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Anil Kumar

Birla Institute of Technology & Science

Guofeng Ye

Shandong University

Xianfeng Gu

SUNY Stony Brook

Yuehao Wang

University of Rhode Island

Gongqin Sun

University of Jinan

See next page for additional authors

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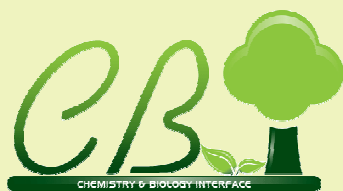
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Authors

Anil Kumar, Guofeng Ye, Xianfeng Gu, Yuehao Wang, Gongqin Sun, and Keykavous Parang



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Research Paper

Synthesis of Pyrazolo[3,4-*d*]pyrimidine Derivatives and Evaluation of their Src Kinase Inhibitory Activities

Anil Kumar,^{a,*} Guofeng Ye,^b Xianfeng Gu,^b Yuehao Wang,^c Gongqin Sun,^c Keykavous Parang^{b,*}

^a Department of Chemistry, Birla Institute of Technology and Science, Pilani 333031; India

^b Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, RI, 02881, USA

^c Department of Cell and Molecular Biology, University of Rhode Island, Kingston RI 02881

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Abstract: A series of pyrazolo[3,4-*d*]pyrimidine derivatives was synthesized and evaluated for the Src kinase inhibitory activities. Compound **6e** and **10c** exhibited inhibition of Src kinase with an IC₅₀ value of 5.6 and 5.1 μM, respectively. Hydroxamate derivative **15a** was found to be a metal-mediated inhibitor for human Csk with an IC₅₀ value of 2.0 μM showing 56-fold selectivity over Src kinase inhibition.

Introduction

The Src family of tyrosine kinases (SFKs) is comprised of nine tyrosine kinases *viz.* Src, Lck, Fyn, Yes, Hck, Blk, Fgr, Lyn and Yrk. SFKs have important role in signaling pathways that control a diverse spectrum of biological activities, such as cell division, growth factor signaling, differentiation, survival, adhesion, migration, and invasion.¹ Src tyrosine kinase expression is frequently elevated in a number of tumors including colon, breast, prostate, lung, ovary, and pancreas compared with the adjacent normal tissues. Src kinase is a key modulator of cancer cell invasion and metastasis.² Src also plays a crucial role in osteoclast function where key function of the enzyme is to

promote the rapid assembly and disassembly of the podosomes, and regulation of vesicle transport and secretion of proteases.³ Thus, Src has become an attractive therapeutic target for the design of new therapeutic agents.⁴ A number of Src kinase inhibitors have shown potential as both anti-proliferative and anti-invasive agents in preclinical studies in different solid tumors.⁵ Phenylpyrazolopyrimidine derivatives (PP1 and PP2) (Figure 1) have been reported as SFK inhibitors.⁶ We evaluated a series of 3-phenylpyrazolopyrimidine derivatives and 1,4-disubstituted triazoles for their c-Src kinase inhibitory activity.⁷

In continuation of our efforts towards the development of c-Src kinase inhibitors,⁸ herein we describe synthesis of novel pyrazolo[3,4-*d*]pyrimidine derivatives by structural modification of pyrazolo[3,4-

Corresponding Author* E-mail: anilkumar@pilani.bits-pilani.ac.in

d]pyrimidine scaffold (Figure 1) and evaluation of their Src tyrosine kinase inhibitory activity. The purpose of this study was to establish the structure-activity relationship to optimize pyrazolo[3,4-*d*]pyrimidine derivatives as potential Src kinase inhibitors.

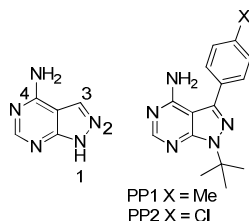
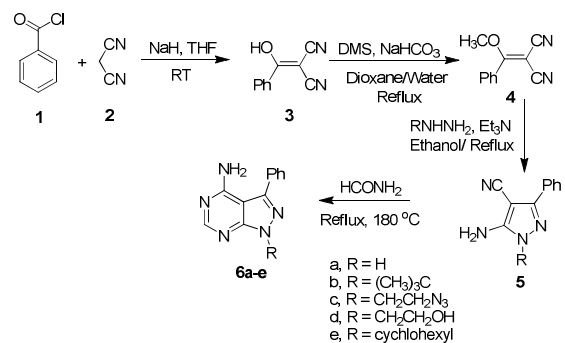


Figure 1. Pyrazolo[3,4-*d*]pyrimidine scaffold, PP1, and PP2 as Src kinase inhibitors.

