Received Date : 12-Mar-2014

Revised Date : 24-Apr-2014

Accepted Date : 17-May-2014

Article type : Research Article

Synthesis and Evaluation of a New Series of 3,5-bis ((5-bromo-6-methyl-2-*t*-aminopyrimidin-4-yl)thio)-4*H*-1,2,4-triazol-4-amines and their Cyclized Products "Pyrimidinylthio pyrimidotriazolothiadiazines" as 15- Lipoxygenase Inhibitors

Running tittle: 3,5-bis(substituted pyrimidinylthio) triazolamines

Keywords: 3,5-bis((5-bromo-6-methyl-2-*t*-aminopyrimidin-4-yl)thio)-4*H*-1,2,4-triazol-4-amine, heterocyclization, synthesis, 15-LO, pyrimidinylthio, inhibitors, docking

Tayebe Asghari[a], Mehdi Bakavoli[a*], Mohammad Rahimizadeh[a], Hossein Eshghi[a], Sattar Saberi[a] Azam Karimian[a,] Farzin Hadizadeh[b*], Moreteza Ghandadi[c]

[a]Department of Chemistry, School of Sciences, Ferdowsi University of Mashhad, 91775-1436 Mashhad, Iran

[b]Biotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

[c]Department of biotechnology, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

*Corresponding authors: Mehdi Bakavoli, School of Sciences, Ferdowsi University of Mashhad, Iran; email: mbakavoli@yahoo.com Tel: + 98-511- 8797022; fax: + 98-511-8796416;
Farzin Hadizadeh, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran; email: hadizadehf@mums.ac.ir; Fax: +98-511-8823251

Abstract

A series of new 3,5-bis((5-bromo-6-methyl-2-*t*-aminopyrimidin-4-yl)thio)-4*H*-1,2,4-triazol-4-amines and their cyclized products "pyrimidinylthio pyrimidotriazolothiadiazines" were designed, synthesized, and evaluated as potential inhibitors of 15-lipoxygenase (15-LO). Their syntheses started by initial condensation of 2:1 equivalents of pyrimidine with triazole and subsequent nucleophilic displacement of the chlorine atoms with secondary amines and finally cyclocondensation in the presence of $NaNH_2$. The compounds **4d** and **4f** showed the best IC_{50} of 15-LO inhibition (IC_{50} = 9 and 12 μM, respectively). Compounds **4a-g** were docked into 15-LO. We suggest that the hydrogen bonds in quaternary nitrogen of piperazine ring of compounds **4d** and **4f** appears to play major role in lipoxygenase inhibition by this set of synthesized analogs and hydrophobic nature of this protein's binding site should be considered in ongoing investigations.

Introduction

Triazoles and their derivatives play important roles in medicinal, agricultural and industrial fields (1-3). These compounds are known to exhibit antibacterial and antifungal (4), analgesic (5), anticancer and antimicrobial (6), anticonvulsant (7), anti-inflammatory properties (8). Some of the modern day drugs with triazole nucleus are Ribavirin (antiviral agent), Alprazolam (anxiolytic agent), Fluconazole, Itraconazole (antifungal agent) and Rizatriptan (antimigrane

agent). The commonly known systems are triazoles fused with pyridines (9), pyridazines (10), pyrazines (11), imidazoles (12) and triazines (13).

Triazolothiadiazines are a class of heterocyclic compounds that have been described as anti-inflammatory (14), anticandidal (15), analgesic (16), antibacterial, anticancer (17) and antiviral agents (18). Moreover Pyrimidine-fused heterocycles often appear as the core of biologically active compounds (19-21) such as anticancer (22), antiviral (23), anti-inflammatory (24), RET Kinase Inhibitors (25). A literature survey reveals that there are not many examples of triazolethiadiazine fused with pyrimidine reported. Bside more recently, 15-lipoxygenase (15-LO) has emerged as an attractive target for therapeutic intervention (26). 15-LO has been implicated in the progression of certain cancers (27, 28) and chronic obstructive pulmonary disease (COPD) (27). Evidence for the inhibition of 15-LO in the treatment of vascular disease is, however, most compelling (29). Both transgenic and knockout studies implicate a role for 15-LO in atherogenesis (30,31). The enzyme is abundantly expressed in macrophages residing within the atherosclerotic lesion (26). Keeping this in mind, and in continuation of our recent studies (32, 33) on the enzyme inhibitory activity of heterocyclic compound against soybean 15-lipoxygenase, we considered the synthesis of new derivatives of 3,5-bis((5-bromo-6-methyl-2-*t*-aminopyrimidin-4-yl)thio)-4*H*-1,2,4-triazol-4-amines and their cyclized products pyrimidinylthio pyrimidotriazolothiadiazines.

Method and material

Chemistry

Melting points were taken on an Electrothermal type 9100 melting point apparatus and are uncorrected. The IR spectra were obtained in KBr disks on an Avatar 370 FT-IR Thermo-Nicolet spectrometer (ν_{max} in cm^{-1}). The ^1H NMR spectra were recorded on a Bruker AC at 100 MHz,

400 MHz using tetramethylsilane (TMS) as an internal reference. Chemical shift values were given in ppm, at room temperature using $CDCl_3$, DMSO-d_6 or CD_3COCD_3 as a solvent; chemical shifts were reported in parts per million (ppm) downfield from TMS; multiplicities were abbreviated as: s: singlet; d: doublet; q: quartet; m: multiplet; br s: broad singlet. The Mass spectra were obtained on a Varian Mat CH-7 and Agilent 5973 instruments at 70 eV. Elemental analysis was performed on a Thermo Finnigan Flash EA microanalyzer.

3,5-bis((5-bromo-2-chloro-6-methylpyrimidin-4-yl)thio)-4H-1,2,4-triazol-4-amine (3):

To a solution of 4-amino-1,2,4-triazolidine-3,5-dithione **2** (10 mmol, 1.48) and Et_3N (26 mmol, 3.2 ml) in (40 ml) DMF was added 5-Bromo-2,4-dichloro-6-methylpyrimidine **1** (20 mmol, 4.84 gr). The solution was stirred for 2h at room temperature and then was poured into water (150 mL). The resulting precipitate was filtered off and washed with water (2 × 50 mL) and diethyl ether (20 mL), respectively. After drying the solid, 5.50 gr of **3** were obtained as a crude product. Yield: 98%, m.p. 246-247ºC; ^{1}H NMR (100 MHz, DMSO-d_6): δ 2.56 (s, 6H, $2CH_3$), 6.12 ppm (s, 2H, NH_2, D_2O exchangeable); IR: ν 3332, 3252, 2958, 1523, 755cm^{-1}; MS (*m/z)* 556 (M^+), 558 (M^++2); Anal. Calcd. for $C_{12}H_8Br_2Cl_2N_8S_2$: C, 25.78; H, 1.44; N, 20.04; S, 11.47. Found: C, 25.97; H, 1.48; N, 20.35; S, 11.28.

The general procedure for preparation (4a–g) compounds

The appropriate secondary amine (30 mmol) was added to a stirred mixture of compound **3** (10 mmol, 5.59 g) in acetonitrile` (100 mL), and the solution was heated under reflux for 30-60 min. Progress of the reaction was monitored by TLC using chloroform:methanol (30:1). The solid obtained on cooling was filtered and recrystallized from ethanol.

