Identification and Characterization of the First Selective Y$_4$ Receptor Positive Allosteric Modulator

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S *Supporting Information*

ABSTRACT: The human Y$_4$ receptor (Y$_4$R) and its cognate ligand, pancreatic polypeptide (PP), are involved in the regulation of energy expenditure, satiety, and food intake. This system represents a potential target for the treatment of metabolic diseases and has been extensively investigated and validated in vivo. Here, we present the compound tBPC (*tert*-butylphenoxycyclohexanol), a novel and selective Y$_4$R positive allosteric modulator that potentiates Y$_4$R activation in G-protein signaling and arrestin3 recruitment experiments. The compound has no effect on the binding of the orthosteric ligands, implying its allosteric mode of action at the Y$_4$R and evidence for a purely efficacy-driven positive allosteric modulation. Finally, the ability of tBPC to selectively potentiate Y$_4$R agonism initiated by PP was confirmed in mouse descending colon mucosa preparations expressing native Y$_4$R, demonstrating Y$_4$R positive allosteric modulation in vitro.
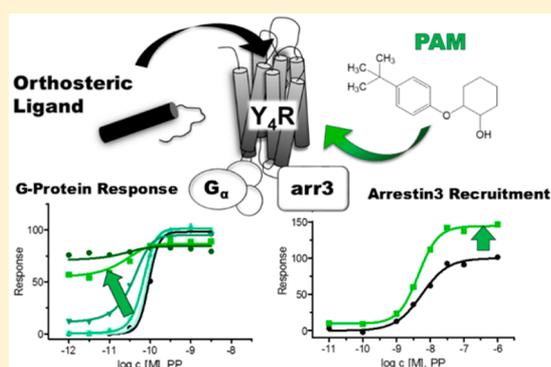
INTRODUCTION

The human Y$_4$R belongs to the neuropeptide Y (NPY) receptor family, a multireceptor/multiligand system consisting of four different receptors (Y$_1$R, Y$_2$R, Y$_4$R, Y$_5$R) and the three peptide ligands NPY, peptide YY (PYY), and pancreatic polypeptide (PP). Neuropeptide Y receptors and ligands are critically involved in the regulation of energy metabolism and thus represent potential drug targets with respect to the increasing global health problem of overweight and obesity.[1,2] The receptors differ in their tissue expression pattern and in the binding/activation by the peptide ligands. Y$_1$R and Y$_2$R subtypes are highly expressed in central nervous regions and bind preferably NPY and PYY. The Y$_5$R is present in central nervous tissue and binds all three peptide ligands with high affinities.[3] In contrast, the Y$_4$R is predominantly expressed in the gastrointestinal tract and at much lower levels in the brain.[4,5] This receptor shows a high preference for PP but can also be activated by NPY and PYY with lower potency.[6] Several studies revealed the strong influence of PP and the Y$_4$R as a trigger of satiety after food intake and regulation of energy expenditure, leading to reduced food intake and loss of weight.[7−12] Subcutaneous or intravenous administration of PP reduces food intake in humans, revealing the in vivo efficacy of this system as a pharmacological target.[13−15]

To investigate this therapeutic potential, different reviews have highlighted the potential of nonpeptidic small molecule compounds to target the Y$_4$R and to extend the pharmacological toolbox; however none have been reported to date.[16,17]

Allosteric modulators offer several advantages over classical agonists. With the potential to modulate G-protein-coupled receptors (GPCRs) without major intrinsic effects, they enable a fine-tuning of the receptor activation by the endogenous ligand, maintaining spatial and temporal activation patterns.[18,19] Small molecule allosteric modulators also offer an alternative for the development of selective receptor ligands, as demonstrated for several GPCR families.[20,21] We recently identified niclosamide and structurally related compounds as the first Y$_4$R small molecule ligands with positive allosteric modulator (PAM) activity.[22] However, those compounds were not fully selective for the Y$_4$R versus Y$_1$R, Y$_2$R, and Y$_5$R, and niclosamide has also been shown to modulate mGluRs.[23]

