Chem Biol Drug Des 2011; **78:** 438–444

Research Article

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doi: 10.1111/j.1747-0285.2011.01167.x

Safety and Efficacy of New 3,6-diaryl-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine Analogs as Potential Phosphodiesterase-4 Inhibitors in NIH-3T3 Mouse Fibroblastic Cells

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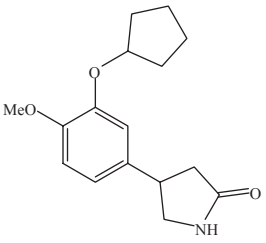
A novel series of potential phosphodiesterase-4 (PDE-4) inhibitors, 6-(3-(cyclopentyloxy)-4-methoxyphenyl)-3-aryl-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazines, were developed. Different concentrations of the synthesized compounds were tested on cultured NIH-3T3 cells to determine their safety and efficacy in NIH-3T3 mouse fibroblastic cells in comparison with rolipram, a selective PDE-4 inhibitor. The viability of cells was determined by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. The PDE inhibition rate was measured indirectly by determination of concentrations of extracellular cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) using enzyme-linked immunoassay technique. The results showed that all tested compounds caused a marked increase in the concentration of cAMP, whereas the concentration of cGMP stayed approximately unchanged. The cytotoxic IC_{50} of all synthesized compounds was approximately twofold greater than their required concentration for inhibition of PDE-4 (in terms of elevation of cAMP), and thus, these structures could be used to develop potent and safe inhibitors of PDE-4 enzyme.

Key words: cAMP, cGMP, drug discovery, phosphodiesterase inhibitor

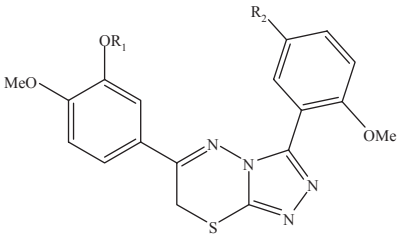
Received 2 June 2010, revised 3 June 2011 and accepted for publication 27 June 2011

Cyclic nucleotide phosphodiesterases (PDEs) are the enzymes in charge for the hydrolysis of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) playing a key role in many physiological and pathological processes in mammalian organs. PDE inhibitors have revealed numerous worthy pharmacological properties in various organs like cardiovascular, central nervous system, respiratory tract, gastrointestinal tract (GI), and as antioxidants (1–4). PDE-4 inhibitors are especially useful for the treatment of inflammation in illnesses like asthma and chronic obstructive pulmonary disorders (COPD, emphysema, and bronchitis), as well as for the treatment of depression, psychosis, and memory problems (5). As a result, diverse synthetic PDE-4 inhibitors are under development (5–7) and several natural PDE inhibitors have been derived from medicinal plants (4).

As the development of PDE-4 inhibitors, such as rolipram **1** (Figure 1), and structurally related compounds are restricted by some side effects, the development of unique, powerful, and selective PDE-4 inhibitors with lower side effects is an urgent need. Recently, several alkoxy-substituted 3,6-diphenyl-7*H*-[1,2,4]triazolo[1,3,4]thiadiazines, including **2** (Figure 1), as potent PDE-4 inhibitors, have been reported (5–7). To extend the structure activity relationship (SAR) of this series of compounds, we report the synthesis, efficacy, and safety of some new 6-(3-(cyclopentyloxy)-4-methoxyphenyl)-3-aryl-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazines **3** (Figure 1) while aryl= (2-methoxyphenyl in **6a**), (3-methoxyphenyl in **6b**), (p-Tolyl in **6c**), and (Phenyl in **6d**) tested in NIH-3T3 cells. Preliminary SAR very much suggests that these compounds are among the catechol class of PDE-4 inhibitors; hence, rolipram as a potent and selective PDE-4 inhibitor from this class was used for comparison. Previous study showed that company of a non-large 3,4-functionality placed at the 5 position of the 3,6-dihydro-2*H*-1,3,4-thiadiazine ring, but not the phenyl ring attached at the 3 position of the 1,2,4-triazole, is essential (7). The main purpose of this study was to study the safety and efficacy of 6-(3-(cyclopentyloxy)-4-methoxyphenyl)-3-aryl-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazines in inhibition of cAMP- or cGMP PDE in NIH-3T3 cells.

