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Multiple tyrosine metabolites are GPR35 agonists

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Both kynurenic acid and 2-acyl lysophosphatidic acid have been postulated to be the endogenous agonists of GPR35. However, controversy remains whether alternative endogenous agonists exist. The molecular targets accounted for many nongenomic actions of thyroid hormones are mostly unknown. Here we report the agonist activity of multiple tyrosine metabolites at the GPR35. Tyrosine metabolism intermediates that contain carboxylic acid and/or catechol functional groups were first selected. Whole cell dynamic mass redistribution (DMR) assays enabled by label-free optical biosensor were then used to characterize their agonist activity in native HT-29. Molecular assays including β-arrestin translocation, ERK phosphorylation and receptor internalization confirmed that GPR35 functions as a receptor for 5,6-dihydroxyindole-2-carboxylic acid, 3,3′,5′-triiodothyronine, 3,3′,5-triiodothyronine, gentisate, rosmarinate, and 3-nitrotyrosine. These results suggest that multiple tyrosine metabolites are alternative endogenous ligands of GPR35, and GPR35 may represent a druggable target for treating certain diseases associated with abnormality of tyrosine metabolism.

G-protein-coupled receptors (GPCRs) represent the largest family of cell surface receptors within the human genome, and are historically a rich source of drug targets for small molecule modulation and therapeutic intervention[1,2]. Orphan GPCRs whose natural ligands and functions are unknown represent potential new targets for drug discovery[3]. Identification of native ligands for these receptors is crucial to understand their physiological and pathophysiological functions. GPR35 was first identified to be a class A orphan GPCR consisting of 309 amino acids[4]. GPR35b, a splicing variant that contains an N-terminal extension of 31 amino acids, was later discovered in gastric cancer cells[5]. GPR35 is expressed in various tissues including stomach, gastrointestinal tissues, and several types of immune cells[6]. Up-regulation of GPR35 has been found in human mast cells upon stimulation with IgE antibodies[7], human macrophages treated with the environmental contaminant polycyclic aromatic hydrocarbon benzo[a]pyrene[8], failing heart cells[9], and gastric cancer cells[5]. Further, deletion of GPR35 gene may, at least partly, be responsible for phenotypic resemblance to Albright hereditary osteodystrophy, as well as for brachydactyly mental retardation syndrome[10].

Kynurenic acid, a tryptophan metabolite of the kynurenine pathway, has been postulated to be a natural agonist of GPR35[11]. However, controversy remains as to whether it reaches sufficient tissue concentrations to be the endogenous ligand[6]. 2-Acyl lysophosphatidic acid (2-acyl-LPA) was recently proposed as an alternative natural agonist of GPR35[12]. However, it still remains to be proven where 2-acyl-LPA is the endogenous agonist of GPR35, given that it is difficult to separate its action via GPR35 from those via other endogenous LPA receptors, multiple of which are often co-expressed in native cells[13]. Here, we set out to determine potential GPR35 agonist activity of tyrosine metabolites using dynamic mass redistribution (DMR) assay in conjunction with Tango β-arrestin assay. This work was motivated by (1) our recent discovery that certain tyrosine derivatives, tyrphostins including tyrphostin-51 and tyrphostin-25, are potent GPR35 agonists[14]; (2) the fact that many of tyrosine metabolites including catecholamines have been long recognized to be native ligands of several families of GPCRs[15]; and (3) the unknown molecular targets accounting for many nongenomic actions of thyroid hormones[16]. We were primarily focused on carboxylic acid- and/or catechol-containing tyrosine metabolism intermediates, based on our recent structure activity relationship analysis of several classes of novel GPR35 agonists[14,17]. DMR assays enabled by label-free optical biosensor are pathway-unbiased yet pathway-sensitive, permitting detecting the agonist activity of ligands with wide pathway coverage and high sensitivity[18–21]. The GPR35 Tango assay directly probes the agonist activity of ligands at the GPR35 by measuring alteration in gene expression directly linked to the GPR35 activation-mediated β-arrestin translocation[14,17]. We found that GPR35 functions as a receptor for several metabolites in tyrosine metabolism pathway including gentisate, 5,6-dihydroxyindole-2-carboxylic acid (DHICA), 3,3′,5′-triiodothyronine (reverse T3), 3,3′,5-triiodothyronine (T3), dihydrocaffeic acid and rosmarinate. 3-Nitrotyrosine, a product of tyrosine nitration, was also found to be a GPR35 agonist.

Results

Pharmacological characterization of know GPR35 agonists. We first characterized in detail the pharmacology of known GPR35 agonists including cromolyn disodium[7], kynurenic acid[11], zaprinast[22], pamoic acid[23], and niflumic acid[24], as well as known GPR35 antagonist SPB05142[23]. DMR agonist profiling using HT-29 cells showed that all known GPR35 agonists triggered a robust DMR signal in a dose dependent manner. At the saturating doses, all agonists led to a DMR sharing similar characteristics, suggesting that they activate the same target, GPR35. The dose response of zaprinast in HT-29 cells was showed in **Fig. 1a**. Conversely, SPB05142 up to 1 mM led to little or no DMR signal in HT-29 cells (**Fig. 1b**). The non-linear regression analysis, based on the maximal amplitude, led to a potency rank order of pamoic acid (EC$_{50}$, 2.1 nM) > zaprinast (0.14 μM) > cromolyn disodium (0.52 μM) > niflumic acid (1.11 μM) ≫ kynurenic acid (152 μM) (**Fig. 1c**, **Table 1**). The efficacy to trigger DMR was similar, but not identical, for these agonists, suggesting that these agonists were divergent in their functional selectivity.

Pharmacological profiling using Tango assays in U2OS-GPR35-*bla* cell line gave rise to almost identical potency rank order: pamoic acid (2.69 μM) > zaprinast (6.12 μM) > cromolyn disodium (9.67 μM) > niflumic acid (41.6 μM) ≫ kynurenic acid (> 500 μM) (**Fig. 1d**). The efficacy, based on the maximal response after normalized to the maximal response of zaprinast within the same plate, was found to be pamoic acid (108%) ~ zaprinast (100%) ~

cromolyn disodium (97%) > kynurenic acid (>60%) > niflumic acid (39%). As expected, SPB05142 was inactive in Tango assays.

Furthermore, SPB05142 was found to dose-dependently inhibit the DMR arising from 10 nM pamoic acid, 500 nM zaprinast, 2 μM cromolyn disodium, 8 μM niflumic acid, or 0.5 mM kynurenic acid, leading to an apparent IC$_{50}$ of 3.97±0.24 μM, 10.4±0.9 μM, 13.5±1.6 μM, 13.2±1.4 μM, and 4.41±0.47 μM (2 independent measurements, n = 4), respectively (**Table 1**). Collectively, these results suggest that these ligands are indeed GPR35 agonists, and their pharmacological profiles obtained are in line with what reported in literature.

