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Computer-Aided Discovery of Aromatic L-α-Amino Acids as Agonists of the Orphan G Protein-Coupled Receptor GPR139

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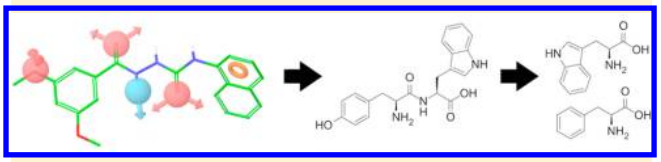


ABSTRACT: GPR139 is an orphan G protein-coupled receptor expressed mainly in the central nervous system. We developed a pharmacophore model based on known GPR139 surrogate agonists which led us to propose aromatic-containing dipeptides as potential ligands. Upon testing, the dipeptides demonstrated agonism in the G_q pathway. Next, in testing all 20 proteinogenic L-α-amino acids, L-tryptophan and L-phenylalanine were found to have EC_{50} values of 220 and 320 μM, respectively, making them the first putative endogenous agonists for GPR139.

INTRODUCTION

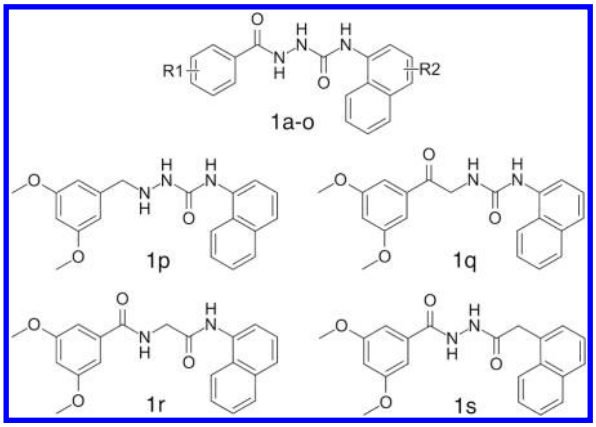
G protein-coupled receptors (GPCRs) form the largest family of cell surface receptors in the human genome[1] and mediate the effect of 30% of currently marketed drugs.[2,3] Determining the endogenous ligands and physiological functions of the remaining ∼130 uncharacterized, so-called orphan GPCRs[4] is of great significance for understanding human physiology and characterization of new therapeutic mechanisms and targets. GPR139 was identified by Gloriam and co-workers as a novel class A GPCR with GPR142 as the only close homologue.[5] The expression pattern of GPR139 is almost exclusively confined to the CNS, mainly to the basal ganglia and the hypothalamus,[6,7] which are involved in movement control[6−8] and regulation of food intake and metabolism.[9] GPR139 is thus a very interesting potential drug target for treatment of e.g. diabetes, eating disorders, obesity, and/or Parkinson's disease.

Two groups have reported small molecule surrogate ligands for GPR139. Hu et al. identified one agonist and two antagonists from a high throughput screening (HTS) campaign.[8] Shi and co-workers reported an agonist identified by HTS and synthesized 20 analogues.[10] In this report, we describe the computer-aided discovery and pharmacological characterization of dipeptides and L-α-amino acids as agonists of GPR139. Elucidating the signaling pathway is a crucial step toward revealing orphan receptor functions. There has been ambiguity regarding the signal transduction mechanism of GPR139 with different reports for G_q,[6,7,10] G_i,[7] or G_s[8] coupling. Here we show that the surrogate agonist 2-(3,5-dimethoxybenzoyl)-*N*-(naphtalen-1-yl)hydrazinecarboxamide (**1a**, Table 1), the identified dipeptides, and L-α-amino acids activate the G_q pathway.

RESULTS AND DISCUSSION

Pharmacophore Model and Structure−Activity Relationships. A GPR139 pharmacophore model was built based on 13 compounds reported by Shi et al.[10] (Table 1).

Table 1. Rationale for Inclusion and Removal of Pharmacophore Elements (Figure 1) with Potency and Efficacy (E_{max} relative to 1a) of the Reported Compounds[a]



pharmacophore element	cmpd	R_1	R_2	EC_{50} (nM)	E_{max}
reference	**1a**	3,5-diMeO	H	39	100
A1 removal and **A2** inclusion	**1f**	3-MeO	H	1000	100
	1h	3,4,5-triMeO	H	63	76
A3 inclusion	**1p**			>10 000	ND[b]
D5 inclusion	**1q**			3300	100
D6 removal	**1r**			180	98
D7 removal	**1s**			88	95
H8, **H9** and **R10** removal	**1e**	3,5-diEt	H	ND	6
R11 inclusion and Excluded volumes	**1j**	3,5-diMeO	4-Br	ND	73
	1k	3,5-diMeO	4-CN	ND	20
	1m	3,5-diMeO	4-Cl	ND	64
	1n	3,5-diMeO	5-Br	nd	54
	1o	3,5-diMeO	7-MeO	nd	50

[a]The compounds were reported by Shi et al.[10] [b]Not determined.

