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Synthesis and Ability of New Ligands for G Protein-Coupled Receptors 17 (GPR17)

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Literature Search F
Funds Collection G

ABCE 1 **Tongyou Zhuo**
AG 2 **Shengxue Zhou**
C 3 **Wei Zhang**
C 4 **Catia Lambertucci**
C 4 **Rosaria Volpini**

1 School of Food Technology, Jilin Agricultural Science and Technology College, Jilin City, Jilin, P.R. China
2 College of Chinese Medicine, Jilin Agricultural Science and Technology College, Jilin City, Jilin, P.R. China
3 Changchun Beihua Pharmaceutical Co., Jilin City, Jilin, P.R. China
4 University of Camerino, Camerino, Italy

Corresponding Author: Shengxue Zhou, e-mail: shengxuezhou741@163.com

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Background: GPR17 is believed to be a novel target for the development of new therapeutic approaches to human stroke and multiple sclerosis. Hence, the selection of GPR17 ligands may be a potent way to reduce the progression of ischemic damage.





Material/Methods: New potential ligands for GPR17, mono-, di-, and triphosphate adenosine nucleotides substituted at N6-position with a methyl and a cyclopentyl group were synthesized. The ability of new ligands to bind GPR17 was evaluated using frontal affinity chromatography-mass spectrometry (FAC-MS) method. Cangrelor, MRS2179, and uridine diphosphate were selected as the reference compounds.

Results: The new triphosphate derivatives 9 and 10 were considered as the new GPR17 ligands. The compound 10 was eluted with breakthrough time (bt) between cangrelor and MRS 2179 (compound 10, bt=12.25; cangrelor, bt=24.55, and MRS 2179, bt=7.10), while the breakthrough volume of compound 9 was similar to that of MRS 2179 (compound 9, bt=7.53 and MRS 2179, bt=7.10).

Conclusions: N6-cyclopentylATP 10 is medium-high affinity ligand of GPR17, while the corresponding N6-methyl derivative 9 is a medium affinity ligand similar to MRS 2179. Hence, the new N6-cyclopentylATP 10 might be a good candidate for the pharmacological characterization of GPR17.

MeSH Keywords: **Affinity Labels • Chromatography, Affinity • Gas Chromatography-Mass Spectrometry**

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Background

G protein-coupled receptors (GPCRs) are most common cell surface proteins, and regulate the extracellular and intracellular signal transduction [1]. GPCRs have been considered to be associated with the biological responses of nervous systems, and are thought to be involved in some disorders and diseases such as neurological disorders, inflammatory diseases, and cancers [2]. Currently, GPCRs are considered as appealing pharmaceutical targets; however, it is well known that most GPCRs have too little of a natural ligand and several of them are “orphan” [1–3]. Thus, it is essential to search for or synthesize the potent and selective ligands of GPCRs.

GPR17, one of these receptors, is closely related to 2 different receptor families [4]: the purinergic P2Y² responding to nucleotides, and cysteinyl leukotrienes (CysLTs) receptors responding to cysteinyl leukotrienes [5,6]. GPR17 is highly present in the brain, kidney, and heart with ischemic damage, which is considered as a potent target for the treatment of human stroke, multiple sclerosis, and trauma [6,7]. Based on this hypothesis, GPR17 knockdown or GPR17 antagonists have been reported to protect against brain damage [8]. Hence, the selection of GPR17 ligands may be a potent way to reduce the progression of ischemic damage. However, the ligands that are currently known to bind to GPR17 also interact with other P2Y or CysLT receptors [9], which limits the development of new GPR17-based pharmacological strategies. Because few studies have reported the synthesis of new ligands for GPR17 and then evaluated its characteristics using frontal affinity chromatography-mass spectrometry (FAC-MS) method [10,11], it is necessary to find new and potent ligands for GPR17.

In the present study, we synthesized new GPR17 ligands, which started from the cangrelor structure ($IC_{50}=0.7$ nM) [6], and new adenosine nucleotides substituted at N⁶-position with a methyl or a cyclopentyl group. Furthermore, the ability to bind GPR17 was evaluated using FAC-MS method to identify the degree of affinity [12].

