Bioorganic & Medicinal Chemistry Letters 18 (2008) 6206–6209

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

The design, synthesis and biological evaluation of 7-alkoxy-4-heteroarylamino-3-cyanoquinolines as dual inhibitors of c-Src and iNOS

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ARTICLE INFO

Article history:
Received 22 March 2008
Revised 11 September 2008
Accepted 1 October 2008
Available online 5 October 2008

Keywords:
c-Src
iNos
Dual inhibitor
Anti-cancer

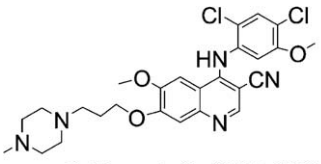
ABSTRACT

Because both *c*-Src and iNOS are key regulatory enzymes in tumorigenesis, a new series of 4-heterocycle amine-3-quinolinecarbonitriles as potent dual inhibitors of both enzymes were designed, synthesized and evaluated as multiple targets agents in cancer therapy. All compounds were evaluated by two related enzyme inhibition assays and an anti-proliferation assay in vitro. The results showed that most compounds inhibited *c*-Src and iNOS well. The best compound **8** inhibited both enzymes with the IC_{50} values of 34.8 nM and 26.7 μM. Several compounds also showed moderate anti-proliferation at 10 μM against colon and liver cancer cell lines.

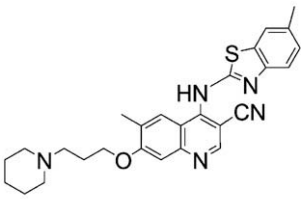
Src family kinases (SFKs) belong to the non-receptor protein tyrosine kinases (PTKs), and they transfer the γ-phosphoryl group from ATP to the hydroxyl group of specific tyrosine (Tyr) residues on substrate proteins.[1] SFKs express predominantly in differentiated cell types and regulate many fundamental cellular processes, including cell growth, differentiation, cell shape, migration, survival, and specialized cell signals.[2] They were also found to be over-activated in various human cancers, including colon,[3] pancreatic,[4] lung,[5] and ovarian cancers.[6] These observations have prompted many investigations of *c*-Src inhibitors aimed toward curing cancer, for example bosutinib (SKI-606, **1**) of Wyeth has been evaluating in a Phase III clinical trial in the treatment of CML.[7,8] CPU-Y020 (2) is another efficient Src inhibitor reported by our group which is under preclinical evaluations (Fig. 1).[9]

iNOS is the inducible hypotype of nitric oxide synthase (NOS), and is expressed in a wide array of cells and tissues, for example, macrophages,[10] Kupffer cells, hepatocytes,[11] neutrophils, pulmonary epithelium, colonic epithelium, and vasculature.[12] Unfortunately, iNOS also expresses highly in various neoplastic diseases.[13] The role of iNOS during tumor development is highly complex and remains obscure. However, there is no doubt that the tumor metastatic activity, host defense mechanisms, and the level of differentiation are all correlated with over-expressed iNOS.[14]

The attempt to suppress two distinct cancer related-enzymes with a single agent was an attractive idea. It is reasonable that the dual inhibitors of both *c*-Src and iNOS were more effective for blocking multiple signaling pathways in cancer intervention



1, Bosutinib (SKI-606)



2, CPU-Y020

Figure 1. The structures of bosutinib (SKI-606, **1**) and CPU-Y020 (**2**).

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doi:10.1016/j.bmcl.2008.10.006

compared with the single inhibitors and this would be beneficial to overcome drug resistance. With the goal of identifying such a new anti-cancer strategy, a new class of dual inhibitors of *c*-Src and iNOS were designed following scaffold hopping principles.[9]

We chose the potent *c*-Src inhibitor template 4-aniline-3-quinolinecarbonitrile as the leading scaffold, and made functional hopping from the conventional 4-aniline group to some multifunctional heterocycle amines, like 2-aminothiazole and its derivatives, etc (**3–6**, Fig. 2), which had been demonstrated as selective inhibitors against iNOS previously.[15–17] In order to retain both the 4-aniline substituent as the molecule heads of Src inhibitors and 2-aminothiazole's structural characters, 2,6-diaminobenzothiazole and 2,5-diaminobenzimidazole were chosen as the hopped groups that were introduced into the leading scaffold (Fig. 3). The relatively more active 6′-amino group was connected to the 4-position of 3-quinolinecarbonitrile and the 2′-amino group was directed out of the molecule.