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Compound **1a** was used as a reference, and all its potential pharmacophore elements (Figure 1A) were evaluated against the binding site. The relevance of R12 is unclear as all analogues have a naphthalene moiety in the corresponding position. R12 was excluded here because we wanted to match also single aromatic (corresponding to R11) and nonaromatic fused rings. The final pharmacophore model (Figure 1B) thus contains three hydrogen bond acceptor elements (A2−4), one hydrogen bond donor element (D5), and one aromatic element (R11) together representing the key interactions required for GPR139 ligand activity. Finally, because compounds **1j**, **1k**, **1m**, **1n**, and **1o** all have lower activity due to substituents on the naphthalene, excluded volumes were added to the positions of the naphthalene substituents.

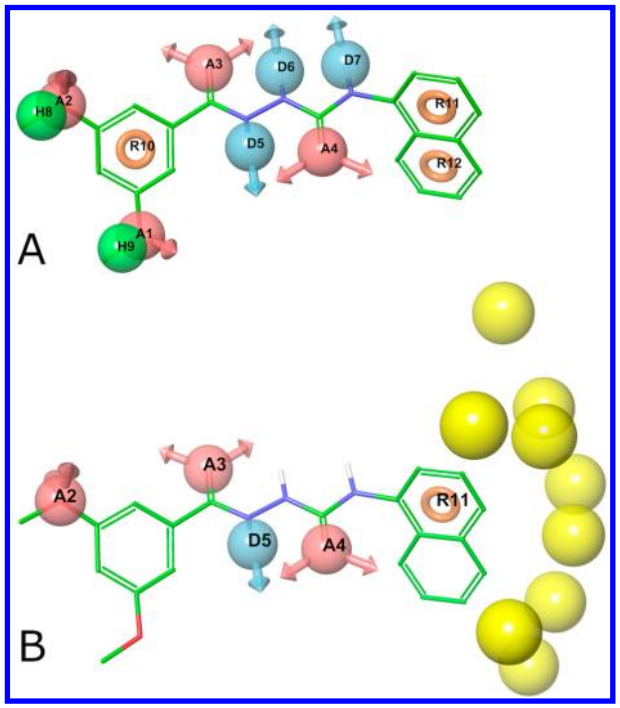

Figure 1. (A) All pharmacophore elements considered in the model building overlaid on the reference compound **1a**. Elements were included or excluded based on the structure−activity relationships of compounds previously reported by Shi et al.[10] (Table 1). (B) Final pharmacophore model containing five features: three hydrogen bond acceptors (red, A2−4), a hydrogen bond donor (blue, D5), and an aromatic ring (orange, R11). Excluded volumes (yellow spheres) were added to the pharmacophore model to represent positions occupied by functional groups unique to the lower activity compounds **1j**, **1k**, **1m**, **1n**, and **1o** from Shi et al.[10]

the analogue structure−activity relationships (higher or lower activity). We found that only one methoxy group on the dimethoxybenzoyl is needed for activity by comparing **1a** and **1f**. Furthermore, **1h** indicates that a third methoxy group does not increase activity. Whereas the SAR indicates that two methoxy groups are beneficial, we selected only one of them (corresponding to A2) to increase the likelihood that the pharmacophore model would match a larger set of new scaffolds. We found that A3 is important and included it because **1p** lacks the corresponding functional group and has significantly lower activity (>10 μM) than **1a**. A4 was included because all compounds contain functional groups corresponding to this acceptor element. D5 is significant and was included as **1q** lacks the corresponding functional group and has lower activity. D6 and D7 were found not to be important and removed because **1r** and **1s** lack the corresponding functional groups, respectively, but retain high activity. The hydrophobic elements H8 and H9 were discarded because of the lower activity of **1e**. All the compounds from Shi et al.[10] contain three aromatic rings. R10 was removed because the lower activity of **1e**, showing that polar substituents on the benzoyl contribute more to activity than the aromaticity of the ring itself. R11 was included because the naphthalene-substituted compounds **1j**, **1k**, **1m**, **1n**, and **1o** have lower activity, indicating the importance of a tight packing of the naphthalene moiety in

