Multiplex G Protein–Coupled Receptor Screen Reveals Reliably Acting Agonists and a Gq-Phospholipase C Coupling Mode of GPR30/GPER1

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ABSTRACT

G protein–coupled receptors (GPCRs) constitute the most versatile family of pharmacological target proteins. For some “orphan” GPCRs, no ligand or drug-like modulator is known. In this study, we have established and applied a parallelized assay to coscreen 29 different human GPCRs. Three compounds, chlorhexidine, Lys-05, and 9-aminocadidine, triggered transient Ca²⁺ signals linked to the expression of GPR30. GPR30, also named G protein–coupled estrogen receptor 1 (GPER1), was reported to elicit increases in cAMP in response to 17β-estradiol, 4-hydroxytamoxifen, or G-1. A thorough analysis of the activated signaling cascade revealed a canonical Gq-coupled pathway, including phospholipase C, protein kinase C and ERK activation, receptor internalization, and sensitivity to the Gq inhibitor YM-254890. When expressed in different cell lines, the localization of a fluorescent GPR30 fusion protein appeared variable. An efficient integration into the plasma membrane and stronger functional responses were found in HEK293 and in MCF-7 cells, whereas GPR30 appeared mostly retained in endomembrane compartments in Cos-7 or HeLa cells. Thus, conflicting findings may result from the use of different cell lines. The newly identified agonists and the finding that GPR30 couples to Gq are expected to serve as a starting point for identifying physiologic responses that are controlled by this GPCR.

SIGNIFICANCE STATEMENT

This study has identified and thoroughly characterized novel and reliably acting agonists of the G protein–coupled receptor GPER1/GPR30. Applying these agonists, this study demonstrates that GPR30 couples to the canonical Gq-phospholipase C pathway and is rapidly internalized upon continuous exposure to the agonists.

Introduction

Representing one of the largest and most versatile groups of pharmacologically addressable target proteins, nonolfactory G protein–coupled receptors (GPCRs) are among the most intensively studied signaling proteins in mammals (Sriram and Insel, 2018). Thus, tremendous efforts have been undertaken to identify physiologic and pharmacologic modulators of hitherto “orphan” GPCRs, with many substantial successes being reported within the last decades (Laschet et al., 2018; Hauser et al., 2020). Nonetheless, almost 100 orphan GPCRs are still awaiting the unequivocal assignment of cognate agonists and physiologic and pathophysiological functions governed by them. There are also “deorphanized” GPCRs whose assigned agonists or sites of expression are not reliably reproducible by other laboratories (Laschet et al., 2018), leading to partly confusing results pertaining to postulated receptor functions and proposed benefits of their modulation by pharmacological intervention. Finally, although not deorphanizing GPCRs by identifying a physiologic agonist, screening activities with or without prior virtual in silico prescreening have been instrumental in identifying drug-like compounds that exert agonistic effects or an inverse agonism toward individual orphan GPCRs.

We have established a Ca²⁺ influx-based academic screening infrastructure to identify cation channel-modulating activities in various compound libraries, comprising Food and Drug Administration–approved drugs, bioactive natural compounds, toxins, and chemically diverse drug-like compounds. To expand the range of investigated target structures, we conducted a first screening on GPR34, a GPCR whose deorphanization as a lysophosphatidyl-L-serine receptor (Sugo et al., 2006) has been questioned (Ritscher et al., 2012). Although screening results were valid, this screen failed to

ABBREVIATIONS: [Ca²⁺], intracellular Ca²⁺ concentration; CFP, cyan fluorescent protein; DAG, diacylglycerol; EPAC, exchange protein activated by cAMP; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FRET, Förster resonance energy transfer; GPCR, G protein–coupled receptor; GPER1, G protein–coupled estrogen receptor 1; HBS, HEPES-buffered saline; InsP3, inositol-1, 4, 5-trisphosphate; PIP2, phosphatidylinositol-4, 5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PTX, pertussis toxin; YFP, yellow fluorescent protein.
identify novel modulators. We therefore decided to explore the possibility to set up a highly parallelized coscreening that may become more successful in identifying novel agonists of orphan or poorly validatedGPCRs in an unbiased fashion.

Typically, a high-throughput screening for GPCR agonists or antagonists requires the generation of a cell line that stably expresses a recombinant GPCR of interest, either alone or together with promiscuously coupling Gz15/16 subunits or with chimeric G-protein α subunits that form a complex with endogenously expressed β/γ subunits. Gz15/16 or chimeric Gαq/11 subunits can be activated by GPCRs even if the receptor would otherwise couple to the Gi or Gs families of heterotrimeric G proteins (Liu et al., 1995; Offermanns and Simon, 1995). At the effector side, Gz15/16 or chimeric Gαq/11 subunits activate phospholipases C (PLC), giving rise to formation of inositol-1,4,5-trisphosphate (InsP3) and Ca2+ release via InsP3 receptors that can be detected by means of fluorescent indicator dyes with exceptionally high signal-to-noise ratio (Kostenis et al., 2005). In preliminary cotransfection experiments, we added increasing numbers of GPCR-encoding plasmid constructs to transfection mixes that also contained expression plasmids that encode Gα15 and Gα16. Since functional signals were robustly detectable in a 384-well screening format with up to 15 coexpressed GPCR constructs, including Gi-coupling FP1 for mylmal peptide receptor, YFP-tagged ETB endothelin receptor, and Gα15/Gα16-encoding cDNA plasmids using the transfection reagent Lipofectamin 2000 (Invitrogen, Thermo Fisher Scientific). The total cDNA plasmid amount per transfection was 9.6 or 10.2 μg, and each single plasmid construct was added at an amount of 600 ng or 300 ng (GPER1, GPR4, GPR12, GPER1, Gα15, Gα16). A reduced cDNA amount was selected for the four GPCR isoforms because they suppressed signals of control receptors when transfected at higher amounts. For focal laser-scanning microscopy and single-cell intracellular Ca2+ concentration ([Ca2+]i) imaging experiments, cells were plated onto 25-mm poly-L-lysine–coated glass coverslips. If required, these cells were transiently transfected with 2 μg of the respective pcDNA construct applying jetPEI Polypus (PEQLAB, Erlangen, Germany) 24 hours after cell plating. To obtain a stably human GPR30-expressing HEK293 cell line (HEK293;GPR30-YFP), cells were transiently transfected, and the growth medium was supplemented with 1 mg/ml geneticin. Stably transfected colonies were generated with a limiting dilution method and verified by fluorescence microscopy and functional assays. All cells were maintained at 37°C and in a 5% CO2-aerated, humidified atmosphere.

