BF0801, a novel adenine derivative, inhibits platelet activation via phosphodiesterase inhibition and P2Y12 antagonism

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Summary
Though antiplatelet drugs are proven beneficial to patients with coronary heart disease and stroke, more effective and safer antiplatelet drugs are still needed. In this study we report the antiplatelet effects and mechanism of BF0801, a novel adenine derivative. BF0801 dramatically inhibited platelet aggregation and ATP release induced by ADP, 2MeS-ADP, AYPGKF, SFLLRN or convulxin without affecting shape change in vitro. It also potentiated the inhibitory effects of adenosine-based P2Y12 antagonist AR-C69931MX or phosphodiesterase (PDE) inhibitor IBMX on platelet aggregation. The cAMP levels in both resting and forskolin-stimulated platelets were increased by BF0801 suggesting its PDE inhibitor activity, which is further confirmed by the concentration-dependent suppression of BF0801 on the native and recombinant PDE.

Similar to AR-C69931MX, BF0801 drastically inhibited 2MeSADP-induced adenylyl cyclase inhibition in platelets indicating its P2Y12 antagonism, which may have therapeutic advantage as a potential antithrombotic drug.

Keywords
Antiplatelet, adenine derivative, cAMP, P2Y12, phosphodiesterase

Introduction
Arterial thrombotic diseases, such as heart attacks and stroke, are the leading cause of morbidity and mortality worldwide. Platelet activation triggered by the spontaneous disruption of atherosclerotic plaque or the endothelium injury caused by percutaneous coronary intervention (PCI) and the consequent intravascular arterial thrombogenesis is the common pathological basis of heart attacks and stroke; therefore antiplatelet drugs are effective for prevention and treatment of coronary artery disease and stroke. Currently, aspirin, a cyclooxygenase inhibitor, clopidogrel and prasugrel, thienopyridine class of the P2Y12 receptor antagonists, fibrinogen receptor antagonists, and cilostazol, a phosphodiesterase (PDE) inhibitor, are the mainly clinically used antiplatelet drugs. Among these antiplatelet drugs for arterial thrombotic diseases, P2Y12 receptor antagonists are most successfully and most widely used for coronary heart disease and stroke (1), while cilostazol is used mainly in peripheral arterial occlusion and is under clinical evaluation in patients undergoing PCI (2). Though antiplatelet drugs are proven to be beneficial to the patients with coronary heart disease, stroke and peripheral arterial disease, the outcome is still not satisfactory. Intensive efforts are devoted to develop novel antiplatelet agents with improved efficacy and safety.

Among the numerous platelet agonists, thrombin, collagen and thromboxane A2 (TXA2) are strong platelet activators while adenosine diposphate (ADP) is a weak activator. ADP is stored in dense granules at molar concentration and released during platelet activation induced by thrombin, collagen or TXA2. The released ADP works as a positive feedback mediator and therefore plays a key role in platelet activation, although itself it is a weak platelet activator.

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ADP activates platelets via co-activation of two G-protein coupled receptors: Gq-coupled P2Y1 induces platelet shape change through phospholipase Cβ activation, calcium mobilisation (3, 4), while Gi-coupled P2Y12 leads to platelet aggregation (5), inhibition of adenyl cyclase, the consequent decrease of intraplatelet cAMP, PI3 kinase activation and Rap1B activation (6). Coactivation of P2Y1 and P2Y12 is essential for ADP-induced platelet aggregation (7). Of the two ADP receptors, P2Y1 is ubiquitously expressed in a myriad of tissues, while P2Y12 is mainly distributed in brain and platelets, and has been intensively investigated in platelets. The central role of P2Y12 in platelet activation, pathological process in arterial thrombogenesis (6) and its tissue-specific distribution renders P2Y12 the most successful target to develop novel P2Y12 receptor antagonists as antiplatelet agents.

In our pursuit to develop novel P2Y12 receptor antagonists as antiplatelet agents, we synthesized a series of novel chemicals based on the structure of P2Y12 receptor antagonists under clinical trial and development as antithrombotic drugs, and tested their antiplatelet activity. Among dozens of chemicals screened, we found propionic acid 2-(6-amino-2-(propylthio)-purin-9-yl)-ethyl ester (BF0801, Fig. 1A), an adenine derivative, exhibited most potent inhibition on platelet aggregation induced by ADP. The antiplatelet activity of BF0801 was further confirmed using platelets stimulated by other agonists and by dosing rats in ex vivo study. When the mechanism was explored, to our surprise, we found that BF0801 exerted its antiplatelet roles via PDE inhibition in addition to P2Y12 receptor antagonism.

