ACS Medicinal Chemistry Letters

Letter

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Discovery of a Negative Allosteric Modulator of $GABA_B$ Receptors

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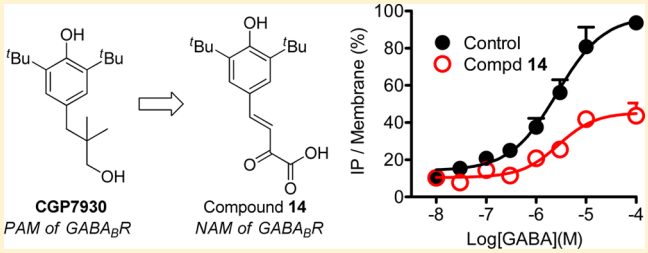
Supporting Information

ABSTRACT: Initialized from the scaffold of CGP7930, an allosteric agonist of $GABA_B$ receptors, a series of noncompetitive antagonists were discovered. Among these compounds, compounds **3**, **6**, and **14** decreased agonist GABA-induced maximal effect of IP3 production in HEK293 cells overexpressing $GABA_B$ receptors and Gq_{i9} proteins without changing the EC_{50}. Compounds **3**, **6**, and **14** not only inhibited agonist baclofen-induced ERK1/2 phosphorylation but also blocked CGP7930-induced ERK1/2 phosphorylation in HEK293 cells overexpressing $GABA_B$ receptors. The results suggested that compounds **3**, **6**, and **14** are negative allosteric modulators of $GABA_B$ receptors. The representative compound **14** decreased GABA-induced IP3 production with IC_{50} of 37.9 μM and had no effect on other GPCR Class C members such as mGluR1, mGluR2, and mGluR5. Finally, we showed that compound **14** did not bind to the orthosteric binding sites of $GABA_B$ receptors, demonstrating that compound **14** negatively modulated $GABA_B$ receptors activity as a negative allosteric modulator.



KEYWORDS: *$GABA_B$ receptors, negative allosteric modulator, CGP7930, α-keto acid*

γ-Aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the mammalian central nervous system (CNS).[1] It activates two classes of receptors: ionotropic $GABA_A$ and $GABA_C$ receptors and metabotropic $GABA_B$ receptors. $GABA_B$ receptors are widely expressed in the brain where they regulate synaptic activity both at the pre- and postsynaptic levels.[2] The $GABA_B$ receptors belong to the class C G protein-coupled receptors (GPCRs) and are functional as heterodimers composed by two subunits, GB1 and GB2.[3−8] The GB1 subunits contain the orthosteric binding sites that bind both agonists (e.g., GABA and baclofen) and competitive antagonists (e.g., CGP54626), whereas GB2 subunits are responsible for escorting GB1 subunits to the cell surface and for activating the $G_{i/o}$-protein.[7−11]

$GABA_B$ receptors have been implicated in a variety of neurological and psychiatric disorders including nociception, cognitive impairment, epilepsy, spasticity, and drug addiction.[12] The therapeutic effect of $GABA_B$ receptors agonist, Lioresal (also known as baclofen), a drug used to treat spasticity in multiple sclerosis patients, is muscle relaxation.[12,13] Its usage is limited for treatment of neurological and psychiatric disorders because of this unwanted, adverse side effect and also its poor ability to penetrate the blood−brain barrier (BBB).[14,15] Positive allosteric modulators (PAMs), which interact with GB2 subunit transmembrane domain, can potentiate the activity of $GABA_B$ receptors, with improved selectivity and safety.[16−25] CGP7930 and GS39783 (Figure 1) are pioneering PAMs of $GABA_B$ receptors reported in 2001[26] and 2003.[27] They have been proven effective in enhancing the potency of GABA both *in vitro* and *in vivo*,[28] but their developments were restricted to some extent due to their relative low potencies (in the μM range) and genotoxicity.[29] On their basis, more potent and safer PAMs including NVP-BHF177,[29] BHFF[30] (Figure 1), and

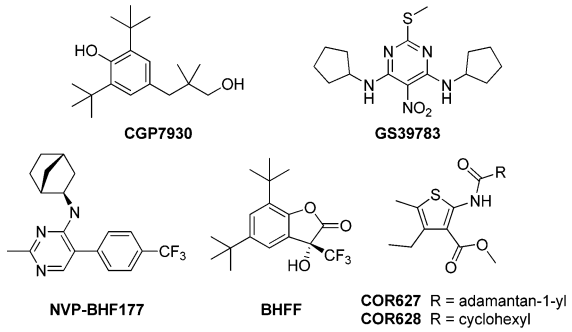


Figure 1. Representative positive allosteric modulators of $GABA_B$ receptors.

