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LPAR1 Modulator Paper

Title

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 mobilization, 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.

KEYWORDS

Negative Allosteric Modulator, LPA_1 Receptor, G-protein signalling, Backscattering Interferometry, Fibrosis

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; Fukishima et al., 1998) functionally couples to $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$ signalling pathways and can activate β-arrestin (Fukishima et al., 2015; Stoddard & 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; Ohashi & 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 (Allanore 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 1x 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 1x PBS and harvested by scraping. Cells were collected by centrifugation and washed several times with ice cold 1x 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, 1x Complete EDTA free Protease Inhibitor Cocktail (Roche)) at 1 x 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 (1,000 x g for 5 min at 4°C). Membranes were harvested from the supernatant by centrifuging at 75,000 x 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 Mobilization 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 (1x 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 200x in DMSO at 10 µM top concentration with ½ log dilutions, stamped into assay buffer to prepare a final 10x (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 1µM (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 200x in DMSO at 10 µM top concentration ½ log dilutions, stamped into assay buffer (1x HBSS, 20 mM HEPES pH 7.4, 0.1% Fatty Acid Free BSA, 0.5 mM IBMX) to prepare a final 4x (2% DMSO) working stock. 3 µl of compound was added per well of the assay plate. 3,000 cells in 3 µl of assay buffer were then added to each well and incubated at room temperature for 5 min. 3µl 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 800x 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 8x 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 4x working stock. [^{35}S]-GTPγS was prepared at 1.2 nM (4x stock) in assay buffer. GDP was prepared at 40 µM (4x stock) in assay buffer. Membranes from LPA_1 receptor expressing recombinant Chem-1 cells (Millipore #HTS089M) were prepared in assay buffer/0.4% saponin. All reagents were dispensed into 96-well plates in the following order: 25 µl membranes, 25 µl GDP, 25 µl [^{35}S]-GTPγS, and 25 µl ligand/compound and incubated at 30°C for 30 min. The reaction was stopped with the addition of 100 µl ice cold assay buffer to all wells. Samples were harvested onto filter plates using a

Brandell cell harvest and dried in a 37°C non-humidified incubator for 120 min. 40 µl/well of Perkin Elmer Betaplate Scintillation Cocktail was added to each well and the plates read on a Microbeta Counter (Perkin Elmer).

2.4.4. β-Arrestin-2 Recruitment Assay

Compounds were tested at DiscoverX (Freemont, CA, USA.). PathHunter CHO-EDG2 cells (DiscoverX) were plated overnight in PathHunter Cell Plating Reagent 18 (DiscoverX). For the determination of agonist activity cells were exposed to titrations of compound for 90 min at 37°C; for determination of antagonist activity cells were exposed to titrations of compound for 30 min at 37°C before exposure to 500 nM LPA for 90 min at 37°C. Assays were terminated with the addition of PathHunter detection reagents as per manufacturer's instructions and the resulting luminescence measured using an Envision plate reader (Perkin Elmer).

2.4.5. Activated RhoA Assay

3.5×10^5 MeT-5A cells/well in 1 ml of full media were seeded into 12 well plates and incubated for 24 hs. Cells were washed twice with 1x PBS and then serum starved for 24 hs in 0.5% charcoal stripped serum media. Cells were incubated with compound for 1 h before being challenged with 10 µM LPA for 1 min. The level of activated RhoA was measured using Activated RhoA GLISA kits (Cytoskeleton) according to the manufacturer's protocol. Briefly,

cells were rapidly processed by rinsing with ice cold 1x PBS before being harvested in ice-cold lysis buffer, clarified by centrifugation and snap frozen in liquid nitrogen. The protein level present in each sample was determined. The protein concentration of thawed samples was equalised by the addition of ice cold buffer before being applied to wells of the ELISA plate (approximately 50 µg). The baseline was determined using lysis buffer alone and freshly prepared purified activated RhoA was used as a positive control. The resultant luminescence present in the processed plates was measured using an Envision plate reader (Perkin Elmer).

