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Molecular Pharmacology

Distinct Signaling Patterns of Allosteric Antagonism at the $P2Y_1$ Receptor*

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Running title:

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Abbreviations: Ap4A, diadenosine tetraphosphate; BPTU, 1-(2-(2-(*tert*-butyl)phenoxy)pyridin-3-yl)-3-(4-(trifluoromethoxy)phenyl)urea; cAMP, 3′,5′-cyclic adenosine monophosphate; DMEM, Dulbecco's modified Eagle's medium; DMR, dynamic mass redistribution; ERK, extracellular-signal-regulated kinase; GO6983, 3-[1-[3-(dimethylamino)propyl]-5-methoxy-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione; GPCR, G protein-coupled receptor; HBSS, Hank's Balanced Salt Solution; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; IP-1, inositol 1-phosphate; 2MeSADP, 2-methylthioadenosine 5′-diphosphate trisodium salt; MRS2365, [[(1*R*,2*R*,3*S*,4*R*,5*S*)-4-[6-amino-2-(methylthio)-9*H*-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid monoester; MRS2500, (1′*R*,2′*S*,4′*S*,5′*S*)-4-(2-iodo-6-methylamino-purin-9-yl)-1-[(phosphato)-methyl]-2-(phosphato)-bicyclo[3.1.0]hexane; UBO-QIC, L-threonine, (3*R*)-*N*-acetyl-3-hydroxy-L-leucyl-(α*R*)-α-hydroxybenzenepropanoyl-2,3-didehydro-*N*-methylalanyl-L-alanyl-*N*-methyl-L-alanyl-(3*R*)-3-[[(2*S*,3*R*)-3-hydroxy-4-methyl-1-oxo-2-[(1-oxopropyl)amino]pentyl]oxy]-L-leucyl-*N*,*O*-dimethyl-, (7→1)-lactone.

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Abstract. Traditionally, GPCR antagonists are classified as competitive or non-competitive and surmountable or insurmountable based on functional antagonism. $P2Y_1$ receptor ($P2Y_1R$) structures showed two antagonists binding to two spatially distinct sites: nucleotide MRS2500 (orthosteric, contacting the helical bundle) and urea BPTU (allosteric, on the external receptor surface). However, the nature of their $P2Y_1R$ antagonism has not been characterized. Here we characterized BPTU antagonism at various signaling pathways activated by structurally diverse agonists. BPTU rightward-shifted the concentration-response curves of both 2MeSADP and MRS2365 (5′-diphosphates) in some signaling events, such as ERK1/2 and label-free), in a parallel manner without affecting the maximum agonist effect (E_{max}), but antagonized insurmountably (suppressed agonist E_{max}) in signaling events such as GTPγS binding and β-arrestin2 recruitment. However, with dinucleotide Ap4A as an agonist, BPTU suppressed the E_{max} insurmountably in all signaling pathways. By comparison, MRS2500 behaved as a surmountable antagonist rightward-shifting concentration-response curves of all three agonists in a parallel manner for all signaling pathways measured. Thus, we demonstrated a previously undocumented phenomenon that $P2Y_1R$ antagonism patterns could vary in different signaling pathways, which could be related to conformational selection, signaling amplification and probe dependence. This phenomenon may apply generally to other receptors considering that antagonism by a specific ligand is often not compared at multiple signaling pathways. Thus, antagonism can be surmountable or insurmountable depending signaling pathways measured and agonists used, which should be of broad relevance to drug discovery and disease treatment.

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Introduction

The $P2Y_1$ receptor ($P2Y_1R$), a G protein-coupled receptor (GPCR), is activated by the endogenous agonist ADP to facilitate platelet aggregation (Savi et al., 1998) and serves as an important drug target (Jacobson et al., 2015). Considerable effort has been devoted to research on this receptor, including its recent structural determination by X-ray crystallography (Zhang D et al., 2015). The $P2Y_1R$ structures featured the high-affinity nucleotide antagonist (1′*R*,2′*S*,4′*S*,5′*S*)-4-(2-iodo-6-methylamino-purin-9-yl)-1-[(phosphato)-methyl]-2-(phosphato)-bicyclo[3.1.0]hexane (MRS2500, Supplemental Figure 1) bound in a pocket formed within the seven transmembrane (TM) domains and more external than most small ligands of rhodopsin-like GPCRs. However, an allosteric antagonist 1-(2-(2-(*tert*-butyl)phenoxy)pyridin-3-yl)-3-(4-(trifluoromethoxy)phenyl)urea (BPTU), a hydrophobic diaryl-urea derivative that arose from a program to develop antithrombotic drugs (Chao et al., 2013), bound on the external receptor surface at the phospholipid membrane interface in contact with TMs 1-3 (Zhang D et al., 2015). This represented the first example in the GPCR field of a ligand located outside the helical bundle or loop regions. BPTU was demonstrated to increase the dissociation rate of an agonist radioligand, [^{3}H]2-methylthioadenosine-5′-diphosphate ([^{3}H]2MeSADP), initiated by the competitive antagonist MRS2500, thus confirming the allosteric property of BPTU pharmacologically. Furthermore, $P2Y_1R$ site-directed mutagenesis supported the conclusion that the binding sites of the nucleotide antagonist and negative allosteric modulator were completely mutually exclusive. However, the functional antagonism by this unique allosteric modulator has not been characterized.

As with other GPCRs, the $P2Y_1R$ is coupled to multiple G protein-dependent and independent effectors (Zhang D et al., 2015; Hoffmann et al., 2008). In the present study, we set out to

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examine the nature of $P2Y_1R$ antagonism by BPTU at various signaling pathways, including Gq/11-mediated production of inositol phosphates (IP), [^{35}S]guanosine 5′-O-(3-thiotriphosphate) (GTPγS) binding to Gq/11, Gq/11- or β-arrestin2-mediated ERK1/2 stimulation, β-arrestin2 recruitment, and $P2Y_1R$ internalization. The present study demonstrated that BPTU rightward-shifted the concentration response curves of both 2MeSADP and MRS2365 in some signaling pathways, such as ERK1/2 activity and Label-free dynamic mass redistribution (DMR), in a parallel manner without affecting the maximum agonist effect (E_{max}), but suppressed the agonist E_{max} in signaling events, GTPγS binding, β-arrestin2 recruitment and β-arrestin2-mediated receptor internalization. However, when using a dinucleotide Ap4A (diadenosine tetraphosphate) as an agonist, BPTU suppressed the E_{max} in all signaling pathways in an insurmountable pattern. By comparison, the orthosteric agonist MRS2500 was shown to behave as a surmountable antagonist shifting concentration-response curves of all three agonists in all signaling pathways measured. Considering that the nature of antagonism at various signaling pathways by a specific antagonist has not been extensively examined previously, these findings could represent a general phenomenon of antagonism with respect to signaling pathways of other GPCRs and even other membrane receptor classes, and should be of broad relevance to drug discovery and disease treatment.

Materials and Methods

Chemical compounds cited in this article:

MRS2500 (PubChem CID: 44448831); BPTU (PubChem CID: 11510579); Ap4A (PubChem CID: 11957521); 2MeSADP (PubChem CID: 121990); MRS2365 (PubChem CID: 73755043); GO6983 (PubChem CID: 3499); GTPγS (PubChem CID: 1764); UBO-QIC (PubChem CID: 14101198).

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3-[1-[3-(Dimethylamino)propyl]-5-methoxy-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione (GO6983), 2MeSADP, MRS2365 and MRS2500 were obtained from Tocris (St. Louis, MO). Ap4A was from Sigma (St. Louis, MO). The allosteric antagonist BPTU was synthesized at NIDDK, NIH and was kindly provided by Dr. E. Kiselev. Reagents for the IP-One HTRF assay were obtained from Cisbio Bioassays (Bedford, MA). Reagents for ALPHAScreen assays and [^{35}S]GTPγS (1250 Ci/mmol) were from PerkinElmer (Waltham, MA). [^{3}H]2MeSADP (7.5 Ci/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). 1321N1 astrocytoma cells expressing the human $P2Y_1R$ were from T.K. Harden (University of North Carolina, Chapel Hill, NC). PathHunter U2OS (human osteosarcoma) cells expressing the recombinant human $P2Y_1R$ and an engineered β-arrestin2 and reagents for the DiscoveRx PathHunter β-arrestin assays were obtained from DiscoveRx (Fremont, CA). ON-TARGETplus human β-arrestin2 siRNA-SMARTpool was from Dharmacon (LaFayette, CO). All other materials were from standard commercial sources (Sigma, St. Louis, MO, unless noted) and of analytical grade.

Inositol 1-Phosphate Assay

Inositol 1-phosphate (IP-1), which is a metabolite of inositol 1,4,5-trisphosphate and downstream of G_q signaling, was detected by the IP-One Tb HTRF kit (Cisbio Bioassays, Bedford, MA) as described previously (Violin et al., 2010; Rajagopal et al., 2011). Briefly, after overnight growth, cells were pretreated with an antagonist for 20 min before the addition of agonist and incubated for another 60 min. Detection reagents were added as instructed by the manual from the manufacturer. The assay plates were read on a Mithras LB940 reader (Berthold Technologies, Oak Ridge, TN) or a PerkinElmer EnSpire plate reader using a time-resolved fluorescence ratio (665/620 nm).

