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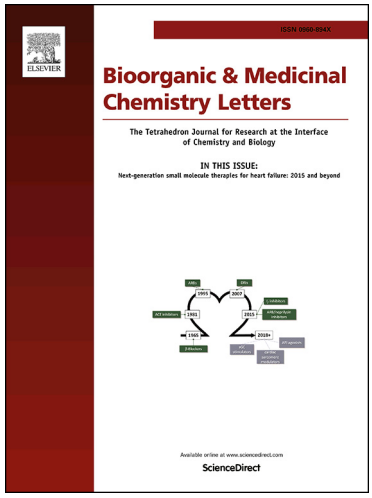

Efficient optimization of pyrazolo[3,4-*d*]pyrimidines derivatives as c-Src kinase inhibitors in neuroblastoma treatment

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Efficient optimization of pyrazolo[3,4-*d*]pyrimidines derivatives as c-Src kinase inhibitors in neuroblastoma treatment

Alessio Molinari [a], Anna Lucia Fallacara [a], Salvatore Di Maria [a], Claudio Zamperini [a], Federica Poggialini [a], Francesca Musumeci [b], Silvia Schenone [b], Adriano Angelucci [c], Alessandro Colapietro [c], Emmanuele Crespan [d], Miroslava Kissova [d], Giovanni Maga [d], Maurizio Botta [a,e,f,*].

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ABSTRACT

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The proto-oncogene c-Src is a non-receptor tyrosine kinase which is involved in the regulation of many cellular processes, such as differentiation, adhesion and survival. c-Src hyperactivation has been detected in many tumors, including neuroblastoma (NB), one of the major causes of death from neoplasia in infancy. We already reported a large family of pyrazolo[3,4-*d*]pyrimidines active as c-Src inhibitors. Interestingly, some of these derivatives resulted also active on SH-SY5Y NB cell line. Herein, starting from our previous Free Energy Perturbation/Monte Carlo calculations, we report an optimization study which led to the identification of a new series of derivatives endowed with nanomolar K_i values against c-Src, interesting antiproliferative activity on SH-SY5Y cells and a suitable ADME profile.

The interest in protein kinases has increased in the last two decades with the entry into the market of the first protein kinase inhibitor in 2001. Many studies identified protein tyrosine kinases (TKs) as attractive targets for therapeutic strategies, highlighting the role of different TKs, including c-Src,[1] in cancer. This non-receptor TK belongs to the Src Family Kinase and has been widely reported to be endowed with a strong proto-oncogenic activity.[2] The over-expression or aberrant activation of c-Src was detected in a large variety of human cancers such as breast,[3] prostate,[4] hepatic cancer[5] and neuroblastoma (NB).[6] Furthermore, c-Src was found to stimulate cell proliferation, migration, invasion as well as angiogenesis.[7] A wide number of compounds demonstrating to be active against c-Src were identified as potential therapeutic options in NB treatment.[8] Dasatinib (Figure 1), a potent dual c-Src/Abl inhibitor, is currently being evaluate in Phase II clinical trials for high-risk NB.[9] NB is a rare tumor affecting the sympathetic nervous system. It is the most common extracranial solid tumor in childhood and is one of the major causes of death from neoplasia in infancy.[10] It may occur anywhere along the sympathetic ganglia. Most primary tumors (65%) occur within the abdomen, with at least half of these arising in the adrenal medulla. Even if the treatment of early stage NB patients has shown favorable outcomes, and a substantial improvement in the treatment of certain subsets of patients has been observed, the long-term survival is still less than 40%.[11] Treatment options for this disease include surgery, chemotherapy and radiotherapy.[12] However, severe side effects and poor prognosis characterize the current treatment strategies, that are still far from satisfactory, highlighting the real need of novel therapeutic approaches.

