Journal of Enzyme Inhibition and Medicinal Chemistry

ISSN: 1475-6366 (Print) 1475-6374 (Online) Journal homepage: informahealthcare.com/journals/ienz20

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To cite this article: Sebla Dincer, Kadir Taylan Cetin, Arzu Onay-Besikci & Süreyya Ölgen (2013) Synthesis, biological evaluation and docking studies of new pyrrolo[2,3-*d*] pyrimidine derivatives as Src family-selective tyrosine kinase inhibitors, Journal of Enzyme Inhibition and Medicinal Chemistry, 28:5, 1080-1087, DOI: 10.3109/14756366.2012.715288

To link to this article: https://doi.org/10.3109/14756366.2012.715288

Published online: 07 Sep 2012.

Journal of Enzyme Inhibition and Medicinal Chemistry, 2013; 28(5): 1080–1087

ISSN 1475-6366 print/ISSN 1475-6374 online
DOI: 10.3109/14756366.2012.715288

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RESEARCH ARTICLE

Synthesis, biological evaluation and docking studies of new pyrrolo[2,3-*d*] pyrimidine derivatives as Src family-selective tyrosine kinase inhibitors

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Abstract

In this study, the synthesis and potential enzyme interactions of new Pyrrolo[2,3-*d*]pyrimidine derivatives along with their inhibitory activity against SFK enzymes such as Fyn, Lyn, Hck, and c-Src were reported. The results indicated that compounds were slightly active of tested SFK enzymes in comparison with **PP2** for Fyn, **A-419259** for Lyn and **CGP77675** for c-Src. Compound *N*-((2-amino-4-oxo-4,7-dihydro-3*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)methyl)-4-(3,4-dimethoxyphenyl)butanamide **(5)** was identified as a non-selective slight inhibitor against Fyn, Lyn and c-Src. However, compounds did not show any inhibitory effects on Hck. Docking studies were performed to analyze the binding mode of compounds against SFKs. The best interaction was obtained between compound **5** and the active site of Fyn and c-Src enzymes in comparison with reference compounds **PP2** and **CGP77675**, respectively.

Keywords: Pyrrolopyrimidine derivatives, inhibition of SFK, docking analysis, synthesis, selectivity

Introduction

Src family kinases (SFKs) are involved in a broad spectrum of cellular processes, such as cell growth, differentiation, survival, adhesion, and migration[1,2]. Many evidences showed that abnormal SFK signalling participates in the pathogenesis of many diseases including cancers, osteoporosis, inflammation-mediated bone loss, rheumatoid arthritis, Alzheimer's and Parkinson's disease[3–6]. The activation of SFK in human cancers may occur through a variety of mechanisms that include domain interaction and structural remodelling in response to various activators or upstream kinases and phosphatases[7]. The Src family of cytoplasmic protein tyrosine kinases includes Src, Lck, Fyn, Lyn, Hck, Fgr, Blk, Yrk, and Yes[8]. The prototype member of SFKs, c-Src (Src) is frequently overexpressed and activated in a variety of epithelial and non-epithelial cancers such as colorectal, breast, ovarian, lung and pancreatic cancer and melanomas[9–11]. Src also plays prominent roles in invasion, tumour progression and development of metastasis, therefore Src is a promising target for cancer theraphy[12–15]. Lck is a positive activator in T-cell signalling (TCR), and its activation is involved in autoimmune diseases such as rheumatoid arthritis, organ transplant rejection and asthma. TCR signals initiated by Lck ultimately lead to gene regulation events triggering cytokine release, proliferation and survival of antigen-specific T cell thereby amplifying specific immune responses[16]. Hck plays an important role in signalling process of inflammation[17]. Hck is a phagocyte-specific proto-oncogene of the Src family and is expressed as two isoforms, p59Hck and p61Hck. It plays a critical role in Bcr/Abl-chronic myeloid leukaemia and is able to transform fibroblasts *in vitro*. However, the tumourigenic activity of Hck and the respective oncogenic functions of Hck isoforms have not been examined[18]. Lyn is

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(Received 06 June 2012; revised 13 July 2012; accepted 14 July 2012)

