**Project Title: MLSCN Assay for Ligands of GPR30 and Classical Estrogen Receptors**

**Grant Number: 1X01MH077627-01**

**Screening Center Name: New Mexico Molecular Libraries Screening Center**

**Principal Investigator of Screening Center: Larry A. Sklar, Ph.D**

**Assay Submitter and Institution:**

**Eric R. Prossnitz**
**University of New Mexico**

**PubChem Bioassay Identifier (AID): 1210, 1211, 1212, 1213, 1219, 1221, 1223, 1224, 1226**

**Assay Target: GPR30**

**Probe PubChem Compound Identifier (CID): 3136844**

**Mode of Action: Competitive inhibitor of ligand binding**

**Specific Aim: To identify small molecule ligands specific for GPR30 or the classical estrogen receptors (ERα and ERβ).**

## Significance and Rationale:

### Target: Transmembrane Estrogen Receptor GPR30/GPER.

Estrogen exerts numerous diverse physiological effects in humans. It is involved in the growth, development and homeostasis of numerous tissues, including mammalian female reproduction and breast development. In addition, estrogen regulates skeletal physiology, (cardio)vascular function, the central nervous system as well as the immune system. Estrogen stimulation initiates numerous signaling pathways that can lead to various cellular responses from adhesion and migration to survival and proliferation to cardiovascular and neuro-protection to organogenesis to angiogenesis to cancer. In addition, stress responses, feeding patterns, sleep cycles and temperature homeostasis are regulated by estrogen.

In the late 1990's, an orphan GPCR was cloned by 4 groups [1-4]. The GPCR identified in these studies was expressed in numerous tissues throughout the body. In 2000, Filardo et al. identified a potential agonist for this GPCR [5]. GPR30-associated activation of MAP kinases (Erk1/2) by estrogen in breast cancer cell lines was reported. ER antagonists, ICI 182,780 and tamoxifen, at high concentrations, were also capable of mediating Erk activation. Erk activation proceeded through a pertussis toxin-sensitive pathway that involved the transactivation of EGFRs through the release of cell-surface heparin-bound EGF. The upregulation of c-fos and other genes by estrogen and phytoestrogens has been shown in breast cancer and other cells. In 2005, additional evidence was provided that GPR30 is a novel estrogen-binding and-responsive receptor with unique properties relative to the classical estrogen receptors [6, 7]. Recent reviews describe the state of our knowledge regarding this receptor [8, 9].

### Known Ligands:

Estrogen (17β-estradiol, E2) is a critical hormone in the human body. Estrogen represents one of a family of steroid hormones, which also includes progesterone, testosterone, cortisol/glucocorticoids and aldosterone/ mineralocorticoids, that control many aspects of mammalian physiology. Additional physiological estrogen agonists, estrone (E1) and estriol (E3), also mediate biological functions.

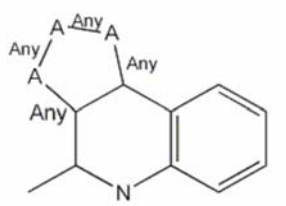
Estrogen-like activity can be found in a large variety of sources, both natural and manmade. These include phytoestrogens/isoflavinoids, from plants and fungi, as well as xenoestrogens, which include a variety of pesticides, polychlorinated biphenyls and plasticizers. The majority of these compounds are thought to exert their effects through the classical nuclear estrogen receptor(s). However, estrogen receptors unrelated to ERα and ERβ, but associated with membranes, such as GPR30, have been proposed to mediate some of the effects of xenoestrogens. Until recently, no ligands specific to GPR30 were known. In order to distinguish between the actions of classical estrogen receptors and GPR30, we recently described in a previous report and publication, an agonist of GPR30, G-1, that shows high selectivity for GPR30 over ERα/β [10]. To complement the pharmacological properties of G-1, we subsequently sought to identify an antagonist of GPR30 that displays similar selectivity against the classical ERs.

## Assay Description, Implementation and Screening:

PubChem Bioassay Name: GPR30 Binding

List of PubChem Bioassay Identifiers (AIDs): 1213,1219, 1224

PubChem Primary Assay Description (AIDs): 1213

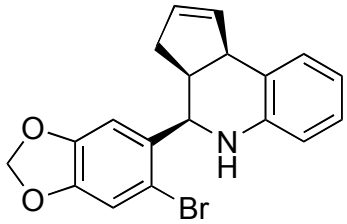



**G scaffold core**

In order to minimize the biomolecular screening required, we employed virtual screening to rapidly identify structures of interest for subsequent biological screening. We performed a substructure search of the MLSMR for compounds containing the core scaffold of the previously identified GPR30 agonist G-1 (G scaffold, where "A" means any atom and "Any" any kind of bond). The search identified 64 molecules, of which 57 were obtained from the SMR. To accomplish the primary biomolecular screening for GPR30 antagonism, we utilized calcium mobilization in GPR30 expressing cells and tested for the ability of the compound to block subsequent cellular activation by estrogen. In addition, biomolecular analyses were performed to determine binding affinities for ER$\alpha$ and ER$\beta$; functional activity for ER$\alpha$, ER$\beta$ and GPR30 in PI3K assays was also assessed.