Here, we present the first highly selective PAM for the Y$_4$R: (4-*tert*-butyl)phenoxycyclohexanol (tBPC). This compound displays positive allosteric modulation of the Y$_4$R in G-protein activation and arrestin3 (arr3) recruitment. Finally, the ability of tBPC to potentiate the native PP response was confirmed in
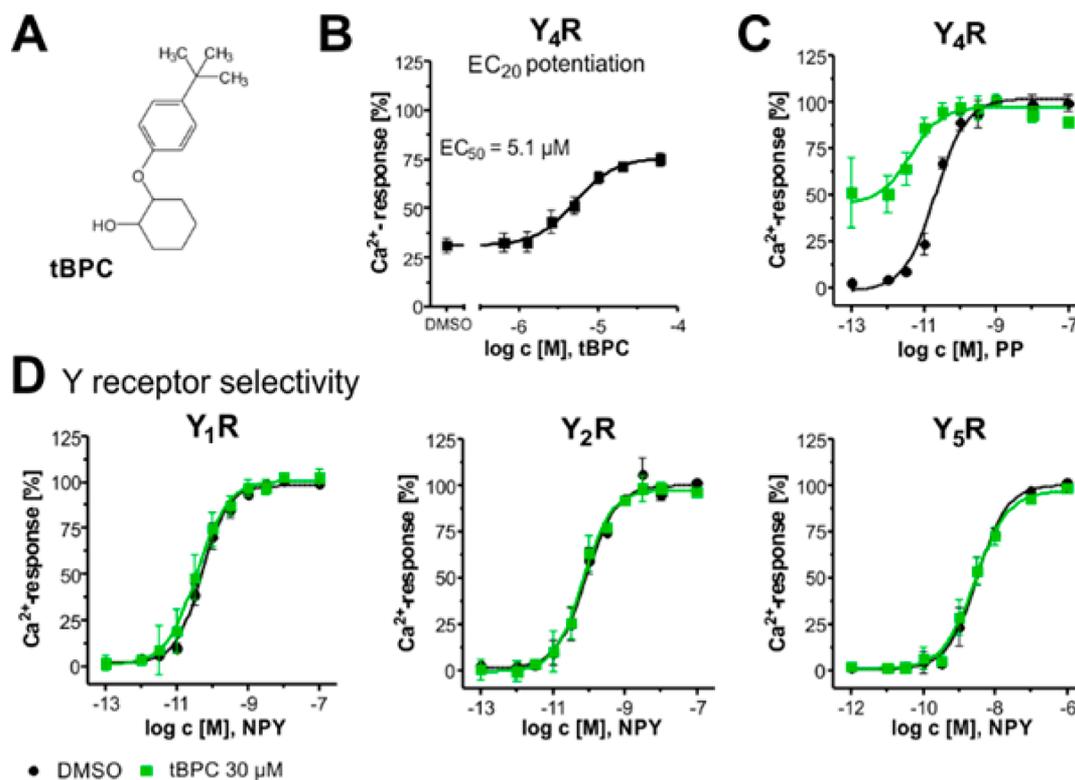
Figure 1. Identification and Y receptor selectivity of tBPC: (A) structure of tBPC; (B) potentiation of a PP EC_{20} signal response by increasing concentrations of tBPC; (C) influence of 30 μM tBPC on the activation of the Y_4R by PP; (D) effect of 30 μM tBPC on the activation of the Y_1R, Y_2R, and Y_5R by NPY. Receptor activation was measured in a Ca^{2+}-flux assay with COS7 cell lines stably expressing one Y receptor subtype−eYFP fusion protein ($Y_{1,2,4,5}R$-eYFP) and the chimeric G-protein ($\Delta 6G\alpha_{qi4\text{-myr}}$). Data represent the mean ± SEM from two to four independent experiments.
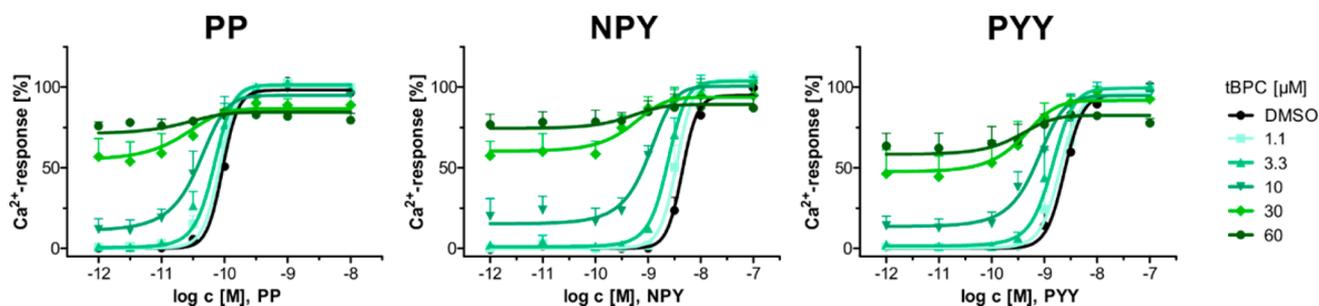
Figure 2. PAM effect of increasing concentrations of tBPC on the Y_4R G-protein activation by PP, NPY, and PYY. Y_4R activation was measured by Ca^{2+} flux assays with stably transfected COS7_Y_4R-eYFP_$\Delta 6G\alpha_{qi4\text{-myr}}$ cells. Data represent the mean ± SEM from three to four independent experiments.

mouse descending colon mucosal preparations, known to endogenously express the Y_4R.

RESULTS AND DISCUSSION

Identification of tBPC as a Y_4R Selective PAM in a Ca^{2+} Flux Based HTS. Because of the clinical potential of the Y_4R but the lack of small molecule agonists or PAMs for this receptor, our efforts first focused on the identification of suitable candidates. The compound tBPC, (4-*tert*-butyl)-phenoxycyclohexanol, (Figure 1 A) was identified in a Ca^{2+} flux based high-throughput screen (HTS) using stably transfected COS7_Y_4R-eYFP_$\Delta 6G\alpha_{qi4\text{-myr}}$ cells, as recently described for the Y_4R PAM niclosamide.[22] tBPC potentiated the Y_4R signal response to an EC_{20} concentration of PP with a potency of 5.1 μM (pEC_{50} = 5.3 ± 0.1) and an E_{max} of 76% of the maximum PP system response (Figure 1 B). Furthermore,

30 μM tBPC displayed agonistic activity at the Y_4R (Figure 1 C). The screening of 385 structurally related compounds, selected by a substructure search in the Vanderbilt University compound library based on tBPC-related scaffolds (Supporting Information Figure 1A−C), identified eight further compounds with Y_4R-PAM activity. Interestingly, a (4-*tert*-butyl)- or (4-isopropyl)phenoxy structure was present in all of the active compounds (Supporting Information Figure 1D), which demonstrates the importance of this motif for biological activity. In contrast, the other region of the molecule tolerated more diverse structures and thus possibilities for modification and optimization (Supporting Information Figure 1D). As tBPC was the most active compound of those tested, this molecule was selected for further characterization.

