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Letters

Pyrazolo[3,4-*d*]pyrimidines Endowed with Antiproliferative Activity on Ductal Infiltrating Carcinoma Cells

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Abstract: Novel 1,4,6-trisubstituted pyrazolo[3,4-*d*]pyrimidines are reported with preliminary in vitro activity data indicating that several of them are potent inhibitors (better than the reference compound) of Src phosphorylation of the breast cancer cells 8701-BC, known to overexpress Src. The ability of such compounds to significantly reduce 8701-BC cell proliferation suggests that this scaffold could be a promising lead for the development of antitumoral agents able to block Src phosphorylation of breast cancer cells.

Over the past 3 decades, breast cancer has been the most common malignancy among Western women, and the incidence rates of first primary breast cancer have been increasing over time.[1] Meanwhile, the survival rates of breast cancer patients have increased substantially, but the incidence rates of second primary breast cancer are still very high especially among women with a first primary that was either lobular or medullary.[2] The major cellular changes involved in the conversion of normal to malignant breast epithelial cells are the progressive loss of the stationary epithelial phenotype and the acquisition of a mesenchimal-like phenotype, correlated with the ability to migrate and invade surrounding and distant tissues.[3] Ductal infiltrating carcinoma (DIC) of the breast is the most common and potentially aggressive form of cancer.[4] The 8701-BC cell line[5] was established from a primary DIC, that is, before the clonal selection of the metastatic process. This cell line maintains a number of properties in culture that are typical of the mammary tumor cells and is known to overexpress Src tyrosine kinase (TK).[6] Such TK is the prototype member of the nonreceptor Src family of protein tyrosine kinases (PTK).[7] Src is activated following engagement of many different classes of cellular receptors and participates as a convergence point in different signaling pathways.[8,9] In this regard, Src is a critical component of the signaling cascades initiated by TK-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 PTK receptor,[10,11] both of which are involved in cancer. Finally, Src overexpression and activation have been correlated with a large number of growth-regulatory processes where Src participates. The process of activation of Src is mediated by the phosporylation of the tyrosine 416. On this basis, inhibitors of Src phosphorylation process may halt uncontrolled tumor cell growth and play an important role as new therapeutic agents for the treatment of cancer.

In light of the morbidity and invasiveness of breast cancer, there remains a deep ongoing interest in the identification of potent compounds with specific inhibitory activity against breast cancer cells. Hence, in the course of our drug discovery programs, we were intrigued by the potency and selectivity of PP1 and PP2 (1-(*tert*-butyl)-3-(4-methylphenyl)-4-aminopyrazolo[3,4-*d*]pyrimidine and 1-(*tert*-butyl)-3-(4-chlorophenyl)-4-aminopyrazolo[3,4-*d*]pyrimidine, respectively) toward the Src family tyrosine kinase.[12−14] This experimental evidence, combined with our previous experience in the synthesis of such bicyclic compounds and their ana-

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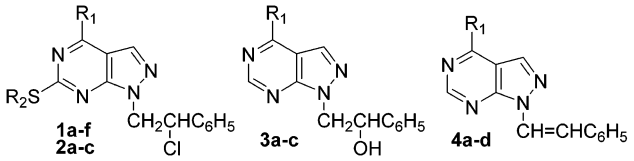
Table 1. Activity Data (Expressed as IC_{50} Values) of the New Pyrazolopyrimidines toward 8701-BC Cells



compd	R_1	R_2	IC_{50} (μM)[a]
PP2			61.8 ± 4.4
1a	1-piperidinyl	Me	67.5 ± 1.2
1b	4-morpholinyl	Me	64.2 ± 1.8
1c	$NH(CH_2)_2OC_2H_5$	Me	59.8 ± 3.1
1d	NHC_4H_9	Me	63.8 ± 2.1
1e	$NHCH_2C_6H_5$	Me	53.2 ± 1.2
1f	$NH(CH_2)_2C_6H_5$	Me	31.2 ± 0.5
2a	1-piperidinyl	Et	NA
2b	4-morpholinyl	Et	79.8 ± 3.0
2c	NHC_4H_9	Et	38.8 ± 0.5
3a	4-morpholinyl		NA
3b	NHC_4H_9		NA
3c	1-piperidinyl		NA
4a	1-piperidinyl		NA
4b	4-morpholinyl		58.8 ± 0.7
4c	NHC_4H_9		80.5 ± 6.8
4d	1-hexahydroazepinyl		93.7 ± 0.6

[a] IC_{50} values were determined on exponentially growing 8701-BC cells incubated with each of the compounds (0−100 μM) for 72 h using the MTT assay.[19] The IC_{50} values are the mean ± SEM of three independent experiments performed in duplicate. NA = not active at 100 μM.

logues,[15] led us to design and synthesize a new class of pyrazolo[3,4-*d*]pyrimidines (**1**−**4**). They were submitted to preliminary in vitro biological studies demonstrating that they are potent inhibitors of the breast cancer cell line 8701-BC via inhibition of Src phosphorylation, even when compared with the reference compound PP2. In addition, on the basis of the experimental evidence that Src kinase has been found to be active in many tumors,[16−18] its inhibitors could become useful anticancer agents for many types of cancer. As a consequence, the pyrazolopyrimidine derivatives disclosed here could represent a profitable tool to inhibit cancer cell growth through the blocking of the Src phosphorylation mechanism.

