Synthesis and biological activities study of novel phthalimides and phenylpyrazolo[1,5-α]pyrimidines

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Abstract
Phosphodiesterase II (PDE2) is mainly distributed in brain and heart cells, and it is a potential therapeutic target for the treatment of central nervous system (CNS) diseases such as Alzheimer’s disease. Based on the structure of the existing PDE2 inhibitor BAY60-7550, a series of novel phthalimides and phenylpyrazolo[1,5-α]pyrimidines have been designed and prepared. Furthermore, after evaluating their inhibitory activity toward PDE2, compound 7-oxo-N-phenethyl-5-phenyl-4,7-dihydropyrazolo[1,5-α]pyrimidine-3-carboxamide is found to have the optimum inhibitory potential (IC50: 1.82 ± 0.29 μM). Discovery Studio software used to simulate the structure–activity relationship between this compound and the PDE2 protein crystal 4HTX to illustrate the binding modes, which provides favorable guidance for the further development of effective PDE2 inhibitors.

Keywords
inhibitors, phenylpyrazolo[1,5-α]pyrimidines, phosphodiesterase II, phthalimides

Introduction
It is well known that cAMP and cGMP, as important secondary messengers, affect the physiological activities of the human body, and, importantly, their imbalance in cell concentration in the body may lead to a variety of diseases.1-3 Previous studies have shown that the activity of phosphodiesterases (PDEs) directly affects the expression concentration of cAMP and cGMP in vivo.4 PDEs are a large family of polygenes, including 11 different subtype structures, which have different substrate specificities, enzyme kinetics, and cell distribution. They also have similar cell structures, show their own homology, and determine the specificity of the substrate or inhibitor.5-7 Among them, PDE2 is a dual-substrate enzyme that can hydrolyze cAMP and cGMP. Although cAMP and cGMP are expressed in the brain, heart, liver, platelets, and T cells, the expression level is the highest in the brain regions,8-10 which indicates that PED2 plays an important role in the emotional, cognitive, and other behavior of humans.11 Therefore, PDE2 inhibitors have the potential to treat a variety of diseases, such as Alzheimer’s disease, angina pectoris, vasodilatation, heart failure, hypertension, and arrhythmia.12-20 However, due to poor pharmacological activity, low blood–brain barrier permeability, and poor metabolic stability, no PDE2 inhibitor has been approved for clinical use. Therefore, the development of novel PDE2 inhibitors is an important research goal.

BAY 60-7550 (Figure 1) is an imidazole–triazine compound with the high selectivity and activity toward PDE2. Moreover, it is often used as an important standard in PDE2 bioactivity tests to investigate other PDE2 inhibitors.21,22 Considering the structural–activity relationship between BAY60-7550 and PDE2 protein (PDB ID: 4HTX, http://www.rcsb.org), a series of phthalimide and phenylpyrazolo[1,5-α]pyrimidine PDE2 inhibitors have been designed and...
preparing. Furthermore, the IC50 values were determined by the biological activity detection method. The structure–activity relationship between the optimum product and 4HTX was simulated by the Discovery Studio software to illustrate the possible binding modes and for the further development of effective PDE2 inhibitors.

Results and discussion

Synthetic pathways

The synthetic strategy toward phthalimide PDE2 inhibitors is depicted in Scheme 1(a), employing ethyl furan and maleic anhydride as the starting materials. Using 4-ethylisobenzofuran-1,3-dione (II) and amines, including 3,4-dimethoxybenzylamine, 3-aminopyrazole, and 3-(benzyloxy)aniline, the desired phthalimide PDE2 inhibitors 1a–c were obtained in good yields through an acylation process. Furthermore, the synthetic strategy for phenylpyrazole pyrimidine PDE2 inhibitors 2 is shown in Scheme 1(b). As expected, the intermediate (IV) could be easily prepared through a two-step reaction involving cyclization and hydrolysis.

Next, the coupling reaction between intermediate (IV) and various amines (benzylamine, phenethylamine, n-butylamine, n-hexylamine, (tetrahydrofuran-2-yl)methanamine, and furfurylamine) provided the desired phenylpyrazolo[1,5-a]pyrimidine PDE2 inhibitors 2a–f in moderate yields.

