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European Journal of Medicinal Chemistry 39 (2004) 939–946
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
www.elsevier.com/locate/ejmech

Original article

Antiproliferative activity of new 1-aryl-4-amino-1*H*-pyrazolo[3,4-*d*]pyrimidine derivatives toward the human epidermoid carcinoma A431 cell line

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Received 10 March 2004; received in revised form 1 June 2004; accepted 21 July 2004

Available online 07 October 2004

Abstract

Synthesis and biological evaluation of a new class of 1-aryl-4-amino-1*H*-pyrazolo[3,4-*d*]pyrimidine derivatives are reported. A preliminary cellular assay system using the tumor cell line A431 responding to epidermal growth factor (EGF) for its growth, shows that the new compounds are potent inhibitors of cell growth. The inhibition of tumor cell proliferation is not associated with blockage of EGF receptor (EGFR), but substantially due to the interference with the signalling pathway at the level of Src tyrosine kinase and at the level of the downstream effector signal mitogen activated protein kinases (MAPKs), ERK1-2.

Keywords: Pyrazolo-pyrimidine; Antiproliferative activity; Tyrosine kinase; A431 cells

1. Introduction

The search for new anticancer chemotherapeutic agents continues to be an active area of research at many pharmaceutical companies [1–4]. In the last 10 years, there has been a growth in the field of research around inhibitors of tyrosine kinases as potential cancer therapeutics. Determining the role that these key signalling enzymes play in the proliferation and spread of cancer has been critical to the identification of new biological targets with new mechanisms of action. Protein tyrosine kinases (PTKs) utilize ATP to phosphorylate specific tyrosine residues within the sequence of functional proteins, thus mediating the transmission of mitogenic signals and numerous other cellular events [5], including cell proliferation, migration, differentiation, metabolism and immune response.

PTKs can be divided into two classes: the transmembrane growth factor receptor TK and the cytoplasmatic TK. In particular, the cytoplasmatic protein c-Src is a ubiquitous nonreceptor TK which is overexpressed or activated in different types of tumors [6], osteoporosis [7], and stroke [8]. Selective inhibition of c-Src function represents an important field of research for treatment of these diseases. As an example, Src phosphorylation inhibition may stop uncontrolled tumor cell growth and play a crucial role in the treatment of cancer. In addition to Src, a family of enzymes that has attracted a significant amount of interest has been the growth factor tyrosine kinase receptors, specially the EGF receptor [9].

During the past decade, many tyrosine kinase inhibitors have been described and reviewed in the literature [1,2,10–13], some of them belonging to the pyrazolo[3,4-*d*]-pyrimidine class. As an example, approaches aimed at identifying ATP-competitive small molecules led Traxler and coworkers to find pyrazolo[3,4-*d*]pyrimidines showing potent inhibitory activity toward EGFR tyrosine kinase [1,17],

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doi:10.1016/j.ejmech.2004.07.010

while Hanke et al. [14], Shokat [15] and Hirst [16] reported pyrazolo[3,4-*d*]pyrimidines as selective inhibitors of Src family. Moreover, purine derivatives such as olomoucine and roscovitine, structurally related to the pyrazolo[3,4-*d*]pyrimidine compounds, exhibited a moderate activity but good selectivity toward several CDKs kinases [31]. To summarize, 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 Src, to CDK serine-threonine kinase.

On the basis of this knowledge, we decided to synthesize new 4-aminopyrazolo[3,4-*d*]pyrimidines, structurally related to the above mentioned inhibitors, but possessing a 2-chloro-2-phenylethyl or a stryryl chain on the N1 of the pyrazole ring [18]. The antiproliferative activity of such compounds was assessed on the human epidermoid carcinoma A431 cell line, in comparison to AG-1478 (Chart 1), chosen as the reference compound. Moreover, to study at the molecular level the mechanism of action of the above compounds, their influence on signalling molecules (namely, Src and ERK1-2) involved in the EGF-induced cell proliferation, was also investigated.

2. Chemistry

1*H*-Pyrazolo[3,4-*d*]pyrimidines **1a–l** and **2a–f** were synthesized as follows (Scheme 1). The starting product was the ethyl ester of 5-amino-1-(2-hydroxy-2-phenylethyl)-1*H*-pyrazole carboxylic acid **3**, prepared following our published procedure [19].

Reaction of **3** with formamide in excess and heating at 200 °C for 8 h afforded the pyrazolo[3,4-*d*]pyrimidinone **4** which was purified by dissolving the crude in NaOH 2 M, boiling with coal, followed by precipitation with acetic acid.

The chlorination with Vilsmeier complex ($POCl_3$: DMF/1:1) in $CHCl_3$ at reflux for 8 h afforded the dihalogenated derivative **5**, bearing a chlorine atom in position 4 of the pyrimidine ring and on the side chain on the N1 of the pyrazole ring. Product **5** was purified by a simple filtration on Florisil® column.

