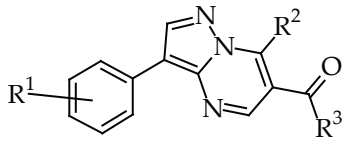
Chapter 5

Novel 3,6,7-substituted pyrazolopyrimidines as positive allosteric modulators for the hydroxy-carboxylic acid receptor 2 (GPR109A)

This chapter is based on Blad CC, van Veldhoven JP, Klopman C, Wolfram DR, Brussee J, Lane JR, IJzerman AP, J Med Chem **2012**; 55(7): 3563-7.

Supporting information: Tables S1-S3, figures S1 and S2, additional notes, and biological methods are included at the end of this chapter, and on http://pubs.acs.org supporting information on the synthesis and characterization of chemical compounds is also available.

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.

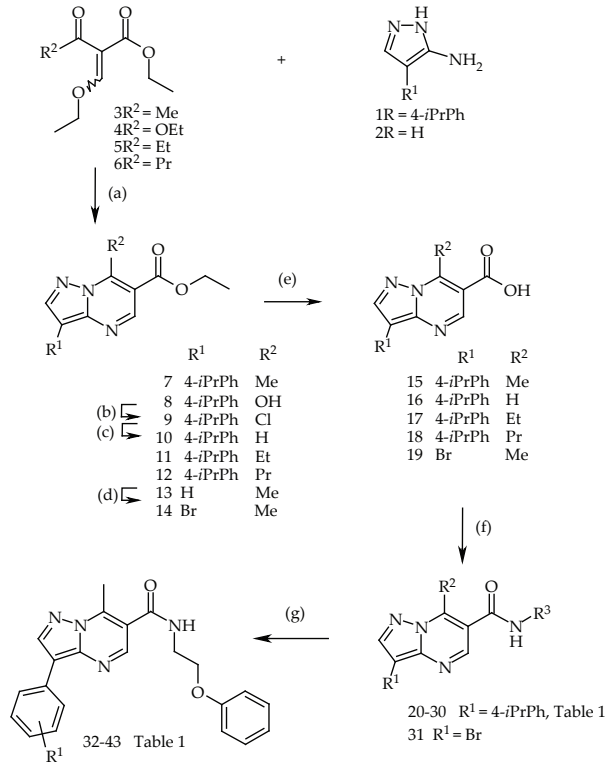
Abbreviations

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

Introduction

Hydroxy-Carboxylic 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 anti-lipolytic action of nicotinic acid (NA) was reported [3-5] and 3-hydroxybutyrate was identified as its endogenous ligand in 2005 [6] (for a review [1, 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 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

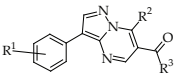


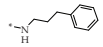
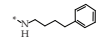
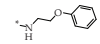
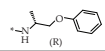
[a]Reagents and conditions: (a) EtOH, reflux, 3 h; (b) $POCl_3$, N,N-dimethylaniline, reflux, 3 h; (c) NaOAc, 5% Pd/C, H_2 2.5 bar, RT, 1 h; (d) NBS, DCM, 0 °C, 1.5 h, RT, 16 h; (e) LiOH, H_2O/MeOH/THF, RT, 16 h; (f) R^3-NH_2, EDC*HCl, DCM, RT, 4 h; (g) R^1-$B(OH)_2$, 31, $Pd(Ph_3P)_4$, Na_2CO_3, toluene/H_2O,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

Scheme 1. Synthetic route to the substituted pyrazolo[1,5-a]pyrimidine-carboxamides **20-43**[a]

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 compound **2** were 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]

Table 1. Different measures for HCA$_2$ receptor activation in [^{35}S]-GTPγS binding assays



Nr.	R^1	R^2	R^3	[^{35}S]- GTPγS binding		
				%E_{max} cpd. alone (SEM)[a]	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 of the test compound alone (100% is the E_{max} at 100 μM nicotinic acid); b) the shift in EC_{50} of nicotinic acid (NA) in the presence of 10 μM of the test compound (1 is without test compound and values less than unity indicate increased affinity of nicotinic acid, e.g. 0.1 means a 10-fold shift); c) the E_{max} reached at 100 μM nicotinic acid (NA) in the presence of 10 μM of the test compound (100% is without test compound); d) also reported by Shen et al [10] (**26**, racemate only). Values are means (± SEM) of 3 independent experiments performed in duplicate.

to give the pyrazolo[1,5-a]pyrimidine **10**. The 3-position of the pyrazolo[1,5-a]pyrimidine compound **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 resulted smoothly 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 of the test compound alone; b) the shift in EC_{50} value of nicotinic acid in the presence of 10 μM of the test compound; c) the E_{max} reached at 100 μM nicotinic acid in the presence of 10 μM of the test compound (100% is without test compound).