Materials and methods

Regents and chemicals

BF0801 was synthesized by Institute of Materia Medica, Beijing University of Chemical Technology. ADP was purchased from Chrono-Log (Havertown, PA, USA). 2-methylthio-ADP (2MeS-ADP), apyrase grade VII, human fibrinogen, forskolin, IBMX, epinephrine, cAMP, and aspirin were from Sigma (St Louis, MO), PAR-1 activating hexapeptide (SFLRN-amide) and PAR-4 activating hexapeptide (AYPGKF-amide) were synthesized by Shanghai Bootech BioScience & Technology (Shanghai, China). Convulxin was purchased from Centerchem (Norwalk, CT, USA). AR-C69931MX was a gift from AstraZeneca (Loughborough, UK). [3H]cAMP was from PerkinElmer (Waltham, MA, USA). cAMP 125I, cGMP 125I radioimmunoassay kits were provided by Isotope Laboratory of Shanghai University of Traditional Chinese Medicine (Shanghai, China). Sodium nitroprusside was purchased from Beijing DCPC (Beijing, China). Cilostamide and MRS2179 were from Tocris Bioscience (Ellisville, MO, USA). Erythro-9-(2-hydroxy-3–nonyl) adenine (EHNA) and 4-[[3,4-(Methylenedioxy) benzyl]amino]-6– chloroquinazoline (MBCQ) were from Biomol (Plymouth Meeting, PA, USA). Phosphodiesterase type 2A (PDE2A) and phosphodiesterase type 3A (PDE3A) were from BPS Bioscience (San Diego, CA, USA). Anti-Akt and anti-phospho-Akt (Ser473) antibodies were from Cell Signaling Technology (Beverly, MA, USA).

Figure 1: BF0801 inhibited ADP-induced platelet activation. A) Structure of BF0801. B) BF0801 concentration-dependently inhibited 10 μM ADP-induced platelet aggregation in aspirin-treated human washed platelets. C) BF0801 (300 μM) abolished ATP release. Aggregation and ATP release were simultaneously recorded in non-aspirin treated human washed platelets. BF0801 (300 μM) pretreatment substantially inhibited platelet aggregation and abolished ATP release induced by ADP (10 μM). D) Dose-dependent inhibition by BF0801 on platelet aggregation induced by ADP (10 μM), with IC50 of 63.3 μM. Each bar was the average of three experiments ± SEM from three different donors. Tracings shown are representative of at least three experiments using platelets from different donors. DMSO was used as a vehicle control.
Animals

Mice deficient in P2Y12 (9, 10) were generated as described. Wild-type littermates were used as controls. Animal procedures were carried out in accordance with institutional guidelines after Fudan University Animal Care and Use Committee approved the study protocol.

Preparation of human platelets

All experiments using human subjects were performed in accordance with the Declaration of Helsinki and approved by the Institutional Review Board Fudan University. Only healthy volunteers without taking aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) for at least 14 days were recruited and informed consent was obtained before blood collection. Washed platelets were prepared as described previously (11–13).

Preparation of mouse platelets and rat platelets

Blood was collected from the abdominal aorta of pentobarbital sodium anesthetised mice into syringes containing 100 µl ml⁻¹ Whites anticoagulant (49.4 g L⁻¹ sodium citrate, 24.6 g L⁻¹ glucose), pH 6.4, 0.1 µg ml⁻¹ PGE₁, and 1 U ml⁻¹ apyrase. Washed platelets were prepared and resuspended in Tyrode’s buffer at a final concentration of approximately 10⁶ platelets µl⁻¹ as described previously (14).

Male Sprague-Dawley (SD) rats weighing 250 – 350 g (Animal Center of Fudan University) were anesthetised by intraperitoneal injection 10% chloral hydrate (400 mg kg⁻¹). At time zero, BF0801 (50 mg kg⁻¹) or vehicle was injected into the tail vein. Blood (6.3 ml) was drawn into tubes containing 0.7 ml 3.8 % sodium citrate 5 minutes (min) later by cannulation of common carotid artery and immediately centrifuged (300 x g, 10 min) at room temperature to get PRP. Platelet number was adjusted to 5 x 10⁸ ml⁻¹ using platelet-poor plasma (PPP).

Measurement of platelet aggregation

Platelet aggregation was analysed using lumi-aggregometer (Model 400VS, Chrono-Log) (11–13) under stirring conditions (900 rpm) at 37°C. Agonists were added to initiate aggregation with or without preincubation with antagonists for 3 min. The chart recorder (Model 707, Chrono-Log) was set for 1 cm min⁻¹. The baseline was set using Tyrode’s buffer or PPP as blank. For ADP- or 2MeSADP-induced aggregation in human washed platelets, 1 mg mL⁻¹ fibrinogen was added (11, 12).

Measurement of cAMP and cGMP in human platelets

Human blood (36 ml) drawn into tubes containing 6 ml ACD solution was centrifuged at 300 x g for 10 min to generate PRP. Platelets were further pelleted by centrifugation at 900 x g for 10 min and were homogenised in 1 ml ice-cold 30 mM phosphate-buffered saline (PBS) containing 0.1% Triton X-100 using ultrasonic homogeniser (10 times for 5 seconds [s] with interval of 5 s, 50 Hz) (Model UR-200P, Tokyo, Japan). Platelet homogenate was centrifuged at 12,000 x g for 20 min at 4°C using Eppendorf Centrifuge 5415 R (Hamburg, Germany). The supernatant was transferred to a tube and stored at −80°C until use or subjected to PDE activity assay.

