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Research article

Novel triazine-tyrosine hybrids containing thiyazol or pyridine fragment as anti-multiple sclerosis agents: Design, synthesis, biological evaluation, and molecular docking study

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ABSTRACT

In this study novel triazine-tyrosine hybrids containing thiazole or pyridine fragments were introduced as anti- Multiple Sclerosis agents. The compounds were designed according to the structure of the Sphingosine-1-phosphate receptor subtype 1 (S1P1) modulator, fingolimode. At first, docking studies was performed using crystal structures of S1P1 and Sphingosine-1-phosphate receptor subtype 3 (S1P3) to theoretically identify the selectivity of the compounds towards the S1P1 isoform. The docking results showed better binding energy (lower ΔG_b) and therefore higher selectivity for S1P1 receptor than S1P3 receptor. Subsequently the designed compounds were synthesized according to proper chemical reactions and structurally analyzed with FTIR and NMR spectrophotometers. Considering the importance of the S1P1 receptor in release of lymphocytes and therefore inflammation produced in Multiple Sclerosis disease, the synthesized compounds were investigated to study lymphocyte reduction in an animal model. Compound (**8e**) with 2-mercaptobenzothiazole substitution at doses of 1 and 3 mg/kg showed significant reduction effect on the percentage of lymphocytes (68.80 %, 56.75 %) compared to the fingolimod (65.73 %, 20.66 %), as the positive control group.

1. Introduction

Multiple sclerosis (MS) as a most common disabling disease is increasing worldwide and represents a fast-growing neurodegenerative condition [1,2]. Although multifaceted gene–environment interactions play an important role in occurrence of this disease but the primary cause and mechanisms of MS remain still opaque. MS is considered as autoimmune disorder which in its pathology autoreactive cells cross the blood–brain–barrier into the CNS, facilitating the damage of myelin and oligodendrocytes, ultimately resulting in gliosis, neuro-axonal damage and inflammation. Continuous inflammatory and degenerative changes in the CNS leading to

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progress of disease and debilitate the symptoms of this disease [3,4]. The main strategy in the MS treatment is the control of the inflammation, which leads to the destruction of myelin and axons in CNS, and thus stop the progression of the disease [5].

One of the important endogen ligands involved in inflammatory processes is Sphingosine 1-phosphate (S1P) [6]. This signaling phospholipid through interactions with a class of five G protein-coupled receptors (GPCRs), known as the S1P, mediated the migration, cell proliferation, and immune cell trafficking [7]. So, the agents that are capable of disturbing the interactions of S1P with its receptors may have utility as therapeutics in inflammatory disorders such as MS [8].

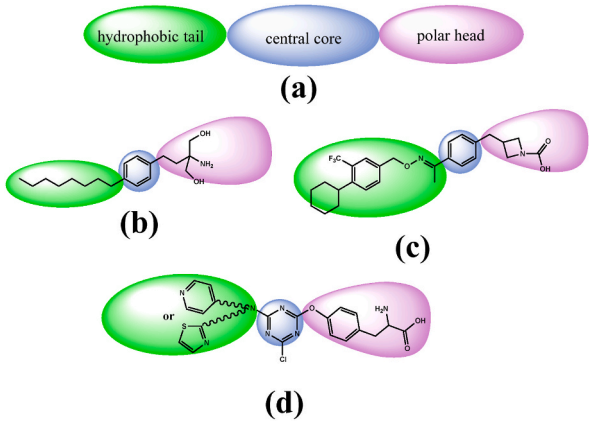
FTY720 (fingolimod) (Scheme 1b) was the first sphingolipid mimetic drug approved for the treatment of relapsing-remitting MS. The active form of the drug, which is formed through phosphorylation in the body, is able to act as a potent agonist for four of the five isoforms of S1P receptor (S1P1, S1P3, S1P4, and S1P5), while in vivo its immunosuppressive effect is due to its interactions with S1P1 which consequently preventing lymphocyte egress from secondary lymphoid organs [9,10]. However, fingolimod as a nonselective S1P receptor modulator is believed to cause chronotropic side effects because of its interaction with S1P3 [11,12]. After fingolimod, Siponimod was approved as a specific modulator for the S1P1 receptor, which is structurally similar to fingolimod (Scheme 1c) [13] Although the cardiovascular effects of this drug are less compared to fingolimod, but it also has a series of side effects such as headache, high blood pressure, increased levels of liver enzymes and so on [14]. Therefore, scientists and pharmaceutical companies are still searching for new drug candidates with improved efficacy and fewer side effects for MS treatment.

According to previous study, the general structure of fingolimod and other S1P1 modulators consist of a polar head, a central core and a hydrophobic tail (Scheme 1a) [15–17]. This study aims to development of novel structurally S1P1 receptor modulator with a potential use as therapeutic agents against MS. In the design of these compounds, the tyrosine amino acid is considered as the polar head of the molecule, which placed on the triazine core, and various aromatic amine or thiol are used as the hydrophobic tail (Scheme 1d). Subsequently, docking studies is performed using crystal structures of S1P1 and also S1P3 receptors to theoretically identify the selectivity of the compounds towards the S1P1 isoform. Then the designed compounds are synthesized, purified and their structures confirmed using different spectroscopic methods. Since S1P1 receptor plays an important role in the immunosuppressive effects, lymphocyte reduction effect of synthesized compounds is investigated on animal models.

2. Experimental

2.1. Molecular docking

The molecular docking study of designed compounds **8a-8f** with S1P1 and S1P3 binding pocket was evaluated by employing AutoDock 4 [18]. Crystallographic structure of S1P1 and S1P3 (PDB ID: 7EO2 and PDB ID: 7EW2) was retrieved from RCSB Protein Data Bank (https://www.rcsb.org/). Co-crystal ligands (fingolimod and siponimod) and also unwanted H_2O molecules were deleted using Discovery Studio Visualizer 4.5(BIOVIA, San Diego, CA, USA). The structure of the ligands (**8a-8f**) was drawn using Chem Draw Ultra 12.0 software and were transferred into Hyperchem software to optimize by PM3 semi-empirical force field using (Version8-Hyperchem, Hypercube, Inc). Using AutoDockTools 1.5.6, polar hydrogens were added to proteins and prepared ligands and also Kollman and Gasteiger charges were assigned to them, respectively. After that PDBQT file of the proteins and ligands were generated. For two proteins a grid box with 60 × 60 × 60 dimensions and 0.375 Å spacing was applied and its center was chosen as the co-crystal ligand center. AutoDock 4.2 with its default search and docking parametersm, except the number of runs (50), was used for studying the ligands interactions. By re-docking of co-crystallized ligands back into respective enzymes S1P1 and S1P3 with root mean square deviation (RMSD) of below 1 Å values, the molecular docking protocol was validated.