Gentisate, an intermediate of tyrosine degradation pathway, is a GPR35 agonist. Phenylalanine is an essential amino acid and the precursor of tyrosine. Both amino acids share a common pathway of degradation which occurs in liver and ultimately leads to fumarate and acetoacetate, accounting for the glycogenic and ketogenic nature of both amino acids, respectively[25] (**Fig. 2a**). Since HT-29 was previously showed to endogenously express GPR35 at relatively high level[14,17], we first examined the DMR signals of several molecules in this pathway in HT-29 using DMR assays. Results showed that at 1 mM only gentisate resulted in a robust DMR signal, while homogentisate led to a much smaller DMR, and other molecules including L-phenylalanine, DL-phenylalanine, L-tyrosine, DL-tyrosine, 4-hydroxy-phenylpyruvate, 2-hydroxy-3-(4-hydroxy-phenyl)propenoate, and fumarate were inactive (**Fig. 2b, Table 1**).
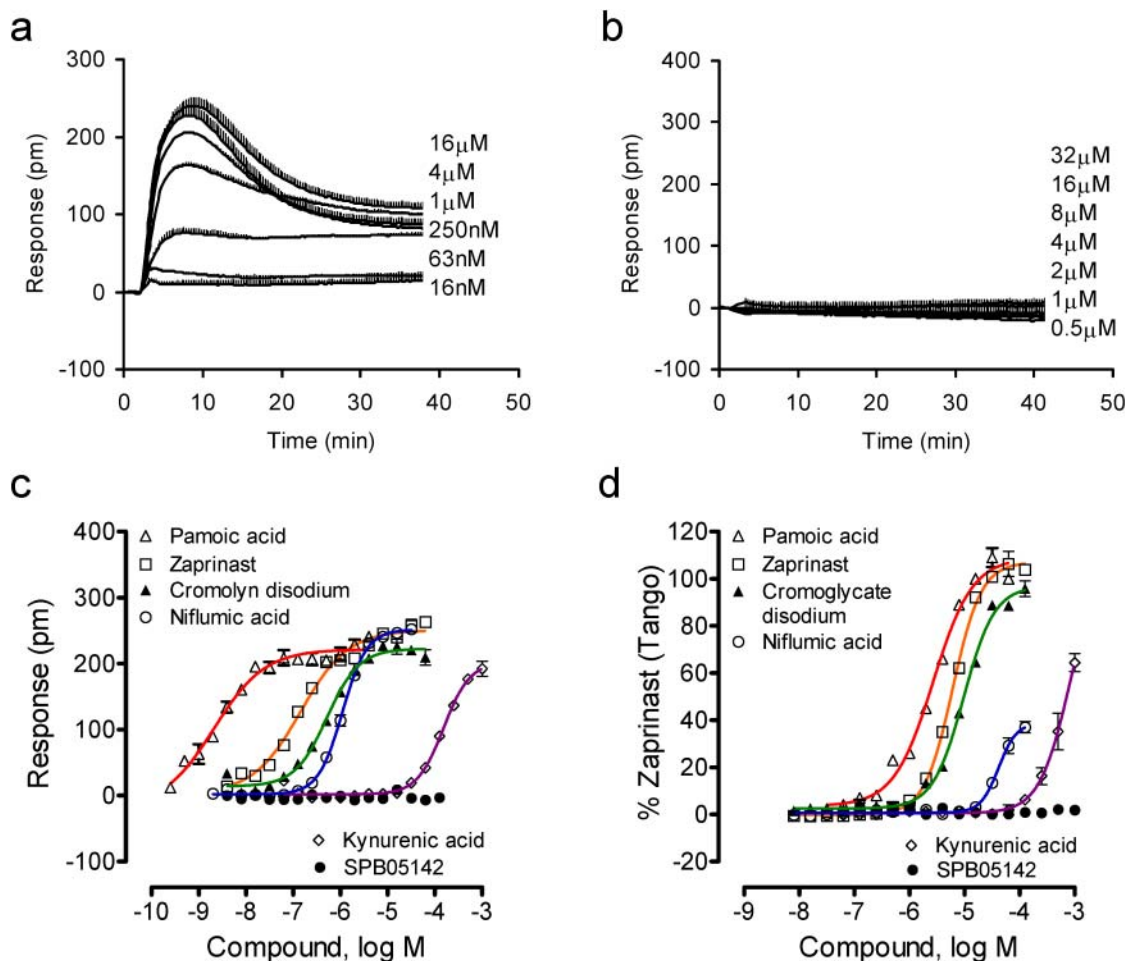

Figure 1 | Pharmacological characterization of known GPR35 agonists. (a) Real time DMR dose response of zaprinast. (b) Real time DMR dose response of SPB05142. (c) The DMR amplitudes of known GPR35 ligands as a function of their doses. (d) The Tango responses of known GPR35 ligands as a function of their doses. The Tango responses were normalized to the maximal response of zaprinast within the same plate. Except for zaprinast (8 independent measurements, each in duplicate, n = 16), all other data represents mean±s.d. from 2 independent measurements, each in duplicate (n=4).

Table 1 | Compounds and their pharmacological characteristics. The EC_{50} was obtained using DMR agonism and Tango assays, while the antagonist IC_{50} was for SPB05142 to block the DMR of each agonist at its EC_{80}, and their efficacy was the maximal responses of each ligand measured using DMR and Tango assays*

	EC_{50} (µM)		IC_{50} (µM)**	Efficacy	
Compound	DMR	Tango	Antagonist	DMR (pm)	Tango (% Zaprinast)
Zaprinast	0.14±0.01	6.12±0.51	10.4±0.9	250±17	100
Pamoic acid	0.0021±0.0002	2.69±0.21	3.97±0.24	222±14	108
Niflumic acid	1.11±0.09	41.6±3.9	13.2±1.4	223±11	39
Cromolyn disodium	0.52±0.04	9.67±0.71	13.5±1.6	252±16	97
Kynurenic acid	152±17	>500	4.41±0.47	203±13	>60
Homogentisate	>500	Inactive	No inhibition	Off-target	
Gentisate	69.6±7.1	>500	6.65±0.49	309±19	>20
L-DOPA	>500	Inactive	7.46±0.54	Off-target	
DHICA	24.2±2.7	22.9±1.6	9.17±0.70	310±11	75
Dihydrocaffeic acid	342±41	186±21	3.92±0.31	293±12	46
Rosmarinate	29.4±2.7	169±17	2.22±0.19	320±11	63
DOPAC	>500	106±7	4.60±0.31	>200	44
DHPG	239±37	563±67	1.97±0.15	310±12	18
3-iodo-L-tyrosine	>500	Inactive	3.62±0.0.27	>50	
3,5-Diiodo-L-tyrosine	304±45	Inactive	2.78±0.19	254±19	
T3	50±5	513±61	3.25±0.21	205±15	40
Reverse T3	5.89±0.39	108±9	2.84±0.23	276±12	108
L-Thyroxine (T4)	>500	>500	2.07±0.17	>100	>20
3-Nitro-L-tyrosine	142±27	>500	6.74±0.39	330±13	>20
L-Tyrosine	Inactive	Inactive			
DL-Tyrosine	Inactive	Inactive			
L-Phenylalanine	Inactive	Inactive			
DL-Phenylalanine	Inactive	Inactive			
4-hydroxy-pheylpyruvate	Inactive	Inactive			
α-methyl-L-DOPA	Inactive	Inactive			
4-Hydroxyphenylacetate	Inactive	Inactive			
Homovanillic acid	Inactive	Inactive			
Ethyl homovanillate	Inactive				
3-Methoxytyramine	Inactive	Inactive			
Sodium fumarate	Inactive	Inactive			

*The data represents mean±s.d. from 2 independent measurements (n=4).
**The IC_{50} was obtained for each agonist at a fixed dose: 10 nM pamoic acid, 500 nM zaprinast, 2 µM cromolyn disodium, 8 µM niflumic acid, 0.5 mM kynurenic acid, 0.5 mM gentisate, 1 mM L-DOPA, 64 µM DHICA, 0.5 mM dihydrocaffeic acid, 32 µM rosmarinate, 1 mM DOPAC, 1 mM DHPG, 1 mM 3-iodo-L-tyrosine, 1 mM 3,5-diiodotyrosine, 16 µM rT3, 50 µM T3, 1 mM T4, and 0.5 mM 3-nitro-L-tyrosine.

The DMR signal of gentisate was dose dependent (**Fig. 2c**), and exhibited characteristics almost identical to those of several known GPR35 agonists including zaprinast, kynurenic acid and tyrphostin-51 reported recently[14]. Nonlinear regression analysis revealed that gentisate gave rise to a monophasic dose response, which was best fitted by a single-phase sigmoidal curve, leading to a single EC_{50} of 69.6±7.1 µM (2 independent measurements, n=4) (**Fig. 2d**). The maximal DMR amplitude induced by gentisate at saturating doses was comparable to those of known GPR35 full agonists including zaprinast and tyrphostin-51[14], but was greater than that of kynurenic acid (**Table 1**). Furthermore, gentisate at high doses also resulted in detectable FRET signal arising from β-arrestin translocation via GPR35 in the engineered U2OS-GPR35-*bla* cells (**Fig. 2e**). Together, these results suggest that gentisate is a GPR35 agonist.

DHICA, an intermediate of melanin biosynthesis pathway, is a GPR35 agonist. Eumelanin is a common type of melanin pigment, which is synthesized from tyrosine *in vivo* through three chromophoric phases, the red pigment L-dopachrome, the purple intermediate melanochrome, and the broad absorption eumelanin[26]. L-dopachrome is converted into 5,6-dihydroxyindole via a spontaneous decarboxylation and into DHICA via an enzymatic tautomerization (**Fig. 3a**). We examined the ability of both L-DOPA and DHICA to trigger DMR signals in HT-29. Results showed that L-DOPA led to a dose-dependent DMR, but did not reach a saturated level up to 1 mM (**Fig. 3b**). In contrast, DHICA resulted in a clear dose-dependent and saturable DMR (**Fig. 3c**). Interestingly, the DMR of DHICA at saturating doses was distinct from those induced by gentisate, characterized by the lack of the early peak of the gentisate DMR. Nonlinear regression analysis of the dose response of DHICA, based on the DMR amplitudes at 50 min poststimulation, yielded a single EC_{50} of 24.2±2.7 µM (2 independent measurements, n=4) (**Fig. 3d**). Furthermore, DHICA also resulted in robust FRET signal arising from β-arrestin translocation in U2OS-GPR35-*bla* cells (**Fig. 3e**). The dose response of DHICA obtained using Tango assay was biphasic – at the low dose range the DHICA response was saturable, yielding an EC_{50} of 22.9±1.6 µM and a maximum of 75% of the zaprinast response (2 independent measurement, n =4); but at the two highest doses the FRET signal started to decrease. In contrast, L-DOPA up to 1 mM was inactive in Tango assays. Together, these results suggest that DHICA was a moderately potent GPR35 agonist.