2.4.6. Back-Scattering Interferometry (BSI) Binding Assay

All assays were performed by Molecular Sensing Inc (Nashville, TN, USA). Membranes were prepared as previously described from RH7777-hLPA$_1$ receptor expressing and untransfected RH7777 cells (wild type) and were diluted 20-fold in PBS, pH 7.4, 0.36% DMSO. The compounds were diluted from 10 mM in DMSO to the final maximum stock concentration of 36 µM with PBS, pH 7.4, 0.42% sucrose. A 3-fold serial dilution was performed with the compounds and refractive index-matched assay buffer, PBS, pH 7.4, 0.42% sucrose, 0.36% DMSO. The compound serial dilutions were mixed 1:1 with either the LPA$_1$ receptor or wild type membrane fractions in a 96-well polypropylene microplate (Eppendorf, Hauppauge, NY). After incubation at room temperature for 120 min, the plate was analysed on a pre-commercial prototype BSI instrument. Binding signal was expressed as the phase shift in the interference

fringe patterns on a CMOS camera as measured in milliradians. The data from the wild type membrane fractions was subtracted from the LPA_1 receptor expressing fractions point by point to obtain the specific binding interaction of the compounds with the LPA_1 receptor. For competition experiments the competing ligand was incubated at 2x the final desired concentration with the target for 30 min prior to the addition of the titrated ligand of interest.

2.5. Data Analysis

All data is plotted as means ± S.E.M. Functional data was fitted using a four parameter, logistic curve equation to estimate compound IC_{50} values. Binding data was fitted using a comparison of a one-site versus a two-site binding model to determine K_d values for each compound. In all cases Graphpad Prism 7.04 software (San Diego, CA, USA) was used.

3. Results

3.1 TAK-615 binds the LPA_1 receptor with high affinity

Having identified TAK-615 following a high throughput screening campaign directed towards inhibitors of the LPA_1 receptor we wanted to confirm if TAK-615 was able to bind to the human LPA_1 receptor. BSI is a label free homogeneous analytical technique that detects changes in the refractive index of samples measured in radians. The signal generated in a reference sample that

lacks the LPA_1 receptor is compared to the signal generated in a test sample containing the receptor. By refractive index matching the buffer using DMSO with the compound being assessed, any detection of refractive index change associated with compound concentration is prevented. Differences that are detected are taken to be due to an interaction between the target protein and compound. Binding isotherms can be generated by plotting radians against compound concentration. Using BSI we were able to show that the binding data for TAK-615 was best described by a two-site model with estimated K_d high affinity (K_dHi) of 1.7 ± 0.5 nM and K_d low affinity (K_dLo) of 14.5 ± 12.1 nM (Fig. 1A). For consistency we also used BSI to estimate the binding affinities of SAR-100842 and BMS-986202 to the human LPA_1 receptor (Fig. 1B & C respectively). The data for SAR-100842 was best fitted using a two-site model (K_dHi 2.2 ± 0.8 nM, K_dLo 921 ± 50 nM) whilst a one-site binding model (K_d 2.7 ± 0.4 nM) best described data generated with BMS-986202.

3.2 *In vitro* functional profiling of TAK-615, SAR-100842 and BMS-986202

Ligand engagement of the LPA_1 receptor leads to the activation of multiple signalling pathways such as $G\alpha_q$, $G\alpha_i$ and β-arrestin. When seeking to determine if a compound shows differential modulation of signalling pathways relative to other compounds, it is vital to assess the activity of all compounds against all signalling pathways arising from receptor ligand engagement. We

compared the inhibitory effects of TAK-615, SAR-100842 and BMS-986202 in cellular assays to build up a pharmacological profile for each compound.

3.2.1 Calcium mobilisation

A calcium mobilization assay in an RH7777 cell line exogenously expressing the LPA_1 receptor was used to assess the impact of compounds on $G\alpha_q$ signal transduction (Fig. 2A). Cells were pre-incubated with compound before stimulation with a single non-maximal concentration of LPA (1000 nM). Unexpectedly in our assay SAR-100842 showed weak inhibitory activity (25-30% at 10 µM). TAK-615 was only able to partially inhibit the LPA response (60% at 10 µM, IC_{50} of 91 ± 30 nM). BMS-986202 was the only compound tested that fully inhibited the LPA response (100% inhibition, IC_{50} of 18 ± 2.2 nM).