Scheme 1. Structures of (a) general structure of S1P1 modulators (b) Fingolimod, (c) Siponimod, (d) designed triazine-tyrosine hybrids containing thiazole or pyridine derivatives.

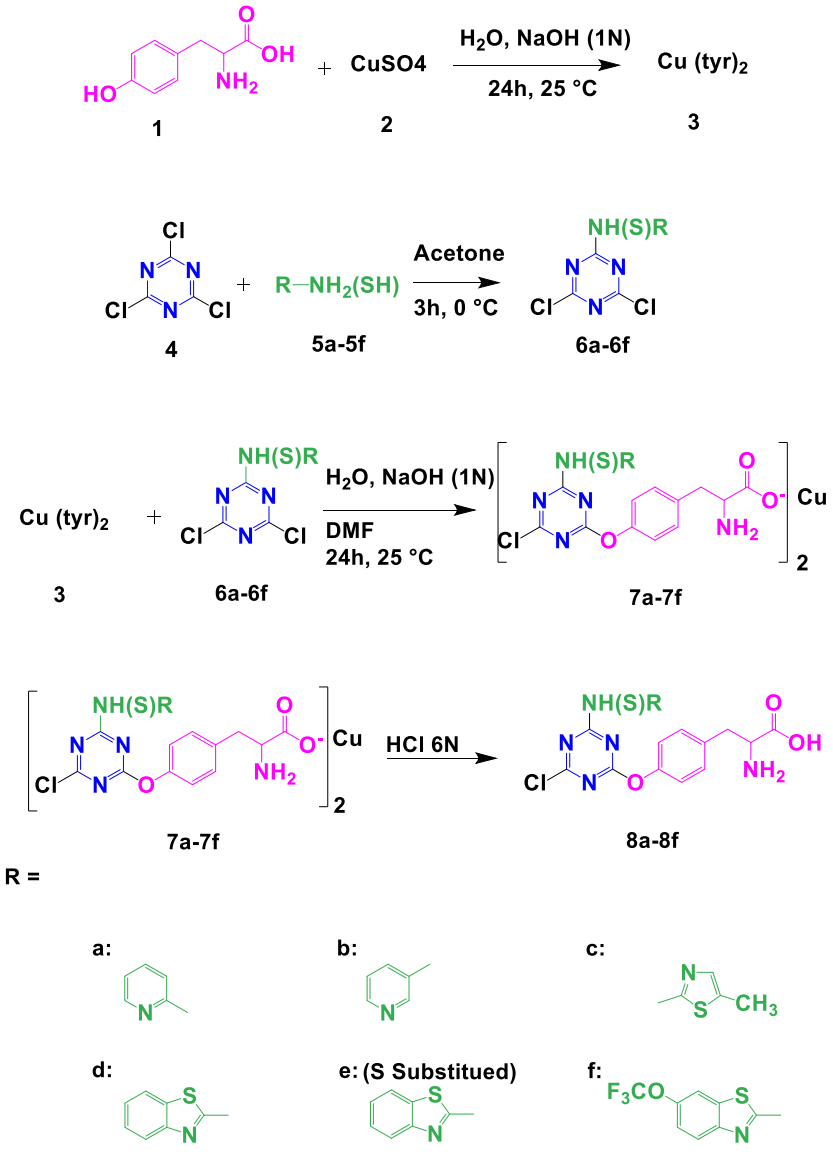
2.2. Material and method

All solvents and reagents were purchased from Sigma Aldrich (USA) and Merck (Germany) and used without further purification. Merck silica gel 60, F254, was used as a pre-coated aluminum plates for monitoring the reactions and spots were detected by CAMAG UV Cabinet. Melting points of the synthesized compounds were determined with electrothermal melting point apparatus (Electrothermal 9200, USA) and are uncorrected. The IR spectra of the compounds in 400–4000 cm^{-1} were recoded Rey light WQF-510 IR/PerkinElmer 1420 Infrared instrument (USA) using KBr pellets from. ^{1}H and ^{13}C nuclear magnetic resonance (^{1}H and ^{13}CNMR) spectra were recorded on a Bruker FT-400 in Dimethyl sulfoxide-D6 (DMSO-d6) and chemical shifts are reported as δ (parts per million, ppm) using Trimethylsilane as internal standard.

2.2.1. Synthesis of triazine-tyrosine hybrids containing thiazole or pyridine derivatives

The desired compounds were prepared according to the pathway depicted in Scheme 2.

2.2.1.1. Synthesis of Cu (Tyrosine)$_2$ (3). To a suspension of L-tyrosine (**1**, 0.2 g, 1.1 mmol)) in 10 mL water, aqueous solution of NaOH 1.0 M was added dropwise with vigorous stirring until the complete dissolution of tyrosine. Then aqueous solution of copper sulfate pentahydrate (**2**) (0.137 g, 0.55 mmol) was added and the pH of the solution was adjusted to 7 and the solution was stirred with a



Scheme 2. General procedure for the synthesis of compounds **8a-8f**.

stirrer for one day, then the precipitates were washed with cold water and dried [19].

2.2.1.2. Di (2-amino-3-(4-hydroxyphenyl) propanoate) Cu (II)(3). Yield: 91 %, blue solid, mp: >245 °C (decomposed) (mp: 250 °C [19]). FT-IR (KBr, cm^{-1}) ν: 3403 (OH), 3295 (NH_2), 1580 (C=O), 1400 (C-O^-), 939 (Cu-O), 827 (Cu-N).

2.2.1.3. Synthesis of dichlorotriazine derivatives (6a-6f). In order to synthesize **6a-6f** compounds, a solution of different aromatic amines (**5a-5f**, 2.7 mmol) in acetone (5 ml) was added dropwise to the solution of cyanuric chloride (**4**, 0.5 g, 2.7 mmol) in acetone (5 ml) and the resulting solution was stirred for 3 h at 0 °C. After completion of the reaction confirmed the by TLC, the obtained precipitation was filtered and washed with cold acetone and then dried [20].