Cell Proliferation Experiments and Immunoblot Analyses. The antiproliferative effect of the new pyrazolopyrimidines was evaluated on the 8701-BC breast cancer cell line (kindly provided by Prof. I. Pucci-Minafra, University of Palermo, Italy) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay,[19] in comparison with the activity of PP2, chosen as the reference compound (Table 1). The mechanism of action of the new compounds was not investigated at the molecular level yet. Consequently, it is unclear if the studied compounds interacted with the phosphorylation of Src through a mechanism similar to or different from that of PP2, known as a potent and selective inhibitor of the Src family. However, the structural similarity between PP2 and our compounds led to the suggestion that the last could share with PP2 the same mechanism of Src phosphorylation inhibition. Following this hypothesis, the activity of the new compounds was compared to that of PP2. Such a comparison showed that seven of the newly synthesized compounds (namely, **1b**−**f**, **2c**, and **4b**, corresponding to about 50%) were characterized by activity values ranging between 31.2 (**1f**) and 64.2 μM (**1b**), comparable or higher to that of PP2 (61.8 μM, Figure 1).

A summary of activity data suggested the following. (1) The most active compounds belonged to subclasses **1** and **2**, bearing both an alkylthio substituent at C6 and a chlorophenylethyl side chain at N1. However, the styryl derivative bearing a morpholino ring at C4 (**4b**) was found to be more active than the congeneric compounds **1b** and **2b**. On the other hand, **4a** was found to be inactive, similar to the corresponding piperidino derivative belonging to the subclass **2** (namely, **2a**). (2) A butyl chain was found as the optimal substituent within the ethylthio subclass, while only the phenylethyl chain led to a better result among the thiomethyl derivatives **1**. (3) Both the deletion of the thioalkyl chain at C6 and the transformation of the chlorine atom of the N1 chlorophenylethyl side chain with the corresponding hydroxy group led to **3** with lower or insignificant activity.

To further investigate the antiproliferative activity of these compounds, the inhibitory effects of **1a**, **1e**, **1f**, and **2c** toward the phosphorylation of Src (Tyr416) were measured on cells treated with 100 nM epithelial growth factor (EGF). Immunoblot analysis, performed using phospho-specific antibodies to Src (Tyr416), clearly showed an inhibition of the Src activation−phosphorylation of 8701-BC cells exposed to the studied compounds, with respect to the control. In particular, **1a**, **1e**, and **1f** were found to inhibit Src phosphorylation better than the reference compound PP2 (Figure 2). In contrast, although **2c** retained appreciable inhibitory activity, it was found to be less potent than PP2. The occurrence, at the same time, of a reduction of Src phosphorylation and an inhibition of cell proliferation led us to assume that our compounds were able to inhibit proliferation through inhibition of Src phosphorylation, since the latter mediates a cascade leading to the stimulation of cell proliferation and mobility. Moreover, independent experiments have shown that all these compounds do not interfere with the phosphorylation of EGF receptor TK.[20]

The proapoptic activity of **1a**, **1e**, **1f**, and **2c** was also tested using a poly-ADP-ribose-polymerase (PARP) assay (Roche Diagnostics, Milano, Italy). Immunoblot analysis of uncleaved and cleaved PARP after a 3 h treatment with the specified compound indicated a significant increase of the cleaved PARP after treatment with **1a** and **2c**, similar to that found for the reference compound PP2 (Figure 3). On the other hand, for **1e** and **1f**, the increase of the cleaved fraction of PARP was less intense and the amount of the uncleaved protein was lower in comparison to that found in the experiments with **1a** and **1c**. Enhancement of the cleaved PARP level demonstrated that cells were induced in apoptosis. In particular, **1a** and **2c** showed a proapoptotic effect toward 8701-BC comparable to that found for the reference compound and more relevant with respect to that of **1e** and **1f**, even if they showed a better inhibitory activity on Src. This may be interpreted as a more pronounced cytostatic effect rather than a proapoptic activity. However, it should be also consid-