**Fluorometric [Ca2+]i Imaging**. All fluorometric Ca2+ assays were performed in HEPES-buffered saline (HBS), containing 132 mM NaCl, 6 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 5.5 mM d-glucose, and 10 mM HEPES, adjusted to pH 7.4 with NaOH. For measurements in a Ca2+-free buffer, we used a modified HBS in which CaCl2 was omitted, and 200 μM EGTA were added before readjusting the pH.

For the primary screen, to generate concentration-response curves or in multwell assays applying various GPCR signaling inhibitors, a custom-made fluorescence imaging plate reader built into a robotic liquid-handling station (Freedom Evo 150, Tecan, Männedorf, Switzerland) was used as previously described (Häfner et al., 2019). To this end, transiently or stably transfected HEK293 cells were detached with trypsin and loaded with 4 μM fluo-4/AM (Invitrogen) in HBS, containing 1% bovine serum albumin for 30 minutes at 37°C. Cells were washed by centrifugation, resuspended in HBS, and dispensed into pigmented, clear-bottom 384-well plates (Greiner, Frickenhausen, Germany). After mounting plates onto the fluorescence imaging plate reader, fluorescence signals were continuously recorded with a Zyla 5.5 camera (Andor, Belfast, UK) and under the control of Micromanager software (Edelstein et al., 2016). Fluo-4 was excited at 480–490 nm by using a projection of a light pipe–homogenized array of light-emitting diodes, and emission was detected through a 515-nM long-pass filter. After recording an initial baseline in each experiment, compounds of Selleckchem library or serially prediluted agonists or modulators were pipetted
with a 96-tip multichannel arm (MCA96, Tecan) in 4 quadrant steps (Q1 to Q4). Finally, fluorescence intensities were calculated from image stacks for each single well with ImageJ software, corrected for background signals and normalized to the initial intensities (F/F<sub>0</sub>). Generation of concentration-response curves was done by fitting Hill equations (minimal effect E<sub>min</sub>, maximal effect E<sub>max</sub>, EC<sub>50</sub>, and Hill coefficient n) to the data.

Single-cell [Ca<sup>2+</sup>] imaging analyses were performed on an inverted epifluorescence microscope (Carl Zeiss, Jena, Germany) and calibrated as described (Lenz et al., 2002). To this end, HEK293, COS-7, HeLa, or MCP-7 cells seeded onto 25-mm coverslips were loaded with 4 μM fura-2/AM (AAT Bioquest) for 30 minutes at 37°C in HBS, containing 0.2% bovine serum albumin. Then, cells were rinsed, and coverslips were mounted in an HBS-filled bath chamber and sequentially excited at wavelengths of 340 nm, 385 nm, and 390 nm. Fluorescence emission was detected at 505–550 nm with a cooled charge-coupled device camera. After baseline recording, chlorhexidine, Lys05, 9-aminoacridine, or thapsigargin were added, and Ca<sup>2+</sup> signals were monitored. Note, when 9-aminoacridine was applied, cells were pressed as free-floating cells with a 100x/1.46 alpha Plan-Apochromat objective but on an inverted epifluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a 25x/0.8 Plan-Neofluar objective. Fluorescence was excited at 410 nm, and images were sequentially obtained through emission bandpass filters at 450–490 nm and 530–600 nm mounted in a motorized filter wheel (Lambda 10-2, Sutter Instruments, Novato, CA). For analysis, emission ratios of background-corrected signals were calculated and finally normalized to initial signals. To determine statistical differences after the addition of chlorhexidine, Lys05, or EGF in comparison with basal FRET signals, photobleaching was corrected by fitting the data of unstimulated controls to a single exponential decay function.

Results

Identification of Novel GPR30 Agonists by a Multi-plexed GPCR Screen. To identify novel agonists that act on orphan or poorly characterized GPCRs, we simultaneously transfected HEK293 cells with two groups of various GPCRs (Fig. 1A) and subjected them to a medium-throughput Ca<sup>2+</sup>-assay, applying 4770 compounds of the Selleckchem bioactive compound library. The two distinct transfection mixes were carried out to further increase the number of screened target structures and also served as a controlscreen to omit compounds that exert off-target effects or stimulate receptors and ion channels expressed by the parental HEK293 cell line. Since some of the orphan GPCRs might not couple to G proteins that activate PLC and Ca<sup>2+</sup> release from the endoplasmic reticulum (ER), we additionally cotransfected the cells with the murine G protein α subunit G<sub>12</sub> and its human counterpart G<sub>14</sub>. As previously described, both G<sub>12</sub> and G<sub>14</sub> both only redirect receptor signaling of G<sub>x</sub> or G<sub>y</sub>-coupled receptors to PLC activation and subsequent Ca<sup>2+</sup> mobilization but also enhance PLC stimulation mediated by G<sub>q</sub> or G<sub>12</sub>-coupled GPCRs (Offermanns and Simon, 1995). To control for the efficiency of G<sub>12</sub>/G<sub>14</sub>-dependent conversion of signaling toward Ca<sup>2+</sup> signals, we added the cDNA encoding the FP<sub>1</sub> formyl peptide receptor and the YFP-tagged ET<sub>6</sub> endothelin receptor as positive controls into each transfection mix. The screening was valid when transfected HEK293 cells expressed the YFP-fused ET<sub>6</sub> receptor, and each transfected and fluo-4/AM-loaded cell batch responded with a substantial Ca<sup>2+</sup> signal upon the addition of 10 nM endothelin-1 and 100 nM fMLP (data not shown). During acute addition of compounds of the Selleckchem library at a final concentration of 20 μM to cells that were transfected with mixture 1 (Fig. 1, B–E), we recorded GPCR-characteristic immediate and transient fluorescence signals in wells that received chlorhexidine, Lys05, or 9-aminoacridine. This prompted us to identify the cDNA plasmids in transfection mixture 1 that confer the responsiveness to these compounds. After two rounds of generating cDNA mixes with lower complexity of added GPCR- and G protein–encoding plasmids, we revealed the single human GPER1-encoding plasmid to correlate with the responses to all three compounds independently of coexpressed G<sub>12</sub>/G<sub>14</sub>.
At a concentration of 10 μM, none of the three ligands caused a substantial Ca$^{2+}$ mobilization in cells that expressed any of the other coscreened receptors in the presence or in the absence of coexpressed Gα15 and Gα16. Interestingly, published GPR30 agonists such as 17β-estradiol, 4-hydroxytamoxifen, fulvestrant, hydroxytyrosol, quercetin, oleuropein, niacin, or niacin amide, which were all included in the used compound library, did not elicit agonist-like properties during the primary screening.