phosphorylated in a KIT-independent manner in neoplastic mast cells (MCs) and it was shown that inhibitors such as dasatinib and bosutinib disrupt Lyn-driven oncogenic signalling in neoplastic MC[19]. Among the SFKs, excess Fyn activity in the brain is associated with conditions such as Alzheimer's and Parkinson's diseases. Therefore inhibition of Fyn kinase may help counteract these nervous system disorders[20]. Elevated levels of Fyn in fibroblast cells exhibited a cancer-like phenotype with increased anchorage-independent growth and prominent morphologic changes. Fyn is overexpressed in various cancers, including glioblastoma, head, neck, and prostate cancer. It is also involved in melanoma induces morphogenic transformation, alteration of mitogenic signals and stimulation of cell growth and proliferation[21]. Most of the kinase activity inhibitors possess at least two aromatic rings, which interfere with ATP binding[22,23]. Several small molecule inhibitors of SFKs are currently investigated in clinical trials. These Src inhibitors include heterocyclic ATP analogs, pyrazolo[2,3-*d*] pyrimidines, pyrrolo[2,3-*d*]pyrimidines, pyrido[2,3-*d*] pyrimidines, quinolines, and olomucines[24]. Here, the information about SFK enzymes inhibitors pyrrolo and pyrazolo[3,4-d]pyrimidine were summarized, since both skeletons are bioisosteres. Pyrazolo[3,4-d]pyrimidine derivatives, **PP1** and **PP2** belong to traditional ATP analogues (Figure 1), which could inhibit c-Src, Lck and Fyn enzymes of SFK, showing IC_{50} values in the range of 4–6 nM[22,25–28]. Amide derivatives of **PP1** and **PP2**, which bearing a C3 un-substituted phenyl ring were found potent inhibitor of Fyn with similar potency, with IC_{50} values of 6.5 nM[28]. Pyrrolopyrimidine compounds **CGP76030** and **CGP77675**, were reported to possess significant inhibitory activities against c-Src, Lck and Yes at nanomolar levels in osteoporosis and cancer models, both *in vitro* and *in vivo* (Figure 1)[16,29]. Other pyrrolo[2,3-*d*] pyrimidine compound **PKI166**[30], inhibits c-Src with an IC_{50} value of 130 nM and **A-419259**[31,32] inhibits c-Src, Lck and Lyn with IC_{50} values of 9, <3 and <3 nM, respectively (Figure 1). Compound (S)-6-(4-(2-(dimethylamino) ethoxy)phenyl)-5-phenyl-*N*-((tetrahydrofuran-2-yl) methyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-amine[16] was found as Lck inhibitor with IC_{50} of <10 nM (Figure 1).

In this work, we have prepared new Pyrrolo[2,3-*d*] pyrimidine derivatives and evaluated for their inhibitory activities and selectivities against SFKs, namely Fyn, Lyn, Hck and c-Src. (Scheme 1). For this aim, structurally different type of amide derivatives are randomly designed to find new promising candidate molecules for specific SFKs inhibition. Subsequently, docking analyses were performed to evaluate binding properties and establishing relationships between biological activity-binding affinities of compounds.

Materials and methods

Chloroacetonitrile, methylformate, 2,6-diamino-4-hydroxy pyrimidine, sodium methoxide, sodium acetate, 10% Pd/C, 4-(3,4-dimethoxyphenyl)butyric acid, (4-methylthio)benzoic acid, 5-methylthiophene-2-carboxylic acid, (4-chlorophenoxy)acetic acid (from Sigma-Aldrich, St. Louis, MO, USA); ethanol, hydrochloric acid, isopropyl alcohol, sodium hydroxide, sulphuric acid, dimethylsulfoxide (from Merck, Darmstadt, Germany), Fyn, Lyn, and Hck kinases (Millipore, MA, USA), c-Src kinase (Invitrogen, NY, USA) and ProFluor Src-family kinase assay kit, 8 plate (Promega, Madison, USA) were purchased. Melting points were measured with a capillary melting point apparatus