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ERK1/2 Stimulation

The method used was essentially as previously described (Gao et al., 2011, 2014). 1321N1 astrocytoma cells or U2OS cells expressing the human $P2Y_1R$ (30,000 cells/100 µl) were seeded in a 96-well plate in complete growth medium. After cell attachment, medium was removed and cells were serum-starved overnight in 90 µl serum free medium. For Gαq-mediated ERK1/2 stimulation, cells were stimulated with agonist for 5 min. For β-arrestin2-mediated ERK1/2 stimulation, cells were incubated with agonist for 30 min following a pretreatment with the broad-spectrum PKC inhibitor GO6983 (10 µM, Tocris, St. Louis, MO) for 20 min (Hoffmann et al., 2008; Lefkowitz and Shenoy, 2005; Reiner et al., 2009). In both cases, cells were pretreated with an antagonist 20 min before the addition of agonist. After agonist treatment, the medium was removed and cells were lysed with 1× lysis buffer (20 µl) (PerkinElmer AlphaScreen SureFire p-ERK1/2 (Thr202/Tyr204) Assay Kit). Lysate (4 µl/well) was transferred to a 384-well ProxiPlate Plus (PerkinElmer). Acceptor beads were diluted 1:50 in a 1:5 mixture of activation buffer in reaction mix and added to the 384-well plate (5 µl/well). The plate was sealed and incubated for 2 h at room temperature. Donor beads (2 µl) diluted 1:20 in dilution buffer were added, and the plate was incubated for another 2 h at room temperature. The plate was measured using an EnVision multilabel reader using standard AlphaScreen settings. Lipofectamine 2000 (Thermo Fisher) was used for the transfection of β-arrestin2 siRNA (final concentration, 100 nM) according to the manufacturer's manual.

[^{35}S]GTPγS binding assay

The preparation of membranes from U2OS cells expressing human $P2Y_1R$ was as previously described (Gao et al., 2011). [^{35}S]GTPγS (PerkinElmer, Boston, MA) binding was carried out in

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duplicate or triplicate by incubation for 30 min at 25°C in 200 µl buffer containing 50 mM Tris HCl (pH 7.4), 1 mM EDTA, 1 mM $MgCl_2$, 1 µM GDP, 1 mM dithiothreitol, 100 mM NaCl, 0.2 nM [^{35}S]GTPγS, 0.5% bovine serum albumin, test agonists and membrane suspension (10 µg protein/tube). Antagonists were added 20 min before the addition of agonists. The reaction was stopped by rapid filtration through Whatman GF/B filters (Brandel, Gaithersburg, MD), pre-soaked in 50 mM Tris HCl, 5 mM $MgCl_2$ (pH 7.4). The filters were washed twice with 3 ml of the same buffer, and the retained radioactivity was measured using liquid scintillation counting. Non-specific binding of [^{35}S]GTPγS was measured in the presence of 10 µM unlabelled GTPγS.

Label-free DMR measurement

Label-free DMR measurement was performed on a PerkinElmer EnSpire (Waltham, MA, USA) multimode plate reader based on the EPIC optical biosensor technology using resonance waveguides. For the measurements, 50 µl of $P2Y_1R$-expressing U2OS cells ($2x10^4$ cells/each well) were seeded into 96-well EnSpire cell assay microplates. The microplates were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C overnight. Immediately before the experiments, the cells were washed three times with assay buffer (HBSS buffer containing 20 mM HEPES, Mediatech, Manassas, VA), and were allowed to equilibrate in the EnSpire multimode reader for one h. After measuring baseline data, 10 µl of the test compounds were added and the cellular response was recorded continuously for 60 min.

β-Arrestin2 Recruitment Assays

The β-arrestin2 recruitment to the $P2Y_1R$ was assessed by DiscoveRx PathHunter β-arrestin assay (Fremont, CA) as described previously (Gao et al., 2008, 2014). In this assay, the GPCR is fused in frame with the small enzyme fragment ProLink™ and co-expressed in U2OS cells

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stably expressing a fusion protein of β-arrestin2 and the larger, N-terminal deletion mutant of β-galactosidase (enzyme acceptor). GPCR activation stimulates binding of β-Arrestin2 to the ProLink-tagged GPCR and forces complementation of the two enzyme fragments, resulting in the formation of an active β-gal enzyme. This interaction leads to an increase in enzyme activity that can be measured using chemiluminescent PathHunter® Detection Reagents. For the measurement of $P2Y_1R$-mediated recruitment, PathHunter U2OS cells expressing the human $P2Y_1R$ were grown in 96-well plates for 24 h in DMEM medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 μmol/ml glutamine. Cells were first treated with antagonists for 20 min and then treated with agonists for 60 min before adding detection reagents (mixture of 1 part Galacton Star substrate with 5 parts Emerald II™ Solution, and 19 parts of PathHunter Cell Assay Buffer), and incubated at room temperature for 60 min before luminescence was measured.

$P2Y_1$ Receptor Internalization

$P2Y_1R$ internalization was assessed using a PathHunter eXpress Activated GPCR Internalization Assay as instructed by the manufacturer (DiscoverX, Fremont, CA). In this assay, PathHunter U2OS cells are engineered to co-express an untagged GPCR, enzyme acceptor (EA) tagged β-Arrestin2, and a ProLink tag localized to the endosomes. Activation of the untagged GPCR induces β-Arrestin recruitment, followed by internalization of the Receptor/Arrestin-EA complex in PK-tagged endosomes, which forces complementation of the two β-galactosidase enzyme fragments, forming functional enzyme that hydrolyzes substrate to generate a chemiluminescent signal. The method used for this assay was similar to that described in the β-arrestin2 recruitment assay, except the agonist incubation time was 180 min.

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Data and Statistical Analysis.

Apparent binding affinities in radioligand binding (Supplemental Methods), according to the formula $K_i = IC_{50}/(1 + [radioligand]/K_d)$, and functional parameters were calculated using Prism 7.00 software (GraphPAD, San Diego, CA, USA). Statistical significance of the differences was assessed using a Student's t test (between two conditions) or a One-Way Analysis of Variance (ANOVA) followed by appropriate post hoc testing. Differences yielding $P < 0.05$ were considered as statistically different.

Results

As described previously (Zhang D et al., 2015), BPTU and MRS2500 bind to structurally distinct sites on the $P2Y_1R$. Therefore, we expected different patterns of antagonism of $P2Y_1R$-mediated signaling. Since the $P2Y_1R$ is a Gq-coupled receptor, we first measured IP-1 production in 1321N1 astrocytoma cells expressing the recombinant human $P2Y_1R$. Figures 1a and 1b show that both MRS2500 and BPTU produce a rightward shift of the agonist concentration-response curves. The K_B values for MRS2500 and BPTU calculated from the Schild analysis are 0.86 ± 0.19 and 6.83 ± 1.22 nM, respectively, and their respective slopes are 0.90 ± 0.08 and 0.95 ± 0.06. Thus, the allosteric property of BPTU cannot be manifested using a Schild analysis.

It has been shown previously that β-arrestin2 mediates $P2Y_1R$ internalization (Reiner et al., 2009). Thus, we examined the antagonism by MRS2500 and BPTU in agonist-induced and β-arrestin2-mediated receptor internalization in U2OS cells. Figures 1c and 1d show that MRS2500 shifts the agonist response curve for receptor internalization to the right in a parallel manner with a slope of 1.06 ± 0.05 and a K_B of 1.12 ± 0.23 nM (Table 1). BPTU concentration-dependently suppressed the maximal agonist effect. Thus, apparently, the allosteric antagonist

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BPTU behaves in a completely different pattern in antagonizing Gq-mediated and β-arrestin2-mediated events, whereas the competitive antagonist MRS2500 behaves similarly for both pathways. The results led us to speculate that BPTU has different antagonistic pattern on $G\alpha_{q/11}$- and β-arrestin2-mediated signaling, whereas the antagonism by MRS2500 for these two branches of signaling (G protein and β-arrestin) is in a similar pattern.

To further prove that BPTU has distinct patterns of antagonism at two signaling pathway branches, we next examined the effect of BPTU on agonist-induced and Gq/11-mediated stimulation of ERK1/2 activity, a known $P2Y_1R$-mediated function (Sellers et al., 2001). Figures 2a and 2b show that the stimulation of ERK1/2 activity for 5 min using 2MeSADP (1 μM) is completely PKC-sensitive and β-arrestin2-insensitive, suggesting a Gq/11- but not β-arrestin2-mediated mechanism (Lefkowitz and Shenoy, 2005; Reiner et al., 2009; Wei et al., 2003). 2MeSADP concentration-dependently induced ERK1/2 stimulation corresponding to an EC_{50} value of 1.74 ± 0.44 nM. Both MRS2500 and BPTU right-shifted agonist response curve in a parallel manner (Figures 2c and 2d), corresponding to K_B values of 0.91 ± 0.13 and 6.66 ± 1.37 nM, respectively. The respective slopes are 1.03 ± 0.11 and 0.95 ± 0.07. Again, the allosteric property of BPTU could not be demonstrated with this assay and the pattern of antagonism by MRS2500 and BPTU were indistinguishable, i.e. both produced parallel rightward shift with slopes close to unity. Thus, the degree of allostery by BPTU for this G protein-dependent pathway is very high, such that by standard criteria of antagonism, its allosteric nature is undetectable. To prove how BPTU inhibits $P2Y_1$ signaling in cells that do not overexpress the recombinant human $P2Y_1$ receptor, the antagonism by BPTU of agonist-induced ERK1/2 activity in HEK293 cells endogenously expressing the human $P2Y_1$ receptor was examined. Supplemental Figure 3

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shows that BPTU rightward shifted the agonist response curve to the right in a parallel manner without affecting the maximum agonist effect.