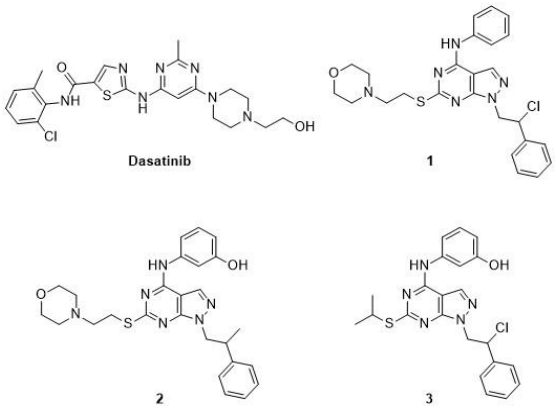

Figure 1. Molecular structures of dasatinib, compounds **1**, **2** and **3**.

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In this context, many pyrazolo[3,4-*d*]pyrimidines reported by us demonstrated to inhibit c-Src, induce apoptosis and reduce cell proliferation in different types of solid tumors,[13] including NB.[14]

These compounds bind the c-Src activated form and act with an ATP-competitive mechanism due to the isosterism of the pyrazolo[3,4-*d*]pyrimidine ring with the adenine scaffold of ATP, the natural phosphorylating agent used by TKs.[15] SAR studies led us to the identification of the hit compound **1**, which showed a favorable profile in terms of both biological activity and ADME properties. No differences were observed in activities of the two enantiomers against the isolated c-Src enzyme. In a previous work, the X-ray structure of the catalytic domain of c-Src bound to compound **1** has been reported: the crystal structure confirmed the predicted binding mode of **1** (PDB code: 4O2P), revealing the presence of two fundamental hydrogen bonds, one between the aniline moiety and the backbone of Thr338, and one between the N2 nitrogen of pyrazole ring and Met341.[16] Starting from these crystallographic data, a first optimization process guided by a Monte Carlo Free Energy Perturbation (MC/FEP) analysis was applied. MC/FEP results allowed the determination of the best sites and substituents able to increase binding affinity toward c-Src, leading to the design and synthesis of compounds **2** and **3** (Figure 1), both bearing a 3-aminophenolic chain at C4 position, and characterized by a potent inhibitory activity against the isolated enzyme (K_i = 0.07 and 0.01 μM, for **2** and **3**, respectively) and an interesting antiproliferative on NB cells.[16,17]

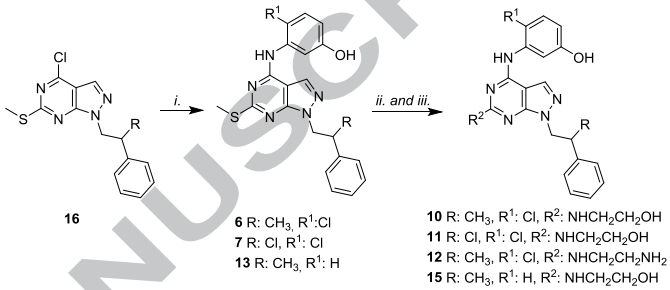
Herein, we report the continuation of our first process of decoration on the aniline ring, exploiting the previously reported data of MC/FEP analysis. Indeed, the computational study suggested a favorable ortho substitution of a hydrogen with a chlorine atom (ΔΔG: 4.8 Kcal/mol).[16] Based on these results, we decided to synthesize a new set of pyrazolo[3,4-*d*]pyrimidines **4-12** bearing in C4 a 3-amino-4-chlorophenolic group. Compounds **13-15**, which do not possess a chlorine atom on the phenyl ring at C4 position, have been also synthesized. Moreover, different chains were introduced at C6 position of the pyrimidine ring to expand the SAR knowledge and to modulate ADME properties. Early assessment of pharmaceutical properties such as solubility, metabolic stability, and permeability has become a key step in the drug discovery process, since it is estimated that 40% of potential drug candidates fail to reach the market due to their poor physicochemical properties.[18] For these reasons, *in vitro* ADME properties were assessed for the synthesized compounds. Then, all compounds were tested *in vitro* assays to determine their c-Src inhibitory activity and cellular viability on SH-SY5Y cells. Furthermore, IC_{50} values were determined for most promising compounds after 72h of treatment, and cell cycle analysis was performed on the most active molecule **11**.