Bioassays

Protein expression and purification. In order to express the catalytic domain (residues 580–941), the recombinant pET15b-PDE2A plasmid was subcloned and purified by the previously reported method. Subsequently, the plasmid was transferred into Escherichia coli strain BL 21 (CodonPlus) to grow in 2XYT medium at 37 °C until the absorbance (A) at 600 nm was 0.6–0.8. Next, 0.1 mmol L−1 of isopropyl-β-D-1-thiogalactopyranoside was added to induce the PDE2A protein expression at 15 °C for 20 h. The next day, the bacteria were harvested at 8000 r/min at 4 °C, the supernatant was discarded, and the pellet was saved. The lysis solution (20 mmol L−1 Tris 8.0, 300 mmol L−1 NaCl, 15 mmol L−1 imidazole, and 1.0 mmol L−1 mercaptoethanol) was added according to the bacterial weight (1:5) to suspend the bacteria and the mixture was then sonicated. The centrifuged supernatant was mixed with a nickel column. Then, the nickel column was washed with 10 mL each of 5, 20, and 50 mmol L−1 imidazole solutions to elute impurities and dialyzed overnight to remove excess imidazole with 3.0 mL of elution buffer containing 150 mmol L−1 of imidazole (20 mmol L−1 Tris 8.0, 50 mmol L−1 NaCl, 150 mmol L−1 imidazole, and 1.0 mmol L−1 mercaptoethanol). The purified protein was detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the protein concentration was measured by the bicinchoninic acid method.

Enzymatic assays

The enzymatic activities of 9 compounds were assayed using Bio-cAMP as the substrate. Briefly, a reaction mixture of 20 mmol L−1 of Tris HCl, 10 mmol L−1 of MgCl2, 0.5 mmol L−1 of DTT, 3H-cAMP or 3H-cGMP (20000–40000 cpn 3H-cAMP or 3H-cGMP per assay), and proteins were incubated at 25 °C for 15 min. The reaction was then terminated by the addition of ZnSO4 and precipitated out by Ba(OH)2. The compound was dissolved in with DMSO, and the possible systematic errors of the method were evaluated through the different contents of DMSO. Finally, BAY60-7550, a typical PDE2 positive inhibitor, was selected to determine its activity and to calculate the accuracy of the IC50 verification method. The hydrolysis rate of H-cAMP or H-cGMP expressed the inhibitory activity of the compound. Seven concentrations of inhibitors were used for measuring the IC50 values. Each measurement was repeated three times, and the IC50 values were calculated by nonlinear regression. BAY 60-7550 was used as the reference compound for PDE2 enzyme activity detection.

Analysis of biological activity results

The AlphaScreen kit method was used to detect the activity of the nine synthesized compounds, which were diluted at seven concentrations, and three sets of parallel experiments were performed. The IC50 values were obtained by fitting calculations using the software GraphPad Prism 7. The results are given in Table 1.

First, according to the IC50 values of phthalimide compounds 1a–c, we found that changing the nitrogen substituents did not improve their activity. From molecular structure analysis, the phenylpyrazolo[1,5-a]pyrimidine can be considered as a rigid structure, similar to the imidazolopyrimidine in BAY 60-7550. Meanwhile, phenyl and nitrogen-linked groups in phenylpyrazolo[1,5-a]pyrimidine are similar to the dimethoxybenzene and alkylbenzene groups in BAY60-7550. Next, we determined the IC50 values of compounds 2a–f and 7-oxo-N-phenethyl-5-phenyl-4,7-dihydropyrazolo[1,5-a]pyrimidine-3-carboxamide (2a) exhibits the optimum inhibitory potential toward PDE2 (IC50: 1.82 ± 0.29 μM).

Molecular docking

Discovery Studio software is a comprehensive molecular simulation platform. The working principle is based geometric
complementarity, chemical environment complementarity, and energy complementarity. If there is an interaction between the ligand and the receptor, then molecular docking can be evaluated and we can obtain the structure–activity relationship and scoring results of the interaction between the molecule and the protein. According to the results, we can judge whether the binding between the molecule and the protein is good or not, and further guide modifications of the compound structure. In this paper, a semi-flexible CDOCKER docking is used to simulate the binding mode of the synthesized compound and the PDE2 protein 4HTX.
According to the analysis of the structure–activity relationship of BAY60-7550 and PDE2 protein 4HTX, we found that the carbonyl oxygen on the pyrimidinone ring forms a hydrophilic region with GLN812 and GLN859. The hydrophobic region is mainly due to the imidazotriazine skeleton, while the amino acids of ILe826, Phe862, Leu770, Leu809, Tyr655, and Met847 can form $\pi$–$\sigma$, $\pi$–$\pi$ and $\pi$–alkyl stacking interactions with this core skeleton, and promote the PDE2 effects of BAY60-7550 (Figure 2).