3.2.2 Modulation of cellular cAMP

Activation of the LPA_1 receptor inhibits the activity of adenylate cyclase through $G\alpha_i$ activation. RH7777 cells exogenously expressing human LPA_1 receptor were incubated with compounds before being challenged with a sub-maximal concentration of LPA (90nM). In this assay decreasing HTRF ratio values follow increasing levels of cellular cAMP. If the $G\alpha_i$ signalling from the LPA_1 receptor had been inhibited this would be revealed by the subsequent activation of adenylate cyclase by forskolin and a decrease in the HTRF ratio. BMS-986202 was able to

fully inhibit the LPA driven $G\alpha_i$ signalling with an IC_{50} of 34 ± 3 nM (Fig. 2B). In contrast to the calcium flux data generated in the same cell line SAR-100842 was also able to fully inhibit $G\alpha_i$ signalling and with a similar potency to BMS-986202 (IC_{50} 52.5 ± 12 nM). TAK-615 was observed to have no inhibition (Fig. 2B), the HTRF ratio remains high so cellular cAMP is low indicating that the LPA_1 receptor is active. To confirm these observations, it was decided to test all three compounds in another orthogonal assay.

3.2.3 [^{35}S]-GTPγS Assay

The use of a radioactively labelled non-hydrolysable GTP analogue, such as [^{35}S]-GTPγS, is a commonly used method to monitor the effect of compounds on receptor mediated G-protein activation. It is generally accepted that this assay predominantly measures $G\alpha_i$ subunit activity because of its relatively high expression levels in cells and high basal rates of GDP-GTP exchange (Milligan, 2003). The finding that the $G\alpha_i$ subunit undergoes rearrangement rather than dissociation from the Gβγ complex upon activation (Bünemann et al., 2003) is also a contributing factor. We tested compounds in a GTPγS assay that was available at Millipore (Fig. 2C). TAK-615 appeared to show no inhibitory activity in agreement with the data generated in the cAMP assay. BMS-986202 and SAR-100842 inhibited the response to LPA equally well albeit with lower potencies (IC_{50}s of 1100 ± 600 nM and 244 ± 80 nM respectively) than those

observed in the cAMP assay. This is likely to be partly a result of using a 10-fold greater stimulatory concentration of LPA in this assay (940 nM) compared to the cAMP assay (90 nM), and that it uses membranes derived from a different cell background (derived from Chem-1 cells expressing the LPA_1 receptor). This shift in sensitivity is probably a contributing factor to the shallow Hill slopes (-0.62 and -0.56 respectively) and large error associated with the IC_{50}. The observed decrease in assay sensitivity relative to the cAMP assay could mean that any weak inhibitory activity of TAK-615 would not be observed. Given that BMS-986202 and SAR-100842 show similar activity to each other in this assay and the cAMP assay the data indicates that TAK-615 has no inhibitory activity on LPA_1 receptor mediated $G\alpha_i$ signalling.

3.2.4. β-arrestin assay

LPA_1 receptor mediated G-protein signalling subsequently leads to recruitment and activation of β-arrestin (Fukishima et al., 2015; Stoddard & Chun 2015). It is plausible to expect that if a compound inhibited a G-protein signalling pathway that this should also be observed by a subsequent decrease in agonist stimulated β-arrestin recruitment. Compounds were tested in antagonist mode by DiscoverX in their PathHunter LPA_1 receptor assay (Fig. 2D). BMS-986202 (IC_{50} 9 ± 1 nM) and SAR-100842 (IC_{50} 31 ± 8.5 nM) fully inhibited the LPA mediated response, whilst TAK-615 was only partially able to inhibit the response to ~40% at 10 µM with an IC_{50} of 23 ± 13 nM.

3.2.5. Activated RhoA assay

Engagement of the LPA_1 receptor by LPA also leads to the activation of the $G\alpha_{12/13}$ signalling cascade. To complete the *in vitro* profile of the compounds it was decided to compare the ability of all three compounds to inhibit this signalling pathway. This is most commonly assessed by measuring the level of activated RhoA present in a cell using an ELISA based methodology. We found that the RH7777-hLPA_1R cell line used internally for monitoring calcium flux and changes in cellular cAMP levels was not a suitable cellular model for use in this assay. Instead the human mesothelium MeT-5A cell line, which predominantly expresses the LPA_1 receptor (data not shown), was used as a measurable increase in activated RhoA in response to LPA stimulation. The inhibitory activities of all three compounds were first tested in this cell line using a cAMP assay. All three were found to behave consistently with the data reported above (section 3.2.2.) (Supplementary data). MeT-5A cells were incubated with compound either at 0.1, 1 or 10 µM for 1 h prior to challenge with 10 µM LPA for 1 min before being processed for analysis. All three compounds showed a significant inhibition of $G\alpha_{12/13}$ signalling at 1 and 10 µM (Fig. 3).