**Validation of Chlorhexidine, Lys05, and 9-Aminoacridine as GPR30 Agonists.** For hit validation, we subcloned the purchased cDNA of human GPER1 into a custom-made pcDNA3-YFP vector to visualize the receptor expression and its cellular localization by monitoring the YFP fluorescence of the C-terminally tagged fusion protein. The generated plasmid construct (hGPR30-YFP) was used to generate a stably transfected HEK293 cell line (HEK293hGPR30-YFP). The major subcellular localization of heterologously expressed hGPR30 seemed to be in the plasma membrane, with minor amounts of the YFP-tagged receptor protein residing in the endoplasmic reticulum as detected by confocal laser-scanning microscopy in living cells (Fig. 2A).

To assess the potency and efficacy of the newly identified GPR30 agonists, we loaded HEK293hGPR30-YFP with fluo-4/AM and measured the Ca$^{2+}$ responses in our fluorescence imaging plate reader during the application of serially diluted agonist concentrations. Lys05 exerted the highest potency, with an EC$_{50}$ of 270 nM, followed by the most efficient agonist chlorhexidine (EC$_{50}$ 5750 nM). Since 9-aminoacridine is a strongly fluorescent dye, and to monitor for possible unspecific Ca$^{2+}$ signals, we also applied the three compounds to the parental HEK293 cell line. We did not obtain any detectable rise in [Ca$^{2+}$]i when we incubated these cells with chlorhexidine, Lys05, or 9-aminoacridine within a concentration range of 0.02–40 μM (Fig. 2B). Next, we imaged GPR30 activation in adherent single GPR30-overexpressing HEK293 cells to estimate the levels of [Ca$^{2+}$]i reached by the addition of 5 μM chlorhexidine and Lys05, with the latter reaching
somewhat lower maximal F/F₀ signals in multiwell assays. During stimulation, all cells responded with uniform and transient [Ca²⁺]ᵢ signals that peaked after 5–15 seconds and almost decayed to baseline levels within 120 seconds, thereby excluding toxic effects on the cells. With regard to peak [Ca²⁺]ᵢ, the higher efficacy of chlorhexidine compared with Lys05 was confirmed by the calibrated single-cell [Ca²⁺]ᵢ analysis. Single-cell Ca²⁺ imaging with 9-aminoacridine required the use of the indicator dye fluo-4 because the fluorescence excitation spectrum of 9-aminoacridine interferes with that of fura-2 (Fig. 2D). It should, therefore, be regarded as qualitative rather than quantitative data. When comparing concentration-response curves obtained in multiwell assays with fluo-4–loaded HEK293hGPR30-YFP cells, we found that F/F₀ values levels at saturating 9-aminoacridine concentrations ranged between those of chlorhexidine and Lys05. In parental HEK293 cells, 5 μM of the respective compounds failed to induce detectable increases in [Ca²⁺]ᵢ in cell suspensions or in single-cell assays (Fig. 2, B–F). To test whether the identified agonists may act in an allosteric manner in relation to each other, we generated concentration-response curves with and without the addition of different submaximally effective concentrations of the respective other agonists. In case of a positive allosteric modulation, we would expect a shift of half-maximally effective concentrations to lower values. In none of the tested combinations did we observe such signs of allosteric modulation (Supplemental Fig. 3).
partial inhibition of \( [\text{Ca}^{2+}] \) signals, which was also evident when applied in combination with chlorhexidine (Supplemental Fig. 3, A and C).

Since none of the previously reported GPR30 agonists appeared active in the primary multiplexed GPCR screening, we selected some relevant and most frequently used GPR30-activating drugs (Rosano et al., 2016) and generated concentration-response curves using our stably transfected HEK293\( _{\text{GPR30-YFP}} \) cell line as well as parental HEK293 cells. Surprisingly, only 4-hydroxytamoxifen and niacin amide led to an increase in fluorescence intensities when added at concentrations higher than 10 \( \mu \text{M} \) (Supplemental Fig. 4). Since they did so in HEK293\( _{\text{GPR30-YFP}} \) cells, and also in untransfected parental HEK293 cells, we consider them as presumably hGPR30-unrelated background signals. Other reported GPR30 agonists, including 17\( \beta \)-estradiol and G-1, which have been described to mobilize \( \text{Ca}^{2+} \) in MCF-7 and SKBr3 cells (Ariazi et al., 2010), were inactive in HEK293\( _{\text{GPR30-YFP}} \) cells as well as in parental HEK293 cells over the entire range of applied concentrations (Supplemental Fig. 4, A and B). If previously reported GPR30 agonists were biased to induce a Gq-coupling mode of the receptor, \( [\text{Ca}^{2+}] \) \( _{i} \) assays might require a Gq priming (Pfeil et al., 2020) or coexpression with the promiscuously coupling G proteins to detect a receptor activation by these agonists. However, neither Gq priming via stimulation of an endogenous muscarinic receptor with 100 \( \mu \text{M} \) carbachol nor coexpression of Gz15 and Gz16 led to \( \text{Ca}^{2+} \) signals upon challenging GPR30-expressing HEK293 cells with 17\( \beta \)-estradiol, G-1, or several other reported GPR30 agonists (Supplemental Fig. 4). Notably, the same Gq priming procedure or coexpression of Gz15 and Gz16 enhanced \( \text{Ca}^{2+} \) signals elicited via a Gq-coupled FMLP receptor.