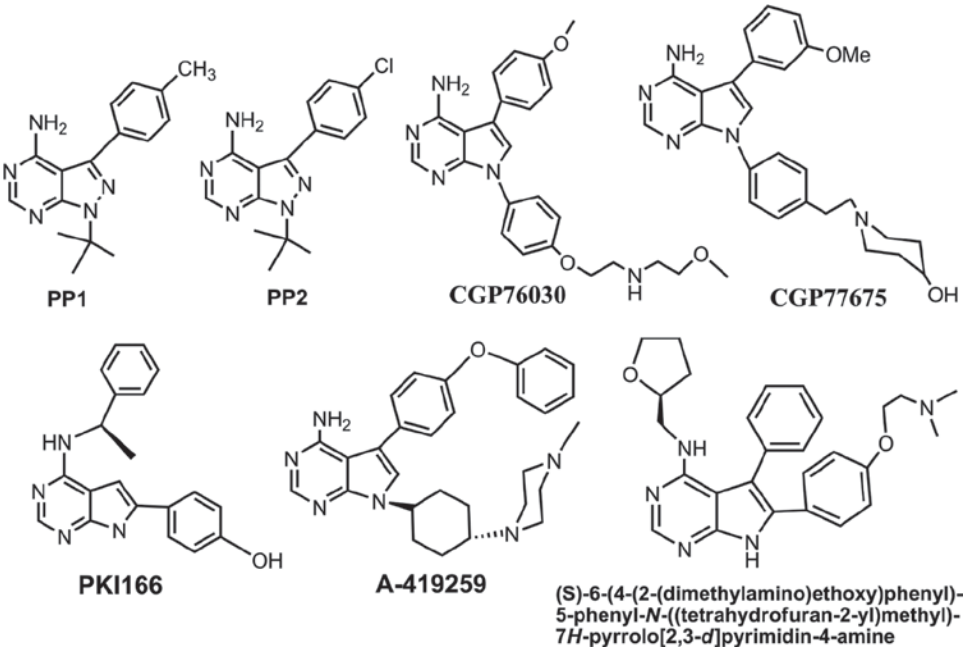
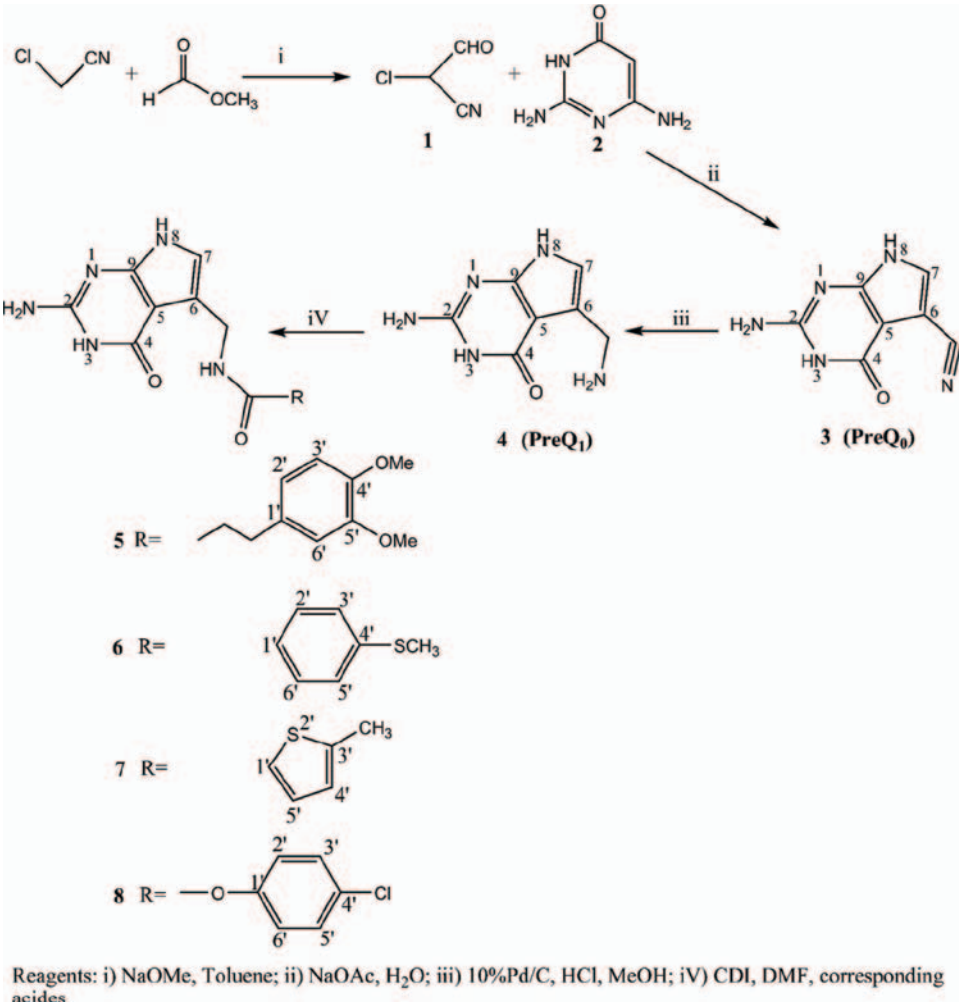


Figure 1. Pyrazolopyrimidine and pyrrolopyrimidine derivatives as SFK inhibitors.



Scheme 1. Synthesis of pyrrolo[2,3-*d*]pyrimidine amide derivatives.

(Electrothermal 9100; Essex, UK). The Nuclear Magnetic Resonance (^{1}H-NMR) spectra were recorded on Varian Mercury 400 NMR spectrometer for 400 MHz (Varian Inc., Palo Alto, CA, USA). The chemical shift values were expressed in parts per million (ppm) relative to tetramethylsilane as an internal standard and signals were reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). Mass spectra were recorded on a Waters ZQ Micromass LC-MS spectrometer (Waters Corporation, Milford, MA, USA). Perkin Elmer S-55 Spectrofluorimetry (from Perkin Elmer, Shelton, CT, USA) was used to measure fluorescence of phosphorylation reaction for enzyme activity studies. Analytical TLC was carried out on Merck 0.2 mm precoated silica gel (60 F-254) aluminium sheets (from Merck, Darmstadt, Germany), with visualization by irradiation with a UV lamp. The flash column chromatography was accomplished on silica gel 60 (230–400 mesh, Merck, Darmstadt, Germany). Elemental analysis was taken on a Leco-932 CHNS-O analyzer (St. Joseph, MI, USA).