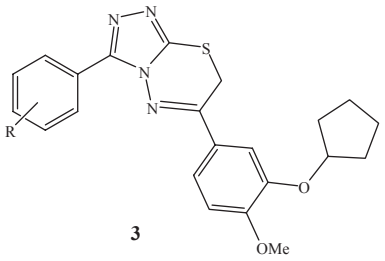


1



2

R_1 = OMe,Cyclopentyl
R_2 = H,OMe



3

3a: R = 2-OMe
3b: R = 3-OMe
3c: R = 4-Me
3d: R = H

Figure 1: Rolipram **1**, alkoxy-substituted 3,6-diphenyl-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazines **2**, some new 6-(3-(cyclopentyloxy)-4-methoxyphenyl)-3-aryl-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazines **3**.

Methods

General chemistry

Melting points were determined on a Kofler hot stage apparatus. The IR spectra were recorded on a Shimadzu 470 spectrophotometer (potassium bromide disks). ^{1}H-NMR spectra were recorded on a Varian unity 400 spectrometer, and chemical shifts were expressed as δ (ppm) with tetramethylsilane as internal standard. MS spectra were obtained with a Finnigan MAT TSQ-70 spectrometer (Finnigan Mat, Bremen, Germany). The purity of synthesized compounds was confirmed by thin layer chromatography using various solvents of different polarities.

Preparation of substituted 6-(3-(cyclopentyloxy)-4-methoxyphenyl)-3-(aryl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine (6a–d)

Ethanol (50 mL) was added to a mixture of substituted 4-amino-5-(aryl)-2,3-dihydro-1,2,4-triazine-3-thion (**5a–d**) (11 mmol) and 2-bromo-1-(3-(cyclopentyloxy)-4-methoxyphenyl)ethanone (11.5 mmol). The reaction mixture was refluxed for 4–5 h. The solvent was removed under reduced pressure, and the residue was treated with HCL (2N, 100 mL). The precipitate was filtered, and the filtrate was treated with NaOH (2N, 100 mL). The product was filtered and crystallized from ethanol–water to give the substituted 6-(3-(cyclopentyloxy)-4-methoxyphenyl)-3-(aryl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine (**6a–d**) in 81% yield.

6-(3-(cyclopentyloxy)-4-methoxyphenyl)-3-(2-methoxyphenyl)-7*H*-[1,2,4]triazolo [3,4 *b*][1,3,4]thiadiazine (6a)

White solid; Yield 82%; mp: 153.9-155.7 °C; IR (KBr, per cm): 3425.2, 2970.9, 1669; ^{1}HNMR ($CDCl_3$, 400 MHz) δ: 1.52-2.05 (m, 8H), 3.93 (s, 3H), 3.94 (s, 3H), 4.90 (s, 2H), 4.85 (m, 1H), 6.91 (d, 1H), 7.06 (m, 2H), 7.49 (t, 1H), 7.56 (s, 1H), 7.68 (d, 1H), 7.87 (d, 1H); ^{13}CNMR (125 MHz, $CDCl_3$) δ: 24.08, 32.73, 41.21, 55.94, 56.14, 80.60, 110.60, 111.89, 112.69, 113.48, 120.70, 123.23, 127.96, 130.29, 133.06, 147.91, 155.1, 157.75, 163.62, 164.63, 173.33; MS m/z [%]: 436 [M^+] (44), 404 (11), 368 (100), 353 (47), 336 (31).

6-(3-(cyclopentyloxy)-4-methoxyphenyl)-3-(3-methoxyphenyl)-7*H*-[1,2,4]triazolo [3,4-*b*][1,3,4]thiadiazine (6b)

White solid; Yield 87%; mp: 147.3-178.8 °C; IR (KBr, per cm): 3440.0, 2951.9, 1607; ^{1}HNMR ($CDCl_3$, 400 MHz) δ: 1.8-2.05 (m, 8H), 3.86 (s, 3H), 3.93 (s, 3H), 3.97 (s, 2H), 4.83 (m, 1H), 6.93 (d, 1H), 7.03 (d, 1H),7.36 (s, 1H), 7.38 (t, 1H), 7.64 (s, 1H), 7.76 (d, 2H); ^{13}CNMR (125 MHz, $CDCl_3$): 22.79, 24.2, 32.81, 55.43, 56.14, 80.65, 110.96, 112.29, 113.46, 116.11, 120.63, 120.95, 125.69, 127.46, 129.43, 148.1, 148.32, 152.18, 153.14, 153.67, 159.61; MS m/z [%]: 436 [M^+] (83), 404 (22), 368 (100), 353 (77), 336 (46).