**Inhibition of GPR30-Triggered \( \text{Ca}^{2+} \) Signals by Gq, PLC, and InsP\(_3\) Receptor Inhibitors.** Signaling of Gq/Gi-coupling GPCRs typically triggers a \( \text{Ca}^{2+} \) release from InsP\(_3\)-sensitive \( \text{Ca}^{2+} \) stores. To strengthen the assumption that chlorhexidine, Lys05, and 9-aminoacridine mediate \( \text{Ca}^{2+} \) release from InsP\(_3\)-sensitive stores, we repeated microfluorometric single-cell \( [\text{Ca}^{2+}] \) \( _{i} \) analyses using a \( \text{Ca}^{2+} \)-free bath solution. As expected, the \( \text{Ca}^{2+} \) mobilization response remained detectable under this condition, with response amplitudes of about two- to 2.5-fold compared with the respective basal levels (Fig. 3). Moreover, we depleted these stores by preincubating cells for 5 minutes with 2 \( \mu \text{M} \) thapsigargin, an inhibitor of endoplasmic reticulum \( \text{Ca}^{2+} \)-ATPases. In thapsigargin-treated HEK293\( _{\text{GPR30-YFP}} \) cells, chlorhexidine, Lys05, or 9-aminoacridine failed to elicit \( [\text{Ca}^{2+}] \) \( _{i} \) signals, indicating that their signaling critically relies on the canonical PLC- and InsP\(_3\)-dependent \( \text{Ca}^{2+} \) mobilization pathway. To characterize GPR30 signaling in more detail, we incubated HEK293\( _{\text{GPR30-YFP}} \) cells either with the Gq inhibitor YM-254890 (1 \( \mu \text{M} \)), the phospholipase C inhibitor U-73122 (10 \( \mu \text{M} \)) or its inactive analog U-73343 (10 \( \mu \text{M} \)), and with the InsP\(_3\) receptor inhibitor 2-APB (100 \( \mu \text{M} \)) for 5 minutes. Then, GPR30 was challenged with the three new identified agonists. In contrast to solvent-treated control cells, \( \text{Ca}^{2+} \) rises were prevented when applying the inhibitors. U-73343 showed a very slight reduction of the maximal control signal during activation by Lys05 or 9-aminoacridine (Fig. 4). Since GPR30-dependent \( [\text{Ca}^{2+}] \) \( _{i} \) signals were fully abrogated by the Gq inhibitor YM-254890, we assume that GPR30 activates phospholipase C, and \( \text{Ca}^{2+} \) mobilization at least in part via a Gq-coupling component. Since a combined Gq/Gi-coupling mode may be sensitive to YM-254890 as well (Pfeil et al., 2020), we used a pretreatment with pertussis toxin (PTX), which uncouples GPCRs from Gq/11 proteins and, thus, disrupts receptor signaling via Gq. To this end, we transiently cotransfected HEK293 cells with GPR30 and the FP\(_3\) and ET\(_B\) receptors as positive controls for exclusively (FP\(_3\)) or predominantly (ET\(_{1}\)) Gq-coupling receptors. The Gq-coupled muscarinic M\(_3\) acetylcholine receptor that is endogenously expressed in our HEK293 cell line served as a control for a PTX-resistant pathway. After treatment of cells with 100 ng/ml PTX or its solvent for 18–20 hours added to the culture medium, responses elicited by the GPCR agonists were analyzed in multiwell \( \text{Ca}^{2+} \) measurements. PTX-treated cells did not respond to 100 nM fMLP, and endothelin-1 (10 nM) signals were diminished by about 50% compared with untreated controls, thereby confirming the coupling behavior of pure Gq or mixed Gq/Gi coupling known for formyl peptide and endothelin receptor type B receptors. Activation of GPR30, applying either 5 \( \mu \text{M} \) chlorhexidine, Lys05, or 9-aminoacridine, as well as M\(_3\) receptor activation by 100 \( \mu \text{M} \) carbachol were unaffected by pretreatment with PTX (Fig. 5). We therefore conclude that hGPR30 genuinely couples to the Gq-signaling cascade when challenged with the newly identified agonists.

**Activation of GPR30 Results in Phosphoinositide Hydrolysis and Dic酰lglycerol Formation.** In general, agonist binding to a Gq-coupling receptor results in an immediate activation of phospholipases C that hydrolyze PIP\(_2\) to InsP\(_3\) and diacylglycerol (DAG). Since Revankar et al. (2005) described an inefficacy of PLC inhibitor U73122 on blocking GPR30-initiated calcium mobilization and thereby suggested a divergent signaling pathway, we extended the analysis of hGPR30 coupling to PLC, applying more direct methods. PIP\(_2\) hydrolysis and DAG formation can be monitored by subcellular translocation of well characterized biosensor proteins. The plasma membrane PIP\(_2\) content is reflected by the association of a CFP-fused pleckstrin homology domain of PLC-\( _{1} \) [CFP-PLC-\( _{1}(\text{PH}) \)] to the plasma membrane. Upon PLC-mediated PIP\(_2\) hydrolysis, CFP-PLC-\( _{1}(\text{PH}) \) translocates to the cytosol. Conversely, a translocation of CFP-tagged, DAG-sensitive novel protein kinase C \( \varepsilon \) isofrom (PKCe-CFP) from the cytosol to the plasma membrane indicates DAG formation by PLC.

We transfected a CFP-PLC-\( _{1}(\text{PH}) \)-encoding plasmid into HEK293\( _{\text{GPR30-YFP}} \) cells and imaged the CFP-PLC-\( _{1}(\text{PH}) \) distribution during the addition of chlorhexidine or Lys05 by confocal microscopy and by epifluorescence time-lapse microscopy (Fig. 6, A–F). Within a few seconds after agonist application, the plasma membrane association of CFP-PLC-\( _{1}(\text{PH}) \) was markedly reduced and partly recovered within the following 5 minutes. Measurements of DAG formation were also performed by a translocation assay. Accordingly, we tracked transiently transfected PKCe-CFP, which was recruited from the cytosol to the plasma membrane upon the application of chlorhexidine or Lys05 (Fig. 6, G–L). Finally, we transiently expressed CFP-PLC-\( _{1}(\text{PH}) \) and PKCe-CFP in parental HEK293 and repeated the experiments. None of the proteins or agonists exerted detectable changes in the distribution of the biosensor proteins in the absence of GPR30 expression, whereas stimulation of a cotransfected histamine H\(_1\) receptor served as a positive control (Supplemental Fig. 5).
Cyclic Adenosine Monophosphate Does Not Rise after Stimulation with GPR30 Agonists. The discovery of GPR30 as a putative membrane-resident estrogen receptor started with the observation that estrogen could activate adenyl cyclase activity in MCF-7 cells, giving rise to the production of cAMP (Aronica et al., 1994). Later on, the GPR30 cDNA was cloned out of this cell line (Carmeci et al., 1997). Finally, Thomas et al. (2005) identified GPR30 as a G protein–coupled membrane receptor that is directly activated by estradiol and triggered increases in cytosolic cAMP concentrations. The cAMP generation mediated by GPR30 could, likewise, be confirmed by applying other agonists like G-1 or tamoxifen (Mo et al., 2013). Hence, we determined the cAMP formation in HEK293GPR30-YFP cells during stimulation with chlorhexidine or Lys05. We applied the well established EPAC-derived cAMP biosensor to sensitively monitor cAMP formation in living cells in a time-resolved manner (Klarenbeek et al., 2015). Since elevation of cAMP would cause an ERK substrate by an increase in the intramolecular FRET efficiency, caused by photobleaching of the FRET acceptor (Fig. 7, B and C). FRET signals upon the addition of 5 μM 17β-estradiol or G-1 did not display discernible differences to those observed after the addition of HBS buffer. Likewise, the addition of chlorhexidine and Lys05 gave rise to FRET signals that were not different from those in solvent-treated controls (Fig. 7, A and C). To assess whether a coupling to the cAMP cascade may be restricted to certain cell types, we repeated assays in MCF7 cells that have been reported to express GPR30 (Carmeci et al., 1997). Neither 17β-estradiol nor G-1 induced a cAMP response as indicated by a coexpressed EPAC-derived cAMP biosensor (Supplemental Fig. 6). We thus exclude a predominant coupling of hGPR30 to the Gαs-adenyl cyclase/cAMP pathway when challenged with previously reported or newly identified GPR30 agonists.