Preparation of cAMP-PDE extracts from human platelets

Human blood (36 ml) drawn into tubes containing 6 ml ACD solution was centrifuged at 300 x g for 10 min to generate PRP. Platelets were further pelleted by centrifugation at 900 x g for 10 min and were homogenised in 1 ml ice-cold 30 mM phosphate-buffered saline (PBS) containing 0.1% Triton X-100 using ultrasonic homogeniser (10 times for 5 seconds [s] with interval of 5 s, 50 Hz) (Model UR-200P, Tokyo, Japan). Platelet homogenate was centrifuged at 12,000 x g for 20 min at 4°C using Eppendorf Centrifuge 5415 R (Hamburg, Germany). The supernatant was transferred to a tube and stored at −80°C until use or subjected to PDE activity assay.

Assay for cAMP-PDE activity in human platelets

The assay for cAMP-PDE activity was performed using HPLC (18, 19). DMSO vehicle, 100 μM IBMX or different concentrations of BF0801 were added to 200 μl assay buffer (137 mM NaCl, 2.7 mM KCl, 8.8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 1 mM CaCl₂, 1 mM MgCl₂, 10 μM cAMP, pH 7.4). Reaction was initiated by adding 10 μl PDE extracts prepared from human platelets as described above. In inactivated enzyme reaction system PDE extracts were boiled for 3 min before being added. After incubation at 37°C for 30 min, the reaction was stopped by boiling the mixture for 3 min. The reaction mixture was cooled on ice, followed by centrifugation at 12,000 x g for 30 min at 4°C. The cAMP in the supernatant was analysed by HPLC (Kromasil 4.6 x 150 mm, Eka-chemicals, Bohns, Sweden). The amount of cAMP was determined using a standard curve. The converted cAMP was measured as residual cAMP amount in inactivated enzyme reaction system minus residual cAMP amount in enzyme reaction system treated by different agents. The inhibition of PDE activity was calculated using the following formula: % inhibition of PDE activity = (1 – converted cAMP in enzyme reaction system treated by different agents / converted cAMP in untreated enzyme reaction system) x 100%.

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Assay for cAMP-PDE activity using recombinant PDEs

Similarly, the effects of BF0801 on the recombinant PDE2A, PDE3A were investigated in the PDE activity assay buffer as described above except for using 10 μl of 30 mM PBS containing 0.1 μg PDE2A or PDE3A instead of 10 μl PDE extracts.

Immunoblot detection of Akt phosphorylation in human platelets

Platelets were stimulated with agonists under stirring condition for the appropriate time, and the Akt phosphorylation was measured as previously described (16).

Atomic force microscopy (AFM) of interaction between ADP and P2Y_{12} receptor

Experiments were performed in phosphate buffered saline using a MFP-3D-BIO atomic force microscope (Asylum Research, Santa Barbara, CA, USA) integrated with an IX71 inverted microscope (20) (Olympus, Tokyo, Japan). Silicon nitride cantilevers (MLCT, Veeco Probes, Santa Barbara, CA, USA) were soaked in acetone for 5 min, UV-irradiated for 15 min, and then incubated in 2% (v/v) (3-Mercaptopropyl) trimethoxysilane in acetone for 15 min, followed by rinsing three times with ethanol and six times with PBS. The cantilevers were then incubated in NHS-PEG5000-MAL solution (50 mM in sodium borate buffer) for 120 min and rinsed with sodium borate buffer for six times. Finally, the cantilevers were incubated in ADP (500 ng/μl, 20 μl) overnight and unbound ADP was removed by rinsing in PBS (21).

Traces recorded the interaction between P2Y_{12} receptor expressing CHO-K1 cells and ADP in the presence of different ADP receptor antagonists, BF0801 or vehicle control, respectively. The breakage (unbinding) forces were defined as the magnitude of maximal force relative to the baseline upon tip dissociation from the sample obtained with a compression force at speeds of 3 μm/s and conditions that minimised contact (0.2 s contact time and 100 pN indentation force). Frequencies of observing positive interactions between tip and cell surface were between 20 and 30%. The specificity of the cantilever-bound ADP-P2Y_{12} receptor interactions was confirmed by a reduction in the frequency of interaction to 5% when an unmodified cantilever was used.

Cantilevers were calibrated using thermal method with a spring constant of around 20 pN/nm.

Statistical analysis

All data are expressed as mean ± SEM. Differences between the groups were analysed by ANOVA followed by a Newman–Keuls test using GraphPad Prism version 4.0. P-values less than 0.05 were considered statistically significant.

Results

Inhibitory effect of BF0801 on ADP-induced platelet aggregation and ATP release

We synthesised a series of novel chemicals based on the existing P2Y_{12} receptor antagonists with antiplatelet activities under clinical trial and development. Among them, BF0801 (Fig. 1A), an adenosine derivative, exhibited maximal inhibitory effects on platelet aggregation induced by ADP. To further confirm the antiplatelet effects of BF0801 and explore its putative P2Y_{12} receptor antagonist mechanism, we investigated the antiplatelet effects of BF0801 in human washed platelets pretreated with aspirin and stimulated with higher concentration of ADP. As shown in Figure 1B, BF0801 concentration-dependently inhibited platelet aggregation induced by 10 μM ADP with an IC_{50} of 63.3 μM (Fig. 1D). As a putative P2Y_{12} receptor antagonist, BF0801 inhibited platelet aggregation without affecting shape change. In non-aspirin treated human washed platelets, pretreatment for 3 min with 300 μM BF0801 drastically inhibited ADP-induced platelet aggregation and abolished adenosine triphosphate (ATP) release (Fig. 1C).