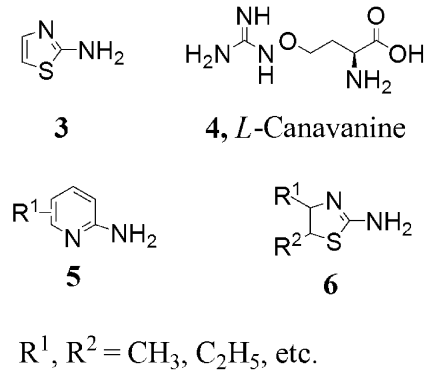


Figure 2. The selective iNOS inhibitors and scaffolds.

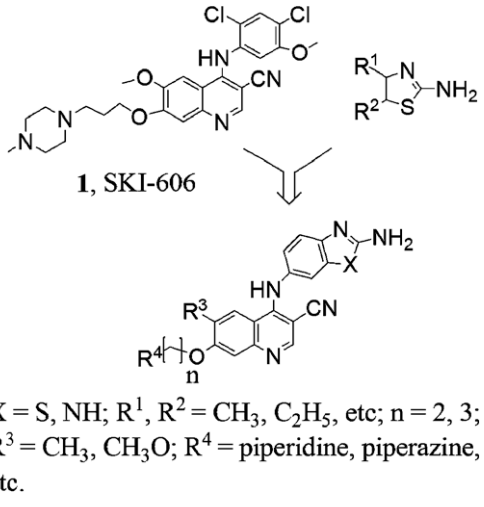


Figure 3. The design of the dual inhibitors of Src and iNOS.

The preparation of the designed compounds was carried out smoothly. 2,6-Diaminobenzothiazole **7a** and 2,5-diaminobenzimidazole **7b** were synthesized by selective nitration at 6-position of 2-aminobenzothiazole and 5-position of 2-aminobenzimidazole,[18] and the nitro group intermediates were reduced by iron powder (Scheme 1) to achieve the heterocycle diamine **7**. The 3-quinolinecarbonitrile core was prepared using the modified cyclic method as described by us previously.[9] Then the two main fragments were connected in refluxing isopropanol. After that, the protective group was removed and an alkaline side chain at C-7 position were introduced (Scheme 2).

The compounds were tested in two related enzyme inhibition assays to determine their inhibition activities on Src and iNOS: a Protein Tyrosine Kinase Inhibition Assay (*c*-Src, and Tyrosine Kinase Assay Kit, Invitrogen) and a iNOS Inhibition Assay (mouse macrophage ANA-1, and Nitric Oxide Synthase Assay Kit, Beyotime China) were carried out following the reported methods.[19–21]

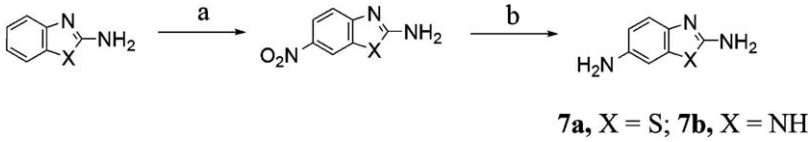
The IC_{50} values of compounds **8–18** (Table 1) clearly showed that most compounds inhibited *c*-Src and iNOS with the IC_{50} values range in the domains of 20–80 nM and 10–50 μM, respectively. However, the Src inhibition activities turned to be less potent than SKI-606 (**1**, Table 1)[7] for all their IC_{50} values were more than 20 nM. The iNOS inhibition activities were similar to those typical iNOS inhibitors, such as 2-aminothiazole (**3**)[16] and L-canavanine (**4**, Table 1).[17] Most of the IC_{50} values towards iNOS ranged 10–50 μM.