Structure-activity relationships

Compounds **20**-**23** show that linker lengths of 2 or 3 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 approximately 130% for all four compounds. Introduction of an ether function in the linker (**24**) resulted in a higher activity in the [^{35}S]-GTPγS binding assay. So, the compound acted as an agonist in its own right, while the EC_{50} value of nicotinic acid was increased approximately 25-fold without a change in its E_{max} value. 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 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} value 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, compounds **32**-**43**). A methyl substituent on the para position (**32**) resulted in more agonism and potentiation of nicotinic acid compared to methyl substituents on the *meta* (**33**) or *ortho* (**34**) positions. Compounds with ethyl (**35**) and *tert*-butyl (**36**) substituents had activities highly similar to the isopropyl-substituted compound **24**, except that **35** and **36** significantly increased

the E_{max} value 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 deminished activity. The 3,4-dichloro-substituted compound (**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 3 independent experiments are discussed below. Compound 24 reached 71±17% receptor

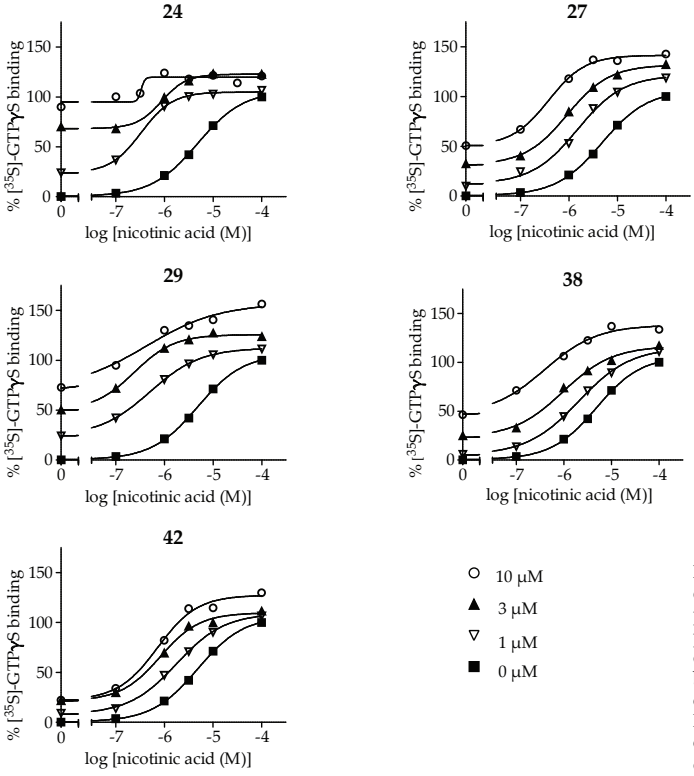


Figure 1. Dose-response curves of nicotinic acid in the presence of 0, 1, 3 and 10 μM pyrazolopyrimidines. The data are from [^{35}S]-GTPγS binding assays performed on HEK-HCA_2 membranes. Representative graphs from one experiment performed in duplicate.

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 μM and 1 μM, 24 increased [^{35}S]-GTPγS binding to 51±14% and 30±5% respectively, and caused shifts in the EC_{50} value of nicotinic acid of approximately 5-fold for both concentrations. Compound 27 caused modest increases in both the potency (2-fold at 1 μM, 5-fold at 3 μM and 7-fold at 10 μM) and the E_{max} values (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) values. 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} value 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, compound 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.