It has been shown previously that $P2Y_1R$ internalization and desensitization are modulated by different mechanisms (Reiner et al., 2009). Thus, we examined the similarity or differences in antagonism by MRS2500 and BPTU in agonist-induced β-arrestin2 recruitment in addition to and β-arrestin2-mediated receptor internalization. In addition to Gq/11-mediated downstream signaling events, $P2Y_1R$ activation is known to cause robust recruitment of β-arrestin2, but not β-arrestin1 (Hoffmann et al., 2008; Reiner et al., 2009). However, the pattern of antagonism by various antagonists at this signaling pathway is unknown. Therefore, we compared the antagonism by MRS2500 and BPTU of the agonist-induced β-arrestin2 recruitment using the PathHunter protein complementation assay, a widely accepted method (Violin et al., 2010; Gao et al., 2008, 2011, 2014). Figure 2e shows that MRS2500 shifts the agonist concentration-response curve to the right in a parallel manner with a slope of 0.89 ± 0.05 and a K_B of 0.85 ± 0.08 nM. However, unlike its effect on IP-1 or ERK1/2 stimulation, BPTU suppressed maximal agonist effect in a concentration-dependent manner and at 10 μM completely blocked the agonist effect (Figure 2f). These results again support that BPTU behaves differently at two branches of signaling pathways.

It is known that β-arrestin2, in addition to its role in receptor desensitization and internalization, may mediate signaling event such as ERK1/2 phosphorylation that is independent of G proteins (Lefkowitz and Shenoy, 2005). Therefore, we tested this possibility with the $P2Y_1R$ expressed in 1321N1 astrocytoma cells by stimulation of cells with 2MeSADP following the pretreatment with the PKC inhibitor GO6983 for 20 min (Shenoy et al., 2006; Wei et al., 2003). We first

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measured the time-course of agonist-induced ERK1/2 stimulation. Figure 3a shows that, in the presence of the PKC inhibitor GO6983, 2MeSADP does not induce stimulation of ERK1/2 activity at 5 min, but stimulates the maximal ERK1/2 phosphorylation at 30 min. The ERK1/2 activity at 30 min was completely abolished by β-arrestin2 siRNA demonstrating a β-arrestin2-mediated effect (Figure 3a). Thus, in the following experiments, we measured the agonist concentration-response and the antagonism by MRS2500 and BPTU under this condition. Figure 3b shows that agonist 2MeSADP concentration-dependently induces a robust stimulation of ERK1/2 activity corresponding to an EC_{50} value of 21.3 ± 2.9 nM, which was completely abolished by β-arrestin2 siRNA. By contrast, β-arrestin2 siRNA did not affect the concentration-response curve of 2MeSADP-induced ERK1/2 stimulation at 5 min in the absence of GO6983 (Figure 2b), further confirming the difference between Gq- and β-arrestin2-stimulated ERK1/2 activity. Interestingly, unlike the effect of BPTU in blocking agonist-induced β-arrestin2 translocation and receptor internalization, it rightward-shifted agonist-induced and β-arrestin2-mediated ERK1/2 activity in a parallel manner in a way similar to that of MRS2500 (Figures 3c and 3d). Thus, the atypical negative allosteric modulator BPTU behaves differently even in different events mediated by β-arrestin2, suggesting that each signaling event is possibly mediated via a specific receptor conformation or a β-arrestin2 conformation or both (Shukla et al., 2008; DeWire et al., 2007), and BPTU is able to block receptors in a pattern that is possibly dependent on specific conformations. The potencies and slopes for inhibition by MRS2500 and BPTU from Schild analyses are listed in Table 1. However, the results from the antagonism of β-arrestin2-mediated ERK1/2 activity are against our initial notion that BPTU behaves differently at two branches of signaling pathways.

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The above results raised that possibility that differences between MRS2500 and BPTU in some signaling pathways, and different patterns of antagonism by BPTU in different pathways might be due to the influences of residence time (Klein Herenbrink et al., 2016; Guo et al., 2014), signaling amplification (Hepler et al., 2014), probe dependence (Kenakin, 2008; Ehlert, 2013) and cell background (Kenakin, 2009), etc., in addition to the possibility of BPTU's conformational selective antagonism in different pathways (Edelstein and Changeux, 2016).

To probe those possibilities, we examined if the incubation time with BPTU may or may not affect the different patterns of antagonism. As described in Materials and Methods, in β-arrestin2-mediated receptor internalization assay, the total incubation time for BPTU is 200 min (20 min of BPTU alone and 180 min together with an agonist). In Gαq-mediated ERK1/2 phosphorylation, the total incubation time for BPTU is only 25 min (20 min alone and 5 min together with an agonist). Thus, the antagonist BPTU was pre-incubated at various time points in the assay of ERK1/2 phosphorylation assay (up to 3 h, which is comparable to the total incubation time of BPTU in the receptor internalization assay). Supplemental Figure 3 shows that the prolonged incubation time does not change the pattern of antagonism by BPTU or the EC_{50} value of agonist, 2MeSADP. The EC_{50} values of 2MeSADP in the presence of BPTU (20, 30, 60, 120 and 180 min) are 2.2 ± 0.4, 1.5 ± 0.5, 2.1 ± 0.2, 1.6 ± 0.4 and 2.3 ± 0.5 nM, respectively, which are not significantly different from Control (1.8 ± 0.3 nM) ($P > 0.05$, One-Way ANOVA with Multiple Comparisons (Dunnett), N=3)).

In the following experiments, in order to address the impact from signaling amplification, probe dependence, and cell backgrounds, we measured both branches of signaling pathways using the same cell type, U2OS cells, and compared signaling pathways with amplification (ERK1/2 and Label-free measurement) and without or with limited amplification (GTP binding and β-arrestin2

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recruitment) of signaling using several agonists of distinct chemical structures (2MeSADP, MRS2365, and Ap4A).

We first compared the initial steps of the two signaling branches, guanine nucleotide binding (Figure 4) and β-arrestin2 recruitment (Figure 5), which can be considered as without or only with limited signaling amplification. Figures 4 and 5 show that both 2MeSADP and MRS2365 are potent agonists in stimulation of [^{35}S]GTPγS binding (Figure 4a) and β-arrestin 2 recruitment (Figure 5a). The dinucleotide Ap4A was shown as a partial agonist, with maximum agonist efficacy was about 20% and 30% of that of 2MeSADP in [^{35}S]GTPγS binding and β-arrestin2 recruitment assays, respectively (Figures 4a and 5a). In both assays, or both branches of signaling pathways, by using three agonists of distinct structures, it was demonstrated that MRS2500 is a surmountable, whereas BPTU is an insurmountable antagonist. Thus, apparently, there is a possibility that signaling amplification plays a role in the different patterns of antagonism by BPTU, as the results from the initial step of both signaling branches demonstrated that BPTU is insurmountable and MRS2500 is surmountable.

We then compared two later signaling steps following the receptor activation, Gαq-mediated and β-arrestin2-mediated ERK1/2 phosphorylation. Again, both 2MeSADP and MRS2365 are potent and full agonists in both pathways measured (Figures 6a and 7a). Ap4A was shown to be a full agonist and high-efficacy partial agonist (65% of the maximum effect of 2MeSADP) in Gαq-mediated and β-arrestin2-mediated ERK1/2 phosphorylation, respectively. Both 2500 and BPTU are surmountable antagonists against both 2MeSADP and MRS2365 in both branches of signaling pathways measured (Figures 6 and 7). However, interestingly, MRS2500 and BPTU were shown to be a surmountable (Figures 6e and 7e) and an insurmountable (Figures 6f and 7f)

antagonist, respectively, when using the dinucleotide Ap4A as an agonist. Thus, BPTU behaves differently in antagonizing the same signaling event induced by different agonists.

To further test the impact of signal amplification and agonist dependency on the patterns of antagonism, we performed a Label-free DMR assay, a kind of end-point measurement. Figure 8a shows that Ap4A is a partial agonist compared with 2MeSADP and MRS2365. The agonist effect induced by the selective $P2Y_1R$ agonist MRS2365 is completely UBO-QIC-sensitive, suggesting a Gαq-mediated, not β-arrestin2-mediated event. Again, both MRS2500 and BPTU are surmountable antagonists when tested against 2MeSADP and MRS2365 (Figures 8b and 8c). MRS2500 and BPTU are a surmountable and an insurmountable antagonist, respectively, when tested against the dinucleotide, Ap4A.