Material and Methods

Instruments and materials

The characteristics of compounds were evaluated by melting points (MPs) and nuclear magnetic resonance (NMR). MPs were measured in open tubes using a Büchi apparatus and were not corrected. ¹H NMR spectra was recorded with a Varian Gemini 200 MHz and a Varian Mercuri 400 MHz spectrometer (δ in parts per million and *J* in Hz). D₂O was added to confirm the exchangeable protons. A Varian Mercuri 400 MHz spectrometer was used to obtain the ³¹P NMR spectra at room temperature.

Elemental analyses were performed on an elemental analyzer (Model EA 1108, Fisons) and the theoretical values were limited within $\pm 0.4\%$. Exact mass analyses were performed on a Hewlett Packard 1100 series, quadrupole electrospray ionization-mass spectrometer (ESI-MS, Hewlett Packard, Waldbronn, Germany). Thin-layer chromatography (TLC) analyses were performed on silica-coated TLC plates (silica gel 60 F-254, Merk, Darmstadt, Germany). Silica gel 60 (Merk) was used for column chromatography, while Sephadex DEAE A-25 (Sigma, St. Louis, MO, USA) was used for ionic exchange chromatography.

Synthesis

6-Iodopurineriboside (2)

Adenosine (1500 mg; 1.87 mmol) was dissolved in 6 mL dry dimethyl formamide (DMF), and then the suspension was stirred at room temperature with the supplement of diiodomethane (13 mL) and isoamyl nitrite (5 mL). The reaction mixture was heated at 60°C in an oil bath and the reaction was completed within 1 h. The volatiles were evaporated; the crude extract was chromatographed on a silica gel column and eluted with a gradient of CHCl₃-CH₃OH (98: 2–95: 5, v/v). Lastly, compound 2 was obtained as pure product with 34% yield. MP: (MeOH): 156–158°C; ¹H NMR (DMSO-d₆) δ 3.62 (m, 2H, CH₂-5'), 3.97 (m, 1H, H-4'), 4.17 (m, 1H, H-3'), 4.57 (m, 1H, H-2'), 5.10 (t, 1H, OH), 5.25 (d, 1H, OH), 5.57 (d, 1H, OH), 5.99 (d, 1H, *J*=5.2 Hz, H-1'), 8.65 (s, 1H, H-2), 8.90 (s, 1H, H-8). Anal. (C₁₀H₁₁N₄O₄) C, H, N.

N⁶-Methyladenosine (3)

Compound 2 (500 mg; 1.32 mmol) was added to a solution of methylamine dissolved in dry tetrahydrofuran (10 mL, 3.6 N) at –20°C in a steel vial. The mixture was reacted for 30 min at –20°C and then for 1 h at room temperature. The volatiles were evaporated; the crude extract was chromatographed on a silica gel column with an elution of CHCl₃-CH₃OH 85: 15. Compound 3 was obtained with 88% yield. MP: (CH₃CN) 130–132°C; ¹H NMR (DMSO-d₆) δ 2.95 (br s, 3H, CH₃), 3.60 (m, 2H, CH₂-5'), 3.95 (m, 1H, H-4'), 4.13 (m, 1H, H-3'), 4.60 (m, 1H, H-2'), 5.17 (d, 1H, OH), 5.41 (m, 2H, 2 X OH), 5.87 (d, 1H, *J*=6.2 Hz, H-1'), 7.80 (m, 1H, NH), 8.21 (s, 1H, H-2), 8.33 (s, 1H, H-8). Anal. (C₁₁H₁₅N₅O₄) C, H, N.