Inhibitory effect of BF0801 on platelet aggregation and ATP release induced by convulxin, SFLLRN, and AYPGKF

BF0801 inhibited ADP-induced platelet aggregation without affecting shape change, suggesting its putative P2Y_{12} antagonism activity. P2Y_{12} receptor participates in platelet activation induced by other platelet agonists including collagen, thrombin and thromboxane. Hence, we examined the antiplatelet effects of BF0801 on platelet aggregation induced by convulxin, a GPVI agonist. In aspirinated human washed platelets, 100 ng ml^{-1} convulxin-induced platelet aggregation partially depends on P2Y_{12} receptor activation resulting from released ADP (22). As expected, BF0801 concentration-dependently inhibited platelet aggregation induced by 100 ng ml^{-1} convulxin, but not platelet shape change (Fig. 2A.1). Similarly, platelet aggregation induced by PAR-1 agonist SFLLRN (10 μM) or PAR-4 agonist AYPGKF (100 μM) was also inhibited by BF0801 in a concentration-dependent manner, without affecting shape change (Fig. 2A.2 and A.3). We used the maximal concentration of SFLLRN and lower (sub-maximal) concentration of AYPGKF to induce platelet aggregation in aspirinated human washed platelets (23). Under such conditions platelet aggregation is partially dependent on P2Y_{12} activation stimulated by secreted ADP and can be partially inhibited by P2Y_{12} receptor antagonist. Therefore the inhibition of BF0801 on platelet aggregation induced by SFLLRN and AYPGKF is in line with its putative P2Y_{12} re-
ceptor antagonist activity. Furthermore, pretreatment of platelets for 3 min with 300 μM BF0801 slightly inhibited ATP release induced by 10 μM SFLLRN (Fig. 2B.2), and almost abolished ATP release induced by 100 ng ml⁻¹ convulxin as well as 100 μM AYPGKF (Fig. 2B.1 and 2B.3).

BF0801 potentiates the suppression of AR-C69931MX and IBMX on platelet aggregation induced by convulxin, SFLLRN and AYPGKF

P2Y₁₂ receptor antagonists have been reported to slightly inhibit convulxin-induced platelet aggregation by blocking the positive feedback of released ADP from dense granules (24). As anticipated, 100 nM AR-C69931MX, a P2Y₁₂ receptor antagonist, only marginally inhibited aggregation induced by 100 ng ml⁻¹ convulxin, which was consistent with the previous report (24). Interestingly, 300 μM BF0801 markedly inhibited 100 ng ml⁻¹ convulxin-induced platelet aggregation. Moreover, this inhibitory effect was much stronger than that of 100 nM AR-C69931MX and there was an obvious synergy between BF0801 and AR-C69931MX on platelet activation induced by convulxin (Fig. 3A.1). The synergy between BF0801 and AR-C69931MX against platelet activation suggests BF0801 exerts its antiplatelet role via another mechanism in addition to P2Y₁₂ antagonism or through a different mechanism because we used the maximal concentration of AR-C69931MX to antagonise P2Y₁₂ receptor, which is 250 times higher than the IC₅₀ of AR-C69931MX to inhibit ADP-induced platelet aggregation (25). Similar synergistic inhibition was noticed when platelet activation was induced by SFLLRN or AYPGKF (Fig. 3A.2 and A.3).

IBMX has been shown to partially inhibit thrombin-induced platelet aggregation at 6 μM (26). At much higher concentration, 100 μM and 300 μM, we got similar inhibition on platelet aggregation induced by SFLLRN and AYPGKF (see Supplementary Fig. 1 available online at www.thrombosis-online.com) suggesting 100...
μM IBMX is providing maximal inhibition. We found 300 μM BF0801 and 100 μM IBMX synergistically suppressed convulxin, SFLRN or AYPGKF induced platelet aggregation of human washed platelets (Figure 3B.1, B.2 and B.3), hinting the existence of a mechanism in addition to PDE inhibition for BF0801, consistent with its putative P2Y12 antagonist mechanism.

**Inhibitory effect of BF0801 on platelet activation induced by co-activation of Gz plus Gq**

In aspirin-treated washed platelets, preincubation with AR-C69931MX followed by stimulation with 2MeSADP (a stable and more potent ADP analogue) plus epinephrine can induce aggregation similar to ADP-induced aggregation; this is because of the coactivation of both Gq- and Gz-signaling pathways (7). We found that aggregation induced by co-stimulation of Gq plus Gz was abolished by 300 μM BF0801 (see Supplementary Fig. 2 available online at www.thrombosis-online.com). Such finding further corroborates the existence of a mechanism distinct from P2Y12 receptor underlying the antiplatelet role of BF0801. Taken together, we herein propose that BF0801 exert its antiplatelet role via PDE inhibition in addition to P2Y12 receptor antagonism.