The binding affinity of BPTU at the $P2Y_1R$ has been previously reported (Zhang D et al., 2015). In the present study, we used more concentrations in order to examine the pattern of displacement against the agonist radioligand [^{3}H]2MeSADP, such as the Hill slope. Both MRS2500 and BPTU completely displaced equilibrium [^{3}H]2MeSADP binding with slopes close to one (Table 1). BPTU, but not MRS2500, concentration-dependently accelerated the dissociation of [^{3}H]2MeSADP from the $P2Y_1R$ (Supplemental Figure 4).

Discussion

The present study demonstrated that an antagonist can right-shift agonist concentration-response curves in a parallel manner in some signaling pathways with linear Schild plot and slope close to unity, while it suppresses the maximal agonist response in an insurmountable pattern in some other pathways, depending on the signaling pathways, extent of signaling amplification and agonists used (Supplemental Table 1). Allosteric modulators have been suggested to have the

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tendency of being biased (Edelstein and Changeux, 2016; Gao and Jacobson, 2013), but it has not been well documented previously that an allosteric antagonist can be surmountable in one pathway but insurmountable in another pathway.

The dinucleotide Ap4A behaves as a partial agonist in [^{35}S]GTPγS binding, β-arrestin2 recruitment, β-arrestin2-mediated ERK1/2 stimulation and Label-free DMR, but is a full agonist in Gq-mediated ERK1/2 stimulation. Both 5′-diphosphate agonists appear to be full agonists in all activities measured. Because the second nucleoside moiety of the dinucleotides is proposed to reside outside the principle mononucleoside binding site in P2YRs (Jacobson et al., 2015), the bitopic nature of Ap4A suggests functional modulation by this moiety to reduce the intrinsic full agonist response. This partial agonist is particularly susceptible to insurmountable antagonism by BPTU. The probe dependence of BPTU's antagonism is consistent with the hypothesis that signaling amplification, induced by the full agonists and associated with progression of the signaling cascade, is needed to overcome BPTU's allosteric nature to shift the antagonism from insurmountable to an apparent surmountable manner. BPTU inhibition of IP accumulation and Gq-mediated ERK1/2 activation may be surmountable since there is a lot of signal amplification in the first assay and may need only a fraction of total signaling to activate PKC in the latter assay.

The degree of $P2Y_1R$ reserve has been suggested to play a role in the agonist efficacy of ATP but not ADP (Palmer et al., 1998). It remains to be examined if the agonist efficacies of 2MeSADP, MRS2365 and AP4A, as well as the antagonistic patterns by BPTU, are susceptible to the degree of receptor reserve. The antagonism of 2MeSADP-induced ERK1/2 phosphorylation by BPTU at three different cell types with variable expression levels shows similar patterns, suggesting that receptor reserve may not play a major role in the surmountable nature of BPTU at this specific

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signaling pathway. However, it remains to be seen if the insurmountable nature of BPTU at other pathways and against other agonists is related to degree of receptor reserve or not.

Biased agonism has been relatively well studied in recent years (Lefkowitz and Shenoy, 2005; Wisler et al., 2014). G protein-biased agonists of glucagon-like peptide-1 receptors were suggested to provide a novel therapeutic approach to Type 2 diabetes (Zhang H et al., 2015). Propranolol acting at the β_2-adrenergic receptor is an inverse agonist in cAMP accumulation, but it is a partial agonist in ERK1/2 phosphorylation (Azzi et al., 2006; Galandrin and Bouvier, 2006). β-Arrestin pathway-selective angiotensin 1 (AT1) receptor agonists may promote cell growth via an arrestin-mediated mechanism despite their antagonism of G protein signaling (Kendall et al., 2014). Also, the proteinase-activated receptor 2 (PAR2) ligand GB88 selectively inhibits G(q/11)/Ca^{2+}/PKC signaling, but activates cAMP, ERK, and Rho pathways (Suen et al., 2014). Some of those earlier studies suggest that both agonists and antagonists may behave differently in distinct signaling pathways.

Biased antagonism at multiple signaling pathways has not been extensively explored, although it has been suggested that this is a potential area of therapeutic interest (Kenakin, 2014). For example, Muniz-Medina et al (2008) demonstrated that allosteric antagonists of CCR5, maraviroc and aplaviroc, have a dramatic difference in potency in blocking HIV entry and CCR5 internalization. Nadeau-Vallee et al. (2015) reported that some IL-1 inhibitors inhibit c-jun and RhoGTPase/Rho-associated coiled-coil-containing protein kinase but not NFκB signaling, which could prevent inflammation-related conditions without potential side effects caused by balanced antagonists.

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It was reported that β-arrestin2 but not β-arrestin1 is critical in $P2Y_1R$ desensitization and internalization (Hoffmann et al., 2008; Reiner et al., 2009). Also, $P2Y_1R$ desensitization and internalization are mediated by different phosphorylation sites and kinases (Reiner et al., 2009). The present study also demonstrated the greater $P2Y_1R$ agonist potency in β-arrestin2 translocation compared to β-arrestin2-mediated receptor internalization and β-arrestin2-mediated ERK1/2 stimulation. Independent ERK1/2 signaling mediated via β-arrestin2 and Gq also may suggest that a distinct 'active' $P2Y_1R$ conformation is coupled to each effector (Wisler et al., 2014; Edelstein and Changeux, 2016). The different patterns of antagonism at different signaling pathways, and against events induced by different agonists, raise the possibility of BPTU as conformationally selective antagonist, which, in theory, could be more therapeutically advantageous and resulting in fewer side effects.

It is known that GPCRs can adopt multiple conformations that have signaling implications (Kahsai et al., 2011). Shukla et al. (2008) showed that β-arrestins can also adopt multiple conformations to inhibit classical G protein signaling and to initiate distinct β-arrestin-mediated signaling (Shukla et al., 2008; Nobles, 2011). Thus, distinct receptor conformations induced or stabilized by different ligands can promote distinct and functionally specific conformations in arrestins, which could partly explain the differential antagonism by BPTU at three β-arrestin2-mediated events demonstrated in the present study. To our knowledge, the antagonism of various β-arrestin signaling pathways is still a largely unexplored area. It seems that the different patterns of antagonism may also be related to different extent of signaling amplification in different pathways induced by different agonists.

Traditionally, GPCR antagonist affinity has been considered constant, regardless of different agonists used in different assays. Recent evidence suggested that an antagonist can show

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different affinities if measured under distinct experimental conditions (Kenakin, 2014; Baker et al., 2007). One reason that has been ascribed to this phenomenon is the possible involvement of more than one binding site on the receptor. However, this assumption so far is only based on pharmacological data but not on structurally elucidated information showing distinct binding sites in a receptor. The present study is the first clear, pharmacological example to show differential antagonism resulting from the binding to two structurally distinguished sites on the receptor. Thus, antagonists may not only have different affinities in distinct measurement conditions, they may also show completely different patterns of antagonism in different signaling pathways which have not been demonstrated previously. Even in different events mediated via β-arrestin2, BPTU showed different potencies and patterns of antagonism, suggesting both receptors and β-arrestin2 can adopt multiple conformations.

In classical pharmacology, Schild analysis of antagonist-induced shifts of agonist concentration-response curves has long been used to define the competitive property of antagonists (Schild, 1949; Arunlakshana and Schild, 1959; Kenakin, 1984; Wyllie and Chen, 2007). It is often assumed that if the pattern of a rightward shift is parallel with a slope close to one, competitive antagonism is defined. On the other hand, the definition of noncompetitive or allosteric antagonism is often based on nonlinear Schild plots, insurmountable inhibitory effects and/or the incomplete displacement of radioligand binding. However, the present study demonstrates that neither is a strict criterion for characterizing the mode of antagonism. Thus, the conventional definition of antagonist action has to be combined with knowledge of the binding site(s) involved and signaling pathways measured, as demonstrated in the present study. Also, allosteric modulators may resemble competitive antagonists in both competitive binding and functional assays. It might additionally require one of the two criteria, structurally elucidated or

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pharmacologically demonstrated by an effect on the dissociation rate of an orthosteric agonist, to define a novel antagonist in terms of competitive or allosteric action.

A strict comparison of the pharmacological consequences of antagonists binding to different sites on a specific receptor is rare in the literature, partly due to the fact that only limited information is currently available for diverse ligand binding sites on a receptor. Most of the previous structural information derived from crystallized GPCR complexes indicated that both competitive antagonists and allosteric antagonists bind at or near the pocket formed by seven TM domains, not exclusively on the external surface of the receptor. However, it should be noted that only very few allosteric antagonists have been used in crystallization compared with the total population of GPCR antagonists available. Even for the urea class of $P2Y_1R$ antagonists related to BPTU, the binding location was initially predicated by molecular modeling to be within the helical bundle (Chao et al., 2013; Qiao et al., 2013). Thus, potentially biased functional antagonism remains to be explored for other $P2Y_1R$ antagonists and for diverse antagonists, especially allosteric modulators, of other GPCRs. Also, it remains to be seen if the pattern of antagonism at platelet aggregation induced by various $P2Y_1R$ agonists, which is an end-point functional measurement, by this unique allosteric antagonist is surmountable or insurmountable.