3,5-bis((5-bromo-6-methyl-2-morpholinopyrimidin-4-yl)thio)-4H-1,2,4-triazol-4-amine(4a):

White powder; yield: 89%; m.p. 248-249 ºC; ^{1}H NMR (100 MHz, $CDCl_3$): δ 2.46 (s, 6H, $2CH_3$), 3.48-3.57 (m, 8H, $4CH_2N$), 3.57-3.62 (m, 8H, $4CH_2O$), 5.02 ppm (s, 2H, NH_2, D_2O exchangeable); IR (KBr disc): ν 3328, 3271, 2973,2916,2847, 1543, 770cm^{-1}.; MS (*m/z)* 658

(M^+), 660 (M^++2); Anal. Calcd. for $C_{20}H_{24}Br_2N_{10}O_2S_2$: C, 36.37; H, 3.66; N, 21.21; S, 9.71. Found: C, 36.55; H, 3.62; N, 21.51; S, 9.36%.

3,5-bis((5-bromo-6-methyl-2-(piperidin-1-yl)pyrimidin-4-yl)thio)-4H-1,2,4-triazol-4-amine (4b):

White powder; yield: 91%; m.p. 242 ºC; ^{1}H NMR (100 MHz, $CDCl_3$): δ 1.20-1.82 (m, 12H, $6CH_2$), 2.40 (S, 6H, $2CH_3$), 3.21-3.60 (m, 8H, $4CH_2N$), 5.02 ppm (s, 2H, NH_2, D_2O exchangeable); IR (KBr disc): ν 3338, 3260, 2931, 2851, 1555, 768cm^{-1}.; MS (*m/z)* 654 (M^+), 656 (M^++2); Anal. Calcd. for $C_{22}H_{28}Br_2N_{10}S_2$: C, 40.25; H, 4.30; N, 21.34; S, 9.77. Found: C, 40.48; H, 4.46; N, 21.18; S, 9.91%.

3,5-bis((5-bromo-6-methyl-2-(4-methylpiperidin-1-yl)pyrimidin-4-yl)thio)-4H-1,2,4-triazol-4-amine (4c):

White powder; yield: 89%; m.p. 179 ºC; ^{1}H NMR (100 MHz, $CDCl_3$): δ 0.91 (d, J = 6 Hz, 6H, $2CH_3$), 1.10-1.30 (m, 2H, 2CH) 1.36-1.89 (m, 8H, $4CH_2$), 2.41 (s, 6H, $2CH_3$), 2.51-2.92 (m, 4H, equatorial hydrogens of $2CH_2N$), 3.92-4.41 (m, 4H, axial hydrogens of $2CH_2N$), 5.03 (s, 2H, NH_2, D_2O exchangeable); IR (KBr disc): ν 3329, 3256, 2947, 2922 , 1547, 1515 , 772cm^{-1}; MS (*m/z)* 682 (M^+), 684 (M^++2); Anal. Calcd. for $C_{24}H_{32}Br_2N_{10}S_2$: C, 42.11; H, 4.71; N, 20.46; S, 9.37. Found: C, 42.53; H, 4.78; N, 20.47; S, 9.51%.

3,5-bis((5-bromo-6-methyl-2-(4-methylpiperazin-1-yl)pyrimidin-4-yl)thio)-4H-1,2,4-triazol-4-amine (4d):

White powder; yield: 88%; m.p. 267-268ºC; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.27 (s, 6H, $2CH_3$) 2.32 (app. t, 8H, $4CH_2N$), 2.38 (s, 6H, $2CH_3$), 3.46 (app. t, 8H, $4CH_2N$), 5.13 ppm (s, 2H, NH_2, D_2O exchangeable); IR (KBr disc): ν 3354, 3268, 2966, 2937, 1547, 775cm^{-1}.; MS (*m/z)* 684

(M^+), 686 (M^+ + 2); Anal. Calcd. for $C_{22}H_{30}Br_2N_{12}S_2$: C, 38.49; H, 4.40; N, 24.48; S, 9.34 Found: C, 38.59; H, 4.31; N, 24.44; S, 9.05%.

3,5-bis((5-bromo-6-methyl-2-(4-phenylpiperazin-1-yl)pyrimidin-4-yl)thio)-4H-1,2,4-triazol-4-amine (4e):

White powder; yield: 87%; m.p. 199-201 ºC; ^{1}H NMR (100 MHz, $CDCl_3$): δ 2.50 (s, 6H, $2CH_3$), 3.01 (app. t, 8H, $4CH_2N$), 3.60 (app. t, 8H, $4CH_2N$), 5.10 (s, 2H, NH_2, D_2O exchangeable), 6.75-7.02 (m, 6H, aromatic), 7.12-7.41 ppm (m, 4H, aromatic); IR (KBr disc): ν 3333, 3252, 2953, 2908, 1598, 1548, 758cm^{-1}.; MS (*m/z)* 808 (M^+), 810 (M^++2); Anal. Calcd. for $C_{32}H_{34}Br_2N_{12}S_2$: C, 47.41; H, 4.23; N, 20.73; S, 7.91. Found: C, 47.63; H, 4.34; N, 20.75; S, 7.77%.

3,5-bis((5-bromo-6-methyl-2-(4-ethylpiperazin-1-yl)pyrimidin-4-yl)thio)-4H-1,2,4-triazol-4-amine (4f):

White powder; yield: 91%; m.p. 220 ºC; ^{1}H NMR (100 MHz, $CDCl_3$): δ 1.16 (t, J=7Hz, 6H, $2CH_3$), 2.44 (s, 6H, $2CH_3$), 2.50-2.57 (m, 12H, $4CH_2N$,$2CH_2$), 3.56 (app. t, 8H, $4CH_2N$), 5.12 ppm (s, 2H, NH_2, D_2O exchangeable); IR (KBr disc): ν 3358, 3280, 2966, 2945 , 2810 , 1545, 767cm^{-1}.; MS (*m/z)* 712 (M^+), 714 (M^++2); Anal. Calcd. for $C_{24}H_{34}Br_2N_{12}S_2$: C, 40.34; H, 4.80; N, 23.52; S, 8.97 Found: C, 40.73; H, 4.77; N, 23.10; S, 8.37%.

3,5-bis((5-bromo-6-methyl-2-(pyrrolidin-1-yl)pyrimidin-4-yl)thio)-4H-1,2,4-triazol-4-amine (4g):

White powder; yield: 90%; m.p. 245-246 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.75–1.86 (m, 8H, 4CH2), 2.47 (s, 6H, 2CH3), 3.00 (m, 4H, $2CH_2N$), 3.47 (m, 4H, $2CH_2N$), 5.20 (s, 2H, NH_2, D2O exchangeable); IR (KBr disc): ν 3330, 3268, 2966, 2870, 1557, 769cm−1; MS (m/z) 626 (M^+), 628 (M^++2). Anal. Calcd for $C_{20}H_{24}Br_2N_{10}S_2$: C, 38.23; H, 3.85; N, 22.29; S, 10.21. Found: C, 38.18; H, 3.87; N, 21.97; S, 10.01%.

The general procedure for preparation of (5a–g) compounds:

A mixture of each of compounds **4a-g** (10 mmol), $NaNH_2$ (30mmol) in dry acetonitrile (100 mL) was heated under reflux for 15-60 min. Progress of the reaction was monitored by TLC using chloroform:methanol (15:1) or ethylacetate:n-hexane (15:9). The mixture was cooled and the solvent was removed under reduced pressure. Then, a solution of acetic acid (1 mL) in water (20 mL) was added to the residue and the resulting precipitant was filtered off and recrystallized from ethanol.

