

Identification of Novel G Protein–Coupled Receptor 143 Ligands as Pharmacologic Tools for Investigating X-Linked Ocular Albinism

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PURPOSE. GPR143 regulates melanosome biogenesis and organelle size in pigment cells. The mechanisms underlying receptor function remain unclear. G protein–coupled receptors (GPCRs) are excellent pharmacologic targets; thus, we developed and applied a screening approach to identify potential GPR143 ligands and chemical modulators.

METHODS. GPR143 interacts with β -arrestin; we therefore established a β -arrestin recruitment assay to screen for compounds that modulate activity. Because GPR143 is localized intracellularly, screening with the wild-type receptor would be restricted to agents absorbed by the cell. For the screen we used a mutant receptor, which shows similar basal activity as the wild type but traffics to the plasma membrane. We tested two compound libraries and investigated validated hits for their effects on melanocyte pigmentation.

RESULTS. GPR143, which showed high constitutive activity in the β -arrestin assay, was inhibited by several compounds. The three validated inhibitors (pimozide, niclosamide, and ethacridine lactate) were assessed for impact on melanocytes. Pigmentation and expression of tyrosinase, a key melanogenic enzyme, were reduced by all compounds. Because GPR143 appears to be constitutively active, these compounds may turn off its activity.

CONCLUSIONS. X-linked ocular albinism type I, characterized by developmental eye defects, results from GPR143 mutations. Identifying pharmacologic agents that modulate GPR143 activity will contribute significantly to our understanding of its function and provide novel tools with which to study GPCRs in melanocytes and retinal pigment epithelium. Pimozide, one of three GPR143 inhibitors identified in this study, maybe be a good lead structure for development of more potent compounds and provide a platform for design of novel therapeutic agents.

Keywords: G protein–coupled receptor 143, GPR143 ligands, pharmacologic tools, ocular albinism, OA1, pimozide

G protein–coupled receptors (GPCRs) are key for signaling pathways because they can respond to ligands by activating transduction cascades. Although most GPCRs bind molecules outside the cell and activate intracellular pathways, GPR143 is atypical and is intracellularly localized.¹ In pigment cells, such as epidermal melanocytes and RPE, GPR143 traffics to the membrane of melanosomes,² which are specialized organelles where melanin is synthesized. GPR143 is also expressed in the central nervous system and some peripheral tissues (kidney, spleen, and lung).³

GPR143 mutations are associated with X-linked ocular albinism type 1 (OA1), which is characterized by loss of visual acuity, nystagmus, strabismus, iris translucency, photophobia, misrouting of the optic tract resulting in loss of stereoscopic vision, retinal hypopigmentation, and foveal hypoplasia.^{4,5} Skin pigmentation is typically not affected or only slightly reduced; however, subcellular abnormalities, such as presence of giant melanosomes (macromelanosomes), reduction in melanosome number, and alteration of melanosome motility, were observed in both epidermal melanocytes and RPE.^{6,7}

Macromelanosomes, formed by overgrowth of single organelles in pigment cells of OA1 patients, indicate that GPR143 may regulate melanosome size by blocking import of melanin-related proteins (MRPs) to mature melanosomes.^{8,9} GPR143 also regulates transcription of melanosomal genes, including tyrosinase that catalyzes several reactions during melanin synthesis, through modulation of the microphthalmia-associated transcription factor (MITF). It thus forms a feedback loop being both an MITF regulator and target.¹⁰ In addition, GPR143 may ensure delivery of MRPs to melanosomes rather than lysosomes by regulating multivesicular body (MVB) fusion. Exogenous GPR143 expression inhibited MVB-lysosome fusion. In pigment cells, this may allow preferential delivery to melanosomes,¹¹ a hypothesis consistent with the observation that GPR143 mutations are less consequential in the skin than in eyes where lysosomal activity, which is crucial in RPE for degradation of rod outer segments, may be disrupted.⁸

Levodopa (L-DOPA) and dopamine have been proposed as GPR143 ligands. They were initially shown to bind GPR143 at high concentrations (L-DOPA K_d of 9.35 μ M, dopamine K_d of



2.39 μM) and activate intracellular calcium release through $G_{q/11}$ protein coupling in transfected Chinese Hamster Ovary (CHO) cells.¹² Conversely, Hiroshima et al. found that L-DOPA has a much lower affinity for GPR143 (K_d of 79.1 μM) and does not cause calcium release in CHO cells, only in RPE-derived cells.¹³ Topologic orientation of GPR143 suggests that ligands bind from the melanosomal lumen.^{14,15} The association of GPR143 with L-DOPA and its precise role in signaling remain under debate.

RPE-regulated dopamine and L-DOPA levels are critical during development of the neural layer of the retina, which contains photoreceptor cells required for light absorption and ganglions.^{16,17} Reduction of L-DOPA levels due to disruption of melanin synthesis may underlie perturbed optic tract development. Thus, developmental eye defects are present in all forms of albinism, regardless of the mutated gene.¹⁸ In the case of OA1, L-DOPA-mediated GPR143 signaling may be inhibited.³ Furthermore, L-DOPA stimulation promotes GPR143- $G_{q/11}$ protein coupling, although GPR143 also coprecipitates with G_{α_o} , G_{α_i} , and G_{β} subunits of heterotrimeric G proteins in melanocyte extracts.^{1,12} A constitutively active GPR143 colocalized with β -arrestin 2 and 3 in transfected COS7 cells.¹⁹ Thus, multiple binding proteins, including tyrosinase,²⁰ may modulate GPR143 function.