Y Receptor Selectivity. High selectivity of tBPC for the Y_4R is important because of the opposing roles of Y_1R and Y_5R

versus Y_2R and Y_4R in the regulation of energy metabolism and food intake.[2,24] The effect of tBPC on the Y_1R, Y_2R, and Y_5R was investigated in Ca^{2+} flux assays using stably transfected COS7_$Y_{1/2/5}R$_$\Delta6G\alpha_{qi4\text{-}myr}$ cell lines, expressing one specific Y receptor subtype and the chimeric G-protein. Receptor activation was measured in the presence of 30 μM tBPC and in solutions containing equivalent concentrations of DMSO (Figure 1D). NPY was used because it acts as a natural ligand for Y_1R, Y_2R, and Y_5R. The high concentration of 30 μM tBPC was chosen because it induced a maximum potentiation of the PP response at the Y_4R (Figure 1B,C). In contrast, treatment with 30 μM tBPC had no effect on the efficacy (E_{max}) or potency (EC_{50}) of the NPY signal response at the Y_1R (pEC_{50} DMSO 10.3 ± 0.1; tBPC 10.4 ± 0.1), Y_2R (pEC_{50} DMSO 10.0 ± 0.1; tBPC 10.2 ± 0.1), and Y_5R (pEC_{50} DMSO 8.5 ± 0.1; tBPC 8.5 ± 0.1). These results demonstrate the high Y_4R selectivity of tBPC in the human Y receptor family.

tBPC Potentiates Y_4R Activity in G-Protein Signaling and Arrestin3 Recruitment. The allosteric modulation of the Y_4R was investigated in two different signaling pathways, the classical G-protein activation and arrestin3 (arr3) recruitment. First, G-protein activation was examined in more detail using stably transfected COS7_Y_4R-eYFP_$\Delta6G\alpha_{qi4\text{-}myr}$ cells that were previously used in the HTS for the identification of tBPC. The allosteric effect on the Y_4R activation was investigated for increasing tBPC concentrations (Figure 2), which allowed the quantification of the allosteric effect by using an operational model of allosterism.[25] With this approach, the intrinsic agonism (τ_B), the dissociation constant (K_B) of tBPC, and the strength of the allosteric modulation on potency and efficacy of the ligand signal response (cooperativity factor $\alpha\beta$) were determined (Table 1). Furthermore, the modulation of

Table 1. Global Fitting Analysis of Ca^{2+} Flux Data from Figure 2[a]

	$\alpha\beta$ (log $\alpha\beta$ ± SEM)	τ_B (log τ_B ± SEM)	pK_B ± SEM
PP	2.0 (0.30 ± 0.2)	2.6 (0.41 ± 0.1)	4.4 ± 0.2
NPY	7.9 (0.90 ± 0.2)	2.9 (0.46 ± 0.1)	4.4 ± 0.2
PYY	6.2 (0.79 ± 0.2)	2.3 (0.36 ± 0.1)	4.4 ± 0.2

[a]Data from Figure 2 were analyzed using the operational model of allosterism of Aurelio et al.[25]

tBPC was investigated for the peptide ligands PP, NPY, and PYY to consider probe dependence effects. tBPC shifted the concentration−response curves of PP, NPY, and PYY to lower EC_{50} values and therefore displayed a positive cooperativity for the Y_4R activation by all three peptide ligands (PP $\alpha\beta$ = 2.0, NPY $\alpha\beta$ = 7.9, PYY $\alpha\beta$ = 6.2; Table 1). Along with the PAM activity, tBPC displayed an agonistic activity (τ_B = 2.6–2.9) with increasing concentrations (Figure 2) and is thus classified as an Y_4R ago-PAM. This intrinsic agonism of tBPC might be caused by a potentiation of the slight constitutive activation of G-protein pathways, which has been described for the Y_4R.[26] The dissociation constant of tBPC was determined with pK_B = 4.4 ± 0.18 (Table 1). Thus, these experiments revealed the PAM activity of tBPC to potentiate G_i-mediated signaling at the Y_4R.

It was recently shown that the arr3 pathway is important in the Y_4R signaling and internalization.[27] Therefore, arr3 recruitment was chosen as an alternative signaling pathway to investigate the PAM activity and potential signaling bias effects of tBPC at the Y_4R. The receptor−arr3 interaction was