4-(5-bromo-4-methyl-6-((6-methyl-8-morpholino-5H-pyrimido[5,4-e][1,2,4]triazolo[3,4-b][1,3,4]thiadiazin-3-yl)thio)pyrimidin-2-yl)morpholine (5a):

White powder; yield: 75%; m.p.> 300 ºC (dec.); ^{1}H NMR (100 MHz, CD_3COCD_3); δ 2.20 (s, 3H, CH_3), 2.40 (s, 3H, CH_3) 3.35-3.50 (m, 8H, $4CH_2N$), 3.50-3.70 (m, 8H, $4CH_2O$), 7.85 ppm (br s, 1H, NH, D_2O exchangeable); IR (KBr disc): ν 3260, 3166, 2958, 1611cm^{-1} ; MS (*m/z)* 578 (M^+), 580 (M^++2) Anal. Calcd. for $C_{20}H_{23}BrN_{10}O_2S_2$: C, 41.45; H, 4.00; N, 24.17; S, 11.07 Found: C, 41.54; H, 4.10; N, 23.93; S, 10.89%.

3-((5-bromo-6-methyl-2-(piperidin-1-yl)pyrimidin-4-yl)thio)-6-methyl-8-(piperidin-1-yl)-5H-pyrimido[5,4-e][1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (5b):

White powder; yield: 71%; m.p. 270 ºC (dec.); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.30-1.70 (m, 12H, $6CH_2$), 2.32 (s, 3H, CH_3), 2.43 (s, 3H, CH_3), 3.38 (app. t, 4H, $2CH_2N$), 3.70 (t, J=5.2Hz, 4H, $2CH_2N$) 7.80 ppm (br s, 1H, NH, D_2O exchangeable); IR (KBr disc): ν 3260, 3170, 2931, 1631cm^{-1}.; MS (*m/z)* 574 (M^+), 576 (M^++2); Anal. Calcd. for: $C_{22}H_{27}BrN_{10}S_2$: C, 45.91; H, 4.73; N, 24.34; S, 11.14. Found: C, 45.33; H, 4.81; N, 24.06; S, 10.88%.

3-((5-bromo-6-methyl-2-(4-methylpiperidin-1-yl)pyrimidin-4-yl)thio)-6-methyl-8-(4-methylpiperidin-1-yl)-5H-pyrimido[5,4-e][1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (5c):

White powder; yield: 74%; m.p. 220 ºC; ^{1}H NMR (100 MHz, $CDCl_3$): δ 0.80- 1.10 (m, 6H, $2CH_3$), 1.31-1.85 (m, 10H, 2CH and $4CH_2$), 2.30 (s, 3H, CH_3), 2.42 (s, 3H, CH_3), 2.50-2.90 (m, 4H, equatorial hydrogens of $2CH_2N$), 4.10-4.70 (m, 4H, axial hydrogens of $2CH_2N$), 7.80 ppm (br. s, 1H, NH, D_2O exchangeable); IR (KBr disc): ν 3280, 2946, 2923, 1635cm^{-1}.; MS (*m/z)* 602 (M^+), 604 (M^++2); Anal. Calcd. for $C_{24}H_{31}BrN_{10}S_2$: C, 47.76; H, 5.18; N, 23.21; S, 10.62. Found: C, 47.37; H, 5.09; N, 23.04; S, 10.36%.

3-((5-bromo-6-methyl-2-(4-methylpiperazin-1-yl)pyrimidin-4-yl)thio)-6-methyl-8-(4-methylpiperazin-1-yl)-5H-pyrimido[5,4-e][1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (5d):

White powder; yield: 75%; m.p.> 300 ºC (dec.); ^{1}H NMR (100 MHz, $CDCl_3$); δ 2.20-280 (m, 20H, $4CH_3$, $4CH_2N$,), 3.40-3.90 (m, 8H, $4CH_2N$), 8.12 ppm (br s, 1H, NH, D_2O exchangeable); IR (KBr disc): ν 3284, 2934, 1585 cm^{-1}.; MS (*m/z)* 604 (M^+), 606 (M^++2) Anal. Calcd. for $C_{22}H_{29}BrN_{12}S_2$: C, 43.63; H, 4.83; N, 27.76; S, 10.59. Found: C, 43.31; H, 4.93; N, 27.45; S, 10.61%.

3-((5-bromo-6-methyl-2-(4-phenylpiperazin-1-yl)pyrimidin-4-yl)thio)-6-methyl-8-(4-phenylpiperazin-1-yl)-5H-pyrimido[5,4-e][1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (5e):

White powder; yield: 73%; m.p. 240 ºC (dec);^{1}H NMR (100 MHz, $CDCl_3$): δ 2.36 (s, 3H, CH_3), 2.47 (s, 3H, CH_3), 2.85-3.31 (m, 8H, $4CH_2N$), 3.45-4.01 (m, 8H, $4CH_2N$), 6.69-7.08 (m, 6H, aromatic)**,** 7.08-7.41(m, 4H, aromatic), 8.11 ppm (br s, 1H, NH, D_2O exchangeable); IR (KBr disc): ν 3245, 2953, 2913, 1598cm^{-1}.; MS (*m/z)* 728 (M^+), 730 (M^++2); Anal. Calcd. for $C_{32}H_{33}BrN_{12}S_2$: C, 52.67; H, 4.56; N, 23.03; S, 8.79. Found: C, 52.39; H, 4.91; N, 23.18; S, 8.43%.

3-((5-bromo-2-(4-ethylpiperazin-1-yl)-6-methylpyrimidin-4-yl)thio)-8-(4-ethylpiperazin-1-yl)-6-methyl-5H-pyrimido[5,4-e][1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (5f):

White powder; yield: 70%; m.p. 260 ºC (dec); ^{1}H NMR (100 MHz, $CDCl_3$): δ 0.92- 1.27 (m, 6H, $2CH_3$), 2.06-2.69 (m, 18H, $2CH_3$, $4CH_2N$, $2CH_2$), 3.29-3.98 (m, 8H, $4CH_2N$) 8.50 ppm (br. s, 1H, NH, D_2O exchangeable); IR (KBr disc): ν 3248, 2966, 1642 cm^{-1}.; MS (*m/z)* 632 (M^+), 634 (M^++2) Anal. Calcd. for $C_{24}H_{33}BrN_{12}S_2$: C, 45.49; H, 5.25; N, 26.53; S, 10.12. Found: C, 45.23; H, 5.40; N, 26.66; S, 10.05%.

3-((5-bromo-6-methyl-2-(pyrrolidin-1-yl)pyrimidin-4-yl)thio)-6-methyl-8-(pyrrolidin-1-yl)-5H-pyrimido[5,4-e][1,2,4]triazolo[3,4-b][1,3,4]thiadiazine (5g):

White powder; yield: 77%; m.p.> 300 °C (dec.); ^{1}H NMR (100 MHz, $CDCl_3$): δ 1.68–1.92 (m, 8H, $4CH_2$), 2.10 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 2.98–3.42 (m, 8H, $4CH_2N$), 8.12 (s, 1H, NH, D2O exchangeable). IR (KBr disc): ν 3268, 3190, 2968, 1615cm−1; MS (*m/z)* 546 (M+), 548 (M^++2); Anal. Calcd for $C_{20}H_{23}BrN_{10}S_2$: C, 43.87; H, 4.23; N, 25.58; S, 11.71. Found: C, 43.52; H, 4.26; N, 25.60; S, 11.39%.

