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Brief Article

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Novel 3,6,7-Substituted Pyrazolopyrimidines as Positive Allosteric Modulators for the Hydroxycarboxylic Acid Receptor 2 (GPR109A)

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Supporting Information

ABSTRACT: A number of pyrazolopyrimidines were synthesized and tested for their positive allosteric modulation of the HCA_2 receptor (GPR109A). Compound **24**, an efficacious and potent agonist and allosteric enhancer of nicotinic acid's action, was the basis for most other compounds. Interestingly, some of the compounds were found to increase the efficacy of the endogenous ligand 3-hydroxybutyrate and enhance its potency almost 10-fold. This suggests that the pyrazolopyrimidines may have therapeutic value when given alone.

INTRODUCTION

Hydroxycarboxylic acid receptor 2 (HCA_2)[1] or GPR109A is a G-protein-coupled receptor (GPCR) that was first identified in 2001.[2] Two years later its involvement in the antilipolytic action of nicotinic acid (NA) was reported[3−5] and 3-hydroxybutyrate was identified as its endogenous ligand in 2005[6] (for reviews, see refs 1 and 7). A broad range of synthetic ligands (agonists only) for this receptor has been developed.[7−9] An intriguing class among those are substituted pyrazolopyrimidines, as recently reported by Shen and colleagues.[10] These compounds act as agonists, some with potencies comparable to that of nicotinic acid, but were also suggested to bind allosterically to HCA_2. One compound in particular (**25** in the present study) was shown to behave as a positive allosteric modulator (PAM) as well, by significantly enhancing the potency of nicotinic acid at HCA_2 (∼100-fold at 1 μM). We decided to further investigate the pyrazolopyrimidines by synthesizing a new series of derivatives and evaluating their activity on HCA_2 in several [^{3}H]nicotinic acid and [^{35}S]GTPγS binding assays.

CHEMICAL SYNTHESIS

The synthetic route used to obtain the pyrazolo[1,5-*a*]-pyrimidine-6-carboxamides **20**−**30** and **32**−**43** is depicted in Scheme 1. The 1*H*-pyrazol-5-amine **1**[10] or the commercially available **2** was ring closed with the respective ethyl 2-(ethoxymethylene)-3-oxoate[11] (**3**, **5**, or **6**) or the available diester **4** in EtOH at reflux temperature, resulting in the ethyl pyrazolo[1,5-*a*]pyrimidine-6-carboxylates (**7**, **8**, and **11**−**13**)[10,12,13] in good yields. The 7-hydroxypyrazolopyrimidine **8** was converted into the 7-chloro analogue (**9**) in the presence of $POCl_3$[13] followed by palladium-catalyzed reductive dechlorination[13] to give the pyrazolo[1,5-*a*]pyrimidine **10**. The 3-position of the pyrazolo[1,5-*a*]pyrimidine **13**[14] was functionalized using NBS[15] to the versatile 3-bromo analogue **14**. Subsequently hydrolysis of the ethyl esters **7**, **10**−**12**, and **14** with LiOH smoothly resulted in the corresponding acids (**15**−**19**). The final pyrazolo[1,5-*a*]pyrimidine carboxamides **20**−**30** and the 3-bromo building block **31** were synthesized from the corresponding acids (**15**−**19**) and a range of amines by use of coupling reagent EDC·HCl. Finally a Suzuki reaction with building block **31** and various arylboronic acids, under microwave conditions, yielded the 3-aryl compounds **32**−**43**.[16]

BIOLOGICAL EVALUATION AND DISCUSSION

To evaluate the compounds, we performed [^{3}H]nicotinic acid binding assays (see Supporting Information Table S1) and [^{35}S]GTPγS binding assays (Table 1) on HEK-HCA_2 membranes. In Table 1 the following results are reported: (a) the percentage of [^{35}S]GTPγS binding in the presence of 10 μM test compound alone; (b) the shift in EC_{50} of nicotinic acid in the presence of 10 μM test compound; (c) the E_{max} reached at 100 μM nicotinic acid in the presence of 10 μM test compound (100% is without test compound).