## Center Summary of the Primary Screen:

A combination of virtual screening of the MLSMR and a biomolecular calcium mobilization assay was used to identify potential novel small molecule antagonist(s) of GPR30. Screening of the 57 G-scaffold containing compounds from the MLSMR yielded 8 that showed inhibition of estrogen-mediated calcium mobilization in cells expressing GPR30. The compounds did not promote calcium mobilization when added alone and did not inhibit the subsequent mobilization of intracellular calcium by ATP through purinergic GPCRs. Of particular interest was one compound that closely resembled G-1 but lacked the ethanone functionality of the molecule. Based on the structural overlap of G-1 with estrogen and in particular the similar though not identical spacing of oxygen atoms at the extremes of the molecules, we speculated that the keto (ethano) functionality of G-1 might play an important role in the activation of GPR30 through the formation of critical hydrogen bonds with the receptor. This potential antagonist (designated G15) was subsequently selected for chemical synthesis and further biological characterization.



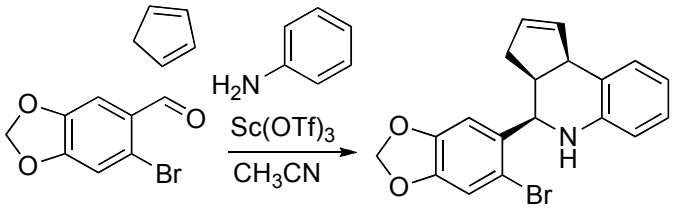

Structure of G15

## Probe Optimization:

### General Chemistry Description:

Based on the primary screening results of compounds containing the G-scaffold, we synthesized the G-1 derivative lacking the ethanone group, designated G15. The tetrahydro-3H-cyclopenta[c]quinoline scaffold of G-1 is synthetically accessible via the versatile three-component Povarov cyclization. We evaluated a series of reaction conditions employing protic and Lewis acid catalysts to optimize reaction rate, yield, and diastereoselectivity for the construction of G-1-like derivatives. Our optimized one-step procedure employing the catalyst $Sc(OTf)_3$ [11] in acetonitrile resulted in rapid reaction times, high product yield and enhanced selectivity favoring the syn diastereomeric products. The synthesis of G15 from aniline, 6-bromopiperonal and cyclopentadiene is illustrated in **Scheme 1**. Precipitation from dichloromethane/methanol gave analytically pure G15 in yields exceeding 85% as a racemic mixture of syn diastereomers. The stereochemistry is distinguished by the $^1$H-NMR coupling pattern of H-4 (4.65 ppm) with a coupling constant of 3.2 Hz that is characteristic for the syn orientation of the cyclopentene ring and phenyl group. The product was fully characterized by NMR spectroscopy, and HPLC-MS with positive ion detection showed the correct molecular ion ($MH^+$) as well as a single peak by UV (PDA/$l_{max}$= 294 nm) detection.

**Scheme 1. Synthesis of GPR30 Antagonist G15**



## Chemical Synthesis and Characterization:

### Synthesis and characterization of G15-C (4-(6-Bromo-benzo[1,3]dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinoline)

A catalytic amount of $Sc(OTf)_3$ (0.049 g, 0.1mmol, 10 mol%) in anhydrous acetonitrile (1 mL) was added to a mixture of 6-bromopiperanal (0.229 g, 1.00 mmol), aniline (0.093 g, 1.0 mmol) and cyclopentadiene (0.33 g, 5.0 mmol) in acetonitrile (3 mL). The reaction was stirred at ambient temperature (~23˚C) for 3 h with monitoring of product formation by thin layer chromatography using 80% hexane/ethyl acetate eluent.($R_f$ 0.6). The volatiles were removed *in vacuo*. The residue was dissolved in methylene chloride (10 mL) and then precipitated by drop-wise addition of methanol (5 mL), filtered and washed with additional methanol to give the product G15-C as a colorless solid (321mg, 87%): mp 178-180° C; FT-IR (**Fig. 1**) (KBr, $cm^{-1}$) 3338(w), 2887(w), 1605(w), 1587(s), 1473(s). $^1H$ NMR (**Fig. 2**) (300 MHz, DMSO-$d_6$) δ 7.23 (s, 1H), 7.13 (s, 1H), 6.97 (d, *J* = 7.9 Hz, 1H), 6.87 (ddd, *J* = 7.3, 7.3, 1.3 Hz, 1H), 6.68 (dd, *J* = 7.9, 1.3 Hz, 1H), 6.60 (ddd, *J* = 7.3, 7.3, 1.3 Hz, 1H), 6.1 (d, *J* = 0.9 Hz, 1H), 6.06 (d, *J* = 0.9 Hz, 1H), 5.88-5.82 (m, 1H), 5.60-5.53 (m, 2H), 4.65 (d, *J* = 3.2 Hz, 1H), 3.98 (d, *J* = 9.8 Hz, 1H), 3.07-2.95 (m, 1H), 2.48-2.38 (m, 1H), 1.69-1.59 (m, 1H). $^{13}C$ NMR (**Fig. 3**) (75 MHz, $CDCl_3$) δ 147.5, 147.2, 145.3, 134.6, 134.0, 130.3, 129.0, 126.3, 126.2, 119.4, 116.1, 113.0, 112.9, 108.1, 101.7, 56.7, 46.1, 42.2, 31.4. HRMS calcd for $C_{19}H_{16}BrNO_2$ $(M+H)^+$ 370.0443; found 370.0435. Anal ($C_{19}H_{15}BrNO_2$) C, H, N.