*2.2.1.3.1. 4,6-Dichloro-N-(pyridine-2-yl)-1,3,5-triazin-2-amine **(6a)**.* Yield: 67 %, yellow solid, mp: 256–260 °C (mp: 258–260 °C [20]).FT-IR (KBr, cm^{-1}) ν: 3200 (NH), 3017 (CH Aromatic), 1621 (CN Aromatic), 1647-1452 (CC Aromatic), 761 (CCl).

*2.2.1.3.2. 4,6-Dichloro-N-(pyridine-3-yl)-1,3,5-triazin-2-amine **(6b)**.* Yield: 74 %, yellow solid, mp: >125 °C (decomposed).FT-IR (KBr, cm^{-1}) ν: 3213 (NH), 3099 (CH Aromatic), 1572 (CN Aromatic), 1632-1448 (CC Aromatic), 773 (CCl); ^{1}H NMR (400 MHz, DMSO) δ: 8.19–8.17 (br, 1H, NH), 7.97–7.89 (m, 1H, Ar-CH), 7.03–7.01 (m, 1H, Ar-CH), 6.99–6.83 (m, 2H, Ar-CH).

*2.2.1.3.3. N-(4,6-dichloro-1,3,5-triazin-2-yl)-5-methylthiazole-2-amine **(6c)**.* Yield: 51 %, yellow solid, mp: 157–160 °C, FT-IR (KBr, cm^{-1}) ν: 3298 (NH), 3099 (CH Aromatic), 2891 (CH Aliphatic), 1570 (CN Aromatic), 1618-1484 (CC Aromatic), 771 (CCl); ^{1}H NMR (400 MHz, DMSO) δ: 7.13 (s, 1H, NH), 6.49 (s, 1H, Ar-CH), 2.22 (s, 3H, CH_3).

*2.2.1.3.4. N-(4,6-dichloro-1,3,5-triazin-2-yl) benzo [d] thiazol-2-amine **(6d)**.* Yield: 66 %, yellow solid, mp: 225–229 °C, FT-IR (KBr, cm^{-1}) ν: 3200 (NH), 3030 (CH Aromatic), 1578 (CN Aromatic), 1650-1465 (CC Aromatic), 778 (CCl); ^{1}H NMR (400 MHz, DMSO) δ: 9.05–9.02 (br,1H, NH), 7.95–7.15 (m,4H, Ar-CH).

*2.2.1.3.5. 2-((4,6-Dichloro-1,3,5-triazin-2-yl) thio) benzo [d] thiazol **(6e)**.* Yield: 60 %, white solid, mp: 207–210 °C (mp: 209–210 °C [21]), FT-IR (KBr, cm^{-1}) ν: 3088 (CH Aromatic), 1556 (CN Aromatic), 1650-1481 (CC Aromatic), 770 (CCl).

*2.2.1.3.6. N-(4,6-dichloro-1,3,5-triazin-2-yl)-6-(trifluoromethoxy) benzo [d] thiazol-2-amine **(6f)**.* Yield: 70 %, yellow solid, mp: >210 °C (decomposed).FT-IR (KBr, cm^{-1}) ν: 3245 (NH), 3078 (CH Aromatic), 1532 (CN Aromatic), 1610-1480 (CC Aromatic), 1252 (C-O), 770 (CCl); ^{1}H NMR (400 MHz, DMSO) δ: 8.35–8.34 (br,1H, NH), 7.86–7.25 (m,3H, Ar-CH).

2.2.1.4. Synthesis of Cu (Tyrosine)$_2$-triazine derivatives (7a-7f). For the synthesis of **7a-7f** compounds, 0.03 g (0.07 mmol) of compound (**3**) in water (10 ml) containing 5 ml of NaOH (1N) was added dropwise to the 0.035 mmol of **6a-6f** derivatives in N,N-Dimethylformamide (5 ml) and stirred in room temperature for 24 h. After confirming the reaction by TLC, the obtained precipitations were separated with filter paper and washed with water and acetone. The resulted products dried and used for next step.

*2.2.1.4.1. Di (2-amino-3-(4-((4-chloro-6-(pyridine-2-ylamino)-1,3,5-triazin-2-yl) oxy) phenyl) propanoate) Cu (II) **(7a)**.* Yield: 64 %, green solid, mp: >175 °C (decomposed). FT-IR (KBr, cm^{-1}) ν: 3323 (NH), 2935 (CH Aliphatic), 1582 (C=O), 1539 (CN Aromatic), 1600-1444 (CC Aromatic), 1338 (C-O), 857, 965(Cu-O), 803 (CCl).

*2.2.1.4.2. Di (2-amino-3-(4-((4-chloro-6-(pyridine-3-ylamino)-1,3,5-triazin-2-yl) oxy) phenyl) propanoate) Cu (II) **(7b)**.* Yield: 57 %, green solid, mp: >185 °C (decomposed). FT-IR (KBr, cm^{-1}) ν: 3254 (NH), 2860 (CH Aliphatic),1585 (C=O), 1538 (CN Aromatic), 1620-1418 (CC Aromatic), 1113 (C-O), 960 (Cu-O), 761 (CCl).

*2.2.1.4.3. Di (2-amino-3-(4-((4-chloro-6-((5-methlthiazol-2-yl) amino)-1,3,5-triazin-2yl) oxy) phenyl) propanoate) Cu (II) **(7c)**.* Yield: 65 %, green solid, mp: >154 °C (decomposed). FT-IR (KBr, cm^{-1}) ν: 3402 (NH), 2912 (CH Aliphatic),1580 (C=O), 1539(CN Aromatic),1666-1469 (CC Aromatic), 1219 (C-O), 840 (Cu-N), 777 (CCl).

*2.2.1.4.4. Di (2-amino-3-(4-((4-benzo [d]thiazol-2-ylamino)-6-chloro-1,3,5-triazin-2yl) oxy) phenyl) propanoate) Cu (II) **(7d)**.* Yield: 60 %, green solid, mp: >195 °C (decomposed). FT-IR (KBr, cm^{-1}) ν: 3222 (NH), 2955 (CH Aliphatic), 1570 (C=O), 1555 (CN Aromatic), 1650-1468 (CC Aromatic), 1224 (C-O), 845,960 (Cu-O), 781 (CCl).