Regioselective substitution of the C4 chlorine of compounds **5** with an excess of various primary and secondary amines, in toluene at rt, afforded the desired products **1a–l** in good yield.

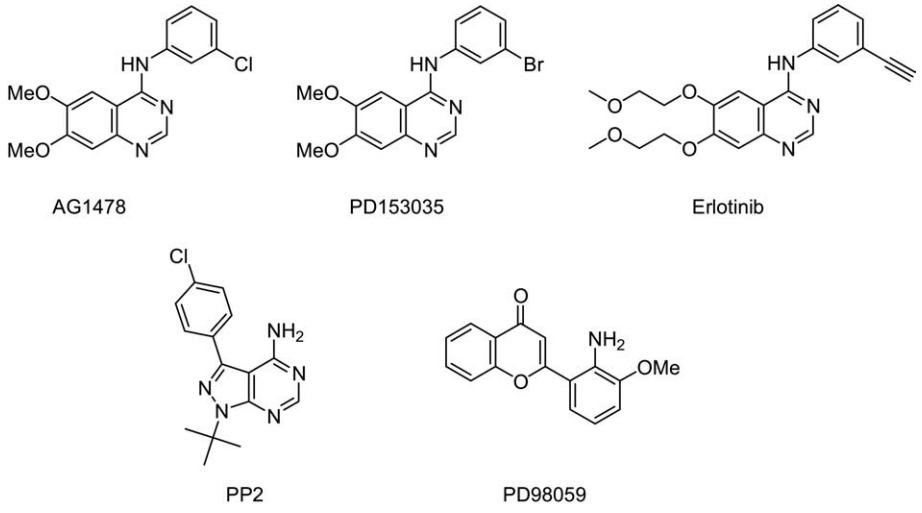
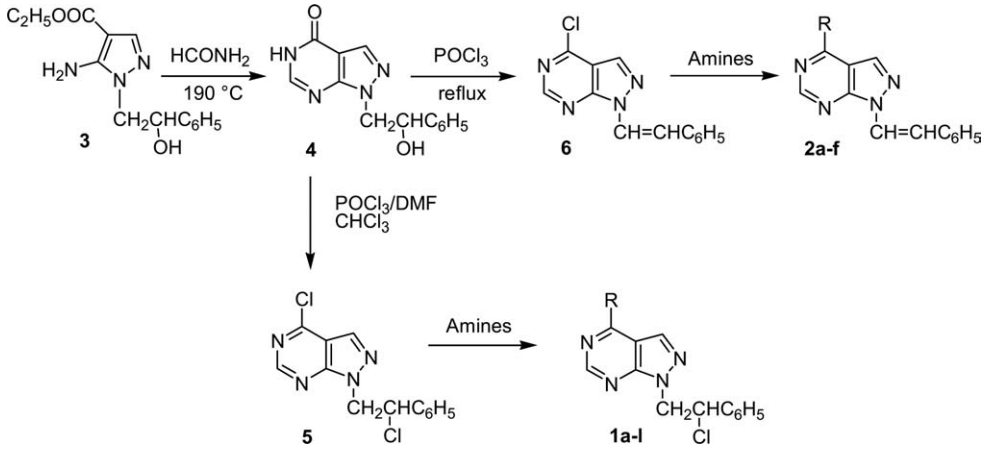


Chart 1. Structure of the reference compounds cited ou used in this study.



Scheme 1.

It is interesting to point out that the chlorine atom at the side chain of all of the new compounds has never been substituted by the amino group. This finding was proved by the ^{1}H NMR chemical shifts of the CH_2CH side chain protons, which resonates unchanged at the same position and with the same pattern.

Treatment of **4** at reflux with $POCl_3$ afforded the 1-styryl derivative **6**.

Compound **2a–f** were prepared by reaction of **6** with the proper amine, in toluene at rt.

3. Pharmacology

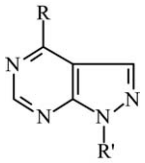
To study their cellular mechanism of action and specificity, compounds **1a–l** and **2a–f** were tested in assay systems using a cell line responding to EGF for its growth. Inhibition of EGF-stimulated cell growth was measured in the human epidermoid carcinoma A431 cells [20], known to overexpress both EGFR and Src.

The growth inhibitory activity of the compounds studied was compared with the activity of the selective EGFR inhibitor AG1478 [21] at the concentration of 50 nM. The inhibitory effect exerted by the compounds on cell proliferation when tested at 50 nM, is reported in Table 1.