| Entry | Compound | Structure | PDE2 IC$_{50}$ (µM) |
|-------|----------|-----------|----------------------|
| 1     | 1a       | ![Image](image1.png) | 9.87 ± 0.43          |
| 2     | 1b       | ![Image](image2.png) | 6.89 ± 1.25          |
| 3     | 1c       | ![Image](image3.png) | >10                  |
| 4     | 2a       | ![Image](image4.png) | 1.82 ± 0.29          |
| 5     | 2b       | ![Image](image5.png) | 2.62 ± 0.39          |
| 6     | 2c       | ![Image](image6.png) | 9.23 ± 0.65          |
| 7     | 2d       | ![Image](image7.png) | >10                  |
| 8     | 2e       | ![Image](image8.png) | 5.67 ± 0.43          |
| 9     | 2f       | ![Image](image9.png) | 4.19 ± 0.52          |

*BAY60-7550 was used as the reference compound with an IC$_{50}$ of 8.4 nM.

*IC$_{50}$ values determined against 3H-cAMP.
To further explain the inhibitory activity of compound 2a, it has been docked into the binding pocket of PDE2 Discovery Studio 2020 (Figure 3). The results show that compound 2a can form hydrogen bond interactions with Gln812, and π–π stacking interactions with Phe862, Ile826, Leu809, Phe830, and Leu770. However, due to the poor hydrogen bond interaction strength of Gln859 and the weakening of the π–π stacking interactions, the inhibitory activity of compound 2a is lower than that of BAY60-7550. Although the inhibitory effect of compound 2a on PDE2 is not as obvious as that of BAY60-7550, it has a certain inhibitory effects on the PDE2 enzyme.

Conclusion

In this study, two series of phthalimides (1a–c) and phenylpyrazolo[1,5-a]pyrimidines (2a–f) have been designed and synthesized as potential PDE2 inhibitors. 1H NMR, 13C NMR, and mass spectrometry were used to confirm the structures of the compounds. All compounds were tested for PDE2 enzyme activity with BAY60-7550 as a reference and Bio-cAMP as the substrate. It was noted that compound 2a has the optimum inhibitory potential toward PDE2 (IC50: 1.82 ± 0.29 μM). In this work, the molecular docking of the synthesized compound and the PDE2 protein 4HTX was performed using a flexible docking software CDocker embedded in Discovery Studio program. This research is expected to provide a basis for the further development of effective PDE2 inhibitors.

Experimental

General

1H NMR spectra were recorded on a BrukerBioSpin GmbH spectrometer at 300 and 400 MHz. 13C NMR spectra were recorded on a BrukerBioSpin GmbH spectrometer at 75 MHz; the coupling constants are given in Hz. High-resolution mass spectrometry (HRMS) was performed using an Agilent 6200 accurate-mass time-of-flight (TOF) liquid chromatography (LC)/mass spectrometry (MS) system with electrospray ionization (ESI). Thin-layer chromatography (TLC) was performed on precoated silica gel F-254 plates (25 mm × 75 mm, Shanghai Pinjia Chemical Co., Ltd), and the samples were visualized with UV light. High-performance liquid chromatography (HPLC) analysis was performed using a SHIMADZU LC-20AB instrument. Melting points were measured with an X4-A microscopic melting point apparatus. All the starting materials and reagents were purchased from commercial suppliers.

Synthesis of compounds (1a–c); general procedures

To a solution of maleic anhydride (30 mmol, 2.94 g) in ether (30 mL) was added ethyl furan (30 mmol, 2.88 g) in a dropwise manner, and the resulting mixture was stirred overnight at room temperature in the dark. The progress of reaction was monitored by TLC until all the starting materials had been consumed. The solution was filtered, and the filter cake was washed with ether (60 mL) to afford I as a white solid27 (5.52 g, 95%). Next, concentrated sulfuric acid (180 mL) was added to a 250-mL round-bottom flask, and compound I was slowly added. The temperature was maintained at 0–5 °C with an ice–salt–water bath, and the mixture was stirred for 2 h, during which time the solution turned black/green. After completion of the reaction, the solution was added dropwise to an ice–water mixture, and a yellow flocculent solid was precipitated. This solid was filtered and dried to give II as a white solid27 (4.34 g, 79%). To a solution of compound II (17 mmol, 3 g) in acetic acid (60 mL) was added the corresponding amine (19.3 mmol) in a dropwise manner. The mixture was stirred for 2 h; during which time the solution turned black/green. After completion of the reaction, the solution was added dropwise to an ice–water mixture, and a yellow flocculent solid was precipitated. This solid was filtered and dried to give II as a white solid27 (4.34 g, 79%).
(PE:EtOAc = 3:1). After 4 h, when the reaction is over, the reaction solution was evaporated under reduced pressure at 70 °C to give a black oily substance. NaHCO₃ solution (4%) was added dropwise until no more bubbles were generated. The product was then recrystallized from hot ethanol to give the desired products 1a–c.