Biology

Linoleic acid and two assay solutions (A and B) were prepared in advance. Solution A was 50 mM 3-(dimethylamino) benzoic acid (DMAB) in a 100 mM phosphate buffer (pH 7.0). Solution B was a mixture of 10 mM 3-methyl-2-benzothiazolinone (MBTH) 3 mL, hemoglobin (5 mg/mL, 3 mL) in 50 mM phosphate buffer at pH 5.0 (25 mL). A linoleic acid solution was prepared by mixing 5 mg of linoleic acid with 50 mg Tween 20 in 3 mL water and then diluting with KOH 100 mM to a final volume of 5 mL. In the standard assay, the sample in ethanol (25 mL), soybean 15-lipoxygenase (SLO) (4000 units/mL in 50 mM phosphate buffer pH 7.0; 25 mL) and phosphate buffer pH 7.0 (50 mM; 900 mL) were mixed in a test tube and preincubation was carried out for 5 min at room temperature. A control test was done with the same volume of ethanol. After the preincubation, linoleic acid solution (50 mL) was added to start the

peroxidation reaction, and, 7 min later, solution A (270 mL) and then solution B (130 mL) was added to start the color formation. Further 5 min later, 200 mL of a 2% SDS solution was added to terminate the reaction. The absorbance at 598 nm was compared with control test. SLO and other chemicals were purchased from Sigma, Aldrich and Merck Co. respectively.

Docking

The mode of interaction between synthesized ligands and lipoxygenase was investigated by docking. 2D structure of chemicals was drowned in ChemDraw ultera 8.0 software and 3D structures were prepared by Hyperchem 7 software (http://www.hyper.com/), using molecular mechanic force filed pre-optimization followed by AM1 semiempirical calculation. The X-ray crystal structure of lipoxygenase (PDB ID: 1IK3) was downloaded from the Protein Data Bank at the Research Collaboratory for Structural Bioinformatics (www.rcsb.org). Further preparations such as polar hydrogen addition and water molecules elimination were done by MOE software. Synthesized chemicals were docked into the binding site of protein by MOE software. All atoms within a 5 Å around the co-crystallized ligand in crystal coordinates of lipoxygenase was selected as binding site. The docking simulations were performed using Triangle matcher placement algorithm in combination with *London dG* scoring function and forcefield as refinement method. For each compound, the top-score docking poses were selected for final ligand–target interaction analysis using LigX module in MOE Software. Validation of docking methodology was first evaluated by docking of co-crystalized ligand into the lipoxygenase binding site.

Results and discussion

Chemistry

The starting material 5-bromo-2,4-dichloro-6-methyl-pyrimidine **1** was prepared according to published method (34). Treatment of compound **1** with 4-amino-1,2,4-triazolidine-3,5-dithione **2**

at room temperature afforded intermediate **3** in which the C-4 chlorine atoms in the pyrimidine rings were replaced selectively by two sulfhydryl groups of ditiol **2** .The foregoing compound **2** was prepared according to the published procedure (35). Subsequent reaction of compound **3** with various secondary amines led to the selective replacement of the C-2 chlorine atoms in the pyrimidine rings to produce the corresponding diheteroaryl sulfide intermediates **4a-g**. The latter compounds subsequently underwent an intramolecular S_NAr reaction in the presence of $NaNH_2$ in boiling acetonitrile to give the desired tricyclic pyrimidinylthio pyrimido[5,4-e]triazolo[5,1-b][1,3,4]thiadiazines **5a-g.** (Scheme 1)

Our attempt for replacement of Br atom of second pyrimidine ring in different solvents and temperature was failed. Structural preference of **5a-g** may be attributed to ring strain of two fused pyrimidothiadiazine rings with triazol. The yield of compounds **4a-g** were increased between 30-40% by changing ethanol to acetonitrile as a solvent. The structure of newly synthesized compounds **5a-g** was confirmed by recording their IR, ^{1}H NMR, elemental analysis and mass spectra. For example, the IR spectrum of compound **4b** exhibited the stretching vibration bands at 3338 and 3260cm^{-1} due to symetrical and asymetrical vibrations of NH_2 group while the IR spectrum of compound **5b** showed a band at 3260cm^{-1} for NH vibration. The ^{1}H NMR spectrum of the asymetrical cyclized product **5b** showed two singlet at δ 2.32 and 2.44 ppm in $CDCl_3$ belonging to methyl groups of the pyrimidine moieties whereas, the ^{1}H NMR spectrum of the symmetrical precursor **4b** showed a singlet at δ 2.46 ppm in $CDCl_3$ for methyl groups of the pyrimidine moieties. The ^{1}H NMR spectrum of the precursor **4b** showed the NH_2 signal at δ 5.02 ppm in CD_3Cl which was removed on adding D_2O. However, the ^{1}H NMR spectrum of the cyclized product **5b** did not show this signal and instead an exchangeable broad singlet peak at δ

7.80 ppm confirming the occurrence of heterocyclisation to **5b**. The mass spectrum of **5b** showed a molecular ion peak at m/z 574 (M^+) corresponding to the molecular formula $C_{22}H_{27}BrN_{10}S_2$.

Biology

The compounds **5a-g** and **4a-g** were evaluated in vitro to determine their activity on 15-LO inhibition, and their inhibitory potencies were compared to 4-methyl-2-(4-methyl piperazinyl)pyrimido[4,5-b]benzothiazine (4-MMPB) with IC_{50} 18µM (31). Compounds **4d** and **4f** were the most potent inhibitors of 15-LO at a concentration of 9 and 12 µM respectively. It was interesting to see that pyrrolidine, morpholine, piperidine, methylpiperidine, and phenylpiperazine analogs didn't show inhibitory activity on 15-LO (IC_{50} > 500 µM) (Table 1). Also Log Dose-response 15-LO activity curves for biologically active compounds **4d**, **4f** and reference **4-MMPB** has been shown in Figure 1.

Docking

In order to investigate the mode of interactions between the lipoxygenase and its inhibitors in a 3D fashion, the compounds were docked into the binding site of lipoxygenase. The validity and quality of docking procedure was evaluated by docking of co-crystalized ligand into the binding site of lipoxygenase. The top binding pose from docking studies shows a similar orientation in the binding pocket to the co-crystallized ligand found in crystal structure (PDB ID 1IK3). The *root mean square deviation* (RMSD) between ligand docked into the binding site and co crystallized ligand in the crystal structure for lipoxygenase was 1.4 Å indicating good ability to reproduce the ligand binding mode observed in the experimental data (Figure 2). Figure 3 is 3D structure of docked chemicals in protein, in this picture red compound is co-crystalysed ligand

and docked analogs represented as line structures colored by atoms. It indicates synthetic ligands are in suitable position between active site of protein. As mentioned in Table 1 compounds **4d** and **4f** derivatives demonstrated the most potent inhibitory effects. Investigating ligand interaction mode of docked analogs by LigX module of MOE software revealed that quaternary nitrogen of piperazine ring in these two compounds have made appropriate site for hydrogen binding. As shown in figure 4,5 there is a hydrogen bond between quaternary nitrogen of piperazine ring and ser-510 in active compounds **4d, 4f.** Inactive compounds **4a**, **b**, **c, g** do not have piperazine ring and quaternary nitrogen and so they cannot have hydrogen bonding to ser -510. In inactive compound **4e** which has a piperazine ring, there is a bulk, electron withdrawing N-phenyl substituent, bigger than N-methyl (**4d**) and N-ethyl (**4f**) , which prevents quaternization of nitrogen of piperazine. So this compound also cannot have hydrogen bond with ser-510 (Figure 6,7). It should be mentioned that presence of hydrophobic amino acids in binding site of lipoxygenase is remarkable (green area in Figure 2 and 3 and lots of hydrophobic amino acids colored as green in Figure 4 and 6 demonstrate this point) and can be considered as opportunity to develop lipoxygenase inhibitors.