4. Results and discussion

A431 cells appeared to be sensitive to the activity of these new compounds. In fact, only four compounds (namely **1b**, **1e**, **1i**, **1j** and **2f**) showed an inhibition activity toward the cell growth lower than 50%, while five molecules (namely **1c**, **1d**, **1h**, **1l** and **2a**) were reported to have activity higher than 86% when compared with the 100% inhibitory activity of the reference compound, AG1478. Regarding compounds **1**, an attempt to rationalize the relationships between the structural features of the C4 substituent and their inhibitory activity suggested that a better inhibition of the A431 cells growth was found for compounds bearing large substituents at that position. As an example, enlargement of the pyrrolidino ring of **1c** (86% inhibition) to a piperidino and 1-hexahydroazepino ring led to compounds **1d** and **1h** with higher activity (128% and 107%, respectively). Moreover, transformation of the piperidino ring of **1d** to a morpholino moiety (**1e**) caused a significant decrease in activity, from 128% to 46%, respectively. Substitution of the morpholino ring with an ethoxyethylamino side chain led to **1f** with enhanced activity (66%) with respect to the parent compound. Introduction of an aromatic moiety at C4, such as a benzylamino group, was favorable for activity (**1l**, 112%), while increasing the size of this group into a phenylethylamino side chain produced a drop in activity (**1i**, 49%). Smaller alkylamino side chains at C4 (namely, cyclopropylamino, propylamino, butylamino, and diethylamino) led to compounds **1a, 1b, 1k**, and **1j**, respectively, with low activity, ranging from 43% to 57%.

Table 1
Inhibitory effect of compounds **1a–l** and **2a–d** on the growth of A431 cells



No.	R	R′	Percent inhibition relative to the reference compound AG1478	EC_{50}
1a	NHcyclopropyl	$CH_2CHClPh$	53	69
1b	NHC_3H_7	$CH_2CHClPh$	46	78
1c	1-pyrrolidinyl	$CH_2CHClPh$	86	26
1d	1-piperidinyl	$CH_2CHClPh$	128	20
1e	4-morpholinyl	$CH_2CHClPh$	46	71
1f	$NH(CH_2)_2OC_2H_5$	$CH_2CHClPh$	66	41
1g	NHcyclohexyl	$CH_2CHClPh$	63	48
1h	1-hexahydroazepinyl	$CH_2CHClPh$	107	15
1i	$NH(CH_2)_2Ph$	$CH_2CHClPh$	49	70
1j	NEt_2	$CH_2CHClPh$	43	59
1k	NHC_4H_9	$CH_2CHClPh$	57	50
1l	$NHCH_2Ph$	$CH_2CHClPh$	112	4.3
2a	NHcyclopropyl	CH=CHPh	146	1.4
2b	$NH(CH_2)_2OC_2H_5$	CH=CHPh	63	46
2c	NHcyclohexyl	CH=CHPh	51	62
2d	1-hexahydroazepinyl	CH=CHPh	58	59
2e	$NHCH_2Ph$	CH=CHPh	64	41
2f	$NH(CH_2)_2Ph$	CH=CHPh	46	78

MTT cell proliferation assay for A431 cell line in the presence with 50 nM test or reference compounds. Cell growth was determined at 48 h. EGF was not added to A431 cell line since its receptor is constitutively activated. Results are expressed as percent values with respect to the reference inhibitor AG1478, whose inhibition activity was set to 100, and were calculated from six data taken from two separate experiments. EC_{50} values were calculated from series separate assays, each performed in quadruplicate.

Substituent at the 1-position of the bicyclic nucleus is worthy of further consideration. Compound **2a**, bearing a cyclopropylamino moiety at C4 and an unsaturated aryl chain (i.e., styryl moiety) at N1 was characterized by an inhibitory profile much more interesting with respect to the corresponding phenyl chloroethyl derivative **1a**. In fact, **2a** showed an inhibitory activity value of 146% (the highest inhibitory activity found among all the new compounds) toward A431 cells growth with respect to the reference compound, while **1a** possessed only 53% toward the same target.

Moreover, **2b–d** showed inhibitory activity lower than the corresponding phenylethyl derivatives **1f–h**. In particular, while activity of **1f** and **2b** were comparable (66% versus 63%), the introduction of bulkier side chains at C4 led to more pronounced loss of activity, when compared to the corresponding phenylethyl compounds. In fact, activity of **1g** was found to be 63% versus a 51% determined for **2c**. Finally, a further enlargement of the cycle at C4 from a cyclohexyl to a 1-hexahydroazepinyl ring led to a deeper decrease in activity, from 107% (**1h**) to 58% (**2d**). Similarly,

an aromatic substituent at C4 led to compounds **2e** and **2f** with lower activity (64% and 46%, respectively) with respect to the corresponding saturated derivatives **1l** and **1i** (112% and 49%, respectively). These findings suggested that a planar system such as the styryl moiety, combined with a small secondary amino group, should be more profitable, with respect to saturated alkyl chains at N1 and bulkier amino groups at C4, to interact with molecular targets leading to the inhibition of A431 cells proliferation [22]. This result was not in agreement with what found for compounds **1a–l** where large substituents at C4 produced an enhancement in activity. To prove this speculation, more compounds with a N1 styryl chain have been planned to be synthesized and tested.