Received: November 17, 2013
Accepted: May 27, 2014
Published: May 27, 2014

ADX71441[31] (structure undisclosed) have been developed, and they showed efficacy in rodent models of nicotine[32] or alcohol addition,[33] stress-induced hypothermia,[30] and overactive bladder,[31] respectively. PAMs with novel scaffolds have also been discovered,[25] the representative compounds COR627 and COR628 (Figure 1) both potentiated the sedative/hypnotic effect of baclofen in DBA mice.[34] Furthermore, we and other groups have shown that CGP7930 alone may activate $GABA_B$ receptors as an allosteric agonist.[9,35,36] However, until now, no negative allosteric modulators (NAMs) of $GABA_B$ receptors have been reported.

In this context, we report the synthesis and characterization of the compounds based on the scaffold of CGP7930. Among these compounds, compounds **3**, **6**, and **14** decreased GABA-induced maximal effect of IP3 production in HEK293 cells overexpressing $GABA_B$ receptors and Gq_{i9} proteins without changing the EC_{50}, thus indicating that compounds **3**, **6**, and **14** may be noncompetitive antagonists. The term "noncompetitive antagonism" can be used to describe two distinct phenomena: one in which the antagonist binds to the active site of the receptor, and one in which the antagonist binds to an allosteric site of the receptor.[37] We further showed that compounds **3**, **6**, and **14** not only inhibited agonist baclofen-induced ERK1/2 phosphorylation but also blocked the allosteric agonist CGP7930-induced ERK1/2 phosphorylation in HEK293 cells overexpressing $GABA_B$ receptors. The antagonist, CGP54626, blocked baclofen-induced ERK1/2 phosphorylation but failed to block CGP7930's effect. These results suggested that compounds **3**, **6**, and **14** may be the antagonists that bind to the allosteric sites of $GABA_B$ receptors as NAMs. Finally, we showed that the representative compound **14** had no effect on other GPCR Class C members such as mGluR1, mGluR2, and mGluR5, demonstrating the specificity of compound **14** toward $GABA_B$ receptors. Moreover, we showed that compound **14** did not bind to the orthosteric binding sites of $GABA_B$ receptors, thus demonstrating that compound **14** negatively modulated $GABA_B$ receptors activity as a negative allosteric modulator.

In order to find PAMs of $GABA_B$ receptor with better activity, we have been devoted to the structure optimization of CGP7930 and obtained the unexpected compound **3** containing a rare hemiacetal group, when synthesizing compound **2** (Figure 2A). We showed that compound **3** inhibited the GABA-induced IP3 (inositol trisphosphate) production in HEK-293 cells overexpressing $GABA_B$ receptors and Gq_{i9} chimeric proteins, while compound **2** acted as a PAM to potentiate GABA-induced IP3 production (Figure 2B). As compound **3** shares the same scaffold with CGP7930, we hypothesized if compound **3** inhibited the GABA-induced IP3 production as a negative allosteric modulator.

To better characterize the inhibition mechanism of compound **3**, we tested the effect of compound **3** (100 μM) on the GABA-induced IP3 production maximum effect and EC_{50}. In HEK293 cells expressing $GABA_B$ receptors and Gq_{i9} chimeric proteins, compound **3** decreased the maximal response of GABA-induced IP3 production without changing the EC_{50} (Figure 2C). This result is consistent with a noncompetitive mode of action. To evaluate the effect of compound **3** on ERK1/2 phosphorylation, HEK293 cells expressing $GABA_B$ receptors were treated by baclofen or CGP7930 with or without compound **3**, we found that compound **3** blocked ERK1/2 phosphorylation either induced by baclofen or CGP7930, without altering the protein expression level (Figure 2D). The fact that $GABA_BR$ antagonist, CGP54626, failed to inhibit CGP7930 induced phosphorylation of ERK1/2[9,35] suggests that compound **3** antagonizes the effect on $GABA_B$ receptors induced by the allosteric agonist CGP7930 to act as a negative allosteric modulator (NAM).