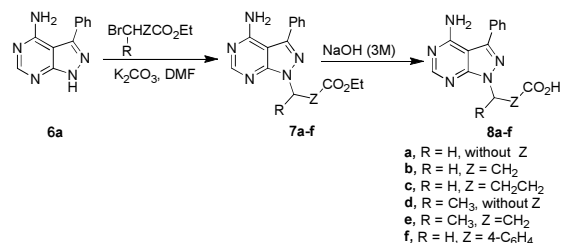
Results and Discussion

Chemistry. The synthesis of *N*¹-substituted 3-phenylpyrazolo[3,4-*d*]pyrimidine derivatives (**6a-e**) was accomplished by sequence of reactions as shown in Scheme 1. Reaction of benzoyl chloride (**1**) with malononitrile (**2**) in the presence of sodium hydride in THF gave benzoylmalononitrile (**3**), which on methylation with dimethylsulfate (DMS, Me₂SO₄) in the presence of NaHCO₃ in dioxane afforded 2-(methoxyphenylmethylene)malononitrile (**4**). Reaction of **4** with different hydrazines *viz.* NH₂NH₂·HCl, *tert*-butylhydrazine, 2-azidoethyl hydrazine, 2-hydroxyethyl hydrazine, and cyclohexyl hydrazine in the presence of triethylamine (Et₃N) afforded corresponding 5-amino-3-phenyl-pyrazolo-4-carbonitriles (**5a-e**). Reaction of **5a-e** with formamide at 180 °C for 24 h afforded pyrazolopyrimidines (**6a-e**).



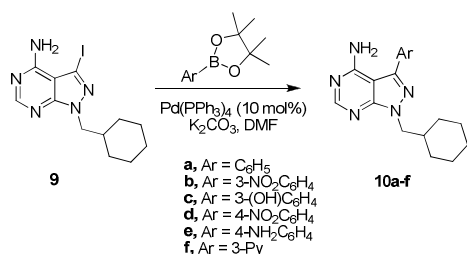
Scheme 1. Synthesis of *N*¹-substituted 3-phenylpyrazolo[3,4-*d*]pyrimidine derivatives.

*N*¹-Carboxylic acid ester pyrazolo[3,4-*d*]pyrimidine derivatives (**7a-f**) were synthesized from the reaction of 3-phenyl-1H-pyrazolo[3,4-*d*]pyrimidine-4-ylamine (**6a**) with ethyl 2-bromoacetate, ethyl 3-bromopropionate, ethyl 4-bromobutyrate, ethyl 2-bromopropionate, ethyl 3-bromobutyrate, or ethyl 4-(bromomethyl)benzoate in the presence of anhyd. K₂CO₃ in dry *N,N*-dimethylformamide (DMF). Only the *N*¹-endocyclic amino group of pyrazolopyrimidine reacted with bromo substituted analogues. Unprotected *N*⁴-exocyclic amine was not reactive under this reaction condition as shown previously.⁹ Ester hydrolysis with sodium hydroxide afforded the corresponding *N*¹-substituted carboxylic acid derivatives of 3-phenylpyrazolo[3,4-*d*]pyrimidine (**8a-f**, Scheme 2). All the synthesized compounds were characterized by NMR and mass spectroscopy.⁷



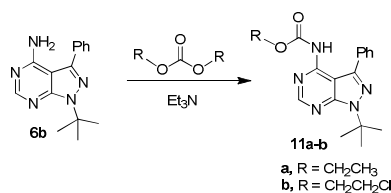
Scheme 2. Synthesis of *N*¹-carboxylic acid ester pyrazolo[3,4-*d*]pyrimidine derivatives.

3-Aryl substituted pyrazolo[3,4-*d*]pyrimidine derivatives containing NH₂, OH, NO₂, and other functional groups at *para* and *meta* positions of the 3-phenyl group (**10a-f**) were synthesized using Suzuki reaction of 1-(cyclohexylmethyl)-3-iodo-1H-pyrazolo-[3,4-*d*]pyrimidin-4-amine (**9**) with different boronic acid derivatives in the presence of Pd(PPh₃)₄ (Scheme 3).



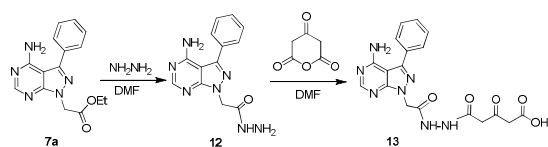
Scheme 3. Synthesis of 3-substituted pyrazolo[3,4-*d*]pyrimidine derivatives.