Moreover, to further investigate their biological properties, EC_{50} values were determined for compounds **1** and **2**. In detail, the best results were obtained for compounds **1d**, **1h**, **1l**, and **2a**, showing an EC_{50} of 20, 15, 4.3, and 1.4 μM, respectively, in comparison to a value of 2.9 μM found for the reference compound AG1478. This result confirmed compound **2a** as characterized by the best biological profile with respect to the remaining new pyrazolo-pyrimidine derivatives.

The above experimental evidence prompted us to test the new compounds for their inhibitory properties on EGF tyrosine kinase. In fact, (1) a relevant structural similarity was shared by our compounds and those reported by Traxler et al. [17] (i.e., the pyrazolo[3,4-*d*]pyrimidine nucleus; the hydrogen bond donor-acceptor motif represented by the amino group at the C4 and the endocyclic N5 nitrogen atom, respectively; the anilido group at the pyrimidine ring of several compounds); (2) while compounds **1d** and **1h** showed a remarkable inhibitory activity toward the growth of A431 cells, the activity of **1l** (4.3 μM) and **2a** (1.4 μM) was comparable to or higher than that found for the reference compound AG1478 (2.9 μM).

The influence of such compounds on the activity of the human EGF tyrosine kinase receptor was evaluated by measuring the phosphorylation of poly GAT (Glu, Ala, Tyr) using a purified A431 cell membrane preparation as the enzyme source [23]. As a result, all the newly synthesized compounds were inactive (data not shown). Compound **1l**, taken as an example, showed only a 2% inhibition of the control enzyme activity, with respect to the reference compound, PD 153035, known as an EGFR inhibitor similar to AG1478 and erlotinib [24,25]. This result suggested that the antiproliferative activity of the studied compounds did not involve inhibition of EGFR.

On the other hand, considering that the A431 cell line is known to overexpress Src, we studied the influence of **1l** and **2a** (chosen on the basis of their high A431 antiproliferative activity) on the phosphorylation of Src, through an in vitro assay on A431 cell lysates. To determine whether such compounds were able to inhibit the phosphorylation of Src at the level of Tyr416, we used a phosphospecific anti-Src (Tyr416) antibody. On the other hand, PP2, previously identified as a Src-selective tyrosine kinase inhibitor [14], was used as the reference compound. As a result, although PP2 was found to be more efficient than **1l** and **2a**, the two compounds were able to inhibit Src phosphorylation with almost the same efficacy of PP2 (Fig. 1).

Moreover, we also investigated signalling molecules involved in the EGF-induced cell proliferation. In further detail, compound **1l** showed an interesting 50% inhibition (with respect to the reference compound staurosporine) on the activity of the human Src kinase receptor, measured by fluorescence polarization, according to the procedure reported by Parker [26]. Moreover, two compounds showing the higher inhibitory activity toward the growth of A431 cells (**1l** and **2a**) were tested (at the concentration of 50 nM) for their effects on the phosphorylation activity of the downstream extracellular receptor kinases 1-2 (ERK1-2) belonging to the family of the tyrosine-threonine mitogen activated protein kinases (MAPKs) and playing a crucial role in the intracellular signalling regulating the cellular growth process mediated by EGF.

As a result (Fig. 2), both **1l** and **2a** completely inhibited ERK1-2 activity induced by EGF and measured as ERK1-2 phosphorylation [27]. It is worth to note that, at the same nanomolar concentration, the selective MEK inhibitor PD 98059 [28] did not affect EGF-induced ERK1-2 phosphorylation. A 100 μM concentration of PD 98059 was required to show an inhibition of ERK1-2 phosphorylation comparable to that induced by a 50 nM concentration of **1l**. In summary, compounds **1l** and **2a** were found to inhibit the tyrosine-threonine MEK kinase activity responsible for ERK phosphorylation. In particular compound **1l** emerged as a potent inhibitor of EGFR dependent tumor cell growth (e.g. A431) and its inhibitory effect appears to be not related to the inhibition of protein tyrosine kinase EGFR, but associated with both the Src and MAPK pathways.

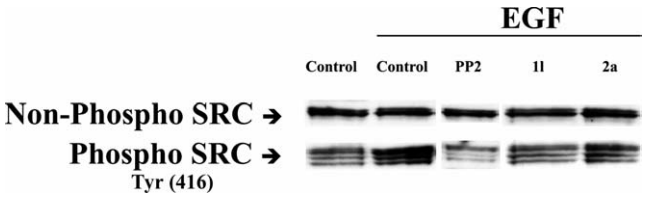


Fig. 1. Phospho-Src inhibition by compounds **1l** and **2a** toward A431 cells. The figure is a representative gel of three similar experiments.