HPLC-MS: Electrospray positive ion (cone voltage 62 V, Capillary Voltage 3 kV).
G15-C (1mg/mL $CH_3CN$, 20 μL) was injected onto a Symmetry® C18 (5 μm, 3.0 X 150 mm, Waters) column and eluted with 60-90% $CH_3CN$ in $H_2O$ (gradient 1.5 % $min^{-1}$) and exhibited a single peak $R_t$= 16.52 min. (**Fig 4**). UV-Vis $\lambda_{max}$ 294 nm (**Fig. 5**). ESI-MS *m/z* (ES+) calcd for $C_{19}H_{16}BrNO_2$ $(M+H)^+$ 370.04; found 370.02 **(Fig. 5).**

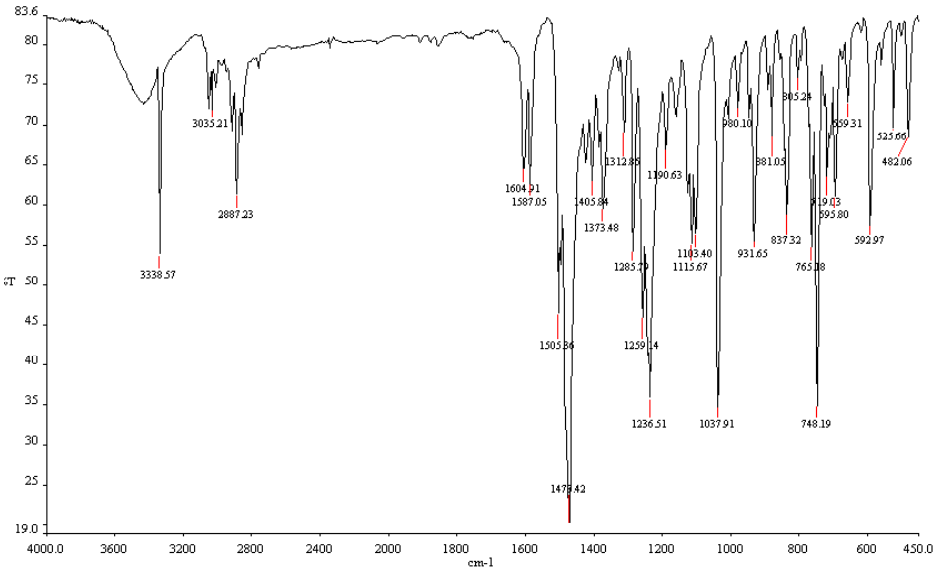



**Fig. 1.** FTIR Spectrum of G15.

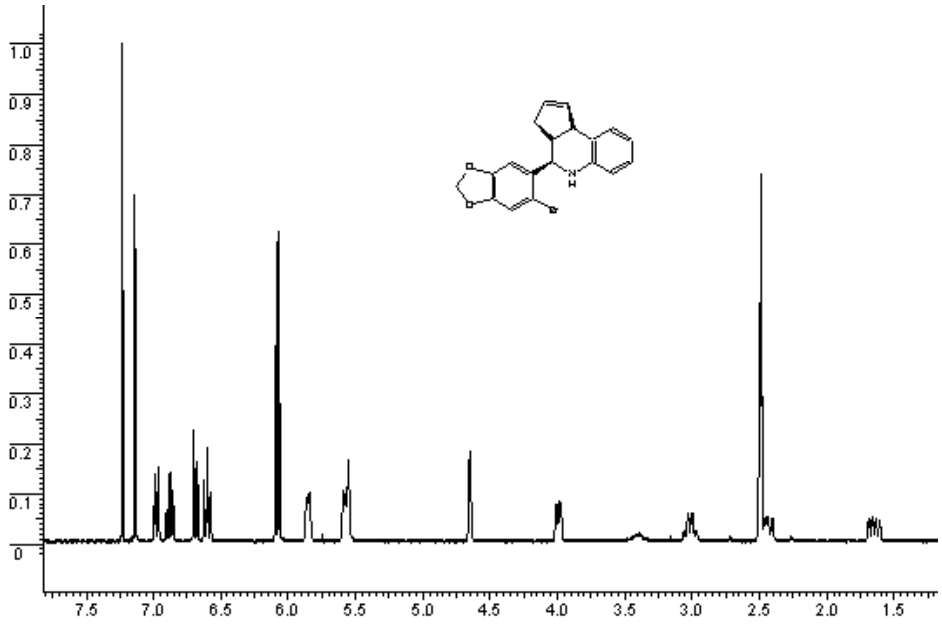



**Fig. 2.** $^{1}$H NMR of G15.

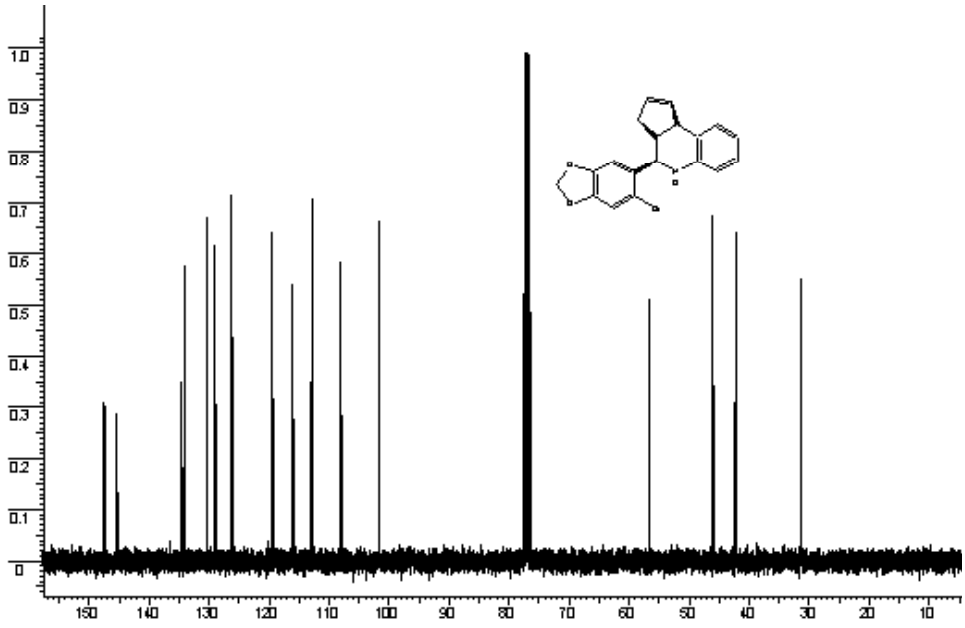