**Effect of BF0801 on cyclic nucleotide levels in platelets**

Thus far, our data suggest that BF0801 exerts its antiplatelet role via possible involvement of PDE inhibition and P2Y12 receptor antagonism. To further elucidate the mechanism of BF0801 against platelet activation, we studied whether BF0801 would affect intracellular cyclic nucleotide levels. BF0801 significantly inhibited 2MeSADP-induced cAMP decrease in forskolin-treated platelets as did AR-C69931MX (Fig. 4A); this data strongly supports the involvement of P2Y12 antagonism as its antiplatelet mechanism. However, BF0801 also slightly inhibited epinephrine-induced cAMP decrease in forskolin-treated platelets and significantly increased cAMP level in platelets pretreated with forskolin (Fig. 4B). These results cannot be explained by P2Y12 receptor blocking alone and concur with PDE inhibition underlying the antiplatelet role of BF0801. In addition to augmenting forskolin-elevated intracellular cAMP, BF0801 also notably increased basal cAMP level in resting platelets similar to PDE inhibitors EHNA and cilostamide (Fig. 4C). Similar to cAMP, intracellular cGMP increase induced by 100 μM sodium nitroprusside (SNP) was also further remarkably enhanced by 100 μM BF0801 (Fig. 4D). Taken together, these findings strongly suggest the involvement of PDE inhibition as well as P2Y12 antagonism in the antiplatelet role of BF0801.

In accordance with its anti-aggregatory synergy with AR-C69931MX or IBMX (Fig. 3), BF0801 demonstrated synergistic inhibition on 2MeSADP-induced cAMP decrease in platelets stimulated with forskolin when used in combination with AR-C69931MX or IBMX (Fig. 4E). These results further strengthen the dual antiplatelet role of BF0801 via PDE inhibition and P2Y12 antagonism.

**BF0801 inhibits the activity of PDE extracted from human platelets**

To provide direct evidence that BF0801 inhibits platelet activation via PDE inhibition, we prepared PDE extracts from human platelets and investigated the effects of BF0801 on the PDE activity. PDE activity was measured by HPLC analysing the residual cAMP content in 200 μl reaction mixture containing platelet PDE extracts, 10 μM cAMP, and different concentrations of BF0801 incubated at 37°C for 30 min. As shown in Figure 5A, at concentrations ranging from 10 μM to 300 μM, BF0801 inhibited the activity of platelet PDE extracts in a concentration-dependent manner as evidenced by concentration-dependent increase in the residual cAMP content in the reaction mixture (Fig. 5B). The maximal inhibition of 300 μM BF0801 on PDE extracts was similar to that of 100 μM IBMX (more than 50% inhibition), a non-specific PDE inhibitor, while AR-C69931MX (100 nM) did not inhibit PDE activity (data not shown).

**BF0801 inhibits activities of recombinant PDEs**

So far, we have shown that BF0801 inhibited the degradation of cAMP and cGMP in platelets (Fig. 4) and confirmed the inhibitory role of BF0801 on platelet PDE extracts (Fig. 5); therefore our next goal was to identify the specific phosphodiesterase inhibited by BF0801. It has been known that human platelets express PDE2A, PDE3 and PDE5 responsible for hydrolysis of cyclic nucleotide in platelets (27). Among them only PDE2A and PDE3 have dual specificity for cAMP and cGMP, while PDE5 specifically degrades cGMP. We have shown that BF0801 non-specifically potentiated the increase of both cAMP and cGMP in platelets (Fig. 4). Therefore we did not evaluate the possible role of BF0801 on PDE5 in this study, though it may also be suppressed by BF0801. PDE3A has been reported to be the main subtype of PDE3 and functions in platelets (28).

Hence, we studied the effect of BF0801 on the activities of the recombinant PDE2A and PDE3A. As showed in Figure 6, BF0801 (100 or 300 μM) exhibited strong inhibition on PDE2A activity. The maximal inhibition of 300 μM BF0801 on PDE2A was similar to that of EHNA, a non-selective PDE2 inhibitor (29) (Fig. 6A). In contrast, BF0801 (100 or 300 μM) demonstrated less inhibition on PDE3A activity compared with the selective PDE3 inhibitor cilostamide (29) (Fig. 6B).

Moreover, to correlate the inhibitory effect of BF0801 on PDE with its antiplatelet activity, we compared the inhibitory effect of BF0801 with other PDE inhibitors including PDE2-specific inhibitor EHNA, PDE3-specific inhibitor cilostamide, and PDE5 inhibitory SFLLRN or AYPGKF induced platelet aggregation of human washed platelets (Figure 3B.1, B.2 and B.3), hinting the existence of a mechanism in addition to PDE inhibition for BF0801, consistent with its putative P2Y12 antagonist mechanism.
Figure 4: Effect of BF0801 on cAMP and cGMP levels in human washed platelets.
A) BF0801 inhibited 2MeSADP-induced cAMP decrease in human washed platelets stimulated with forskolin. B) BF0801 potentiated forskolin-induced cAMP increase and slightly reversed epinephrine-induced cAMP decrease.
C) BF0801 increased basal cAMP in washed human platelets. D) BF0801 potentiated SNP-induced cGMP elevation in washed human platelets. E) BF0801 synergistically potentiated the inhibition of AR-C69931MX or IBMX on 2MeSADP-induced cAMP decrease in human platelets stimulated with forskolin. cAMP levels were measured using chromatography (A, B and E) or radioimmunoassay (C) as described under Materials and methods. Data were expressed as mean ± SEM representing 3–7 separate experiments measured in duplicate. Unless otherwise stated, BF0801 (BF) (300 μM), 2MeSADP (100 nM), forskolin (Fors) (20 μM), epinephrine (Epi) (10 μM), EHNA (200 μM), cilostamide (100 μM), AR-C69931MX (ARC) (100 nM) and IBMX (100 μM) were used.
hibitor MBCQ. All the PDE inhibitors were used at maximal concentrations in our experiment to inhibit the corresponding PDE activity completely. The order of inhibition of different chemicals was: BF0801 > cilostamide > MBCQ > EHNA (Fig. 6C). Such data indicate that dual activity against PDE and P2Y12 may have better antiplatelet effects.