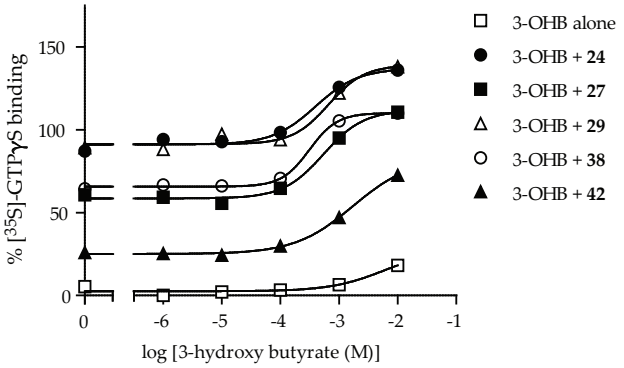


Figure 2. Concentration-response curves of 3-hydroxybutyrate in the presence and absence of 10 μM pyrazolopyrimidine in the presence and absence of 10 μM pyrazolopyrimidine. The data are from [^{35}S]-GTPγS binding assays performed on HEK-HCA_2 membranes. Representative graphs from one experiment performed in duplicate (see also Table S3 in SI).

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 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. It should be noted that 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 approx. 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 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 ≥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, B: 9 MeCN/1 H_2O. High resolution mass spectra were recorded on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) with an electro spray ion source in positive mode resolution R = 60000 at m/z 400 (mass range m/z = 150-2000). Reactions were routinely monitored on 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]pyrimidine carboxamides (20-31)

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

3-(4-Isopropylphenyl)7-methyl-N-(2-phenoxyethyl)pyrazolo[1,5-a] pyrimidine-6-carboxamide (24).

Started from acid 15 (0.33 mmol) and 2-phenoxy-ethylamine 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 $[C_{25}H_{26}N_4O_2+H]^+$ 415.21285, found 415.21274.

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)

According to a modified procedure of Berger [16]. A mixture of 31 (1.0 eq.), the substituted-phenylboronic acid (2.0 eq.), tetrakis(triphenyl-phosphine)palladium(0) (0.03 eq.) and sodium carbonate (3.0 eq.) 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 (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**)*

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 $[C_{22}H_{19}ClN_4O_2+H]^+$ 407.12693, found 407.12679.

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 protein per well in 25 μL) were pre-incubated with 25 μL of 40 μM GDP, in absence or presence of test compound, and 25 μL increasing concentrations of the orthosteric ligand, for 30 min at room temperature. Then, 25 μL [^{35}S]-GTPγS was added (final concentration 0.3 nM) and the mixture was incubated 90 min at 25 °C with constant shaking. The incubation was terminated by filtration over GF/B filterplates on a FilterMate harvester (PerkinElmer). The filters were dried and 25 μL 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, USA). 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 analysed by nonlinear regression to obtain EC_{50} values.

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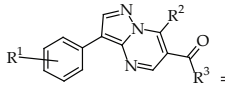
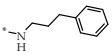
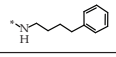
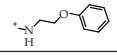
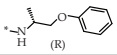
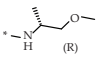
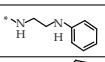
Supporting Information

Table S1. Effects on [^{3}H]-nicotinic acid binding of a series of 3-(aryl)-7-methyl-*N*-(substituted)pyrazolo[1,5-a] pyrimidine-6-carboxamides (measured at 10 μM).



Cpd.	R^1	R^2	R^3	% [^{3}H]-nicotinic acid binding (±SEM)	
				in equilibrium[a]	after 30 min dissociation[b]
20	4-*i*Pr	Me		96 (7.8)	91 (5.9)
21	4-*i*Pr	Me		108 (10.5)	114 (10.9)
22	4-*i*Pr	Me		111 (5.0)	99 (7.7)
23	4-*i*Pr	Me		101 (6.8)	112 (9.6)
24	4-*i*Pr	Me		149 (6.1)	133 (2.3)
25	4-*i*Pr	Me		109 (5.9)	92 (6.2)
26	4-*i*Pr	Me		69 (4.0)	118 (15.7)
27	4-*i*Pr	Me		107 (7.6)	115 (8.1)
28	4-*i*Pr	H		102 (3.4)	108 (18.7)
29	4-*i*Pr	Et		143 (3.2)	152 (1.4)
30	4-*i*Pr	Pr		148 (9.0)	136 (3.8)
32	4-Me	Me		114 (12.1)	130 (2.4)
33	3-Me	Me		117 (9.0)	145 (11.3)
34	2-Me	Me		111 (4.2)	113 (9.1)
35	4-Et	Me		147 (4.4)	148 (23.4)
36	4-*t*Bu	Me		142 (12.0)	147 (11.5)
37	4-OMe	Me		126 (6.4)	144 (14.3)
38	4-O*i*Pr	Me		143 (6.7)	162 (8.2)
39	4-Ph	Me		106 (3.5)	100 (2.0)
40	4-Cl	Me		108 (0.6)	96 (2.6)
41	3-Cl	Me		101 (4.4)	102 (5.3)
42	3,4 diCl	Me		96 (5.3)	101 (2.6)
43	4-CF_3	Me		119 (4.5)	103 (1.7)