Dihydrocaffeic acid, rosmarinate, and DHPG, intermediates of catecholamine degradation pathways, are GPR35 agonists. Catecholamines are catabolized to a series of metabolites through the sequential actions of catecholamine-O-methyltransferase and monoamine oxidase. Since several intermediates in catecholamine degradation pathways contain carboxylic acid and/or catechol groups (**Fig. 4a**), we were interested in the potential agonist activity of these intermediates at the GPR35. DMR agonism profiling showed that four metabolites including dihydrocaffeic
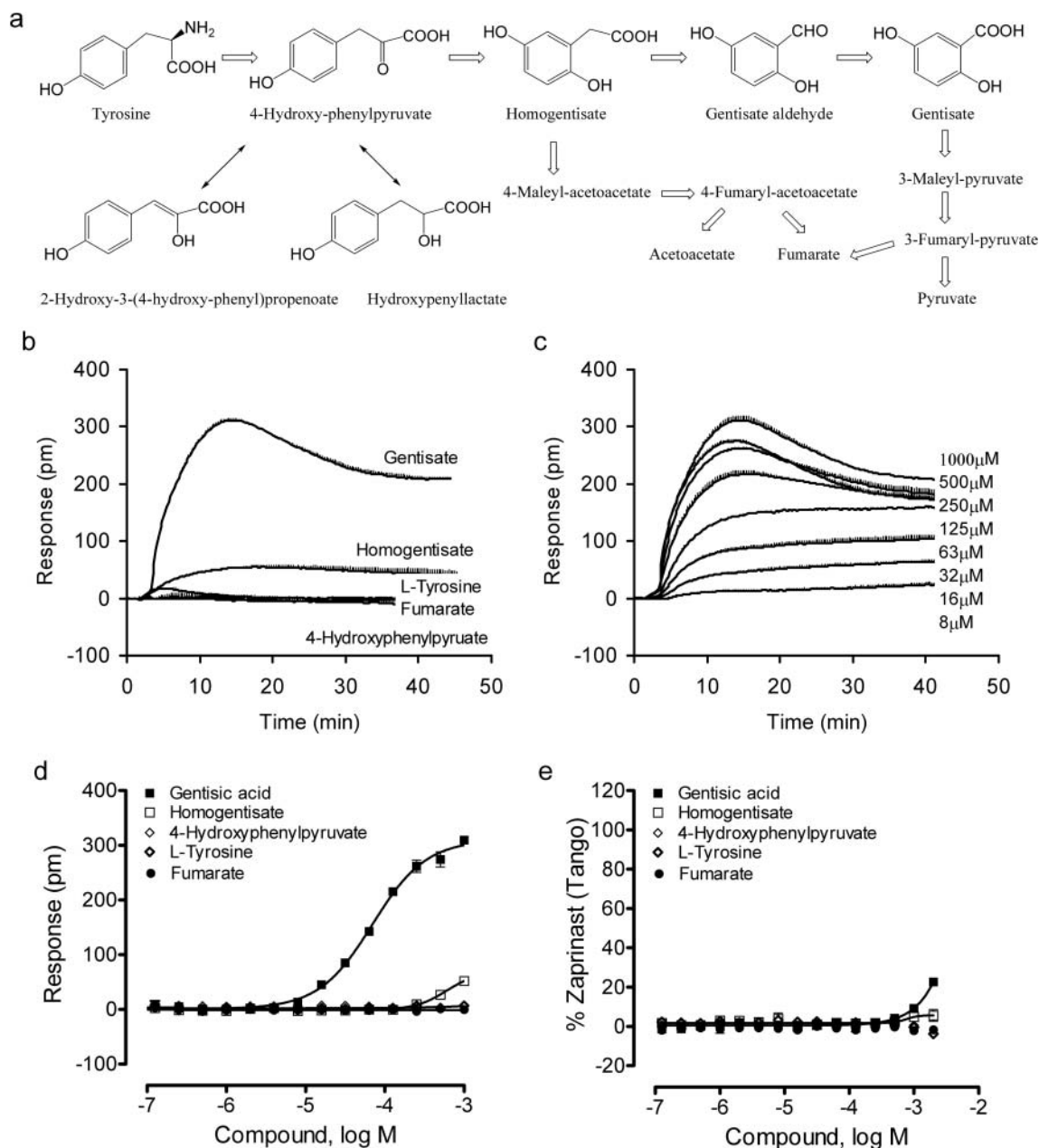
Figure 2 | Gentisate, a tyrosine degradation metabolite, is a GPR35 agonist. (a) Scheme showing the tyrosine degradation pathway. (b) The DMR signals of several metabolites, each at 1 mM, in HT-29 cells. (c) Real time DMR dose responses of gentisate in HT-29. (d) The maximal DMR amplitudes of different molecules as a function of their doses. (e) The β-arrestin translocation dose responses of different molecules. All data represents mean±s.d. from 2 independent measurements, each in duplicate (n=4).

acid, rosmarinate, DOPAC, and DHPG were active in HT-29 cells (Fig. 4b to e, respectively), but α-methyl-L-DOPA, homovanillic acid, ethyl homovanillate, and 3-methoxytyramine were inactive (Table 1). The DMR signals of the four DMR-active metabolites were similar to that of DHICA. Except for DOPAC which up to 1 mM did not saturate its DMR signal, the DMR response was saturable to the increasing doses of dihydrocaffeic acid, rosmarinate, or DHPG, leading to an EC_{50} of 342 ± 41 μM, 29.4 ± 2.7 μM, and 239 ± 37 μM (2 independent measurements, n=4), respectively (Fig. 4f). Furthermore, the four DMR-active metabolites were also able to trigger β-arrestin translocation in U2OS-GPR35-bla cells, with an EC_{50} of 186 ± 21 μM, 169 ± 17 μM, 106 ± 7 μM, and 563 ± 67 μM (2 independent measurements, n=4) for dihydrocaffeic acid, rosmarinate, DOPAC and DHPG, respectively (Fig. 4g). The efficacy was found to be in the order of rosmarinate > dihydrocaffeic acid ~

DOPAC > DHPG (Table 1). Together, these results suggest that these four DMR-active metabolites are GPR35 agonists.