5-(Cyano)-2-aminopyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (PreQ$_0$, compound 3)

Methyl formate was added to a stirred mixture of $NaOCH_3$ in anhydrous toluene at 0°C and chloroacetonitrile was added as dropwise over 30 min at 0°C. The reaction mixture was stirred at 0°C for 3 h. Chloro(formyl) acetonitrile **1** was obtained as an oily compound (6.3 g), which was used without further purification for next step (Scheme 1). NaOAc (0.101 mol, 8.29 g) and 2,6-diamino-4-hydroxy pyrimidine **2** (0.051 mol, 6.37 g) was mixed and heated at 100°C. Compound **1** in water was added to this mixture and it was refluxed for 5 h. The yellow solid was collected and dried in vacuo oven at 50°C for 24 h. Compound **3**[33] was obtained with 36%. M.p. >300°C; ^{1}H-NMR (400 MHz, DMSO-d_6, δ, ppm): 6.40 (s, 2-NH_2, 2H), 7.60 (s, 7-CH, 1H), 10.70 (s, 8 NH, 1H), 11.98 (s, 3-NH, 1H); IR (KBr, cm^{-1}): 3450, 3400, 3100 (N-H signals), 2240 (C≡N), 1620 (C=C); ESI-MS: *m/z,* 176.3 (M+1).

2-Amino-5-(aminomethyl)pyrrolo[2,3-*d*]pyrimidin-4(3*H*)-one (compound 4, PreQ$_1$)

PreQo was suspended in 50 mL MeOH and 50 mL of 1 N HCl. Ten percentage of Pd/C was added. The compound was treated with a hydrogenation gas (3 atm) at room temperature and stirred for 24 h. The precipitate was purified by silica gel column chromatography. Compound **4** was obtained with Yield 95%. M.p. 220–225°C; ^{1}H NMR (400 MHz; DMSO-d_6, δ, ppm): 4.00 (2H, s, CH_2-NH_2), 6.46 (2H,

s, 2-NH_2), 6.78 (1H, s 7-CH), 10.58 (1H, s, 8-NH), 11.21 (1H, s, 3-NH); IR (KBr, cm^{-1}): 3440, 3340, 3225 (N-H signals), 1610 (C=C); LC/MS, (ESI, m/z): 180.5 (M+1), 163.0 (-NH_3, %100).

The general synthesis of 2-amino-pyrrolo[2,3-*d*] pyrimidin-4(3*H*)-one-5-(substituted) amide derivatives (compounds 5–8)

One millimolar appropriate acid was dissolved in DMF and 1.2 mmol CDI was added at 0°C. The mixture was stirred at room temperature for 1 h. One millimolar **PreQ$_1$** was added and the mixture was stirred at 50°C for 12–24 h. The solvent was removed in vacuo and the resulting residue was purified by column chromatography on silica gel (CH_2Cl_2/MeOH, 9.5:0.5) to provide compounds **5**–**8**. Purity of compounds were determined by HPLC and RT (Retention Time) were given as below. Chromatographic conditions; column C18 (Isterra) 5 mm × 25 cm, mobile phase 15 H_2O: 65 CH_3CN: 10 MeOH: 10 formic acid 0.1% in CH_3CN, flow rate: 0.55 mL/min, temperature: 25°C.

N-((2-Amino-4-oxo-4,7-dihydro-3*H*-pyrrolo[2,3-*d*] pyrimidin-5-yl)methyl)-4-(3,4-dimethoxy phenyl) butanamide (5)

Yield 0.086 g, 40%; m.p. 256–258°C; ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 1.77 (2H, m, J = 7.6 Hz, CH_2-CH_2-CH_2), 2.09 (2H, t, J = 7.6 Hz, O=C-CH_2-CH_2), 2.47 (2H, t, J = 7.6 Hz, -CH_2-CH_2-Ar), 3.70 (3H, s, Ar-OCH_3), 3.72 (3H, s, Ar-OCH_3), 4.28 (2H, d, J = 5.2 Hz, -CH_2-NH-C=O), 6.09 (2H, s, 2-NH_2), 6.48 (1H, d, J = 2.4 Hz, CH-7), 6.64 (1H, dd, J = 8.0, 2.0, Ar-H2′), 6.75 (1H, d, J = 2.0 Hz, Ar-H6′), 6.82 (1H, d, J = 8.0 Hz, Ar-H3′), 8.19 (1H, t, J = 5.2 Hz, -CH_2-NH-C=O), 10.35 (1H, s, 8-NH), 10.85 (1H, s, 3-NH); ^{13}C NMR (δ, ppm): 27.9 (-C-*C*-C), 34.9 (O=C-C-C-*C*), 35.7 (-*C*-C-C-Ar), 35.7 (-*C*-NH-), 56.0 (Ar-OCH_3), 56.2 (Ar-OCH_3), 99.0 (C-6′), 112.5 (C-5), 112.8 (C-3′), 114.4 (C-7), 116.2 (C-6), 120.7 (C-2′), 134.9 (C-1′), 147.6 (C-4′), 149.3 (C-5′), 152.2 (C-9), 152.9 (C-2), 160.2 (C-4), 171.9 (NH-*C*=O); IR (KBr, cm^{-1}): 3450, 3375, 3320, 3125 (N-H signals), 1630 (C=O Amide), 1600 (C=C); LC/MS (ESI, m/z): 386.8 (M+1, 100%); HPLC-RT: 3.54 min (pure single peak); $C_{19}H_{23}N_5O_4$, calcd. C 59.21, H 6.02, N 18.17; found C 59.57, H 6.28, N 18.00.