Structure−Activity Relationships. Compounds **20**−**23** show that linker lengths of two or three carbons between the carboxamide and the phenyl group are preferred for agonism and potentiation of nicotinic acid's effects. However, linker length does not appear to influence the E_{max} of nicotinic acid, which was ∼130% for all four compounds. Introduction of an ether function in the linker (**24**) resulted in a higher activity in the GTPγS binding assay. So the compound acted as an agonist in its own right, while the EC_{50} of nicotinic acid was increased approximately 25-fold without a change in its E_{max}. This compound also considerably slowed the dissociation of [^{3}H]nicotinic acid from the receptor and increased the equilibrium specific binding of the radioligand to an extent that was not surpassed by any of the other test compounds (Supporting Information Table S1). Introduction of a methyl substituent on the linker (**25**) was not beneficial in our hands, as opposed to the findings of Shen et al.[10] Removal of the phenyl group yielded **26**, which enhanced nicotinic acid's activity somewhat better. Replacing the ether of **24** by a

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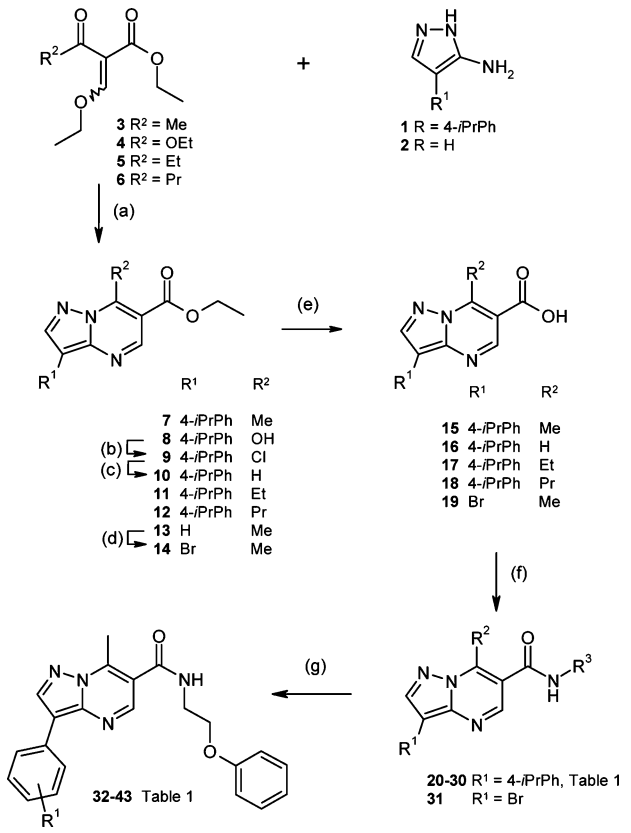
Scheme 1. Synthetic Route to the Substituted Pyrazolo[1,5-*a*]pyrimidinecarboxamides 20−43[a]



[a]Reagents and conditions: (a) EtOH, reflux, 3 h; (b) $POCl_3$, *N*,*N*-dimethylaniline, reflux, 3 h; (c) NaOAc, 5% Pd/C, H_2 2.5 bar, rt, 1 h; (d) NBS, DCM, 0 °C, 1.5 h, rt, 16 h; (e) LiOH, H_2O/MeOH/THF, rt, 16 h; (f) R^3NH_2, EDC·HCl, DCM, rt, 4 h; (g) $R^1B(OH)_2$, **31**, $Pd(Ph_3P)_4$, Na_2CO_3, toluene/H_2O, microwave 150 °C, 2 h.

secondary amine to obtain aniline **27** rendered the compound more active than **25** in all respects.

Next, a small series was synthesized with varying substituents on the 7-position (R^2, Table 1). Absence of the methyl substituent (**28**) severely reduced the activity compared to **24**. The ethyl and propyl substituted compounds (**29** and **30**) behaved highly similar to each other and also to the parent derivative **24** except for the enhancement of nicotinic acid's E_{max} by **29** and **30** but not **24**. Finally, a series of derivatives of **24** with varying substituents on the 3-phenyl ring was then tested (R^1, Table 1, **32**−**43**). A methyl substituent on the para position (**32**) resulted in more agonism and potentiation of nicotinic acid compared to methyl substituents at the meta (**33**) or ortho (**34**) positions. Compounds with ethyl (**35**) and *tert*-butyl (**36**) substituents had activities highly similar to that of the isopropyl-substituted **24** except that **35** and **36** significantly increased the E_{max} of nicotinic acid, which was not affected by **24**. Compounds substituted with methoxy (**37**) or isopropoxy (**38**) were also highly active. These compounds, like methyl derivatives **32** and **33**, seemed to have a more modest agonistic activity compared to their activity as enhancers of nicotinic acid potency. A phenyl derivative (**39**) retained activity in the [^{35}S]GTPγS binding assay. Meta-substitution (**41**), and to a lesser extent, para-substitution (**40**) with chlorine diminished activity. The 3,4-dichloro-substituted **42** behaved very similarly to the 4-chloro derivative **40**. A trifluoromethyl group (**43**) conferred good enhancement of nicotinic acid potency and efficacy, paired with a moderate agonistic activity. To investigate any correlation between the agonistic and modulating effects of the pyrazolopyrimidines, the [^{35}S]GTPγS binding activation and the potency shift of nicotinic acid, both at 10 μM, were plotted against each other (Supporting Information Figure S2), yielding a nonlinear correlation. The plot suggests that the ability of the pyrazolopyrimidine agonists to stabilize active receptor conformations contributes to, or even determines, the modulator strength of the compounds.