N⁶-Cyclopentyladenosine (4)

Compound 2 (500 mg; 1.32 mmol) was added to 10 mL cyclopentylamine. The reaction mixture was stirred for 1 h. The volatiles were evaporated and the crude product was purified on a silica gel column with an elution of cyclohexane-CHCl₃-CH₃OH (50: 40: 10). Compound 4 was obtained with 80% yield. MP: 90–93°C; ¹H NMR (DMSO-d₆) δ 1.64 (m, 6H, H-cyclopentyl), 1.94 (m, 2H, H-cyclopentyl), 3.62 (m, 2H, CH₂-5'), 3.96 (m, 1H,

H-4'), 4.14 (m, 1H, H-3'), 4.59 (m, 2H, H-2' and CH-cyclopentyl), 5.18 (d, 1H, OH), 5.44 (m, 2H, 2 X OH), 5.87 (d, 1H, $J=6.2$ Hz, H-1'), 7.79 (d, 1H, $J=7.2$ Hz, NH), 8.20 (s, 1H, H-2), 8.34 (s, 1H, H-8). Anal. ($C_{15}H_{21}N_5O_4$) C, H, N.

Nucleoside-5'-monophosphates (5-6)

The nucleosides 3 or 4 (0.56 mmol), dissolved in 3.0 mL trimethyl phosphate in turn, were mixed with 4 equivalents of $POCl_3$ (209 μ L, 2.24 mmol). The reaction mixture was stirred for 3 h, and then 3 mL H_2O was added. Next, triethylamine was added dropwise to neutralize the solution. Ion-exchange chromatography was performed to purify the mixture. Compound 5 (N^6 -Methyladenosine Monophosphate, N^6 -MethylAMP) was obtained from compound 3 with 62% yield. 1H NMR (D_2O) δ 2.83 (s, 3H, CH_3), 3.94 (m, 2H, CH_2-5'), 4.20 (m, 1H, H-4'), 4.31 (m, 1H, H-3'), 4.55 (t, 1H, $J=5.4$ Hz, H-2'), 5.87 (d, 1H, $J=6.0$ Hz, H-1'), 7.92 (s, 1H, H-2), 8.19 (s, 1H, H-8). ^{31}P NMR (D_2O) δ -0.14 (s). ESI-MS m/z : 360.0 [M-H]⁻, 721.1 [2M-H]⁻. Compound 6 (N^6 -CyclopentylAMP) was obtained from compound 4 with 76% yield. 1H NMR (D_2O) δ 1.46 (m, 4H, H-cyclopentyl), 1.57 (m, 2H, H-cyclopentyl), 1.85 (m, 2H, H-cyclopentyl), 3.93 (m, 2H, CH_2-5'), 4.18 (m, 2H, H-4' and CH-cyclopentyl), 4.29 (m, 1H, H-3'), 4.54 (t, 1H, $J=5.4$ Hz, H-2'), 5.90 (d, 1H, $J=6.0$ Hz, H-1'), 8.00 (s, 1H, H-2), 8.22 (s, 1H, H-8). ^{31}P NMR (D_2O) δ 1.33 (s). ESI-MS m/z : 414.0 [M-H]⁻, 829.1 [2M-H]⁻.

Nucleoside-5'-diphosphates (7-8) and nucleoside-5'-triphosphates (9-10)

The nucleoside-5'-monophosphates 5 or 6 (0.15 mmol) were added into dry DMF (1 mL), and then the solution was added to 36 μ L tri-*n*-butylamine (0.15 mmol). The mixture was stirred for 20 min, and then evaporated to dryness. After being dissolved in dry DMF (1.4 mL), the suspension was mixed with *N,N'*-carbonyl diimidazole (122 mg, 0.75 mmol) and then reacted for 3 h by agitation. Next, 49 μ L methanol (1.2 mmol) was added and then reacted with the mixture at room temperature for 30 min. Tri-*n*-butylammonium phosphate or bis(tri-*n*-butylammonium) pyrophosphate (6 mL, 0.5 mol) dissolved in DMF were added. After stirring for 14 h, the solvent was removed. Ion-exchange chromatography was performed to purify the mixture dissolved in H_2O . Compound 7 (N^6 -Methyladenosine Diphosphate, N^6 -MethylADP) was obtained from compound 5 with 46% yield. 1H NMR (D_2O) δ 2.94 (s, 3H, CH_3), 4.05 (m, 2H, CH_2-5'), 4.23 (m, 1H, H-4'), 4.38 (m, 1H, H-3'), 4.60 (m, 1H, H-2'), 5.96 (d, 1H, $J=6.0$ Hz, H-1'), 8.09 (s, 1H, H-2), 8.30 (s, 1H, H-8). ^{31}P NMR (D_2O) δ -11.30 (d), -10.79 (d). ESI-MS m/z : 440.0 [M-H]⁻. Compound 8 (N^6 -CyclopentylADP) was obtained from compound 6 with 21% yield. 1H NMR (D_2O) δ 1.49 (m, 4H, H-cyclopentyl), 1.61 (m, 2H, H-cyclopentyl), 1.89 (m, 2H, H-cyclopentyl), 4.03 (m, 2H, CH_2-5'), 4.20 (m, 1H, H-4'), 4.23 (m, 1H, CH-cyclopentyl), 4.45 (m, 1H, H-3'), 4.60 (m, 1H, H-2'), 5.95 (d, 1H, $J=5.6$ Hz, H-1'), 8.06