7-Oxo-N-phenethyl-5-phenyl-4,7-dihydropyrazolo[1,5-a]pyrimidine-3-carboxamide (2a). Using phenethylamine as the starting martial, the desired product 2a was obtained as a white solid (0.16 g, 45%). M.p. >300 °C. 1H NMR (400 MHz, DMSO-d₆) δ 8.68 (s, 1H), 7.97 (s, 1H), 7.85-7.76 (m, 2H), 7.42 (t, J = 3.5 Hz, 3H), 7.30-7.22 (m, 5H), 6.07 (s, 1H), 3.64 (t, J = 6.5 Hz, 2H), 2.88 (t, J = 6.9 Hz, 2H); 13C NMR (75 MHz, DMSO-d₆) δ 163.52, 162.81, 158.80, 157.81, 150.20, 142.68, 140.10, 139.47, 128.91, 128.93, 127.69, 126.58, 126.02, 102.01, 97.35, 95.51, 31.64; HRMS (ESI): m/z [M + H]^+ calculated for C₁₉H₁₈N₄O₂: 359.1430; found: 359.1433. Chromatographic purity: 100% (HPLC).

N-Benzyl-7-oxo-5-phenyl-4,7-dihydropyrazolo[1,5-a]pyrimidine-3-carboxamide (2b). Using benzylamine as the starting martial, the desired product 2b was obtained as a white solid (0.17 g, 55%). M.p. >300 °C. 1H NMR (400 MHz, DMSO-d₆) δ 8.68 (s, 1H), 7.97 (s, 1H), 7.85-7.76 (m, 2H), 7.42 (t, J = 3.5 Hz, 3H), 7.30-7.22 (m, 5H), 6.07 (s, 1H), 3.64 (t, J = 6.5 Hz, 2H), 2.88 (t, J = 6.9 Hz, 2H); 13C NMR (75 MHz, DMSO-d₆) δ 163.52, 162.81, 158.80, 157.81, 150.20, 142.68, 140.10, 139.47, 128.91, 128.93, 127.69, 126.58, 126.02, 102.01, 97.35, 95.51, 31.64; HRMS (ESI): m/z [M + H]^+ calculated for C₁₉H₁₈N₄O₂: 359.1430; found: 359.1433. Chromatographic purity: 100% (HPLC).

N-Butyl-7-oxo-5-phenyl-4,7-dihydropyrazolo[1,5-a]pyrimidine-3-carboxamide (2c). Using n-butylamine as the starting martial, the desired product 2c was obtained as a white solid (0.17 g, 55%). M.p. >300 °C. 1H NMR (400 MHz, DMSO-d₆) δ 8.70 (s, 1H), 7.98 (d, J = 7.4 Hz, 2H), 7.93 (s, 1H), 7.52-7.34 (m, 3H), 6.07 (s, 1H), 3.32 (t, J = 10.0 Hz, 2H), 1.59-1.49 (m, 2H), 1.43-1.40 (m, 2H), 0.93 (t, J = 7.2 Hz, 3H); 13C NMR (75 MHz, DMSO-d₆) δ 163.44, 158.91, 157.72, 150.15, 142.61, 139.41, 129.55, 128.93, 127.03, 127.44, 121.69, 124.47, 120.19, 114.91, 114.77, 69.95, 24.33, 15.45; HRMS (ESI): m/z [M + H]^+ calculated for C₁₉H₂₀N₂O₂: 345.1273; found: 345.1278. Chromatographic purity: 100% (HPLC).