N-[(2-Amino-4-oxo-4,7-dihydro-3*H*-pyrrolo[2,3-*d*] pyrimidin-5-yl)methyl]-4-(methylthio) benzamide (6)

Yield 0.030 g, 16%; m.p. 238–240°C; ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 4.25 (3H, s, SCH_3), 4.46 (2H, d, J = 5.6 Hz, -CH_2-NH-C=O), 6.11 (2H, s, 2-NH_2), 6.55 (1H, d, J = 2.0 Hz, CH-7), 7.28 (2H, d, J = 8.4 Hz, Ar-H3′,5′), 7.77 (2H, d, J = 8.4 Hz, Ar-H2′,6′), 9.27 (1H, t, J = 5.2 Hz, -CH_2-NH-C=O), 10.51 (1H, s, 8-NH), 10.88 (1H, s, 3-NH); ^{13}C NMR (δ, ppm): 14.8 (S*C*H_3), 36.4 (-*C*-NH-), 99.2 (C-5), 114.6 (C-7), 115.8 (C-6), 125.7 (C-3′), 125.9 (C-5′), 128.1 (C-2′), 128.2 (C-6′), 131.4 (C-1′), 142.9 (C-9), 152.5 (C-2), 152.9 (C-4′), 160.7 (C-4), 165.5 (NH-*C*=O); IR (KBr, cm^{-1}): 3450, 3400, 3190, 3145 (N-H signals), 1700 (C=O Amide), 1600 (C=C); LC/MS (ESI, m/z): 330.9 (M+1, 100%); HPLC-RT: 4.75 min (pure single peak); $C_{15}H_{15}N_5O_2S$ calcd. C 54.70, H 4.59, N 21.26; found C 54.98, H 4.33, N 21.45.

N-[(2-Amino-4-oxo-4,7-dihydro-3*H*-pyrrolo[2,3-*d*] pyrimidin-5-yl)methyl]-5-methylthiophene-2-carboxamide (7)

Yield 0.118 g, 35%; m.p. 260–262°C; ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 2.46 (3H, s, thiophene-CH_3), 4.44 (2H, d, J = 5.2 Hz, -CH_2-NH-C=O), 6.15 (2H, s, 2-NH_2), 6.57 (1H, d, J = 2.0 Hz, CH-7), 6.83 (1H, dd, J = 4.0, 1.2 Hz, thiophene-H4′), 7.49 (1H, d, J = 4.0 Hz, thiophene-H5′), 9.19 (1H, t, J = 5.2 Hz, -CH_2-NH-C=O), 10.52 (1H, s, 8-NH), 10.91(1H, s, 3-NH); ^{13}C NMR (δ, ppm): 14.8 (*C*H_3), 36.2 (-*C*-NH-), 99.2 (C-5), 114.6 (C-7), 115.8 (C-6), 127.0 (C-4′), 128.4 (C-5′), 138.4 (C-1′), 144.9 (C-9), 152.5 (C-2), 152.9 (C-3′), 160.7 (C-4), 161.2 (NH-*C*=O); IR (KBr, cm^{-1}): 3400, 3340, 3130 (N-H signals), 1640 (C=O Amide), 1600 (C=C); LC/MS (ESI, m/z): 304.7 (M+1, 100%); HPLC-RT: 4.15 min (pure single peak); $C_{13}H_{13}N_5O_2S$ calcd. C 55.03, H 4.01, N 21.39; found C 54.76, H 3.69, N 21.50.

N-[(2-Amino-4-oxo-4,7-dihydro-3*H*-pyrrolo[2,3-*d*] pyrimidin-5-yl)methyl]-2-(4-chlorophenoxy) acetamide (8)

Yield 0.065 g, 33%; m.p. 200–202°C; ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 4.36 (2H, d, J = 6.0 Hz, CH_2-NH-C=O), 4.49 (2H, s, -O-CH_2-C=O), 6.10 (2H, s, 2-NH_2), 6.53 (1H, d, J = 2.0 Hz, CH-7), 7.00 (2H, d, J = 9.2 Hz, Ar-H3′,5′), 7.35 (2H, d, J = 9.6 Hz, Ar-H2′,6′), 8.88 (1H, t, J = 5.6 Hz, -CH_2-NH-C=O), 10.52 (1H, s, 8-NH), 10.87 (1H, s, 3-NH); ^{13}C NMR (δ, ppm): 35.3 (-*C*-NH-), 67.1 (CH_2OPh), 99.3 (C-5), 114.5 (C-7), 117.4 (C-6), 115.5 (C-2′), 115.9 (C-6′), 129.8 (C-5′), 129.9 (C-3′), 135.8 (C-9), 153.0 (C-2), 153.2 (C-4′), 157.1 (C-1′), 160.5 (C-4), 167.3 (NH-*C*=O); IR (KBr, cm^{-1}): 3420, 3310, 3150 (N-H signals), 1640 (C=O Amide), 1600 (C=C); LC/MS (ESI, m/z): 348.8 (M^+, 100%); HPLC-RT: 4.79 min (pure single peak); $C_{15}H_{14}N_5O_3Cl.H_2O$ calcd. C 49.26, H 4.41, N 19.15; found C 49.13, H 4.68, N 19.27.