ERK Activation Assay. Coupling to the Ras-Raf-MEK-ERK pathway is a typical hallmark of Gαi-coupled receptors. To assess the activity of this cascade in HEK293 cells, we coexpressed GPR30 with a cytosolic FRET-based ERK biosensor protein (cytoERKAR4) that reports the phosphorylation state of an ERK substrate by an increase in the intramolecular FRET efficiency (Keyes et al., 2020). As shown in Fig. 8, A–D, 5 μM chlorhexidine or 5 μM Lys05 caused an increase in FRET signals, with a maximum appearing about 10 minutes after agonist application and reaching an amplitude of about 40%–60% of maximal responses that were exerted by 100 ng/ml recombinant epidermal growth factor. Responses to chlorhexidine or Lys05 were absent in HEK293 cells that expressed the reporter protein but no GPR30 (Fig. 8, E–H).

Rapid Internalization of GPR30 and Potential Coupling to Gq12/13. A characteristic feature of efficient and sustained GPCR activation is given by the receptor internalization, which uncouples the GPCR from G-protein signaling and may
be followed either by terminal degradation in lysosomes or by receptor recycling back to the plasma membrane. To follow these processes, we imaged the subcellular localization of the YFP-fused hGPR30 during prolonged agonist application by using confocal laser-scanning microscopy. Upon the addition of 5 μM chlorhexidine or Lys05 that cause comparable amplitudes of Ca\(^{2+}\) responses, a clustered (“patchy”) distribution and first distinct internalization patterns were already observable 2 minutes.

**Fig. 4.** GPR30-induced Ca\(^{2+}\) release is suppressed by Gq, PLC, and InsP\(_3\) receptor inhibitors. (A) Representative fluorescence imaging plate reader analyses of fluo-4-loaded HEK293-hGPR30-YFP cell suspensions after 5 minutes of preincubation without (HBS control) and with 1 μM YM-254890 (Gq inhibitor), 100 μM 2-APB (IP\(_3\) receptor inhibitor), 10 μM U-73122 (PLC inhibitor), or U-73434 (inactive U-73122 analog). GPR30 was activated either by chlorhexidine (left panel), Lys05 (middle panel), or 9-aminoacridine (right panel). Shown are the kinetics of relative fluorescence intensities during stimulation, each condition as duplicate. Pipetting artifacts caused during compound application are not deleted. (B) Aggregated data (means and S.D.; n = 3 independent biologic experiments performed in technical duplicates each) of peak fluorescence intensities (F\(_{\text{Peak}}\)/F\(_0\)) measured as shown in (A). All blockers acting on Gq-mediated Ca\(^{2+}\) release were effective (# or *, P < 0.05 by one-way ANOVA with Dunn-Sidak post hoc test). Note that U-73434 only suppressed a minor fraction of Lys05- and 9-aminoacridine–induced responses, and effects on chlorhexidine-stimulated cells were statistically not significant (n.s.).

**Fig. 5.** Effect of PTX on GPR30-induced [Ca\(^{2+}\)]\(_i\) signals. Similar fluorescence imaging plate reader experiments as in Fig. 4 but with HEK293 cells that transiently expressed human GPR30, the ET\(_B\) receptor, and the FP1 formyl peptide receptor. Six hours after transfection, PTX (100 ng/ml) was added to the culture medium and incubated overnight to deactivate G\(_{\alpha}\) proteins by ADP ribosylation. The next day, cells were loaded with fluo-4 and subjected to multiwell Ca\(^{2+}\) imaging. (A and B) Shown are time courses of [Ca\(^{2+}\)]\(_i\), obtained in a representative experiment during the addition of 5 μM 9-aminoacridine, 5 μM chlorhexidine, 5 μM Lys05, or the control agonists 100 μM carbachol, 10 nM endothelin-1, and 100 nM fMLP. Orange lines: data from four wells containing PTX-treated cells; black lines: four wells with untreated control cells from same transfection day. To exclude confounding effects of different cell numbers in single wells, the [Ca\(^{2+}\)]\(_i\) was calibrated in each well at the end of the experiment. To this end, 0.04% Triton X-100 and 10 mM EGTA were sequentially added to expose the indicator to high (1 mM) and to very low (<20 nM) Ca\(^{2+}\) concentrations, respectively. (C) Statistical analysis of PTX-treated (black bars) and -untreated cells (white bars). Shown are means and S.D. (n = 4 independent biologic experiments, each performed in duplicates) of maximal [Ca\(^{2+}\)]\(_i\) increases (Δ[Ca\(^{2+}\)]\(_i\)) after adding the indicated agonists. Differences (#P < 0.05, Dunn–Sidak post hoc test) were only registered during activation of G\(_i\)-coupling ET\(_B\) or FP\(_1\) receptors but not after stimulating endogenous muscarinic acetylcholine receptors or after applying hGPR30 agonists. n.s., statistically not significant.
after agonist application (Fig. 9, A and B). Within 10–20 minute, hGPR30-YFP was almost quantitatively removed from the plasma membrane in chlorhexidine- or Lys05-treated cells. 9-Aminoacridine seemed to be slower acting, but within 20 minutes, GPR30 followed the same clustered distribution and substantial internalization as seen with the other two agonists (Fig. 9C). In the transmitted light channel, a second finding while imaging chlorhexidine- or Lys05-stimulated HEK293 cells overexpressing hGPR30 was a marked membrane blebbing. To visualize the blebbing events independently of hGPR30 clustering and internalization, we stably transfected HEK293hGPR30-YFP cells with an expression plasmid that encodes a CAAX-box–modified and thereby membrane-targeted CFP. During hGPR30 internalization mediated by chlorhexidine or Lys05, the CFP-CAAX protein indicated distinct bleb-like bulges in the plasma membrane (Fig. 10) that may reflect a rearrangement of the cortical actin network (Charras, 2008). Since GPCR coupling to Gα12/13 is known to affect the actin cytoskeleton through the downstream effectors RhoA and ROCK (Purvanov et al., 2014; Vanderboor et al., 2020), we applied the ROCK inhibitors Y-27632 or fasudil to affirm this assumption. Both blockers prevented bleb formation (data not shown). Thus, we propose that, besides Gαq,