Modulation of Nicotinic Acid Mediated Receptor Activation. We next examined the effects at 1, 3, and 10 μM of five selected enhancers on the concentration−effect curves for nicotinic acid in [^{35}S]GTPγS binding assays. Figure 1 shows the results of a representative experiment, and the average values obtained from three independent experiments are discussed below. Compound **24** reached 71 ± 17% receptor activation at 10 μM without any nicotinic acid present, and nicotinic acid further increased activation to 109 ± 6%, with a 24-fold increased potency compared to control.

At concentrations of 3 and 1 μM, **24** increased [^{35}S]GTPγS binding to 51 ± 14% and 30 ± 5%, respectively, and caused shifts in the EC_{50} of nicotinic acid of ∼5-fold for both concentrations. Compound **27** caused modest increases in the potency (2-fold at 1 μM, 5-fold at 3 μM, and 7-fold at 10 μM) and the E_{max} (120 ± 3% at 1 μM, 129 ± 3% at 3 μM, and 139 ± 10% at 10 μM) of nicotinic acid, which seemed to follow the increase in [^{35}S]GTPγS binding due to agonist activity alone (13 ± 2% at 1 μM, 31 ± 2% at 3 μM, and 41 ± 6% at 10 μM). Compound **29** was highly efficacious in all aspects: an agonist in its own right (28 ± 3%, 53 ± 5%, and 69 ± 7% receptor activation at 1, 3, and 10 μM, respectively) and a positive allosteric modulator of nicotinic acid's EC_{50} (5-, 9-, and 13-fold shifts) and E_{max} (122 ± 7%, 129 ± 6%, and 151 ± 6% of control). Both **38** and **42** were in every respect less potent and efficacious than **29**. As an agonist, **38** was more potent and efficacious than **42**, causing more receptor activation at 1 μM (15 ± 6% vs 5 ± 3%), 3 μM (27 ± 2% vs 16 ± 3%), and 10 μM (50 ± 3% vs 31 ± 5%). The modulation of the EC_{50} of nicotinic acid was similar at 1 μM (both 2-fold) and 3 μM (5-fold vs 4-fold), but at 10 μM **38** was the more active compound again (11-fold vs 5-fold shift). Both compounds increased the E_{max} of nicotinic acid similarly (139 ± 12%, 139 ± 6% at 10 μM). However, **42** showed a relatively low potency compared to **38**, since no effect was seen at 1 μM (compared to 111 ± 4% for **38**) and only a small effect at 3 μM (114 ± 3% for **42** compared to 123 ± 5% for **38**). For EC_{50} values and E_{max} values of **24**, **29**, **30**, **32**, **35**, **36** and **38**, tested again in the absence of nicotinic acid, see Table S2 in Supporting Information. Some further observations are discussed on page S7 of Supporting Information.

Positive Allosteric Modulation of 3-Hydroxybutyrate Potency and Efficacy. Allosteric modulators that enhance the potency of nicotinic acid on the HCA_2 receptor are of interest clinically, since they could greatly reduce the daily dose of nicotinic acid when used in combination therapy. Clearly the pyrazolopyrimidines fit this picture, but many of them can also activate HCA_2 on their own. In vivo, the activity of the endogenous ligand 3-hydroxybutyrate (3-OHB) may also be enhanced by the pyrazolopyrimidines, but this cannot be assumed a priori, since allosteric enhancement is probe dependent.[17] Therefore, 3-OHB dose−response curves were