Synthesis of compounds (2a–f); general procedures

A mixture of 3-amino-4-ethoxybenzylpyrazole (25 mmol, 3.82 g) and ethyl benzoyl acetate (25 mmol, 4.74 g) was heated in acetic acid (15 mL) at 120 °C for 12 h. After the completion of the reaction as evident by TLC, the solution was cooled and filtered. The residue was washed with acetic acid, filtered, and dried to give III as a beige crystalline powder (38%). To a solution of compound III (1.2 g) in a mixed solvent consisting of EtOH (32 mL), THF (16 mL), and H₂O (40 mL) was added KOH (10.16 mmol, 0.57 g) at 45 °C. After stirring for 10 min, the solid was almost completely dissolved. After maintaining the temperature at 50 °C for 3 h, KOH (6.24 mmol, 0.35 g) was added again and the temperature was raised to 80 °C. After 3 h, KOH (3.56 mmol, 0.2 g) was added, and the mixture was allowed to react overnight. The reaction was stopped and the solution was then cooled to room temperature. HCl (1.0 mol L⁻¹) was slowly added dropwise to adjust the pH to weak acidity, and a white crystalline solid was precipitated. After suction filtration, the filter cake was washed with deionized water and dried to obtain IV as a white crystal compound (72%). A solution of compound IV (1.0 mmol, 0.25 g) in DMF (10 mL) was treated with EDCI (1.2 mmol, 0.23 g), HOBT (1.2 mmol, 0.16 g), and DIEA (1.2 mmol, 0.15 g). After 1 h, the corresponding amine (1.2 mmol) was added dropwise, and the mixture was stirred overnight at 25 °C. The solution was transferred dropwise to the saturated NaHCO₃ aqueous solution, and a white flocculent solid was formed. The solid was filtered and dried to give the desired product 2a–f.
N-Hexyl-7-oxo-5-phenyl-4,7-diarylpyrazolo[1,5-a]pyrimidine-3-carboxamide (2d). Using n-hexylamine as the starting material, the desired product 2d was obtained as a white solid (0.17 g, 50%). M.p. >300 °C. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.72 (s, 1H), 8.01 (d, $J = 7.47$ Hz, 2H), 7.79 (s, 1H), 7.48-7.43 (m, 3H), 6.11 (s, 1H), 3.35 (t, $J = 12.0$ Hz, 2H), 1.59-1.55 (m, 2H), 1.46-1.42 (m, 2H), 1.35-1.24 (m, 4H), 0.86 (t, $J = 7.2$ Hz, 3H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ 163.43, 158.90, 157.73, 150.16, 142.62, 139.43, 129.52, 128.89, 127.02, 102.19, 91.67, 38.32, 31.60, 30.01, 26.88, 22.60, 14.39; HRMS (ESI): m/z $[M + H]^+$ calcd for C$_{19}$H$_{15}$N$_4$O$_3$: 335.1066; found: 335.1069. Chromatographic purity: 98.5% (HPLC).

7-Oxo-5-phenyl-N-[(tetrahydrofuran-2-yl)methyl]-4,7-diarylpyrazolo[1,5-a]pyrimidine-3-carboxamide (2e). Using (tetrahydrofuran-2-yl)methanamine as the starting material, the desired product 2e was obtained as a white solid (0.20 g, 59%). M.p. >300 °C. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 9.03 (s, 1H), 8.15 (d, $J = 7.1$ Hz, 2H), 7.97 (s, 1H), 7.46 (d, $J = 8.5$ Hz, 3H), 6.14 (s, 1H), 3.99 (m, 1H), 3.91 (d, $J = 7.9$ Hz, 1H), 3.76-3.50 (m, 3H), 1.97 (m, 4H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ 163.47, 158.93, 157.34, 150.24, 142.65, 139.06, 129.55, 128.82, 127.14, 101.97, 91.37, 78.13, 67.90, 42.24, 28.65, 26.02; HRMS (ESI): m/z $[M + H]^+$ calcd for C$_{18}$H$_{19}$N$_4$O$_3$: 339.1379; found: 339.1384. Chromatographic purity: 100% (HPLC).

N-(Furan-2-ylmethyl)-7-oxo-5-phenyl-4,7-diarylpyrazolo[1,5-a]pyrimidine-3-carboxamide (2f). Using furfurylamine as the starting material, the desired product 2f was obtained as a white solid (0.19 g, 57%). M.p. >300 °C. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 9.14 (s, 1H), 8.07-7.88 (m, 3H), 7.69 (s, 1H), 7.46-7.41 (m, 3H), 6.56-6.32 (m, 2H), 6.13 (s, 1H), 4.55 (s, 2H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ 163.16, 158.94, 157.55, 152.93, 150.23, 142.98, 142.65, 138.93, 129.62, 128.96, 127.06, 111.03, 107.41, 101.67, 91.65, 35.91; HRMS (ESI): m/z $[M + H]^+$ calcd for C$_{18}$H$_{19}$N$_4$O$_3$: 339.1066; found: 335.1069. Chromatographic purity: 100% (HPLC).

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