Regarding the molecular mechanisms of insurmountable antagonism, the slow dissociation of an antagonist-receptor complex or long residence time has been traditionally considered as a major mechanism (Guo et al., 2014; Klein Herenbrink et al., 2016). Allosteric sites, distinct conformations, and receptor internalization have also been used as alternative explanations. Insurmountable antagonism is also related to signaling amplification (Hepler et al., 2014), probe dependence (Ehlert, 2013), cell types and cell background (Kenakin, 2009). Our results suggest that the insurmountable nature of BPTU can only be demonstrated in some signaling pathways,

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and by using some agonists to initiate the receptor activation, which seems to be related to signaling amplification, agonist probes and possibly degree of receptor reserve. Thus, to define the nature of a ligand in terms of "surmountable" or "insurmountable", one has to measure multiple signaling pathways by using agonists of diverse or distinct structures. Although both MRS2500 and BPTU inhibit [^{3}H]2MeSADP binding completely in a similar pattern based the displacement binding curves, inhibition by MRS2500 is probably via the direction competition of the same binding pocket, whereas the inhibition by BPTU is indirectly via the acceleration of [^{3}H]2MeSADP dissociation rate. The exact, detailed mechanism of inhibition remains to be defined.

In summary, the present study illustrates a previously undocumented example to challenge the dogma in traditional pharmacology in defining competitive vs. allosteric antagonism. First, a parallel rightward shift of agonist concentration-response curves and complete inhibition of radioligand binding may or may not be evidence in support of competitive antagonism. Second, the nature of functional antagonism for various signaling pathways may be different in terms of being competitive and noncompetitive (or surmountable or insurmountable), which could be especially true for allosteric antagonists and for the largely uncharacterized antagonism at various β-arrestin pathways. Furthermore, the present study demonstrated a clear example of the implications of signaling amplification and probe dependence in the discovery and characterization of $P2Y_1R$ allosteric modulators, which could be a general phenomenon for other receptors considering the antagonism at various signaling pathways was largely unexplored. The findings suggest that an antagonist can be surmountable or insurmountable depending signaling pathways measured and agonists used, which should be of broad relevance to drug discovery and disease treatment.

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Authorship Contributions

Participated in research design: Gao, Jacobson

Conducted experiments and data analysis: Gao

Wrote or contributed to the writing of the manuscript: Gao, Jacobson

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Figure Legends

Figure 1. Comparison of antagonism of 2MeSADP-induced IP-1 formation via Gq/11 proteins and receptor internalization mediated by β-arrestin2. IP-1 accumulation was measured in $P2Y_1R$-expressing 1321N1 astrocytoma cells in the presence or absence of MRS2500 (MRS; A) and BPTU (B). Cells were pretreated with MRS or BPTU for 20 min before the addition of agonist and incubated for an additional 60 min. Receptor internalization was measured using DiscoverX PathHunter U2OS cells, which were pretreated with MRS2500 (C) or BPTU (D) for 20 min followed by addition of 2MeSADP for incubated for 180 min. Results were expressed as mean ± SD from three independent experiments performed in duplicate. The K_B values of MRS2500 and BPTU and slopes from Schild analysis from three separate experiments are listed in Table 1.

Figure 2. Comparison of antagonism of 2MeSADP-induced ERK1/2 phosphorylation mediated via $G_{q/11}$ and β-arrrestin2 recruitment. A. Agonist-induced ERK1/2 stimulation in 1321N1 astrocytoma cells expressing the $P2Y_1R$ is mediated via PKC. Cells were either treated with 2MeSADP for 5 min or treated with PKC inhibitor GO6983 (10 μM) for 20 min before the addition of 2MeSADP for another 5 min. $^{\#\#}$Significantly different from other two groups ($P <$ 0.01, One-Way ANOVA with post-hoc test (Turkey), N=3). B. Gq/11-mediated ERK1/2 activity is insensitive to β-arrestin2 siRNA. C. and D. Agonist concentration-response curves in the absence or presence of MRS (MRS2500, A) or BPTU (B). Cells were pretreated with MRS (A) or BPTU (B) for 20 min before the addition of agonist and incubated for another 5 min. **E.** and **F.** β-arrrestin2 recruitment was measured using DiscoverX PathHunter U2OS cells, which were pretreated with MRS2500 (C) or BPTU (D) for 20 min followed by addition of 2MeSADP and incubated for 60 min. Results were expressed as mean ± SD from three separate experiments

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performed in duplicate or triplicate. The K_B values of MRS2500 and BPTU and slopes from Schild analysis are listed in Table 1.

Figure 3. A. β-arrestin2-mediated ERK1/2 stimulation is completely diminished by β-arrestin2 siRNA. β-arrestin2 siRNA-transfected or control 1321N1 astrocytoma cells expressing the $P2Y_1R$ were incubated with the PKC inhibitor GO6983 (10 μM) for 20 min and then treated with 1 μM 2MeSADP for the indicated time points. The level of β-arrestin2 gene expression was knocked down by 94.6% measured using real-time PCR as described earlier (Gao et al., 2014). #Significantly different from control value at time 0 ($P < 0.01$, One-Way ANOVA with Multiple Comparisons (Dunnett), N=3). **B.** Concentration-response of 2MeSADP-induced and β-arrestin2-mediated ERK1/2 stimulation (30 min) in the presence of the PKC inhibitor GO6983 (10 μM) and in the presence or absence of β-arrestin2 siRNA. **C**. and **D**. Comparison of antagonism by MRS (C) and BPTU (D) of β-arrestin2-mediated ERK1/2 stimulation. MRS (C) or BPTU (D) was added together with GO6983 (10 μM) and incubated for 20 min before the addition of agonist and incubated for another 30 min. Each data point represents the mean ± SD from three experiments. The K_B values and slopes yielded by Schild analysis from three independent experiments are listed in Table 1.

Figure 4. A. Agonist-induced [^{35}S]GTPγS binding to membrane preparations from $P2Y_1R$-expressing U2OS cells. The maximum stimulation by 2MeSADP was expressed as 100%. B. Effect of MRS2500 and BPTU on Ap4A-stimulated [^{35}S]GTPγS binding. In order to make the curves discernible, the maximum [^{35}S]GTPγS binding stimulated by Ap4A is expressed as 100% (about 15-20% of the efficacy of 2MeSADP). C. Antagonism of 2MeSADP-induced [^{35}S]GTPγS binding by MRS2500. D. antagonism of 2MeSADP-induced [^{35}S]GTPγS binding by BPTU. E. antagonism of MRS2365-induced [^{35}S]GTPγS binding by MRS2500. F. Antagonism of

2MeSADP-induced [^{35}S]GTPγS binding by BPTU. Results were expressed as mean ± SD from three independent experiments.

Figure 5. Concentration-response for β-arrestin2 recruitment induced by various agonists in the absence or presence of MRS2500 (C, E, G) or BPTU (D, F, H). PathHunter U2OS cells expressing the human $P2Y_1R$ were first treated with antagonists for 20 min and then treated with agonists for 60 min before the termination of reaction by adding detection reagents. Data shown are from three independent experiments of similar results performed in duplicate or triplicate. In Figure 6G and Figure 6H, the maximum [^{35}S]GTPγS binding stimulated by Ap4A is expressed as 100%, in order to make the curves discernible.

Figure 6. A. Agonist-induced and Gq/11-mediated ERK1/2 stimulation in U2OS cells. B. antagonism of 2MeSADP-induced ERK1/2 stimulation by MRS2500, BPTU and the PKC inhibitor GO6983 (GO). C and D. antagonism of MRS2365-stimulated ERK1/2 activity by MRS2500 (C) and BPTU (D). E and F. antagonism of Ap4A-stimulated ERK1/2 activity by MRS2500 (E) and BPTU (F). Cells were either treated with agonists for 5 min or treated with antagonists or PKC inhibitor GO6983 (10 μM) for 20 min before the addition of agonists for another 5 min. Each data point represents the mean ± SD from three experiments.

Figure 7. A. Agonist-induced and β-arrestin2-mediated ERK1/2 stimulation in U2OS cells. B. antagonism of 2MeSADP-induced ERK1/2 stimulation by MRS2500, BPTU and the PKC inhibitor GO6983. C and D. antagonism of MRS2365-stimulated ERK1/2 activity by MRS2500 (C) and BPTU (D). E and F. antagonism of Ap4A-stimulated ERK1/2 activity by MRS2500 (E) and BPTU (F). Cells were first treated with the PKC inhibitor GO6983 (GO; 10 μM) and/or antagonists for 20 min before the treatment with agonists for another 30 min. Maximum

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stimulation by Ap4A was expressed as 100%, in order to make various curves discernible. Each data point represents the mean ± SD from three experiments.

Figure. 8. Label-free DMR measurement of agonist potencies and antagonism by MRS2500 and BPTU. A. measurement of agonist response and the effect of the Gαq/11 inhibitor UBO-QIC (UBO). B. antagonism of 2MeSADP-induced response by MRS or BPTU. C. antagonism of MRS2365-induced response by MRS or BPTU. D. antagonism of Ap4A-induced response by MRS or BPTU; Ap4A-induced maximum response was expressed as 100%, in order to discern various concentration-response curves. U2OS cells expressing the $P2Y_1R$ cells were pretreated with inhibitors for 20 min followed by the addition of agonists and measured for 60 min. Results are expressed as mean ± SD from three independent experiments performed in duplicate or triplicate.