The preparation of 6-thiomethyl derivatives **6,7,13** and 6-amino derivatives **10-12** and **15** is reported in Scheme 1. The opportune 4-chloro derivative **16**[13] was reacted with an excess of 3-aminophenol or 3-amino-4-chlorophenol in EtOH under reflux affording, through an aromatic nucleophilic substitution, compounds **6,7,13**. The latter were oxidized using *m*-chloroperbenzoic acid in dichloromethane to obtain a mixture of sulfone and sulfoxide, which was subsequently treated with ethanolamine or ethylenediamine in DMSO/1-butanol to give derivatives **10-12** and **15**. Preparation of the reactant 3-amino-4-chlorophenol and compounds **4,5,8,9** and **13,14** is reported in the Supporting Information.

All synthesized compounds were initially tested in a cell-free enzymatic assay to evaluate their affinity toward c-Src, as shown in Table 1. The molecules inhibit c-Src with K_i values in the nanomolar range and compounds bearing the 3-amino-4-chlorophenolic group are generally more active than the C4 not halogenated derivatives, as predicted by FEP/MC analysis (for instance, compare 3-amino-4-chlorophenolic derivatives **6**, **9** and **10**, with the correspondent not halogenated compounds **13**, **14** and **15**). Remarkably, the most active compounds **7** and **9** possess a K_i value of 3.5 nM,and show 20 and ~3 fold increased activities than the previous generation derivatives **2** and **3**, respectively.

A wide-ranging SAR evaluation on this class of derivatives suggest that the insertion of a chlorine atom on the ortho position of the aminophenolic ring represents a favorable substitution for increasing the binding affinity towards c-Src, regardless of C6 substitution, and that compounds chlorinated on the N1 side chain generally show a better activity compared to the methyl analogues.

Scheme 1. Synthesis of 6-thiomethyl derivatives **6**,**7**,**13** and 6-amino derivatives **10-12** and **15**



Reagents and conditions: *i*. opportune aminophenol, EtOH, reflux, 8 h; *ii*. *m*CPBA, DCM, 0 °C to room temperature, 1 h; *iii*. NHR_2, DMSO/1-butanol, 90 °C, 8 h.

Even if the developed computational approach afforded compounds endowed with an increased binding affinity towards c-Src compared to hits **1** and **2**, it was essential to evaluate the ability of these new pyrazolo[3,4-*d*]pyrimidines to reach the cytoplasmic district in which our target enzyme is expressed. To this aim, and to estimate water solubility and metabolic stability, *in vitro* ADME properties were assessed. Parallel artificial membrane permeability assay (PAMPA) was performed for all compounds. The inhibitors showed acceptable values of permeability, indicating their potential to get across cellular membranes. The low water solubility is a problem connected to the pyrazolo[3,4-*d*]pyrimidine scaffold. Unfortunately, in the newly synthesized compounds, the introduction of the polar ethanolamine moiety improved the water solubility only for compound **15**, that, on the other hand, is one of the less active inhibitors. However, this issue can be overtaken by preparing suitable inhibitors formulation.[17,19]

All compounds provided good values of metabolic stability in human liver microsomes. The oxidized and dechlorinated rate of metabolite formation has been calculated exploiting a protocol previously applied by us (Table 1).[20]

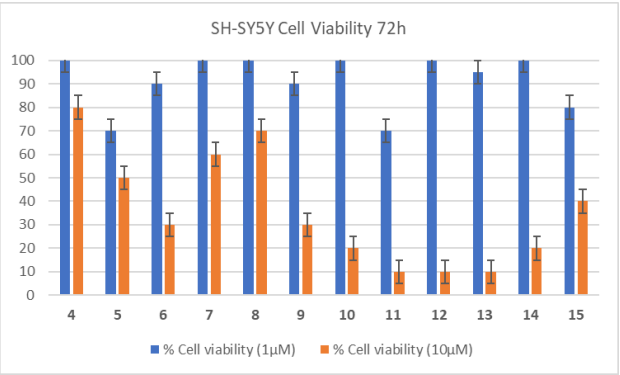


Figure 2. Cell viability rate of derivatives **4**-**15** (1-10 μM) on SH-SY5Y cells at 72 hours after treatment.

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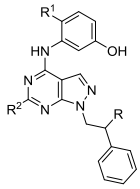
Table 1. Biological data and *in vitro* ADME properties of compounds **2-15**.