Lack of synergistic inhibition of BF0801 and IBMX on AYPGKF-induced platelet aggregation in P2Y12 null mice

To decipher the P2Y12 receptor blocking mechanism in the antiplatelet role of BF0801, we employed P2Y12 knockout mice in our study. We first confirmed the P2Y12 deficiency in the P2Y12 knockout mice we used. As shown in Figure 7A.1, platelets from P2Y12 knockout mice lost their response to 100 nM 2MeSADP for platelets aggregation (30), while the same concentration of 2MeSADP-induced robust aggregation in platelets from the wild-type littermates. Using these mice, we further found that the synergistic inhibition of BF0801 and IBMX on AYPGKF-induced platelet aggregation found in human platelets (Fig. 3B.3) was observed only in wild-type mice (Fig. 7A.2) but not in P2Y12 knockout mice (Fig. 7A.3). In P2Y12 knockout mice, IBMX alone demonstrated similar inhibition on AYPGKF-induced platelet aggregation as IBMX plus BF0801 (Fig. 7A.3). These data support that BF0801 also works as a P2Y12 receptor antagonist to exert its antiplatelet roles.

BF0801 inhibited Akt phosphorylation

Akt is an important downstream signal molecule in platelet P2Y12-Gi signalling pathway. Akt is activated and phosphorylated upon P2Y12 receptor activation in platelets. P2Y12 receptor antag-
Figure 6: PDE inhibition specificity of BF0801 and its anti-aggregatory role in comparison with other PDE inhibitors.

A) BF0801 (BF) concentration-dependently inhibited the activity of recombinant PDE2A with similar efficacy as EHNA, a selective PDE2 inhibitor. B) Relatively weak inhibition of BF0801 on PDE3A. Data were expressed as mean ± SEM representing three separate experiments measured in duplicate. C) The anti-aggregatory efficacy of BF0801 (300 μM) in comparison with other specific PDE inhibitors including EHNA (200 μM), cilostamide (100 μM), and MBCQ (100 μM). At the respective maximal concentrations, BF0801 exhibited maximal inhibition on platelet aggregation in human washed platelets stimulated with 2MeS-ADP (100 nM). Tracings shown are representative of three separate experiments using platelets from different donors.

Figure 7: P2Y12 antagonism anticipated in the antiplatelet effect of BF0801. A) BF0801 synergistically potentiated the inhibition of IBMX on platelet aggregation induced by AYPGKF in wild-type mouse platelets but not in P2Y12 knockout mouse platelets. 2MeSADP (100 nM), BF0801 (BF) (300 μM), IBMX (100 μM) and AYPGKF (200 μM) were used. Tracings shown are representative of experiments run on two different days using platelets from 11 different knockout (KO) mice and eight wild-type (WT) mice. B) BF0801 concentration dependently inhibited Akt phosphorylation in aspirin-treated human washed platelets induced by 2MeSADP. Platelets preincubated with DMSO, AR-C69931MX (ARC) or BF0801 were stimulated at 37°C for 5 min with 100 nM 2MeSADP. Equal amounts of proteins were separated by SDS-PAGE, Western blotted, and probed for anti-phospho-Akt (Ser473) or anti-Akt antibody. The results shown are representative of three experiments.
onist inhibits Akt phosphorylation in platelets (31). Hence, we sought to explore whether BF0801, as a P2Y12 receptor antagonist, influences Akt phosphorylation. As shown in Figure 7B, similar to P2Y12 receptor antagonist ARC-69931MX, in the range of 30 – 300 μM, BF0801 concentration-dependently inhibited 100 nM 2MeS-ADP-induced Akt phosphorylation in human washed platelets. Interestingly, at 300 μM BF0801 had a much more marked effect in reducing Akt phosphorylation than ARC 69931MX. PDE inhibition has been reported to attenuate Akt phosphorylation (32), the better effect of BF0801 than AR-C69931MX in reducing Akt phosphorylation may be explained by its dual activity of P2Y12 antagonism and PDE inhibition.

**BF0801 inhibited interaction between ADP and P2Y12 receptor**

Direct force measurements of interaction between ADP and single P2Y12-expressing CHO-K cell were carried out by AFM. As shown in Figure 8, similar to P2Y12 receptor antagonist ARC-69931MX, BF0801 significantly inhibited the interaction between ADP and P2Y12 receptor expressed in CHO-K1 cells, while MRS2179, a P2Y1 antagonist of platelet ADP receptor, did not affect the interaction between ADP and P2Y12 receptor.