6-(3-(cyclopentyloxy)-4-methoxyphenyl)-3-p-tolyl-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine (6c)

Light yellow solid, Yield 85%; mp: 196.1–201 °C; IR (KBr, cm^{-1}): 3540.0, 2851.9, 1607; ^{1}HNMR ($CDCl_3$, 400 MHz) δ: 1.68–2.13 (m, 8H), 2.42 (s, 3H), 3.93 (s, 3H), 3.96 (s, 2H), 4.83 (m, 1H), 6.93 (d, 1H, J = 8.2 Hz), 7.28 (d, 1H, J = 8 Hz) 7.36 (d, 1H, J = 8.2 Hz), 7.62 (s, 1H, H^2), 8.05 (d, 1H, J = 8 Hz); ^{13}CNMR (125 MHz, $CDCl_3$): 22.81, 24.13 29.69, 32.79, 56.13, 80.65, 111.00, 112.33, 120.92, 123.52, 125.80, 128.18, 129.16, 140.38, 148.29, 151.95, 152.95, 153.58; MS m/z [%]: 422 [M^+] (3), 404 (20), 368 (100), 353 (77), 336 (49), 325 (10), 307 (5).

6-(3-(cyclopentyloxy)-4-methoxyphenyl)-3-phenyl-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine (6d)

White solid; Yield 88%; mp: 192.7–195.6 °C; IR (KBr, per cm): 3725.2, 2967.9, 2862, 1660; ^{1}HNMR ($CDCl_3$, 400 MHz) δ: 1.8-2.1 (m, 8H), 3.86 (s, 3H), 3.94 (s, 3H), 3.98 (s, 2H), 4.82 (m, 1H), 6.94 (d, 1H), 7.37 (s, 1H), 7.49 (m, 2H), 7.63 (s, 1H), 7.89 (s, 1H), 8.18 (d, 2H); ^{13}CNMR (125 MHz, $CDCl_3$): 24.5, 34.9, 36.3, 55.94, 56.2, 79.3, 114.50, 111.89, 121.8, 126.9, 127.48, 128.8, 129.3, 130.7, 146.7, 148.0, 152.23, 164.6; MS m/z [%]: 406 [M^+] (49), 388 (2), 368 (3.1), 338 (100), 323 (64), 295 (10).

Cell line

The NIH-3T3 mouse fibroblastic cell line was purchased from the Institute of Pasteur at Tehran and was cultured as monolayers on polystyrene culture dishes and maintained at 37 °C in a humidified atmosphere with 5% CO2 in air. NIH-3T3 cells were grown in RPMI medium with L-glutamine, supplemented with 10% fetal bovine serum and 1% antibiotic, 100 units of penicillin G/mL, and 100 μg of streptomycin/mL. Cells were seeded at 24 multi-well plate, and four wells were harvested using trypsin each day until stationary phase was reached. The population doubling time of these cells (26 h) was determined by cell counting.

Treatments

Concerning estimated ED_{50} of rolipram (0.3 μM) (8), five concentrations of 0.05, 0.1, 0.5, 1, and 5 μM were used as control positive groups. As well by considering the average ED_{50} of substituted 3,6-diphenyl-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazines (20 μM) (7), concentrations of 1, 10, 20, 40, and 80 μM of each synthesized compound were prepared in dimethylsulfoxide (DMSO). The cell monolayers in exponential growth were harvested by means of 0.25% trypsin, and at that point, single-cell suspensions were acquired by frequent pipetting. The number of cells was calculated by a hemocytometer, and single-cell suspensions having cell mass of 1×10^3 cells per 100 μL medium/well were added to 96-well plates. Then, cells were protected overnight at 37 °C before treatment. All of the set concentrations of rolipram and created compounds were added to three wells of each plate, together with a group of control which did not receive any drugs. Afterward, the cells were kept warm at 37 °C. At 1, 6, and 24 h after treatment, and the culture medium of every one well of a 96-well plate was moved to a microtube for measurement of cAMP and cGMP concentrations, and then, (3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazoliumbromide (MTT) assay was performed.