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Table 1. Inhibitory effects of the competitive antagonist MRS2500 and the allosteric antagonist BPTU on various $P2Y_1R$-mediated signaling pathways.

	MRS2500		BPTU		2MeSADP	
	K_B or K_i (nM)	slope	K_B or K_i (nM)	slope	EC_{50} (nM)	-logEC_{50}(CIs)
Gq signaling						
IP-1[a]	0.86 ± 0.19	0.94 ± 0.08	6.83 ± 1.22	0.95 ± 0.06	4.83 ± 1.12	8.58 (8.86-8.20)
ERK1/2[a]	0.91 ± 0.13	1.03 ± 0.11	6.66 ± 1.37	0.95 ± 0.07	1.74 ± 0.44	8.80 (9.04-8.85)
GTP binding[b]	5.68 ± 1.22	0.96 ± 0.05	NA[c]	NA[c]	4.62 ± 1.09	8.31 (8.58-8.04)
β-arrestin2 signaling						
Recruitment[b]	0.85 ± 0.08	0.89 ± 0.05	NA[c]	NA[c]	7.81 ± 2.03[f]	8.01 (8.19-7.84)
Internalization[b]	1.12 ± 0.23	1.06 ± 0.05	NA[c]	NA[c]	25.8 ± 3.3[f]	7.62 (7.83-7.41)
ERK1/2[a]	7.41 ± 2.23	1.10 ± 0.09	28.2 ± 6.4	1.00 ± 0.06	21.3 ± 2.9	7.68 (7.80-7.56)
Radioligand binding[b,e]						
	4.90 ± 1.32	1.05 ± 0.08	116 ± 17	1.04 ± 0.03	2.13 ± 0.32[d]	
-log K_i (CIs)	8.28 (8.44-8.12)		6.73 (7.30-6.16)			

Data were analyzed using Prism 7.00. Results are expressed as mean ± SEM from at least three independent experiments performed either in triplicate or in duplicate. Ligand binding affinities and EC_{50} values of 2MeSADP were expressed in both nM and log scale (CIs). K_B values were converted from log scale (Schild analysis) to nM. The values from individual experiments were calculated separately, and then the mean and SEM of those values were reported. The -logEC_{50}(CIs) or -log K_i (CIs) were calculated with one fit of all the data. NA, not applicable. [a]1321N1 cells expressing the human $P2Y_1R$. [b]U2OS cells expressing the human $P2Y_1R$. [c]Antagonism by BPTU in these three assays is insurmountable, and thus K_B values and slopes are not applicable. [d]K_d value from saturation binding. [e][^{3}H]2MeSADP (2 nM) binding to membranes prepared from U2OS cells expressing the human $P2Y_1R$ (K_i or K_d, nM). [f]The potencies of 2MeSADP in β-arrestin2 recruitment and receptor internalization are significantly different ($P < 0.05$, student's test, N=3).

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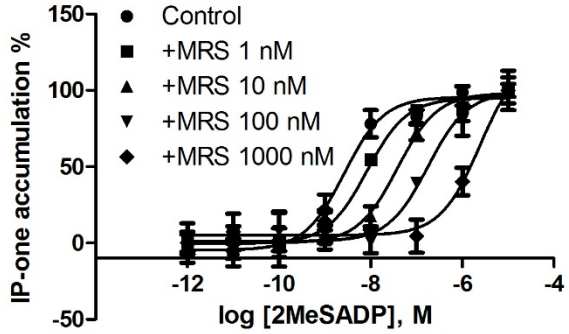
Figure 1

a.



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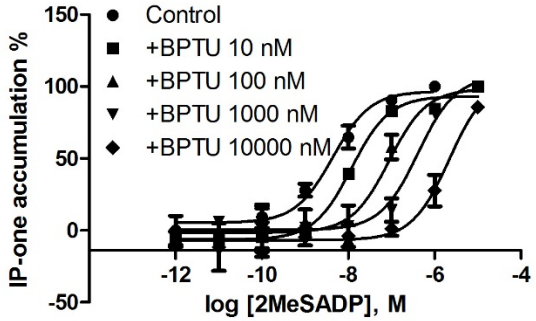

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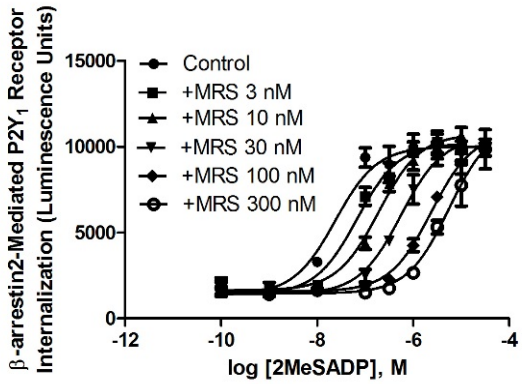

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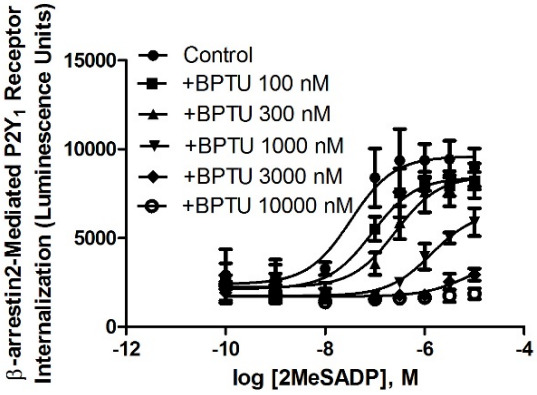