3.2.6. Summary of *in vitro* functional profiling

The collective data from each assay suggests that each compound exhibits a different *in vitro* pharmacological profile. BMS-986202 fully inhibited the LPA response associated with $G\alpha_q$,

$G\alpha_i$ and β-arrestin signal transduction. SAR-100842 was able to fully inhibit $G\alpha_i$ and β-arrestin signalling with a similar potency to BMS-986202 but interestingly appeared in our calcium flux assay system to have no effect upon $G\alpha_q$ signalling. In contrast TAK-615 showed partial inhibition of $G\alpha_q$ and β-arrestin signalling with no observable effect upon LPA driven decreases in cellular cAMP. All compounds however, showed significant and similar inhibition of $G\alpha_{12/13}$ signalling. TAK-615 appears to have a distinct functional pharmacological profile at the LPA_1 receptor. It significantly inhibits LPA driven $G\alpha_{12/13}$ signalling at 1 and 10 μM, as do BMS-986202 and SAR-100842, but displays limited or no efficacy against other signal transduction pathways arising from activation of the LPA_1 receptor.

3.3 Compounds bind to the LPA_1 receptor with different modalities

We had observed using BSI that all three compounds could bind to the LPA_1 receptor. Each compound however, elicited a different functional inhibition profile on the LPA signalling pathways measured. We were interested to know what effects on compound binding there would be in the presence of a high concentration of LPA.

In the presence of 10 μM LPA the BSI signal shifted leftward indicating an apparent increase in affinity of TAK-615, which was still best described using a two-site binding model (K_dHi 0.11 ± 0.1 nM, K_dLo 5.4 ± 6 nM) (Fig. 4A). The increase in apparent affinity is suggestive of there

being positive binding co-operativity between TAK-615 and LPA. The combined binding and functional data that we have generated supports the hypothesis that TAK-615 may act as a negative allosteric modulator (NAM) at the LPA_1 receptor.

SAR-100842 also showed binding co-cooperativity with LPA (Fig. 4B). Interestingly in the presence of 10 μM LPA the data best fitted a one-site binding model with a K_d of 1.3 ± 0.2 nM). It is worth noting that this is comparable to the K_d estimated for the high affinity site determined in the absence of LPA. This data coupled with the functional data presented previously suggests that SAR-100942 also acts as a NAM at the LPA_1 receptor but with different functional consequences on LPA stimulated LPA_1 receptor signalling to those observed with TAK-615. No measurable signal could be obtained for BMS-986202 in the presence of a high concentration of LPA (Fig. 4C). As BMS-986202 also inhibited all functional responses measured (Fig. 2) this suggests that it acts as an orthosteric antagonist of the LPA_1 receptor.

3.4 Compounds do not bind to the LPA_1 receptor at the same site

Having identified that TAK-615 and SAR-100842 appeared to act as NAMs at the LPA_1 receptor but with different functional consequences, we used BSI to investigate whether the binding sites of each compound overlapped. Each compound was titrated against the LPA_1 receptor in the presence of a fixed concentration of one of the other compounds in turn. The assay methodology required that membranes were pre-incubated with 10 μM of one compound prior to addition of

varying concentrations of a second compound. The high concentration of the first compound is expected to saturate its binding site. The data generated for TAK-615 was best fitted using a one-site binding model when incubated in the presence of either SAR-100842 (K_d 5 ± 1.8 nM) or BMS-986202 (K_d 2.1 ± 0.44 nM) (Fig. 5A). We also found that in the presence of TAK-615, the binding signal of SAR-100842 was best described by a one-site model (K_d 7.8 ± 2 nM) but that BMS-986202 had no obvious effect (Fig. 5B).

