Bioorganic & Medicinal Chemistry Letters 22 (2012) 4148–4152

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Thieno[3,2-*b*]thiophene-2-carboxylic acid derivatives as GPR35 agonists

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ARTICLE INFO

Article history:
Received 21 February 2012
Revised 4 April 2012
Accepted 10 April 2012
Available online 21 April 2012

Keywords:
GPR35
Thieno[3,2-*b*]thiophene-2-carboxylic acid
Agonists

ABSTRACT

The optimization of a series of thieno[3,2-*b*]thiophene-2-carboxylic acid derivatives for agonist activity against the GPR35 is reported. Compounds were optimized to achieve β-arrestin-biased agonism for developing probe molecules that may be useful for elucidating the biology and physiology of GPR35. Compound **13** was identified to the most potent GPR35 agonist, and compounds **30** and **36** exhibited the highest efficacy to cause β-arrestin translocation.

GPR35 is an orphan G protein-coupled receptor (GPCR) implicated in inflammation, pain, cardiovascular diseases, and metabolic disorders.[1] Several known GPR35 agonists have been reported in literature; these include clinically used drugs. Cromolyn and dicumarol, the two anti-asthma drugs with unknown mechanism of action, were recently identified to be GPR35 agonists with moderate potency.[2] Bumetanide and furosemide, two loop diuretics used for treating cardiovascular disease, were also found to be GPR35 agonists.[3] Using label-free phenotypic profiling, we had found that GPR35 is a target of entacapone,[4] a catechol-*O*-methyl transferase inhibitor drug for the treatment of Parkinson's disease, as well as the anti-nociception niflumic acid.[5] We also discovered that certain abundant natural phytochemicals including myricetin, morin, and ellagic acid,[5] and gallic acid[6] are GPR35 agonists. Furthermore, GPR35 has been found to be up-regulated in several types of inflamed cells.[1,2,7] Therefore, these findings provide rationales for proposing activation of GPR35 as a therapeutic target with opportunity for development in certain diseases including inflammatory disorders.

Ligand-directed functional selectivity, or biased agonism, describes the ability of distinct ligands to differentially activate one of the vectorial pathways mediated through a receptor.[8] The biased agonism is often inferred from the relative potency and efficacy to modify one signaling molecule/event over another. As a result, many ligands may give rise to assay readout-dependent potency and efficacy, suggesting that the efficacy for many ligands is collateral.[9] Biased agonism, once thought to be an artifact of recombinant technology, has been now amply demonstrated to be a natural cellular control mechanism,[10,11] and is believed to have therapeutic impacts.[12] Previously, we had discovered several thieno[3,2-*b*]thiophene-2-carboxylic acid (TTAC) derivatives as GPR35 agonists.[13] These chemicals were originally designed and synthesized for non-linear optics and organic transistor applications. To expand pharmacological tools for GPR35, we synthesized a series of novel TTAC analogs, and characterized their agonist activity against the GPR35.

TTCA presents several positions for structure–activity relationship (SAR) modifications (Fig. 1). Since we have previously shown that its carboxylic acid group is critical for its agonist activity at the GPR35,[13] we decided to focus on analogs bearing modifications at other positions of TTCA.

Compounds **4**, **8**, **10**, **11**, **13** and **14** were synthesized from perbromothiophene as outlined in Scheme 1. The procedure commences with C-methylation of perbromothiophene using butyllithium (*n*-BuLi) and methyl benzesulfonate to produce **1**, the Friedel–Crafts acylation (C-acylation) with $AlCl_3$ and acetyl chloride (CS_2) to produce **2**, the ketone-based ring closure reaction with ethyl 2-mercaptoacetate using 18-crown-6 as the catalytic agent to produce intermediate **3**, and finally hydrolysis to produce **4**. Compound **8** was synthesized using lithiation followed by C-acylation of perbromothiophene, then the ketone-based ring closure, bromination with *N*-bromosuccinimide (NBS), and hydrolysis

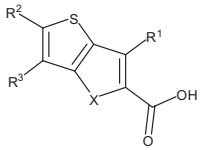

Figure 1. Structures of TTCA analogs. YE210: X = S, $R^1 = CH_3$, R^2 = H, R^3 = Br.