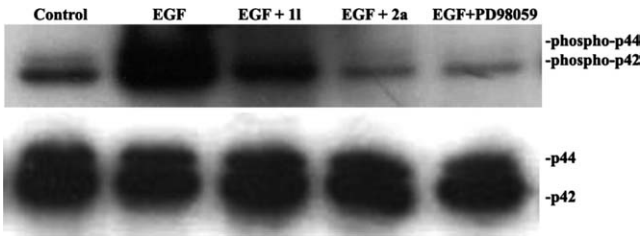


Fig. 2. Effect of compounds **1l** and **2a** on ERK1/2 activity in the A431 cell line. Western blot for phospho-ERK 1/2 (phospho-p44/p42) and total p44/p42 in A431 cell line exposed for 1 h to compound **1l** or **2a** at 50 nM or PD 98059 at 100 μM and then stimulated for 10 min with 50 ng/ml EGF. A representative gel out of three is shown.

Finally, it is interesting to point out that compound **1l** and **2a** did not show cell toxicity up to 10 μM concentration.

In conclusion, the new pyrazolo-pyrimidines showed an interesting antiproliferative effect toward A431 cells, mainly due to the interference with ERK1-2, and to a partial inhibition of the Src phosphorylation. 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 gain further insight on the relationships between structure and activity of such compounds, biological tests for the evaluation of their activity and selectivity on a panel of tumor cell lines have been also planned and results will be reported in due time.

5. Experimental protocols

5.1. Chemistry

Starting materials were purchased from Aldrich-Italia (Milan).

Melting points were determined with a Büchi B 540 apparatus and are uncorrected. IR spectra were recorded in KBr with a Perkin-Elmer 398 spectrophotometer. ^{1}H NMR spectra were recorded in $CDCl_3$ solution on a Varian Gemini 200 (200 MHz) instrument, chemical shifts (δ) are reported in ppm relative to TMS as internal standard; J in Hz. ^{1}H patterns are described using the following abbreviations: s = singlet, d = doublet, dd = double doublet, t = triplet, q = quartet, sext = sextet, m = multiplet, br = broad.

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

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

5.1.1. 1-(2-Hydroxy-2-phenylethyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one **4**

A suspension of **3** (2.75 g, 10 mmol) in formamide (10 g, 333 mmol) was heated at 190 °C for 8 h and then poured in H_2O (300 ml). The crude was filtered and purified by dissolving in 2 M NaOH (100 ml), boiling with coal, followed by precipitation with glacial acetic acid. The solid was filtered and recrystallized from absolute ethanol to give **4** (1.79 g, 70%) as a white solid, mp 270–271 °C. ^{1}H NMR: δ 4.25–4.55 (m, 2H, CH_2), 5.05–5.15 (m, 1H, CHO), 5.65 (d, 1H, OH, disappears with D_2O), 7.25–7.45 (m, 5H Ar), 8.05 (s, 1H, H-3), 8.10 (s, 1H, H-6), 12.15 (br s, 1H, NH, disappears with D_2O). IR cm^{-1}: 3400 (NH), 3245–2500 (OH), 1740 (C=O).

5.1.2. 4-Chloro-1-(2-chloro-2-phenylethyl)-1H-pyrazolo[3,4-d]pyrimidine **5**

The Vilsmeier complex, previously prepared from $POCl_3$ (6.13 g, 40 mmol) and anhydrous dimethylformamide (DMF) (2.92 g, 40 mmol) was added to a suspension of **4** (2.56 g, 10 mmol) in $CHCl_3$ (20 ml).

The mixture was refluxed for 7 h. 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 Mesh), using CH_2Cl_2 as eluant, to afford the pure product **5** (2.2 g, 75%) as a white solid, mp 105–106 °C. ^{1}H NMR δ 4.78–4.97 and 5.02–5.18 (2dd, 2H, CH_2), 5.50–5.59 (m, 1H, CHCl), 7.25–7.48 (m, 5H Ar), 8.18 (s, 1H, H-3), 8.75 (s, 1H, H-6).

5.1.3. General procedure for 1-(2-chloro-2-phenylethyl)-1H-pyrazolo[3,4-d]pyrimidines 4-amino substituted **1a–l**

To a solution of **5** (2.93 g, 10 mmol) in anhydrous toluene (20 ml), the proper amine (40 mmol) was added, and the reaction mixture was stirred at room temperature for 36 h. After it was extracted with H_2O (10 ml), the organic phase was dried under reduced pressure; the oil residue crystallized by adding absolute ethanol (10 ml) to give products **1a–l**.

1a. White solid, mp 194–195 °C, yield 90%. ^{1}H NMR: δ 0.73–0.87 and 0.98–1.10 (2m, 4H, $2CH_2$), 2.88–3.02 (m, 1H, CH), 4.71–4.83 and 4.95–5.08 (2dd, 2H, CH_2N), 5.53–5.62 (m, 1H, CHCl), 6.68 (br s, 1H, NH, disappears with D_2O), 7.25–7.52 (m, 5H Ar), 8.19 (s, 1H, H-3), 8.32 (s, 1H, H-6). IR cm^{-1}: 3410 (NH).

