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Identification of compounds acting as negative allosteric modulators of the LPA_1 receptor

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ABSTRACT

The Lysophosphatidic Acid 1 Receptor (LPA_1 receptor) has been linked to the initiation and progression of a variety of poorly treated fibrotic conditions. Several compounds that have been described as LPA_1 receptor antagonists have progressed into clinical trials: 1-(4-{4-[3-methyl-4-({[(1R)-1-phenylethoxy]carbonyl}amino)-1,2-oxazol-5-yl]phenyl}phenyl)cyclopropane-1-carboxylic acid (BMS-986202) and 2-{4-methoxy-3-[2-(3-methylphenyl)ethoxy]benzamido}-2,3-dihydro-1H-indene-2-carboxylic acid (SAR-100842). We considered that as LPA_1 receptor function is involved in many normal physiological processes, inhibition of specific signalling pathways associated with fibrosis may be therapeutically advantageous. We compared the binding and functional effects of a novel compound; 4-({(Cyclopropylmethyl)[4-(2-fluorophenoxy)benzoyl]amino}methyl}benzoic acid (TAK-615) with BMS-986202 and SAR-100842. Back-scattering interferometry (BSI) was used to show that the apparent affinity of TAK-615 was enhanced in the presence of LPA. The binding signal for BMS-986202 was not detected in the presence of LPA suggesting competition but interestingly the apparent affinity of SAR-100842 was also enhanced in the presence of LPA. Only BMS-986202 was able to fully inhibit the response to LPA in calcium mobilisation, β-arrestin, cAMP, GTPγS and RhoA functional assays. TAK-615 and SAR-100842 showed different inhibitory profiles in the same functional assays. Further binding studies indicated that TAK-615 is not competitive with either SAR-100842 or BMS-986202, suggesting a different site of binding. The results generated with this set of experiments demonstrate that TAK-615 acts as a negative allosteric modulator (NAM) of the LPA_1 receptor. Surprisingly we find that SAR-100842 also behaves like a NAM. BMS-986202 on the other hand behaves like an orthosteric antagonist.

1. Introduction

Oleoyl-L-α-Lysophosphatidic acid (LPA) describes a subset of small bioactive lysophospholipids that exert their biological effects through a family of six known G-protein coupled receptors (GPCRs), designated LPA_{1-6} (Choi et al., 2010). The LPA_1 receptor (An et al., 1997; Fukushima et al., 1998) functionally couples to $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$ signalling pathways and can activate β-arrestin (Fukushima et al.,

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2015; Stoddard and Chun, 2015). It is involved in the regulation of a wide range of cellular functions including proliferation, migration, survival and differentiation (Aikawa et al., 2015). There is growing evidence linking aberrant LPA_1 receptor signalling to a variety of poorly treated pathophysiological conditions. Increased levels of LPA and LPA_1 receptor signalling activity have been implicated in the development of neuropathic pain (Inoue et al., 2004; Ma et al., 2009; Halder et al., 2013) and the LPA_1 receptor has also been implicated in the initiation and progression of kidney (Pradère et al., 2007) and lung (Tager et al., 2008) fibrosis. Genetic deletion of the LPA_1 receptor provided protection in a bleomycin model of scleroderma (Castelino et al., 2011). Fibrotic conditions are poorly treated clinically, and programs have been initiated to develop compounds that target the LPA_1 receptor (Kihara et al., 2015). As a result, compounds with inhibitory activity toward the LPA_1 receptor and efficacy in pre-clinical models of fibrosis have been published (Qian et al., 2012; Gan et al., 2011; Castelino et al., 2011; Swaney et al., 2010; Ohasi and Yamamoto, 2015). Compounds exemplified in these studies display activities against other LPA receptors making it difficult to interpret the relative contribution that LPA_1 receptor inhibition plays in alleviating fibrosis in these models. It is suggested that fibroblasts migrate to sites of fibrosis and become activated by LPA via the LPA_1 receptor accelerating the development of fibrosis (Miyabe et al., 2014; Tang et al., 2014). Cell migration in response to LPA stimulation of the LPA_1 receptor has been mapped to the activation of RhoA (Sugimoto et al., 2006; Hao et al., 2007).

LPA signalling plays a vital role in the normal physiological responses of many bodily processes. Although LPA_1 receptor antagonists have been shown to have beneficial effects in several disease states we hypothesised that negatively modulating a specific subset of the signalling pathways may be more beneficial. We compared: the in vitro pharmacological profile of 4-({(Cyclopropylmethyl)[4-(2-fluorophenoxy)benzoyl]amino}methyl}benzoic acid (TAK-615) with two previously described LPA_1 receptor antagonists, 2-{4-methoxy-3-[2-(3-methylphenyl)ethoxy]benzamido}-2,3-dihydro-1H-indene-2-carboxylic acid (SAR-100842) (Khanna et al., 2014) and -(4-{4-[3-methyl-4-({[(1R)-1-phenylethoxy]carbonyl}amino)-1,2-oxazol-5-yl]phenyl} phenyl)cyclopropane-1-carboxylic acid (BMS-986202) (also known as BMS-986020 and AM152). BMS-986202 which is related to AM095 (Swaney et al., 2011; Castelino et al., 2011) is currently in Phase II clinical trials for idiopathic pulmonary fibrosis. SAR-100842 has some clinical efficacy reported from a Phase IIA clinical trial for diffuse cutaneous systemic sclerosis (Allanorem et al., 2015). The long-term impact of these broad LPA_1 receptor antagonists on normal physiological responses remains to be determined.