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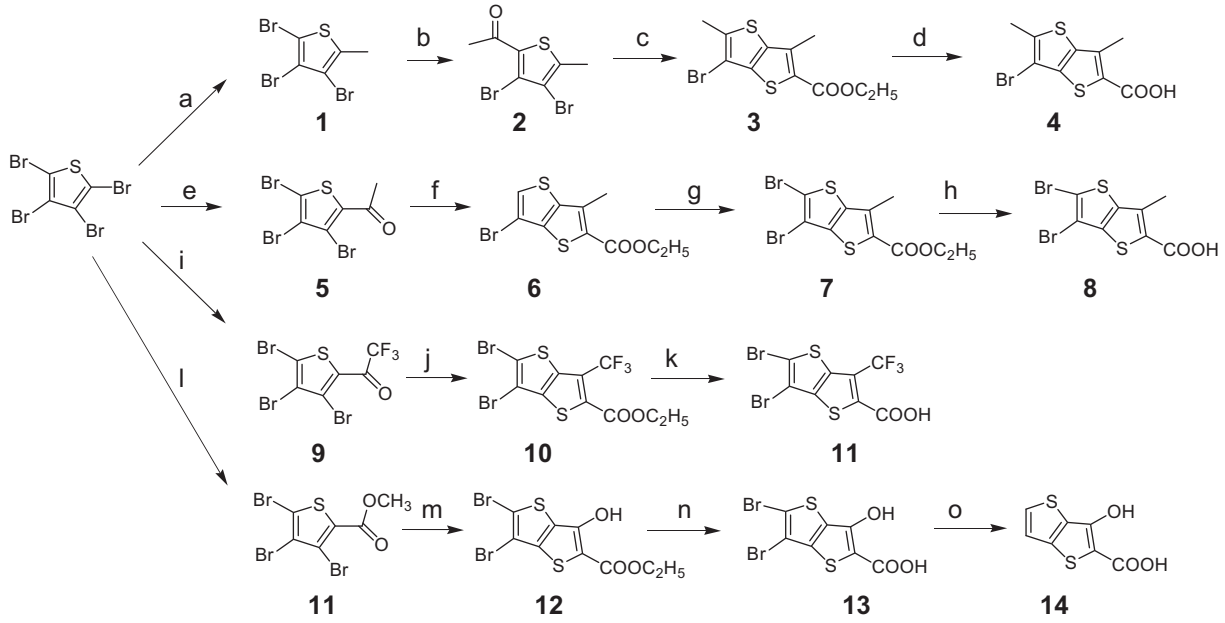
http://dx.doi.org/10.1016/j.bmcl.2012.04.057



Scheme 1. Synthesis of TTAC analogs. Reagents and conditions: (a) *n*-BuLi, THF, 0 °C; methyl benzenesulfonate, THF, rt, 95%; (b) $AlCl_3$, CS_2, 0 °C; AcCl, rt, 53%; (c) ethyl 2-mercaptoacetate, K_2CO_3, catalytic amount of 18-crown-6, DMF, 70 °C, 55%; (d) LiOH, EtOH/H_2O, 40 °C, 31%; (e) $AlCl_3$, CS_2, 0 °C; AcCl, 40 °C, 55%; (f) ethyl 2-mercaptoacetate, K_2CO_3, catalytic amount of 18-crown-6, DMF, 70 °C, 31%; (g) NBS, DMF, rt, 31%; (h) LiOH, EtOH/H_2O, 40 °C, 36%; (i) *n*-BuLi, THF, −78 °C; 2,2,2-trifluoro-*N*-methyoxy-*N*-methylacetamide, rt, 80%; (j) ethyl 2-mercaptoacetate, sodium bis(trimethylsily)amide, toluene/THF, rt, 50%; (k) LiOH, THF, 70 °C, 90%; (l) *n* BuLi, THF, −78 °C; ethyl carbonochloridate, H_2O, rt, 50%; (m) ethyl 2-mercaptoacetate, sodium bis(trimethylsily)amide, toluene/THF, rt, 50%; (n) LiOH, THF, 70 °C, 90%; (o) *n* BuLi, −78 °C, THF; 6 N HCl, 20%.