1b. White solid, mp 142–143 °C, yield 88%. ^{1}H NMR: δ 1.04 (t, J = 7.0, 3H, CH_3), 1.76 (sx, J = 6.0, 2H, CH_2), 3.58 (q, J = 6.0, 2H, CH_2Nprop), 4.68–4.82 and 4.92–5.06 (2dd, 2H, CH_2N), 5.53–5.62 (m, 2H, CHCl), 5.90–6.20 (br s, 1H, NH, disappears with D_2O), 7.25–7.52 (m, 5H Ar), 7.90 (s, 1H, H-3), 8.35 (s, 1H, H-6). IR cm^{-1}: 3415 (NH).

1c. White solid, mp 161–162 °C, yield 75%. ^{1}H NMR: δ 1.95–2.25 (m, 4H, $2CH_2$), 3.78 (t, 4H, $2CH_2$Npyrr), 4.68–4.82 and 4.95–5.06 (2dd, 2H, CH_2N), 5.53–5.62 (m, 1H, CHCl), 7.22–7.52 (m, 5H Ar), 7.92 (s, 1H, H-3), 8.36 (s, 1H, H-6).

1d. White solid, mp 148–149 °C, yield 72%. ^{1}H NMR: δ 1.62–1.85 (m, 6H, $3CH_2$), 3.95–4.07 (m, 4H, $2CH_2$Npip), 4.67–4.82 and 4.92–5.08 (2dd, 2H, CH_2N), 5.53–5.63 (m, 1H, CHCl), 7.25–7.52 (m, 5H Ar), 7.94 (s, 1H, H-3), 8.35 (s, 1H, H-6).

1e. White solid, mp 132–133 °C, yield 70%. ^{1}H NMR: δ 3.79–3.89 and 3.91–4.04 (2m, 8H, $4CH_2$ morph), 4.71–4.83 and 4.94–5.08 (2dd, 2H, CH_2N), 5.53–5.62 (m, 1H, CHCl), 7.24–7.52 (m, 5H Ar), 7.94 (s, 1H, H-3), 8.37 (s, 1H, H-6).

1f. White solid, mp 119–120 °C, yield 65%. ^{1}H NMR: δ 1.24 (t, J = 7.0, 3H, CH_3), 3.56 (q, J = 7.0, 2H, $CH_3\underline{CH_2}O$), 3.68 (t, 2H, $\underline{CH_2}O$), 3.83 (q, 2H, CH_2NH), 4.71–4.83 and 4.95–5.06 (2dd, 2H, CH_2N), 5.52–5.60 (m, 1H, CHCl), 5.80 (br s, 1H, NH, disappears with D_2O), 7.26–7.50 (m, 5H Ar), 7.91 (s, 1H, H-3), 8.39 (s, 1H, H-6). IR cm^{-1}: 3439 (NH).

1g. White solid, mp 157–158 °C, yield 71%. ^{1}H NMR: δ 1.19–1.58, 1.62–1.95 and 2.04–2.20 (3m, 10H, $5CH_2$), 3.95–4.15 (m, 1H, $\underline{CH}NH$), 4.69–4.81 and 4.91–5.08 (2dd, 2H, CH_2N), 5.51–5.61 (m, 1H, CHCl), 7.12–7.62 (m, 5H Ar), 7.86 (s, 1H, H-3), 8.38 (s, 1H, H-6), 8.79 (br s, 1H, NH, disappears with D_2O). IR cm^{-1}: 3403 (NH).

1h. White solid, mp 134–135 °C, yield 55%. ^{1}H NMR: δ 1.55–1.78 and 1.80–2.03 (2m, 8H, 4CH_2), 3.75–4.04 (m, 4H, 2CH_2N), 4.68–4.82 and 4.93–5.08 (2dd, 2H, CH_2N), 5.52–5.65 (m, 1H, CHCl), 7.27–7.57 (m, 5H Ar), 7.88 (s, 1H, H-3), 8.36 (s, 1H, H-6).

1i. White solid, mp 102–103 °C, yield 81%. ^{1}H NMR: δ 3.02 (t, J = 7.0, 2H, CH_2), 3.91 (q, J = 7.0, 2H, $\underline{CH_2}$NH), 4.70–4.85 and 4.95–5.10 (2dd, 2H, CH_2N), 5.51–5.64 (m, 2H, CHCl + NH, 1H disappears with D_2O), 7.22–7.55 (m, 10H Ar), 7.87 (s, 1H, H-3), 8.40 (s, 1H, H-6). IR cm^{-1}: 3420 (NH).