Table 1: Concentrations of cAMP after 1, 6, and 24 h of exposure of NIH-3T3 cells to different concentration of **6a–6d** in comparison with those of rolipram

Groups		cAMP concentration (pmol/cell) Time (h)		
		1	6	24
Drug name	Concentration (μM)	Mean ± SEM (percent of increase)	Mean ± SEM (percent of increase)	Mean ± SEM (percent of increase)
Rolipram	0.05	76.3 ± 2.3* (58)	75.6 ± 15.9* (72)	117.3 ± 0.9* (76)
Rolipram	0.1	78 ± 2* (59)	80.1 ± 4.5* (73)	132.3 ± 13.9* (78.8)
Rolipram	0.5	145.3 ± 1.3* (78)	136 ± 2.5* (84)	201.6 ± 6.17* (86)
Rolipram	1	164.6 ± 2.9* (80)	166.6 ± 13.3* (87)	222.6 ± 8.17* (87)
Rolipram	5	220 ± 6.4* (85)	247.3 ± 6.9* (91)	309.3 ± 18.3* (90)
6a	10	85 ± 18.9* (62)	49.1 ± 3.7* (56.8)	40.6 ± 1.45 (31)
6a	20	99.6 ± 2.7* (68)	60.6 ± 1.2* (65)	41 ± 2.08 (31)
6a	40	115.6 ± 2.3* (72)	106.3 ± 5* (80)	51 ± 1.7 (45)
6b	10	104.2 ± 2.1* (69)	110 ± 4.9* (80)	43.6 ± 1.45 (35)
6b	20	123.3 ± 1.15* (74)	136.6 ± 7.3* (84)	95 ± 4.6* (70)
6b	40	132.6 ± 2.9* (76)	157.3 ± 8.8* (86)	173.3 ± 58* (83)
6c	10	124.1 ± 17* (74)	110 ± 4* (80)	35.3 ± 2.02 (20)
6c	20	157.5 ± 4.5* (80)	162.3 ± 12.5* (87)	38.6 ± 1.6 (27)
6c	40	159.4 ± 4.7* (80)	206.6 ± 7.4* (89)	68.3 ± 2.02* (59)
6d	10	139.5 ± 5.57* (77)	106.6 ± 4.2* (80)	48.3 ± 1.76 (42)
6d	20	165.6 ± 7.8* (81)	156.6 ± 6.6* (86)	79.6 ± 5.3* (64)
6d	40	239.8 ± 2.4* (86)	189.6 ± 9.3* (88)	130 ± 5.7* (78)
Control	–	31.6 ± 7.3	21.2 ± 5	28 ± 3.5

*Significantly different from control group at $p < 0.05$.

Safety test

After transmitting the medium of each well into a microtube, 100 μL of fresh medium and 20 μL of 5 mg/mL MTT dissolved in phosphate-buffered saline were added. One set of wells were allocated as the control group which contained an adjusted planting cell number in log phase in the culture medium holding 0.1% DMSO. Cells were incubated for 3.5 h at 37 °C. Subsequently, media was removed and 150 μL DMSO was added to each well of the 96-well plates to solubilize formazan crystals. The absorbance was measured at the test wavelength of 570 nm and the reference wavelength of 690 nm by a microplate reader. The percentage of viable cells was calculated using the formula: percent of control = [1 − (A570 test/A570 cont)] × 100 where A570 test = absorbance of test sample, A570 cont = absorbance of control sample. Dose–response curves were plotted from percentage of control values against concentrations of inhibitors as μM (*x*-axis, log scale). The values of 50% inhibition of cell proliferation (IC_{50}) were calculated by locating the *x*-axis values corresponding to one-half the absorbance values of control containing an adjusted seeding cell number and 0.1% DMSO without addition of any inhibitors (Table 1).

Measurement of cAMP and cGMP concentrations

Medium of each well was transferred to a microtube at certain intervals after treating with inhibitors. For precipitation of interfering proteins, 10 μL of trichloroacetic acid was added to each sample. Then, microtubes were put in the ice for 15 min and centrifuged subsequently at 11 490 ***g*** for 10 min. The acid content was neutralized with KOH 2 μM. Microtubes were kept at −20 °C. The ELISA kit from Bender MedSystems (Vienna, Austria) was used to assay non-acetylated samples. The optical densities were determined at 405 nm. The concentrations of cAMP or cGMP were calculated according to the standard curve generated in each assay. The results of each group were normalized by dividing the calculated cyclic nucleotides concentrations by the number of viable cells. The base of test was on the competition between the enzyme conjugate and the cAMP or cGMP in the sample for a narrow number of binding sites.

Statistical analysis

Results were expressed as mean ± SEM and analyzed by Student's *t*-test. Statistical significance level was defined at $p < 0.05$. StatsDirect statistical software version 2.7.8 was used.