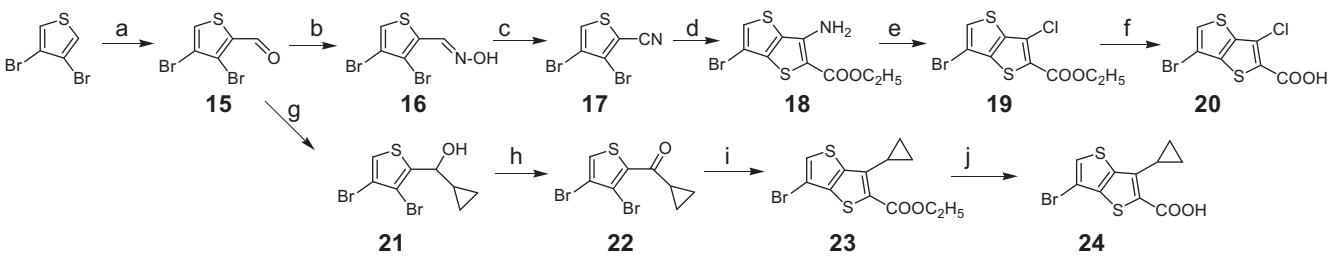
in a sequential manner. Compound **11** was synthesized using the introduction of trifluoroacetyl through C-acylation of perbromothiophene to produced **9**, the ketone-base ring closure to produce intermediate **10**, and then hydrolysis to produce **11**. Compound **13** was synthesized using the introduction of methoxycarbonyl through C-acylation to perbromothiophene, the ring closure, and then hydrolysis. Compound **14** was produced from **13** through debromination using *n*-BuLi and 6 N HCl.

Compounds **20** and **24** were synthesized according to Scheme 2. The synthesis of compound **20** begins with C-acylation of 3,4-dibromothiophene to produce aldehyde intermediate **15**, then aldehyde oxime formation to produce **16**, conversion of oxime to nitrile **17**, coupling followed by condensation to form intermediate **18**, diazonium salt formation followed by chlorination to form **19**, and hydrolysis to produce **20**. The synthesis of compound **24** was done using addition of Grignard reagents to aldehyde **15** to produce **21**, oxidization to form ketone **22**, the ring closure action to produce ester **23**, and hydrolysis to produce **24**.

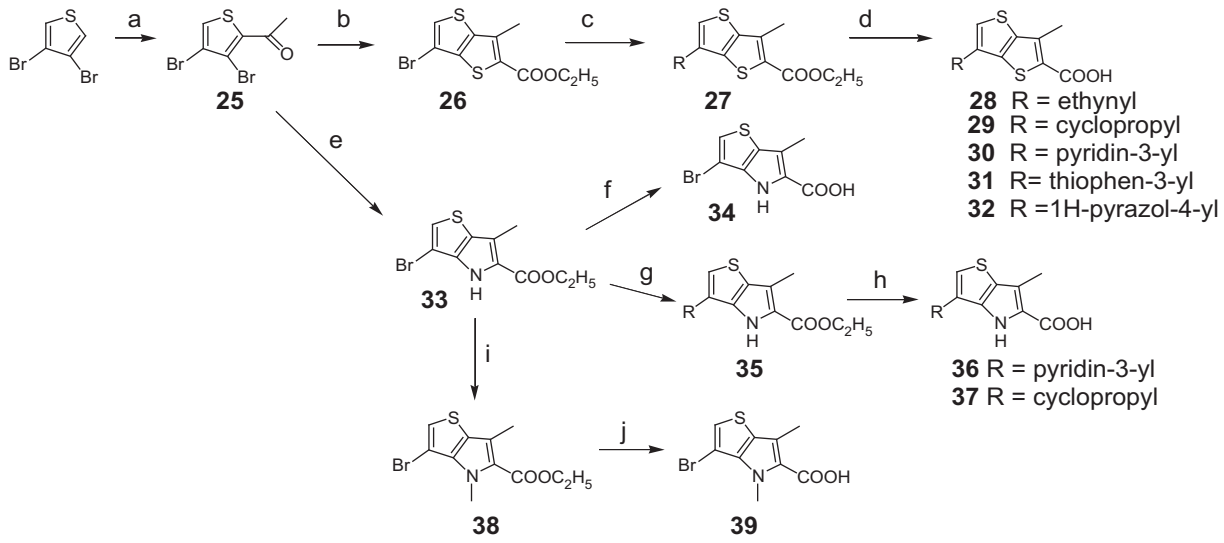
Compounds **28**–**32**, **34**, **36** and **37** were synthesized according to Scheme 3. The synthesis of compounds **28**–**32** commences with C-acylation of 3,4-dibromothiophene to the form ketone **25**, the ring closure to form the ester **26**, Hiyama cross-coupling or Suzki cross-coupling to produce **28**, or **29** to **32**, respectively. Compound **34** was produced from ketone **25** through consensation/coupling/deformylation cascade process, and then hydrolysis. Compounds **36** and **37** were synthesized from the ester **33** through Suzuki cross-coupling and sequential hydrolysis. Compound **39** was synthesized from the ester **38** through N-methylation and sequential hydrolysis.

