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APPLICATION NOTE

Ligand Characterization of Mas-Related GPCR-X2 via Cell-Based Assays: Assessing the Regulation of Mast Cell Function and Implications in Safety Pharmacology

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INTRODUCTION

Effectors of mast cell degranulation (MCD) such as histamine, serotonin, heparin, and certain cytokines principally drive IgE-mediated allergic reactions. On the other hand, non-immune mediated activation of mast cells has been identified to cause pseudo-allergic Adverse Drug Reactions (ADR), due in part to multiple receptor expression profiles on their surfaces. Furthermore, the recognition of a wide range of activating ligands, including certain classes of therapeutics targeting mast cell receptors warrants a closer examination into their safety pharmacological roles. Among other receptor classes, orphan GPCRs have emerged as active proponents in non-immunogenic mast cell activation profiles with possible roles in pseudo-allergic or anaphylactoid reactions. As a result, efforts in drug discovery and development have focused on developing safer therapeutics aimed at reducing anaphylactoid reactions. For instance, a study by McNeil *et al.* (2015) has formed the basis of exploring the role of orphan receptors in non-IgE mediated occurrence of histamine-like symptoms associated with secretagogues and therapeutic drugs.

A relatively new subfamily of GPCRs associated with the MAS1 oncogene was identified based on transcriptome analysis by Dong *et al.* (2001). The encoding genes were termed as Mas-Related genes (MRGs) and the receptors as MAS-related GPCRs (MRGPR). One of its members, MrgprX2 encoded by the gene MRGPRX2, has been identified to be promiscuous and activated by several synthetic small molecules and peptides including U.S. Food and Drug Administration (FDA) approved drugs. While a role for the former "orphan" MrgprX2 was established in 2015, subsequent studies have identified MrgprX2 receptor-mediated agonism to be implicated in causing adverse reactions associated with clinical candidate molecules during their development (Grimes *et al.* 2019).

Overall, these studies have since necessitated more complex risk assessments and targeted safety testing for drugs in development to minimize ADRs, including anaphylaxis and allergic reactions.

RECEPTOR INTERACTIONS AND CLINICAL RELEVANCE

Mast cells are principal players in IgE-, and more recently, MrgprX2-mediated anaphylactoid reactions. They can be activated by immunogenic effectors and by several types of receptors including Toll-like receptors (TLR), protease-activated receptors (PARs), opioid receptors, c5a complement, and T-cells depending on localization and type of mast cell (Hennino *et al.* 2006, Spoerl *et al.* 2017) opening up routes along non-immunogenic activation related pathways (Figure 1.).