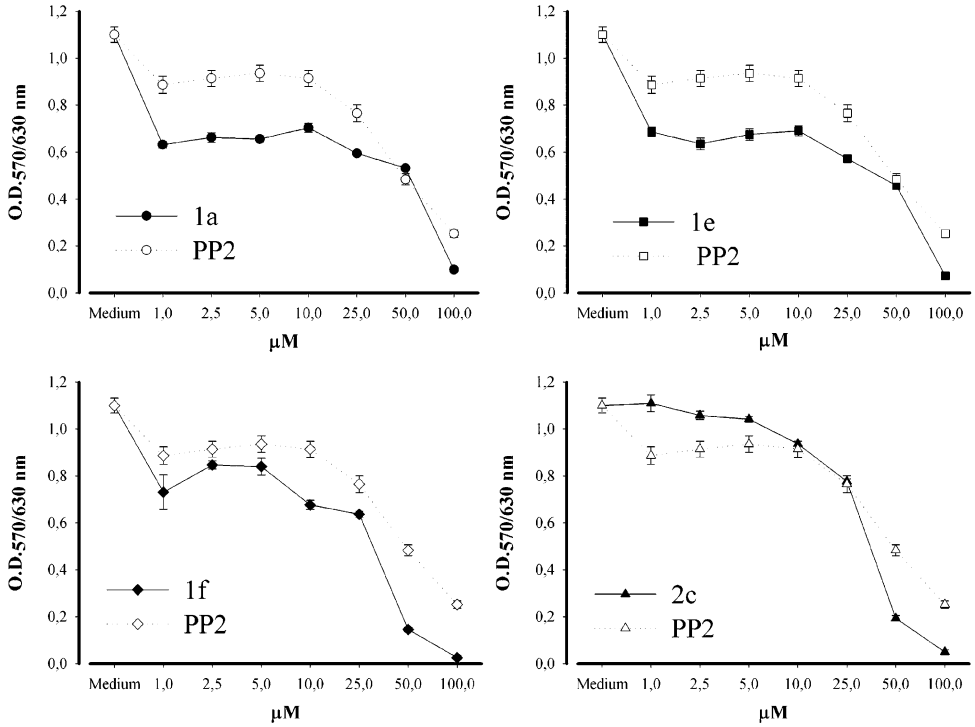


Figure 1. Inhibitory effects of **1a**, **1e**, **1f**, and **2c** on 8701-BC cells measured by the MTT assay. Values are the mean ± SEM of three independent experiments performed in duplicate.

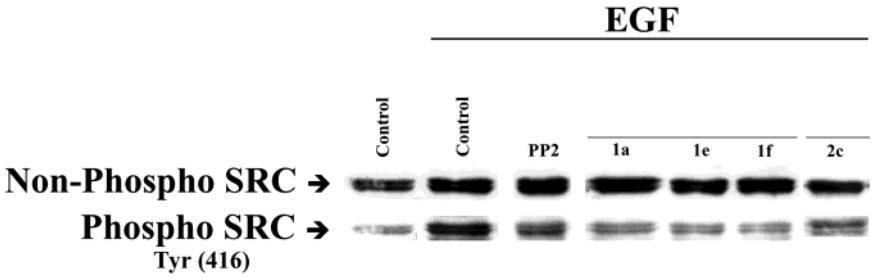


Figure 2. Phospho-Src inhibition by **1a**, **1e**, **1f**, and **2c** toward 8701-BC cells. Immunoblot analysis was performed using phospho-specific antibodies to Src (Tyr416). Filters were additionally reprobed with specific non-phospho anti-Src antibodies after stripping: (lane 1) cell control; (lane 2) cells treated for 15 min with EGF (100 nM); (lane 3−7) cells challenged for 3 h in the presence of 10 μM of the specified compound and then treated for 15 min with EGF (100 nM). Results are representative of three independent experiments.

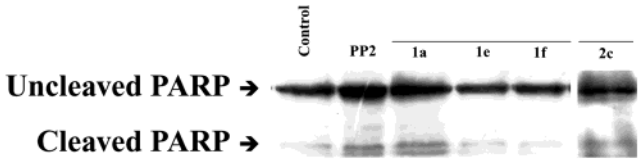


Figure 3. Detection of cleaved PARP in 8701-BC cell extracts. Immunoblot analysis was performed using anti-PARP specific antibodies. An antirabbit secondary antibody conjugated with peroxidase and a peroxidase substrate revealed the presence of PARP cleavage products in apoptotic cells. The antibody recognized both the uncleaved PARP (113 kDa) and the larger cleaved fragment (85 kDa): (lane 1) control cells; (lanes 2−6) cells treated for 3 h with 10 μM of the specified compound. Results are representative of three independent experiments.

ered that the time of cellular exposure to the compounds may be relevant for apoptotic induction.

In conclusion, considering the morbidity of breast cancer and the characteristics of the cell line chosen for this study, along with the antiproliferative activity exerted on these cells by the reported compounds (particularly **1f** and **2c**) when compared with one of the most potent and selective Src inhibitors known (PP2), we think that such compounds deserve great consideration. As a consequence, since, to the best of our knowledge, these are the first reported compounds with such an antiproliferative activity toward the 8701-BC cell line, **1f** and **2c** have been chosen as leads for more focused experiments in the field of anticancer agents acting via Src phosphorylation inhibition.[21] Nevertheless, it is important to point out that further investigations are required to evaluate the selectivity of the new compounds toward different cancer cell lines. In addition, studies aimed at identifying at the molecular level the mechanism of action of such compounds are currently ongoing. Results of such experiments will be reported in due time.

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Supporting Information Available: Experimental details (chemistry and pharmacology). This material is available free of charge via the Internet at http://pubs.acs.org.

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