**Fig. 3.** $^{13}$C NMR of G15.

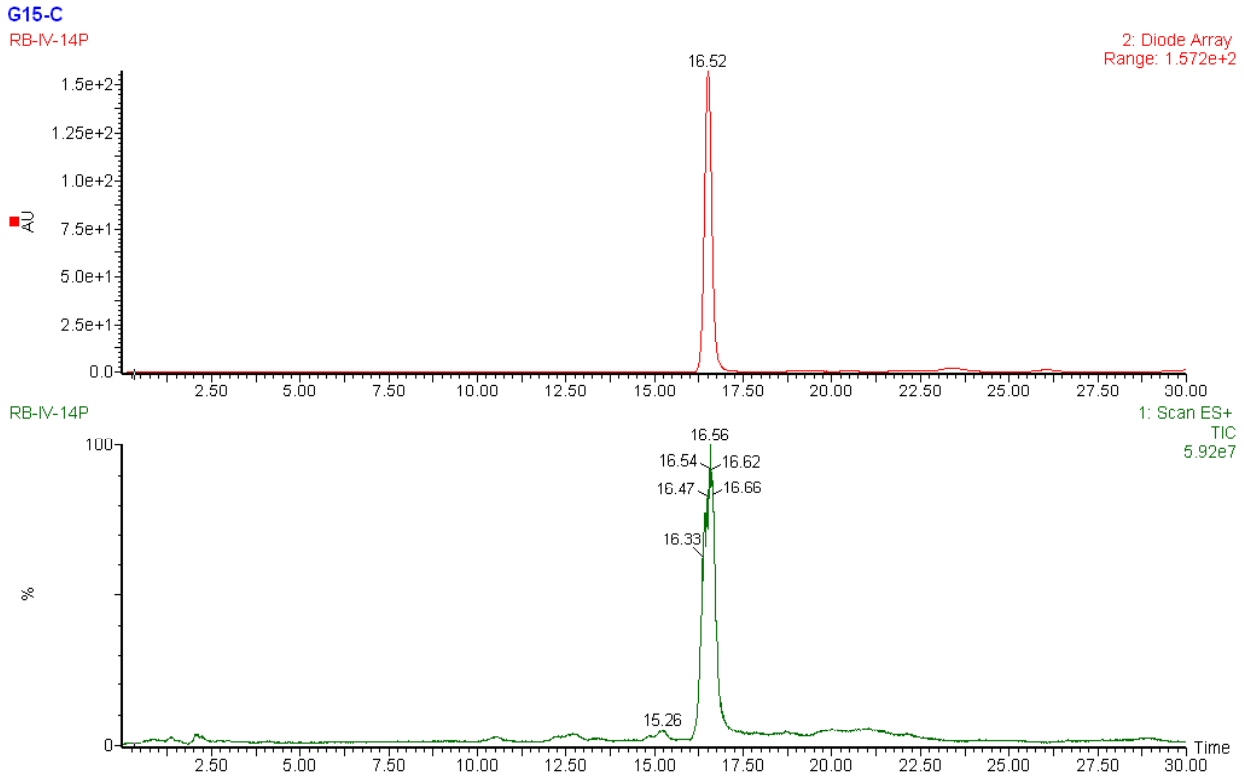



**Fig. 4.** HPLC PDA (top) and ESI-MS (ES+) TIC (bottom) of G15.

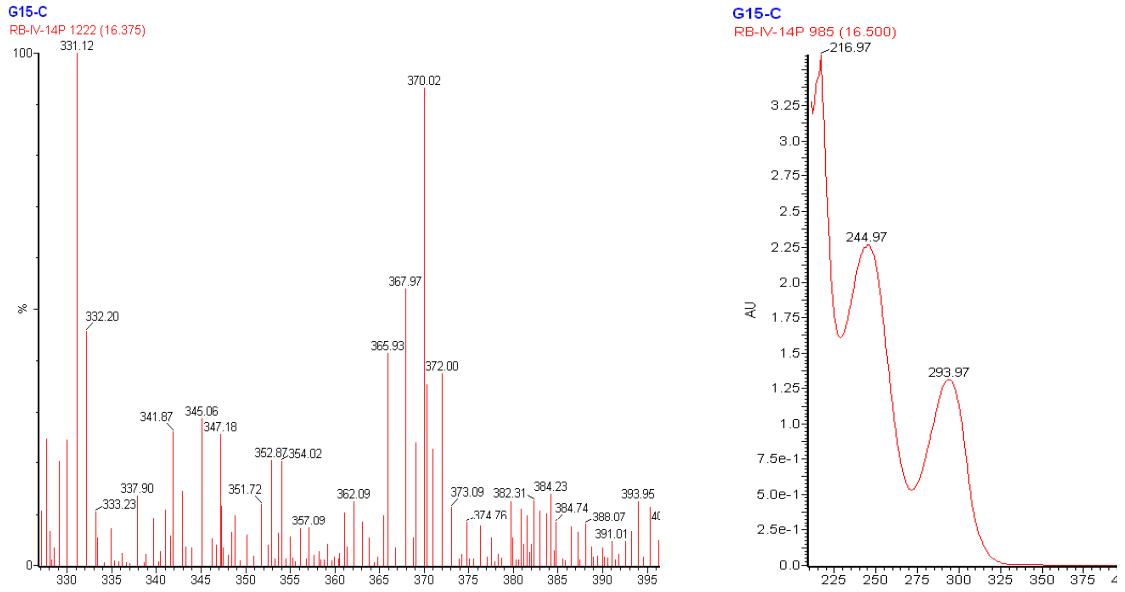



**Fig. 5.** ESI-MS (left) and UV-Vis (right) of G15.

## Activity and Specificity:

## Cellular activity:

Competitive binding assays using endogenous GPR30 and a novel radiolabeled iodine-125 derivative (probe report in preparation), demonstrated that G15 binds to GPR30 with an affinity of approximately 20 nM (**Fig. 6**). This compares to an affinity for G-1, utilizing the same assay, of approximately 7 nM, similar to our previously reported affinity of G-1 for recombinant GPR30 of 11 nM [10] and reported affinities for 17β-estradiol between 3-6 nM [6, 7]. Thus removal of the ethanone moiety of G-1 resulted in a decrease in binding affinity of 2-3 fold. Additional competitive binding studies to assess interactions with ERα and ERβ revealed that like G-1, G15 displays no substantial binding (<~15%) to ERα or ERβ at concentrations up to 10 μM, where estrogen competes with a Ki of approximately 0.3-0.5 nM, suggesting a selectivity >10,000 (**Figs. 6**). These results reveal that G15, like G-1, displays high affinity for GPR30 with minimal binding to ERα and ERβ (Ki >> 10 μM).