Modification of NH₂ group at C-4 position of pyrazolo[3,4-*d*]pyrimidine was achieved by the reaction of **6b** with diethyl carbonate and *bis*(2-chloroethyl)carbonate to give **11a-b** (Scheme 4).



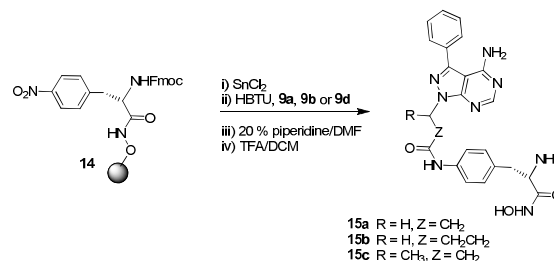
Scheme 4. Synthesis of 4-amino substituted analogs of pyrazolo[3,4-*d*]pyrimidine.

Synthesis of hydrazone derivative (**12**) was achieved by the reaction of **7a** with hydrazine hydrate in DMF. Compound **12** underwent reaction with 2H-pyran-2,4,6-(3H,5H)-trione to afford **13** (Scheme 5).



Scheme 5. Synthesis of hydrazone derivatives (**12** and **13**).

The carboxylic acid derivatives (**8a**, **8b** and **8d**) were used for the synthesis of the corresponding hydroxamates (**15a-c**) using solid-phase synthesis employing Fmoc-based chemistry (Scheme 6). Fmoc-4-nitrophenyl alanine was coupled with hydroxylamine Wang resin in the presence of HBTU and *N*-methylmorpholine (NMM) to yield polymer-bound hydroxamate derivative (**14**). The nitro group of **14** was reduced with SnCl₂ and resulting amino group was coupled with **8a**, **8b** and **8d** using HBTU as a coupling agent in NMM. The deprotection of the Fmoc group with 20% piperidine in DMF followed by treatment with TFA afforded **15a-c**.



Scheme 6. Synthesis of hydroxamate derivatives of 3-phenylpyrazolopyrimidine.

Tyrosine Kinase Inhibitory Activity. Protein tyrosine kinase activities of all the compounds were determined by measuring phosphorylation of poly-E₄Y (average MW: 35kD) by the kinase using a standard radiometric PTK activity assay as described previously.¹⁰ The radioactive kinase assay was performed using active p60-c-Src (Upstate Cell Signaling, Cat. No. 14-117) and polyE₄Y as an artificial substrate. The assays were done in duplicate and repeated at least three times. Control reactions lacking polyE₄Y were included for each enzyme concentration to correct for any nonpolyE₄Y-specific phosphorylation. The percentage of inhibition was plotted as a function of the compound. The concentration and IC₅₀ values (the concentration of a compound that caused

50% inhibition) were obtained from such a plot. The inhibitory potencies of the synthesized compounds against active c-Src are given in Table 1.

Table 1. Src kinase inhibitory activity.

Comp. No.	IC ₅₀ (μM) ^a	Comp. No.	IC ₅₀ (μM) ^a
6a	- ^b	8e	71
Control (6b)	0.47	8f	- ^b
6c	6.2	10a	6.5
6d	180	10b	260
6e	5.6	10c	5.1
7a	160	10d	- ^b
7b	- ^b	10e	28
7c	16	10f	56
7d	11	11a	38
7e	12	11b	690
7f	- ^b	12	275
8a	250	13	280
8b	220	15a	112
8c	293	15b	46
8d	- ^b	15c	155

^aIC₅₀ is the concentration required to produce 50% inhibition in the phosphorylation of polyE₄Y by active c-Src (average of triplicate experiments).

^bNo inhibition was observed up to 500 μM.

Compound **6b** (IC₅₀ = 0.47 μM), a close analog of PP1 lacking one methyl group, was used as a control and showed potent Src kinase inhibitory activity with an IC₅₀ value of 0.47 μM. 3-Phenylpyrazolo[3,4-*d*]pyrimidine derivatives substituted at N¹ endocyclic amine with a carboxylic acid (**8a-f**, **13**), carboxylic acid esters (**7a-f**), and hydroxamate (**15a-c**) exhibited inhibitory potencies with IC₅₀ values of 12 to >500 μM. 3-Phenylpyrazolopyrimidine derivative **8a** substituted with an alkyl carboxylic acid at N¹-endocyclic amine, exhibited weak inhibitory potency (IC₅₀ = 250 μM). A similar pattern was observed by the attachment of alkyl carboxylic acids

containing a longer alkyl chain, as shown in **8b-d** and **8f**.