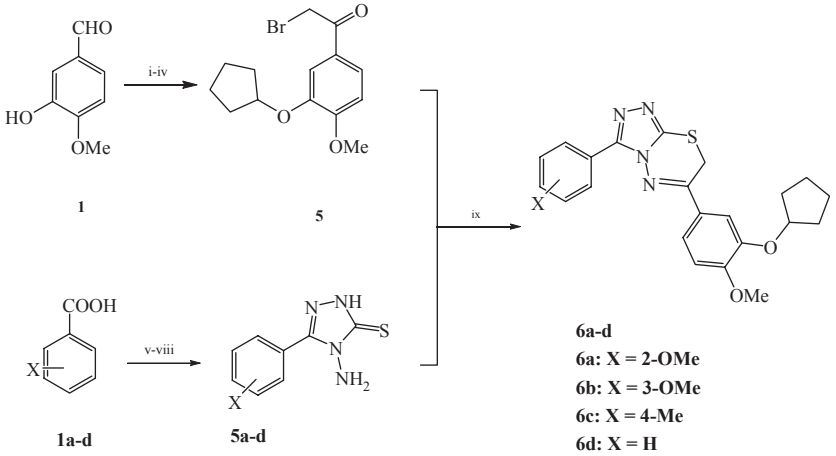
Results

Chemical synthesis

Reaction sequence employed for the synthesis of substituted 6-(3-(cyclopentyloxy)-4-methoxyphenyl)-3-(aryl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine (**6a–d**) is shown in Scheme 1. The starting material substituted benzoic acid (**1a–d**) was converted into substituted ethyl benzoates (**2a–d**). Substituted arylhydrazides (**3a–d**) were achieved by refluxing (**2a–d**) with hydrazine hydrate in methanol. Compound (**3a–d**) on reaction with carbon disulfide in methanolic potassium hydroxide yielded potassium 3-aryldithiocarbazates (**4a–d**) in worthy yield and was right used for the next stage without additional purification. The triazoles **5** were synthesized by refluxing (**4a–d**) with hydrazine hydrate. Compression of (**5a–d**) with phenacyl bromide in ethanol under reflux state provided replaced 6-(3-(cyclopentyloxy)-4-methoxyphenyl)-3-(aryl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine (**6a–d**) in good yield.

Cell proliferation

NIH-3T3 cells were multiplied quickly with a cell doubling time of 26 h. To prompt adequate cell death, the dose of rolipram was increased up to 500 μM. Rolipram was less cytotoxic than other synthesized compounds with IC50 values of 160, 353, and 500 μM after 1, 6, and 24 h of exposure, respectively.



Scheme 1. Synthesis of substituted 6-(3-(cyclopentyloxy)-4-methoxyphenyl)-3-(aryl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine (**6a-d**). Conditions: (i) cyclopentyl bromide (1.5 equiv), K_2CO_3 (1.2 equiv), DMF, 60 °C, 12 h, 84%; (ii) MeMgBr (1.3 equiv), Et_2O, 0–25 °C, 1 h, 76%; (iii) MnO_2, Et_2O, 0–25 °C, 4 h, 73%; (iv) $CuBr_2$, EtOAc, reflux, 3.5 h, 74%; (v) cat. H_2SO_4, MeOH, rt, 12 h; (vi) hydrazine, EtOH, reflux 12 h.; (vii) KOH, EtOH; then CS_2 rt, 12 h; (viii) hydrazine monohydrate, H_2O, reflux, 3 h, then Conc. HCl; (ix) EtOH, reflux °C, 4 h.

Table 2: Concentrations of cGMP after 1, 6, and 24 h of exposure of NIH-3T3 cells to different concentration of **6a–6d** in comparison with those of rolipram