Both T3 and reverse T3, intermediates of thyroid hormone synthesis pathway, are GPR35 agonists. Thyroid hormones including T4 and T3 are tyrosine-derived hormones produced by follicular cells of the thyroid gland and are primarily responsible for regulation of metabolism, growth and development[27]. As a storage hormone and the most abundant, T4 is produced through enzymatic cleavage of the precursor thyroglobulin. Appropriately 40% of T4 is converted to the most active hormone T3, and the remaining 60% of T4 goes to the inactive reverse T3 (Fig. 5a). DMR agonism profiling showed that all five thyroid-related molecules tested were able to trigger DMR in HT-29 cells. 3-Iodo-L-tyrosine at the highest dose examined (1 mM) led to a small but
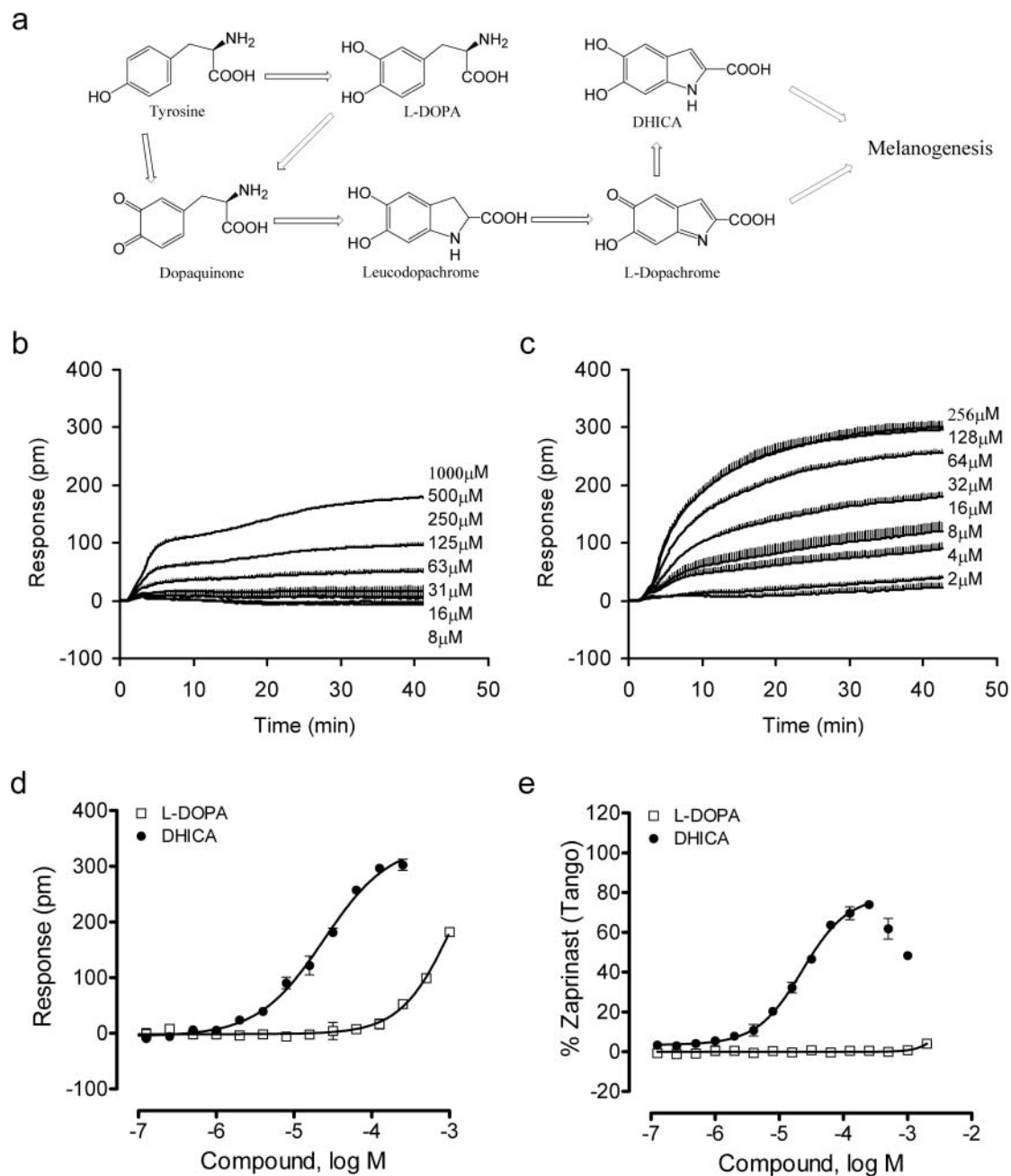
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Figure 3 | DHICA, an intermediate of melanin biosynthesis pathway, is a GPR35 agonist. (a) Scheme showing the melanin biosynthesis pathway. (b) Real time DMR dose responses of L-DOPA. (c) Real time DMR dose responses of DHICA. (d) The maximal DMR amplitudes of both molecules as a function of their doses. (e) The β-arrestin translocation dose responses of both molecules. All data represents mean±s.d. from 2 independent measurements, each in duplicate (n=4).

detectable DMR (**Fig. 5b**). 3,5-Diiodo-L-tyrosine, T3 and reverse T3 all led to a clear dose-dependent DMR (**Fig. 5c to e**), and T4 appeared to be less potent (**Fig. 5f**). The DMR of 3,5-diiodo-L-tyrosine, T3 or reverse T3 at its respective saturating doses exhibited characteristics that are intermediate between gentisate and DHICA. The potency rank order to trigger DMR in HT-29 cells was found to be reverse T3 (EC_{50}, 5.89±0.39 µM) > T3 (50±5 µM) > 3,5-diiodo-L-tyrosine (304±45 µM) (2 independent measurements, n=4) (**Fig. 5g**). Similarly, except for 3-iodo-L-tyrosine, all other four thyroid molecules were found to be active in causing β-arrestin translocation in U2OS-GPR35-*bla* cells (**Fig. 5h**). Remarkably, reverse T3 acted as a full agonist similar to zaprinast, while T3 was a partial agonist to result in β-arrestin translocation. Together, these results suggest that both reverse T3 and T3 are GPR35 agonists.

Nitrotyrosine, a product of tyrosine nitration, is a GPR35 agonist. Nitrotyrosine is a product of tyrosine nitration mediated by reactive nitrogen species including peroxynitrite anion and nitrogen dioxide, and is considered a biomarker of NO-dependent, reactive nitrogen species-induced nitrative stress[28]. DMR agonism profiling showed that 3-nitrotyrosine triggered a robust DMR signal in HT-29 cells, whose characteristics were almost identical to gentistate (**Fig. 6a**). Nonlinear regression analysis of the monophasic dose response of 3-nitro-L-tyrosine showed that it exhibited a relatively low potency with an EC_{50} of 142±27 µM (2 independent measurements, n=4) (**Fig. 6b**). Tango assays confirmed that 3-nitro-L-tyrosine was able to result in β-arrestin translocation in U2OS-GPR35-*bla* cells (**Fig. 6c**). Together, these results suggest that 3-nitro-L-tyrosine is a GPR35 agonist.
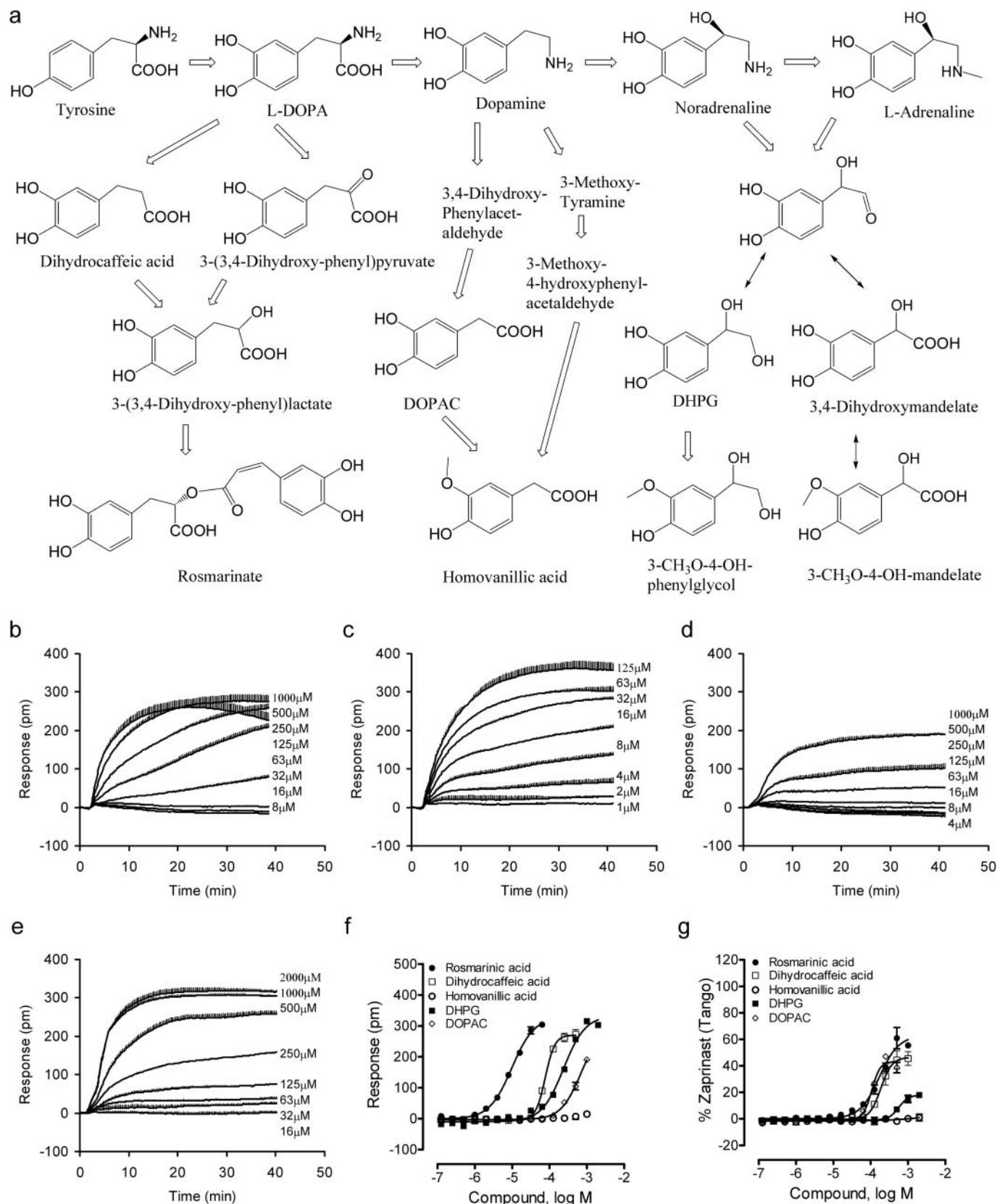
Figure 4 | Dihydrocaffeic acid, rosmarinate, DOPAC, and DHPG, four intermediates of L-DOPA and catecholamine degradation pathways, are GPR35 agonists. (a) Scheme showing L-DOPA and catecholamine degradation pathway. (b to e) Real time DMR dose responses of dihydrocaffeic acid (b), rosmarinate (c), DOPAC (d) and DHPG (e). (f) The maximal DMR amplitudes of different molecules as a function of their doses. (g) The β-arrestin translocation dose responses of different molecules. All data represents mean±s.d. from 2 independent measurements, each in duplicate (n=4).
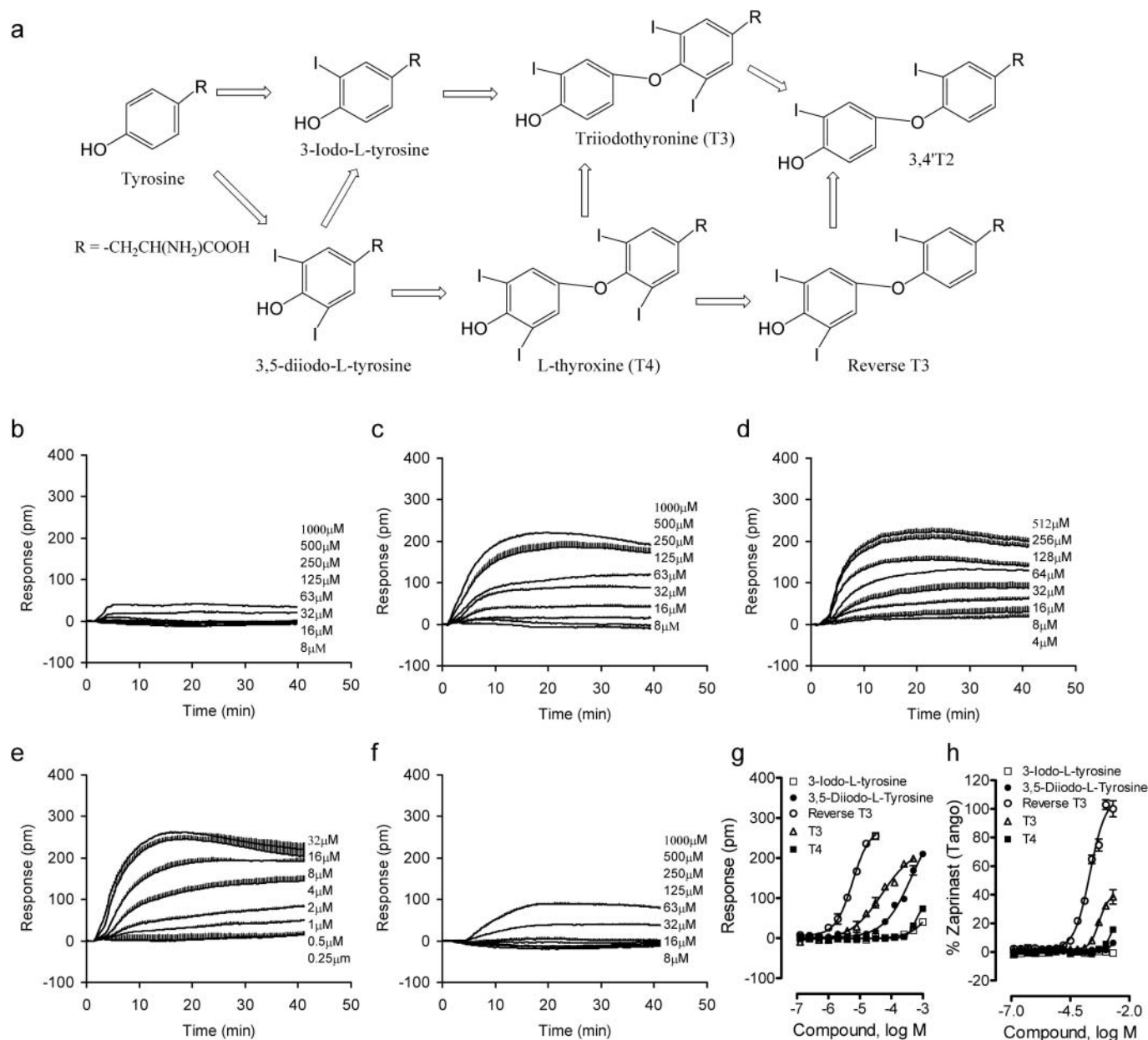
Figure 5 | Thyroid molecules are GPR35 agonists. (a) Scheme showing thyroid hormone biosynthesis pathway. (b to e) Real time DMR dose responses of 3-iodo-L-tyrosine (b), 3,5-diiodo-L-tyrosine (c), T3 (d), reverse T3 (e), and T4 (f). (g) The maximal DMR amplitudes of different molecules as a function of their doses. (h) The β-arrestin translocation dose responses of different molecules. All data represents mean±s.d. from 2 independent measurements, each in duplicate (n=4).