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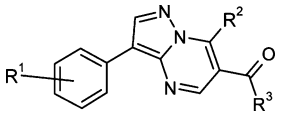
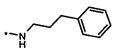
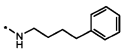
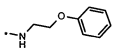
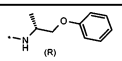
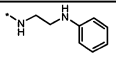
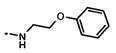
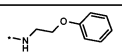
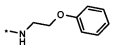
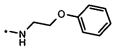
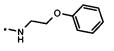
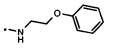
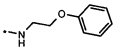
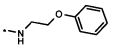
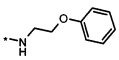
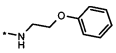
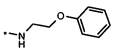
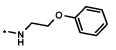
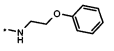
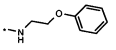
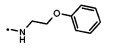
Table 1. Different Measures for HCA_2 Receptor Activation in [^{35}S]GTPγS Binding Assays[e]



Nr	R^1	R^2	R^3	[^{35}S]- GTPγS binding		
				%E_{max} comp. alone[a] (SEM)	EC_{50} shift NA[b]	%E_{max} + NA (SEM)[c]
20	4-*i*Pr	Me		3 (1)	0.60	129 (9)
21	4-*i*Pr	Me		23 (1)	0.32	135 (5)
22[d]	4-*i*Pr	Me		22 (8)	0.36	134 (7)
23	4-*i*Pr	Me		1 (2)	1.07	128 (4)
24[d]	4-*i*Pr	Me		71 (17)	0.04	109 (6)
25[d]	4-*i*Pr	Me		5 (2)	0.53	115 (9)
26[d]	4-*i*Pr	Me		22 (0.4)	0.22	100 (1)
27	4-*i*Pr	Me		41 (6)	0.15	139 (10)
28	4-*i*Pr	H		10 (3)	0.62	106 (7)
29	4-*i*Pr	Et		69 (7)	0.08	151 (6)
30	4-*i*Pr	Pr		58 (20)	0.17	151 (9)
32	4-Me	Me		52 (4)	0.13	113 (7)
33	3-Me	Me		17 (2)	0.25	119 (5)
34	2-Me	Me		3 (1)	0.58	91 (6)
35	4-Et	Me		64 (9)	0.13	153 (15)
36	4-*t*Bu	Me		80 (5)	0.09	141 (4)
37	4-OMe	Me		20 (3)	0.24	129 (3)
38	4-O*i*Pr	Me		50 (3)	0.09	139 (2)
39	4-Ph	Me		41 (4)	0.16	139 (6)
40	4-Cl	Me		42 (9)	0.16	141 (14)
41	3-Cl	Me		16 (6)	0.37	126 (13)
42	3,4-diCl	Me		31 (5)	0.19	139 (12)
43	4-CF_3	Me		47 (6)	0.13	147 (8)

[a]Percentage of [^{35}S]GTPγS binding in the presence of 10 μM test compound alone (100% is the E_{max} at 100 μM nicotinic acid). [b]Shift in EC_{50} of nicotinic acid (NA) in the presence of 10 μM test compound (1 is without test compound, and values less than unity indicate increased affinity of nicotinic acid, e.g., where 0.1 means a 10-fold shift). [c]E_{max} reached at 100 μM nicotinic acid (NA) in the presence of 10 μM test compound (100% is without test compound). [d]Also reported in ref 10 (**26**, racemate only). [e]Values are the mean (±SEM) of three independent experiments performed in duplicate.

recorded in the presence of five compounds, and four of these increased the potency of 3-hydroxybutyrate approximately 8-fold (Figure 2 and Supporting Information Table S3). The modulators caused an increase in the intrinsic efficacy of the endogenous ligand as well. In a [^{35}S]GTPγS binding assay 3-OHB was previously reported as a high-efficacy partial agonist for HCA_2,[18] whereas it behaved as a low-efficacy partial agonist with ∼30% intrinsic efficacy in our hands. This may have to do with lower levels of receptor expression in our preparation, as we explicitly selected clones for physiological rather than high expression levels.

Future Perspectives and Conclusion. With the expanded ligand repertoire reported in this study other functional assays with the pyrazolopyrimidines can be performed, including ERK