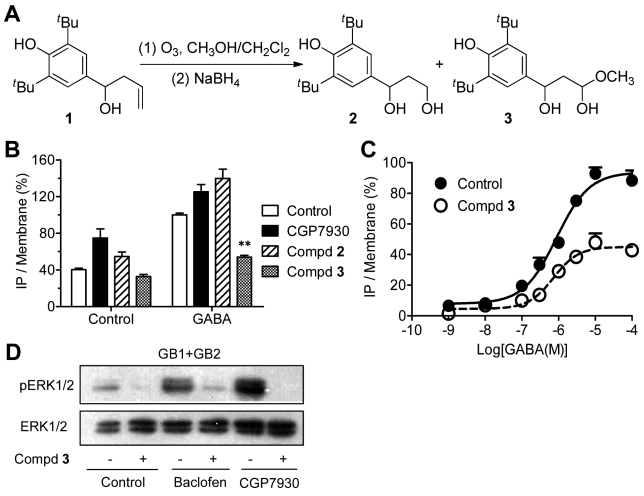


Figure 2. Discovery and biological evaluation of compound **3**. (A) Synthesis of compounds **2** and **3**. (B) Effect of CGP7930 and its analogues (100 μM) on GABA (1 μM)-induced IP3 formation in HEK293 cells overexpressing $GABA_B$ receptors (GB1 + GB2) and Gq_{i9} chimeric proteins. (C) Dose−response curves for GABA-induced IP3 formation in HEK293 cells overexpressing $GABA_B$ receptors (GB1 + GB2) and Gq_{i9} chimeric proteins in the absence (●) or presence (○) of compound **3** (100 μM). (B,C) Data are the mean ± SEM from three independent experiments. $^{**}p < 0.01$, versus treated with GABA in the absence of compounds. (D) Effect of compound **3** on either baclofen (100 μM) or CGP7930 (100 μM)-stimulated ERK1/2 phosphorylation in HEK293 cells overexpressing $GABA_B$ receptors (GB1 + GB2).

Before the mechanistic investigation of compound **3**, we found the inhibiting ability of compound **3** was fading during the repeated activity confirmation lasted for several months, which supported the following observation of partial decomposition of compound **3** stock solution in DMSO utilizing the liquid chromatography−mass spectrometry (LC−MS) analysis. After the routine column chromatography isolation and structure identification, four compounds **4**−**7** were acquired (Figure 3A), with the recovery of compound **3**. We further compared the activities of compounds **4**−**7** with compound **3** (Figure 3B). On HEK293 cells expressing $GABA_B$ receptors and Gq_{i9} chimeric proteins, compound **6** inhibited the GABA-induced IP3 production to a similar extent of compound **3**, while the other three displayed little effect compared to the control.

Compound **6** has a conjugated aldehyde group, which is prone to react with nucleophilic reagents such as amines and thiols in the cells. Does the activity of compound **6** result from the nonspecific reaction with nucleophiles like GABA? To answer this question, we assessed the activity of compound **6** analogues as compounds **8**−**12** (Figure 3C). The result showed that only compound **8** displayed moderate activity, among these three compounds possessing conjugated carbonyl group (Figure 3D). Compound **12** also showed moderate activity, which contains the same substituted phenyl group as compound **6** but has the allylic alcohol group instead of conjugated aldehyde. Compound **11** had little activity due to the deletion of two *tert*-butyl groups on the phenyl group.

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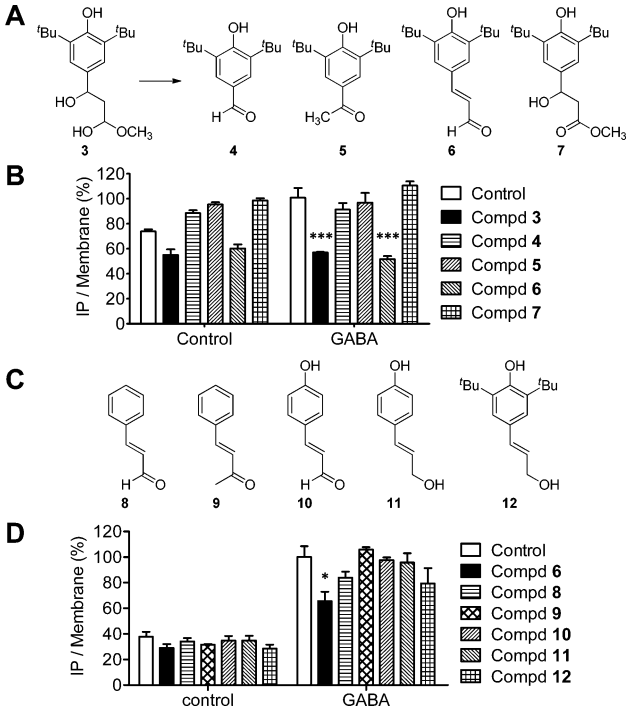