Agonist activity is specific to the activation of GPR35. Given that many compounds may exhibit polypharmacology and DMR measurement is integrative in nature, a compound-induced DMR may contain contributions from multiple targets/pathways which the compound activates[19]. Thus, we examined the specificity of the DMR signals of the twelve DMR-active compounds to the activation of GPR35 using DMR antagonist assays. Each compound was assayed at a dose close to its respective EC_{50} to EC_{80} in the presence of SPB05142. Results showed that except for DOPA whose DMR was partially inhibited, SPB05142 dose-dependently inhibited the DMR arising from all other eleven agonists in HT-29 cells, resulting in similar potency (**Fig. 7, Table 1**). It is worthy noting that for gentisate, dihydrocaffeic acid, rosmarinic acid, DOPAC and DHPG, there were residual DMR signal in the presence of 64 μM SPB05142. Nonetheless, these results suggest that the DMR of L-DOPA is partially due

to the activation of GPR35, and the DMR of all other ligands are mostly specific to the activation of GPR35.

Representative GPR35 agonists result in receptor internalization and ERK phosphorylation. Since GPR35 activation is known to cause receptor internalization, we further investigated representative ligands. Results showed that under unstimulated conditions GPR35 was primarily located at the cell surface (**Fig. 8a**). Stimulation of HT-29 with 50 μM gentisate, 2 mM L-DOPA, 50 μM DHICA, 500 μM DOPAC, 5 μM reverse T3, 200 μM T4, or 50 μM 3-nitrotyrosine all led to obvious internalization of GPR35 (**Fig. 8b** to **h**).

Since ERK phosphorylation is a hallmark of the activation and signaling of many GPCRs including GPR35[14,17,23], we examined the ability of representative ligands to result in ERK phosphorylation. Results showed that stimulation of HT-29 cells with all
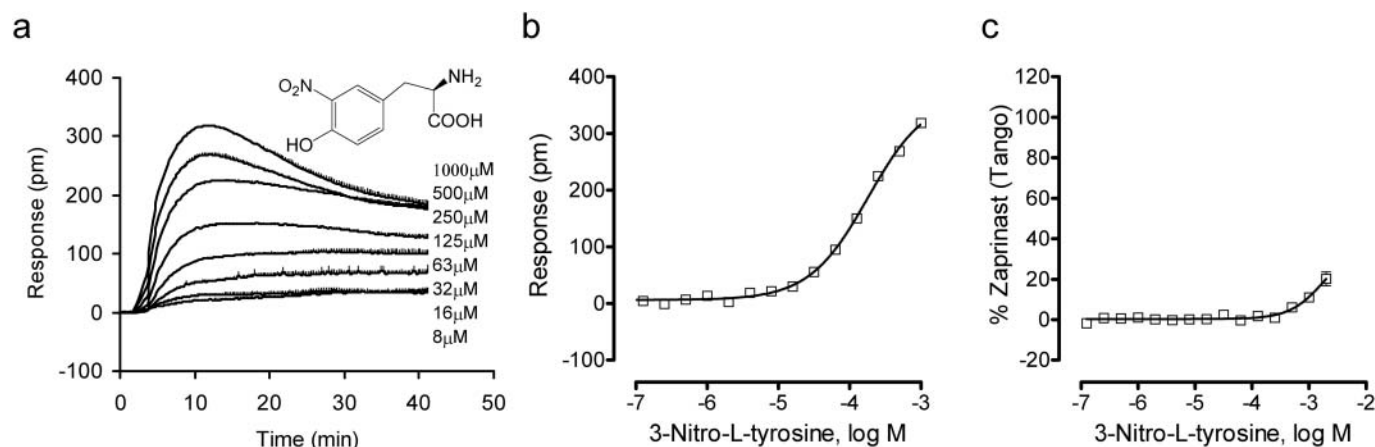
Figure 6 | 3-Nitro-L-tyrosine, a product of tyrosine nitration, is a GPR35 agonist. (a) Real time DMR dose responses of 3-nitrotyrosine and its structure. (b) The maximal DMR amplitudes of 3-nitrotyosine as a function of its doses. (c) The β-arrestin translocation dose response of 3-nitrotyrosine. All data represents mean±s.d. from 2 independent measurements, each in duplicate (n=4).

ligands tested led to robust phosphorylation of ERK (**Fig. 9**). Together, these results suggest that these tyrosine metabolites are GPR35 agonists.