In conclusion hydrogen bonds in quaternary nitrogen of piperazine ring of compounds **4d** and **4f** appears to play major role in lipoxygenase inhibition by this set of synthesized analogs and hydrophobic nature of this protein's binding site should be considered in ongoing investigation.

Conclusion

A series of 3,5-bis((5-bromo-6-methyl-2-*t*-aminopyrimidin-4-yl)thio)-4*H*-1,2,4-triazol-4-amines and their cyclized products pyrimidinylthio pyrimidotriazolothiadiazines were synthesized and

evaluated as potential 15-LO inhibitors. Among all these synthesized compounds only two derivatives **4d** and **4f** with IC_{50} 9 and 12 μM respectively showed strong potency for 15-LO inhibition. The mode of interactions between the lipoxygenase and its inhibitors **4a-g** in a 3D fashion showed that hydrogen bonds in quaternary nitrogen of piperazine ring of compounds **4d** and **4f** appears to play a major role in lipoxygenase inhibition.

Acknowledgement

We are grateful to department of chemistry, Ferdowsi University of Mashhad, Iran for financial Support. (Grant No 25466/3)

Conflict of Interest

The authors confirm there is no conflict of interest.

References

1. Snelders E., Huis in 't Veld R.A.G., Rijs A.J.M.M., Kema G.H.J., Melchers W.J.G., Verweij P.E. (2009) Possible environmental origin of resistance of aspergillus fumigatus to medical triazoles. Appl Environ Microbiol;75:4053-4057.

2. Menegola E., Broccia M.L., Renzo F.D., Giavini E. (2006) Postulated pathogenic pathway in triazole fungicide induced dysmorphogenic effects. Reprod Toxicol;22:186–195.

3. Bentiss F., Jama C., Mernari B., Attari H.E., Kadi L.E., Lebrini M., Traisnel M., Lagrenee M. (2009) Corrosion control of mild steel using 3,5-bis(4-methoxyphenyl)-4-amino-1,2,4-triazole in normal hydrochloric acid medium. Corros Sci;51:1628–1635.

4. Chohan Z.H., Sumrra S.H., Youssoufi M.H., Hadda T.B. (2010) Metal based biologically active compounds: design, synthesis, and antibacterial/antifungal/cytotoxic properties of triazole-derived schiff bases and their oxovanadium(IV) complexes. Eur J Med Chem;45:2739-2747.

5. Turan-Zitouni G., Kaplancikli Z.A., Erol K., Kilic F.S. (1999) Synthesis and analgesic activity of some triazoles and triazolothiadiazines. Farmaco;54:218–223.

6. Sztanke K., Tuzimski T., Rzymowska J., Pasternak K., Kandefer-Szerszen M. (2008) Synthesis, determination of the lipophilicity, anticancer and antimicrobial properties of some fused 1,2,4-triazole derivatives. Eur J Med Chem;43:404-419.

7. Siddiqui N., Ahsan W. (2010) Triazole incorporated thiazoles as a new class of anticonvulsants: design, synthesis and in vivo screening. Eur J Med Chem;45:1536–1543.

8. Labanauskas L., Udrenaite E., Gaidelis P., Brukštus A. (2004) Synthesis of 5-(2-,3- and 4-methoxyphenyl)-4*H*-1,2,4-triazole-3-thiol derivatives exhibiting anti-inflammatory activity. Il Farmaco;59:255–259.

9. Sadana A.K., Mirza Y., Aneja K.R., Prakash O. (2003) Hypervalent iodine–mediated synthesis of 1-aryl=hetryl-1,2,4-triazolo[4,3-a] pyridines and 1-aryl=hetryl 5-methyl-1,2,4-triazolo[4,3-a]quinolines as antibacterial agents. Eur J Med Chem;38:533–538.

10. Bussolari J.C., Panzica R.P. (1999) Synthesis and anti-HIV evaluation of 2',3'-dideoxy imidazo- and *v*-triazolo[4,5-d]pyridazine nucleosides. Bioorg Med Chem;7:2373–2379.

11. Yao G., Haque S., Sha L., Kumaravel G., Wang J., Engber T.M., Whalley E.T., Conlon P.R., Chang H., Kiesman W.F., Petter R.C. (2005) Synthesis of alkyne derivatives of a novel triazolopyrazine as A 2A adenosine receptor antagonists. Bioorg Med Chem Lett;15:511–515.

12. Neochoritis C., Tsoleridis C.A., Stephanidou J.S. (2008) 1-Arylaminoimidazole-2-thiones as intermediates in the synthesis of imidazo[2,1-b][1,3,4]thiadiazines. Tetrahedron;64:3527–3533.

13. Vu C.B., Shields P., Peng B., Kumaravel G., Jin X., Phadke D., Wang J., Engber T., Ayyub E., Petter R. (2004) Triamino derivatives of triazolotriazine and triazolopyrimidine as adenosine A2a receptor antagonists C. Bioorg Med Chem Lett;14:4835–838.

14. El Shehry M.F., Abu-Hashem A.A., El-Telbani E.M. (2010) Synthesis of 3-((2,4-dichlorophenoxy)methyl)-1,2,4-triazolo(thiadiazoles and thiadiazines) as anti-inflammatory and molluscicidal agents. Eur J Med Chem;45:1906–1911.

15. Altıntop M.D., Kaplancıklı Z.A., Turan-Zitouni G., Özdemir A., İşcan G., Akalın G., Yıldırım Ş.U. (2011) Synthesis and anticandidal activity of new triazolothiadiazine derivatives. Eur J Med Chem;46:5562-5566.

16. Khan I., Ibrar A., Abbas N. (2013) Triazolothiadiazoles and triazolothiadiazines-biologically attractive scaffolds. Eur J Med Chem;63:854-868.

17. Holla B.S., Rao B.S., Sarojini B.K., Akberali P.M., Kumari N.S. (2006) Synthesis and studies on some new fluorine containing triazolothiadiazines as possible antibacterial, antifungal and anticancer agents. Eur J Med Chem;41:657–663.

18. Holla B.S., Akberali P.M., Shivananda M.K. (2001) Studies on nitrophenylfuran derivatives, part XII: synthesis, characterization, antibacterial and antiviral activities of some nitrophenylfurfurylidene-1,2,4-triazolo[3,4-b]-1,3,4-thiadiazines. Farmaco;56:919–927.

19. Gangjee A., Lin X., Queener S.F. (2004) Design, synthesis, and biological evaluation of 2,4-diamino-5-methyl-6-substituted-pyrrolo[2,3-*d*]pyrimidines as dihydrofolate reductase inhibitors. J Med Chem;47:3689–3692.

20. Cossrow J., Guan B., Ishchenko A., Jones J.H., Kumaravel G., Lugovskoy A., Peng H., Powell N., Raimundo B.C., Tanaka H., Vessels J., Wynn T., Xin Z. (2009) Heterocyclic compounds useful as RAF kinase inhibitors. Patent US20090005359.