Figure 3. Discovery and biological validation of compound **6**. (A) Structures of the four compounds isolated from decomposition products of compound **3**. (B) Effects of compounds **3**−**7** (100 μM) on GABA (1 μM)-induced IP3 formation in HEK293 cells overexpressing $GABA_B$ receptors (GB1 + GB2) and Gq_{i9} chimeric proteins. (C) Structures of compound **6** analogues. (D) Effects of compound **6** and its analogues (100 μM) on GABA (1 μM)-induced IP3 formation in HEK293 cells overexpressing $GABA_B$ receptors (GB1 + GB2) and Gq_{i9} chimeric proteins. (B,D) Data are the mean ± SEM from three independent experiments. $^*p < 0.05$ and $^{***}p < 0.001$ versus treated with GABA in the absence of compounds.

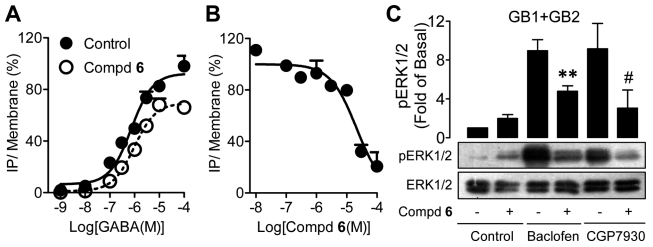

Figure 4. Biological evaluation of compound 6. (A) Dose−response curves for GABA (1 μM)-induced IP3 formation in HEK293 cells overexpressing $GABA_B$ receptors and Gq_{i9} chimeric proteins in the absence (●) or presence (○) of compound **6** (100 μM). (B) Dose−response curves for compound **6**-inhibited IP3 formation in HEK293 cells overexpressing $GABA_B$ receptors (GB1 + GB2) and Gq_{i9} chimeric proteins in the presence of GABA (1 μM). (A,B) Data are the mean ± SEM from three independent experiments. (C) Effect of compound **6** (100 μM) on baclofen (100 μM) or CGP7930 (100 μM)-stimulated ERK1/2 phosphorylation in HEK293 cells overexpressing $GABA_B$ receptors (GB1 + GB2). $^{**}p < 0.01$ versus treated with baclofen in the absence of compounds, and $^{\#}p < 0.05$ versus treated with CGP7930 in the absence of compounds.

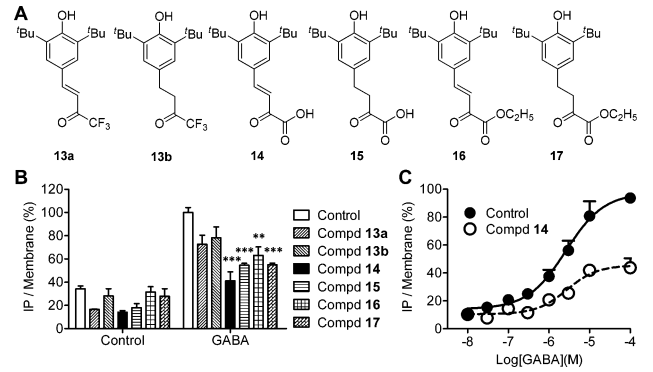

Figure 5. Structure and biological evaluation of compounds **13a**−**17**. (**A**) Structures of CGP7930 analogues containing highly electrophilic groups. (B) Effects of compounds **13a**−**17** (100 μM) on GABA (1 μM)-induced IP3 formation in HEK293 cells overexpressing $GABA_B$ receptors and Gq_{i9} chimeric proteins. (C) Dose−response curves for GABA-induced IP3 formation in HEK293 cells overexpressing $GABA_B$ receptors (GB1 + GB2) and Gq_{i9} chimeric proteins in the absence (●) or presence (○) of compound **14** (100 μM). (B,C) Data are the mean ± SEM from three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus treated with GABA in the absence of compounds.