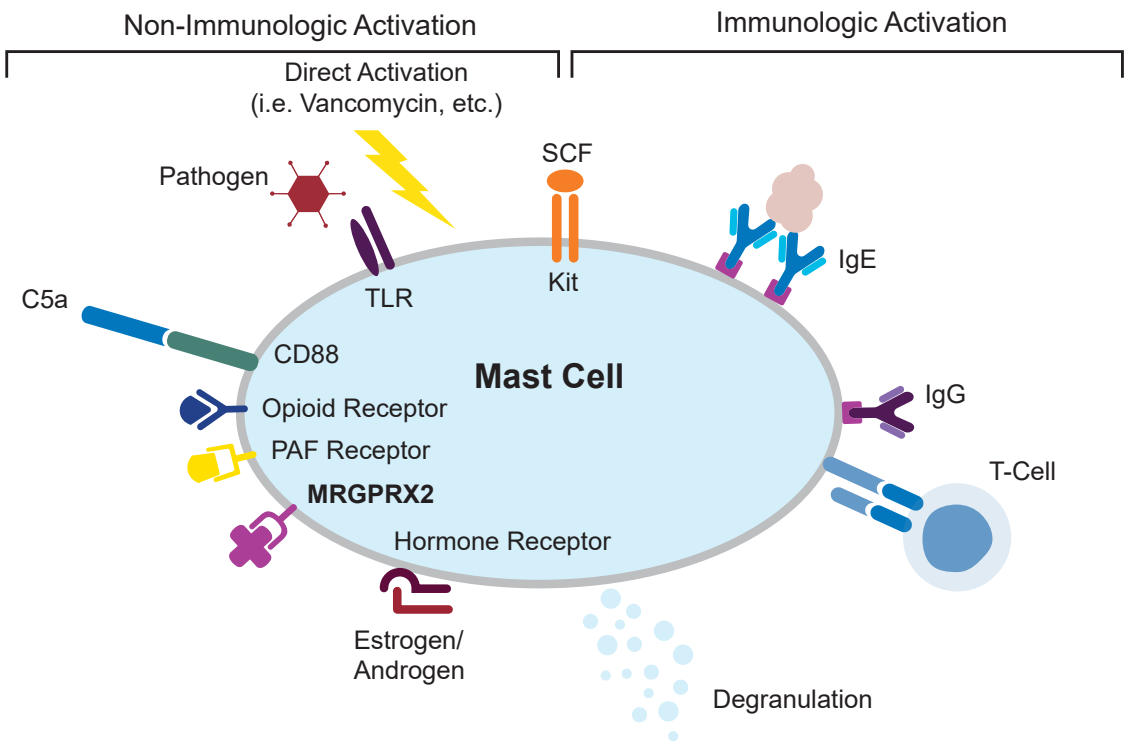


Figure 1. Mast cell degranulation including non-immunological activation route (adapted from Spoerl *et al.* 2017).

Historically and despite significant efforts, the exact function and role of the MrgprX2 orphan receptor remained elusive given its extensive mast cell expression profile. Previously, studies with the human MrgprX2 receptor had revealed surrogate ligand pairings, but were unsuccessful in linking the receptor to its biological function; primarily due to the lack of understanding of orthologue receptor genetics. The catalyst for the recent focus on this receptor was a study from Johns Hopkins University (McNeil *et al.* 2015) that elucidated a physiological role for the receptor for the first time. The group focused on the mouse orthologue gene, recapitulating the same pharmacology observed with the human MrgprX2 receptor and their knockout studies confirmed a link between receptor expression to mast cell function and anaphylaxis.

A few other studies emerged to better understand the receptor's link to drug-induced anaphylaxis. Of particular mention is a study led by Dr. Andrew Brown's team at GlaxoSmithKline (GSK), which identified a series of orthologue receptors, typically of pharmacological interest during drug development, with a bioinformatics approach (Grimes *et al.* 2019). Subsequently, recombinant expression studies and compound profiling, including Eurofins DiscoverX's β-arrestin recruitment and calcium signaling assays, provided a rigorous characterization platform of compound pharmacology.

In retrospect, a series of drug discovery programs, where anaphylaxis in animals had impeded development, the (drug) candidates were shown to exhibit MrgprX2 pharmacology at the corresponding orthologue gene. Notably, these programs studied diverse therapeutic types. Further analysis and insight correlated diverse pharmacology to a ligand molecule's net charge or pH rather than any specific peptide sequence or structural features. Indeed, the promiscuity of the MrgprX2 receptor supported Dr. Brown's hypothesis that it functions as a sensor for exogenous and potentially harmful (viral) peptides, invoking a mast cell host response (Grimes *et al.* 2019). This is analogous to the function of the formyl peptide receptor (FPR1), a promiscuous receptor without an established cognate ligand, sensing patterns of bacterial peptides.

NEED FOR SAFETY LIABILITY SCREENS

Receptor activation towards regulating mast cell degranulation has a purported key safety liability, as observed in studies such as that of Lafleur *et al.* 2020. Since assays with mast cells are challenging (principally due to access to insufficient cells and difficulty creating viable cultures), their study characterized an extended panel of established and novel ligands with an emphasis on investigating the pharmacology of compounds (peptide) charge.