The purpose of our study was to establish an assay that allows high-throughput screening of compound libraries to find pharmacologic tools with which to investigate GPR143 function. We sought to identify compounds that either activate or inhibit receptor signaling. Due to its intracellular localization, hydrophobic and/or high-molecular-weight compounds may not reach GPR143. Thus, we generated a mutant GPR143 (plasma membrane [pm]-GPR143) that traffics to the plasma membrane.²¹ Screening assays are typically performed with cell lines expressing recombinant proteins while lacking the endogenous proteins. Thus, we used CHO cells, which do not express GPR143 endogenously. A widely applied assay for library screening is the β -arrestin recruitment assay, because many ligands also show (biased) agonism in this assay.^{22–26} The assay is well suited for orphan GPCRs, because a positive control is not essential.^{27,28} GPR143 colocalizes with β -arrestin¹⁹; therefore, we hypothesized that the β -arrestin recruitment assay was suitable for high-throughput screening. CHO β -arrestin cell lines expressing either wild-type (wt-GPR143) or pm-GPR143 were thus established. Two compound libraries were screened for GPR143-specific ligands and hit compounds investigated for their effects in melanocytes.

MATERIALS AND METHODS

For detailed information about cell culture media, supplements, chemicals, and other materials, see Supplementary Materials.

Cloning, Cell Culture, and Transfection

Wt- and pm-GPR143 cloning was reported previously.²⁰ For more details, see Supplementary Materials.

Immunostaining

Transfected CHO cells were cultured, fixed, and stained as described in the Supplementary Information. Slides were analyzed with a Nikon A1 Spectral confocal microscope (Pharmaceutical Institute, University of Bonn, Bonn, Germany) and NIS Element Advanced Research software 4.0 (Nikon Instrument, New York, USA).

β -Arrestin Assay and High-Throughput Screening

The β -arrestin recruitment assay is a cell-based method (DiscoverX, Birmingham, UK) for detecting GPCR activation after ligand stimulation. In brief,²⁹ the day before the assay, CHO β arrestin cells transfected with wt or mutant GPR143 (20,000 cells per well) were seeded in a 96-well plate in 90 μL F12 medium with 10% fetal calf serum, 100 U/mL penicillin G, and 100 $\mu\text{g}/\text{mL}$ streptomycin and cultivated overnight. Then, 10 μL diluted compounds was added, and plates were incubated at 37°C for 90 minutes. F12 medium was used as negative control. The final dimethyl sulfoxide (DMSO) concentration per well was 1%. Afterward, the PathHunter detection reagent (DiscoverX) was added to the cell plate (50 $\mu\text{L}/\text{well}$) and incubated 1 hour at room temperature in the dark, and finally chemoluminescence was detected. The high-throughput screening was performed adding 1 μL 1 mM DMSO diluted compound in 99 μL F12 medium in each well to test the compounds at 10 μM and keep the 1% DMSO final concentration.

Melanin Assay

Melanin assays were performed as previously described.³⁰ In brief, melan-a cells were seeded in 10-cm² dishes (60,000 cells per dish) and cultivated overnight at 37°C with 10% CO₂. Different concentrations of compounds (pimozide, niclosamide, and ethacridine lactate) were added in each dish and incubated for 5 days as described above. On day 3, the cell medium was exchanged for fresh medium containing the compounds. Cells were treated with vehicles (DMSO and ethanol) as negative controls, whereas propylthiouracil (PTU, 300 μM final concentration) and 3-isobutyl-1-methylxanthine (IBMX, 150 μM final concentration) were used to decrease and increase, respectively, the pigmentation of melan-a cells. Afterward, cells were washed with PBS, scraped off the dishes with lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 5 mM EDTA, and protease inhibitor) and centrifuged 20 minutes at 14,000g at 4°C. The supernatant was transferred in a fresh tube, and the protein concentration was determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Grand Island, NY, USA). The cell pellet was washed twice with a solution 1:1 ethanol/ether and let dry under the fume hood. A water solution of 2 N NaOH was added to the dried pellets and warmed up to 80°C to dissolve the melanin. A fixed volume of melanin solution (100 to 200 μL) was transferred to a 96-well plate, and the absorbance was read at 490 nm. Independent experiments were performed in triplicates.

Tyrosinase Activity in Cell Lysates

The tyrosinase assays were performed as previously reported.^{30,31} In brief, melan-a cell lysates were used to determine the effect of compounds on tyrosinase activity.² The reaction mixture contained 1 mM cold tyrosine, 0.3 $\mu\text{Ci}/\text{sample}$ [³H]-tyrosine, 1 mM L-DOPA, 30 μg protein lysate, and the proper compound concentration.

To determine the effect of the compounds on tyrosinase in the cell, lysates were derived either from melan-a cells treated with compounds (no compound was added in the reaction mixture) or untreated melan-a cells.

The reaction was incubated at 37°C for 60 minutes and then separated on a Dowex column (Sigma-Aldrich Corp., St. Louis, MO, USA). Radioactivity of the eluted tritiated water released during the enzymatic hydroxylation of tyrosine to L-DOPA was quantified in a liquid scintillation analyser (LS6500; Beckman Coulter, Indianapolis, IN, USA). Data were normalized to the

tyrosinase hydroxylase activity in absence of compounds, set as 100%. Independent experiments were performed in triplicates.

Assays

Detailed descriptions of further assays and data analysis are provided in the Supplementary Materials. Calcium mobilization assays³² and cAMP assays³³ were performed as previously described.

Viability assays. Melan-a cells were seeded in a 96-well plate overnight. Cells were dosed at indicated concentrations and incubated for 72 hours at 37°C/10% CO₂. Viability was determined using the Cell-Titer 96 Cell Proliferation Assay (MTS; Promega, Madison, WI, USA). Cells treated with vehicle alone were used as controls to determine relative viability. Four independent experiments were performed.