2. Materials and methods

2.1. Materials

Oleoyl-L-α-LPA was purchased from Sigma (L7260). All other standard laboratory chemicals were obtained from Sigma unless stated otherwise. Cell culture media and supplements were obtained from Thermo Fisher unless stated otherwise.

2.2. Cell culture

The rat buffalo hepatoma cell line, MCA-RH 7777 (RH7777), was obtained from ECACC (Cat. 90021504). RH7777 cells over-expressing either the human LPA_1 (RH7777-hLPA_1R) receptor were generated 'in-house' and maintained in DMEM supplemented with 1× non-essential amino acids, 25 mM HEPES, pH 7.4, 10% Foetal Clone III and 250 μg/ml G418 at 37 °C, 5% CO_2. The human mesothelium cell line, MeT-5A, was obtained from ATCC (CRL-9444). These cells were maintained in M199 medium supplemented with 1.5 g/l $NaHCO_3$, 3.3 nM Epidermal Growth Factor (PeproTech, USA), 400 nM Hydrocortisone, 870 nM Zinc-free bovine Insulin (Roche, Basal, Switzerland) 3.87 μg/l H_2SeO_3, 20 mM HEPES, 10% Foetal Bovine Serum and 1 ml/l Trace elements B liquid (MediaTech). Foetal Clone III was obtained from GE Healthcare.

2.3. Crude membrane preparation

Cells were gown to near confluency in trays, washed with 1× PBS and harvested by scraping. Cells were collected by centrifugation and washed several times with ice cold 1× PBS. Cells were suspended in ice cold Complete Bomb Buffer (10 mM PIPES, pH 7.3, 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1 mM ATP, 0.1 mM PMSF, 1× Complete EDTA free Protease Inhibitor Cocktail (Roche)) at 1×10^7 cells/ml and kept on ice for 10 min. Cells were placed into a suitable pressure container and the solution saturated with nitrogen at 500–600 pounds per square inch for 20 min at 4 °C. Simultaneous disruption and collection of the cellular material was achieved by the slow release of pressure via a collection tube. The resultant homogenate was neutralized with 0.1 M EDTA and 0.1 M EGTA. Large cellular debris was removed by a low speed centrifugation (1000×*g* for 5 min at 4 °C). Membranes were harvested from the supernatant by centrifuging at 75,000×*g* for 60 min at 4 °C. The membrane pellet was homogenised in ice cold 20 mM HEPES pH 7.4 and the protein concentration determined. The protein concentration was adjusted to 2 mg/ml and the membrane homogenate stored at − 80 °C in 20 mM HEPES pH 7.4, 10% sucrose, 1% BSA.

2.4. In vitro studies

2.4.1. Calcium mobilisation assay

10,000 cells/well in 25 μl of media were seeded into collagen coated black clear bottomed 384 well plates (Corning Biocoat #354667) and incubated overnight at 37 °C, 5% CO_2. On the day of assay, media was replaced with Calcium 5 dye (Molecular Devices) prepared in assay buffer (1× HBSS, 25 mM HEPES, pH 7.4, 0.1% fatty acid free BSA, 1.25 mM Probenecid) and allowed to load for 60 min at 37 °C. Compounds were prepared at 200× in DMSO at 10 μM top concentration with ½ log dilutions, stamped into assay buffer to prepare a final 10× (5% DMSO) working stock. Compound was added to the cells to give a final top concentration of 10 μM, ½ log dilutions and incubated for 25 min. LPA to a final concentration of 1 μM (EC_{80}) was added in a FLIPR Tetra (Molecular Devices) and changes in fluorescence recorded (excitation wavelengths of 470–495 nm and emission wavelengths of 515–575 nm).

2.4.2. cAMP accumulation assay

Compounds were prepared at 200× in DMSO at 10 μM top concentration ½ log dilutions, stamped into assay buffer (1× HBSS, 20 mM HEPES pH 7.4, 0.1% Fatty Acid Free BSA, 0.5 mM IBMX) to prepare a final 4× (2% DMSO) working stock. 3 μl of compound was added per well of the assay plate. 3000 cells in 3 μl of assay buffer were then added to each well and incubated at room temperature for 5 min 3 μl of LPA (final concentration of 90 nM (EC_{90})) followed by 3 μl of forskolin (final concentration of 1 μM) in assay buffer was added to each well and incubated at room temperature for 60 min. Changes in cAMP, expressed as a change in the fluorescence ratio, in each well were determined using a HTRF Dynamic cAMP assay (Cisbio #62AM4PEC) according to the manufacturer's protocol. The resulting fluorescence was measured on an Envision plate reader (Perkin Elmer) (excitation wavelength 320 nm and emission wavelengths at 620 and 665 nm).

2.4.3. GTPγS assay

Compounds were profiled in a [^{35}S]-GTPγS assay at Millipore (St Charles, MO, USA). Compounds were prepared at 800× in DMSO at 10 μM top concentration, 1:3 dilutions, and stamped into assay buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$) to prepare an 8× final concentration stock solution. Compounds were mixed 1:1 either in assay buffer (agonist plate) or 7.5 μM LPA (antagonist plate) to give a 4× working stock. [^{35}S]-GTPγS was prepared at 1.2 nM (4× stock) in