Molecular modeling studies indicate that the carboxylic acids in these compounds have unfavorable electrostatic interaction with the side chain of Asp386 (Figure 2). This residue is in the proximity of the cavity occupied by the ATP binding site inhibitors. When the methyl group was incorporated on the β-carbon of the side chain of alkyl carboxylic acid in **8e**, a higher inhibitory potency (IC₅₀ = 71 μM) was resulted (**8e** versus **8b**); this may be due to the enhanced hydrophobic interaction by the methyl group with the hydrophobic cavity. Inhibitory potency of the ethyl ester derivatives **7a** and **7c-e** were improved slightly compared to their acid analogues **8a** and **8c-e**, suggesting that the short carboxylic acids as *N*₁-substituents have unfavorable electrostatic interaction with Asp386 that is reduced in ester conjugates.



Figure 2. Binding of a 3-phenylpyrazolopyrimidine to Src (1FMK) (10) and its proximity to aspartic acids located in the kinase domain.

Among 3-substituted pyrazolo[3,4-*d*]pyrimidine derivatives, 3-(3'-hydroxyphenyl)-N¹-methylcyclohexyl pyrazolo[3,4-*d*]pyrimidine (**10c**) exhibited significantly improved inhibitory potency (IC₅₀ = 5.1 μM). Similarly 3-phenyl-N¹-cyclohexyl derivative **6e** exhibited inhibition of Src kinase with an IC₅₀ value of 5.6 μM.

These results indicate that the introduction of a hydrophobic group at N^1 -position and a small functional group on the phenyl ring capable of hydrogen bonding, such as OH, as shown in compounds **6e** and **10c**, respectively, improve the inhibitory potency for c-Src kinase. This may be due to the rigid structure of cyclohexyl group in **6e** that fits well in the hydrophobic pocket of c-Src. Structure-activity relationship studies suggested that the incorporation of bulky groups at N^1 and N^4 -amino groups are not tolerated as shown in compounds **15a-c** and **11a-b**. It has been previously reported that tethering bulky chemical groups to PP1 through its exocyclic amine (N^4) yields weak inhibitors¹¹, owing to the unfavorable interactions¹² with a structurally conserved amino acid residue 338 that contains a bulky side chain in all known eukaryotic protein kinases. Substitution at N^4 in 3-phenyl-pyrazolopyrimidine derivatives with esters or larger groups in **11a** and **11b** significantly reduced the inhibitory potency, therefore confirming the previous results.

We have previously shown that hydroxamates can act as metal-mediated inhibitors of Csk.¹³ Thus, we evaluated hydroxamate compounds **15a**, **15b** and **15c** for inhibition of human Csk. To determine the inhibition of Csk by hydroxamate compounds, 0.2 mM CoCl_2 was also added to the reaction mixture. It was found that **15a**, **15b**, and **15c** act as potent metal-mediated inhibitors with IC_{50} values of 2.0, 2.7 and 5.0 μM , respectively. Thus, conjugating hydroxamate on the phenyl ring in **15a-c** enhanced the inhibitory potency approximately 2- to 4.75-fold higher when compared to tyrosine hydroxamate (IC_{50} = 9.5 μM). Moreover, while compound **15a** was a poor inhibitor of c-Src kinase (IC_{50} = 112 μM), it showed 56-fold selectivity towards Csk (IC_{50} = 2.0 μM) in the presence of CoCl_2 . These compounds can be further

optimized to develop selective inhibitors of Csk.

Conclusions

In conclusion, we have synthesized a series of pyrazolo[3,4-*d*]pyrimidine derivatives and evaluated them for the c-Src kinase inhibitory activities. Among all the compounds, **6b** (IC_{50} = 0.47 μM) was the most potent derivative. Compounds **6e**, **10a**, and **10c** exhibited modest inhibition of Src kinase with IC_{50} values of 5.1-6.5 μM , respectively. Structure-activity relationship studies suggested that the incorporation of bulky groups at N^1 and N^4 -amino groups were not tolerated. The data provides insight for further optimization of these compounds by incorporation of smaller hydrophobic groups at N^1 or hydrophobic aryl groups with hydrogen bonding substituents at C_3 -position of pyrazolopyrimidine scaffold. Hydroxamate derivatives of pyrazolo[3,4-*d*]pyrimidine were found to be selective inhibitors of Csk. Taken together, these results suggest that further exploration and optimization of functional groups and their positioning in pyrazolo[3,4-*d*]pyrimidine derivatives could lead to more potent Src inhibitors.

Acknowledgements

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