Western Blot Analysis

Western blot analysis was performed as previously reported.²⁰ For details, also see Supplementary Materials.

RESULTS

Characterization of Wild-Type and Plasma Membrane-Targeted GPR143

We first developed a cell-based GPR143 expressing system for screening compound libraries. CHO cells were used, because they express very few GPCRs, are easy to transfect, and are well suited for multiple cell-based assay systems. GPR143 is intracellularly localized and topologically oriented toward the lysosomal/melanosomal lumen, constituting a challenge for screening. The GPR143 amino acid sequence contains two sorting signals that confer intracellular localization.²¹ By mutating these sequences (L223A-L224A, W329A-E330A), we generated a double mutant GPR143 (pm-GPR143) that localizes to the plasma membrane²⁰ with the putative binding site facing the extracellular space and accessible to ligands in the culture medium. Because the mutations affected only the signal sequences, we predicted that they would not interfere with downstream signaling and GPCR function would remain intact.

Ligand-bound GPR143 recruits β -arrestin, which is involved in agonist-mediated desensitization and signal transduction of GPCRs.¹⁹ We transfected β -arrestin-expressing CHO lines with either wt- or pm-GPR143 vectors and established stable lines. Western blot analysis confirmed expression (Fig. 1a) and immunostaining verified subcellular localization (Fig. 1b). Although the wt receptor is intracellularly expressed in the perinuclear region and in cytoplasmic vesicles, pm-GPR143 primarily colocalizes with the plasma membrane marker cadherin at the cell surface (Fig. 1b).

A β -arrestin recruitment assay suitable for high-throughput screening, that specifically identifies GPCR ligands, was then performed. Briefly, the assay evaluates GPCR ligand binding by monitoring β -galactosidase activity. β -Galactosidase is split into a smaller ProLink (PL) fragment linked to the C terminus of GPR143 and a large fragment fused to β -arrestin. On ligand activation, β -arrestin is recruited to GPR143, and β -galactosidase is complemented and can hydrolyze a substrate producing a chemiluminescent signal. Untransfected cells were used as controls to test ligand specificity. A major advantage of the β -arrestin assay is that it is well suited for orphan receptors because a positive control is not necessary. When high basal activity is observed, the assay is suitable to find not only potential agonists but also antagonists.²⁷ wt-GPR143-express-

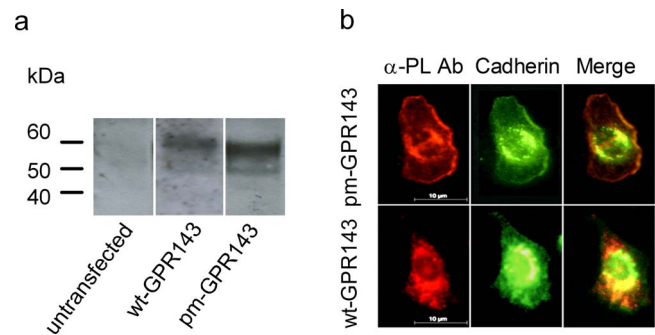


FIGURE 1. Expression and subcellular localization of CHO cells expressing wt- or pm-GPR143. (a) Western blot analysis: 30 μ g lysates were separated in 10% SDS-PAGE and transferred onto nitrocellulose. The anti-ProLink tag (mouse monoclonal) antibody was used as primary and rabbit anti-mouse horseradish peroxidase-coupled antibody was used as secondary antibody. (b) Immunofluorescence: cells were fixed with methanol/acetone and stained with primary antibodies anti-ProLink tag (mouse monoclonal) and anti-cadherin (rabbit polyclonal). Secondary antibodies anti-mouse-Alexa594 (red) and anti-rabbit-Alexa488 (green) were used.

ing cells showed high basal activity compared with untransfected CHO cells, as did the mutant receptor, thus confirming that both receptors are functional and the mutations do not affect receptor activity (Fig. 2a). A similar high basal activity was also found in the PathHunter CHO-K1 GPR143 β -arrestin orphan GPCR cell line by DiscoverX.

GPR143 Stimulation With L-DOPA

The β -arrestin CHO cell lines expressing the wt- or pm-GPR143 were used to investigate activation of various signaling pathways by the proposed GPR143 ligand L-DOPA.¹² CHO cell lines expressing wt- and pm-GPR143 showed high basal activation using the β -arrestin assay (Fig. 2a). Stimulation of GPR143 with L-DOPA at different concentrations induced only a minor increase in β -arrestin recruitment compared with the control. The increase was not concentration dependent, and the calculated signal-to-noise ratio was only 1.12 for pm-GPR143 and 1.25 for wt-GPR143. According to the manufacturer, only compounds reaching a signal-to-noise ratio >3 can be considered “valuable agonists.” The intracellular calcium release associated with GPR143 G_{q/11} protein coupling was also measured after L-DOPA stimulation. No increase in intracellular calcium was observed in either CHO line after L-DOPA treatment compared with the control (Fig. 2b; Supplementary Fig. S1a). Finally, the signaling pathways associated with G_i and G_s protein coupling were assessed. If L-DOPA induced coupling of GPR143 with G_i protein, a reduction in intracellular cAMP concentration is expected. However, no significant difference was measured after L-DOPA stimulation in comparison with the cAMP accumulation due to forskolin treatment (Fig. 2c; Supplementary Fig. S1b). In contrast, coupling of GPR143 to G_s protein after L-DOPA stimulation would cause an increase of intracellular cAMP accumulation. L-DOPA treatment of pm-GPR143-expressing cells did not induce any rise in cAMP concentration (Fig. 2c). To further confirm our findings, we also tested an L-DOPA ester (melevodopa), which did not have any effect on GPR143 activation (Supplementary Fig. S2).