Fig. 6. Imaging of CFP-PLC–δ1(PH) and PKC–ε-CFP translocation in HEK293hGPR30-YFP cells induced by chlorhexidine and Lys05 addition. HEK293hGPR30-YFP Cells were transiently transfected with cDNA plasmids encoding the pleckstrin homology domain of phospholipase C–δ1 N-terminally fused to CFP [CFP-PLC–δ1(PH); A–F] or a protein kinase C ε C-terminally fused to CFP (PKC–ε-CFP; G–L). (A, D, G, and J) Confocal microscopy images of the CFP-fused translocating biosensor proteins in unstimulated (left panels) and in chlorhexidine- or Lys05-stimulated cells (right panels). Scale bars, 20 μM. (B, E, H, and K) Typical time courses of relative changes of PLC–δ1(PH) or PKC–ε fluorescence signals measured by time-lapse epifluorescence microscopy in regions of interest defined over the plasma membrane (Fpm) and the cytosol (Fcyt) of the same cells, expressed as ratios and normalized to the initial values. Shown are data from five to six independent experiments, each comprising four to nine measured cells. (C, F, I, and L) Statistical comparison (#P < 0.05, Student’s t test with unpaired data) of strongest PLC–δ1(PH) or PKC–ε-CFP translocation effects seen in GPR30-expressing (GPR30) or in parental HEK293 (control) cells after the addition of chlorhexidine (C and I) or Lys05 (F and L) observed in n = 5 to 6 independent experiments each.
of chlorhexidine or Lys05 to elicit functional Ca\textsuperscript{2+} signals, we performed single-cell 

In this study, we identified three novel GPR30 agonists by performing an unbiased multiplexed screen of 31 GPRCs, including numerous orphan members. Our screening assay was based on measurement of intracellular Ca\textsuperscript{2+} concentrations with coexpressed G-protein \( \alpha \) subunits 15/16 to enforce a convergence of GPCR signaling toward PLC activation with subsequent Ca\textsuperscript{2+} signals. Until now, GPR30 has been described as a receptor that induces cAMP production via a \( G_s \) coupling, hGPR30 may also activate heterotrimeric G proteins of the G\textsubscript{12/13} family, which is a frequently observed dual-coupling pattern for \( G_s \)-linked GPCRs.

**Transiently Transfected GPR30 Pattern and Calcium Signals Differ in Various Cell Types.** In the past, the results of GPR30 localization assays revealed conflicting patterns with reports on a predominant localization in the endoplasmic reticulum or in the plasma membrane (Revankar et al., 2005; Bologa et al., 2006; Funakoshi et al., 2006; Filardo et al., 2007; Otto et al., 2008). Since variations in the cellular localization patterns may depend on the cell types they are investigated in, we transiently transfected the expression plasmid encoding YFP-tagged hGPR30 into HeLa, COS-7, and MCF-7 cells, which endogenously express GPR30. Surprisingly, the plasma membrane localization of transiently expressed hGPR30-YFP in HEK293 cells was less prominent than in our stable HEK293hGPR30-YFP cell line and only observable in cells that express only small amounts of the fusion protein (Fig. 11A). When compared with the other cell lines, HEK293 cells followed by MCF-7 were visually the cell type with the highest abundance of hGPR30-YFP in the plasma membrane. In COS-7 and in HeLa cells, hGPR30-YFP seemed to be retained in the endoplasmic reticulum, as indicated by the reticular pattern and nuclear membrane residence of intracellularly retained hGPR30-YFP proteins (Fig. 11, D and G).

To examine whether the cellular distribution affects the efficacy of chlorhexidine or Lys05 to elicit functional Ca\textsuperscript{2+} mobilization signals, we performed single-cell [Ca\textsuperscript{2+}]\textsubscript{i} imaging experiments in all investigated cell types. After transient transfection of GPR30, chlorhexidine and Lys05 elicited a rise in basal [Ca\textsuperscript{2+}]\textsubscript{i} in all cell lines. In HEK293 and MCF-7 cells, 62%–67% of the transfected cells (as detected by their yellow fluorescence) responded to the addition of chlorhexidine or Lys05 with increases in [Ca\textsuperscript{2+}]\textsubscript{i}, by more than 100 nM over their basal [Ca\textsuperscript{2+}]\textsubscript{i}. In HeLa cells, rises in [Ca\textsuperscript{2+}]\textsubscript{i} occurred in 40%–64% of transfected cells but often with a delayed response when compared with HEK293 or MCF-7 cells (Fig. 11, H and I). A functional GPR30 activation in COS-7 cells by chlorhexidine or Lys05 could only provoked in 16%–25% of GPR30-expressing cells (Fig. 11, E and F). This observation was in line with the observed higher efficiency in plasma membrane targeting of the protein in HEK293 or MCF-7 cells compared with COS-7 and HeLa cells. Parental HEK293 (Fig. 2) or parental COS-7 and HeLa cells (data not shown) did not respond to the two GPR30 agonists. Parental MCF-7 cells showed transient and inhomogeneous [Ca\textsuperscript{2+}]\textsubscript{i} signals to the addition of 5 \( \mu \)M chlorhexidine that were also obtained when applying 0.05% DMSO as solvent control (Supplemental Fig. 7). Notably, agonist-induced [Ca\textsuperscript{2+}]\textsubscript{i} signals in transiently hGPR30-YFP–overexpressing MCF-7 signals were more uniform and stronger than in the untransfected or mock-transfected parental cell line. Taken together, we conclude that the newly identified GPR30 agonists, albeit elicting more variable results in transiently transfected cells, were effective specific for GPR30 regardless of which cell line we used. Furthermore, diverse findings in past could rely on the use of various cell types in different laboratories.