Except for **8**, **10**, **11**, **13** and **14** that were synthesized internally, the rest compounds were synthesized by BioDuro Co. (Beijing, China), a CRO company. All internal synthesized compounds have purity greater than 98%, and the rest compounds with purity greater than 95%.

We employed both label-free dynamic mass redistribution (DMR)[14,15] and Tango β-arrestin translocation assays[16] to characterize the pharmacological activity of TTAC analogs at GPR35. DMR assay is based on whole cell phenotypic responses,[14] while Tango is an endpoint measurement of gene reporter activity linked to the GPR35 activation-mediated β-arrestin translocation.[4,5] The



Scheme 2. Synthesis of TTAC analogs. Reagents and conditions: (a) LDA, THF, N_2, −78 °C; DMF, rt, 89.5%; (b) hydroxylamine HCl, $NaHCO_3$, EtOH/H_2O, rt, 90.2%; (c) acetic anhydride, 120 °C, 59.5%; (d) ethyl 2-mercaptoacetate, K_2CO_3, catalytic amount of 18-crown-6, DMF, 60 °C, 74%; (d) *tert*-butyl nitrite, CuCl, acetonitrile, 75 °C, 69.8%; (e) LiOH, EtOH/H_2O, 70 °C, 25%; (f) LDA, THF, −78 °C; DMF, −78 °C, 89.5%; (g) bromocyclopropane, magnesium, THF, N_2, 0 °C, 21.8%; (h) PCC, CH_2Cl_2, rt, 83.3%; (i) ethyl 2-mercaptoacetate, K_2CO_3, catalytic amount of 18-crown-6, DMF, 60 °C, 56%; (j) LiOH, EtOH/H_2O, 70 °C, 23%.



Scheme 3. Synthesis of TTAC analogs. Reagents and conditions: (a) $AlCl_3$, CH_2Cl_2, 0 °C; AcCl, 0 °C, 98%; (b) ethyl 2-mercaptoacetate, K_2CO_3, catalytic amount of 18-crown-6, DMF, 80 °C, 65%; (c) ethyltrimethylsilane, cyclopropylboronic acid, pyridin-3-ylboronic acid, thiophrn-3-ylboronic acid, or 1*H*-pyrazol-4-ylboronic acid for **28**–**32**, respectively, CuI, $Pd(dppf)_2Cl_3$, CH_2Cl_2, Et_3N, N_2, 100 °C, 31–74%; (d) LiOH, EtOH/H_2O, rt, 12–50%; (e) ethyl 2-isocyanoacetate, CuI, Cs_2CO_3, N_2, 80 °C, 52%; (f) LiOH, EtOH/H_2O, rt, 37%; (g) pyridin-3-ylboronic acid or cyclopropylboronic acid for **36** or **37**, respectively; K_2CO_3, $Pd(dppf)_2Cl_2$, dioxane/H_2O, N_2, 100 °C, 75–80%; (h) LiOH, EtOH/H_2O, rt to 40 °C, 24–35%; (i) NaH, THF, 0 °C; CH_3I (**30**), THF, rt, 70%; (j) LiOH, EtOH/H_2O, rt, 37%.

dose responses were performed in two independent measurements, each in duplicate. SAR analysis was carried out based on relative potency and efficacy of compounds tested.