In vitro assay for Src tyrosine kinase activity

Kinase activity (Src-family kinases, Hck, Lyn, Fyn, and c-Src) and the effect of the molecules were determined by ProFluor Src-Family Kinase Assay Kit (Promega). A titration assay was performed for each kinase to determine the amount of the enzyme that results in approximately 80% phosphorylation as suggested by the manufacturer[34]. The compounds were dissolved in 10% DMSO and tested at 1, 10 and 100 μM concentrations. Briefly, the molecules were mixed with a reaction buffer that included a specific substrate for Src-family kinases (R110), a control substrate and the kinase. The reaction was initiated by the addition of ATP. After incubating the 96-well reaction plate at 22°C for 1 h, protease solution was added to each well and the plate was incubated for 1 h. The fluorescence of the liberated R110 was read at an excitation wavelength of 485 nm and emission wavelength of 530 nm. The strength of fluorescence is inversely correlated with

the kinase activity. The Src kinase activity is measured as the difference between the total activity of no vehicle (DMSO) and the activity of enzyme in the presence of DMSO. Percentage inhibition value of compounds was determined as the mean of triplicate measurements.

Molecular modelling

For this study, X-ray crystal structure of Fyn-saturosporine complex (PDB entry 2DQ7), Lyn-PP2 complex (PDB entry 2ZV9), Hck-PP1 complex (PDB entry 1QCF), and c-Src-CGP77675 complex (PDB entry 1YOL) were downloaded from Protein Data Bank (PDB). Solvents of molecules were deleted and hydrogen atoms were added to obtain the docking grid the active site was defined using Autogrid. The grid size was set to $40 \times 40 \times 40$ points with grid spacing of 0.375 Å. The grid box was placed on the centre of the ligand from the corresponding crystal structure complexes. 2D structures of inhibitors were established by using ChemBioDrawUltra 11.0 and minimized with ChemBio3DUltra 11.0. To be exported to Autodock, the minimized structures were converted into the mol2 file format. The structures of proteins and ligands were combined using the Autodock 4.2.1 and Vina 1.0.2 software package (The Scripps Research Institute, La Jolla CA, USA). The rigid root and rotatable bonds were defined using ADT. The reliability of the docking protocol was first tested by simulations of the binding mode of known SFK inhibitors (PP1, PP2, CGP77675, and A419259). The results were ranked by the scoring functions of ligand-fit, and analyzed for relatively lower binding energy, and potential in forming hydrogen bonds with SFK kinases. The ligand was fully optimized inside the binding site during the docking simulations. The conformation with the lowest predicted binding free energy of the most occurring binding modes in SFKs active pockets were evaluated to binding properties and relationships between biological activity-binding affinities of compounds. Representation binding modes of compounds were shown by using PyMOL Molecular Graphics System (DeLano Scientific LLC, 2008).

Results and discussion

The starting compound **1** was synthesized with the reaction of methyl formate and chloroacetonitrile in anhydrous toluene (Scheme 1). To obtain compound **3 (PreQ$_0$)**, 2,6-diamino-4-hydroxy pyrimidine **2** was reacted with compound **1** in presence of sodium acetate. **PreQ$_1$** (compound **4**) was prepared by reduction of cyano group of **PreQ$_0$** to amine using H_2/Pd in 1 M HCl[33]. The synthesis of amide derivatives of pyrrolo[2,3-*d*]pyrimidine (compounds **5**–**8**) were achieved by the reaction of **PreQ$_1$** and corresponding acid derivatives in presence of 1,1′-carbonyldiimidazole (CDI) in DMF. The structural elucidations of compounds were established by ^{1}H-NMR, IR, HPLC and MS. The proton numbering for NMR interpretation is indicated on the formula in Scheme 1.

The inhibitory activities of compounds were evaluated via measurement the strength of fluorescence, which are inversely correlated with the kinase activity. For the primary assay, compounds were tested against Lyn, Fyn, Hck and c-Src of SFK enzymes at 0.1, 0.01 and 0.001 mM concentrations. In these concentrations range, compounds inhibited the enzymes Fyn (Figure 2), Lyn (Figure 3) and c-Src (Figure 4) by 18–46, 12–42, and 14–71%, respectively. These results indicated that compounds are slightly active inhibitors of the tested

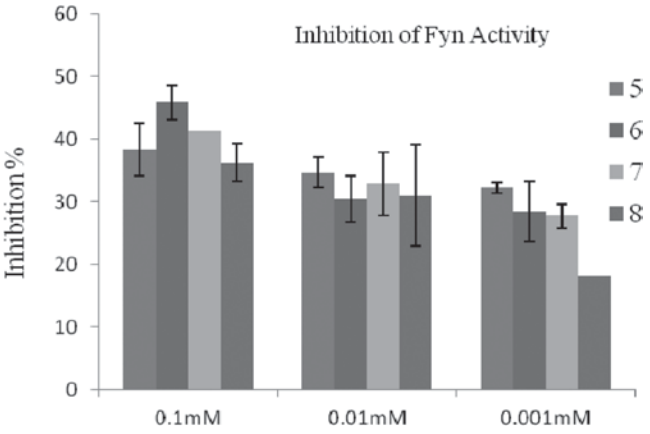


Figure 2. The inhibitory effect of the molecules at 0.1, 0.01 and 0.001 mM concentrations on Fyn activity.