quantified using a BRET assay system in HEK293 cells transiently expressing the Y_4R-Rluc8 and venus-arr3 fusion proteins.[27,28] Arr3 recruitment was investigated by two different approaches to examine ligand concentration response curves (Figure 3A−C) and the kinetics of Y_4R arr3 recruitment by continuous measurement (Figure 3D, E; Supporting Information Figure 2). The results of these kinetic experiments demonstrate that arr3 recruitment in response to ligand stimulation reached a stable equilibrium after approximately 15 min (Supporting Information Figure 2). Therefore, ligand concentration−response curves were detected as end point measurements after 30 min incubations. Treatment with tBPC increased the E_{max} of Y_4R arr3 recruitment for the activation by PP, NPY, and PYY. Furthermore, the compound had no effect on the potency of PP activation but slightly reduced the EC_{50} of NPY and PYY (Figure 3A−C, Table 2). These data reveal a PAM effect of tBPC predominantly on the maximum activation (E_{max}) in the Y_4R arrestin pathway, which was confirmed in the kinetic BRET experiments (Figure 3). Furthermore, the kinetic measurements allowed the quantification of the arr3 recruitment rate K (in [NetBRET min^{-1}]), which was significantly increased in the presence of 30 μM tBPC for all three ligands (Figure 3D,E; Supporting Information Figure 2). Remarkably, the arr3 recruitment rates for NPY and PYY were increased to K-values of 1.0 NetBRET min^{-1}, which was the same rate reached for high concentrations of the native ligand PP (100 nM), probably representing the maximal Y_4R activation in this assay.

The differences observed in tBPC's modulation of G-protein activation versus arrestin3 recruitment, especially with respect to the intrinsic agonism of tBPC and the potency of PP, NPY, and PYY (Table 2), could be caused by the different mechanisms underlying these pathways. In contrast with the rapid G-protein activation, arr3 recruitment after receptor activation is much slower and dependent on GRK (G-protein-coupled receptor kinase) phosphorylation of motifs in the C-terminal tail of the receptor, which regulate the internalization and desensitization of the Y_4R.[27] Importantly, the stimulation of the Y_4R by tBPC (30 μM) alone did not induce internalization of the receptor (Supporting Information Figure 4), corroborating the results of the BRET assay and ensuring the preservation of the arr3 signal sensitivity. In addition, the different sensitivity of the Ca^{2+} flux (G-protein activation) and the BRET (arr3 recruitment) assay systems might contribute to the differences in tBPC modulation pattern. Agonism of positive allosteric modulators is predominantly detected in sensitive assays that measure intracellular second messengers directly (cAMP, Ca^{2+} flux) or kinase phosphorylation (ERK1/2), as extensively shown for PAMs of various GPCRs.[19,29,30] Under conditions of low stimulus-response coupling efficacy or low tissue sensitivity (i.e., low receptor expression), the agonism of an allosteric ligand might not be apparent and the allosteric modulation would mainly be observed in the potency and efficacy of the signal response.[19,31−33] Overall, arr3 recruitment assays displayed a slightly stronger PAM effect of tBPC for the lower affinity ligands NPY and PYY compared to PP, which is consistent with the higher cooperativity ($\alpha\beta$) of tBPC observed in the Ca^{2+}-flux assays (Table 1). These probe dependent effects have been described for a variety of GPCRs.[32,34,35] However, these results raise the question of whether the PAM activity of tBPC could cause an unnatural activation of the Y_4R by NPY or PYY in vivo and what might be the consequences of this. So far, data are lacking for a
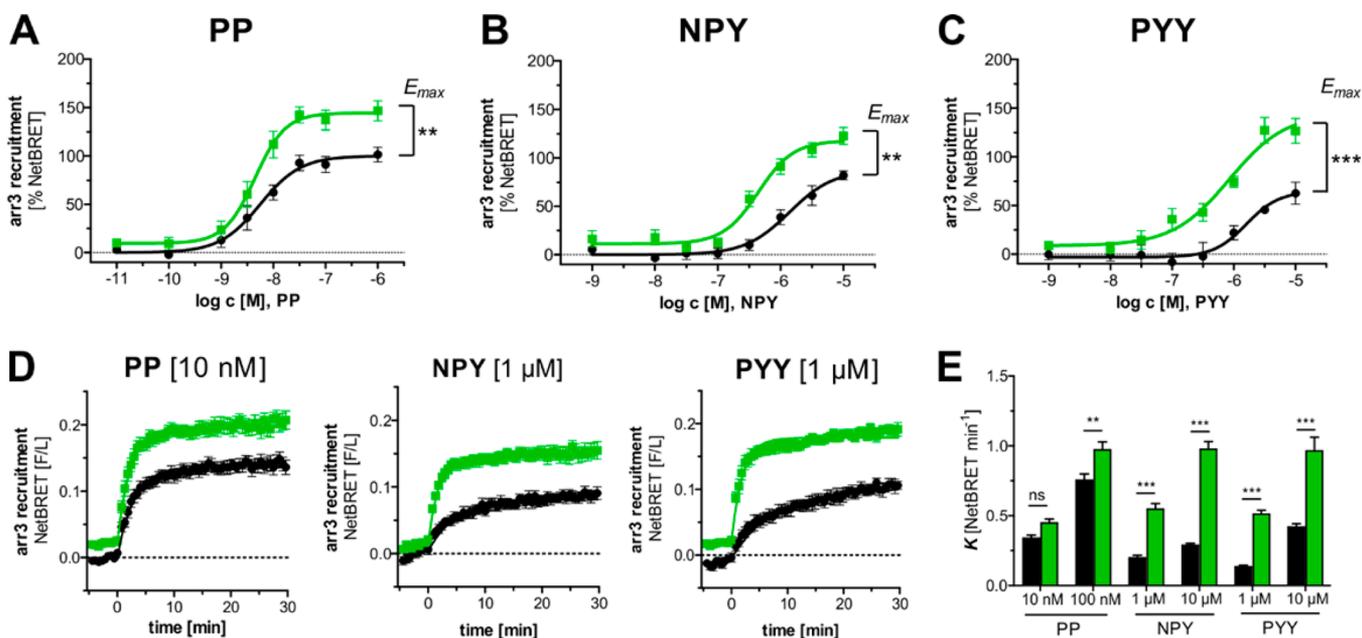
Figure 3. Potentiation of Y_4R−arrestin3 recruitment by tBPC. Arrestin3 (arr3) recruitment was measured using a BRET assay in transiently transfected HEK293_Y_4R-Rluc8_venus-arr3 cells in the presence of 30 μM tBPC (green box) or DMSO control (black dot). (A−C) Concentration−response curves after 30 min ligand stimulation. (D) Representative traces of kinetic measurements and (E) determined rate parameters K (NetBRET min^{-1}) of Y_4R arr3 recruitment. Data show the mean ± SEM from four to six independent experiments. Statistical analysis was performed using two-way ANOVA and Bonferroni's post-test (∗∗)$P < 0.01$; (∗∗∗) $P < 0.001$.