(s, 1H, H-2), 8.30 (s, 1H, H-8). ^{31}P NMR (D_2O) δ -10.94 (d), -6.39 (d). ESI-MS m/z : 246.5 [M-2H]⁻, 494.0 [M-H]⁻. Compound 9 (N^6 -Methyladenosine Triphosphate, N^6 -MethylATP) was obtained from compound 5 with 32% yield. 1H NMR (D_2O) δ 2.92 (s, 3H, CH_3), 4.05 (m, 2H, CH_2-5'), 4.21 (m, 1H, H-4'), 4.41 (m, 1H, H-3'), 4.60 (m, 1H, H-2'), 5.95 (d, 1H, $J=6.0$ Hz, H-1'), 8.09 (s, 1H, H-2), 8.29 (s, 1H, H-8). ^{31}P NMR (D_2O) δ -21.47 (t), -10.22 (d), 7.47 (d). ESI-MS m/z : 259.5 [M-2H]⁻, 519.9 [M-H]⁻. Compound 10 (N^6 -CyclopentylATP) was obtained from compound 6 with 29% yield. 1H NMR (D_2O) δ 1.47 (m, 4H, H-cyclopentyl), 1.58 (m, 2H, H-cyclopentyl), 1.87 (m, 2H, H-cyclopentyl), 4.05 (m, 2H, CH_2-5'), 4.20 (m, 2H, H-4' and CH-cyclopentyl), 4.38 (m, 1H, H-3'), 4.59 (m, 1H, H-2'), 5.93 (d, 1H, $J=6.4$ Hz, H-1'), 8.05 (s, 1H, H-2), 8.27 (s, 1H, H-8). ^{31}P NMR (D_2O) δ -22.07 (t), -10.37 (d), 9.56 (d). ESI-MS m/z : 286.5 [M-2H]⁻, 574.0 [M-H]⁻.

Phosphorylation reactions

TLC was performed to monitor the reactions, and mobile phase was composed of 30% *i*Propanole- H_2O - NH_4OH with a proportion of 5.5: 1: 3.5. The ion-exchange chromatography was performed to purify the nucleotides by the equilibration of H_2O and the elution of gradient of $H_2O/0.5$ mol NH_4HCO_3 .

Preparation of GPR17 – immobilized artificial membrane (IAM) columns

The human GPR17 receptor gene was inserted into the pcDNA3.1 vector and termed pcDNA3.1-GPR17h. For membrane preparation, human astrocytoma cells (1321N1) were cultured, and then transfected with the identified pcDNA3.1-GPR17h or pcDNA3.1 empty vector by FuGene 6 (Roche, Basel, Switzerland). After transfection for 48 h, the cells were collected and homogenized in cell lysis buffer (500 mM NaCl, 10 μ g/mL aprotinin, 100 μ g/mL phenylmethylsulfonyl fluoride, 100 μ M benzamidine, 5 mM 2-mercaptoethanol, 10 μ g/ml leupeptin, 50 μ g/mL tosyl-amido-2-phenylethylchloromethyl ketone, and 100 μ M ATP). Then, after first centrifugation at 700 g for 5 min, the precipitate was discarded. Next, after second centrifugation at 100 000 g for 30 min, the precipitate was resuspended in 10 ml Hepes buffer (20 mM, pH 8.0). As a result, after being rotated at 150 rpm for 18 h at 4°C, the resuspension was centrifuged and the supernatant was acquired. For membrane immobilization, the supernatant was mixed with IAM particles with 12- μ m particle size and 300-Å pore size (Regis Chemical, Morton Grove, IL, USA). After being rotated gently at 150 rpm for 1 h at room temperature, the suspended particles were dialyzed by Hepes buffer for 2 days (changed every 24 h), and then centrifuged (700 rpm, 3 min) at 4°C. The supernatant was discarded, while the precipitate was collected and washed with 10 ml ammonium acetate (5 mM, pH 7.4, Fluka, Buchs, Switzerland). Again, after centrifugation, the precipitate was resuspended in 1 ml ammonium acetate (5 mM, pH 7.4), and then added to a