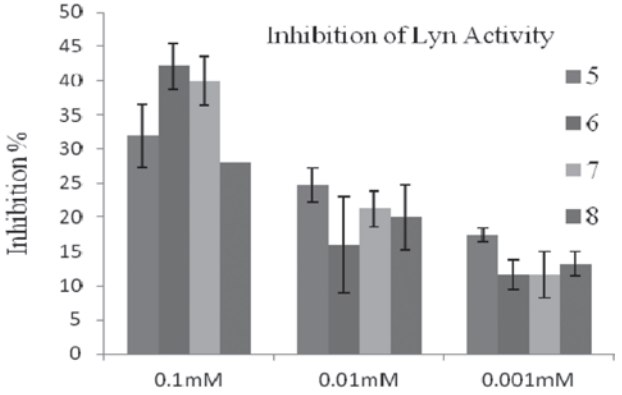


Figure 3. The inhibitory effect of the molecules at 0.1, 0.01 and 0.001 mM concentrations on Lyn activity.

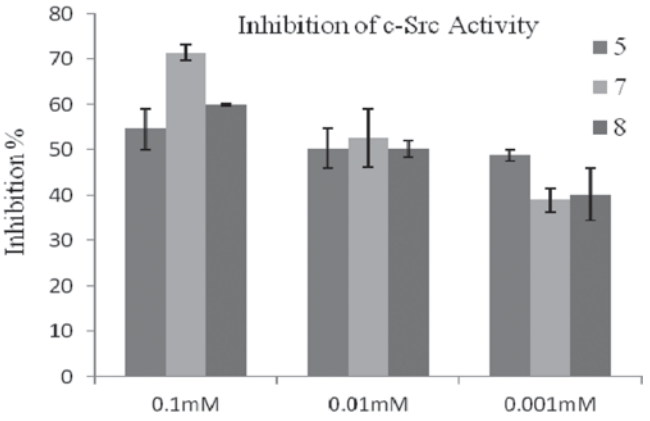


Figure 4. The inhibitory effect of the molecules at 0.1, 0.01 and 0.001 mM concentrations on c-Src activity.

Table 1. Inhibition of SFKs (c-Src, Fyn and Lyn) by compounds **5–8** and reference compounds.

		% c-Src inhibition[b]			% Fyn inhibition[b]			% Lyn inhibition[b]		
Comp. No	IC_{50} values[a]	0.1 mM	0.01 mM	0.001 mM	0.1 mM	0.01 mM	0.001 mM	0.1 mM	0.01 mM	0.001 mM
5		54±4	50±4	50±1 (21 μM)[c]	38±4	35±2	32±1	32±1	25±1	17±2
6		14±4	4±4	25±2	46±3	30±4	28±5	42±4	16±2	12±2
7		71±2	53±6 (16 μM)[c]	39±2	41±0	33±5	28±2	40±1	21±3	12±3
8		60±1	50±2	40±6	36±3	31±8	18±0	28±1	20±1	13±2
PP2	0.5 nM (Fyn)									
A-419259	0.3 nM (Lyn)									
CGP77675	0.4 nM (c-Src)									

[a]Activity data of reference compounds were calculated as nM level.
[b]Average (from three independent experiments) % inhibitory effects of compounds at 0.1, 0.01 and 0.001 mM concentrations.
[c]IC_{50} values of compounds were given as μM level.