When a 2′,6′-diaminobenzothiazole group was at C-4 position, and a methoxyl group was at C-6 position, compound **18** showed the best Src inhibition activity with an IC_{50} of 20.7 nM while its iNOS inhibition activity was only 105 μM. But when the C-6 methoxyl group was changed into a methyl group, the compound **11**'s Src inhibition activity decreased to 129 nM while its iNOS inhibition activity increased to 17.3 μM. When n equaled 3 and the terminal alkaline group piperazine was changed to 3′,5′-dimethylpiperazine, compound **13** showed the IC_{50} values of 36.6 nM and 89.1 μM towards Src and iNOS.

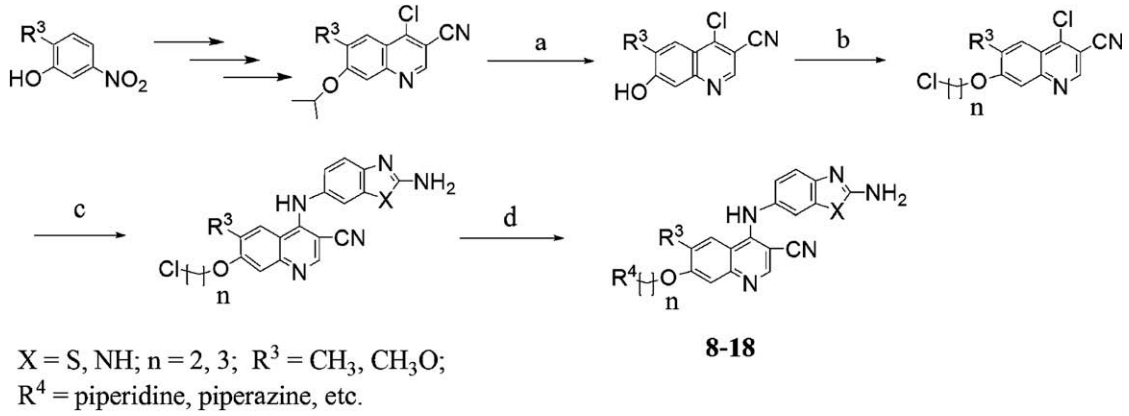
Other C-7 alkaline substituted compounds like compounds **9**, **10** and **12**'s Src inhibition activities were 3- to 5-fold weaker than compound **13** while their iNOS inhibition activities were more potent than compound **13**. When n equaled 2 and the terminal alkaline group was piperidine, compound **8** showed IC_{50} values of 34.8 nM and 26.7 μM towards the two enzymes and that was better compound **13**.

The changed 4-heteroaromatic amines of these compounds affected the inhibition activity towards Src kinase. When the 4-anilino group was changed into 2′,5′-diaminobenzoimidazole, compound **16** and **17** only showed weaker inhibition against Src kinase with IC_{50} values of 143 and 159 nM. But it was interesting that compounds **16** and **17** showed better inhibition activities against iNOS with the IC_{50} values of 13.2 and 24.8 μM compared with the 2′,6′-diaminobenzothiazole substituted compounds.

In general, most compounds could inhibit both enzymes in vitro screening, while compound **8** showed the best activities against both enzymes with the IC_{50} values of 34.8 nM and 26.7 μM, respectively. The new compounds were more potent against Src than their activities against iNOS, as indicated by the IC_{50} values in

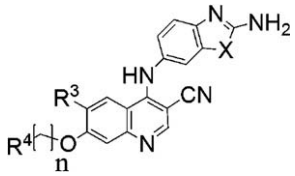


Scheme 1. Reagents: (a) HNO_3, H_2SO_4; (b) Fe, HCl, ethanol.



Scheme 2. Reagents and conditions: (a) $AlCl_3$, CH_2Cl_2, 0–5 °C; (b) 1-bromo-3-chloropropane or 1-bromo-2-chloropropane, K_2CO_3, DMF; (c) 2,6-diaminobenzothiazole or 2,5-diaminobenzimidazole, isopropanol, pyridine hydrochloride, reflux; (d) *N*-methylpiperazine, K_2CO_3, DMF or similar amines and reaction conditions.