First, we recorded the dose responses of all TTAC analogs in native HT-29 cells using DMR agonist assays. The real time DMR signals of representative compounds, **8** and **13**, are showed in Figure 2a and b, respectively. Compound **10** was inactive (Fig. 2c), consistent with our previous observation that the carboxylic acid group is essential for the agonist activity of TTAC analogs.[13] All remaining compounds led to a clear dose-dependent DMR whose characteristics were similar to YE210 (6-bromo-3-methylthieno[3,2-*b*]thiophene-2-carboxylic acid),[13] as well as other known GPR35 agonists including zaprinast, pamoic acid, and kynurenic acid.[4] These compounds were thus termed DMR active compounds. The dose responses were best fitted with a monophasic sigmoidal non-linear regression, leading to a single EC_{50} for each compound (Fig. 3c, Table 1). We further determined the specificity of a compound-induced DMR to GPR35 using DMR antagonist assay. The antagonist assay employed a known GPR35 antagonist, CID 2745687 (also known as SPB05142),[13,17] to examine its ability to inhibit a compound-induced DMR. Results showed that CID 2745687 dose-dependently blocked the DMR induced by all DMR-active compounds, when they were assayed at a fixed dose. Furthermore, the potency of CID 2745687 was found to be similar to block the DMR of these compounds (Table 1). The dose-dependent inhibition of representative compounds, YE210, **8** and **13**, by CID 2745687 were shown in Figure 2d. These results suggest that these DMR-active TTAC analogs are GPR35 agonists.

Second, we measured the ability of TTAC analogs to cause β-arrestin translocation using Tango assay in the engineered U2OS-GPR35-*bla* cell line. This cell line stably expresses two fusion proteins: human GPR35 linked to a Gal4-VP16 transcription factor via a TEV protease site, and β-arrestin/TEV protease fusion protein. The cell line also stably expresses the β-lactamase reporter gene under the control of a UAS response element. The activation of GPR35 by agonists leads to the recruitment of β-arrestin-TEV protease fusion protein to the activated GPR35, leading to cleavage of Gal4-VP16 transcription factor from the receptor by the protease. The transcription factor then translocates to the nucleus and activates the expression of β-lactamase. Such a β-arrestin translocation is often specific to the GPR35 activation, given that the test compound is not fluorescent.[5] All Tango assay results were reported after normalized to the maximal response of zaprinast within the same plate. Results showed that as expected, compound **10** was inactive. All DMR-active compounds were found to be also active in Tango assays. The dose responses were also best fitted with a monophasic

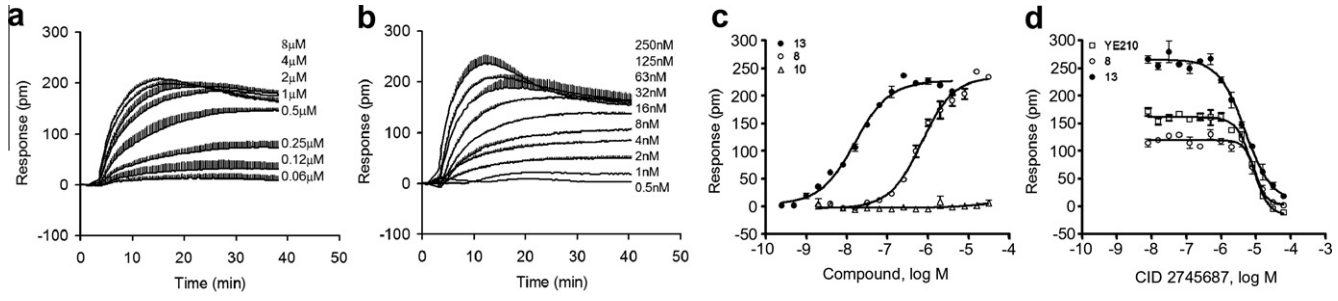


Figure 2. The dose responses of TTAC analogs. (a,b) Real time dose DMR responses of **8** and **13** in HT-29 cells, respectively. (c) The DMR amplitudes of **8**, **10**, and **13** as a function of their doses. (d) The dose-dependent inhibition of compound-induced DMR by CID 2745687. Compound concentration was 250 nM, 2 μM and 125 nM for YE210, **8** and **13**, respectively. The DMR amplitudes 10 min poststimulation in HT-29 cells were calculated for all. The data represents mean ± s.d. from two independent measurements, each in duplicate ($n = 4$).