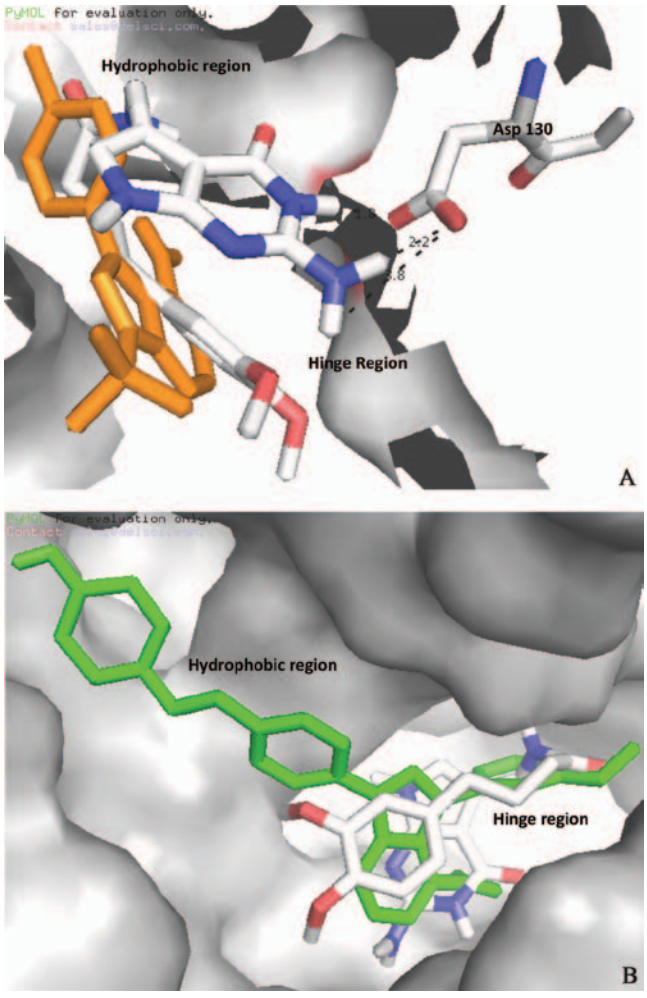


Figure 5. (A) Comparison of compound **5** and **PP2** (orange) with Fyn binding site; (B) Comparison of compound **5** and **CGP77675** (green) with c-Src binding site.

SFK enzymes in comparison with **PP2** (IC_{50} = 0.5 nM) for Fyn, **A-419259** (IC_{50} = 0.3 nM) for Lyn and **CGP77675** (IC_{50} = 0.4 nM) for c-Src. Compounds did not show any inhibitory properties against Hck. The best Fyn and Lyn inhibition (Table 1) was obtained with compound **6** as shown by an inhibition rate of 46 and 42% at 0.1 mM concentration, respectively. Moreover, compound **5** showed Fyn inhibitions rates of 35 and 32% at 0.01 and 0.001 mM concentrations, respectively. On the other hand, compound **5** possessed weaker inhibitory effect on Lyn as shown by 25 and 17% inhibitions at 0.01 and 0.001 mM concentrations, respectively. To evaluate the inhibitory activities at lower concentrations, the data confirmed that compound **5** had higher inhibition profiles against Fyn and Lyn. This result revealed that compound **5** was more potent than the other compounds. As seen in Figure 4, compound **7** showed 53% c-Src inhibition 0.01 mM concentration (IC_{50} was determined as 16 μM), whereas compound **5** showed 50% c-Src inhibition at much lower 0.001 mM concentration (IC_{50} was determined as 21 μM). The results indicate that compound **5** is slightly inhibitor of all three kinases, which suggest compound **5** has a non-selective inhibitory properties. While sulphur containing compounds **6** and **7** have similar inhibitory potencies against Fyn and Lyn, compound **6** has not any inhibition against c-Src. However, compound **7** and **8** are better selective inhibitors of c-Src. Overall, these findings suggest that the features of substituents on fifth position might have significant influence on the inhibitory potencies and selectivity profiles of compounds. It is noteworthy to insert new substituents on this position to design more potent and selective pyrrolopyrimidine compounds against SFKs.

Binding conformation and the degree of fit into the SFKs (c-Src, Fyn, Lyn and Hck) of the compounds were evaluated by using Autodock 4.2.1 and Vina 1.0.2. The program successfully reproduced the X-ray coordinates of the inhibitor binding with RMSD values ranging from 1–3 Å and exhibited a similar accuracy with the same compounds when docked into the SFK binding sites. Each docked compound was assigned a score according to its binding mode onto the binding site of enzymes. The results suggest that ligands with highest binding affinities have a better chance of interaction with enzymes and have

also a good probability of acting as inhibitors. From the interaction mode of the compounds with the predicated active site, it has been found that only compound **5** makes three hydrogen bonds with Fyn. One of the hydrogen bound was observed between the H (NH-3 of pyrolopyrimidine ring) and the O (OH) backbone of ASP130 with the distance of 1.8 Å. A couple of hydrogen bonds formed between the amino group at 2nd position of pyrrolopyrimidine ring and C=O of ASP130 with the distances of 2.2 Å and 3.8 Å (Figure 5A). All compounds were found to bind into the active site of Fyn similar to **PP2**, which is known as most potent inhibitor of Fyn but none of them are overlapped well with **PP2**, including compound **5** (Figure 5A). Figure 5B depicted the interactions of compound **5** and **CGP77675** with the active site of c-Src. Compound **5** was found to bind into the ATP binding site of c-Src similar to the crystallized inhibitor **CGP77675** but none of hydrogen bonds involving the pyrrolopyrimidine nucleus and the hinge region were determined. Compounds were not docked well onto the Lyn and Hck in comparison with potent known inhibitors **A419259** and **PP1**, respectively. The docking studies suggested that the flexible or longer side chains of our compounds at 5th position might be the reason for weak binding property and activity in comparison with the reference compounds. As conclusion of above results, it was impossible to identify for each inhibitor a molecular portion that interacts with the same pocket of the SFKs binding site, since inhibitors showed quite different binding modes.

Declaration of interest

This work was partially supported by grants from the University of Ankara Research Fund (BAP-09B3336002).

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