21. Dehnhardt C.M., Venkatesan A.M., Santos E.D., Chen Z., Santos O., Ayral-Kaloustian S., Brooijmans N., Mallon R., Hollander I., Feldberg L., Lucas J., Chaudhary I., Yu K., Gibbons J., Abraham R., Mansour T.S. (2010) Lead optimization of N-3-substituted 7-morpholinotriazolopyrimidines as dual phosphoinositide 3-kinase/mammalian target of rapamycin inhibitors: discovery of PKI-402. J Med Chem;53:798–810.

22. Huang Y.Y., Wang Li.Ya., Chang C.H., Kuo Y.H., Kaneko K., Takayama H., Kimura M., Juang S.H., Wong F.F. (2012) One-pot synthesis and antiproliferative evaluation of pyrazolo [3,4-d]pyrimidine derivatives. Tetrahedron;68:9658-9664.

23. Kifli N., Clercq E.D., Balzarini J., Simons C. (2004) Novel imidazo[1,2-c]pyrimidine base-modified nucleosides: synthesis and antiviral evaluation. Bioorg Med Chem;12:4245-4252.

24. El-Gazzar A.B.A., El-Enany M.M., Mahmoud M.N. (2008) Synthesis, analgesic, anti-inflammatory, and antimicrobial activity of some novel pyrimido[4,5-b]quinolin-4-ones. Bioorg Med Chem;16:3261-3273.

25. Dine´r P., Alao J.P., Söderlund J., Sunnerhagen P., Grøtli M. (2012) Preparation of 3-substituted-1-isopropyl-1H-pyrazolo[3,4-d]pyrimidin-4-amines as RET Kinase inhibitors. J Med Chem;55:4872-4876.

26. Kelavkar U., Glasgow W., Eling T.E. (2002) The effect of 15-lipoxygenase-1 expression on cancer cells. Curr Urol Rep;3:207-214.

27. Kelavkar U.P, Cohen C., Kamitani H., Eling T.E., Badr K.F. (2000) Concordant induction of 15-lipoxygenase-1 and mutant p53 expression in human prostate adenocarcinoma: correlation with Gleason staging. Carcinogenesis;21:1777-1787.

28. Zhu J., Kilty I., Granger H., Gamble E., Qiu Y.S., Hattotuwa K., Elston W., Liu W.L., Oliva A., Pauwels R.A., Kips J.C., Rose V.D., Barnes N., Yeadon M., Jenkinson S., et al. (2002) Gene

expression and immunolocalization of 15-lipoxygenase isozymes in the airway mucosa of smokers with chronic bronchitis. Am J Respir Cell Mol Biol;27:666-677.

29. Zhao L., Funk C.D. (2004) Lipoxygenase pathways in atherogenesis. Trends Cardiovasc Med;14:191-195.

30. Cyrus T., Witztum J.L., Rader D.J., Tangirala R., Fazio S., Linton M.F., Funk C.D. (1999) Distruption of the 12/15 lipoxygenase gene diminishes atherosclerosis in apo E-deficient mice. J Clin Invest;103:1597-1604.

31. Harats D., Shaish A., George J., Mulkins M., Kurihara H., Levkovitz H., Sigal E (2000) Overexpression of 15-lipoxygenase in vascular endothelium accelerates early atherosclerosis in LDL reseptor-deficient mice. Arterioscler Thromb Vasc Biol;20:2100-2105.

32. Bakavoli M., Nikpour M., Rahimizadeh M., Saberi M.R., Sadeghian H. (2007) Design and synthesis of pyrimido[4,5-b][1,4]benzothiazine derivatives, as potent 15-lipoxygenase inhibitors. Bioorg Med Chem;15:2120–2126.

33. Bakavoli M., Seyedi S.M., Shiri A., Saberi S., Gholami M., Sadeghian H. (2013) Synthesis of new derivatives of pyrimido[5,4-e][1,2,4]triazolo[3,4-b][1,3,4]thiadiazine and their enzyme inhibitory activity assessment on soybean 15-lipoxygenase. J Chem Res;48-50

34. Bakavoli M., Nikpour M., Rahimizadeh M. (2006) Molecular iodine promoted synthesis of new pyrazolo [3,4-d] pyrimidine derivatives as potential antibacterial agents. J Heterocycl Chem;43:1327-1329.

35. Sandstrom J.(1961) Cyclizations of thiocarbohydrazide and its mono-hydrazones partIII reactions with carbon disulphide in pyridine. Acta Chem Scand;15:1295-1302.

Figures and Table Legends

Table 1: IC_{50} values for the **4a-g** and **5a-g** compounds.

Scheme 1: Synthesis of tricyclic pyrimidinylthio pyrimido[5,4-e]triazolo[5,1-b][1,3,4]thiadiazines **5a-g.**

Figure 1: Log Dose-response 15-LO activity curves for biologically active compounds **4d** (panel A), **4f** (panel B) and reference **4-MMPB** (panel C).

Figure 2: 3D representation of docked ligand (red color) into binding site of lipoxygenase using MOE program as well as co-crystallized one (blue color) in the crystal structure of lipoxygenase (PDB ID: 1IK3).

Figure 3: Map surface of docked analogs in active site of enzyme. Crystallized ligand is indicated by red and stick lines. Green: hydrophobic; violet: H bonding; blue: Mild polar.

Figure 4: The 2D representation of the interaction between compound **4d** (panel A) and compound **4f** (panel B) in the crystal structure of lipoxygenase (PDB ID: 1IK3) using LigX in MOE.

Figure 5: The 3D position of docked compound **4d** (panel A) and compound **4f** (panel B) in the active site of lipoxygenase (PDB ID: 1IK3), hydrogen bonds are in purple and all hydrogens were removed for clarification, compounds represented as stick and amino acid residues as lines colored by atoms.

Figure 6: The 2D representation of the interaction between compound **4b** (panel A), **4a** (panel B) and **4e** (panel C) and lipoxygenase.

Figure 7: The 3D position of docked compound **4b** (panel A), compound **4a** (panel B) and **4e**(panel C) in the active site of lipoxygenase (PDB ID: 1IK3), hydrogen bonds are in purple and all hydrogens were removed for clarification, compounds represented as stick and amino acid residues as lines colored by atoms.