Table 1
The structures of compounds **8–18** and their enzyme inhibition activities against *c*-Src and iNOS



Compound	X	R^3	*n*	R^4	IC_{50}[a] (nM) *c*-Src	IC_{50}[a,b] (μM) iNOS
8	S	Me	2	Piperidine	34.8	26.7
9	S	Me	3	*N*-methylpiperazine	50.6	37.1
10	S	Me	3	Morpholine	81.1	31.8
11	S	Me	3	Piperazine	129	17.3
12	S	Me	3	4-Methylpiperidine	53.4	12.7
13	S	Me	3	3,5-Dimethylpiperazine	36.6	89.1
14	S	Me	3	Diethylamine	42.0	46.8
15	S	Me	3	3,5-Dimethylpiperidine	267	10.5
16	NH	Me	3	Dimethylamine	143	13.2
17	NH	Me	3	Pyrrolidine	159	24.8
18	S	MeO	3	Piperazine	20.7	105
1 (Bosutinib)					1.8[c]	–
2 (CPU-Y020)					15.4	313
3 (2-Aminothiazole)					–	20[d]
4 (L-Canavanine)					–	65[d]

[a] The IC_{50} values were means of triplet experiments, and the variabilities were within 10%.
[b] iNOS of mouse macrophage ANA-1.
[c] According to Ref. 7, the IC_{50} value of bosutinib for Src was 1.2 nM in an ELISA assay.
[d] According to Refs. 16 and 17, the IC_{50} values of 2-aminothiazole and L-canavanine for iNOS from mouse macrophage ANA-1 were 18 and 60 μM, respectively.

the nanomolar range. This result may be explained as that the scaffold of the series was mainly designed to fit in the hinge region near the ATP binding site in Src kinase domain, and what is more, the agents only possess some inhibition groups against iNOS. Thus, there were no breakthrough findings with respect to iNOS inhibitors.

The anticancer activities of these compounds were also evaluated by a SRB (Sulforhodamine B) anti-proliferation assay.[22] However, there were no predominant compounds discovered according to the assay results. All compounds were less active than SKI-606 in the anti-proliferation screening assay according to the percentages of survival cancer cells at 10 μM treated by the compounds. Only a few compounds, such as compounds **8**, **9** and **15**, exhibited moderate inhibition activities towards the colon cancer HT-29 and liver cancer HepG2 cell lines at 10 μM in vitro assays (Table 2).

Table 2
The percentages of survival cells at 10 μM treated by compounds **8, 9, 15** and bosutinib (**1**) against 5 different human cancer cell lines

Compound	Percentage of survival (%)				
	Lung A549	Stomach AGS	Liver HepG2	Colon HT-29	Prostate PC-3
8	55.39	61.01	37.10	42.70	77.68
9	64.14	68.13	40.45	31.60	59.45
15	53.64	65.46	42.16	60.59	62.99
1 (Bosutinib)	9.32	3.80	9.20	2.84	7.81

The discrepancy between enzymatic and cell activity is likely attributed to the poor cell permeabilities of the compounds. The 2-aminobenzothiazole and 2-aminobenzimidazole at the heads of the molecules act as the 4-heteraromatic amine groups in the designed scaffold exhibit both acidic and alkaline properties in solution, and could be easily protonated in the artificial environment of the cell culture medium, which imitates the natural intercellular substance of the cells. The molecular polarizations of these compounds would be enhanced, and with the extra positive charges on the molecules, it would be difficult for the agents to permeate across the cell membranes. As a result, the anti-proliferation activities of the compounds decreased though they showed moderate enzyme inhibition activities towards both Src and iNOS.

In conclusion, a series of dual inhibitors of Src kinase and iNOS as anticancer agents were proposed in this study. The designed compounds were synthesized and evaluated by related biological assays. Most of these compounds were effective towards both enzymes. Compound **8** showed the best activities against both enzymes with the IC_{50} values of 34.8 nM and 26.7 μM agaisnt Src and iNOS, respectively. Compounds **8**, **9** and **15** also showed moderate inhibition activities on colon and liver cancer cells in vitro screening. However, the anti-proliferation activities of these compounds decreased as compared with SKI-606. Some further modifications with different 4-heteroaromatic amines are underway.

Acknowledgment

Acknowledgments are made to National Natural Science Foundation of China (NSFC Grant No. 30371676) for the financial supports.

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