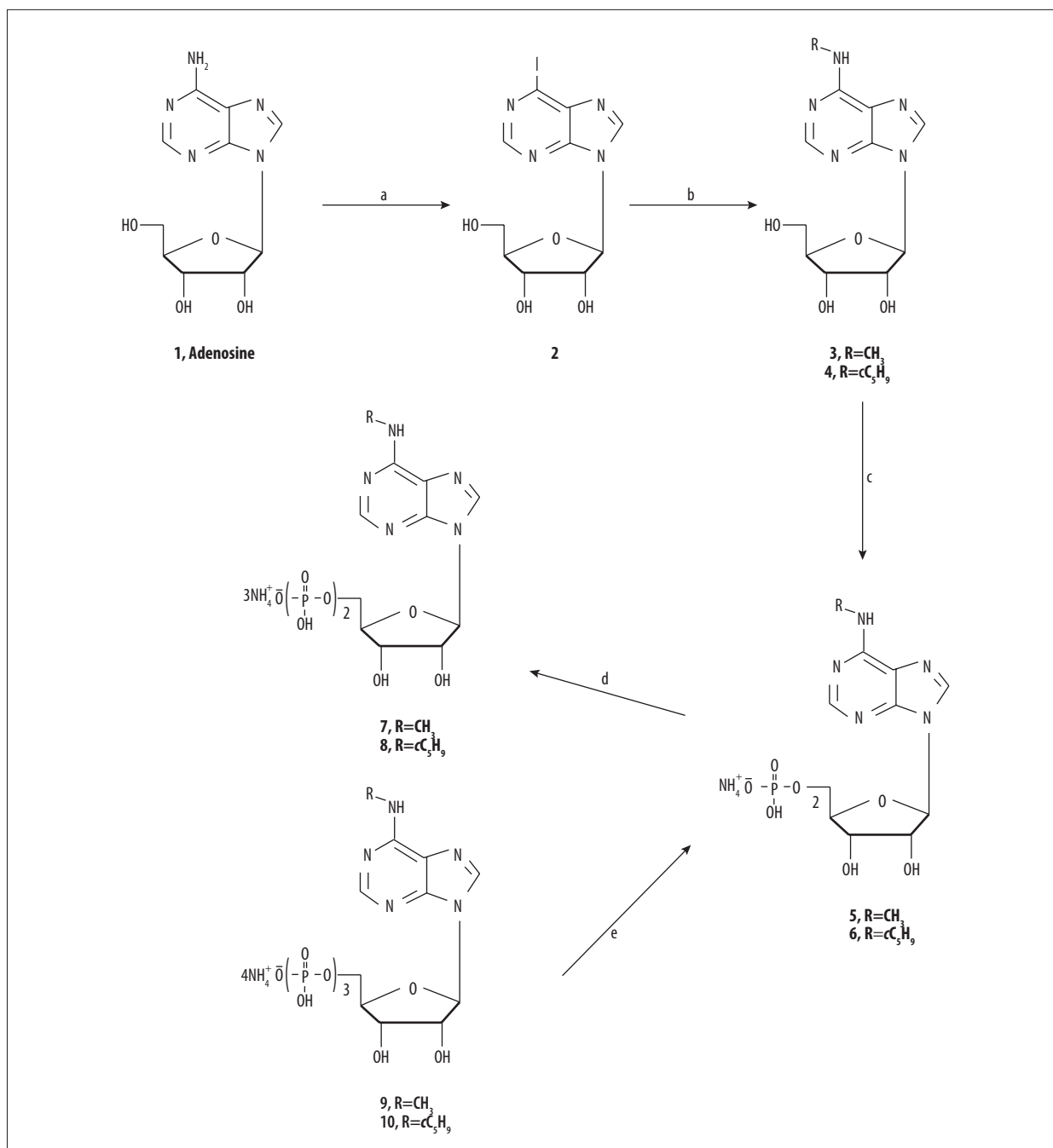


Figure 1. The synthesis scheme of new G-protein-coupled receptor GPR17 ligands. Reaction conditions: a. CH₂I₂, iC₅H₁₁ONO, dimethyl formamide (DMF), 60°C; b. CH₃NH₂/THF, rt; or C₅H₉NH₂, rt; c. POCl₃, (CH₃O)₃PO; d. 1) *n*Bu₃N, DMF, 2) CDI, DMF, 3) (*n*Bu₃NH)(PO₄H₂), DMF; e. 1) *n*Bu₃N, DMF, 2) CDI, DMF, 3) (*n*Bu₃NH)₂(P₂O₇H₂), DMF.

chromatographic glass column (Alltech, Milan, Italy), eventually producing a chromatographic bed.

FAC-MS screening

The FAC experiment was performed on a chromatographic system (Thermo Finnigan, San Jose, CA, USA). This system

included a linear ion trap MS with an ESI ion source, a quaternary gradient Surveyor or MS pump, and a thermostated column oven Surveyor autosampler. The mobile phase consisted of 10% methanol and 90% ammonium acetate, and a flow rate of 500 μL/min was applied in this experiment at room temperature. Then, the insertion of a post-column mobile phase resulted in only 200 μL/min divert to MS. As a result, mass

Table 1. Chemical structures and characteristics of the compounds 9 and 10 as well as the indicators.

Analytes	Structure	Characteristics
9		Molecular weight: 589.3 g/mol
10		Molecular weight: 643.4 g/mol
MRS 2179		GPR17 antagonist Molecular weight: 425.2 g/mol IC ₅₀ =508 nM5
UDP		GPR17 agonist Molecular weight: 404.2 g/mol EC ₅₀ =1.14 μM5
Cangrelor		GPR17 antagonist Molecular weight: 776.4 g/mol IC ₅₀ =0.7 nM5

UDP – uridine diphosphate; IC₅₀ – inhibitory concentration 50; EC₅₀ – effective concentration 50.