a) the percentage of [^{3}H]-nicotinic acid specifically bound to HEK293-HCA_2 membranes at equilibrium in the presence of 10 μM of the test compound (100% is without test compound).
b) the percentage of [^{3}H]-nicotinic acid still bound to HEK293-HCA_2 membranes after 30 minutes of dissociation at 15 °C induced by 10 μM of nicotinic acid in the presence of 10 μM of the test compound (100% is without test compound - values above 100% indicate a slower dissociation rate in the presence of the test compound). Values are means (± SEM) of 3 independent experiments performed in duplicate.

Modulation of [^{3}H]-nicotinic acid binding

In Figure S1-A the effect of 10 μM 24 on the full dissociation curve of the radioligand is shown. The data indicate that in the presence of 24 the dissociation rate is unchanged ($t_{1/2}$ = 30 min for both curves) but that a plateau is reached at 40% specific binding in the presence of the modulator. The control curve reached a plateau at 10%.

For the compounds that markedly increased the binding of [^{3}H]-nicotinic acid in equilibrium assays (Table S1) we attempted to determine an EC_{50} of this effect. However, at 10 μM none of the curves had reached a plateau, and the solubility of the compounds did not permit an increase above this concentration. The curves for three selected compounds, 24, 29 and 38, are shown in Figure S1-B.

Correlation between potentiation of nicotinic acid and allosteric agonism

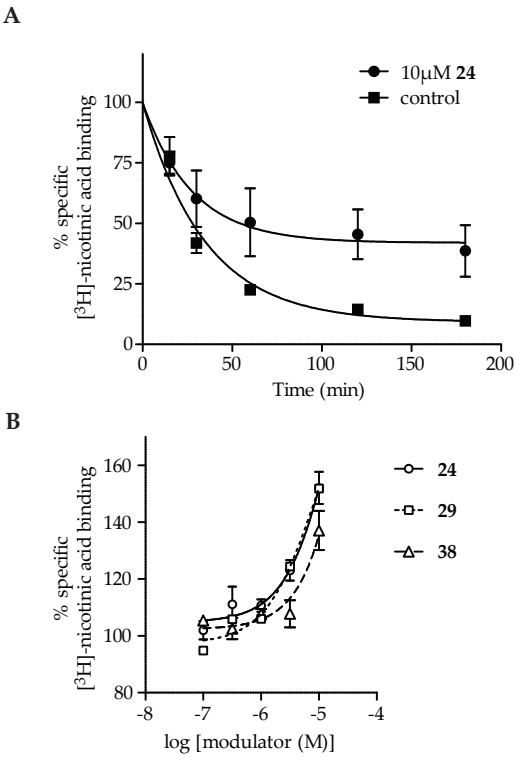


Figure S1. A. Dissociation curves with and without 10 μM **24** at 15 °C. After 3 hours association of 20 nM [^{3}H]-nicotinic acid to HEK-HCA_2 membranes, dissociation was started by addition of 10 μM nicotinic acid. The data points are means ± SEM for 3 independent experiments performed in duplicate. **B.** Binding curves (equilibrium) of compounds **24**, **29** and **38**. The assays were performed using 20 nM [^{3}H]-nicotinic acid and 50 μg HEK-HCA_2 membranes per assay point, and incubation was 1h at 25 °C. The data points are means ± SEM of 3 independent experiments performed in duplicate.

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 (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.