Several targets can represent pharmacological panels in safety liability screens. With appropriate testing methodologies, improved understanding of their pharmacological relevance can be met, which in turn can show the impact of a particular target (and its mechanism-of-action) on the safety liability of the drug at a much earlier stage in the discovery process. In general, it was observed that the outcomes of MrgprX2 activation bear a strong correlation with histamine release in CD34+ stem cell-derived mature human mast cells (La Fleur *et al.* 2020). For that study, the Eurofins DiscoverX's PathHunter® platform was employed to analyze MrgprX2 for both calcium release and β-arrestin recruitment. The study endorsed the use of the cell-based functional assays in safety liability screens as a predictive and phenotypically representative model of mast cell activation.

MRGPRX2 is part of Mas-related gene (MRG) family of GPCRs comprising almost 50 members sub-divided into subfamilies (A-H and X), of which the X-species is specific to primates including humans, macaques, and rhesus monkeys. Transcriptomic analyses have shown that MRGPRX2 to be one of the highest expressed receptors in human mast cells (Motakis *et al.* 2014). Studies related to ligand activation of the MrgprX2 receptor have brought forth its promiscuous nature of exerting unexpected/un-intended histamine-like allergenic effects, necessitating the investigation of interaction of potential ligands with MrgprX2.

In this application note, we independently generated a new set of data with selected agonists, previously identified as MrgprX2 agonists, using calcium mobilization and β-arrestin recruitment assays feasible with the application of the PathHunter MrgprX2 cell line. The goal of this study was to show the high-throughput and time-savings value of using engineered, cell-based assays to study its activation through these screening assays.

MATERIALS

Compound 48/80 (Sigma Aldrich, UK), mastoparan (Tocris Biosciences, Bristol, UK), cortistatin-14 (Genscript, USA), octreotide, indolicidin, and vancomycin (Sigma Aldrich, UK); U2OS (ATCC Cat. No. HTB-96, RRID:CVCL_0042) or HEK 293-MSRII cells; and Eurofins DiscoverX PathHunter CHO-K1 MRGPRX2 β-Arrestin Cell Line (Cat. No. 93-0309C2), AssayComplete™ Cell Plating 0 Reagent (CP0, Cat. No. 93-0563R0A), and PathHunter Detection Kit (Cat. No. 93-0001).

METHODS

Calcium Mobilization Assay

Assay comprised of cell line expansion and propagation, dye loading, and agonist activity assay to determine the mobilization of calcium in the cell.

Cell Handling

U2OS or HEK 293-MSRII cell lines were expanded from freezer stocks according to standard procedures. Cells were seeded in a total volume of 20 µL into black-walled, clear-bottom, Poly-D-lysine coated 384-well microplates, and then incubated at 37°C and 5% CO_2 for the appropriate time prior to testing.

Dye Loading

Assays were performed in 1X Dye Loading Buffer consisting of 1X dye, 1X Additive A, and 2.5 mM Probenecid in HBSS / 20 mM HEPES. Probenicid was prepared fresh. Cells were loaded with dye prior to testing. Media was aspirated from cells and replaced with 20 µL Dye Loading Buffer. Cells were incubated for 45 minutes at 37°C and 5% CO_2.

Agonist Format

For agonist determination, cells were incubated with ligand samples (compound 48/80, mastoparan, cortistatin-14, octreotide, indolicidin, and vancomycin) to induce a response. After dye loading, cells were removed from the incubator, and 10 µL HBSS / 20 mM HEPES was added. Cells were incubated for 15 minutes at room temperature in the dark to equilibrate plate temperature. Intermediate dilution of sample stocks was performed to generate 4X sample in assay buffer. Compound agonist activity was measured on a FLIPR Tetra (MDS). Calcium mobilization was monitored for 2 minutes and 10 µL 4X sample in HBSS / 20 mM HEPES was added to the cells 5 seconds into the assay. Refer to Figure 2. for the assay principle.