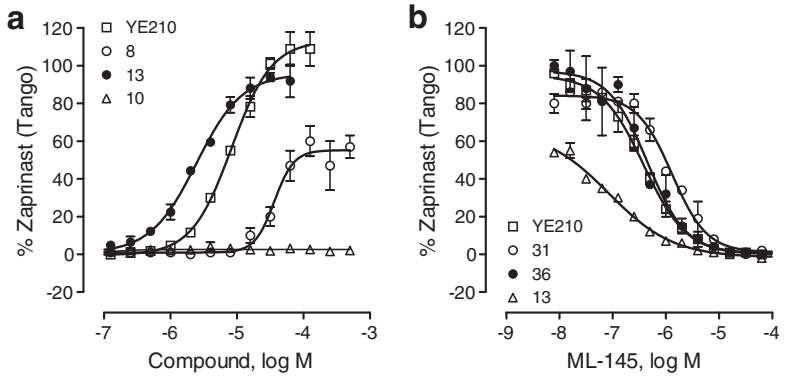


Figure 3. The dose-dependent responses of TTAC analogs obtained using Tango assays. (a) The dose responses of four TTAC analogs, YE210, **8**, **10** and **13**. (b) The CID 2745687 dose-dependent inhibition of the Tango signals induced by **13**, **31**, **36**, and YE210, each at a fixed dose. The compound dose was 10 μM, 100 μM, 64 μM, and 20 μM for compound **13**, **31**, **36**, and YE210, respectively. The data represents mean ± s.d. from two independent measurements, each in duplicate ($n = 4$).

Table 1
Compounds, their EC_{50} and efficacy (that is, the maximal responses) as measured using DMR and Tango assays, and the IC_{50} of CID 2745687 to block the DMR of each compound at a fixed dose*

Compound	EC_{50} (μM)		IC_{50} (μM) antagonist**	Efficacy	
	DMR	Tango		DMR (pm)	Tango (% zaprinast)
Kynurenic acid	152 ± 17	>500	4.41 ± 0.47	202	>60
Zaprinast	0.16 ± 0.02	6.2 ± 0.9	10.5 ± 0.3	269	100
1 (YE210)	0.061 ± 0.004	8.30 ± 0.6	8.06 ± 0.54	290	113
4	1.29 ± 0.11	22.2 ± 1.8	9.80 ± 0.71	268	52
8	0.70 ± 0.05	36.5 ± 3.8	9.61 ± 0.79	232	52
10	Inactive	Inactive			
11	0.51 ± 0.04	31.1 ± 2.5	4.18 ± 0.43	230	52
13	0.016 ± 0.002	2.46 ± 0.19	4.09 ± 0.31	228	96
14	9.78 ± 0.81	340 ± 29	4.77 ± 0.37	159	64
20	1.03 ± 0.09	39.2 ± 2.7	7.82 ± 0.56	262	68
24	2.63 ± 0.18	504 ± 41	13.6 ± 1.2	246	46
28	1.48 ± 0.09	54.8 ± 4.2	8.20 ± 0.72	257	102
29	0.95 ± 0.07	52.4 ± 5.1	8.92 ± 0.65	262	101
30	0.24 ± 0.02	11.8 ± 0.9	10.0 ± 0.9	223	118
31	0.26 ± 0.02	17.6 ± 1.2	8.70 ± 0.53	232	107
32	0.37 ± 0.02	52.5 ± 4.7	1.97 ± 0.12	180	102
34	11.8 ± 0.9	113 ± 12	9.33 ± 0.77	222	24
36	0.63 ± 0.04	26.9 ± 1.9	10.2 ± 1.0	210	120
37	8.37 ± 0.67	119 ± 21	8.45 ± 0.62	228	35
39	5.30 ± 0.42	87.5 ± 7.2	13.5 ± 1.1	290	77