Table 2. Data obtained with column GPR17-IAM-I (20.72 million cells).

Analytes	Breakthrough time (min)	%
UDP	3.20	13.03
MRS 2179	7.10	28.92
9	7.53	30.67
10	12.25	49.90
Cangrelor	24.55	100.00

GPR17 – G protein-coupled receptor 17; IAM – immobilized artificial membrane; UDP – uridine diphosphate.

spectra were generated under constant instrumental conditions in the negative ion mode. During FAC-MS screening runs, the ligand mixture with an equimolar solution was continuously infused. The mixture contained affinity reference compounds in the mobile phase, including cangrelor (Medicines Company, Parsippany, NJ, USA), MRS2179, and uridine diphosphate (UDP) (1 μ M, Sigma).

Results and Discussion

Design and synthesis of new GPR17 ligands

The synthesis scheme of new GPR17 ligands is shown in Figure 1. The synthesis of *N*⁶-alkyl adenosines 3 and 4 was carried out starting from commercially available adenosine 1. After evaporation of volatiles and chromatography on silica gel column, the 6-iodo-purineriboside 2 was obtained. Then, the synthesis of monophosphate derivatives 5 and 6 was achieved by reacting the corresponding compounds 3 and 4 with phosphorous oxychloride in trimethyl phosphate at room temperature [13]. The nucleoside diphosphates 7 and 8, as well as triphosphates 9 and 10, were synthesized, following

a modification of the Hoard-Ott method [14]. This synthesis scheme was similar to that used by Calleri et al. [10]. The new triphosphate derivatives 9 and 10 were considered as the new GPR17 ligands.