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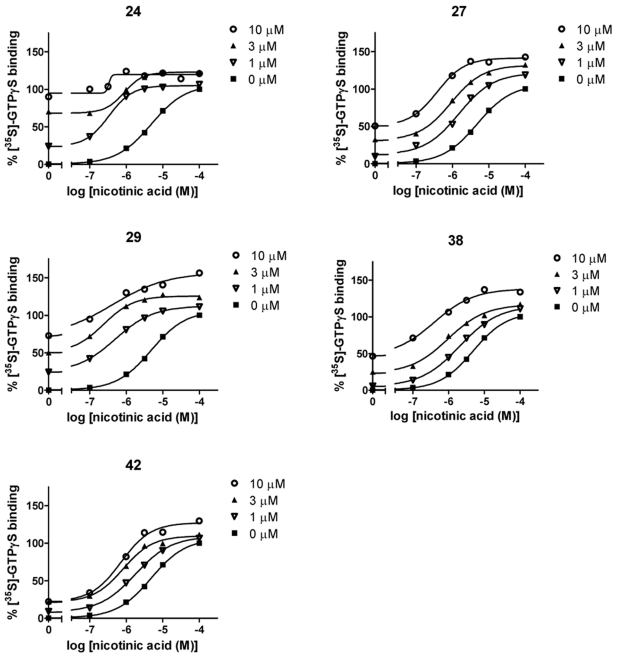

Figure 1. Dose−response curves of nicotinic acid in the presence of 0, 1, 3, and 10 μM pyrazolopyrimidines. The data are from [^{35}S]GTPγS binding assays performed on HEK-HCA$_2$ membranes. Shown are representative graphs from one experiment performed in duplicate.

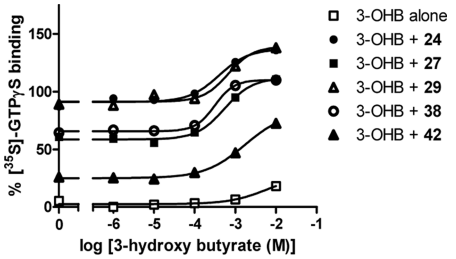

Figure 2. Concentration−response curves of 3-hydroxybutyrate in the presence and absence of 10 μM pyrazolopyrimidine. The data are from [^{35}S]GTPγS binding assays performed on HEK-HCA$_2$ membranes. Shown are representative graphs from one experiment performed in duplicate (see also Table S3 in Supporting Information).

1/2 phosphorylation assays. Activation of ERK 1/2 induced by HCA$_2$ agonists in vitro has been suggested to be predictive of the skin flushing side effect in vivo.[19] Furthermore, cooperativity with probes (orthosteric ligands) that are not structurally related to nicotinic acid should be examined. In the future, mutagenesis studies will hopefully shed light on the binding mode of the allosteric modulators and on how these ligands trigger the changes in receptor activation. In conclusion, we presented several pyrazolopyrimidine derivatives that do not displace [^{3}H]nicotinic acid from HCA$_2$ but are capable of activating this receptor, which indicates an allosteric binding mode. Next to their agonistic effects these compounds potentiate the action of nicotinic acid and the endogenous ligand 3-hydroxybutyrate. Therapeutically, such positive allosteric modulators may represent an interesting alternative in the search for HCA$_2$ receptor ligands.

EXPERIMENTAL SECTION

Chemistry. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV 400 (^{1}H NMR, 400 MHz; ^{13}C NMR, 100 MHz) spectrometer. Chemical shifts (δ) are reported in ppm relatively to Me_4Si. Purity was confirmed to be ≥95% by HPLC performed on a Gilson 306 system (detection at 254 nm) equipped with an analytical C18 column in combination with a gradient of mixture A (1 MeCN/9 H_2O) and B (9 MeCN/1 H_2O). High resolution mass spectra were recorded on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) with an electrospray ion source in positive mode, resolution R = 60000 at m/z 400 (mass range m/z = 150−2000). Reactions were routinely monitored with TLC using Merck silica gel F_{254} plates. Microwave reactions were performed in an Emrys optimizer (Biotage AB). Yields were not optimized. The final products were purified by column chromatography and/or by recrystallization.

General Procedure for the Synthesis of Pyrazolo[1,5-*a*]pyrimidinecarboxamides (20−31). To a solution of the appropriate pyrazolo[1,5-*a*]pyrimidine-6-carboxylic acid (**15**−**19**; see Supporting Information) (1.0 equiv) and the substituted amine (1.2 equiv) in DCM (20 mL per mmol) was added EDC·HCl (1.2 equiv) at room temperature. After 4 h the mixture was concentrated and purified by column chromatography.