*2.2.1.4.5. Di (2-amino-3-(4-((4-(benzo [d] thiazol-2-yl thio)-6chloro-1,3,5-triazin-2-yl) oxy) phenyl) propanoate) Cu (II) **(7e)**.* Yield: 52 %, yellow solid, mp: >200 °C (decomposed). FT-IR (KBr, cm^{-1}) ν: 3218 (NH), 2919 (CH Aliphatic), 1580 (C=O), 1559 (CN Aromatic), 1600-1410 (CC Aromatic), 1116 (C-O), 840 (Cu-N), 718 (CCl).

*2.2.1.4.6. Di (2-amino-3-(4-((4-chloro-6-((6-(trifluoromethoxy) benzo [d] thiazol-2-yl) amino)-1,3,5-triazin-2-yl) oxy) phenyl) propanoate) Cu (II) **(7f)**.* Yield: 67 %, green solid, mp: >240 °C (decomposed). FT-IR (KBr, cm^{-1}) ν: 3301 (NH), 2870 (CH Aliphatic), 1585 (C=O), 1533 (CN Aromatic), 1540-1485 (CC Aromatic), 1234 (C-O), 819 (Cu-N), 780 (CCl).

2.2.1.5. Synthesis of final tyrosine -triazine derivatives containing thiyazol or pyridine derivatives (8a-8f). In this step, 10 ml of hydrochloric acid (6N) was added to 1 mmol of compound **7a-7f** and the reaction mixture was stirred at room temperature for 24 h. After following the completion of the reaction with TLC, the precipitates were filtered and washed with water and dried as compound **8a-8f**.

*2.2.1.5.1. 2-Amino-3-(4-((4-chloro-6-(pyridine-2-ylamino)-1,3,5-triazin-2yl) oxy) phenyl) propanoic acid **(8a)**.* Yield: 60 %, yellow solid, mp: >180 °C (decomposed).FT-IR (KBr, cm^{-1}) ν:3457-2572 (OH, COOH), 3309 (NH_2, NH), 2951 (CH Aliphatic), 1630 (C=O), 1572 (CN Aromatic), 1670-1412 (CC Aromatic), 1285 (C-O), 761 (CCl); ^{1}H NMR (400 MHz, DMSO) δ: 12.09 (s, 1H, COOH), 8.37 (s, 1H, NH), 7.89–6.79 (m, 8H, Ar-CH, and 2H, NH_2), 4.37–4.22 (m, 1H, CH), 3.15–3.09 (dd, J = 20 Hz, J = 4 Hz, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-ppm) δ: 35.4, 55.7, 174.7, 135.3, 129.8, 117.9, 125.4, 129.8, 102.9, 132.5, 154.5, 148.1, 168.5, 150.4, 186.0, 165.5.

*2.2.1.5.2. 2-Amino-3-(4-((4-chloro-6-(pyridine-3-ylamino)-1,3,5-triazin-2yl) oxy) phenyl) propanoic acid **(8b)**.* Yield: 68 %, brown solid, mp: >230 °C (decomposed).FT-IR (KBr, cm^{-1}) ν: 3390-2510 (OH, COOH), 3260 (NH_2, NH), 2954 (CH Aliphatic), 1629 (C=O), 1509 (CN Aromatic), 1617-1445 (CC Aromatic), 1290 (C-O), 772 (CCl); ^{1}H NMR (400 MHz, DMSO) δ: 11.75 (s, 1H, COOH), 8.84–8.71

(br, 1H, NH), 8.68–7.19 (m, 8H, Ar-CH, and 2H, NH_2), 4.32–4.26 (m, 1H, CH), 3.16–3.04 (m, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-ppm) δ: 36.4, 55.7, 174.7, 130.8, 122.8, 125 0.7, 125.4, 129.8, 125.4, 132.5, 143.0, 136.5, 138.5, 172.2, 150.4, 180.1, 168.7.

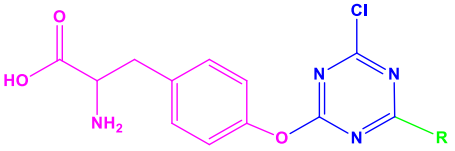
2.2.1.5.3. 2-Amino-3-(4-((4-chloro-6-((5-methlthiazol-2-yl) amino)-1,3,5-triazin-2yl) oxy) phenyl) propanoic acid ***(8c)***. Yield: 48 %, brown solid, mp: >203 °C (decomposed). FT-IR (KBr, cm^{-1}) ν:3394-2525 (OH, COOH), 3264 (NH_2, NH), 2891 (CH Aliphatic),1631 (C=O), 1540 (CN Aromatic), 1666-1469 (CC Aromatic), 1139 (C-O), 781 (CCl); ^1H NMR (400 MHz, DMSO) δ: 11.83 (s, 1H, COOH), 7.99 (s, 1H, NH), 7.52–7.34 (m, 4H, Ar-CH_a), 6.92 (s, 2H, NH_2), 6.70 (s, 1H, Ar-CH_b),4.24–4.22 (d, J = 7 Hz, 1H, CH), 3.11–3.06 (dd, J = 17 Hz, J = 7 Hz, 2H, CH), 2.29–2.24 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-ppm) δ: 37.9, 15.8, 54.7, 174.7, 127.8, 125.4, 130.8, 125.4, 132.5, 168.5, 150.4, 183.0, 165.5, 135.4, 120.5, 158.2.

2.2.1.5.4. 2-Amino-3-(4-((4-benzo [d]thiazol-2-ylamino)-6-chloro-1,3,5-triazin-2yl) oxy) phenyl) propanoic acid ***(8d)***. Yield: 54 %, yellow solid, mp: >134 °C (decomposed). FT-IR (KBr, cm^{-1}) ν: 3317-2619(OH, COOH), 3218 (NH_2, NH), 2930 (CH Aliphatic),1631 (C=O), 1567 (CN Aromatic), 1650-1451 (CC Aromatic), 1223 (C-O), 778 (CCl); ^1H NMR (400 MHz, DMSO) δ: 13.18 (s, 1H, COOH), 8.89 (s, 1H, NH), 8.14 (s, 2H, NH_2), 7.69–7.22 (m, 8H, Ar-CH), 4.04 (m, 1H, CH), 3.10–3.06 (dd, J = 15 Hz, J = 5 Hz, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-ppm) δ: 37.1, 55.7, 174.3, 128.8, 125.1, 124.5, 125.4, 129.6, 125.4, 132.5, 118.3, 121.8, 161.5, 150.4, 186.0, 165.5, 156.2, 130.8, 174.5.