Pharmacological Evaluation of Dipeptides. GPR139 had previously been suggested to be a receptor for peptides,[7] and the central linker of the compounds reported by Shi et al.[10] has a resemblance to a peptide backbone. We therefore screened di- and tripeptides against the pharmacophore model in search of peptide motifs able to bind to GPR139. Among the screening hits were four dipeptides consisting of aromatic amino acids (Figure 2). The dipeptides were purchased, and

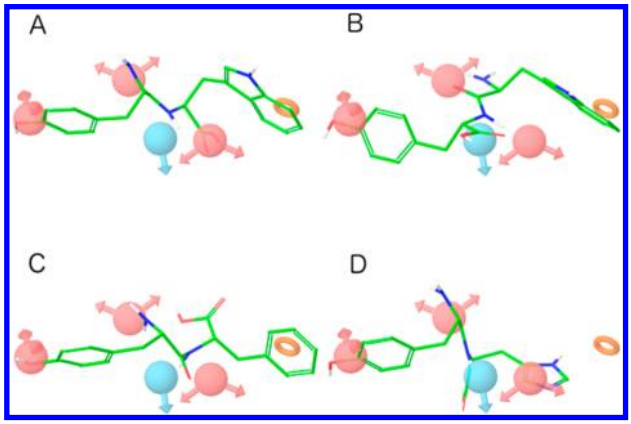

Figure 2. Dipeptides (A) TyrTrp, (B) TrpTyr, (C) TyrPhe, and (D) TyrHis in the pharmacophore model. TyrTrp and TyrPhe satisfy all five pharmacophore elements, but TrpTyr and TyrHis are missing hydrogen bond acceptor and aromatic elements, respectively. This is consistent with the experimental results (Table 2).

their activities were evaluated on Chinese hamster ovary (CHO-K1) cells stably expressing human GPR139. TyrTrp and TyrPhe satisfy all five elements of the pharmacophore model, whereas TrpTyr is missing a hydrogen bond acceptor element and TyrHis is missing an aromatic element. This is consistent with the experimental results, where the highest potencies were observed for TyrTrp and TyrPhe (Table 2).

Pharmacological Evaluation of Amino Acids. To determine the individual contributions of amino acids, the L-isomers of all 20 proteinogenic L-α-amino acids were assayed. All active dipeptides and amino acids were tested for GPR139-specificity using native CHO-K1 cells and found to be specific for the GPR139 receptor. L-Isomers of Trp and Phe were found to be agonists of GPR139 (Figure 3B, Table 2). Notably, no agonist activity was observed for L-tyrosine, although the most potent dipeptide is TyrTrp.

Interestingly, the D-isomers of Trp and Phe showed equal efficacy and potency as the L-isomers (Table 2). This contrasts the other promiscuous amino acid sensors, which have mainly been found to be activated by L-α-amino acids, although the T1R2 receptor is activated by sweet tasting D-amino acids and

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Table 2. Agonist Potency and Maximum Effect (E_{max}) of Amino Acids, Dipeptides, and Neurotransmitters at CHO-K1 Cells Expressing GPR139[a]

Compound	EC_{50} (μM)	pEC_{50} ± SEM	E_{max} ± SEM (%)
1a	0.11	7.0 ± 0.05	100
D-Trp	80	4.1 ± 0.12	120 ± 6.1
L-Trp	220	3.7 ± 0.03	100 ± 5.0
L-Phe	320	3.5 ± 0.06	94 ± 3.0
D-Phe	750	3.1 ± 0.19	90 ± 2.8
TyrTrp	160	3.8 ± 0.03	68 ± 7.2
TyrPhe	180	3.7 ± 0.18	68 ± 4.5
TrpTrp	290	3.5 ± 0.08	39 ± 9.2
ArgTrp	440	3.3 ± 0.05	64 ± 12
PheTrp	490	3.3 ± 0.03	38 ± 1.7
PheArg	590	3.2 ± 0.03	38 ± 4.6
TrpTyr		32 ± 4.1% at 500 μM	
PheTyr		25 ± 4.2% at 500 μM	
HisTyr		21 ± 5.9% at 500 μM	
TyrHis		15 ± 6.0% at 500 μM	
dopamine		4.7 ± 0.5% at 625 μM	
noradrenaline		−6.4 ± 8.0% at 625 μM	
adrenaline		−12 ± 1.8% at 39 μM[b]	
serotonin		6.0 ± 0.5% at 625 μM	

[a] EC_{50} was determined by measurements ($n \geq 3$) of intracellular calcium levels using the Fluo-4 NW calcium assay (Methods). The E_{max} of all tested compounds were normalized to that of **1a**. All proteinogenic L-α-amino acids not included in Table 1 were inactive at 500 μM. [b] Adrenaline was found to be nonspecific in concentrations higher than 39 μM.