**Binding mode of BF0801 to P2Y12 receptor**

So far, our data demonstrate that BF0801 inhibits ADP binding to P2Y12 as a receptor antagonist. To investigate binding mode of BF0801 to P2Y12 receptor, docking simulations were employed to search optimal pose of BF0801 against the receptor model (33) by Glide (Glide, version 5.5, Schrödinger, LLC, New York, NY, 2009). As a result of docking of BF0801 into P2Y12 receptor, we obtained a complex showing an overall binding mode with the ligand accommodated in the pocket formed by the upper part of TM3, TM6, and TM7 and closed by EL2. The conserved residues of the human P2Y12 receptors K174 in TM2 and R256 in TM6, play key role in binding to the ligand by hydrogen bonds, as shown in Figure 8D, which is in agreement with the available pharmacological and mutagenesis data. Furthermore, the amide in the main chain of Cys175 provides additional hydrogen bond to stabilise the pose of the ligand in the active site of the P2Y12 receptor.

**Antiplatelet effect of BF0801 confirmed in ex vivo study by dosing rats**

Finally, when administrated intravenously to rats, BF0801 (50 mg kg<sup>-1</sup>) almost abolished 10 μM ADP or 100 nM 2MeSADP-induced platelet aggregation in PRP after BF0801 intravenous administration for 5 min (see Supplementary Fig. 3 available online at www.thrombosis-online.com). Further observation of one month did not show abnormal physiological change on rats dosed by BF0801 (50 mg kg<sup>-1</sup>) (data not shown).

**Discussion**

The proven benefit of clopidogrel and prasugrel for patients with stroke and heart attack has made P2Y12 receptor the most successful target to develop novel antiplatelet drugs. In this study, we reported a novel adenine derivative targeting both P2Y12 and PDE. BF0801 exerts its antiplatelet effects via P2Y12 antagonism because: (i) BF0801 dramatically inhibited platelet aggregation, but not platelet shape change induced by ADP (Fig. 1); (ii) BF0801 inhibited platelet aggregation and ATP release in human washed platelets stimulated with convulxin, SFLLRN, and AYPGKF (Fig. 2), consistent with the potentiation role of released ADP in platelet activation induced by convulxin, SFLLRN and AYPGKF (22, 23); (iii) BF0801 reversed 2MeSADP-elicted adenylyl cyclase inhibition (Fig. 4A) and Akt phosphorylation (Fig. 7B) in platelets as did AR-C69931MX, a well-characterised P2Y12 receptor antagonist under clinical trial as antiplatelet drug in the name of cangrelor; (iv) BF0801 synergistically potentiated the inhibitory effect of non-specific PDE inhibitor IBMX on convulxin, SFLLRN or AYPGKF-stimulated platelet aggregation (Fig. 3B); (v) IBMX inhibited 2MeSADP-induced cAMP decrease in forskolin-pretreated platelets, whereas BF0801 synergistically potentiated the effects of IBMX (Fig. 4E); (vi) Using platelets from P2Y12 knockout mice we demonstrated that P2Y12 knockout abolished the synergistic inhibition of BF0801 and IBMX on AYPGKF-induced platelet aggregation (Fig. 7A). (vii) Finally and most importantly, AFM measurements showed BF0801 inhibited the interaction of ADP and P2Y12 directly (Fig. 8) as did AR-C69931MX. By comparing the structures of BF0801 and several P2Y12 receptor antagonist antiplatelet agents including AR-C66096 (FPL66096) (34), AR-C67085 (FPL67085), AR-C69931MX, AR-C78511, and AZD6140 (ticagrelor), we found all of them contain 2-propylthioadenine core structure (see Supplementary Figure 4 available online at www.thrombosis-online.com), this provides the structural basis for BF0801 as a P2Y12 receptor antagonist. Docking stimulation result also demonstrates that BF0801 is accommodated in the pocket formed by the upper part of TM3, TM6, and TM7 and closed by EL2 of P2Y12 receptor (Fig. 8D), providing further evidence that BF0801 targets P2Y12 receptor.

In our P2Y12 receptor knockout mouse experiments (Fig. 7A), the concentration of AYPGKF is 200 μM, a low concentration which can be used to evaluate the contribution of P2Y12 receptor in mouse platelet aggregation (23). In the P2Y12-deficient mouse platelets, aggregation induced by 200 μM AYPGKF was less than that in wild-type mouse platelets because of the loss of P2Y12-Gi activation, which is consistent with the previous study (23). The stronger inhibition of BF0801 on aggregation induced by AYPGKF in knockout mice compared with wild-type is due to the deficiency of P2Y12 receptor, which is only partially blocked by BF0801.
even at 300 μM (Fig. 1C). The dramatically synergistic inhibition between BF0801 and IBMX observed in wild-type platelets rather than P2Y12 knockout mouse platelets indicated that BF0801 also targets P2Y12 receptor as an antiplatelet agent.