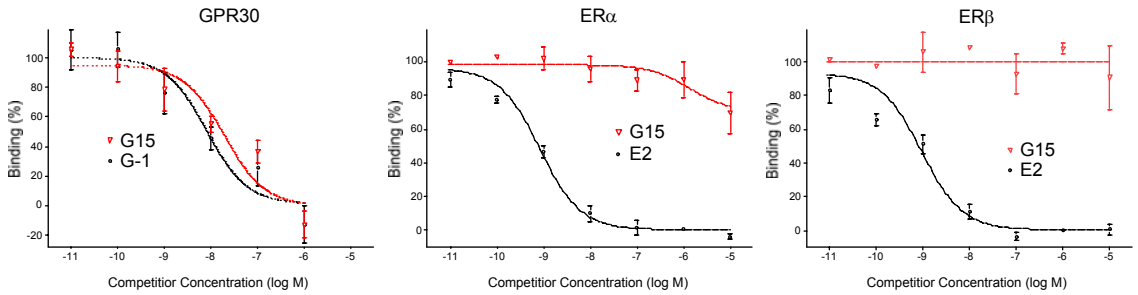



**Fig. 6.** Specificity of G15 for GPR30 compared to ERα and ERβ.

We next examined the functional capabilities of G15 in comparison to estrogen with respect to the rapid mobilization of intracellular calcium. G15 alone induced no calcium response but was able to inhibit the subsequent response to both estrogen and G-1 (**Fig. 7a**). There was no effect on stimulation through purinergic receptors by ATP. Inhibition of the G-1-mediated response occurred with an $IC_{50}$ of approximately 10 nM (**Fig. 7b**).

PI3K activation was assessed via the translocation of a PIP3-binding reporter, PH-Akt-mRFP. Cos cells cotransfected with GPR30 and a PH-mRFP reporter demonstrated nuclear translocation of the reporter upon stimulation by E2 or G-1. Both activities were blocked by the preincubation of the cells with a ten-fold excess of G15 (**Fig. 8**). However, G15 (at 10 μM) had no effect on the stimulation of PI3K by ERα stimulated with E2 (**Fig. 9**).

Finally, we examined the ability of G15 to modulate murine uterine epithelial cell proliferation *in vivo*. Seventeen-day-old sexually immature mice were

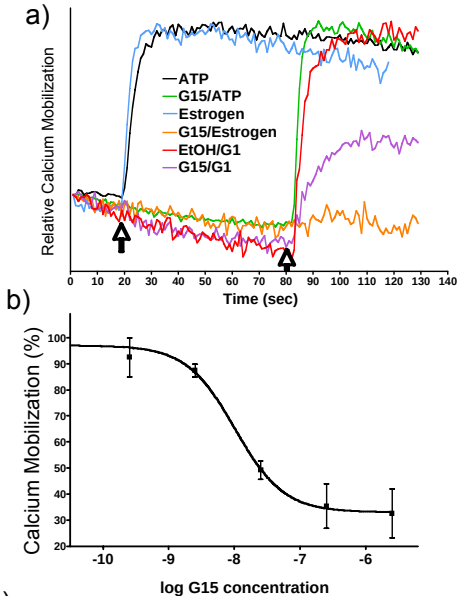



**Fig. 7.** Characterization of GPR30 antagonist G15. **a)** Calcium mobilization in endogenously GPR30-expressing SKBr3 cells. Compounds were added singly or in succession as indicated by the arrows and figure legend. **b)** G15 dose response inhibiting stimulation by 30 nM E2.

injected once with vehicle (sham), E2, G-1, G-15, E2 + G-15, or G-1 + G-15, as indicated (**Fig. 10**). Eighteen hr after the last injection, uteri were harvested, weighed, embedded in paraffin, and sectioned. Where E2 induced approximately 35% of the uterine epithelial cells to exit the $G_o$ phase of the cell cycle (determined from Ki67 expression by immunohistochemistry), G-1 induced approximately 10% of the epithelial cells to proliferate. G15 treatment alone had no significant effect. However, cotreatment with G15 completely abolished the G-1 response and reduced the E2 response by approximately two-thirds.