2.2.1.5.5. 2-Amino-3-(4-((4-benzo [d]thiazol-2-ylthio)-6-chloro-1,3,5-triazin-2yl) oxy) phenyl) propanoic acid ***(8e)***. Yield: 57 %, yellow solid, mp: >205 °C (decomposed). FT-IR (KBr, cm^{-1}) ν: 3397-2499 (OH, COOH), 3395 (NH), 2872 (CH Aliphatic), 1630 (C=O), 1555 (CN Aromatic), 1653-1471 (CC Aromatic), 1232 (C-O), 787 (CCl); ^1H NMR (400 MHz, DMSO) δ: 13.78 (s, 1H, COOH), 8.15–8.03 (br, 2H, NH_2), 7.71–7.29 (m, 8H, Ar-CH), 4.37 (s, 1H, CH), 3.15–3.11 (m, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-ppm) δ: 37.3, 50.7, 121.1, 124.5, 125.3, 129.8, 135.4, 137.8, 150.4, 155.5, 166.6, 169.8, 173.4, 178.7, 194.9.

2.2.1.5.6. 2-Amino-3-(4-((4-chloro-6-((6-(trifluoromethoxy) benzo [d] thiazol-2-yl) amino)-1,3,5-triazin-2-yl) oxy) phenyl) propanoic acid ***(8f)***. Yield: 63 %, yellow solid, mp: >172 °C (decomposed).FT-IR (KBr, cm^{-1}) ν: 3417-2414 (OH, COOH), 3287 (NH, NH_2), 2911 (CH Aliphatic), 1633 (C=O), 1572 (CN Aromatic), 1624-1471 (CC Aromatic), 1238 (C-O), 795 (CCl); ^1H NMR (400 MHz, DMSO) δ: 13.02 (s, 1H, COOH), 8.73 (s, 1H, NH), 8.21 (s, 2H, NH_2), 7.75–7.47 (m, 7H, Ar-CH), 4.33 (s, 1H, CH), 3.43–3.28 (dd, J = 20 Hz, J = 5 Hz, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-ppm) δ: 37.3, 56.7, 174.7, 129.7, 129.8, 125.4, 129.8, 125.4, 114.6, 132.5, 110.2, 104.9, 168.5, 156.7, 150.4, 186.0, 165.5, 145.5, 131.9, 174.5.

Table 1
ΔG_b (kcal/mol), interactions as well as Ki of synthesized compounds with S1P1 and S1P3 receptors calculated by AutoDock.



cod	R	receptor	ΔG_b (Kcal/mol)	K_i	Hydrogen bonds	Hydrophobic interaction
8a	2-aminopyridine	S1P1	−7.36	3.99 μM	Ser 129 Cys 206	Phe125 Leu128,276
		S1P3	1.09	–	–	–
8b	3-aminopyridine	S1P1	−7.32	4.29 μM	Glu121	Phe125 Leu276,213
		S1P3	1.08	–	–	–
8c	2-aminomethylthiazole	S1P1	−6.02	38.43 μM	Glu 294	Phe125 Leu128,272
		S1P3	0.89	–	–	–
8d	2- aminobenzothiazole	S1P1	−7.12	6.08 μM	Glu121 Arg120	Phe125 Leu128,276
		S1P3	0.62	–	–	–
8e	2-mercaptobenzothiazole	S1P1	−7.43	3.6 μM	Glu121	Phe125 Leu272,276
		S1P3	0.27	–	–	–
8f	riluzole	S1P1	−3.89	1.4 mM	Ser 123 Leu93	Leu93,127 Ala 127,130
		S1P3	−6.99	7.51 μM	Tyr92 Phe119	Leu122,189 Ile 96,284
	fingolimod	S1P1	−5.95	43.37 μM	Glu121	Phe125,210 Leu128,272
		S1P3	−5.83	53.44 μM	Ser 99 Glu 115	Phe119 Leu122
	siponimod	S1P1	−9.8	65.34 nM	Arg120 Ser105	Phe125 Leu128,272,297
		S1P3	−8.12	41.3 nM	–	Phe119,260 Leu122,295

(————————) not seen.

2.3. In vivo biological activity (counting lymphocyte cells)

The lymphocyte reduction activity of the desired compounds was analyzed on animal model [22,23]. For this purpose, 6-8-week--old male Wistar rats (160–180 mg) were used. The rats were kept at 22 ± 5 °C on a 12/12-h light/dark cycle for three days. During this time the rats had access to sufficient food and water. The animal experimental procedures and protocols were accepted by the Isfahan University of Medical Sciences, Institutional Research Ethics Committee (Ethical No. IR.MUI.RESEARCH.REC.1399.828). The synthesized compounds and also Fingolimod as a standard drug were injected intraperitoneally to the rats with doses of 0.3, 1 and 3 mg/kg. Normal saline and DMSO were used as the control. Before and 24 h after injection, with the help of an anesthetic agent, the rats were anesthetized and then retro-orbital blood sampling was performed. The blood samples were evaluated to count lymphocyte and red blood cells.

2.3.1. Statistical analysis

Data were represented as the mean ± standard error of mean (SEM). Two-way analysis of variance (ANOVA) followed by Tukey post-hoc test was used by Graphpad prism software to compare the means. P values < 0.05 were considered as significant difference.

3. Results and discussion

3.1. Docking study

The free energies of binding (ΔG_b), inhibition constants (K_i) and hydrogen bond interaction obtained from the docking studies of the compounds with S1P1 and S1P3 using Autodock4 were presented in Table 1. Fingolimod and Siponimod as a refences ligands also were docked to the mentioned enzymes.

According to docking results, generally, the ΔG_b of designed compounds with S1P1 receptor was more negative (better) compared to S1P3 receptor, which indicates the theoretical selectivity of these compounds for S1P1. In addition, the observed interactions to the S1P1 sub-receptor are similar to the interactions of fingolimod and siponimod. In interaction with S1P1 receptor, fingolimod showed a hydrogen bond between the polar side of the molecule with glutamic amino acid 121 (Glu121) and also exhibited hydrophobic interactions with leucine 128 and 272 (Leu128,272) and phenylalanine 125 and 210 (Phe125, 210). The observed interactions are in agreement with previous studies [24,25]. In interaction with S1P1 receptor, siponimod exhibited a hydrogen bond from the polar side of the molecule with asparagine 101 (Asn 101), serine 105 (Ser105), arginine 120 (Arg120) and Glu121, which is similar to the reported interactions in Alizadeh et al.'s study [26]. It also showed hydrophobic interactions with Phe125 and Leu272,297,128,195.