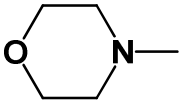
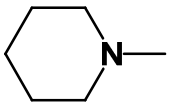
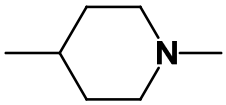
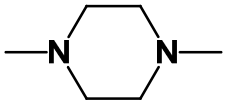
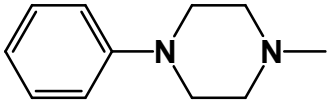
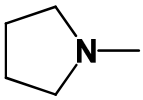
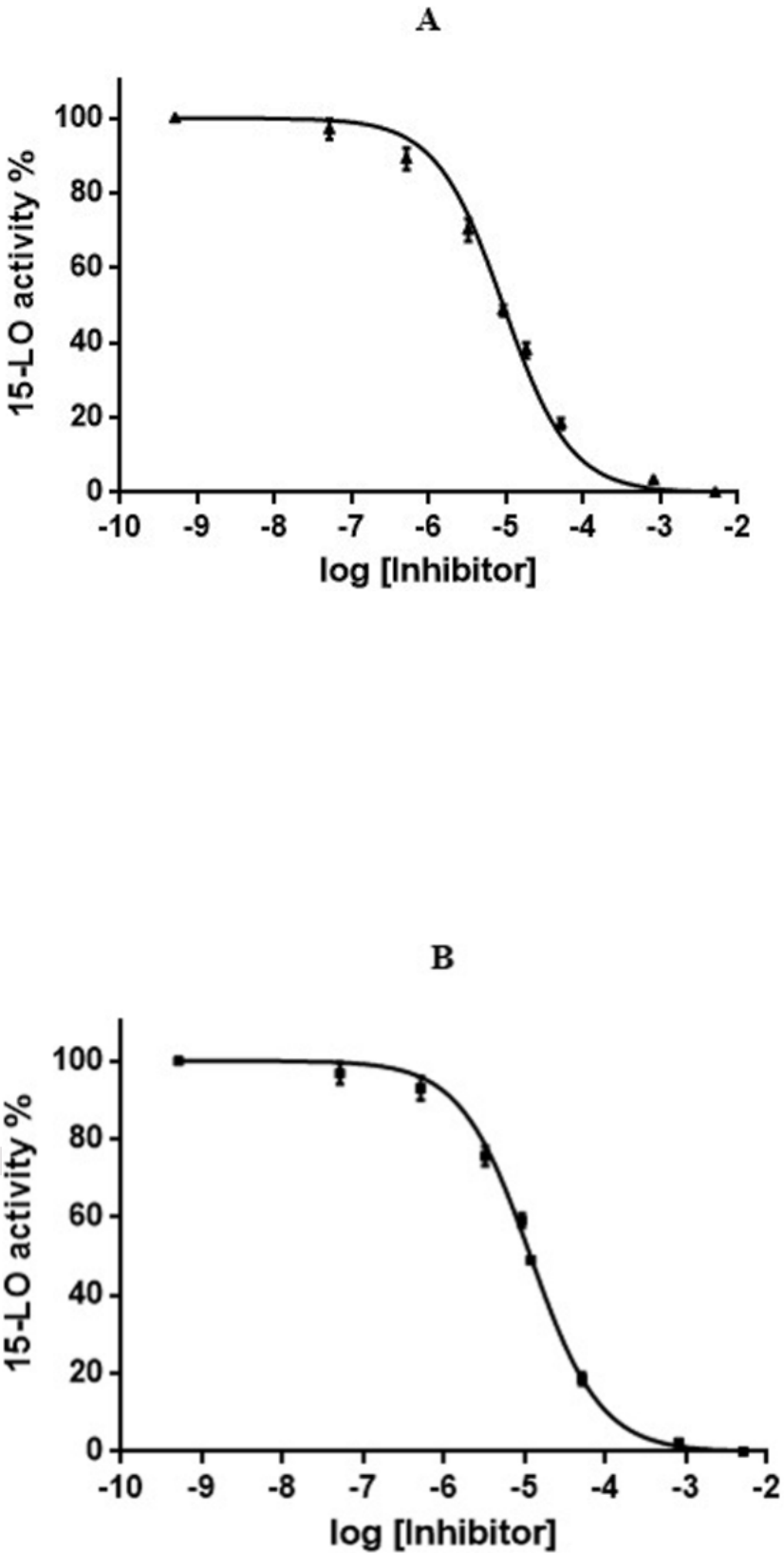
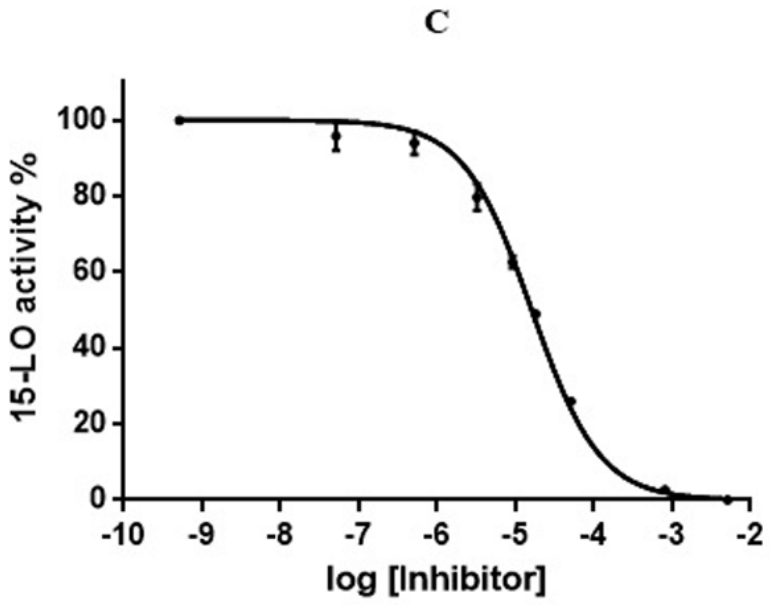
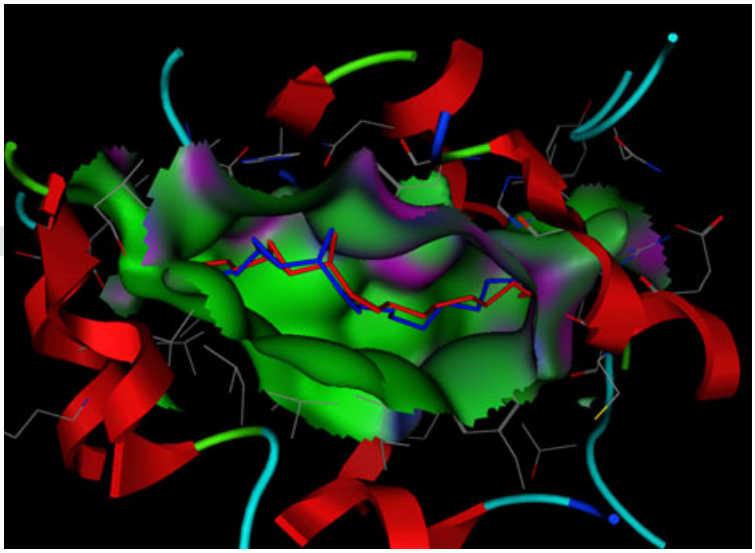
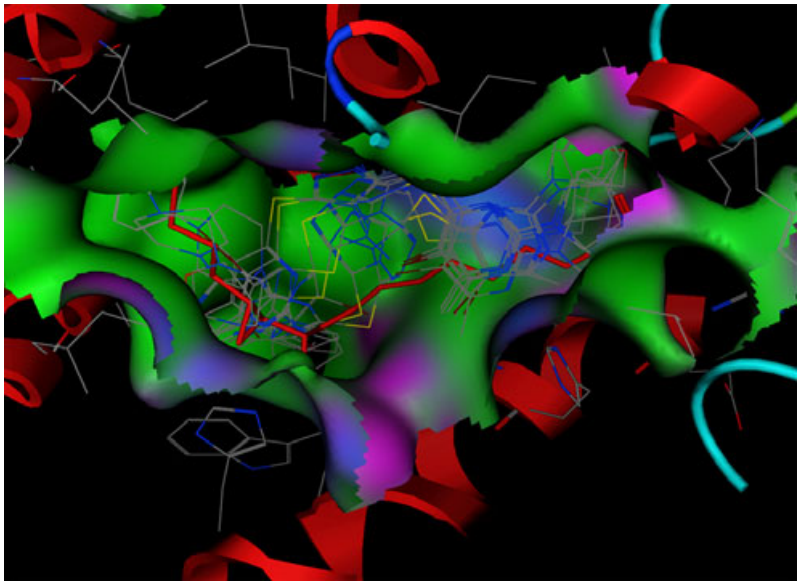
Entry	—NR_2	IC_{50} (μM)
4a		**>500**
5a		**>500**
4b		**>500**
5b		**>500**
4c		**>500**
5c		**>500**
4d		**9**
5d		**>500**
4e		**>500**
5e		**>500**
4f		**12**
5f		**>500**
4g		**>500**
5g		**>500**