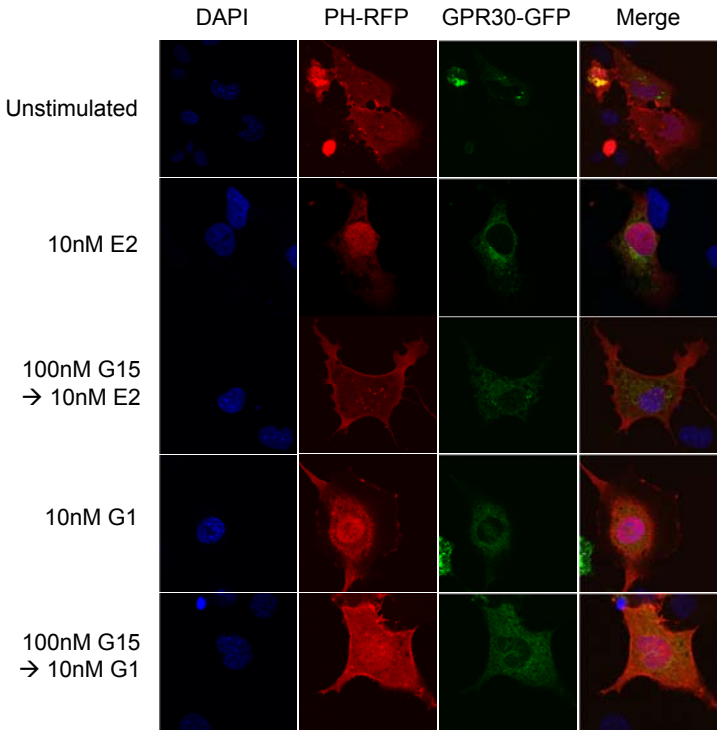



**Fig. 8.** GPR30-transfected COS cells were incubated with the indicated concentration of G15 or vehicle for 15 min prior to 15 min stimulation with E2 or G-1. G15 effectively inhibited both E2 and G-1 stimulated PI3K activation (represented by nuclear localization of the PH-RFP). DAPI; nuclei.

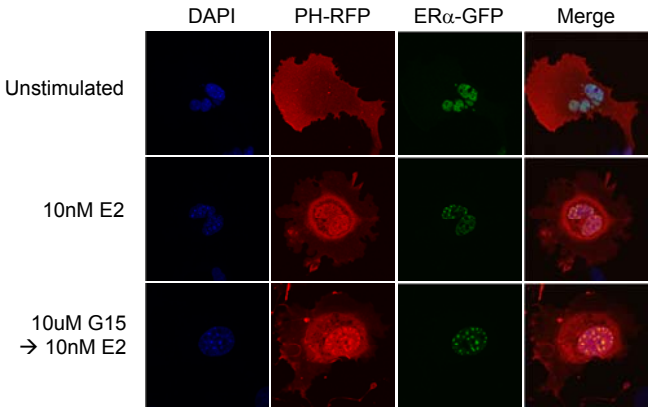



**Fig. 9.** ERα-transfected COS cells were incubated with the indicated concentration of G15 or vehicle for 15 min prior to 15 min stimulation with E2. G15 was unable to inhibit E2-mediated PI3K activation even at 10 μM.

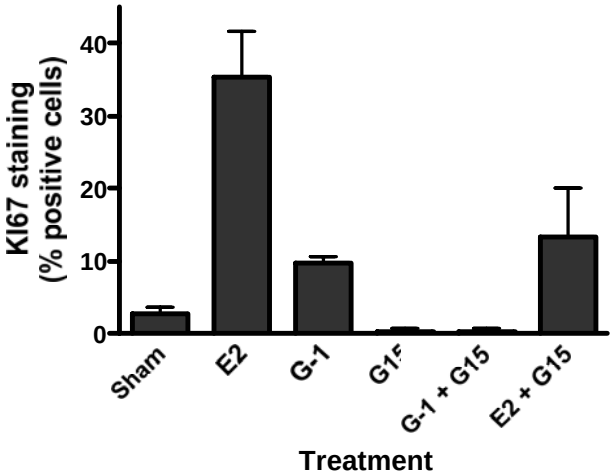



**Fig. 10.** Uterine epithelial cell proliferation. *In vivo* treatment of sexually immature mice with E2, G-1 and G15

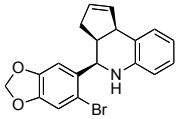
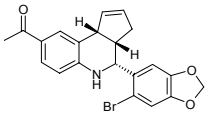
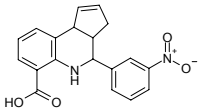
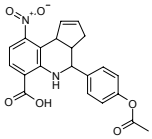
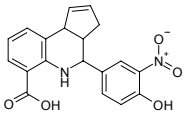
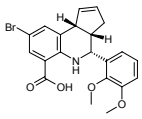
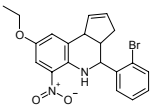
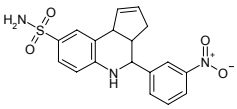
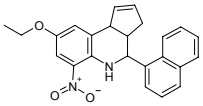
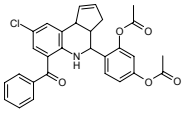
## Center summary of probe properties and recommendations for the scientific use of probe as research tool:

G15 has been identified as a highly selective GPR30 antagonist (with little to no binding/activity towards classical estrogen receptors ER$\alpha$/ER$\beta$). It has an affinity for GPR30 of ~20 nM and shows effectiveness at 100 nM. It is recommended that G-1 be used at concentrations up to 500 nM (lower concentrations are recommended to avoid possible off target effects).