Table 2. Biological Data for Y_4R Modulation by tBPC[a]

	vehicle (DMSO)		tBPC, 30 μM	
ligand	EC_{50} [nM] (pEC_{50} ± SEM)	E_{max} ± SEM [%]	EC_{50} [nM] (pEC_{50} ± SEM)	E_{max} ± SEM [%]
	G-Protein Activation[b]			
PP	0.1 (−10.0 ± 0.04)	100 ± 3	0.03 (−10.4 ± 0.22)	89 ± 4
NPY	5.2 (−8.3 ± 0.04)	100 ± 5	0.4 (−9.4 ± 0.23)	94 ± 5
PYY	2.6 (−8.6 ± 0.08)	100 ± 7	0.5 (−9.3 ± 0.28)	95 ± 7
	Arrestin3 Recruitment[c]			
PP	5.6 (−8.3 ± 0.12)	100 ± 6	4.5 (−8.4 ± 0.09)	144 ± 6∗∗
NPY	1380 (−5.9 ± 0.15)	88 ± 12	443 (−6.4 ± 0.09)	118 ± 7∗∗
PYY	1600 (−5.8 ± 0.20)	66 ± 14	845 (−6.1 ± 0.20)	145 ± 20 ∗∗∗

[a]All data were normalized to the native ligand PP. Statistical analysis was performed using two-way ANOVA and Bonferroni's post-test ((∗∗) $P < 0.01$; (∗∗∗) $P < 0.001$). [b]G-protein activation was measured using a Ca^{2+}-flux assay. [c]Arr3 recruitment was quantified using a BRET assay.

physiological role of NPY or PYY at the Y_4R. In addition to the peripheral expression of the Y_4R in the gastrointestinal tract, the receptor is also expressed in central regions, in the brainstem and the arcuate nucleus.[36] A potential role of NPY on the activation of central Y_4R is possible; since NPY is highly expressed in the brain, colocalization with the Y_4R might occur and the activation of hypothalamic Y_4R was shown to be important for the anorexigenic effect of PP.[2,11] PYY, similar to PP, is predominantly expressed in gastrointestinal regions and released into the circulation after food intake to induce anorexigenic functions.[37,38] Taken together, if tBPC potentiation results in Y_4R activation centrally by NPY or peripherally by PYY, the desired effect of the compound on food intake and finally weight loss may be strengthened. While this possibility is

intriguing, additional in vivo studies are needed to test this hypothesis. However, it is not clear whether the levels of the less potent agonists NPY and PYY are high enough to activate the Y_4R in situ. Nevertheless, potentiation of less potent ligands in a multiligand system has to be considered in the characterization of allosteric modulators in drug discovery.[39]

tBPC Has No Effect on Y_4R Ligand Affinity. The mechanism of the PAM activity was established by radioligand binding assays to examine the relation between modulation of signaling and peptide ligand binding affinity. In these experiments, ^{125}I-PP was used as an orthosteric Y_4R radioligand with membrane preparations from stably transfected HEK293_Y_4R-eYFP cells. The results of equilibrium binding studies demonstrate that increasing tBPC concentrations, up to 60 μM, had no effect on the binding of ^{125}I-PP to the Y_4R (Figure 4A). The lack of a competitive behavior of tBPC to ^{125}I-PP reveals that the compound has no affinity to the orthosteric binding site, which indicates an allosteric mechanism of tBPC. To exclude the possibility that tBPC activity is affected by the ^{125}I-modification of PP in the radiotracer, competition experiments were performed with the unmodified peptides. In these experiments, 60 pM ^{125}I-PP was displaced by increasing concentrations of unlabeled peptide ligands in the presence of vehicle (DMSO 0.3%) or 30 μM tBPC (Figure 4B−D). The compound had no influence on the pK_i values of PP (DMSO 10.2 ± 0.1; tBPC 10.2 ± 0.1), NPY (DMSO 7.9 ± 0.2; tBPC 8.2 ± 0.2), or PYY (DMSO 8.0 ± 0.2; tBPC 8.2 ± 0.2). Furthermore, tBPC had no effect on the binding kinetics of ^{125}I-PP to the Y_4R membrane preparations in association binding experiments (Supporting Information Figure 3B). These results demonstrate that tBPC acts through an allosteric modulation of the Y_4R activation in terms of signaling efficacy rather than ligand binding affinity.