Discussions

Three lines of evidence suggest the presence of alternative endogenous agonists for GPR35. First, we have found that certain tyrosine derivatives, tyrphostins including tyrphostin-51, tyrphostin-23, and tyrphostin-47, are GPR35 agonists with high to moderate potency[14]. Tyrphostins are a family of tyrosine analog compounds, and were originally synthesized as inhibitors of tyrosine kinases[29]. Second, Wang *et al.*[11] found that the tryptophan metabolite kynurenic acid is an agonist of GPR35, but controversy remains as to whether it is the endogenous ligand[6]. We had showed that kynurenic acid was indeed able to result in DMR in HT-29, and to trigger β-arrestin translocation in U2OS-GPR35-*bla* cells, but with quite low potency; its EC$_{50}$ was 152 μM and >500 μM, respectively (Table 1)[14]. Third, tyrosine is metabolized to a diverse array of molecules, many of which are natural agonists for distinct families of receptors. Catecholamines are a family of neurotransmitters including dopamine, norepinephrine and epinephrine. Dopamine is synthesized in neurons and the medulla of the adrenal glands, and is the natural agonist for the five dopamine receptors. Norepinephrine and epinephrine are synthesized in the sympathetic neurons affecting the heart, and are the natural agonists for adrenergic receptors consisting of nine members. L-DOPA, an intermediate in both catecholamines and melanin biosynthesis pathways, was recently discovered to be an agonist of GPR143[30]. Succinate, a product of amino acid catabolism pathway, was found to be an agonist for GPR91[31]. Last but not least, thyroid hormones have been long recognized to be potent agonists for thyroid hormone receptors, a family of four nuclear receptors[27]. Rapid and nongenomic actions of the thyroid hormone T4 have also been discovered[16], and are believed to mediate, at least partly, through a receptor on the integrin $\alpha_v\beta_3$[32].

DMR assay enabled by label-free resonant waveguide grating biosensors is phenotypic in nature, and permits discovery of many, if not all, modes of action of ligand molecules for a receptor[19,21,33]. Using DMR profiling, we found that several tyrosine metabolites including gentisate, DHICA, dihydrocaffeic acid, rosmarinate, DHPG, 3,5-diiodo-L-tyrosine, T3 and reverse T3 are GPR35 agonists but with distinct efficacy and potency. Free 3-nitrotyrosine was also a GPR35 agonist. The potency rank order was reverse T3 (EC$_{50}$, 5.89 μM) > DHICA (24.2 μM) ~ rosmarinate (29.4 μM) > T3 (50 μM) > gentisate (69.6 μM) > 3-nitro-L-tyrosine (142 μM) > DHPG (239 μM) > 3,5-diiodo-L-tyrosine (304 μM) ~ dihydrocaffeic acid

(342 μM); and among them reverse T3 was the most potent GPR35 agonist to result in DMR in HT-29 cells. Tango assays confirmed that all, but 3,5-diiodo-L-tyrosine, of these DMR-active molecules were active to trigger β-arrestin translocation via GPR35 in the engineered cell line; and DHICA was the most potent agonist, and reverse T3 the second. Interestingly, reverse T3 exhibited the highest efficacy, which is comparable to zaprinast.

DHICA and 5,6-dihydroxyindole are two key monomer building blocks of eumelanins, the black to brown pigments found in human skin, hair and eyes. The plasma concentration of DHICA was unknown to us; however, its O-methy derivative, 6-hydroxy-5-methoxyindole-2-carboxylic acid, was found to be 0.51 ng/ml in healthy patients, and can be up to 4.09 ng/ml in patients having melanoma metastases[34]. The DHICA level is often increased upon UV exposure. Considering that local concentration of DHICA may be much higher, the GPR35 agonist activity of DHICA is worthy of further attention and may be of biological relevance.

L-DOPA, the precursor of DHICA, has been shown to have a regulatory role in melanogenesis and other cellular functions[35]. GPR143 was recently proposed to be a membrane receptor target for L-DOPA[30]. L-DOPA was found to have a weak agonist activity at the GPR35, beside the activation of another target in HT-29. Previous cross-linking experiments of cells cultured in low tyrosine media have revealed that L-DOPA binds specifically to a 30 kDa cell surface protein (a molecular weight, M.W., identical to GPR35), besides a 55 kDa cell surface protein (a M.W. close to GPR143)[36]. The DMR of L-DOPA in HT-29 may be due to the co-activation of both GPR143 and GPR35. The expression and function of GPR143 in HT-29 was currently under investigation.

Gentisate is an intermediate of tyrosine catabolism pathway, leading to the production of fumarate and pyruvate. Both eukaryotic and prokaryotic organisms catabolize phenylalanine and tyrosine by a common peripheral pathway which results in homogentisate, a central intermediate prior to gentisate[37]. Gentisate is also an active oxymetabolite of aspirin, resulting in analgesic and anti-inflammatory effects[38]. Although the plasma concentration of endogenous gentisate was unknown to us, it has been detected for gentisate to reach a concentration up to 15 μM in plasma and synovial fluid of patients receiving aspirin therapy for rheumatoid arthritis[39]. Since gentisate is more likely to be formed by disease processes where activated cells produce oxyradicals[38], its local concentration at inflammation sites during similar aspirin therapy could be much higher for febrile patients than healthy individuals. Therefore, we hypothesized that the moderate potency of gentisate to activate GPR35 may be relevant to the clinical effects of aspirin.
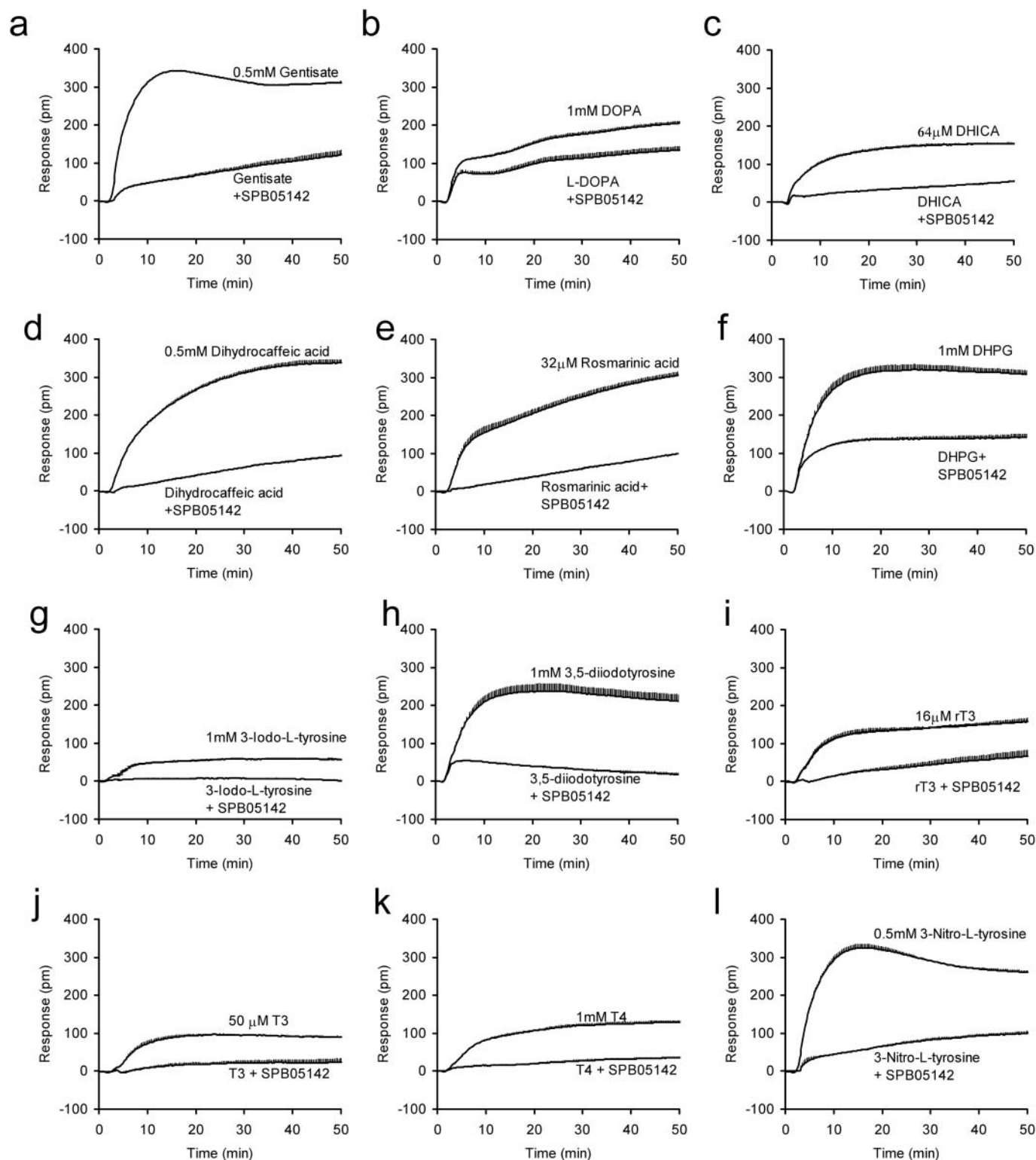
Figure 7 | The DMR characteristics and specificity to GPR35 activation of different ligands. (a to l) The DMR signal of each ligand in the absence and presence of 64 µM SPB05142: (a) gentisate, (b) L-DOPA, (c) DHICA, (d) dihydrocaffeic acid, (e) rosmarinic acid, (f) DHPG, (g) 3-iodo-L-tyrosine, (h) 3,5-diiodo-L-tyrosine, (i) reverse T3, (j) T3, (k) T4, and (l) 3-nitro-L-tyrosine. The concentration of each agonist was indicated in the graph. All data represents mean±s.d. from 2 independent measurements, each in 8 replicates (n=16).