The compounds at the bottom right of the curve are the most potent modulators and also the most active agonists. More to the left are compounds that are not potent or efficacious agonists, but still fairly active as modulators. However, it appears that a 3-fold or greater shift in nicotinic acid potency is always coupled to some agonist activity in this series. Closest to the origin of the graph is a cluster of compounds, 41, 33, 37 and 26. Replacing the 4-isopropyl by a 3-methyl (33), 3-chloro (41) or 4-methoxy (37) appears to decrease the agonist action significantly while preserving appreciable modulator strength. Removal of the phenyl from the 6-position substituent (R^3) followed by methylation (26) seems to have a similar effect.

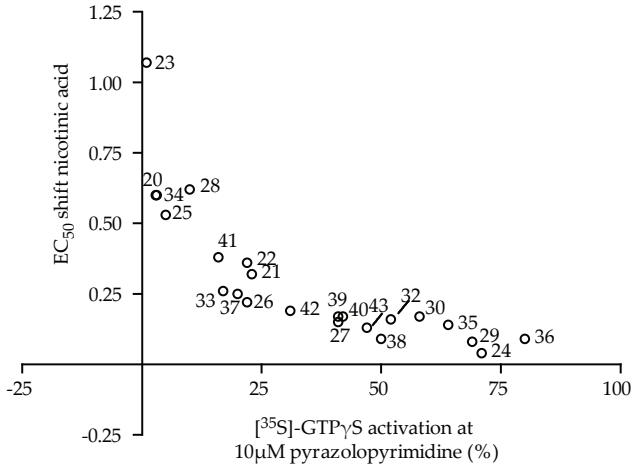


Figure S2. Correlation between potency enhancement of nicotinic acid and agonistic activity of the pyrazolopyrimidines on HCA_2

Modulation of the intrinsic efficacy of nicotinic acid

As described above, many of the active pyrazolopyrimidines are allosteric agonists for HCA_2. In the concentration-effect curves of nicotinic acid with the modulator, this was observed as an increase in the basal receptor activation level. At the same time, many of the same compounds also increased the maximal [^{35}S]-GTPγS binding. In most cases, nicotinic acid still caused an increase in [^{35}S]-GTPγS binding of 100%, identical to the range of the curve in absence of a modulator and typical of a full agonist. However, there were a few cases where the allosteric modulator changed the intrinsic efficacy of nicotinic acid. Thus, in the presence of compounds 24, 32, 36, and, to a lesser extent, 26 and 29, nicotinic acid appeared to be a partial agonist (Table 1). For 36 and 29 this may be due to their high agonist activity and the apparent maximum in E_{max} at approximately 150%, but for the other derivatives this postulated E_{max} ceiling was not reached. For example, in the presence of 10 μM 24, the basal receptor activation was on average 71% and the E_{max} was 109%, which means nicotinic acid only caused a 38% increase in [^{35}S]-GTPγS binding (see Table 1 and Figure 2). At 3 μM 24, nicotinic acid still behaved as a partial agonist with the concentration-effect curve ranging from 51% to 107%, on average.

The lowest concentration of 1 μM 24 had a negligible effect on the intrinsic efficacy of

nicotinic acid: here, the basal [^{35}S]-GTPγS binding was 30% and the maximum was 120%. Compounds 20 and 23 had the opposite effect and made nicotinic acid behave as a superagonist. The effect was highly similar for the two allosteric ligands: in the presence of 10 μM modulator, the basal [^{35}S]-GTPγS binding was unchanged, whereas the E_{max} was increased to approximately 130% (see Table 1 and 2). Thus, the modulators increased the intrinsic efficacy of nicotinic acid by 30%. These enhancers were not tested at lower concentrations.

Pyrazolopyrimidines as allosteric agonists

Further characterization of the most active allosteric agonists in concentration-effect curves (in absence of nicotinic acid) revealed that 24, 35 and 36 have a similar potency to nicotinic acid on HCA_2 (Table S2). The other compounds all seem to have a 2- to 3-fold lower potency, although the difference is not significant. All pyrazolopyrimidines are partial agonists; the most efficacious derivative, 24, has an intrinsic efficacy close to 90%. None of the pyrazolopyrimidines increased [^{35}S]-GTPγS binding in non-transfected HEK cells (data not shown).

Table S2. Agonism of the pyrazolopyrimidines in [^{35}S]-GTPγS assays on HEK-HCA_2 membranes. The values are means (SEM) of 3 independent experiments performed in duplicate.