1j. White solid, mp 161–162 °C, yield 85%. ^{1}H NMR: δ 1.30 (t, J = 7.0, 6H, 2CH_3), 3.74 (q, J = 7.0, 4H, 2CH_2), 4.68–4.83 and 4.85–5.06 (2dd, 2H, CH_2N), 5.52–5.63 (m, 1H, CHCl), 7.15–7.58 (m, 5H Ar), 7.85 (s, 1H, H-3), 8.36 (s, 1H, H-6).

1k. White solid, mp 93–94 °C, yield 80%. ^{1}H NMR: δ 0.98 (t, J = 7.0, 3H, CH_3), 1.35–1.59 (sx, J = 7.0, 2H, CH_2), 1.61–1.79 (quintet, J = 7.0, 2H, CH_2), 3.60 (q, J = 7.0, 2H, CH_2NH), 4.70–4.81 and 4.92–5.08 (2dd, 2H, CH_2N), 5.48–5.63 (m, 2H, CHCl + NH, 1H disappears with D_2O), 7.25–7.55 (m, 5H Ar), 7.90 (s, 1H, H-3), 8.35 (s, 1H, H-6). IR cm^{-1}: 3415 (NH).

1l. White solid, mp 158–160 °C, yield 82%. ^{1}H NMR: δ 4.69–4.80 and 4.93–5.07 (2dd, 2H, CH_2N), 4.82–4.88 (m, 2H, $\underline{CH_2}$NH), 5.51–5.59 (m, 2H, CHCl), 7.25–7.51 (m, 10H Ar), 7.85 (s, 1H, H-3), 8.39 (s, 1H, H-6). IR cm^{-1}: 3425 (NH).

*5.1.4. 4-Chloro-1-(2-phenylvinyl)-1*H*-pyrazolo[3,4-*d*]pyrimidine **6***

$POCl_3$ (14 g, 91 mmol) was added to **4** (2.56 g, 10 mmol), the mixture was refluxed for 12 h and then cooled to room temperature

The excess of $POCl_3$ was removed by distillation under reduced pressure. H_2O (20 ml) was carefully added to the residue and the suspension was extracted with $CHCl_3$ (3 × 20 ml). The organic solution was washed with H_2O (10 ml), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude brown oil was purified by column chromatography (Florisil® 100–200 Mesh), using $CHCl_3$ as the eluant to afford the pure product **6** (1.66 g, 65%) as a white solid: mp 139–140 °C. ^{1}H NMR: δ 7.25–7.61 (m, 6H, 5H Ar + CH=), 8.04 (d, J_{trans} = 14.6, 1H, CH=), 8.29 (s, 1H, H-3), 8.85 (s, 1H, H-6). IR cm^{-1} 1658 (C=C).

*5.1.5. General procedure for 1-(2-phenylvinyl)-1*H*-pyrazolo[3,4-*d*]pyrimidines-4-amino substituted **2a–d***

Compound **2a–d** were prepared according to the synthetic sequence described for compounds **1** starting from product **6**

2a. White solid: mp 220–221 °C, yield 85%. ^{1}H NMR: δ 0.77–0.87 (m, 2H, CH_2), 1.00–1.11 (m, 2H, CH_2), 2.92–3.06 (m, 1H, CH cyclopr.), 6.35 (br. s, 1H, NH, disappears with D_2O), 7.20–7.58 (m, 6H, 5H Ar + CH=), 8.07 (d, J_{trans} = 14.6, 1H, CH=), 8.30 (s, 1H, H-3), 8.38 (s, 1H, H-6). IR cm^{-1}: 3615 (NH), 1655 (C=C).

2b .White solid: mp 115–116 °C, yield 84%. ^{1}H NMR: δ 1.23 (t, J = 7.0, 3H, CH_3), 3.56 (q, J = 7.0, 2H, $CH_3\underline{CH_2}O$), 3.70 (t, 2H, CH_2O), 3.83 (q, 2H, $\underline{CH_2}$NH), 6.08 (br s, 1H, NH, disappears with D_2O), 7.20–7.55 (m, 6H, 5H Ar + CH=), 8.01 (s, 1H, H-3), 8.02 (d, J_{trans} = 14.6, 1H, CH=), 8.44 (s, 1H, H-6). IR cm^{-1}: 3440 (NH), 1662 (C=C).

2c. White solid: mp 187–188 °C, yield 80%. ^{1}H NMR: δ 1.12–1.58, 1.60–1.93, 2.08–2.22 (3m, 11H cycloexyl), 4.08 (br s, 1H, NH, disappears with D_2O), 7.20–7.58 (m, 6H, 5H Ar + CH=), 7.99 (s, 1H, H-3), 8.00 (d, J_{trans} = 14.4, 1H, CH=), 8.41 (s, 1H, H-6). IR cm^{-1}: 3405 (NH), 1658 (C=C).