In all synthesized compounds, the polar head of the molecule was able to established at least one hydrogen bond with the S1P1 receptor. The triazine ring and terminal aromatic amine in these compounds were involved in van der Waals interactions with the hydrophobic amino acids.

The compound with 2-mercaptobenzothiazole substitution (**8e**), which was recognized as the best compound in the biological test, similar to fingolimod showed a hydrogen bond with the Glu121. In this compound, triazine ring formed hydrophobic interactions with Leu 195 and Phe125 and also benzothiazole ring exhibiter hydrophobic interaction with Leu272, Leu276 and Phe 210. According to docking results, the compound **8e** with the ΔG_b of −7.43 (Kcal/mol) showed the best theoretical interaction with the S1P1 receptor (Fig. 1).

3.2. Preparation of novel triazine -tyrosin hybrids containing pyridine or thiazole

In chemistry section of the study, to maintain the amino acid fragment as the polar head of the molecule, this part must be protected. So, the formation of a complex between the amino acid fragment **(1)** and copper **(2)** was used to protect the amino acid group

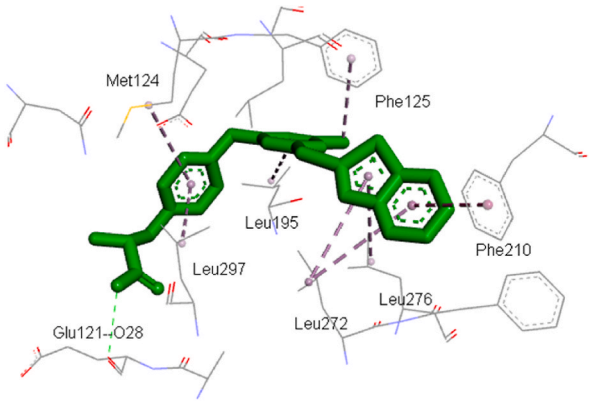


Fig. 1. The binding mode of compounds **8e** in the active site of S1P1.

and also increase the nucleophilic potency of the phenolic hydroxy (Scheme 2). On the other hand, nucleophilic attack of different aromatic amin **(5a-5f)** to cyanuric chloride **(4)** resulted in replacement of one chloro group on triazine ring. It should be mentioned that three chloro groups in cyanuric chloride **(4)** could be replaced in different condition. The first chloro of cyanuric chloride can be substituted at low temperature (below 5 °C), while at room temperature two of them can participate in substitution reactions, and three chloro groups can be replaced with nucleophiles under high temperatures, [27]. In the next step, disubstituted s-triazine compounds (**7a-7f**) were obtained by the reaction of **(3)** with **6a-6f** derivatives and in this way, the amino acid complexed with copper was placed on the triazine ring. Finally, to form the polar head of the molecule, the copper complex was broken in an acidic environment and the amino acid head of the molecule was established in compounds **(8a-8f)**.

3.3. Characterizations of synthesized compounds

Novel triazine -tyrosin derivatives were characterized by spectral techniques such as ^{1}H NMR, ^{13}CNMR and FT-IR techniques. FTIR measures the absorption of infrared radiation, which occurs due to changes in molecular vibrations and dipole moments. It provides information about bond stretching, bending, and functional groups. NMR measures the nuclear magnetic resonance of specific atomic nuclei analyses the coupling pattern between the different nuclei and provides information about the content and purity of a sample as well as its molecular structure [28,29]. In FT-IR spectrum of compound **3,** the observed decrease in the wave number of the carbonyl group (1580 cm^{-1}) compared to the carbonyl group of tyrosine (1628 cm^{-1}) was attributed to the formation of a complex between tyrosine and copper ion. In addition, the observation of new peaks at 937 and 827 cm^{-1} compared to the tyrosine spectrum confirms the presence of metal-oxygen and metal-nitrogen groups (Fig. 2). On the other hand, by insertion different aromatic amine on the triazine ring, the vibrations in 3200–3300 cm^{-1}, related to the NH group, could be observed in the IR spectrum of compound (**6a-6f** except **6e**). In the IR spectra of compounds **7a-7f**, the vibrations related to both tyrosine-copper complex and aromatic amine substituted triazine could be seen. Finally, the observed peak in the region of 2500–3500 in the spectrum of compounds **8a-8f** confirmed the presence of the unprotected acid group. FT-IR spectrum of compound **8e** is shown as an example in Fig. 2.

The ^{1}H NMR spectral data of the synthesized compounds recorded in (D_6) DMSO along with its possible assignments reported in the experimental part. All the aromatic and aliphatic hydrogen related to three connected fragments were found in their expected regions. In final products acidic hydrogen of tyrosine moity was observed around 12.00–13.80 ppm. Aliphatic hydrogen atoms of CH_2 and CH exhibited peaks at 3.00–3.15 and 4.04–4.37 ppm, respectively. Aromatic hydrogens were found in the region at 6.70–8.68 ppm. In derivatives having NH, this group exhibited a broad singlet at 7.99–8.89 ppm. As an example, ^{1}HNMR spectrum of compound **8e** was shown in Fig. 3a. In the ^{13}CNMR spectrum of compounds **8a-8f**, methyne and chiral center were presented around 40 and 50 ppm. The peak at 173–175 was attributed to carbon of carbonyl groups. Aromatic carbone were founded in 120–195 ppm. As an example,