* The data represents mean ± s.d. from two independent measurements ($n = 4$).
** The antagonist IC_{50} was obtained against a specific compound at a fixed dose: **1** (0.5 μM), **4** (8 μM), **8** (2 μM), **11** (2 μM), **13** (0.125 μM), **14** (32 μM), **20** (4 μM), **24** (16 μM), **28** (4 μM), **29** (2 μM), **30** (2 μM), **31** (2 μM), **32** (2 μM), **34** (32 μM), **36** (2 μM), **37** (32 μM), **39** (32 μM).

sigmoidal non-linear regression, leading to a single EC_{50} for each compound (Fig. 3a, Table 1). Further, Tango antagonist assay showed that ML-145 dose-dependently blocked the Tango signals induced by compounds **13**, **31**, **36** or YE210, leading to an IC_{50} of 0.085 ± 0.007 μM, 1.30 ± 0.10 μM, 0.45 ± 0.03 μM, and 0.38 ± 0.03 μM, respectively (two independent measurements, $n = 4$) (Fig. 3b). ML-145 is a potent GPR35 agonist.[18] These assay results further confirmed that all DMR-active compounds were GPR35 agonists.

Third, we performed correlation analysis between the results obtained using DMR agonist assay in HT-29 and Tango assay in the engineered U2OS cells. First, we compared their relative efficacy, the maximal responses after normalized to the maximal responses of zaprinast obtained using both DMR and Tango assays. Results showed that TTAC analogs displayed assay readout-dependent efficacy (Fig. 4a). These compounds can be classified to three categories using zaprinast as the reference compound. The first group includes compounds **11**, **14**, **28** and **29** that gave rise to comparable efficacy between the two assay readouts. The second group includes compounds such as **30**, **32** and **36** that exhibited higher efficacy to cause β-arrestin translocation in the engineered cells than those to result in DMR in the native HT-29 cells. The third group includes compounds such as **34** and **37** which exhibited higher efficacy to trigger DMR than those to induce Tango signal. Compounds **30** and **36** exhibited the highest efficacy to cause β-arrestin translocation.

Comparing their relative potency obtained using the two distinct assays showed that Tango assays generally gave rise to a right-shifted potency, compared to DMR assay results (Fig. 4b). Using YE210 as the reference, we found that most TTAC analogs led to a less right-shifted potency obtained using Tango assay. Compound **4** displayed a moderate potency but the least right-shifted potency, compared to YE210. Compound **13** was the most potent GPR35 agonist among all TTAC analogs tested.

Finally, we examined the ability of compound **30** to cause the internalization of endogenous GPR35 in HT-29 cells using a previously reported protocol.[13] Results showed that similar to zaprinast at 10 μM, compound **30** at 12.5 μM caused significant internalization of GPR35 in the native cell lines (Fig. 5). Together with DMR and Tango assays, this result further confirmed that compound **30** is a potent GPR35 agonist.

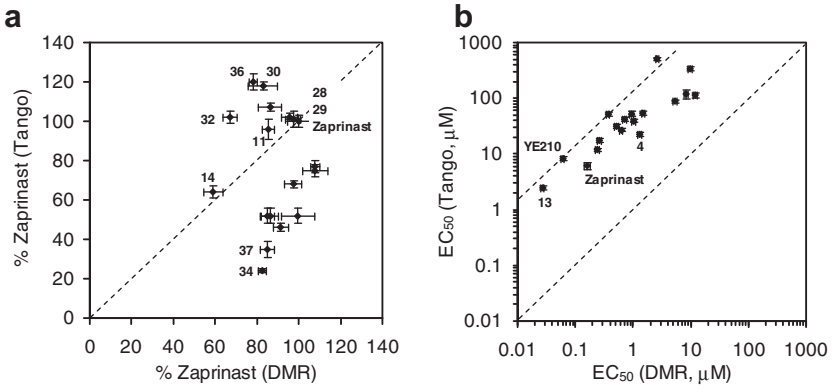


Figure 4. Correlation analyses between DMR and Tango assays. (a) The maximal responses of all agonists after normalized to the maximal response of zaprinast within the same plate. (b) The correlation plot between EC_{50} values of all agonists obtained using DMR and Tango assays. The data represents mean ± s.d. from two independent measurements, each in duplicate ($n = 4$).