				Biological data		*In vitro* ADME			
Cpd	R	R^1	R^2	c-Src K_i (μM)[a]	SY-SH5Y IC_{50}(μM)	Solubility H_2O (μg/mL)[b]	PAMPA Papp 10^{-6} cm/sec	Metab. stability (%)[c]	Metabolite formation (%)[d]
2*	CH_3	H	SCH_2CH_2-4-morpholino	0.07		0.100	6.81	99.0	
3*	Cl	H	$SCH(CH_3)_2$	0.01	8.63±0.7		4.53	93.5	
4	CH_3	Cl	H	0.12					
5	Cl	Cl	H	0.017		0.053	4.39	86.3	M1= 7.6[e] M2= 6.1[f]
6	CH_3	Cl	SCH_3	0.05	6.7±1.9		8.33	92.7	M3= 6.1[g] M3= 1.2[g]
7	Cl	Cl	SCH_3	0.0035			3.23	96.4	M1= 1.08[e] M2= 0.16[f] M3= 0.9[g] M3= 1.35[g]
8	CH_3	Cl	SCH_2CH_2-4-morpholino	0.06		0.031	11.2	97.9	M3= 1.05[g] M3= 1.14[g]
9	Cl	Cl	SCH_2CH_2-4-morpholino	0.0035	6.6±1.8		9.74	96.9	M1= 1.5[e] M2= 0.9[f] M3= 0.7[g]
10	CH_3	Cl	$NHCH_2CH_2OH$	0.023		0.103	0.1	97.9	M3= 2.13[g]
11	Cl	Cl	$NHCH_2CH_2OH$	0.074	3.1±0.3	0.042	2.7	97.2	M3= 2.8[g]
12	CH_3	Cl	$NHCH_2CH_2NH_2$	0.033		0.1	2.2	98.4	M3= 1.6[g]
13	CH_3	H	SCH_3	0.84					
14	Cl	H	SCH_2CH_2-4-morpholino	0.19		0.197	4.44	98.7	M1= 0.5[e] M2= 0.2[f] M3= 0.6[g]
15	CH_3	H	$NHCH_2CH_2OH$	0.3		134.2	0.2	99.4	M3= 0.62[g]

[a]Values are the mean of at least two experiments. [b]Determined by UV/LC-MS. [c]Metabolic stability was assessed using human liver microsomes. [d]Protocol applied[20]. [e]M1= M-HCl+O (-36+16); [f]M2= M-Cl+OH (-35+17); [g]M3= M+OH (+16). *Compounds previously published[16]. Empty cell means not determined.

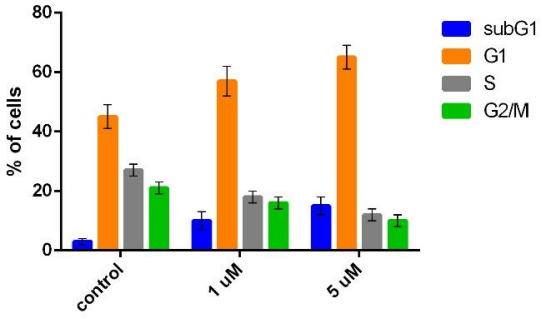


Figure 3. Analysis of cell cycle distribution of SH-SY5Y cells after treatment with 1 and 5 μM of **11**. Cells status was investigated by cytofluorimetric analysis of DNA content, and results were expressed as percentage of cells in each phase of cell cycle respect to total viable cells.

Finally, the inhibition of SH-SY5Y NB cell viability was assessed for all compounds at doses of 1 μM and 10 μM after 72 hours of treatment (Figure 2). Then compounds **6**, **9**, and **11**, showing the best percentage of cell inhibition, were selected for IC_{50} determination. Results, shown in Table 1, indicate an increasing efficacy, especially for compound **11** (IC_{50} = 3.1 μM) which is about three times more active than the reference compound **3** (IC_{50} = 8.63 μM). In order to better characterize the biological effect of **11**, cell cycle analysis was performed. SH-SY5Y cells were treated with increasing concentration of compound (1 and 5 μM), and the percentage of cells in each phase was evaluated by cytofluorimetric analysis of DNA content. Figure 3 reveals a dose-dependent accumulation of cells in G1 phase of cell cycle, according to the results obtained for similar pyrazolo[3,4-*d*]pyrimidine derivatives.[16]