Groups		cGMP concentration (pmol/cell) Time (h)		
		1	6	24
Drug name	Concentration (μM)	Mean ± SEM (percent of increase)	Mean ± SEM (percent of increase)	Mean ± SEM (percent of increase)
Rolipram	0.05	2.3 ± 1.2 (–)	2.6 ± 1.3 (3%)	2.4 ± 0.9 (–)
Rolipram	0.1	2.5 ± 1.1 (–)	2.5 ± 1 (–)	2.5 ± 1.2 (–)
Rolipram	0.5	2.4 ± 0.9 (–)	2.4 ± 0.8 (–)	2.6 ± 1.1 (4)
Rolipram	1	2.5 ± 1.1 (–)	2.5 ± 1.2 (–)	3.3 ± 1.5 (20)
Rolipram	5	2.6 ± 1.1 (4)	2.8 ± 1.2 (12)	3 ± 1.55 (20)
6a	10	2.5 ± 0.9 (–)	2.3 ± 1.1 (–)	2.3 ± 1.1 (–)
6a	20	3 ± 1.3 (20)	3 ± 1.4 (20)	3 ± 1 (20)
6a	40	3.3 ± 1.1 (32)	3 ± 1.5 (20)	3.2 ± 1.6 (28)
6b	10	2.3 ± 0.9 (–)	3 ± 1.4 (20)	2.5 ± 1.2 (–)
6b	20	3.4 ± 1.5 (36)	3.5 ± 1.6 (40)	3.6 ± 1.5 (44)
6b	40	3.6 ± 0.9 (44)	3.6 ± 1.4 (44)	3.5 ± 1.2 (40)
6c	10	3 ± 1.3 (20)	3.5 ± 1.45 (40)	3 ± 1.35 (20)
6c	20	3 ± 1.1 (20)	3.6 ± 1.5 (44)	3.4 ± 1.2 (36)
6c	40	3.4 ± 1.2 (36)	3.6 ± 1.1 (44)	3.5 ± 1.3 (44)
6d	10	2.5 ± 1.1 (–)	2.5 ± 1.2 (–)	2.6 ± 1.3 (4)
6d	20	2.6 ± 1 (4)	2.8 ± 1.3 (12)	3 ± 1.4 (20)
6d	40	2.7 ± 0.9 (8)	3 ± 1.5 (20)	3.1 ± 1.6 (24)
Control	–	2.5 ± 1.2	2.5 ± 1.3	2.5 ± 1.2

Level of cAMP and cGMP

All synthesized compounds produced a major increase in cAMP level (Table 1), although the concentration of cGMP remained roughly unchanged (Table 2). The potency of rolipram and each of four synthesized compounds were calculated as cAMP and cGMP ED_{50} (Table 3). In this regard, **6d** showed specificity for PDE-4 with the lowest cAMP ED_{50} of 13 μM and the highest cGMP ED_{50} of 49.8 μM (Table 3).

Cytotoxicity

The cytotoxicity of **6a** on NIH-3T3 cells was assessed with IC_{50} value of >80 μM, after 24 h. Compound **6b** produced an abrupt rise of response on NIH-3T3 cell line after 6 h of contact. Thus, the IC_{50} reduced from early >80 μM at 1 h to 73 and 40 μM after 6 and 24 h of exposure, respectively. The IC_{50} value of **6b** at 24 h was found 2-fold higher than that of 1 h. Sensitivity of cell line to **6c** was augmented with IC_{50} values of 74, 42, and 41 μM, after 1, 6, and 24 h postexposure, respectively. Compound **6d** was the most cytotoxic among the tested compounds owing to the strong inhibition of cell proliferation with IC_{50} values of 66, 59, and 40 μM, respectively, after 1, 6 and 24 h of exposure. After 1-h exposure, the NIH-3T3 cell line displayed the maximum toxicity in response to 80 μM dose of **6d** and **6c**, whereas the cells showed little response to **6a** and **6b** in the equal duration. After 6 h of experience, the cell reaction to **6b** cytotoxicity amplified especially in upper doses close to 80 μM. By increasing the contact duration, the variance between the cytotoxicity of **6d**, **6c**, and **6b** and that of **6a** appeared in lower doses. As a result, a noteworthy difference was found between toxic effects of **6b**, **6c**, **6d**, and **6a** in doses greater than 30 μM after 24 h (Table 4).

Table 3: ED_{50} values of compounds for inhibition of cAMP- and cGMP PDE after 1, 6, and 24 h of exposure

Groups	Time (h)		
	1	6	24
cAMP (μM)			
Rolipram	1.459	1.2	0.8
6a	22.6	29	38.1
6b	23	24.34	22
6c	23	36.2	21.6
6d	38	27.4	13
GMP (μM)			
Rolipram	Inactive	Inactive	Inactive
6a	44	45.7	48.6
6b	46.2	48.4	48.5
6c	45.3	49.9	46.4
6d	49.7	49.2	49.8

Molecular modeling

The initial model of phosphodiesterase-4d (PDE-4D) for docking studies was built based on the X-ray crystal structure of human PDE-4D with the regulatory domain in complex with 1-(3-nitrophenyl)-3-(pyridin-4-ylmethyl)pyrido[2,3-*d*]pyrimidine-2,4(1*H*,3*H*)-dione which was obtained from the Protein Data Bank (PDB entry 3G4G) (9, 10). The original ligand was removed while water molecules present in the PDB file were maintained in their position. 3D structures of **4d** was generated and optimized by MarvinSketch, Marvin Beans package 5.0.7. AUTODOCK 4 package was used to perform docking simulations, which adopts the hybrid Lamarckian Genetic Algorithm as searching algorithm and allows full flexibility of the