FAC-MS screening of new GPR17 ligands

The ability of the new triphosphate derivatives **9** and **10** (Table 1) to bind GPR17 and 3 known GPR17 ligands (Table 1) was determined using FAC-MS method [11]. The combination of FAC and MS was used for binding studies in 1998 [15]. FAC was used to evaluate the molecular interactions and obtain precise and accurate binding data, while MS was used to identify compounds solely based on mass-to-charge ratio (*m/z*) [16]. Details of the chromatographic experiment were described in the Methods and Materials section of this article. As a result, compounds **9** and **10** were considered as the potential ligands, based on their affinity. The breakthrough times (bt) and the percentage based on cangrelor for all 5 analytes were calculated to compare the chromatographic results of compounds **9** and **10** (Table 2). Consistently, our study also showed the same elution order of the 3 indicators, and their potency at this receptor was that of cangrelor ($IC_{50}=0.7$ nM) >MRS 2179 ($IC_{50}=508$ nM) >UDP ($EC_{50}=1.14$ μ M [11]). We found that the relative breakthrough% times of MRS 2179 and UDP (the less potent indicators) with respect to cangrelor were similar (28.92% and 13.03%, respectively), which is line with a previous study [10]. Interestingly, the relative breakthrough% times of compounds 9 and 10 were 30.67% and 49.90%, respectively.

Also, the compound 10 was eluted with bt between cangrelor and MRS 2179 (compound 10, bt=12.25; cangrelor, bt=24.55, and MRS 2179, bt=7.10), while the breakthrough volume of compound 9 was very similar to that of MRS 2179 (compound 9, bt=7.53 and MRS 2179, bt=7.10). These results indicate that compound 10 could be identified as a medium-high binding ligand, and compound 9 might be a medium binding ligand.

Table 3. Comparison of the data obtained with Column: GPR17-IAM-I (20.72 million cells) and column: GPR17-IAM-II (19.5 million cells).

Analytes	GPR17-IAM-I		GPR17-IAM-II	
	(min)	%	(min)	%
UDP	3.20	13.03	2.69	25.38
MRS 2179	7.10	28.92	3.62	34.15
9	7.53	30.67	3.69	34.81
10	12.25	49.90	5.67	53.49
Cangrelor	24.55	100.00	10.6	100.00

GPR17 – G protein-coupled receptor 17; IAM – immobilized artificial membrane; UDP – uridine diphosphate.

In view of the application of the GPR17-column in FAC-MS experiments, a second GPR17 column (named as GPR17-IAM-II) was used to evaluate the compounds. We then performed a new experiment by infusing a new GPR17-IAM-II column with the solution containing the new synthesized compounds 9 and 10 based on the 3 reference compounds. A comparison of these data achieved by GPR17-IAM-I and GPR17-IAM-II columns is shown in Table 3. The results showed that the *bt* of these 5 compounds based on GPR17-IAM-II column had an overall decrease, and had a similar ranking as the GPR17-IAM-I column. The overall decrease of *bt* might be caused by the different levels of GPR17 expression due to the increased transfection efficiency. All these results further confirm that FAC-MS is a powerful tool for ligand discovery. The data from FAC-MS experiments suggested that lipophilic substituents on the *N*⁶ amino group of purine ring could result in a close interaction with the hydrophobic residues present in the binding pocket. In fact, compounds 9 and 10, bearing a methyl and a cyclopentyl group in *N*⁶ position, bound strongly, as they showed

a rank of order between cangrelor and MRS 2179 with retention times of 30.67% and 49.90%, respectively.

Conclusions

The results show that FAC-MS may be a potent tool for ligand discovery. In addition, *N*⁶-cyclopentylATP 10 is a medium-high affinity ligand of GPR17, while the corresponding *N*⁶-methyl derivative 9 is a medium affinity ligand similar to MRS 2179. Hence, the new *N*⁶-cyclopentylATP 10 might be a good candidate useful for the pharmacological characterization of GPR17. However, experimental data are needed to assess the activity and selectivity of this compound.

Competing interests

The authors declare that they have no competing interests.

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