specific antibody. The membranes were visualized with luminescent substrates. The figure is a representative gel of three similar experiments.

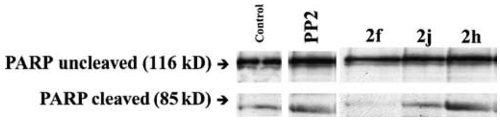


Figure 4. Detection of cleaved PARP in A431 cell extracts. Immunoblot analysis was performed using anti-PARP specific antibodies. An anti-rabbit secondary antibody conjugated with peroxidase and a peroxidase substrate revealed the presence of PARP cleavage products in apoptotic cells. The antibody recognized both the uncleaved PARP (116 kDa) and the larger cleaved fragment (85 kD). Cells were treated for 3 h with 10 μM of the specified compound. Results are representative of three independent experiments.

whether these pyrazolo-pyrimidine compounds were able to inhibit the phosphorylation of Src Tyr416, we used a phosphospecific anti-Src (Tyr416) antibody (Cell Signaling Technology, MA, USA). PP2, used as a reference compound, has been identified as a Src-selective tyrosine kinase inhibitor and has been used extensively to investigate signaling pathways involving Src kinases, including events downstream of the stem cell factor (SCF) receptor c-Kit.[15d] As a result, we found that compounds **2f**, **2h**, and **2j** were able to inhibit Src phosphorylation with almost the same efficacy of PP2 (Fig. 3, Table 2).

Moreover, prompted by the ability of such three compounds in reducing Src phosphorylation, we also tested their pro apoptic activity on a Poly-ADP-Ribose-Polymerase (PARP) assay. PARP is a 116 kDa zinc-dependent protein that binds specifically at DNA strand breaks produced by various genotoxic agents.[25] PARP is also a substrate for certain caspases (e.g., caspase 3 and 7) activated during early stages of apoptosis. These proteases cleave PARP to fragments of approximately 85 and 24 kDa. Detection of the 85 kDa PARP fragment with anti-PARP thus serves as an early marker of apoptosis.[26]

Treatment of A431 cells with **2j** and **2h** (10 μM) caused proteolytic cleavage of PARP, with accumulation of the 85 kDa species and reduction of the full-size 116 kDa protein (Fig. 4), similarly to the effect of PP2. PARP cleavage was apparent within 3 h of treatment with **2j** and **2h**, while **2f** showed no appreciable effect. This fact may be due to the short time of exposure of the A431 cells to the compound.

In conclusion, the antiproliferative effect of the new pyrazolo-pyrimidines toward A431 cells, due to the inhibition of Src phosphorylation, induced apoptotic cell death probably as a consequence of the inhibition of the tyrosine kinase positive signaling pathways. This is in agreement with the increasing evidence that tyrosine kinase oncogenes generate strong viability signals (e.g., stem cell factor) and that turning off a kinase leads to rapid apoptosis. This effect has already been nicely demonstrated for BCR/ABL both in vitro and in vivo.[27,28]

As a consequence, the molecules described in this paper could represent a useful tool to discover new hit compounds to be studied as anticancer agents.[29] For this purpose, while additional efforts are ongoing in our laboratory to gather further insight on the structure–activity relationships of such compounds, biological tests aimed at evaluating the activity of these compounds on tumor cell lines have been also planned and will be reported in due time.

Acknowledgements

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Table 2. Inhibitory effects of compounds **2f**, **2j**, and **2h** on phospho-Src after EGF stimulation of A431 cells measured by immunoblot

	Control	Control + EGF[a]	PP2[a]	**2f**[a]	**2j**[a]	**2h**[a]
Nonphospho-Src	178	167	131	142	159	154
Phospho-Src (Tyr416)	38	104	22	34	53	32
Phospho-Src/nonphospho-Src ratio (%)	21	62	17	24	33	21

Value are expressed as area units of the corresponding gels reported in Figure 3.

[a] A431 cells treated for 15 min with 100 nM EGF.

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