2d. White solid, mp 121–122 °C, yield 72%. ^{1}H NMR: δ 1.53–1.73 (m, 4H, 2CH_2), 1.82–2.08 (m, 4H, 2CH_2), 3.80–4.08 (m, 4H, 2CH_2N), 7.20–7.62 (m, 6H, 5H Ar + CH=), 8.05 (s, 1H, H-3), 8.07 (d, J_{trans} = 14.4, 1H, CH=), 8.43 (s, 1H, H-6). IR cm^{-1}: 1655 (C=C).

2e. White solid, mp 184–185 °C, yield 80%. ^{1}H NMR: δ 4.86 (d, 2H, CH_2), 5.80–5.90 (br s, 1H, NH, disappears with D_2O), 7.20–7.59 (m, 6H, 5H Ar + CH=), 7.99 (s, 1H, H-3), 8.03 (d, J_{trans} = 14.4, 1H, CH=), 8.45 (s, 1H, H-6). IR cm^{-1}: 3430 (NH), 1655 (C=C).

2f. White solid, mp 152–153 °C, yield 78%. ^{1}H NMR: δ 3.04 (t, J = 7.0, 2H, CH_2Ar), 3.93 (q, J = 7.0, 2H, CH_2N), 5.50–5.71(br s, 1H, NH, disappears with D_2O), 7.24–7.60 (m, 6H, 5H Ar + CH=), 8.01 (s, 1H, H-3), 8.05 (d, J_{trans} = 14.0, 1H, CH=), 8.45 (s, 1H, H-6). IR cm^{-1}: 3440 (NH), 1660 (C=C).

6. Pharmacology

6.1. Reagents

Cell culture reagents and materials were from Sigma Chemical. FCS was from Hyclone. Human recombinant EGF were from Calbiochem-Novabiochem. Vybrant MTT cell proliferation assay kit was from Molecular Probe.

6.2. Cytotoxicity

The cytotoxic effect of compound was evaluated by trypan blue exclusion. Cells were stimulated for 1 h at 37 °C with the increasing concentrations (10 nM–10 μM) of the test compound in 1% FCS medium. Cells were then stained with 0.4% trypan blue in phosphate buffer saline (PBS) for 5 min. The number of dead and living cells was counted at the microscope in a blind manner. The percentage of dead cells over the total number of cells was calculated.

6.3. Cell culture

The human epidermoid carcinoma A431 cells were obtained from American Type Culture Collection (Rockville, MD). A431 cells were maintained in culture in DMEM supplemented with 4500 m/l glucose and 10% FCS. Cells were split 1:5 twice a week.

6.4. MTT cell proliferation assay

Cell proliferation was quantified by Vybrant MTT cell proliferation assay kit (Molecular Probe). A431 cells (2.5×10^3) were seeded in 96 multiwell plate with 10% serum for 5 h, stabilised for 16 h and then exposed to tested compounds (50 nM) for 48 h. The medium was then removed and the cells were incubated for 4 h with fresh medium in the presence of 1.2 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Live cells reduce MTT to a strongly pigmented formazan product. After solubilization in DMSO, the absorbance of the formazan was measured with a microplate absorbance reader (Tecan) at 540 nm. The growth inhibitory activity of the compounds studied was compared with the activity of the selective EGFR inhibitor AG1478 at the concentration of 50 nM.

6.5. Western blotting for Src phosphorylation activity

Cells were cultured at a concentration of 20^3 cells/ml in the presence of 10 μM of the compound **1l**, **2a** or the reference compound PP2, 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.

6.6. Western blotting for ERK1-2 kinase activity

A431 Cells (3×10^6) were seeded in a 6 cm plate with 10% serum overnight, starved for 24 h, pretreated with 50 nM of compounds **1l** and **2a** for 1 h and then stimulated with 50 ng/ml EGF for 10 min at 37 °C. As positive control, 100 μM PD 98059, a selective inhibitor of MEKK kinase activity, the kinase upstream to ERK1-2, was used. Proteins were extracted and 20 μg of lysate for each experimental point were subjected to 10% SDS-polyacrilammide gel electrophoresis and transferred to a PDVF membrane. Western blot analysis was performed as described [30]. The mouse anti-phosphop42/p44 antibody 1:1000 (Cell Signaling Technology) or rabbit anti-p42/p44 (1:1000) (Cell Signaling Technology) for the determination of corresponding kinase levels were incubate with the membranes for 18 h. Blots were incubated with HRP-goat anti-mouse antibody or HRP-goat anti-rabbit antibody, respectively (Upstate Biotechnology) and the bands visualized with an enhanced chemiluminescence system (Amersham Pharmacia Biotech). Band intensities were measured using the ScioImage sotware package.

Acknowledgements

Financial support from Italian MIUR (Cofin 2002, grant 2002038577_002) (M.B.), the Italian Association for Cancer Research (AIRC) (M.Z.), and MIUR (Cofin 2001, grant 2001068248_005) (M.Z.) are gratefully acknowledged. M. B. thanks the Merck Research Laboratories for the 2002 Academic Development Program Chemistry Award.

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