Dihydrocaffeic acid and rosmarinic acid are two metabolites in DOPA degradation pathway. Rosmarinic acid is also believed to be an active constituent of *Danshen*, the dried root of *Salvia miltior-rhiza*, which is a commonly used traditional Chinese medicine for the treatment of cardiovascular and cerebrovascular diseases[40,41].

Here we found that rosmarinic acid exhibited much higher potency to activate GPR35 than dihydrocaffeic acid did. The agonist activity at the GPR35 of rosmarinic acid may be relevant to its clinical feature, since it share such a molecular mode of action with furosemide. Furosemide, a clinically used loop diuretic to
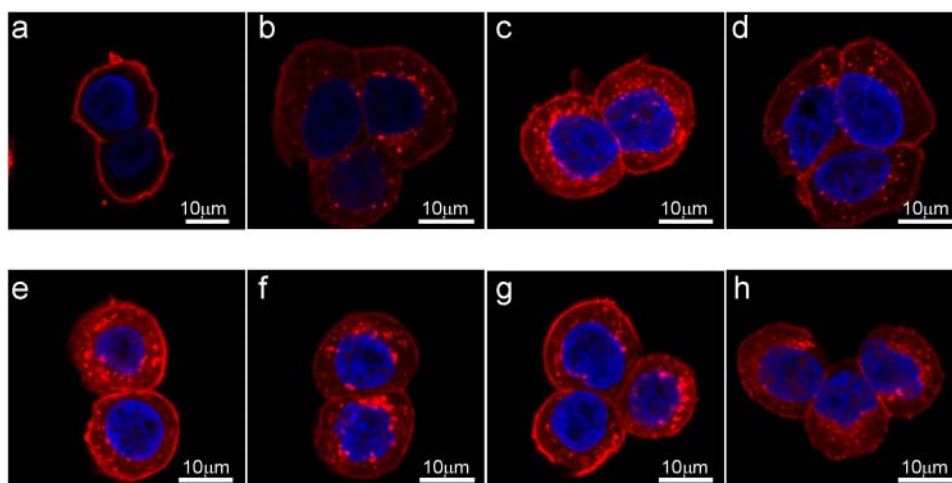
Figure 8 | GPR35 agonists trigger receptor internalization in HT-29 cells. Representative confocal fluorescence images of HT-29 under different conditions: (a) treated with the assay vehicle containing 0.1% DMSO; (b to h) treated with various ligands at a fixed dose: (b) 50 μM gentisate, (c) 2 mM L-DOPA, (d) 50 μM DHICA, (e) 0.5 mM DOPAC, (f) 5 μM reverse T3 (rT3), (g) 200 μM T4, and (h) 50 μM 3-nitrotyrosine. The images were obtained after compound treatment for 1 hr, 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.

treat congestive heart failure and edema, was recently identified to be a GPR35 agonist[42].

Thyroid hormone biosynthesis metabolites including T3, reverse T3, 3,5-diiodo-L-tyrosine and T4 were also found to be GPR35 agonists. T4, once produced by the thyroid gland, lasts for weeks in the body and is converted to T3 and reverse T3. Reverse T3 is considered to be not bio-active and can block the actions of T3 at thyroid receptors when produced in excess. Various nongenomic actions of thyroid hormones have been long reported to be originated from cytoplasmic proteins or the cell surface integrin receptor $\alpha_v\beta_3$.[16] Further, much attention was focused on the nongenomic action of both T4 and T3 at a concentration range being close to their physiological free levels of the hormone[16]. Compared to the nongenomic actions reported in literature, these iodothyronines exhibited much lower potency as GPR35 agonists. However, considering the increasing production of reverse T3 in thyroid disorders[43–47] as well as the up-regulation of GPR35 in certain diseases[5,7–9], the GPR35 agonist activity of thyroid hormones may be of biological relevance, and represent a novel molecular mode of action contributing to the increasing list of nongenomic actions of thyroid hormones.

The two metabolites including DOPAC and DHPG in the catecholamine degradation pathways were also found to exhibit agonist activity at the GPR35. Catecholamines are biosynthesized in both neuronal and non-neuronal cells, including the central nervous system, sympathetic nerves, adrenal medulla, gastrointestinal tract, and kidneys. Dopamine is preferentially converted to acid metabolites including DOPAC, and noradrenaline and adrenaline are preferentially converted to alcohol metabolites including DHPG[48]. The plasma concentration of DHPG was reported to be about 5 nM in healthy patients[49]. Thus, DHPG alone may not represent an endogenous ligand of GPR35.

3-Nitrotyrosine is a derivative of tyrosine during nitrative stress. Amassing evidence suggest that several nitrating species including nitrogen dioxide, peroxynitrite and nitrous acid can be produced during inflammation[28]. Free 3-nitrotyrosine can be detected in human plasma and other biofluids and is generally considered a biomarker of nitrative stress[50]. The concentration of endogenous free 3-nitrotyrosine in the plasma is <100 nM under normal physiological conditions[51,52], but can increase by ~100 fold in the joint fluid of rheumatoid arthritis[53] and up to 100 μM in the plasma of patients with septic shock and renal failure[54]. Given that the local concentration of free 3-nitrotyrosine in close proximity to the site of production of nitrating species can be much higher, the agonist activity at the GPR35 of 3-nitrotyrosine may be of biological relevance.

In summary, our study showed that GPR35 is a target of multiple tyrosine metabolites including DHICA, reverse T3, T3, rosmarinate, as well as a product of tyrosine nitration 3-nitrotyrosine. Since each of these metabolites does not reach sufficient plasma concentration
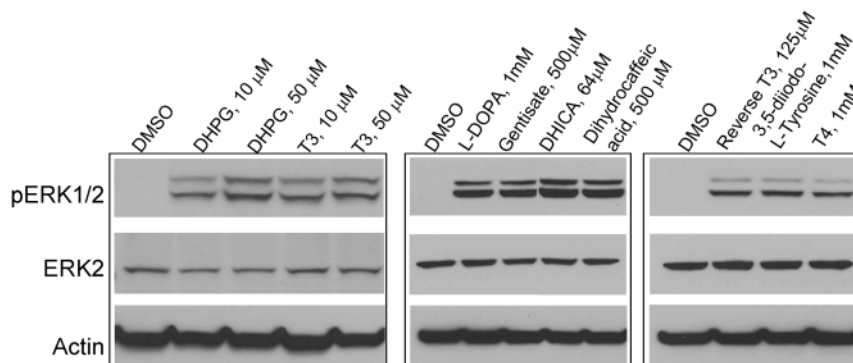

Figure 9 | GPR35 agonists result in robust ERK phosphorylation. ERK phosphorylation induced by representative ligands, each at different doses. The phosphorylated ERK and total ERK were plotted, and actin the control. Before cell lysis, the cells were treated with respective drugs for 30 min. Representative images were obtained from 2 independent measurements.

to fully activate GPR35 on its own, our discovery that multiple metabolites having agonist activity at the GPR35 are enriched within tyrosine metabolism pathway may point to a possibility that some, if not all, of these metabolites work collectively to regulate the physiological and pathophysiological function of GPR35.