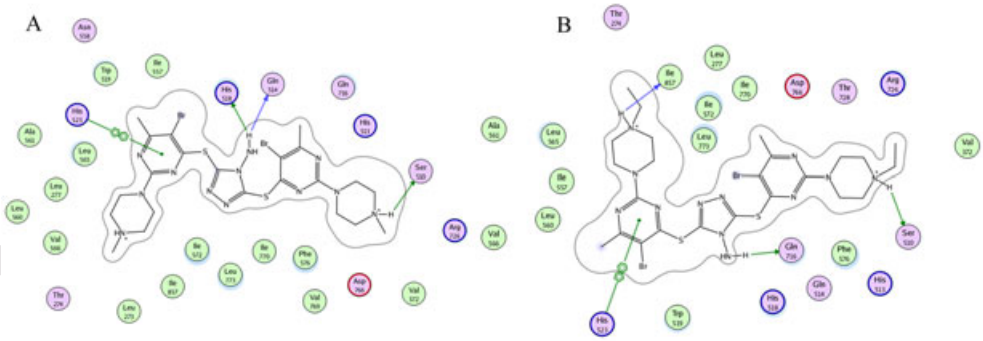
Table 1: IC_{50} values for the **4a-g** and **5a-g** compounds

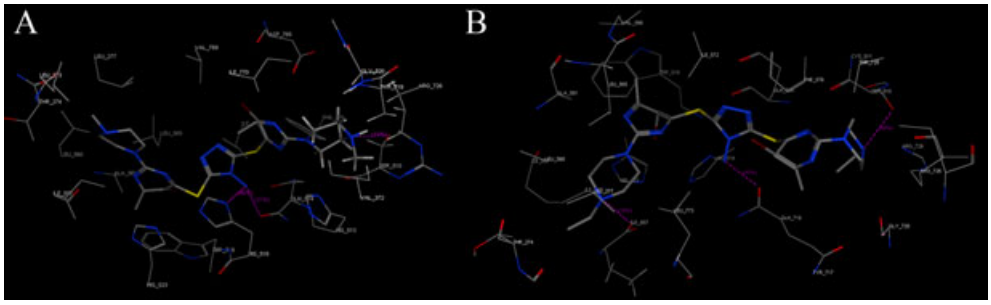


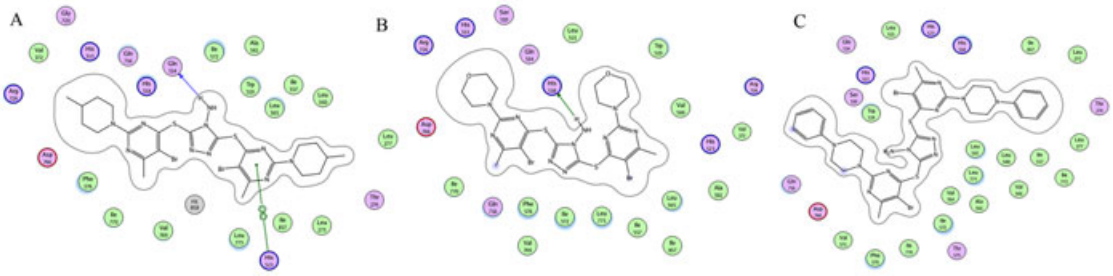


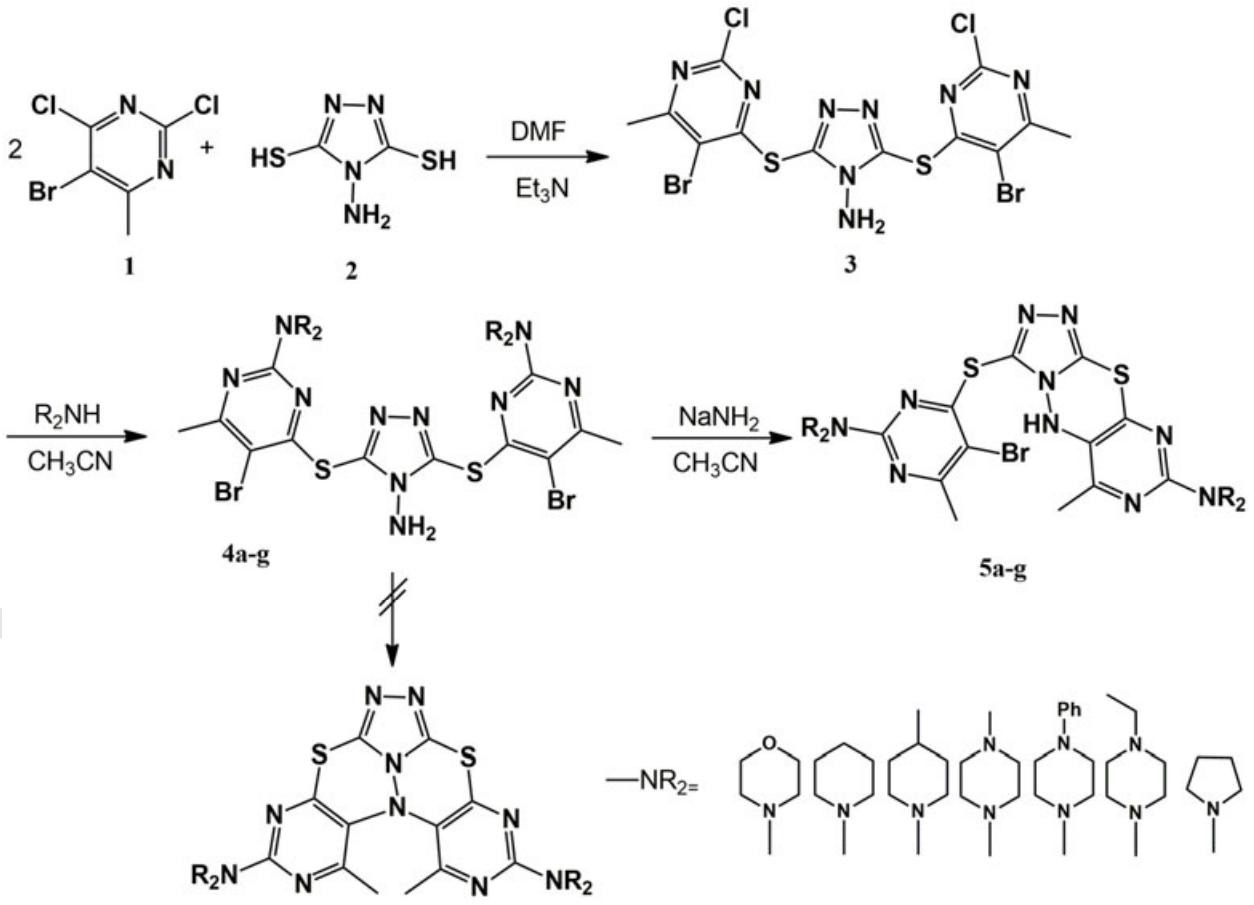












1 2 3 4a-g 5a-g