In summary, we could not validate that L-DOPA is a GPR143 ligand, because the stimulation of the receptor by L-DOPA did not induce significant signaling pathway activation. L-DOPA was therefore not useful as a positive control.

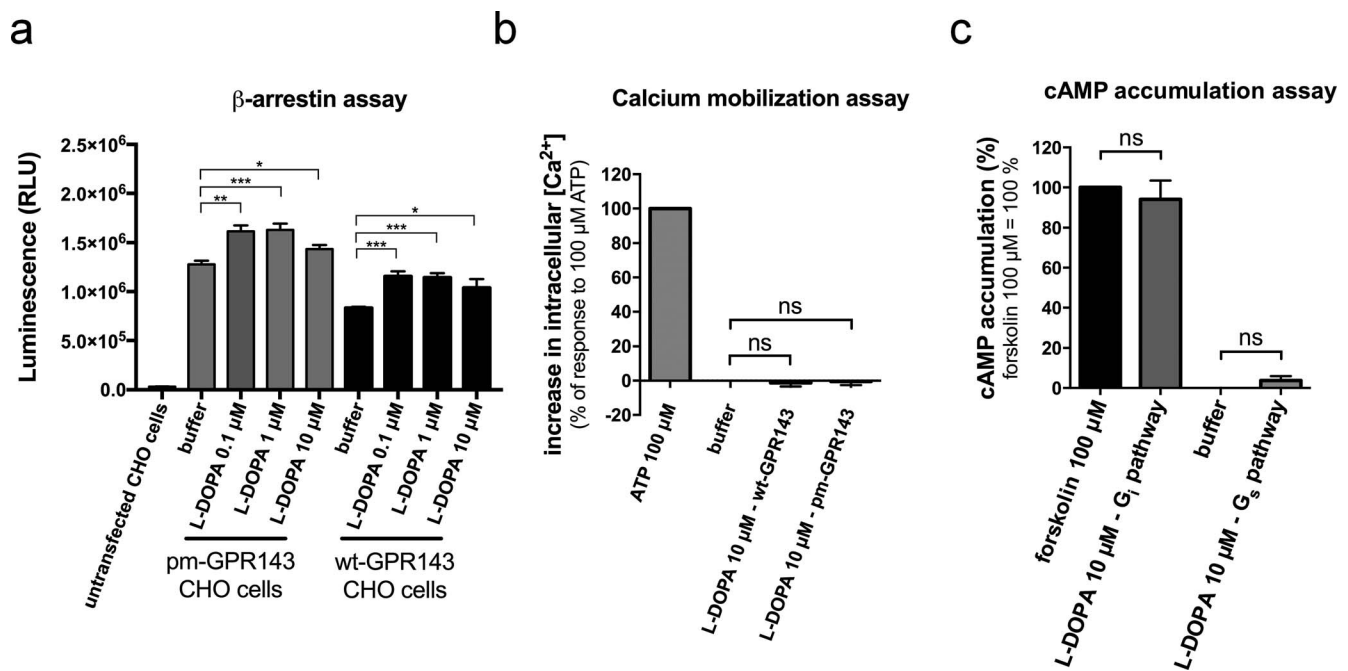


FIGURE 2. L-DOPA does not stimulate GPR143 in transfected CHO cells. (a) β -arrestin assay: L-DOPA was tested at 0.1, 1, and 10 μ M final concentrations at CHO cells expressing the wt- or pm-GPR143, and buffer was used as control. Experiments were performed in duplicate. (b) Calcium mobilization assay: data were normalized to the positive control (100 μ M ATP) and set as 100%, and the buffer control was set at 0%. Experiments were performed in triplicate. (c) cAMP accumulation assay: data were normalized to the positive control (100 μ M forskolin), set as 100%, and to the negative control, buffer, set as 0%. Experiments were performed in triplicate. Data are mean \pm SEM of three independent experiments. Two-tailed *t*-test: ^{ns}not significantly different from the control, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Compound Library Screening

The β -arrestin recruitment assay optimized for GPR143 was used for high-throughput screening of the Tocris library (includes chemically diverse biologically active compounds that modulate activity of GPCRs, ion channels, kinases, enzymes, and transporters) and an in-house drug library that includes approximately 600 marketed drugs. These libraries have been used successfully for screening purposes for other targets (GPCRs and enzymes) to find hit compounds suitable as lead compounds for further optimization or as pharmacologic tools.^{22,34–36}

Due to the high basal activity observed in CHO cells expressing GPR143, investigation of compounds either increasing or decreasing receptor activation was feasible. Each library compound was tested at 10 μ M final concentration, and the corresponding signal-to-noise ratio was calculated (Fig. 3). As a comparison, dopamine, L-DOPA, and L-DOPA methyl ester values were added to the plot (purple, black, and orange diamonds, respectively, Fig. 3a). No compounds reached a signal-to-noise ratio ≥ 3 , and the ones with highest ratios (≥ 1.5) produced nonreproducible values. In contrast, 36 compounds reached a signal-to-noise ratio ≤ 0.6 (threshold suggested by the manufacturer for potential antagonists or inverse agonists). All potential hits were rescreened twice, and three hit compounds from the in-house drug library were identified as potential GPR143 inhibitors with reproducible and consistent values (data not shown). The three compounds were tested against an alternative GPCR with high constitutive activity, and they did not alter its function, underlining the specificity of the interaction between the hit compounds and GPR143 (Table). The hits are drugs currently on the market: pimozone, an antipsychotic, and niclosamide and ethacridine lactate, which are antiseptic compounds (Table). Dose–response curves were obtained with increasing compound concentrations using the

β -arrestin assay (Fig. 4a). Although a full curve was obtained for pimozone, only partial curves were obtained for niclosamide and ethacridine lactate due to solubility and toxicity issues. Compound potencies calculated from these curves were in the low micromolar range: $2.72 \pm 1.17 \mu$ M for pimozone, $3.40 \pm 1.11 \mu$ M for niclosamide, and $26.1 \pm 1.03 \mu$ M for ethacridine lactate. A dose–response curve for pimozone was also obtained with CHO cells expressing wt-GPR143; the EC₅₀ value was in the same range: $4.62 \pm 1.37 \mu$ M (Fig. 4b).