## Appendices

Comparison of selected compounds containing the 4-ring core structure found in G15 with low affinity and minimal specificity for GPR30 and ERs reveals selectivity (**Table I**).

**Table I.**

| PubChem CID | PubChem SID | Structure | MW | ERα ($IC_{50}$) | ERβ ($IC_{50}$) | GPR30 ($IC_{50}$) |
|---|---|---|---|---|---|---|
| 3136844 | 48409616 |  | 370.24 | >1.0E-04 | >1.0E-04 | 2.0E-08 |
| 5322399 | 48409542 |  | 412.28 | >1.0E-04 | >1.0E-04 | 0.7-1.1E-08 |
| 2854262 | 48409568 |  | 336.35 | 8.9E-07 | 1.5E-05 | 4.7E-07 |
| 2900725 | 48409587 |  | 394.39 | 1.1E-05 | 1.4E-05 | 1.4E-05 |
| 4182169 | 48409588 |  | 352.35 | 6.3E-07 | 1.6E-05 | 8.2E-07 |
| 5322461 | 48409599 |  | 430.30 | 6.7E-06 | 4.2E-05 | 1.2E-05 |
| 2871986 | 48409583 |  | 415.29 | ND | ND | ND |
| 2868619 | 48409569 |  | 371.41 | ND | ND | ND |
| 3120364 | 48409579 |  | 386.45 | ND | ND | ND |
| 3843745 | 48409584 |  | 501.97 | ND | ND | ND |

ND – no detectable binding at 10 uM

## References


1. Carmeci, C., Thompson, D.A., Ring, H.Z., Francke, U., Weigel, R.J. (1997) Identification of a gene (GPR30) with homology to the G-protein-coupled receptor superfamily associated with estrogen receptor expression in breast cancer. *Genomics* **45**, 607-17.

2. O'Dowd, B.F., Nguyen, T., Marchese, A., Cheng, R., Lynch, K.R., Heng, H.H., Kolakowski, L.F., Jr., George, S.R. (1998) Discovery of three novel G-protein-coupled receptor genes. *Genomics* **47**, 310-3.

3. Owman, C., Blay, P., Nilsson, C., Lolait, S.J. (1996) Cloning of human cDNA encoding a novel heptahelix receptor expressed in Burkitt's lymphoma and widely distributed in brain and peripheral tissues. *Biochem Biophys Res Commun* **228**, 285-92.

4. Takada, Y., Kato, C., Kondo, S., Korenaga, R., Ando, J. (1997) Cloning of cDNAs encoding G protein-coupled receptor expressed in human endothelial cells exposed to fluid shear stress. *Biochem Biophys Res Commun* **240**, 737-41.

5. Filardo, E.J., Quinn, J.A., Bland, K.I., Frackelton, A.R., Jr. (2000) Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs via trans-activation of the epidermal growth factor receptor through release of HB-EGF. *Molecular endocrinology (Baltimore, Md* **14**, 1649-60.

6. Revankar, C.M., Cimino, D.F., Sklar, L.A., Arterburn, J.B., Prossnitz, E.R. (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* **307**, 1625-30.

7. Thomas, P., Pang, Y., Filardo, E.J., Dong, J. (2005) Identity of an estrogen membrane receptor coupled to a G-protein in human breast cancer cells. *Endocrinology* **146**, 624-32.

8. Prossnitz, E.R., Arterburn, J.B., Smith, H.O., Oprea, T.I., Sklar, L.A., Hathaway, H.J. (2008) Estrogen action via the transmembrane receptor GPR30. *Annu Rev Physiol* **70**.

9. Prossnitz, E.R., Sklar, L.A., Oprea, T.I., Arterburn, J.B. (2008) GPR30: a novel therapeutic target in estrogen-related disease. *Trends in Pharmacological Sciences* **(in press)**.

10. Bologa, C.G., Revankar, C.M., Young, S.M., Edwards, B.S., Arterburn, J.B., Kiselyov, A.S., Parker, M.A., Tkachenko, S.E., Savchuck, N.P., Sklar, L.A., Oprea, T.I., Prossnitz, E.R. (2006) Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nat Chem Biol* **2**, 207-12.

11. Kobayashi, S., Ishitani, H., Nagayama, S. (1995) Lanthanide triflate catalyzed imino Diels-Alder reactions; convenient synthesis of pyridine and quinoline derivatives. *Synthesis*, 1195-1202.