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In summary, starting from in-house crystallographic data and earlier identified compounds **2** and **3**, we applied a previously performed MC/FEP analysis to design a promising family of Src inhibitors. Then, the compounds, characterized by a pyrazolo[3,4-*d*]pyrimidine scaffold and decorated at C4 with the 3-amino-4-chlorophenolic ring, have been synthesized. A chemical variability has been introduced at C6 position to expand the SAR knowledge. All compounds have been tested against c-Src and showed K_i values in the nanomolar range. Interestingly, the most active derivatives **7** and **9** possess a K_i value of 3.5 nM. Then, since c-Src is located into the cytoplasm of cells, the ability of compounds to cross cell membranes to bind their target was assessed by PAMPA. This assay showed proper values of permeability for the majority of selected compounds. Finally, a cell viability assay on SH-SY5Y NB cell line has been performed and IC_{50} has been calculated for the three most active derivatives. Compound **11** resulted about three times more active than the reference **3** (IC_{50} values of 3.1 and 8.63 μM, respectively). Despite this remarkably activity on SH-SY5Y cells, **11** didn't show an optimal PAMPA value, suggesting a possible involvement of an active carrier to the transport into the cell. This hypothesis will be evaluated in our further studies.

Overall, this optimization study led to the identification of promising candidates for NB treatment, which will be further investigated in preclinical studies to assess their pharmacokinetic/pharmacodynamic profile.

Acknowledgments

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Highlights:

- Synthesis of pyrazolo[3,4-*d*]pyrimidines derivatives endowed with nM K_i values.
- Best compound exerted good cytotoxicity activity against neuroblastoma cells.
- Optimization process based on SAR study and ADME profile.

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Graphical Abstract

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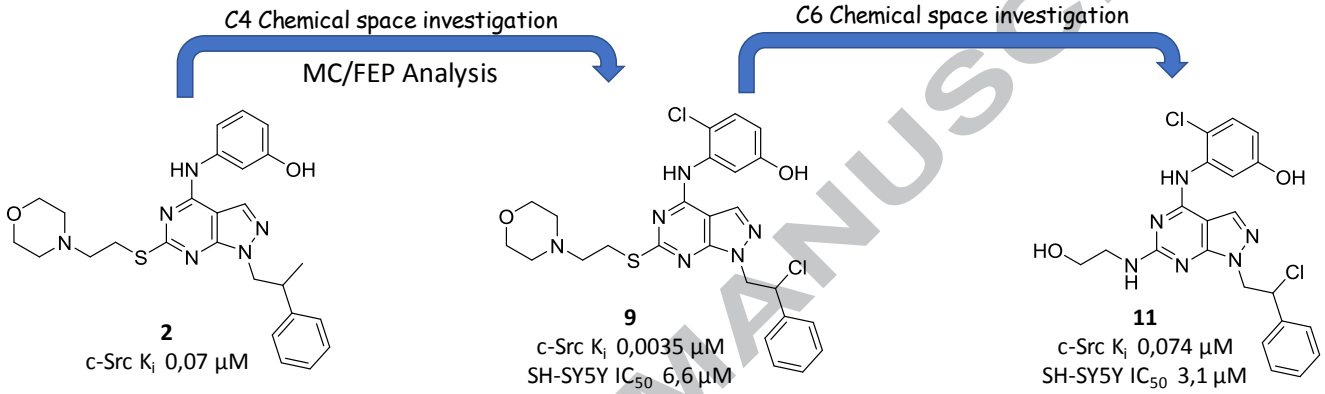
Efficient optimization of pyrazolo[3,4-*d*]pyrimidines derivatives as c-Src kinase inhibitors in neuroblastama treatment

Alessio Molinari[a], Anna Lucia Fallacara[a], Salvatore Di Maria[a], Claudio Zamperini[a], Federica Poggialini[a], Francesca Musumeci[b], Silvia Schenone[b], Adriano Angelucci[c], Alessandro Colapietro[c], Emmanuele Crespan[d], Miroslava Kissova[d], Giovanni Maga[d], Maurizio Botta[a,e,f,*].



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