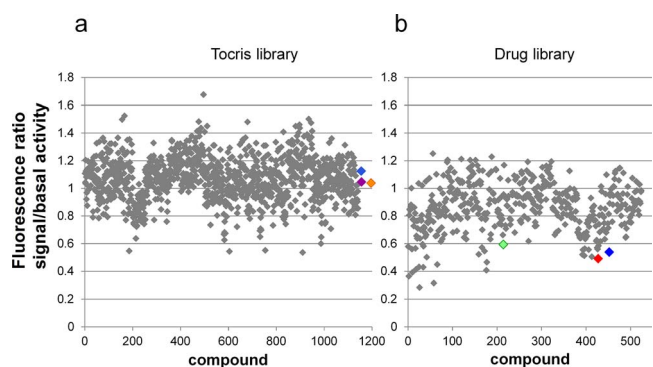


FIGURE 3. High-throughput library screening using the β -arrestin assay. (a) Tocris and (b) in-house drug library screens. CHO cells expressing pm-GPR143 were used. Each point represents a single compound (*n* = 1) tested at 10 μ M final concentration. Plotted values correspond to the signal-to-noise ratio (compound signal/baseline). Compounds are considered potential GPR143 agonists if the signal-to-noise ratio is >3.0 and potential inverse agonists if ≤ 0.6 . Data corresponding to L-DOPA, dopamine, and L-DOPA methyl ester (10 μ M) are shown in black, purple, and orange, respectively. (b) Reproducible hit compounds are highlighted in red (pimozone), green (niclosamide), and light blue (ethacridine lactate).

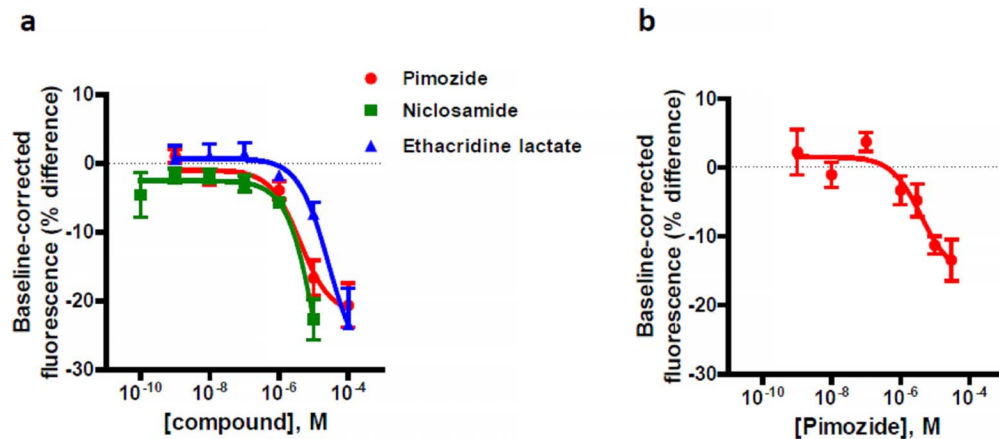


FIGURE 4. Dose-response curves of hit compounds in β -arrestin assay. CHO cells expressing the pm-GPR143 (a) or wt-GPR143 (b) were stimulated with different concentrations of pimoziide, niclosamide, and ethacridine lactate (a) or only pimoziide (b). Data were baseline corrected, normalized, and correspond to mean \pm SEM of three independent experiments performed in duplicates (a) or triplicates (b). Data were analyzed using Graph Pad Prism, and EC_{50} values were calculated as described in the Supplementary Information. EC_{50} values for pm-GPR143 are 2.72 ± 1.17 (pimoziide), 3.40 ± 1.11 (niclosamide), and 26.1 ± 1.0 mM (ethacridine lactate) (a). The EC_{50} value for pimoziide at the wt-GPR143 is 4.62 ± 1.37 μ M (b).

Validation of Hit Compounds

Because the effects of the hit compounds were specific for GPR143 expressed in CHO cells, we tested them in melanoma murine melanocytes. A viability assay was performed to determine an appropriate concentration range that was not toxic. After 3 days of treatment, pimoziide caused toxicity at concentrations higher than 10 μ M, whereas niclosamide caused a 60% loss of viability at 1 μ M. Ethacridine lactate displayed toxicity at concentrations higher than 100 μ M (Fig. 5a).

Melanin assays were performed using melanocyte extracts following 5 days of treatment with increasing compound concentrations. PTU, a tyrosinase inhibitor that reduces melanin synthesis, and IBXM, a phosphodiesterase regulator, which promotes pigmentation, were used as controls (Fig. 5b). Niclosamide and pimoziide dramatically reduced cell pigmentation. Niclosamide (0.5 μ M) caused a 94% reduction in melanin, whereas pimoziide caused a 88% decrease relative to cells treated with DMSO. Ethacridine lactate induced a 39% reduction in pigmentation at 1 μ M and 85% at 5 μ M compared to the control; however, at the highest concentration (7.5 μ M), no change in melanin accumulation was observed. For images of cells after treatment and corresponding pellets see Supplementary Figure S3.