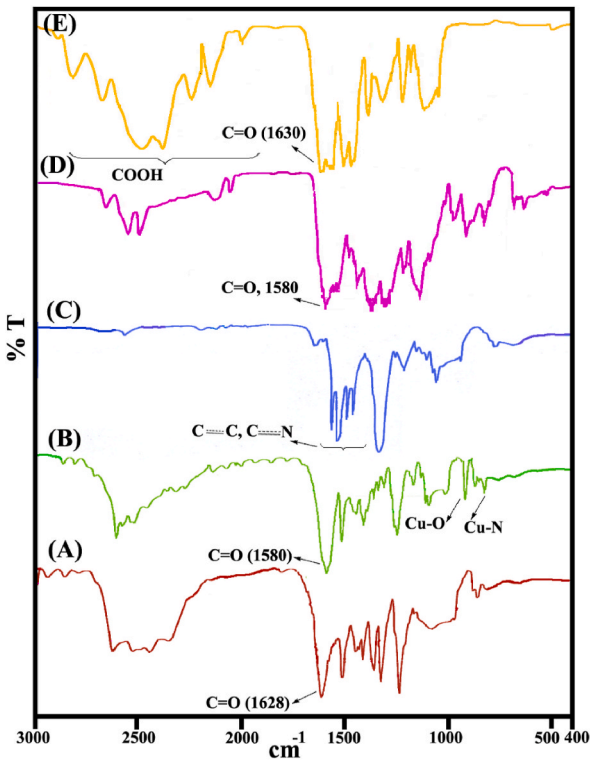


Fig. 2. FT-IR spectrum of compounds (A) tyrosine, (B) Cu-Tyrosine complex, (C) aromatic amine substituted triazine (**6e**), (D) substituted triazine-Tyrosine - Cu derivative (**7a**) and (E). substituted triazine- Tyrosine derivative (**8e**).

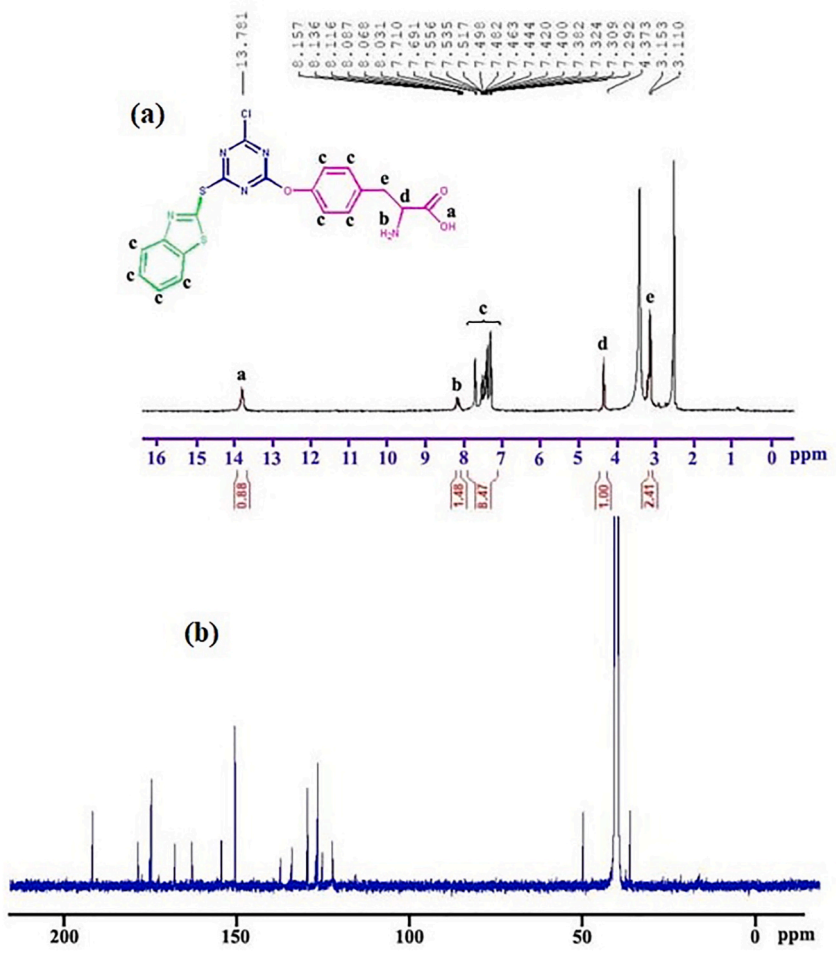


Fig. 3. (a) ^{1}HNMR spectrum of compounds **8e** and (b) ^{13}CNMR spectrum of compounds **8e**.

^{13}CNMR spectrum of compound **8e** was shown in Fig. 3b. In this spectrum the carbone attached to benzothiazole and tyrosine moities were obsedved in 194.9 and 178.7 ppm, respectively.

3.4. *In vivo biological activity (counting lymphocyte cells)*

Mechanism of fingolimod as an approved drug for MS was to prevent the release and migration of lymphocytes to the central nervous system [17,27]. In fact lymphocyte reduction is the main characteristic of fingolimod as S1P1 modulators. According to pervious study, Mehling et al. [30] showed that in MS patients treated with fingolimod, the total number of blood lymphocytes decreases by 60–80 % compared to healthy individuals and MS patients without treatment. In a study, Foster et al. [31] showed that 6 h

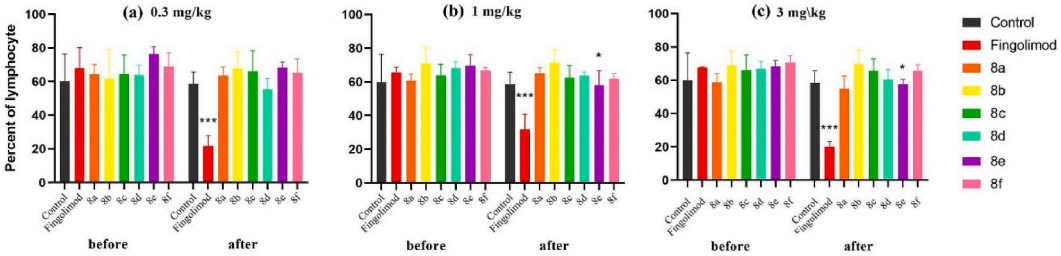


Fig. 4. Lymphocytes percentage before and after the injection of fingolimod and **8a-8f** at (a) 0.3 mg/kg, (b) 1 mg/kg, and (c) 3 mg/kg. Lymphocyte percentage was determined using the flow cytometry method. Data are shown as mean ± SEM. $^{*}P < 0.05$ and $^{***}P < 0.001$ indicate significant differences before and after injection of the same compound and with control.