Methods

Compounds and Reagents. Kynurenic acid, homogentisate, gentisate, L-DOPA (3,4-dihydroxy-L-phenylalanine), DOPAC (3,4-Dihydroxyphenylacetic acid), DHPG (3,4-dihydroxyphenylglycol), 3-iodo-L-tyrosine, 3,5-diiodo-L-tyrosine, reverse T3, T3, T4 (L-thyroxine), 3-nitro-L-tyrosine, L-tyrosine, DL-tyrosine, DL-phenylalanine, DL-phenylalanine, α-methyl-L-DOPA, 4-hydroxyphenylacetate, homovanillic acid, ethyl homovanillate, 3-methoxytyramine, sodium fumarate, and rosmarinate were obtained from Sigma Chemical Co. (St. Louis, MO). Dihydrocaffeic acid (3-(3-(3,4-dihydroxyphenyl)propionic acid) was obtained from Frontier Scientific Services, Inc. (Newark, DE). 4-Hydroxy-phelypyruvate was obtained from UkrOrgSynthesis, Ltd. (Ukraine). Zaprinast was obtained from Tocris Chemical Co. (St. Louis, MO). SPB05142 (CID2745687; methyl-5-[(tert-butylcarbamothioylhydrazinylidene)-methyl]-1-(2,4-difluorophenyl)-pyrazole-4-carboxylate) was obtained from Ryan Scientific, Inc. (Mt. Pleasant, SC). Epic® 384-well biosensor microplates were obtained from Corning Inc. (Corning, NY, USA).

Synthesis and characterization of DHICA. DHICA was custom synthesized by BioDuro Corp. (Beijing, China). Briefly, to a solution of 2-bromo-4,5-dimethoxybenzaldehyde (2.45 g, 10 mmol) in dimethyl sulfoxide (DMSO, 40 mL) was added CuI (200 mg, 1 mmol) and Cs_2CO_3 (6.52 g, 20 mmol) under N_2. After stirring for 10 minutes, ethyl 2-isocyanoacetate (1.25 g, 11 mmol) was added dropwise and stirring continued for 16 hours at 80°C. The reaction was quenched with water (200 mL) and extracted with 80 mL ethyl acetate (EtOAc) three times. After removal of the solvent, the residue was purified by flash column chromatography on silica gel (petroleum ether/EtOAc =5:1) to give ethyl 5,6-dimethoxy-1H-indole-2-carboxylate (1.5 g, 60% yield) as off-white solid.^{1}H NMR (300 MHz, CDCl₃): δ ppm 8.75 (s, 1H), 7.12 (s, 1H), 7.04 (s, 1H), 6.85 (s, 1H), 4.39 (q, 2H), 3.92 (m, 6H), 1.41 (t, 3H). Next, to the solution of ethyl 5,6-dimethoxy-1H-indole-2-carboxylate (120 mg, 0.48 mmol) in EtOH/H_2O(5 mL/5 mL) was added NaOH (77 mg, 1.92 mmol). The mixture was stirred for 16 hours at room temperature. After removal of the solvent, the residue was adjusted to pH 2~3 and filtered, the filter cake was dried to give 5,6-dimethoxy-1H-indole-2-carboxylic acid (80 mg, 75% yield) as off-white solid. Lastly, to the solution of 5,6-dimethoxy-1H-indole-2-carboxylic acid (80 mg, 0.36 mmol) in dichloromethane (DCM, 10 mL) was added BBr₃ (0.1 mL, 1.08 mmol) dropwise at −78°C. The mixture was stirred for 6 hours at room temperature. The mixture was quenched with water (5 mL) and filtered, the filter cake was washed by water and DCM to give DHICA (30 mg, 43% yield) as off-white solid. ^{1}H NMR (300 MHz, DMSO-d6) δ ppm: 12.41 (s, 1H), 11.11 (s, 1H), 8.07 (s, 1H), 8.56 (s, 1H), 6.87 (m, 3H); LCMS (m/z): ES- 192 (M−H). DHICA with a purity of >95% received from the CRO was used directly.

Cell Culture. Human colorectal adenocarcinoma HT-29 was obtained from American Type Cell Culture (Manassas, VA, USA). The cells were cultured in McCoy's 5a Medium Modified supplemented with 10% fetal bovine serum, 4.5g/liter glucose, 2 mM glutamine, and antibiotics at 37°C under air/5% CO_2. Tango™ U2OS-GPR35-bla cells were purchased from Invitrogen, and were cultured according to the protocols recommended by the supplier. Briefly, the cells were passed using McCoy's 5A medium (Invitrogen 16600–082) supplemented with 10% dialyzed fetal bovine serum, 0.1 μM NEAA, 25 μM Hepes (pH 7.3), 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 200 μg/ml zeocin, 50 μg/ml hygromycin, and 100 μg/ml geneticin in a humidified 37°C/5% CO_2 incubator.

Dynamic mass redistribution assay. Epic® system (Corning Inc., Corning, NY), a wavelength interrogation reader system based on resonant waveguide grating biosensors in microtiter plates, was used for dynamic mass redistribution (DMR) assays[20]. The Epic system consists of a temperature-control unit (26°C), an optical detection unit, and an on-board liquid handling unit with robotics. The detection unit is centered on integrated fiber optics, and enables kinetic measures of cellular responses with a time interval of ~15 sec. For whole cell DMR assays, cells were directly seeded in Epic® plates and cultured overnight to form confluent monolayer in the cell culture medium; and the cells were then washed twice using plate washer, and maintained with Hank's balanced salt solution (1x HBSS) and further incubated inside the system for 1 hr before measurements.

For DMR agonism profiling, a 2-min baseline was first established. Immediately after the compound addition using the onboard liquid handler, the cellular responses were recorded in real time for about 1 hr. For DMR antagonist assays, cells were initially treated with a GPR35 antagonist for 1 hr. The baseline was established, and a testing agonist at a fixed dose was added and the cellular responses were then recorded in real time. All EC$_{50}$ or IC$_{50}$ described in the main text were calculated based on the maximal amplitudes of DMR signals within 1 hr poststimulation. Cellular responses induced by the assay buffer with 0.1% dimethyl sulfoxide (DMSO) were used as negative controls. All DMR signals were background corrected.

Tango β-arrestin translocation gene reporter assays. The U2OS-GPR35-bla cell line stably expresses two fusion proteins: human GPR35 linked to a Gal4-VP16 transcription factor through 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. For culturing in microplates, 10000 cells per well were seeded in 384-well, black-wall, clear bottom assay plates with low fluorescence background (Corning), and cultured in Dulbecco's modified eagle medium (Invitrogen, 10569-010) 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 hrs in a humidified 37°C/5% CO_2, and then loaded with the cell permeable LiveBLAzer™ FRET B/G substrate. After the two hour incubation the coumarin to fluorescein ratio was measured using Tecan Safire II microplate reader (Männedorf, Switzerland). The activation of GPR35 by agonists leads to the recruitment of β-arrestin/TEV protease fusion proteins to the activated GPR35. As a result, the protease cleaves the Gal4-VP16 transcription factor from the receptor, which then translocates to the nucleus and activates the expression of β-lactamase. The GPR35 activation increases the coumarin to fluorescein FRET (fluorescence resonance energy transfer) ratio, which was used as a normalized reporter response.

ERK MAPK assays. The p44/42 MAP kinases were examined using Western blotting. Whole cell lysates were collected after the cells were treated with a compound or DMSO for 1 hr. Equivalent gel loading was confirmed by probing with anti-actin body. The total ERK1/2 and phosphorylated ERK1/2 were blotted using respective antibodies.

Receptor internalization assays. HT-29 cells were plated on an 8-well chamber slide (Nalge Nunc International, Rochester, NY, USA) with a seeding density of 10,000 cells per well and incubated at 37°C for 24 hr. Next day, cells were stimulated with a compound or equal amount of DMSO at 37°C for 1 hr. Afterwards, cells were fixed with 4% formaldehyde in 1 x PBS for 15 min, followed by blocking and permeabilization in a buffer containing 4% goat serum, 0.1 % bovine serum albumin (BSA), 0.1% Triton X100 in 1 x PBS for 2 hrs. After 5 min wash with PBS, fixed cells were incubated with the anti-GPR35 (1:500) (anti-GPR35, T-14, intracellular domain) (Santa Cruz biotechnology, Santa Cruz, CA, USA) in 3% BSA/PBS buffer for 24 hrs, followed by incubation with secondary antibody Alexa Fluor® 594 donkey anti-goat IgG (H+L) (1:500) (Invitrogen) in 3% BSA/PBS for 1 hr at room temperature. Cells were finally washed once with PBS and sealed with 1.5 mm thick glass cover-slip (Corning, NY). Dried slides were stored at 4°C until imaging. Confocal imaging was performed with Zeiss confocal microscope Axiovert 40. The specificity of anti-GPR35 was confirmed by the control peptide from the supplier. Staining showed that the control peptide completely blocked the staining of HT-29 cells with the anti-GPR35 antibody. The collected images were analyzed using MacBiophotonics Image J software (http://www.macbiophotonics.ca/downloads.htm).

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Author contributions

Additional information