Tyrosinase Activity

Tyrosinase catalyzes several melanin synthesis reactions including the first converting tyrosine to L-DOPA. To verify target specificity of hit compounds, tyrosinase activity was analyzed in lysates from melanocytes. The tyrosine hydroxylase activity of tyrosinase was first examined in lysates of untreated cells (Fig. 6a) incubated with different concentrations of the three hit compounds (50 and 100 μ M) to determine whether there was direct interaction of the compounds with tyrosinase and whether the reduction in pigmentation observed in the melanin assays was caused by direct enzyme inhibition. As a control, increasing concentrations of cold L-tyrosine were used to compete with tritiated (3 H)-L-tyrosine. The tyrosinase activity in cell lysates incubated with pimoziide, niclosamide, and ethacridine lactate was comparable to the mock-treated lysates, indicating that the compounds do not directly inhibit tyrosine hydroxylase activity (Fig. 6a).

The impact of the compounds on in situ tyrosinase activity was determined using the same extracts used to assay melanin levels (Fig. 5b). In situ activity represents activity of all mature tyrosinase in the cell, which can be affected by multiple factors, including a change in tyrosinase protein levels. Western blot analysis was performed with the same lysates (Fig. 6b). Enzyme activity following pimoziide treatment was similar to vehicle with only a slight down-regulation at the highest concentration (Fig. 6b). Significant inhibition of tyrosinase activity and a reduction in tyrosinase expression were observed in melanocytes treated with niclosamide compared to the vehicle (Fig. 6b), whereas ethacridine lactate induced a reduction in tyrosinase activity only at higher concentrations (>5 μ M).

DISCUSSION

GPR143 is the protein product of the *OAI* gene and mutations at this locus were found to result in ocular albinism type 1 (OAI).³⁷ The pathophysiologic role of GPR143 in pigmented cells is still unclear. Nevertheless, considering the subcellular consequences of GPR143 mutations, the suggested function for the receptor is as a regulator of melanosome biogenesis and maturation.^{8,9} To date, only L-DOPA has been proposed to be a GPR143 endogenous agonist activating the $G_{q/11}$ protein associated pathway.¹²

The aim of our investigation was to identify additional ligands that could serve as pharmacologic tools to study the function of GPR143 and clarify its role in the OAI pathogenesis. We first developed an assay to monitor GPR143 activity and investigated whether L-DOPA is a useful positive control. To facilitate rapid screening that accommodates testing of compounds that cannot enter the cell, the two sorting signal sequences responsible for intracellular localization of GPR143²¹ were mutated generating pm-GPR143, which is expressed at the plasma membrane and therefore accessible from the extracellular space. We established CHO cell lines expressing either the wt- or pm-GPR143 and optimized three cell-based assays to test GPR143 activation through downstream signaling pathways (β -arrestin recruitment, calcium mobilization, and cAMP accumulation).

The β -arrestin recruitment assay, demonstrated that CHO cells expressing wt- and pm-GPR143 displayed high constitutive activation compared with CHO cells that do not express

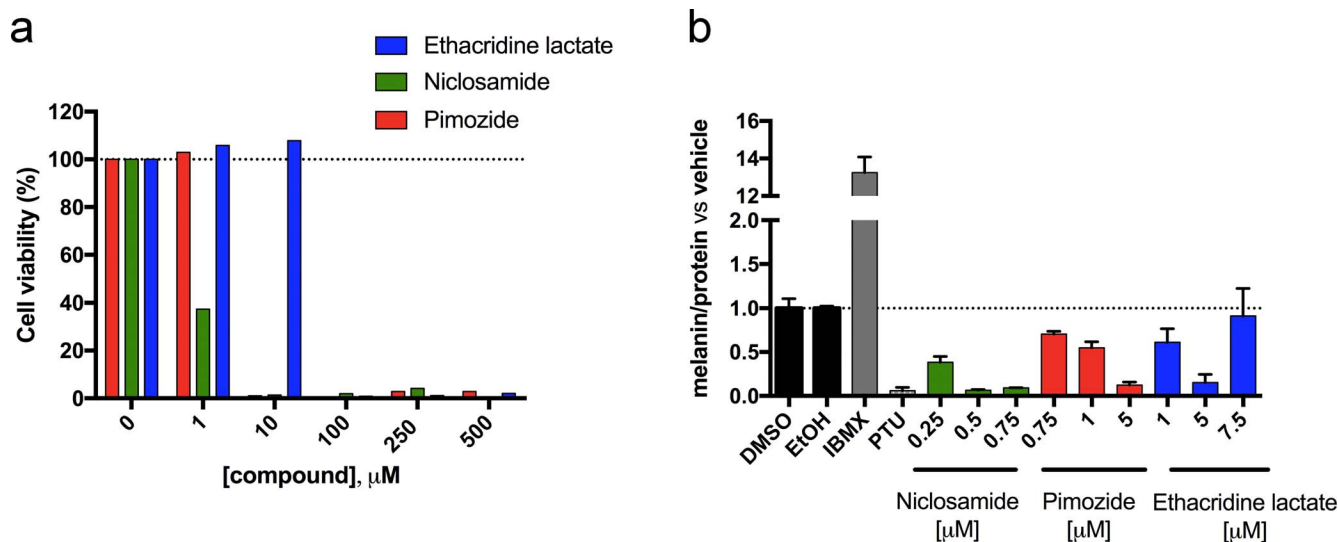


FIGURE 5. Treatment of melanocytes with the hit compounds. (a) MTT assay for cell viability. Melan-a cells were treated with increasing concentrations of pimozide, niclosamide, and ethacridine lactate for 72 hours when cell viability was determined. Data were normalized to viability of cells treated with vehicle (DMSO or ethanol), which was set as 100%. Data represent one of four independent experiments performed in quadruplicate. (b) Melanin assay. Melan-a cells were treated with increasing concentrations of hit compounds. Melanin and protein were extracted and quantified (melanin/protein). Data were normalized to cells treated with vehicle alone (DMSO and ethanol), which was set to 1. PTU and IBMX were used as controls to decrease and increase pigmentation respectively. Data correspond to mean \pm SEM from triplicate samples from one of five representative experiments.