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Figure 2

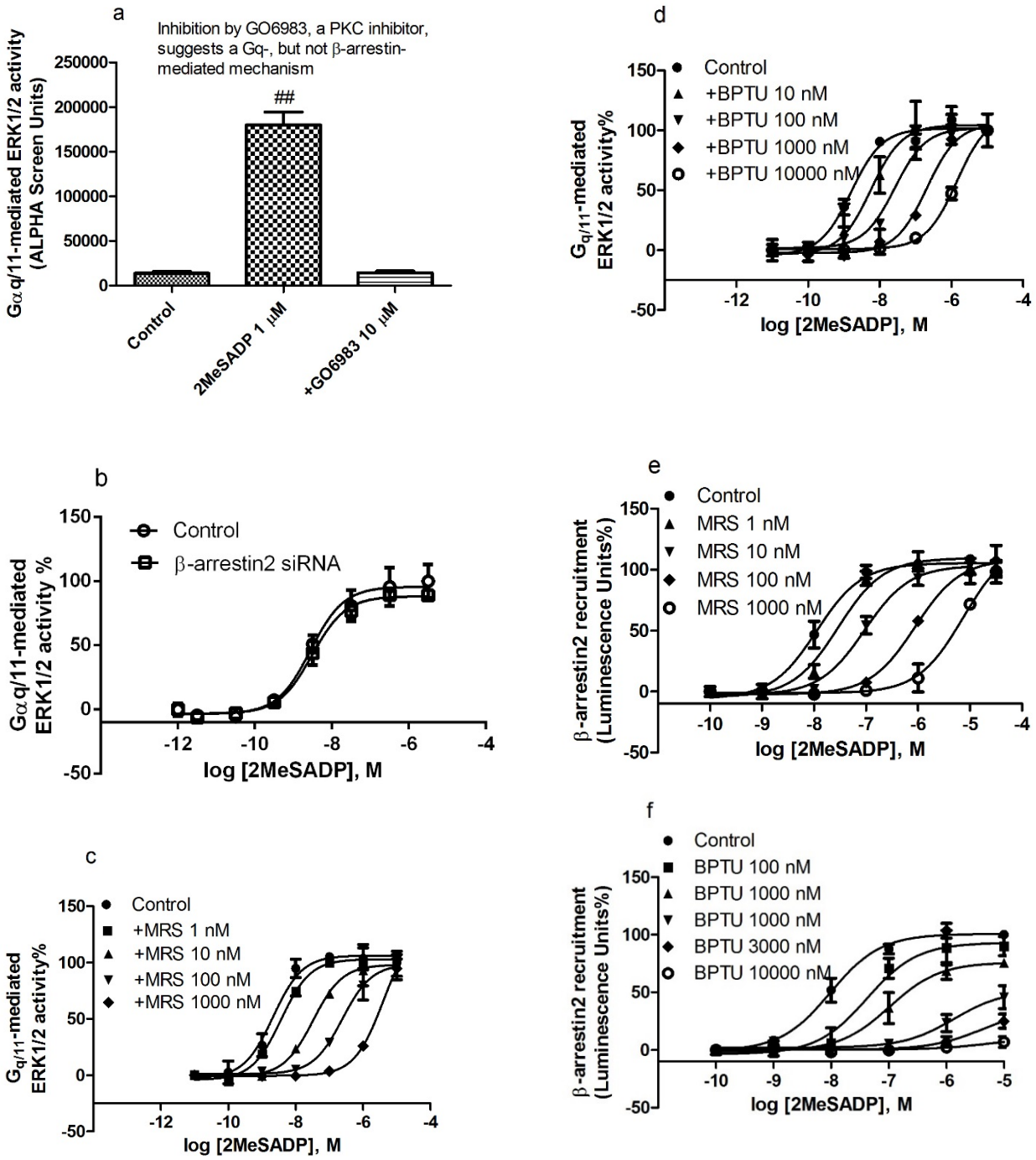

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Figure 3

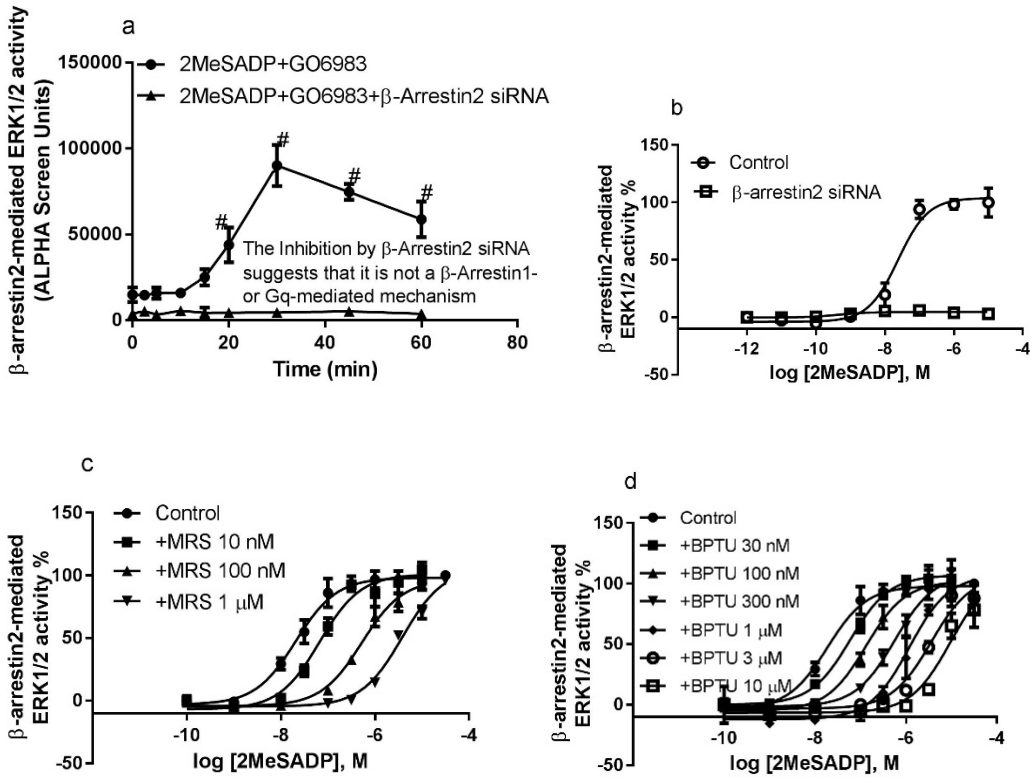

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Figure 4

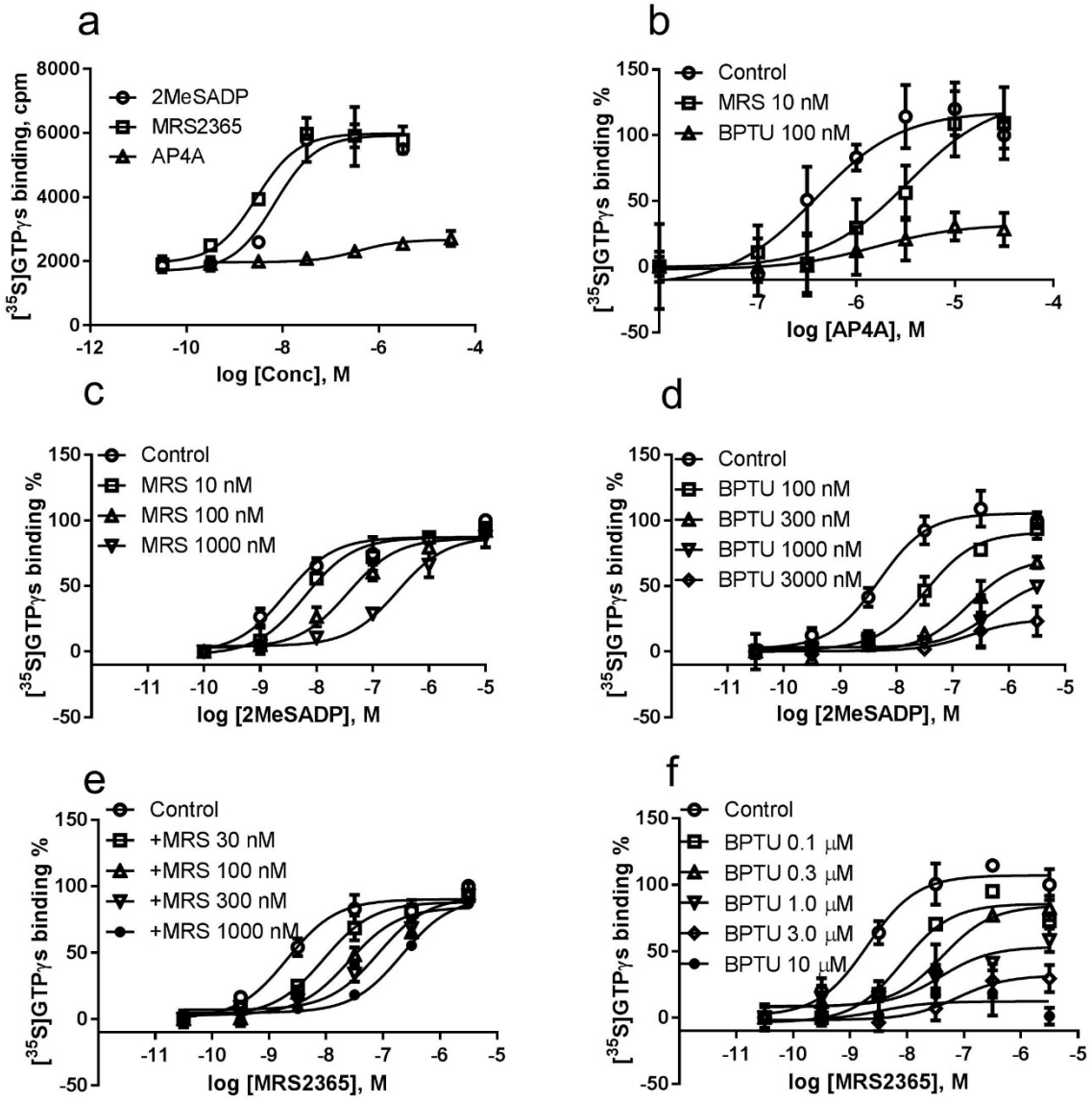