Table 4: Cytotoxicity of tested compounds after 1 and 24 h of exposure of NIH-3T3 cells to different concentration of **6a–6d** in comparison with that of rolipram

	Cytotoxicity (percent of control) (mean ± SEM)		
Concentrations (μM)	After 1 h	After 6 h	After 24 h
Rolipram (0.05)	82 ± 2	80 ± 5	77 ± 4
Rolipram (0.1)	82 ± 3	77 ± 2	76 ± 4
Rolipram (0.5)	82 ± 1	76 ± 2	73 ± 6
Rolipram (1)	82 ± 4	70 ± 2	69 ± 3
Rolipram (5)	79 ± 2	65 ± 3	67 ± 3
Rolipram (25)	76 ± 4	62 ± 5	61 ± 4
Rolipram (125)	69 ± 3	58 ± 3	52 ± 3
Rolipram (250)	61 ± 5	53 ± 5	46 ± 4
Rolipram (500)	50 ± 2	46 ± 7	40 ± 4
6a (1)	81 ± 5	79 ± 5	78 ± 3
6a(10)	81 ± 4	78 ± 3	77 ± 3
6a (20)	78 ± 4.2	74 ± 2	73 ± 5
6a (40)	73 ± 5	68 ± 2	68 ± 3
6a (80)	68.6 ± 1.7	65 ± 1.3	65 ± 5
6b (1)	86 ± 6	80 ± 5	79 ± 5
6b (10)	84 ± 3	72 ± 5	72 ± 4
6b (20)	79 ± 4	64 ± 3	63 ± 3
6b (40)	75 ± 3	57 ± 3	50 ± 3*
6b (80)	74 ± 3	50 ± 2*	45 ± 6*
6c (1)	77 ± 5	78 ± 4	73 ± 5
6c (10)	75 ± 3	63 ± 5	63 ± 7
6c (20)	74 ± 3	57 ± 2	59 ± 6
6c (40)	73 ± 5	51 ± 1	51 ± 4*
6c (80)	46 ± 6	46 ± 4*	43 ± 7*
6d (1)	81 ± 7	74 ± 2	74 ± 9
6d (10)	80 ± 6	69 ± 1	65 ± 5
6d (20)	61 ± 5	64 ± 3	61 ± 4
6d (40)	68 ± 4	61 ± 2	50 ± 5*
6d (80)	42 ± 3	41 ± 5*	41 ± 9*

*Significant difference exists between the cytotoxicity of **6a** (known as the least cytotoxic compound) and the cytotoxicity of **6b**, **6c**, and **6d**.

ligand. Solvation parameters and Kollman charges for all atoms in PDE4D were assigned using AUTODOCK Tools 1.5.4. The grid for energy evaluation was centered at residue Phe196 in PDE4D with grid points in the *x*-, *y*-, *z*-axes set to 50 × 50 × 50 and separated by 0.375 Å. The initial population size and maximum number of energy evaluations were set to 100 and 1.0 × 107, respectively. The docked result within an RMSD of 2 Å was clustered, and the final was selected considering both the embedded empirical binding free energy evaluation and the clustering analysis.

Discussion

On the basis of the recent studies, mercapto- and thione-substituted 1,2,4-triazole ring systems have been reported for having antibacterial, antifungal, and anti-inflammatory properties (11,12).

Substituted 3,6-diphenyl-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazines are potent inhibitors of PDE-4 (5–7). This fundamental structure signifies an innovative chemotype capable of PDE-4 inhibition. Excitingly, the exchanges on the 5 position of the 3,6-dihydro-2*H*-1,3,4-thiadiazine ring are serious functionalities for effective PDE-4 inhibition. However, the phenyl ring attached at the 3 position of the 1,2,4-triazole seems to be less involved in the pharmacophore of this structure. As stated, the 3,4-alkoxy moiety plays a crucial role in many PDE-4 inhibitors, as clarified in the structure of rolipram (7).