GPR143, as previously reported.¹⁹ Thus, pm-GPR143 retains functionality despite the mutations that route it to the plasma membrane. L-DOPA was tested in the β -arrestin recruitment assay against wt- and pm-GPR143, and only a slight increase in

activation was observed at 10 μM . Because the compound was previously reported to have a low affinity for the receptor,^{12,13} higher L-DOPA concentrations were tested. However, the signal did not reach threshold. L-DOPA activation of calcium

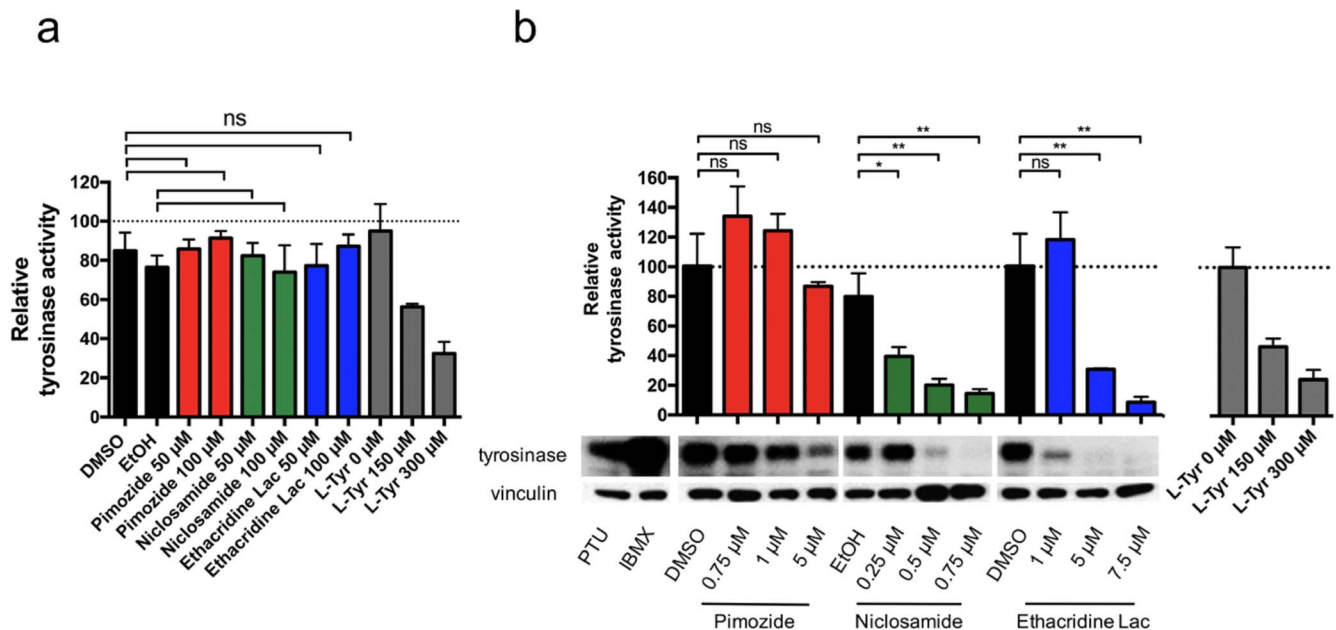
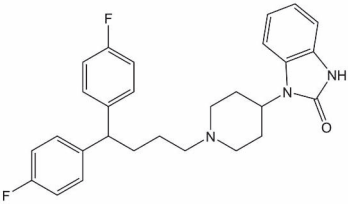
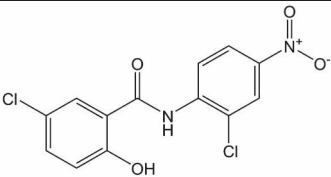
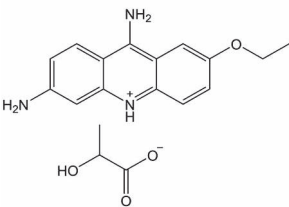


FIGURE 6. Effect of hit compound treatment on tyrosinase activity and expression. (a) Tyrosinase assays performed on lysates from untreated cells incubated with two concentrations (50 and 100 μM) of the hit compounds: pimozide, niclosamide, and ethacridine lactate. Data were normalized to activity of lysates alone (L-Tyr 0 μM), which was set as 100%. Positive controls correspond to L-tyrosine treatments (150 and 300 μM), because unlabeled L-tyrosine competes with [³H] L-tyrosine, and the release of tritiated water decreases. The vehicles (DMSO and ethanol) were tested as control. (b) Tyrosinase assays and Western blot analysis were performed using lysates of melanocytes dosed with the compounds for 5 days (same as in Fig. 5b). L-Tyrosine (150 and 300 μM) and the vehicles (DMSO and ethanol), PTU, and IBMX were used as additional controls. The housekeeping gene vinculin was used as a loading control. Experiments were performed in triplicate. Data are mean \pm SEM of one representative experiment, which was repeated three times with comparable results. Two-tailed *t*-test: ns not significantly different from the control, **P* < 0.05, ***P* < 0.01.