**Discussion**

In this study, we identified three novel GPR30 agonists by performing an unbiased multiplexed screen of 31 GPRCs, including numerous orphan members. Our screening assay was based on measurement of intracellular Ca\textsuperscript{2+} concentrations with coexpressed G-protein \( \alpha \) subunits 15/16 to enforce a convergence of GPCR signaling toward PLC activation with subsequent Ca\textsuperscript{2+} signals. Until now, GPR30 has been described as a receptor that induces cAMP production via a \( G_s \)
pathway (Kanda and Watanabe, 2003; Thomas et al., 2005; Mo et al., 2013). Astonishingly, it has also been described to induce a Ca$^{2+}$ mobilization in a PLC-independent manner (Revankar et al., 2005; Bologa et al., 2006; Ariazi et al., 2010) in both native and in GPR30-overexpressing cells. In addition, GPR30 mediates ERK-1/2 phosphorylation as well as phosphoinositide 3-kinase (PI3K) activation via transactivating epidermal growth factor receptors in breast cancer cell lines or in GPR30-overexpressing COS-7 cells (Filardo et al., 2000; Revankar et al., 2005). Of note, reported GPR30 agonists (Rosano et al., 2016), which are included in our Selleckchem library, failed to elicit increases in [Ca$^{2+}$]i despite favoring promiscuous coupling via G$\alpha$15/16.

By contrast, three compounds that have previously not been reported as GPCR agonists, namely chlorhexidine, Lys05, and 9-aminoacridine, emerged as agonists, with secondary screening results showing that GPR30 was the common target GPCR for all three compounds. Although the newly identified agonists may share structural motifs with an as yet unidentified physiologic GPR30 agonist, the assumption of a unified pharmacophore must not hold true. Notably, the AT$_1$ receptor as closest relative of GPR30 is a peptide receptor.

Our experiments provided several lines of evidence that signaling downstream of GPR30 was conveyed via a canonical G$_q$-linked, PLC-dependent pathway, leading to Ca$^{2+}$ mobilization, recruitment of diacylglycerol-sensitive PKC isoforms, and activation of the ERK pathway. Since previous studies did not show such canonical signaling properties, the different observations may deserve a closer analysis.

Our experiments provided several lines of evidence that signaling downstream of GPR30 was conveyed via a canonical G$_q$-linked, PLC-dependent pathway, leading to Ca$^{2+}$ mobilization, recruitment of diacylglycerol-sensitive PKC isoforms, and activation of the ERK pathway. Since previous studies did not show such canonical signaling properties, the different observations may deserve a closer analysis. In our hands, inhibitors of G$_q$, PLC$_\gamma$, and InsP$_3$ receptors reliably and completely blocked Ca$^{2+}$ signals regardless of which agonist we used. Depletion of InsP$_3$-sensitive Ca$^{2+}$ stores by pretreating GPR30-expressing cells with thapsigargin abolished chlorhexidine-, Lys05-, and 9-aminoacridine–induced Ca$^{2+}$ responses. Other groups negated efficacy of PLC inhibitor U73122 (Revankar et al., 2005) or got variable results when applying IP$_3$ receptor inhibitors 2-APB or xestospong in different cell types (Ariazi et al., 2010). Performing complementary CFP-PLC-δ$_1$ (PH) and PKC$_\varepsilon$-CFP translocation assays that indicate PIP$_2$ hydrolysis and generation of diacylglycerols, respectively, our study demonstrates robust coupling of GPR30 to PLC.

**Fig. 8.** Imaging of GPR30-induced ERK activity by using a FRET-based biosensor. HEK293-GPR30-YFP cells (A) or parental HEK293 (B) cells were transiently transfected with cDNA plasmids encoding a cytosolic ERK activity reporter 4 (cytoEKAR4) to investigate the effect of chlorhexidine and Lys05 on ERK activity. Shown are exemplary measurements of FRET signals obtained from 46–77 single cells (gray lines) and their averaged values (black lines), respectively. As positive controls, experiments with recombinant human epidermal growth factor (EGF) (100 ng/ml), activating endogenously expressed EGF receptors, were performed. The slight linear decline is caused by photobleaching of the FRET acceptor YFP during the experiment. (C and D) For statistical analysis, averaged values were obtained from five to six independent biologic experiments, performed as shown in (A and B) and corrected for photobleaching, and FRET signals before (white bars) and 10 minutes after agonist addition (black bars) were compared. Increases in FRET signals after the addition of chlorhexidine or Lys05 were recorded in HEK293-GPR30-YFP cells (C; #P < 0.05, one-way ANOVA Dunn–Sidák), whereas parental HEK293 cells (D) showed no statistically significant ERK activation (n.s.).
A canonical, PLC-driven Ca\textsuperscript{2+} mobilization response typically consists of a transient increase in [Ca\textsuperscript{2+}]\textsubscript{i}, lasting for about 1 to 2 minutes and terminating in slightly elevated levels. In GPR30-expressing HEK293 cells, we observed such a uniform time course in all imaged cells upon stimulation with the newly identified agonists. In previous studies using 17\beta-estradiol, 4-hydroxytamoxifen, or G-1, single-cell [Ca\textsuperscript{2+}]\textsubscript{i} imaging experiments showed atypical kinetic properties (Revankar et al., 2005; Bologa et al., 2006) or variable kinetic properties depending on the agonists and cell types used (Ariazi et al., 2010). When added at concentrations >10 μM, tamoxifen induced delayed and long-lasting Ca\textsuperscript{2+} signals in GPR30-overexpressing as well as in parental HEK293 cells, confirming earlier reports that found that tamoxifen and its metabolite .
interfere with cytosolic Ca\(^{2+}\) homeostasis in an estrogen receptor or GPR30-independent fashion (Zhang et al., 2000; Bollig et al., 2007; Asp et al., 2013). Our finding that 17\(\beta\)-estradiol and G-1 do not elicit Ca\(^{2+}\) responses in HEK293\(_{hGPR30-YFP}\) cells is in line with observations in heterologously GPR30-expressing CHO-K1 cells (Otto et al., 2008). In MCF-7 cells, the reported GPR30 agonist G-1 induces Ca\(^{2+}\) efflux from the ER that is associated with ER stress and cell death (Vo et al., 2019). When applied at concentrations that activate GPR30, chlorhexidine and Lys05 elicited an increase in metabolic activity reminiscent of the comitogenic activity of numerous G\(_q\)-coupled receptors, including the GPR30-related AT1 angiotensin-II receptor (Forrester et al., 2018). Although the initial characterization of G-1 included data that demonstrate a competition for binding of an estradiol derivative to GPR30, other studies failed to reproduce 17\(\beta\)-estradiol binding to GPR30 (Otto et al., 2008). Similar results were obtained by Pedram et al. (2006). Thus, the specific binding of estrogens to GPR30 is not undisputed.