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Figure 5

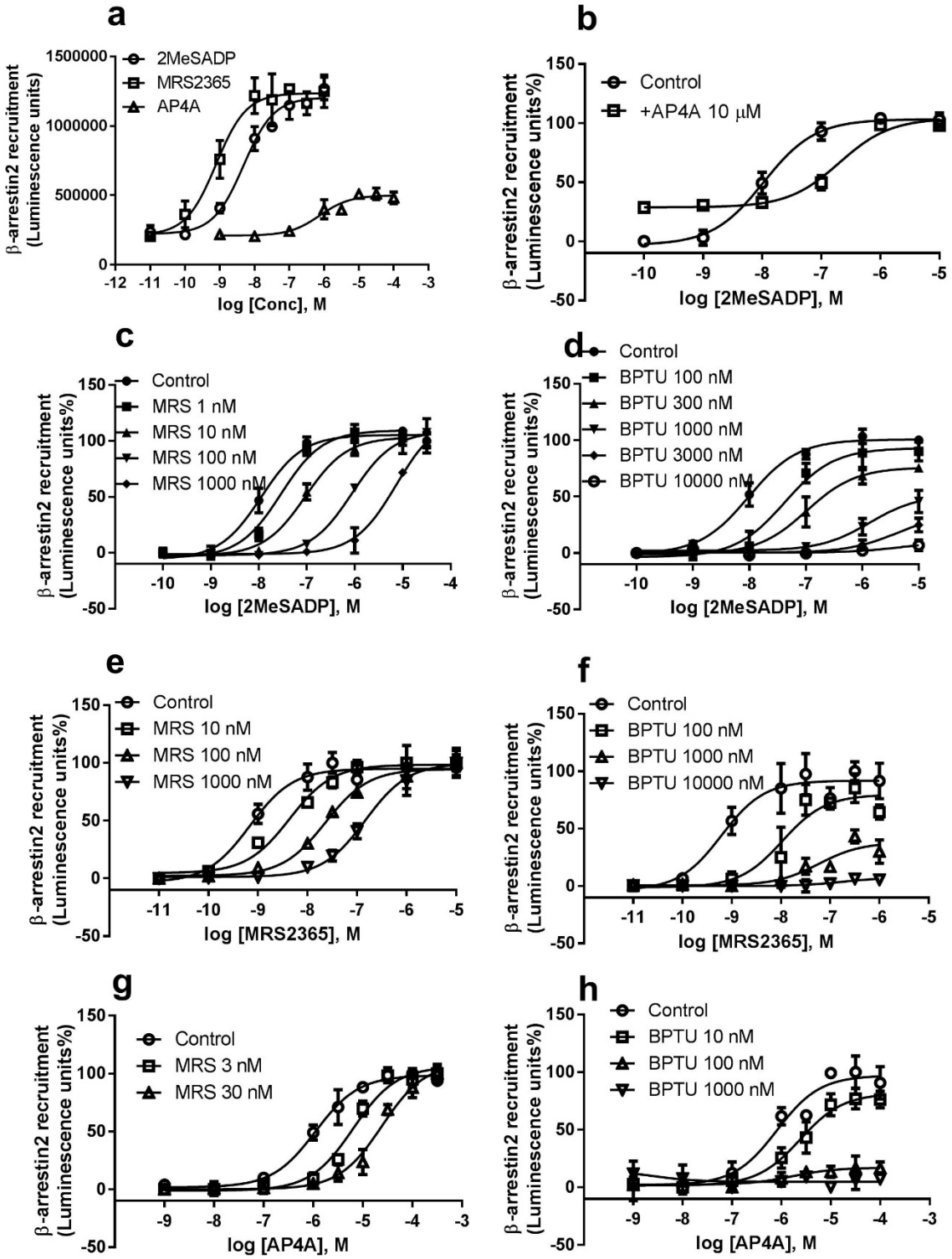

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Figure 6

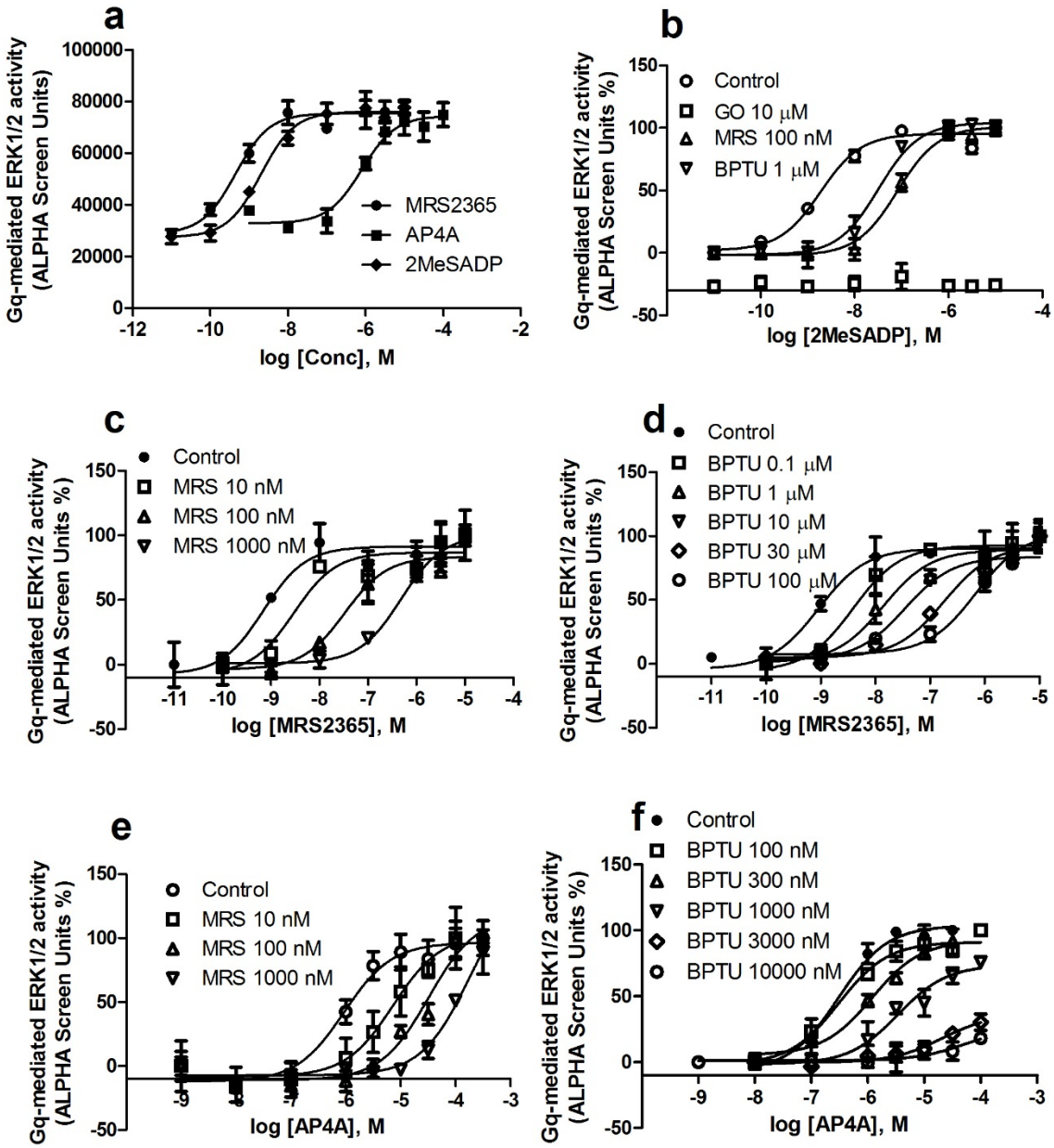

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Figure 7

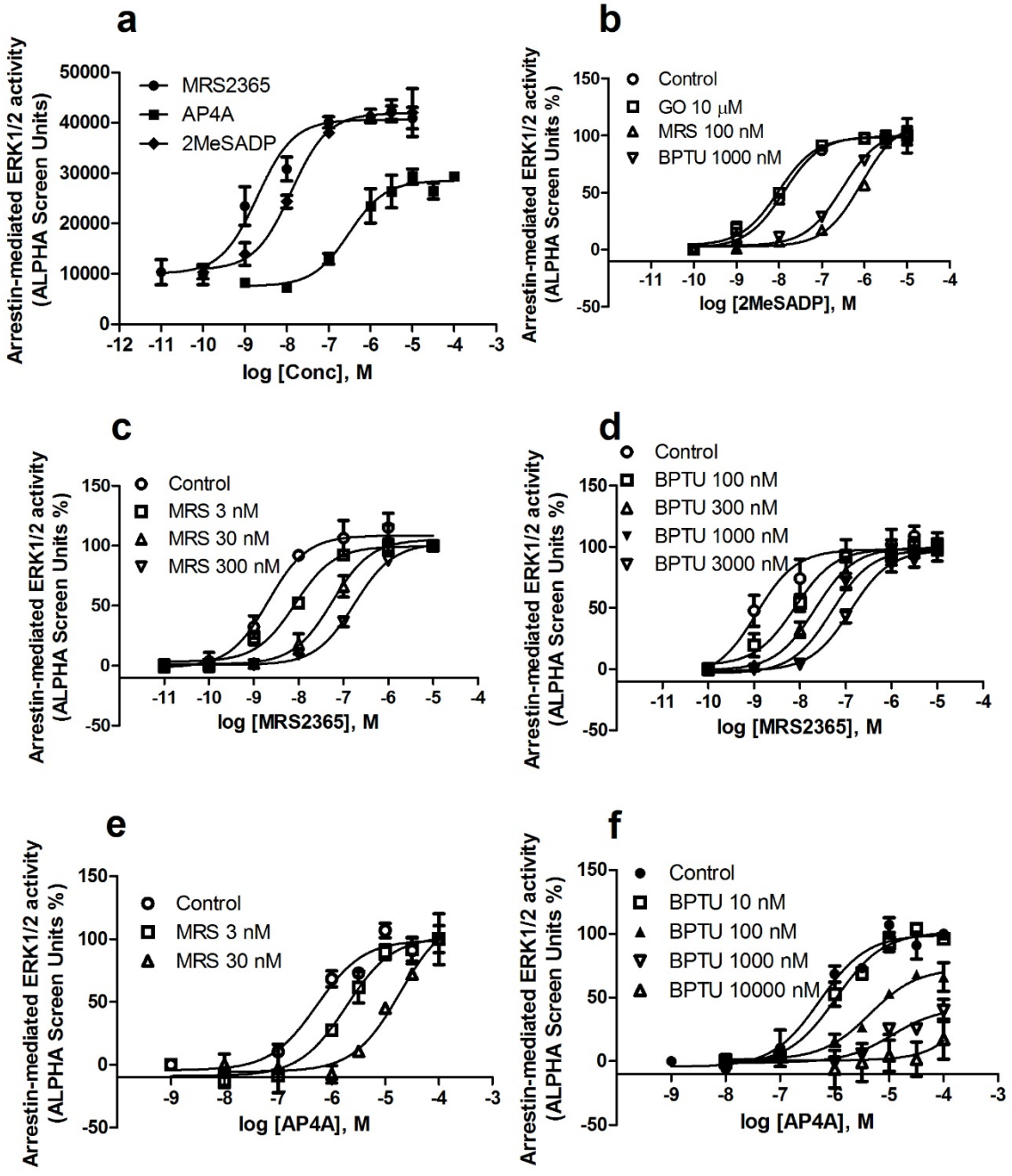

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Figure 8