Interestingly however, in the reciprocal experiment with BMS-986202 the pre-incubation of membranes with SAR-100842 was able to completely inhibit the binding signal (Fig. 5C). We also observed that TAK-615 appeared to decrease the apparent affinity of BMS-986202 (K_d 10.5 ± 3 nM).

4. Discussion

We present *in vitro* data comparing the effect of TAK-615 with two LPA_1 receptor antagonists that have been used in clinical trials (SAR-100842 and BMS-986202). The aim was to understand if the compounds could be differentiated based on their mechanism of action (MOA).

4.1 Differential effects upon LPA_1 receptor signalling

Compounds were profiled in a panel of assays looking at signalling pathways generated upon activation of the LPA_1 receptor. BMS-986202 inhibited all signalling pathways as expected for an antagonist. SAR-100842, although described as a receptor antagonist, failed to show inhibition of calcium mobilisation but showed similar levels of activity to BMS-986202 against other signalling pathways. TAK-615 only showed comparable inhibition against activation of RhoA (Fig. 3). This suggests that the compounds all act differently at the LPA_1 receptor. Ideally, a complete set of assays using a single cellular model system and a single stimulatory concentration of LPA would have been used. This would have enabled a direct comparison of the potency of each compound against each signalling pathway. The differences in assay sensitivity and signal amplification observed in the RH7777-hLPA_1R as well as out-sourcing certain studies made this impossible. Work focused on comparing the relative inhibition on each signalling pathway. No comprehensive studies were conducted to look at any inverse agonism that these molecules might have.

4.2 Compound binding to the LPA_1 receptor

We wanted to confirm that all three compounds could bind to the human LPA_1 receptor. A label free assay system (BSI) was selected to assess the binding of each compound to be independent of the need for compounds to either be competitive or at least demonstrate negative binding co-

operativity with a labelled probe molecule. In addition, radio-labelled LPA tends to exhibit high background which impacts assay robustness. In theory BSI has a very large dynamic range because it detects any change in refractive index. To determine if a binding event has occurred the difference in BSI signal generated by each concentration of compound on membranes expressing the LPA_1 receptor versus control membranes is calculated. Great care is applied in these experiments to control for any signal change that might be due to drift across an assay plate or changes in compound concentration. The only difference in the samples being compared was the presence of the LPA_1 receptor. This provided the confidence to infer that signals were due to an interaction between compound and receptor. A curve fit algorithm was then applied to the resultant values to estimate binding affinities. Data generated for TAK-615 and SAR-100842 were best described with a 2-site model whereas a 1-site model was more applicable for BMS-986202. 2-site binding is observed for agonist molecules interacting with different receptor populations which are associated with G-proteins in either an active or inactive conformation. Binding isotherms for orthosteric antagonists tend to be described by a 1-site binding model. These are observations made using probe molecules that act orthosterically to the ligand binding site. Probes are often selected because they show 1-site binding at nano-molar concentrations, which is convenient for analysis. Binding experiments using a probe molecule examine the effect that an un-labelled compound has on the probe binding interaction. Other binding events

may therefore be missed. Label-free binding techniques may reveal that the binding of more compounds can be described by a 2-site model.

No binding signal was obtained for BMS-986202 in the presence of LPA. Unfortunately, because LPA inserts into the lipid environment of the membranes it was not technically feasible to determine its binding affinity to the LPA_1 receptor by this method. Either the LPA is preventing the binding of BMS-986202 at all concentrations tested or there is no detectable change in BSI signal when BMS-986202 binds in the presence of LPA. In the second scenario because the assay signal is relative to a non-receptor containing control it is highly likely that this could only be achieved if BMS-986202 bound to the same receptor site as LPA so that there was no detectable change in refractive index. Both explanations strongly support the hypothesis that BMS-986202 acts as an orthosteric antagonist of the LPA_1 receptor. It would be predicted that if the amount of LPA present was titrated down, at some point there would no longer be saturation of the binding sites and binding of BMS-986202 would be observed. Either the binding curve of BMS-986202 appears and shifts leftward with decreasing LPA (classic competitive binding) or the binding curve does not shift left and increases in signal only, indicating a population of the receptors is present that is not associated with LPA.