after oral administration of a single dose of fingolimod at dose of 0.3 mg/kg in rats, the number of blood lymphocytes decreased by 70 %. Also, Chiba et al. [32] in their study showed that within 3–24 h after a single oral administration of fingolimod with a dose of 0.1–1 mg/kg, the lymphocytes of rats were reduced by 90 % in blood and lymph and 40 %–80 % in Spleen decreases. In this study peripheral lymphocyte reduction was used as a pharmacodynamic biomarker and the ability of synthesized compounds to reduce lymphocytes was investigated. Fig. 4a-c showed the mean lymphocyte of different groups of rats ($n = 6$) in different doses (0.3, 1 and 3 mg/kg) of **8a-8f** and fingolimod treatment. In 0.3 mg/kg dose, fingolimod with a significant difference ($P < 0.001$) has notable reduction in lymphocyte counts (from 67.93 % to 21.83 %) after 24 h, while no significant decrease of lymphocytes was seen for the synthesized compounds (**8a-8f**) in this dose. In the next groups, treated with the dose of 1 mg/kg, fingolimod and compound **8e** were able to significantly ($P < 0.05$) reduce lymphocytes count from 65.73 % to 31.68 % and 68.80 %–56.90 %, respectively. These two substances also showed a significant ($P < 0.001$) decrease in lymphocyte counts at dose 3 mg/kg after 24 h. In this dose, fingolimod and compound **8e** reduced the lymphocytes from 67.64 % to 20.66 % and 67.85 %–56.85 %, respectively. Additionally, in this study no significant difference in the results of the control group before and 24 h after injection, showing the ineffectiveness of vehicle injection on the measured factors. According to the obtained results, compound **8e** with a significant decrease in the number of lymphocytes at doses 3 and 1 mg/kg compared to fingolimod was the best compound in this study.

Additionally, effect of the synthesized compounds on red blood cells was shown in Fig. 5. As can be seen from Fig. 5a–5c, the significant decreased number of red blood cells was observed in the dose of 0.3 mg/kg of fingolimod (9.22×10^6 to 7.68×10^6) and all the synthesized compounds (9.62×10^6 to 8.02×10^6). With increasing the administration dose to 1 mg/kg, red blood cells were decreased from 9.21×10^6 to 7.7×10^6 for fingolimod which is similar to compounds **8c**, **8d** and **8b** (9.40×10^6 to 7.99×10^6), while compounds **8e**, **8f** and **8a** exhibited a greater reducing effect (9.39×10^6 to 7.52×10^6) at this dose. After injection of 3 mg/kg, fingolimod showed no significant effect on the number of red blood cells, while compound **8d** and **8f** caused a significant ($P < 0.05$) decrease in red blood cells (8.84×10^6 to 7.24×10^6) and also greater reducing effect (9.60×10^6 to 7.45×10^6) was observed for compounds **8a**,**8b** and **8c** ($P < 0.001$).

Concerning the obtained results, fingolimod at doses of 0.3 and 1 mg/kg, reduces the number of red blood cells in rats which was similar to the previous studies [22,33,34], but at a dose of 3 mg/kg, it does not have a significant effect on the number of red blood cells [22]. According to FDA documents [35], using doses of 3, 10 and 30 mg/kg/day of fingolimod did not show a dose-dependent behavior in reducing red blood cells. At a dose of 3 mg/kg, it decreased the number of red blood cells in female rats, but at a dose of 30 mg/kg, the number of red blood cells in male rats increased significantly, while at a dose of 10 mg/kg, it increased the number of red blood cells in both sexes of rats.

4. Conclusion

Novel triazine-tyrosine hybrids containing thiazole or pyridine fragments were designed, synthesized and evaluated as potential anti-MS agents. Docking studies was performed using crystal structures of S1P1 and S1P3 receptors to theoretically identify the selectivity of the compounds towards the S1P1 isoform. The docking results showed better interactions (lower ΔG_b) and therefor higher selectivity for S1P1 receptor than S1P3 receptor. After synthesis of **8a-8f**, theire structures were confirmed through spectrophotometric methods (FT-IR, and NMR). Considering the importance of the S1P1 receptor in release of lymphocytes and therefore inflammation produced in MS disease, the synthesized compounds were investigated to study lymphocyte reduction on animal models. Compound **8e,** with 2-mercaptobenzothiazole substitution, at a dose of 1 and 3 mg/kg showed significant reduction effect on the percentage of lymphocytes (68.80 %, 56.75 %) compared to the fingolimod (65.73 %, 20.66 %) as control group. Further studies are necessary to understand the underlying and implicated mechanisms of observed pharmacologic effects.

Data availability

Data will be made available on request.

Compliance with ethical standards

The study was approved by the Institutional Research Ethics Committee of Isfahan University of Medical Sciences with ethic approval ID: IR.MUI.RESEARCH.REC. 1399.828. The animal experimental practice was carried out in accordance with the international guidelines for laboratory animal use and care (European Directive 2010/63/EU).

CRediT authorship contribution statement

Parvin Asadi: Writing – review & editing, Software, Resources, Methodology, Data curation. **Fateme Mahdie:** Writing – original draft, Software, Investigation, Formal analysis, Data curation. **Ghadamali Khodarahmi:** Writing – review & editing, Validation, Supervision, Conceptualization. **Leila Safaeian:** Writing – review & editing, Visualization, Methodology, Data curation, Conceptualization, Prof. **Farshid Hassanzade:** Writing – review & editing, Visualization, Validation, Conceptualization, Dr.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

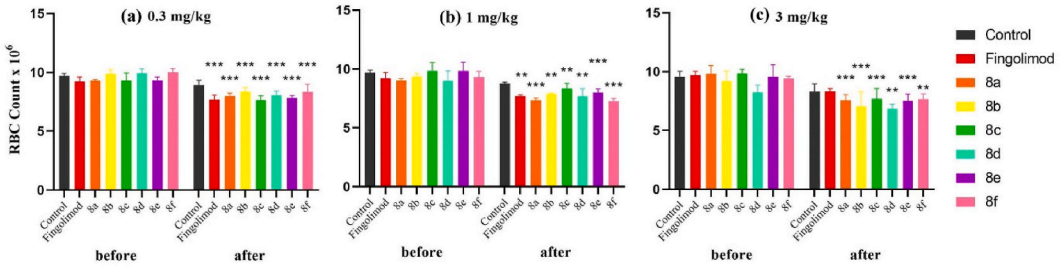


Fig. 5. Red blood cells (RBC) count before and after the injection of fingolimod and **8a-8f** at (a) 0.3 mg/kg, (b) 1 mg/kg, and (c) 3 mg/kg. RBC count was determined using the flow cytometry method. Data are shown as mean ± SEM. **P < 0.01 and ***P < 0.001 indicate significant differences before and after injection of the same compound and with control.

influence the work reported in this paper.

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