Since initial evidence has suggested that activation of GPR30 triggers increases in cAMP concentrations, we wondered whether our GPR30 agonist may exert such a biased agonism toward PLC while maintaining some coupling to the G\(_s\)-cAMP pathway. To test this hypothesis, we used the EPAC-based sensitive cAMP-reporting biosensor. In HEK293\(_{hGPR30-YFP}\) cells, this sensor was capable of detecting cAMP formation triggered by the endogenously expressed adenosine receptor or the \(\beta_2\) adrenoceptor. In the same cells, neither recently reported (17\(\beta\)-estradiol, G-1) nor our newly identified GPR30 agonists chlorhexidine and Lys05 evoked detectable changes in cAMP levels. This observation...

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**Fig. 11.** Subcellular localization and \([\text{Ca}^{2+}]_i\) responses of transiently overexpressed hGPR30 in various cell lines. The cellular localization of human GPR30 and functional responsiveness to chlorhexidine and Lys05 were examined in HEK293 (A–C), COS-7 (D–F), HeLa (G–I), and MCF-7 cells (J–L). (A, D, G, and J) Representative confocal microscopy images of hGPR30-YFP in different cell lines 24 hours after transient transfection. Scale bar, 20 \(\mu\)M. (B, E, H, and K) Fluorometric single-cell \([\text{Ca}^{2+}]_i\), analysis in hGPR30-YFP-expressing HEK293, COS-7, HeLa, or MCF-7 cells was performed as described in Fig. 2C. Shown are exemplary measurements during chlorhexidine (left panels) or Lys05 (right panels) application. (C, F, I, and L) Means and S.D. of the percentage of transfected cells that responded during the application of the indicated hGPR30 agonists with increases in intracellular Ca\(^{2+}\) concentration by more than 100 nM. Data represent four to six independent measurements performed as in (B, E, H, and K).
cortinates findings that COS-7 or HEK293 cells heterologously expressing GPR30 lacked enhanced cAMP formation after treatment with 17β-estradiol or G-1 (Otto et al., 2008; Broselid et al., 2014). Moreover, the latter authors observed a GPR30-mediated, but again 17β-estradiol- or G-1–independent, constitutive inhibition of cAMP formation and increased cAMP levels after siRNA knockdown of native GPR30 in MDCK cells. Recently, it has become clear that some GPCR agonists may shift the signaling toward specific G-protein families or arrestins. This phenomenon is commonly referred to as “biased agonism” (Bock and Bermudez, 2021). Although we cannot exclude that the newly identified GPR30 agonists may exert a biased agonism, the coupling of three chemically distinct agonists toward a canonical Gq pathway argues against such signaling bias.

There are also inconsistent data regarding the subcellular localization of GPR30. Several studies demonstrated that GPR30 is localized to the ER (Revanakar et al., 2005; Bologa et al., 2006; Otto et al., 2008; Lin et al., 2009) or in the Golgi complex (Sakamoto et al., 2007). Other groups found GPR30 integrated into the plasma membrane (Thomas et al., 2005; Funakoshi et al., 2006; Filardo et al., 2007; Mo et al., 2013). Upon expression in different cell lines, we observed a YFP-tagged GPR30 mainly in the ER and in the plasma membrane, but the balance was strongly dependent on the cell type used. A pronounced plasma membrane localization was observable in HEK293 and MCF-7 cells, especially in cells that expressed low amounts of the protein. In COS-7 and HeLa cells, GPR30-YFP mostly accumulated in perinuclear endomembrane compartments. Such cell type– and expression density– dependent effects may explain the diverging observations, especially when using transiently transfected cells.

The identification of reliably acting GPR30 agonists that initiate a canonical Gq- and PLC-linked signaling pathway may pave the way toward an investigation of real physiologic functions of this GPCR and overcoming the state of contradictory results in the field. A genetic knockin mouse model, which harbors a lacZ reporter in the GPR30 locus, has revealed a major expression in vascular endothelial cells of multiple tissues and also in smooth muscle cells and in pericytes of brain vessels (Isensee et al., 2009). Female GPR30 knockout mice showed a blood pressure elevation at the age of 9 months (Mårtensson et al., 1997), this finding implies that GPR30 may play a role in regulating endothelial functions. Furthermore, the ubiquitous expression profile and the similarity to the Gq and G12/13-coupling AT1 receptor (Feng and Gregor, 1997) would fit to a model that GPR30 is expressed in blood vessels. Recently, Tutzauer et al. (2021) attempted to confirm a vasodilatory function of GPR30 by measuring the relaxation of caudal arteries from wild-type as well as from GPR30 knockout mice while treating them with the previously proposed GPR30-specific agonists G-1 and 17β-estradiol. They found that arteries from GPR30-deficient mice relaxed with the same potency and efficacy as in wild-type mice. In addition, various cellular expression models showed no effects of G-1 or 17β-estradiol and prompted them to state that “classifying GPR30 as an estrogen receptor and G-1 as a specific GPR30 agonist is unfounded.” Based on our data, we agree with their conclusion and hope that the discovery of a reliably acting GPR30 agonist and the finding of its coupling to the canonical Gq-phospholipase C signaling pathway may be instrumental in more unambiguously identifying the physiologic functions of GPR30.

Authorship Contributions
Participated in research design: Urban, Schaefer.
Conducted experiments: Urban, Leonhardt.
Performed data analysis: Urban, Schaefer.
Wrote or contributed to the writing of the manuscript: Urban, Schaefer.

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