The ability of allosteric ligands to modulate the signaling efficacy and ligand affinity of GPCRs in an independent manner
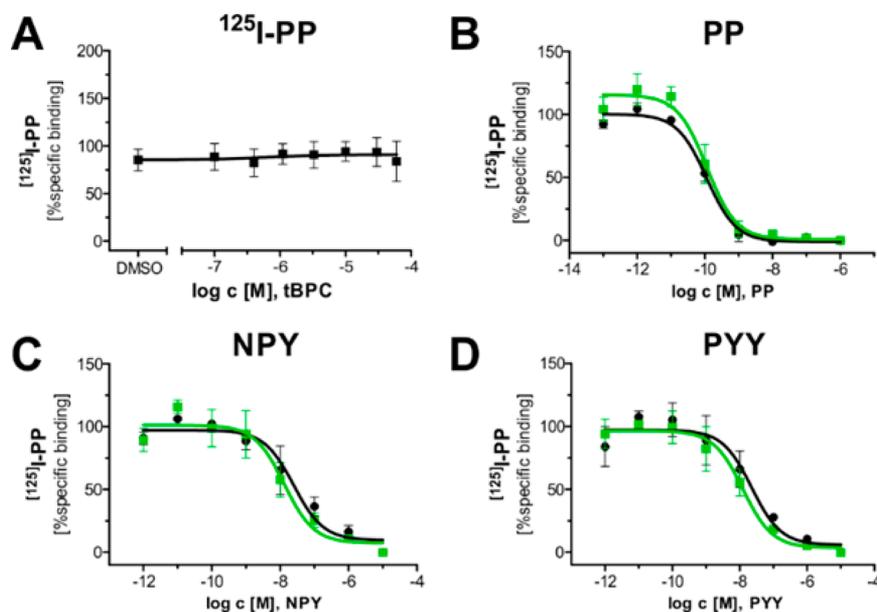
Figure 4. Ligand affinity of Y_4R membrane preparations is not altered by tBPC: (A) equilibrium binding of 20 pM ($0.3K_D$) ^{125}I-PP in the presence of increasing concentrations of tBPC; (B−D) ^{125}I-PP (60 pM) competition binding of PP (B), NPY (C), and PYY (D) in the presence of 30 μM tBPC (green box) vs DMSO (black dot). Data represent the mean $\pm$ SEM from three independent experiments.

has been shown for different compounds and receptor families. Similar to the activity of tBPC at the Y_4, PAMs of the GLP_1R (compound **2**) and the $GABA_B$ receptor (CGP7930) potentiate receptor downstream signaling, without affecting the ligand binding.[39,40] However, allosteric modulators of the CB_1R increase ligand binding but decrease receptor signaling in activation assays, showing the diversity of allosteric effects on GPCRs.[41]

PAM Activity in a Native Y_4R Expressing Tissue Preparation. Functional evidence that tBPC modulation of the Y_4R might be effective in native tissue was revealed by monitoring rat PP responses in mouse descending colon mucosa (Figure 5A). This tissue endogenously expresses the Y_4R, and electrophysiological measurement of vectorial ion transport (measured as changes in short-circuit current, I_{sc}) has shown that rat PP responses are solely mediated through Y_4R.[42,43] In mucosal preparations tBPC had no effect alone (Figure 5B) in contrast with the agonistic activity of tBPC in transfected systems (e.g., Ca^{2+} flux in engineered, over-expressing cell lines, Figure 2). However, tBPC at 30 μM significantly increased the mucosal antisecretory response to PP (at 10 nM), confirming the compound as a Y_4R PAM. These investigations further support the selectivity of tBPC because Y_1R and Y_2R are also expressed in mouse colon mucosa,[42,43] but treatment with tBPC had no effect alone or on subsequent PYY responses or indeed on VIP secretory activity (Figure 5C).

■ CONCLUSION

Only a few allosteric agonists or PAMs are published for peptide GPCRs, compared to the variety of positive and negative allosteric modulators (NAMs) for small molecule GPCRs.[34,44−48] In this study, we present the identification of tBPC, a novel and highly selective PAM of the human Y_4R, a peptide GPCR important in the regulation of food intake and energy metabolism. The characterization of the activity of tBPC in G-protein activation and arrestin recruitment experiments revealed that the positive allosteric modulation of the Y_4R by tBPC is based on a potentiation in signaling efficacy, while
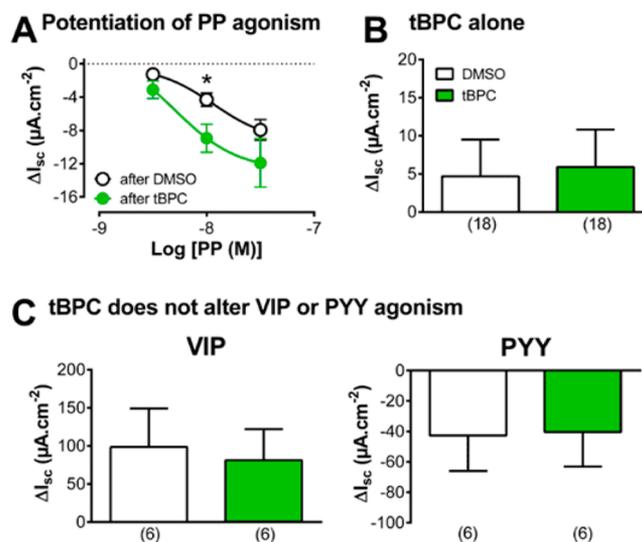
Figure 5. Specific potentiation of rat PP agonism in mouse descending colon mucosa by tBPC. (A) Effect of tBPC (30 μM) on PP responses in colonic mucosa that endogenously express mY_4R. (B) shows the effect of tBPC (30 μM) alone vs its DMSO vehicle control (0.3%). (C) tBPC (30 μM) had no significant effect on either VIP (vasoactive intestinal polypeptide, 10 nM) or subsequent PYY (10 nM) responses. Values are the mean $\pm$ 1 SEM from five to six different colonic specimens (in part A), and n numbers are shown in parentheses in parts B and C. (∗) $P < 0.05$ using Student's t test.