TABLE. Overview of Hit Compounds

Compound	Structure	Medicinal Use	GPR143, S:N ± SEM	Control GPCR, S:N ± SEM
Pimozide		Antipsychotic drug used for treatment of schizophrenia and resistant tic in Tourette syndrome.	0.61 ± 0.04	0.917 ± 0.045
Niclosamide		Anthelmintic teniicide used to treat cestodes infections in humans.	0.63 ± 0.1	0.992 ± 0.108
Ethacridine lactate		Aromatic organic compound based on acridine used primarily an antiseptic. Also used as a second trimester abortifacient.	0.61 ± 0.04	0.928 ± 0.067

The structure and principal effects of the hit compounds from the drug library screening are listed. The signal-to-noise ratio (S:N) of the compounds tested at 10 μ M final concentration on CHO cells expressing the human mutant GPR143 is specified. As a control, the compounds have been tested in CHO cells expressing another GPCR, which has a high basal activity as well. Data correspond to mean \pm SEM of three independent experiments performed in duplicates.

signaling pathway through $G_{q/11}$ protein coupling and cAMP accumulation pathways through G_i and G_s protein coupling were also tested, and no significant differences were observed compared to controls, supporting a previous study.¹³ It is possible that participation of a third factor only present in pigmented cells is necessary for GPR143 activation by L-DOPA. Alternatively, the observed calcium release in ARPE-19 cells after L-DOPA treatment is not a GPR143-specific effect, because several dopamine receptor subtypes are endogenously expressed in those cells, such as D1 and D5 subtypes, and their activation induces intracellular calcium increase.^{38,39} Recent studies indicated that L-DOPA produced by RPE plays a role in retinal development through activation of dopamine receptors present on neural retina cells; however, dopamine receptor targeting has yet to be confirmed.¹⁷ RPE cells differentiate during the first days of eye formation,¹⁶ and during this time, tyrosinase produces L-DOPA, which is then secreted and taken up by retinal neurons. Production of dopamine from L-DOPA in immature retina cells induces activation of dopamine receptors and plays a role in retinal differentiation from early development stages.

Our data suggest that the β -arrestin recruitment assay is the most suitable to analyze GPR143 function given that it shows high constitutive activity. We tested the compounds against pm-GPR143, which is accessible to all compounds. The compound library screening resulted in three reproducible

hit compounds, which decreased constitutive GPR143 activation with potencies in the micromolar range. The hit compounds were pimozide, an antipsychotic drug active on dopamine receptors⁴⁰; niclosamide, an oral anthelmintic drug that is also active against cancer cells⁴¹; and ethacridine lactate, an antiseptic and abortifacient. The effect of the three compounds in the β -arrestin assay was specific for GPR143 because no changes in signal were observed against a second GPCR with high constitutive activity as well.

To investigate the effect of the compounds in pigmented cells expressing endogenous GPR143, wild-type melan-a murine melanocytes were dosed with increasing concentrations of compounds, and accumulated melanin content was quantified. Treatment with the three hit compounds induced a dose-dependent reduction in pigmentation (with the exception of high-dose ethacridine lactate), tyrosinase activity, and expression. Melanin synthesis in melanocytes and RPE is controlled by tyrosinase, which catalyzes tyrosine hydroxylation to L-DOPA and L-DOPAs oxidation to dopaquinone. Thus, we investigated whether the reduction in pigmentation was due to direct inhibition of tyrosinase by the compounds. Incubating melan-a lysates with the compounds did not directly inhibit tyrosinase enzyme activity.

There are several mechanisms through which GPR143 can influence tyrosinase expression. GPR143 regulates transcription of several melanosomal genes, including tyrosinase,

through modulation of the microphthalmia-associated transcription factor (MITF).^{10,42} Niclosamide and ethacridine lactate treatment may inhibit GPR143 and reduce MITF-mediated tyrosinase expression.

The reduction in pigmentation in melan-a cells following pimoziide treatment was not due to a significant reduction in tyrosinase activity. Because pimoziide selectively inhibited the constitutive activity of GPR143, it may induce a reduction in the melanocyte pigmentation by alternative mechanisms. GPR143 may also directly regulate tyrosinase. We have already shown that there is a direct interaction between GPR143 and tyrosinase.²⁰ Disruption of the interaction in the presence of pimoziide may result in a reduction of tyrosinase protein.

In a previous study, GPR143 was inactivated by interference RNA in pigmented MNT-1 melanoma cells, and the melanosome morphology and composition were analyzed when GPR143 expression was silenced.⁴³ It was found that the number of mature melanosomes and melanin content was reduced to 50% compared to the control cells. After 5 days of GPR143 silencing, abnormal enlarged pigmented structures appeared in the cells, and the pigmentation and the number of melanosomes did not increase.⁴³ Thus, pimoziide may decrease GPR143 function and replicate the phenotype resulting from loss of function GPR143 mutations. An alternative explanation would be that pimoziide targets melanocyte dopamine receptors, because this compound has high affinity for different dopamine receptor subtypes, such as D2, D3, and D4.⁴⁴⁻⁴⁶ Melanocytes and RPE express dopamine receptors and dopamine agonists reduce tyrosinase activity in hair follicle melanocytes, diminishing the pigmentation of the cultured cells.

In summary, a β -arrestin recruitment assay, suitable for high-throughput screening, was established. We identified three compounds that decreased constitutive recombinant GPR143 activity and reduced pigmentation of melanocytes, suggesting that they also inhibit endogenous GPR143. Thus, GPR143 may be constitutively active in pigment cells.

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