AFM has increasingly been used to study ligand-receptor interactions either by using purified proteins (35, 36) or, more recently, in situ in living cells (37–39). For instance, interaction of fibronogen ligand and integrin αIIbβ3 receptor in platelets has been visualised by AFM in a physiological environment (40). Using this technology we investigated the interaction between P2Y12 receptor and its physiological ligand ADP in the presence of different ADP receptor antagonists or BF0801; our AFM data demonstrated that

Figure 8: BF0801 inhibited interaction between ADP and P2Y12 receptor. A) Typical atomic force microscopy (AFM) force-displacement measurements of the interaction between ADP and P2Y12 receptor expressed in CHO-K1 cells in the presence of different ADP receptor antagonists, BF0801 or vehicle control. The measurements were acquired with 100 pN indentation force, 0.2 s contact time, and a cantilever retraction speed of 3 μm/s. B) Histograms of individual unbinding forces of ADP-P2Y12 in the presence of different ADP receptor antagonists, BF0801 or vehicle control from force-displacement measurements. The y-axis plots the number of force transitions detected and x-axis is the unbinding force (pN). C) Statistical analysis of unbinding forces of ADP-P2Y12 in the presence of different ADP receptor antagonists, BF0801 (filled bars) or vehicle control (blank bars). Data were expressed as mean ± SEM of the 80 measurements in panel B. D) Predicted binding mode of BF0801 to human P2Y12 receptor. Oxygen atoms are shown in red, nitrogen atoms in blue and sulphur atoms in dark yellow. Carbon atoms of BF0801 and the human P2Y12 receptor are shown in cyan and green, respectively. Side chains of crucial residues in the binding site are shown as stick and labelled. Hydrogen bonds between BF0801 and P2Y12 are depicted in dotted line in yellow. Figures were generated by PyMol. AR-C69931MX (100 nM), MRS2179 (300 nM) and BF0801 (300 μM) were used.
under similar force loading rates, BF0801 dramatically lowered the single molecule unbinding force between ADP and P2Y\textsubscript{12} receptor, similar to AR-C69931MX, a P2Y\textsubscript{12} receptor antagonist (Fig. 8).

Cyclic AMP plays an important role in platelet aggregation. The cAMP level is regulated by adenylyl cyclase and PDE. PDEs are a large group of enzymes that catalyse the hydrolysis of 3',5'-cyclic nucleotides (cAMP and cGMP) to inactive 5'-nucleotides by cleaving a phosphodiesterase bond and therefore affect a myriad of cell functions through regulation of intracellular level of cAMP and cGMP. Though cAMP decrease does not activate platelets, intracellular cAMP increase inhibits platelet aggregation (41–43). Three types of PDEs are found in human platelets. PDE3A is expressed in platelets, as well as in vascular smooth muscle, cardiac myocytes, and oocytes. In addition to platelets, heart, and endothelial cells, PDE2 is also expressed in brain. PDE3 inhibitor cilostazol has been clinically used as an antiplatelet drug safely. BF0801 targets both PDE2 and PDE3, the side effects associated with PDE2 inhibition in brain may be mild due to blood-brain barrier. However, further toxicological study is still needed before BF0801 could be clinically used as an antithrombotic drug.

BF0801 achieved better aggregation inhibition effects than AR-C69931MX in washed platelets when convulxin, SFLLRN or AYPGKF was used to stimulate platelet (Fig. 3A.1, A.2 and A.3). The different action profile between BF0801 and AR-C69931MX prompts us to speculate that BF0801 may inhibit platelet aggregation via a mechanism different from P2Y\textsubscript{12} receptor antagonism alone. We further demonstrate that BF0801 also acts as a PDE inhibitor because: (i) Much stronger inhibition of BF0801 than AR-C69931MX on convulxin-induced aggregation (Fig. 3A.1); (ii) BF0801 synergistically potentiated the anti-aggregatory roles of AR-C69931MX on platelets stimulated with convulxin, SFLLRN or AYPGKF (Fig. 3A); (iii) BF0801 substantially increased both forskolin-elevated and basal cAMP levels in platelets (Fig. 4B and C); (iv) BF0801 strikingly increased the elevated cGMP level elicited by SNP (Fig. 4D); (v) PDE inhibitory activity of BF0801 was confirmed with PDEs isolated from platelets or the recombinant PDEs. The more specific inhibition of BF0801 on PDE2 may be explained by its structural similarity to EHNA (see Supplementary Figure 4 available online at www.thrombosis-online.com), a specific PDE2 inhibitor.

Platelet aggregation involves multiple signal pathways. The clinically used antiplatelet drugs aspirin, clopidogrel and cilostazol inhibit platelet aggregation by their own respective pathways when administered individually and therefore exert moderate platelet inhibition. To obtain more potent platelet inhibition and more satisfactory clinical outcome, triple antiplatelet therapy was recommended for patients undergoing acute coronary syndromes post-PCI (2, 44), or patients who were hyporesponsive to clopidogrel (45). Carriers of the loss-of-function hepatic cytochrome (CYP) 2C19 allele had significantly lower levels of the active metabolite of clopidogrel, diminished platelet inhibition, dual or triple antiplatelet therapy could be an alternative antiplatelet therapy to improve clinical outcome in carriers of CYP2C19 mutant allele (46). A recent study also found that triple antiplatelet therapy consisting of cilostazol, aspirin, and clopidogrel reduced long-term cardiac and cerebral events after PCI (2). PDE2 and PDE3 are expressed in vascular smooth muscle cells and cardiac tissues apart from platelets, blocking these PDEs facilitates the dilation of blood vessels and enhances myocardial contraction to improve blood flow (27). BF0801, a novel dual antiplatelet agent targeting P2Y\textsubscript{12} receptor and PDE may have therapeutic advantage as a potential antithrombotic drug.

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References