ligand affinity remains unaltered. The positive allosteric activity and cooperativity ($\alpha\beta$) of tBPC on the Y_4R are similar to the activity of PAMs for the GLP_1R, which show in vivo efficacy in the modulation of insulin secretion (BETP) and neuro-protection (compound **2**).[39,49,50] Together with the tBPC-induced potentiation of PP responses in mouse colon mucosal preparations, this compound represents the most active and selective PAM for the Y_4R so far and could be used as an in vivo probe for the investigation of this GPCR as a drug target.

■ EXPERIMENTAL SECTION

Ligands. (4-*tert*-Butyl)phenoxycyclohexanol (tBPC) was purchased from HPC Standards (>99% purity). Peptide ligands human PP, porcine NPY, and human PYY were synthesized by solid phase peptide synthesis using Fmoc (fluorenylmethyloxycarbonyl) strategy in a purity of >95%, as described previously.[51] Radioligand human ^{125}I-PP was purchased from PerkinElmer (NEX315). Rat PP (Bachem) was used in mouse colon functional studies.

Plasmids. The cDNA of human Y_1R, Y_2R, Y_4R, and Y_5R was prepared as fusion proteins with a C-terminal eYFP (enhanced yellow fluorescent protein) and cloned into a pVitro2-hygro-mcs vector (Invivogen) as described before.[51] For BRET studies, the pcDNA3 vectors with Y_4R C-terminally fused to a renilla luciferase (Y_4R-Rluc8) and bovine arr3 tagged with a venus fluorescent protein (venus-arr3) were used, as recently described.[52] Coding sequence of the $\Delta 6G_{\alpha qi4-myr}$ was kindly provided by E. Kostenis and cloned in a pVitro2-mcs-neo vector.[53]

Cell Culture. COS7_$Y_{1,2,4,5}$R-eYFP_$\Delta 6G\alpha_{qi4-myr}$ cells were generated through co-transfection of COS7 cells with h$Y_{1,2,4,5}$R-eYFP (pVitro2-mcs-hygro) and $\Delta 6G\alpha_{qi4-myr}$ (pVitro2-mcs-neo) and cultured as described before.[52] The HEK293_Y_4R-eYFP cell line was created and cultured in DMEM/Hams F12 supplemented with 15% FBS and 100 μg/mL hygromycin.[54] HEK293 cells were cultured in DMEM/Hams F12 (Lonza) supplemented with 15% FBS (Lonza). Cell culture was performed at humidified atmosphere at 37 °C and 5% CO_2.

Calcium Flux Assay. Ca^{2+} flux screening assays to identify Y_4R PAMs were performed in a 384-well format using Panoptic (WaveFront Biosciences) as described.[22] Further characterization and Y receptor selectivity studies were performed in a 96-well format. Ca^{2+} flux assays were performed with stably transfected COS7_$Y_{1,2,4,5}$R_$\Delta 6G\alpha_{qi4-myr}$ cells. Cells were seeded in black 96-well plates and incubated overnight. For the assay, cells were incubated with 2.4 μM Fluo2-AM (Abcam) in assay buffer (HBSS (Lonza), 20 mM HEPES (Sigma), 2.5 μM probenecid (Sigma-Aldrich)) for 60 min at 37 °C. Dye solution was aspirated and replaced by assay buffer for the measurement. Fluorescence readout (excitation 485 nm/emission 525 nm) was performed using the FlexStation 3 (Molecular Devices) and a two-addition protocol. Compounds were added after 20 s baseline detection, followed by addition of peptide solutions after 140 s. Signal response was quantified as x-fold over basal and normalized to the control response in the presence of DMSO instead of compound.

Arrestin3 Recruitment Assay. HEK293 cells were grown to 80–90% confluency in 75 cm^2 cell culture flasks and co-transfected overnight with 1 μg of Y_4R-Rluc8 and 11 μg of venus-arr3 using Metafectene Pro (Biontex) according to manufacturer's protocol. The transfection solution was aspirated, and cells were washed with PBS (Lonza) and detached using trypsin/EDTA (Lonza). Cells were resuspended in phenol-red free medium, seeded in white 96-well plates (40 000 cells/well) and incubated overnight. For the BRET measurements, medium was aspirated and replaced with assay buffer (HBSS (Sigma), 25 mM HEPES (Sigma), pH 7.2) containing test compound or DMSO control. Coelenterazine H (DiscoveRx) was added (4.2 μM final concentration), followed by addition peptide solution and BRET measurement in a microplate reader (Tecan Infinite M200) using filter sets Blue1 (luminescence 370–480 nm) and Green1 (fluorescence 520–570 nm) at 37 °C. For kinetic measurements, the basal signal (5 min) was measured after addition of coelenterazine H, followed by 30 min continuous measurement after ligand addition. Full ligand concentration response curves were measured after 30 min incubation at 37 °C.