These results indicate that both the conjugated carbonyl group and the substitutions on phenyl group are essential for the inhibition activity of compound **6** and exclude the possibility that the activity of compound **6** origins from the nonspecific reaction with nucleophiles.

The further biological evaluation showed that compound **6** decreased the maximal response in the GABA-induced IP3 production without changing the EC_{50} of GABA (Figure 4A) and inhibited the GABA-induced IP3 production in a concentration-dependent manner with IC_{50} of 21.3 μM (Figure 4B). Compound **6** also blocked the phosphorylation of ERK1/2 either induced by baclofen or CGP7930 in HEK293 cells overexpressing $GABA_B$ receptors (Figure 4C). These results suggest that compound **6** modulates $GABA_B$ receptors activity as a NAM since it antagonized the phosphorylation of ERK1/2 induced by CGP7930. Although compound **6** displayed weaker activity than compound **3**, its stability and the preliminary structure−activity relationship (SAR) provide us a fresh start for the discovery of more potent NAMs of $GABA_B$ receptors.

The aforementioned activity difference between compounds **6** and **12** indicates that the highly electron-deficient carbonyl group played an important role in the negative allosteric activity of this scaffold. Increasing the electron-deficiency may enhance the activity of these compounds. Thus, we designed and synthesized two types of analogues as compounds **13a**−**17**, containing electrophilic trifluoromethyl ketone group or α-keto acid (ester) group separately (Figure 5A). We showed that compounds **14** and **15** had the best effects to modulate $GABA_B$ receptors activity, comparing to compounds **13a** and **13b** with trifluoromethyl ketone group and their ester form, compounds **16** and **17** (Figure 5B). Since compound **14** is easy to synthesize and its carboxylic acid group facilitates further modification, we performed the functional analysis for this compound. We showed that compound **14** decreased the maximal response in the GABA-induced IP3 production without changing the EC_{50} of GABA (Figure 5C) and inhibited the GABA-induced IP3 production in a concentration-dependent manner with IC_{50} of 37.9 μM (Figure SI, Supporting Information).

To verify the selectivity of compound **14** on $GABA_B$ receptors, we performed the experiment to verify if compound **14** blocked activation of other members of Class C GPCRs such as mGluR1, mGluR2, and mGluR5. Among these Class C GPCRs, mGluR1 and mGluR5 belong to Gq-protein coupled receptors, whereas $GABA_B$ receptors and mGluR2 belong to

dx.doi.org/10.1021/ml500162z | ACS Med. Chem. Lett. 2014, 5, 742−747

Gi/o-protein coupled receptors. We compared $GABA_B$ receptor-induced Ca^{2+} release in HEK293 cells overexpressing $GABA_B$ receptors and Gq_{i9} chimeric proteins with different members of mGluRs-induced Ca^{2+} release in HEK cells overexpressing mGluR1, mGluR2 (with Gq_{i9}), or mGluR5. As shown in Figure 6A, 50 μM of compound **14** inhibited