In the presence of LPA, the binding isotherms of TAK-615 and SAR-100842 shifted leftward indicating an enhancement in apparent affinity which is suggestive of positive co-operativity and is behaviour associated with allosteric interactions. Most striking was the shift of SAR-100842

from a 2-site to a 1-site model. It was considered that this may indicate promotion of a predominant receptor configuration which leads to the broader range of functional inhibition. Additional experiments with different concentrations of LPA would be required to show saturation of the effect, confirm allosteric interaction and to calculate an α value. Combining this observation with the selective inhibition of signalling pathways supports labelling both compounds as NAMs. The different effects of the two NAMs on receptor signalling suggests different conformations of the receptor are stabilised that are permissive for the transduction of different signalling events.

Further binding studies highlight that TAK-615 and SAR-100842 do not show competition or detectable co-operativity towards each other, indicating that they bind to different sites on the LPA_1 receptor that have no obvious impact upon one another, unlike the LPA binding site. The ability of SAR-100842 to block the binding of BMS-986202 but not the other way around is intriguing (Fig. 3B & 3C). The unidirectional nature of the observed inhibition would suggest that the compounds do not bind to the same or overlapping sites on the receptor, though this cannot be entirely ruled out as the binding kinetics had not been investigated and we cannot be certain that equilibrium had been reached. If the compounds are binding at different sites, SAR-100842 could be binding in a manner that promotes a receptor conformation incapable of interacting with BMS-986202. This would be akin to a non-competitive inhibition of binding. Thermodynamically, because we find the affinity of both compounds to be similar, one would

expect that BMS-986202 would have the same effect upon the binding of SAR-100842. As this is not the case there must be a non-thermodynamically driven explanation. The two compounds may be binding in the same general physical location on the receptor which they may access via the same route. In this situation the site at which BMS-986202 binds cannot prevent SAR-100842 from binding but if SAR-100842 is allowed to bind first it can prevent BMS-986202 from binding, i.e. SAR-100842 binds in a manner that sterically hinders the binding of BMS-986202. Radio-labelling each compound would permit confirmation of their binding affinity and check our hypothesis through extended timepoints in competition binding and investigation of their binding kinetics. Additionally, generating crystal structures of the LPA_1 receptor bound with these compounds would also reveal how they bind relative to one another. This would support the above hypothesis and it might cast additional light on how these compounds gain entry to their binding site.

Clinical trials with BMS-986202 (also known as BMS-986020) for the treatment of pulmonary fibrosis were stopped for un-disclosed reasons (Mora et al., 2017). SAR-100842 has some efficacy in a Phase IIA clinical trial for systemic sclerosis (Allanore et al., 2015). Our data suggests that SAR-100842 is a NAM. It may therefore, not be necessary to use an orthosteric antagonist towards the LPA_1 receptor to gain clinical benefit. An orthosteric antagonist may have the potential for more side effects compared to a NAM that can target the most relevant signalling events involved in a pathological situation. For fibrotic indications literature suggests

that one mechanism of importance is an LPA_1 receptor mediated increase in CTGF via a RhoA dependent pathway that promotes fibroblast proliferation (Sakai et al., 2013). This pathway has also been implicated in the inappropriate recruitment of bone marrow derived cells (Tang et al., 2014) that differentiate into fibroblasts and contribute to the development of fibrosis. All three compounds showed equivalent activity in an activated RhoA assay and could therefore all show benefit against CTGF driven fibrotic processes. Our data also highlights the pharmacological differences among the three compounds in each assay system tested. Further investigation is needed to provide insight into how they would behave in a pathological condition. We are aware that we have used different cell lines, assays systems and concentrations of LPA to interrogate the actions of each compound. Comparing the activity of TAK-615, SAR-100842 and BMS-986202 in primary cell systems would show if there is correlation with the functional data presented here. It would also help understand the impact upon disease relevant markers such as CTGF expression in a more physiologically relevant model system.

Author Contributions

J.E., L.D., T.C., L.C., D.B., R.L. designed, performed and analysed experiments: J.E., P.B., P.M., M.B., R.I. designed and analysed experiments: J.E. wrote the paper:

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Supporting Information

Data showing that compounds demonstrated functional inhibition in the MeT-5A cell line is not essential for building the overall message but is important in showing that the MeT-5A cell line is a suitable cellular model for assessing compound activity in a RhoA activation assay. 1 figure supplied.