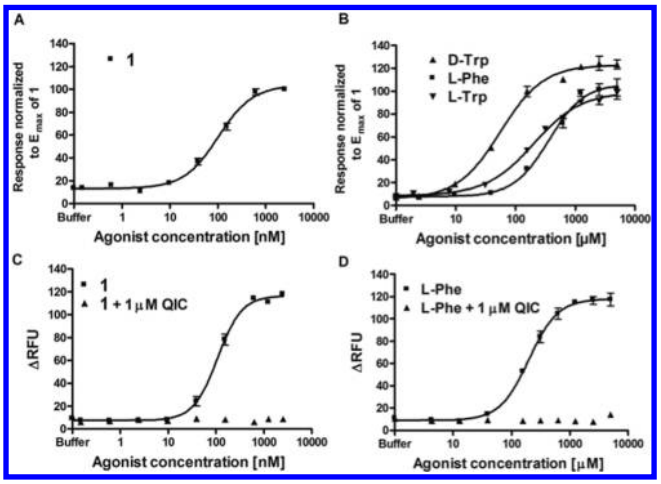

Figure 3. Representative concentration−response curves of (A) **1a** and (B) three proteinogenic α-amino acids. Incubation with UBO-QIC completely abolished the agonist response of **1a** (C) and L-Phe (D). The maximum effect of **1a** was set to 100% and used to normalize all other compounds. Responses are shown as ΔRFU (peak fluorescence units after agonist addition subtracting the basal fluorescence).

not by L-amino acids.[11] The L- and D-isomers of Trp and Phe fit equally well into our pharmacophore model.

L-Trp and L-Phe are precursors of serotonin and dopamine, respectively. GPR139 is expressed in areas of the brain where these neurotransmitters exert their roles in, e.g., metabolism and movement control. We therefore tested dopamine, serotonin, adrenaline, and noradrenaline, which were however found to have no or very limited GPR139 activity (Table 2).

Validation of the Main Signaling Pathway of GPR139.

Our results show that all agonists mobilize intracellular calcium, which is a characteristic G_q pathway activation. To further investigate the signaling pathway of GPR139, we preincubated the cells with the University of Bonn-Gα_q-inhibiting component (UBO-QIC) before applying agonist. UBO-QIC incubation completely abolished the agonist response of **1a** as well as L-Phe (Figure 3C and D). Furthermore, in human embryonic kidney (tsA HEK) cells transiently transfected with GPR139, we observed concentration-dependent Ca^{2+} mobilization when the cells were treated with **1a** (data not shown). Our observations are in agreement with that of Matsuo and co-workers who found that overexpression of GPR139 in 293 EBNA cells induced activation of serum response element luciferase, which was strongly suppressed by the G_q inhibitor YM-254890.[6] Using the same GPR139-expressing cell line as in the present study, Shi et al. reported a series of surrogate agonists activating the G_q pathway.[10] Süsens and colleagues also found evidence of G_q-coupling but, in addition, reported activation of the G_i pathway.[7] In contrast to these studies Hu and co-workers were unable to induce Ca^{2+} mobilization in HEK293 cells transfected with GPR139, whereas the same cells increased cyclic adenosine monophosphate (cAMP) levels, and they thus suggest the receptor to be G_s coupled.[8] Thus, all studies but one have shown G_q-coupling of the GPR139 receptor, which indicate that this is the main signaling pathway of the receptor. However, this does not rule out that GPR139 might be able to activate other pathways depending on the cellular context it is expressed in or that distinct ligands could induce signaling bias.[12]

Süsens and co-workers showed that a small peptide-enriched extract from porcine brain tissue induced an agonist response in GPR139-expressing CHO cells.[7] We found that the two amino acids Trp and Phe are agonists of GPR139 with similar E_{max} as **1a**. At present Trp and Phe are the only known endogenous molecules found to activate GPR139. The physiological concentrations of L-Trp and L-Phe in the brain are 20−70 and 50−100 μM, respectively,[13,14] with plasma levels of 44 ± 7 and 57 ± 8 μM, respectively.[15] These concentrations are in the ranges we found to activate GPR139 (Figure 3B). It is thus plausible that the receptor plays a role in monitoring physiological fluctuations of these amino acids. GPR139 thus joins the calcium-sensing receptor, the GPRC6A receptor, and the T1R1/T1R3 receptor as promiscuous L-α-amino acid sensors, which have recently gained increased interest as potential nutrient sensors.[16]