We synthesized a novel series of inhibitors, with a 3-cyclopentyloxy-4-methoxyphenyl group at the 5 position of the 3,6-dihydro-2*H*-1,3,4-thiadiazine ring. Our findings revealed that such moieties on the 5 position of the 3,6-dihydro-2*H*-1,3,4-thiadiazine ring do not improve the inhibitory effects of these compounds. As a matter of fact' the calculated ED_{50}s of the synthesized compounds (22 μM for **6a**, **6b**, **6c**, and 13 μM for **6d**) are much higher than those of the previously synthesized 3-(2,5-dimethoxyphenyl)-6-(3,4-dimethoxyphenyl)-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazine with ED_{50} of 0.01 μM (7).

Our findings showed that all synthesized compounds cause a substantial intensification in the concentration of extracellular cAMP, whereas the concentration of cGMP stays approximately unaffected. These findings enlightened the possible selectivity of these compounds against cAMP-specific PDE enzymes (Table 3). Among these analogs, **6d** showed the highest potential for elevation of cAMP. Also, the time course effect of **6d** bears a resemblance to that of rolipram. It is obvious that the p-Tolyl moiety on the 5 position of the 3,6-dihydro-2*H*-1,3,4-thiadiazine ring potentiates the activity

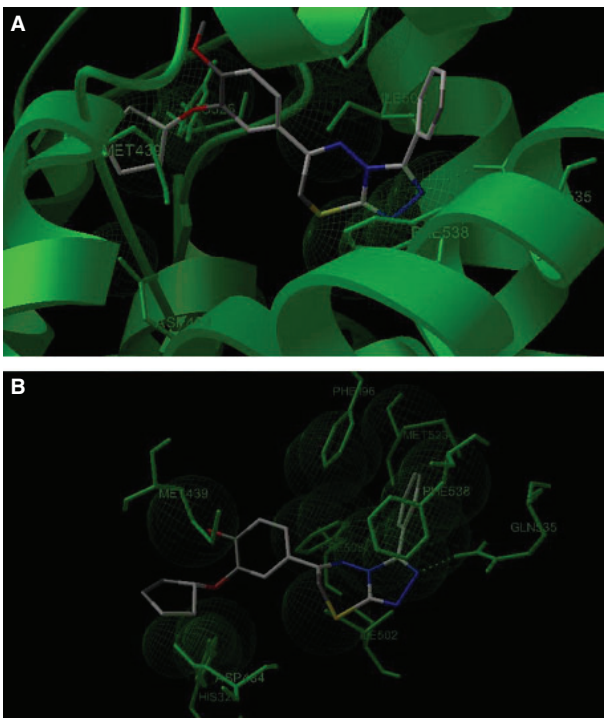


Figure 2: Docking study of compound **4d** (atom type base colored) and the three-dimensional structure of PDE-4D (PDB ID: 3G4G) (A) ribbon view of active site in complex with **4d**, (B) Hydrogen bond is shown with dotted green line between triazol and side chain of Gln535.

of compound **6d**, whereas methoxy substitutions have less effects in terms of SAR. However, the methoxy group will intensify the steadiness of the compound when attached at the 3 position of phenyl ring. Compound **6c** showed two peak elevations in cAMP levels at 1 and 24 h postexposure (Table 3). Interestingly, the cytotoxicity of **6d** was directly proportional to its PDE inhibitory effect. It is worthy to mention that the cytotoxic IC_{50} of all synthesized compounds was approximately twofold greater than their required concentration for inhibition of PDE-4 (in terms of elevation of cAMP) telling that these structures could be used to develop potent and safe inhibitors of PDE-4 enzyme.

To ensure that the elevation of cAMP is mediated through the inhibition of PDE-4 enzyme, the interaction mode of compound **4d** was studied by molecular docking simulation using the AUTODOCK program in MGL Tools package software based on the X-ray crystal structure of PDE-4D (11, 12). As shown in Figure 2, compound **4d** has a nice fit along the active site of PDE-4D. The figures show that **4d** makes several principal interactions along the active site of PDE-4D. Compound **4d** is located in a strong hydrophobic pocket of PDE-4D, in which the two substituted-phenyl groups are involved in hydrophobic interactions with Phe538, Phe506, Met523, and Met439. Moreover, one hydrogen bond was formed by nitrogen of triazol ring with residue Gln535, with bond length of 2.47 Å.

The precise function of 6-(3-(cyclopentyloxy)-4-methoxyphenyl)-3-aryl-7*H*-[1,2,4]triazolo[3,4-*b*][1,3,4]thiadiazines remains to be completed by further investigation into their selectivity for different intact cAMP PDE isoforms.

Acknowledgment

This study was partly supported by a grant from Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences and INSF.

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