	EC_{50} (μM) (SEM)	% EC_{max} (SEM)
nicotinic acid	4.7 (0.5)	100
24	3.0 (0.6)	87 (8)
29	9.0 (6.8)	75 (3)
30	9.2 (6.7)	70 (2)
32	9.7 (6.5)	65 (6)
35	3.8 (1.3)	72 (5)
36	3.0 (0.6)	70 (9)
38	9.9 (6.4)	64 (6)

Allosteric enhancement of the endogenous ligand 3-hydroxybutyrate

Table S3. Potency and intrinsic efficacy (basal activation by modulator minus E_{max}) of 3-hydroxybutyrate (3-OHB) in the presence and absence of 10 μM pyrazolopyrimidine. The data is from [^{35}S]-GTPγS binding assays performed on HEK-HCA_2 membranes. See also Figure 2. The values are means (SEM) of 3 independent experiments performed in duplicate.

	EC_{50} (μM) (SEM)	% efficacy (SEM)
3-OHB alone	3.82 (2.04)	29 (3)
3-OHB+**24**	0.54 (0.14)	39 (6)
3-OHB+**27**	0.62 (0.05)	46 (4)
3-OHB+**29**	0.51 (0.15)	36 (6)
3-OHB+**38**	0.55 (0.16)	51 (8)
3-OHB+**42**	2.58 (1.40)	69 (10)

[^{3}H]-Nicotinic acid (60 Ci/mmol) was purchased from BioTrend (Koeln, Germany). [^{35}S]-GTPγS (1250 Ci/mmol) was purchased from Perkin Elmer (Waltham, MA).

Cell culture and membrane preparation

Human embryonic kidney (HEK) 293T cells stably expressing human HCA_2 were cultured in DMEM supplemented with 10% newborn bovine serum, 0.4 mg/mL G418, 50 IU/mL penicillin and 50 μg/mL streptomycin. The cells were harvested by scraping in cold PBS, centrifuged at 1000g for 10 min and resuspended in cold 50 mM Tris-HCl buffer, pH 7.4. Then a DIAX 900 electrical homogenizer (Heidolph, Schwabach, Germany) was used for 15 sec to obtain cell lysis. The suspension was centrifuged at 100 000g for 20 min at 4 °C and the supernatant was discarded. The pellet was resuspended in Tris-HCl, and the homogenization and centrifugation steps were repeated. The membranes were resuspended in cold assay buffer (50 mM Tris HCl, 1 mM $MgCl_2$, pH 7.4) and the protein content was determined using a BCA assay (Thermo Scientific, Waltham, USA). During membrane preparation the suspension was kept on ice at all times. Membrane aliquots were stored at -80 °C until the day of the assay.

[^{3}H]-nicotinic acid binding assays

Competition assays

Membranes of the HEK293T-HCA_2 cell line (50 μg protein per tube) were incubated 1 hour at 25 °C with 20 nM [^{3}H]-nicotinic acid and increasing concentrations of the test compound in assay buffer (50 mM Tris HCl, 1 mM $MgCl_2$, pH 7.4). The total assay volume was 100 μL. To assess the total binding a control without test compound was included. The non-specific binding was determined in the presence of 10 μM unlabeled nicotinic acid. Final DMSO concentration in all samples was ≤ 0.25%. The incubation was terminated by filtering over GF/B filters using a 24-sample harvester (Brandel, Gaithersburg, USA). The filters were washed 3 times with 2 mL cold buffer (50 mM Tris HCl, pH 7.4). Filters were transferred to counting vials and counted in a Perkin Elmer LSA Tri-Carb 2900TR counter after 2 h extraction in 3.5 ml Emulsifier Safe liquid scintillation cocktail (Perkin Elmer, Waltham, USA).

Dissociation assays

HEK-HCA_2 membranes (50 μg protein per tube) were incubated 3 h at 15 °C with 20 nM [^{3}H]-nicotinic acid in 50 mM Tris HCl, 1 mM $MgCl_2$, pH 7.4, in a total volume of 100 μL. Then, 5 μL nicotinic acid (final concentration 10 μM), in absence or presence of test compound (final concentration 10 μM) was added. In the single point screen dissociation was terminated after 30 min at 15 °C, and for full curves dissociation was measured at different time points. Non-specific binding was determined in the presence of 10 μM nicotinic acid, added prior to the membranes. Otherwise the assay was performed as the competition assay.