Y_4R Membrane Preparations and Radioligand Binding Assays. Y_4R membranes were prepared from stably transfected HEK293_Y_4R-eYFP cells. Cells were suspended in PBS and separated by centrifugation at 1800 rpm, 4 °C for 5 min. The cell pellet was resuspended in Tris buffer (50 mM Tris (Sigma), 50 μM Pefabloc (Sigma), pH 7.5), homogenized in a potter grinder (Potter SB Braun), and centrifuged at 2400 rpm at 4 °C for 20 min. Pelleted fractions were discarded, and the supernatant was centrifuged at 12.000 rpm, 4 °C for 60 min. The resulting crude membrane pellet was resuspended

in HEPES-buffer (25 mM HEPES, 25 mM $CaCl_2$ (Fluka), 1 mM $MgCl_2$ (Fluka), 50 μM Pefabloc (Sigma), pH 7.4) and homogenized using the potter grinder, followed by a centrifugation at 12.000 rpm, 4 °C for 60 min. Membrane pellets were resuspended in HEPES buffer. Protein concentration was determined with the Bradford method.[55] Finally, membrane suspensions were frozen in liquid nitrogen and stored at −20 °C. For binding experiments, 0.3 μg of Y_4R membrane preparation was prepared in HEPES buffer containing vehicle (DMSO) or 30 μM tBPC. Peptide and radioligand (^{125}I-PP) solutions were prepared in aqua dest. containing 0.1% BSA (PAA Laboratories). Radioligand solution was added to all samples. Nonspecific binding was determined for each condition in the presence of 1 μM PP. Samples were incubated for 5 h at room temperature while shaking (200 rpm). Membrane bound ^{125}I-PP species were separated by filtration through GFC filter (PerkinElmer), presoaked with 0.1% polyethylenimine (Sigma) using a MicroBeta 96-well filtermate harvester (PerkinElmer). Membranes were washed with cold PBS and dried for 10 min at 55 °C. MeltiLex scintillation sheets (PerkinElmer) were melted on the membranes, and radioactivity was quantified using a MicroBeta scintillation counter (PerkinElmer).

Electrophysiological Measurement of PP Responses in Mouse Descending Colon Mucosa. Measurement of vectorial ion transport as changes in short-circuit current (I_{sc}) was performed by placing colonic mucosal preparations in Ussing chambers (DVC1000; WPI, Sarasota, FL) as previously described.[42,43] Preparations with exposed areas of 0.14 cm^2 were bathed in oxygenated Krebs−Henseleit solution at 37 °C and voltage-clamped at 0 mV. The resulting I_{sc} was recorded continuously. A stable basal I_{sc} was reached within 20−30 min, after which peptide and tBPC additions were made to the basolateral reservoir. To investigate the effect of tBPC, tissues were pretreated with 30 μM compound or vehicle (DMSO) for 5 min. Tissues then received the well-known secretagogue VIP (10 nM) for 5−10 min to raise epithelial cAMP and consequently I_{sc} levels, thus optimizing subsequent Y_4R-mediated antisecretory responses to single PP additions (at 3, 10, or 30 nM). After a further 20 min, PYY (10 nM) was added as an internal control as these antisecretory responses are known to be Y_1R- and Y_2R-mediated in mouse descending colon.[42,43]

Data Analysis. Data analysis and calculation of concentration−response curves were performed using GraphPad Prism 5.0 software. Global fitting analysis and quantification of allosteric effect of Ca^{2+} flux data were performed using an operational model of allosterism.[25] Calculation of arr3 recruitment rates K was performed using the one-phase association equation. Each signaling experiment was performed in duplicate and at least three times independently.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.7b00976.

> Figure 1 showing selection and screening of tBPC similar compounds; Figure 2 showing tBPC modulation of Y_4R arr3 recruitment kinetics; Figure 3 showing ligand binding control experiments; Figure 4 showing Y_4R internalization and fluorescence microscopy, and section S5 containing fluorescence microscopy information (PDF)
>
> Molecular formula strings and some data (CSV)

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Author Contributions

M.S. designed, performed, and analyzed characterization experiments and wrote the manuscript. I.R.T. and H.M.C. designed, performed, and analyzed electrophysiological measurements. A.G.B.-S. and J.M. designed experiments and analyzed data. C.D.W., Y.D., J.S., and G.S. designed, performed, and analyzed data of primary HTS for compound identification. C.D.W. is an owner of WaveFront Biosciences. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

COS7, African green monkey cells; mGluR, metabotropic glutamate receptor; GRK, G-protein-coupled receptor kinase; GLP_1R, glucagon-like peptide receptor 1; HEK293, human embryonic kidney cells; HTS, high throughput screening; NAM, negative allosteric modulator; NPY, neuropeptide Y; PAM, positive allosteric modulator; PP, pancreatic polypeptide; PYY, peptide YY; tBPC, *tert*-butylphenoxycyclohexanol; VIP, vasoactive intestinal peptide; $Y_{1,2,4,5}R$, human Y receptor subtype 1/2/4/5

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