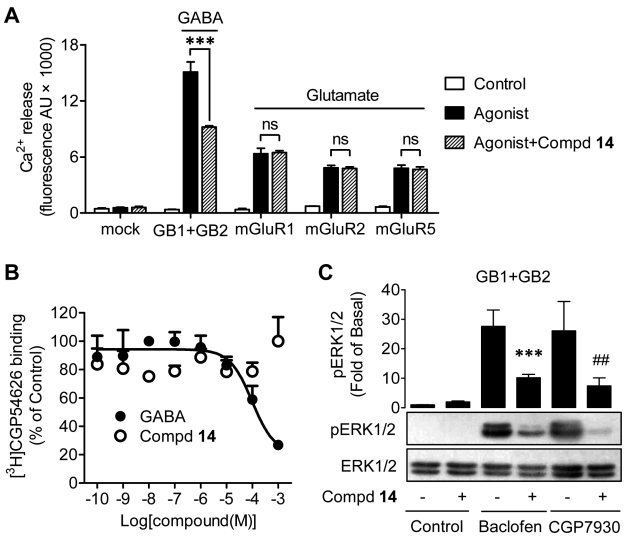

Figure 6. Specificity of compound **14** toward $GABA_B$ receptors as a negative allosteric modulator. (A) Effect of compound **14** (50 μM) on agonist-induced Ca^{2+} release in HEK293 cells overexpressing $GABA_B$ receptors (with Gq_{i9}), mGluR1, mGluR2 (with Gq_{i9}), or mGluR5, respectively. ***p < 0.001 and ns, no effect, versus treated with agonists (GABA (100 μM) for $GABA_B$ receptor and glutamate (10 μM) for mGluR1, 2, and 5 respectively) in the absence of compounds. (B) Effect of compound **14** (○) and GABA (●) on the displacement of the antagonist radioligand [^{3}H]CGP54626 from CHO cells membrane overexpressing $GABA_B$ receptors (GB1 + GB2). The binding of [^{3}H]CGP54626 to membranes from transfected CHO cells was measured in a scintillation proximity assay. (C) Effects of compound **14** (100 μM) on baclofen (100 μM) or CGP7930 (100 μM)-stimulated ERK1/2 phosphorylation in HEK293 cells overexpressing $GABA_B$ receptors (GB1 + GB2). Data are the mean ± SEM from three independent experiments. ***p < 0.001 versus treated with baclofen in the absence of compounds, and $^{\#\#}p$ < 0.01 versus treated with CGP7930 in the absence of compounds.

GABA-induced Ca^{2+} release but had no effect at all on glutamate-induced Ca^{2+} release, demonstrating the specificity of compound **14** on $GABA_B$ receptors. Furthermore, our data in Figure 6A showed that compound **14** only inhibited $GABA_B$ receptor-mediated Gi/o signaling but had no effect on mGluR2-mediated Gi/o signaling, demonstrating that compound **14** had no effect on $GABA_B$ receptors downstream of Gi/o-protein signaling and interacted only with $GABA_B$ receptors at the receptor level.

To verify if compound **14** binds to orthosteric binding sites in GB1, we thus performed radioactivity-labeled binding assay through displacement of [^{3}H]CGP54626 from membranes of CHO cells overexpressing $GABA_B$ receptors.[38] As shown in Figure 6B, [^{3}H]CGP54626 binding was displaced in a concentration-dependent manner by the $GABA_B$ receptors agonist, GABA. In contrast, compound **14** failed to displace [^{3}H]CGP54626 binding even at concentrations up to 100 μM, demonstrating that compound **14** did not bind to orthosteric binding sites in GB1 subunits. Furthermore, CGP54626 blocked baclofen-induced ERK1/2 phosphorylation but failed to block allosteric agonist CGP7930-induced ERK1/2 phosphorylation in HEK293 cells overexpressing $GABA_B$ receptors (Figure SII, Supporting Information). Compound **14** blocked the phosphorylation of ERK1/2 either induced by baclofen or CGP7930 in HEK293 cells overexpressing $GABA_B$ receptors (Figures 6C and SII, Supporting Information). These results demonstrated that compound **14** modulated $GABA_B$ receptor activity as a NAM for $GABA_B$ receptors.

In summary, we have developed a class of noncompetitive antagonists of $GABA_B$ receptors derived from the scaffold of CGP7930. Among them, compound **14** acts as a NAM since it did not bind to the orthosteric binding sites of $GABA_B$ receptors. Whether compound **14** interacts with a GB2 transmembrane domain as CGP7930 remains to be further investigated. The common electrophilic groups shared by these structures indicate a possible specific interaction between ligand and receptor. The fine-tuning of $GABA_B$ receptor activity by a NAM may provide a new strategy for developing novel therapies. More detailed SAR studies focusing on this type of compounds are in progress.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures and characterization of new chemical entities. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by the Ministry of Science and Technology (grant number 2012CB518000), the National Natural Science Foundation of China (NSFC) (grant numbers 31130028, 31225011, and 91213303), the Program of Introducing Talents of Discipline to the Universities of the Ministry of Education (grant number B08029), and the Mérieux Research Grants Program of Institut-Mérieux (to J.-F.L.).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Jean-Philippe Pin and Dr. Philippe Rondard (Institut de Génomique Fonctionnelle, Montpellier, France) for their helpful discussions.

■ ABBREVIATIONS USED

GABA, γ-aminobutyric acid; GPCR, G-protein coupled receptor; PAM, positive allosteric modulator;; NAM, negative allosteric modulator; IP3, inositol trisphosphate; ERK1/2, extracellular-signal-regulated kinases 1/2; SAR, structure−activity relationship

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