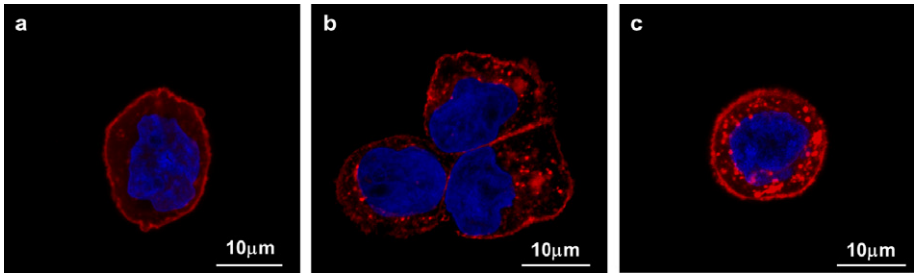


Figure 5. Compound **30** triggered GPR35 internalization in HT-29 cells. Representative confocal fluorescence images of HT-29 under different conditions: (a) treated with the assay vehicle containing 0.1% DMSO; (b) treated with 12.5 μM **30**; (c) treated with 10 μM zaprinast. The images were obtained after compound treatment for 1hr, permeabilized, stained with anti-GPR35, followed by fluorescent secondary antibody. Red: GPR35 stains; Blue: nuclei stains with DAPI. Representative images obtained from 2 independent measurements were used.

In summary, we synthesized a series of TTAC analogs and found that only compound **13** exhibited higher potency than YE210. However, a couple of analogs including **30** and **36** displays higher efficacy than zaprinast to cause β-arrestin translocation. Further, correlation analysis suggests that many ligands displayed biased agonism. Future exploration of SAR may lead to the discovery of TTAC analogs with further improved potency, and more importantly, compounds having β-arrestin biased agonism for GPR35. Considering the importance of β-arrestin in regulating a diverse array of cell signaling pathway downstream GPCRs, such a class of GPR35 agonists may open possibility to further elucidate the biology and physiology of GPR35.

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15. For DMR assays, human colorectal adenocarcinoma HT-29 cells (American Type Cell Culture, Manassas, VA, USA) were seeded at a density of 32,000 cells per well in Epic® 384well cell culture compatible microplates (Corning Inc., Corning, NY, USA), and cultured in McCoy's 5a Medium Modified supplemented with 10% fetal bovine serum, 4.5 g/liter glucose, 2 mM glutamine, and antibiotics at 37 °C under air/5% CO_2. After overnight culture, the confluent cells were washed twice and maintained in the assay buffer (1× HBSS). After incubated inside Epic® system (Corning Inc.) for 1 h, a 2-min baseline was established, compounds were then added using on-board liquid handler and cellular responses were then monitored in real time. For antagonist assays, the GPR35 antagonists used were pre-incubated for 10 min, and then the agonists examined were then added, and the agonist-induced responses were then monitored. All DMR signals were background corrected.
16. For Tango assays, Tango™ GPR35-bla U2OS cells (Invitrogen) were seeded at a density of 10,000 cells per well in 384-well, black-wall, clear bottom assay plates with low fluorescence background (Corning), and cultured in Dulbecco's modified eagle medium (Invitrogen) supplemented with 10% dialyzed fetal bovine serum, 0.1 μM non-essential amino acids, 25 μM Hepes (pH 7.3), 100 U/ml penicillin, and 100 μg/ml streptomycin. After overnight culture, the cells were stimulated with ligands for 5 h in a humidified 37 °C/5% CO_2, and then loaded with the cell permeable LiveBLAzer™ FRET B/G substrate. After the 2 h incubation the coumarin to fluorescein ratio was measured using Tecan Safire II microplate reader (Männedorf, Switzerland). The coumarin to fluorescein ratio was calculated, and all results obtained were then normalized to the zaprinast maximal responses using an intra-plate referencing protocol. The maximal response of zaprinast was set to be 100%.
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