3-(4-Isopropylphenyl)7-methyl-*N*-(2-phenoxyethyl)-pyrazolo[1,5-*a*]pyrimidine-6-carboxamide (24). The synthesis started from acid **15** (0.33 mmol) and 2-phenoxyethylamine to give 90 mg (64%) as a yellow solid. ^{1}H NMR ($CDCl_3$): δ 8.48 (s, 1H), 8.14 (s, 1H), 7.88 (d, J = 8.4 Hz, 2H), 7.29 (d, J = 11.2 Hz, 2H), 7.25 (d, J = 8.0 Hz, 2H), 6.95 (t, J = 7.6 Hz, 1H), 6.87 (d, J = 8.0 Hz, 2H), 6.76 (t, J = 5.6 Hz, 1H), 4.22 (t, J = 5.2 Hz, 2H), 3.82 (q, J = 5.2 Hz, 2H), 2.94 (m, 1H), 2.89 (s, 3H), 1.28 (d, J = 6.8 Hz, 6H). HRMS calcd for $[C_{25}H_{26}N_4O_2 + H]^+$ 415.212 85, found 415.212 74.

General Procedure for the Preparation of 3-(Aryl)-7-methyl-*N*-(2-phenoxyethyl)pyrazolo[1,5-*a*]pyrimidine-6-carboxamides via Suzuki Coupling (32−43). The synthesis was done according to a modified procedure of Berger.[15] A mixture of **31** (1.0 equiv), the substituted phenylboronic acid (2.0 equiv), tetrakis-(triphenylphosphine)palladium(0) (0.03 equiv), and sodium carbonate (3.0 equiv) in toluene (3.0 mL) and H_2O (0.5 mL) was heated in the microwave for 2 h at 150 °C. Water was added, and the organics were extracted with DCM. The organic layer was dried, concentrated, and purified by column chromatography (1% MeOH/DCM). Final products were obtained by recrystallization from MeOH.

3-(4-Chlorophenyl)-7-methyl-*N*-(2-phenoxyethyl)pyrazolo-[1,5-*a*]pyrimidine-6-carboxamide (40). The synthesis started from **31** (0.33 mmol) and (4-chlorophenyl)boronic acid (0.66 mmol). Yield, 82 mg (62%). ^{1}H NMR ($CDCl_3$): δ 8.62 (s, 1H), 8.50 (s, 1H), 7.89 (d, J = 8.4 Hz, 2H), 7.42 (d, J = 8.4 Hz, 2H), 7.31 (t, J = 8.0 Hz, 2H), 7.00 (t, J = 7.6 Hz, 1H), 6.92 (d, J = 8.4 Hz, 2H), 6.42 (s, 1H, NH), 4.21 (t, J = 5.2 Hz, 2H), 3.94 (q, J = 5.6 Hz, 2H), 3.03 (s, 3H). HRMS calcd for $[C_{22}H_{19}ClN_4O_2 + H]^+$ 407.126 93, found 407.126 79.

Biology. [^{35}S]GTPγS Binding Assay. This assay was performed in 96-well format in 50 mM Tris-HCl, 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl, pH 7.4 at 25 °C, with 1 mM DTT, 0.5% BSA, and 50 μg/mL saponin freshly added. HEK-HCA$_2$ membranes (5 μg of protein per well in 25 μL) were preincubated with 25 μL of 40 μM GDP, in absence or presence of test compound, and 25 μL of increasing concentrations of the orthosteric ligand, for 30 min at room temperature. Then 25 μL of [^{35}S]GTPγS was added (final concentration, 0.3 nM) and the mixture was incubated for 90 min at 25 °C with constant shaking. The incubation was terminated by filtration over GF/B filter plates on a FilterMate harvester (PerkinElmer). The filters were dried, and 25 μL of Microscint-20 (PerkinElmer) was added to each filter. After ≥3 h extraction the bound radioactivity was determined in a Wallac MicroBeta TriLux 1450 counter.

Data Analysis. Analysis of the results was performed using Prism 5.0 software (GraphPad Software Inc., La Jolla, CA). Nonlinear regression was used to determine IC_{50} values from competition binding curves. The Cheng−Prusoff equation[20] was then applied to calculate K_i values. [^{35}S]GTPγS curves were analyzed by nonlinear regression to obtain EC_{50} values.

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■ ASSOCIATED CONTENT

Supporting Information

Tables S1−S3, Figures S1 and S2, additional notes, synthesis and characterization of chemical compounds, and biological methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

NA, nicotinic acid; 3-OHB, 3-hydroxybutyrate; [^{35}S]GTPγS, ^{35}S-labeled guanosine 5′-*O*-[γ-thio]triphosphate; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; GDP, guanosine 5′-diphosphate; GPCR, G-protein-coupled receptor; HCA, hydroxycarboxylic acid receptor; HEK, human embryonic kidney cell; PAM, positive allosteric modulator (allosteric enhancer)

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