GPR142 is the closest homologue of GPR139 and is expressed in pancreatic ß-cells.[5] In very recent publications, researchers at Amgen have reported 99 surrogate agonists of GPR142 and 8 inactive analogues.[17−20] The compounds are structurally similar to the GPR139 agonists reported by Shi et al.,[10] and many fit the GPR139 pharmacophore model (46 fit all 5 pharmacophore elements and 103 fit at least 4). It is thus possible that some of these compounds can activate GPR139. The authors refer to unpublished results showing that GPR142 is G_q-coupled and activated by aromatic amino acids with L-Trp being the most potent with an EC_{50} of 0.2−1.0 mM. Furthermore, they refer to unpublished observations that L-Trp dose-dependently increases insulin secretion and improves glucose tolerance in mice and monkeys.[17,18] Collectively, the published GPR142 data and our data indicate that GPR139 and GPR142 are subtypes sharing ligands and a signaling pathway.

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CONCLUSIONS

We found L-Trp and L-Phe to be the first known endogenous agonists of GPR139. We showed that these ligands signal through the G_q pathway. Additional experiments will be essential to clarify the physiological function and therapeutic potential of the agonists and the GPR139 receptor. To this end a GPR139 knockout mouse and/or development of potent antagonists would be highly valuable tools.

METHODS

Pharmacophore Modeling. The pharmacophore model was built using phase 3.3.111[21] based on the compounds in Table 1. Structural conformers were generated with the thorough sampling option. Hypotheses matching the variant AAADR (A hydrogen bond acceptor, D hydrogen bond donor, R aromatic ring) were kept. The activity cutoff was defined as 1 μm, and the top hypothesis matching the elements A2, A3, A4, D5, and R11 was selected after scoring actives and inactives. After excluded volumes were added, the lower activity compounds **1j**, **1k**, **1m**, **1n**, and **1o** (compared to **1a**) were screened against the pharmacophore (including excluded volumes) in search of alternative conformations that evade the excluded volumes, and additional excluded volumes were added to the positions of the naphthalene substituents accordingly. This process was repeated twice. Marvin was used for drawing 2D structures (Marvin 5.12.3, 2013, ChemAxon, www.chemaxon.com).

Pharmacological Assaying. Cell lines and **1a** have previously been reported.[10] Amino acids and monoaminergic neurotransmitters were purchased from Sigma-Aldrich (LAA21-1KT, P1751-5G, T9753, and H8502, H9523, E4375, A7256) and dipeptides from Genscript. Compounds were dissolved in DMSO and subsequently diluted in HEPES buffered HBSS (Invitrogen, 14025, supplemented with 20 mM HEPES) to a final test concentration of max 1% DMSO. DMSO was confirmed not to have any activity by itself at this concentration. Purity of tested compounds was ≥95%, as confirmed by HPLC and MS.

All compounds were tested in a CHO-K1 cell line stably expressing GPR139.[10] The specificity of the compounds was tested using native CHO-K1 cells. The response was considered specific for GPR139 if the compounds induced Ca^{2+} mobilization only in the transfected cells.

Cells were plated in black 96-well plates (30.000 cells/well) with clear bottoms and incubated overnight. The Fluo-4 NW Calcium Assay Kit (Invitrogen, F36206) was performed as described by Mølck and co-workers,[22] however, excluding the BSA and 21 °C incubation steps. Plates were read on a FlexStation 3 Benchtop Multi-Mode Microplate Reader (Molecular Devices) with an excitation filter of 485 nm and emission at 525 nm.

Signaling Pathway Validation. The G_q inhibitor UBO-QIC (corresponding to FR900349—a close analogue of the G_q-inhibitor YM-254890)[23,24] was purchased from the Institute of Pharmaceutical Biology, University of Bonn, Germany. Cells were plated as described above. Two hours before reading the plates, 1 μM UBO-QIC was added to half the wells (remaining wells received HEPES buffer). One hour later the medium was removed, wells were washed with HEPES buffer, and dye was added. Here, 1 μM UBO-QIC was added to half the wells and buffer was added to remaining wells. Plates were read 1 h later as described above.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

CHO, Chinese hamster ovary; GPCRs, G protein-coupled receptors; GPR139, G protein-coupled receptor 139; GPR142, G protein-coupled receptor 142; GPRC6A, G protein-coupled receptor, family C, group 6, subtype A; HEK, Human embryonic kidney; UBO-QIC, University of Bonn-Gα_q-inhibiting component

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