β-ARRESTIN RECRUITMENT ASSAY

5000 Cells/well of the PathHunter CHO-K1 MRGPRX2 β-arrestin cells were seeded with 20 µL AssayComplete CP0 reagent into white 384-well plates (Corning) and incubated overnight (37°C and 5% CO_2). Test agents were serially diluted in DMSO, added to OptiMEM/0.1% BSA (to 5x final), and incubated with 5 µL of cells for 90 minutes at 37°C and 5% CO_2 followed by incubation for 30 minutes at room temperature. Luminescence was detected using an Envision plate reader (Perkin Elmer) after the addition of 12.5 µL PathHunter detection reagent at room temperature for 2 hours. Refer to Figure 3. for the assay principle.

RESULTS

Several agonists (cortistatin-14, indolicidin, compound 48/80, mastoparan, octreotide, and vancomycin) were assessed in PathHunter® MRGPRX2 cell lines for β-arrestin recruitment and calcium release using Eurofins DiscoverX assays.

Measuring intracellular calcium mobilization based on the activation status of specific GPCRs can provide information that uncovers pharmacological rank-orders of lead agonists and calcium signaling flux. The calcium mobilization assay (Figure 2.) uses an inactive dye probe that, when it penetrates the cell membrane, switches to an active form, whose intensity can be measured via fluorescence.

For measuring β-arrestin recruitment, the PathHunter β-arrestin assay (Figure 3.) was used. This assay is based on DiscoverX's proprietary Enzyme Fragment Complementation (EFC) technology, which involves two recombinant β-galactosidase (β-gal) enzyme fragments that act as an enzyme acceptor (EA) and an enzyme donor (ED). Separately, the fragments are inactive, but when combined, they form an active enzyme. In this assay, MrgprX2 cell lines were engineered to co-express the ED, called ProLink™ (PK), tagged to the receptor, MrgprX2, and the EA tagged to β-arrestin. Activation of the MrgprX2-PK by agonist treatment (cortistatin-14) induces β-arrestin-EA recruitment, forcing complementation of the two β-gal enzyme fragments. The resulting functional enzyme hydrolyzes substrate to generate a chemiluminescent signal.

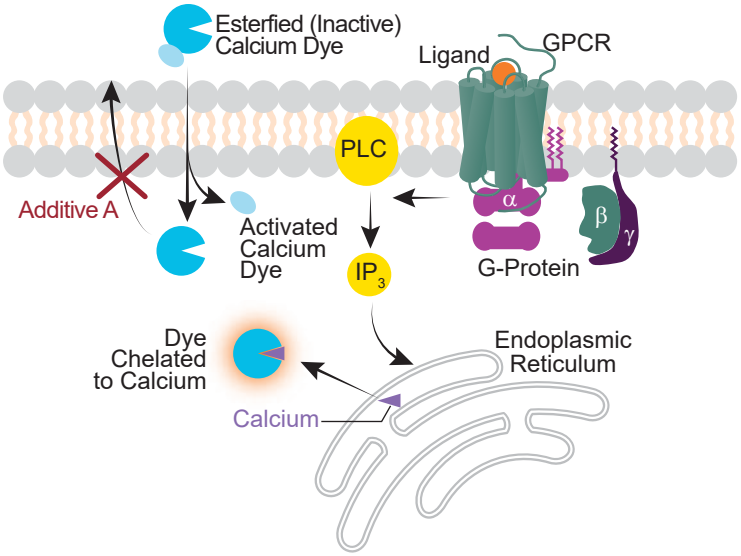


Figure 2. Calcium mobilization assay principle. The calcium mobilization assay (see DiscoverX.com/calcium) is a Calcium NoWash^{PLUS} Assay wherein an esterified (inactive) calcium dye (probe) penetrates the cell membrane and becomes active once inside the cell. The active form of the dye becomes fluorescent after binding to intracellular calcium. Additive A is added to prevent the dye from being released out from the cell. Ligand binding stimulates GPCR activation, resulting in the release of intracellular calcium stores from the endoplasmic reticulum (ER), which leads to an increase in fluorescence in the presence of the activated calcium dye.

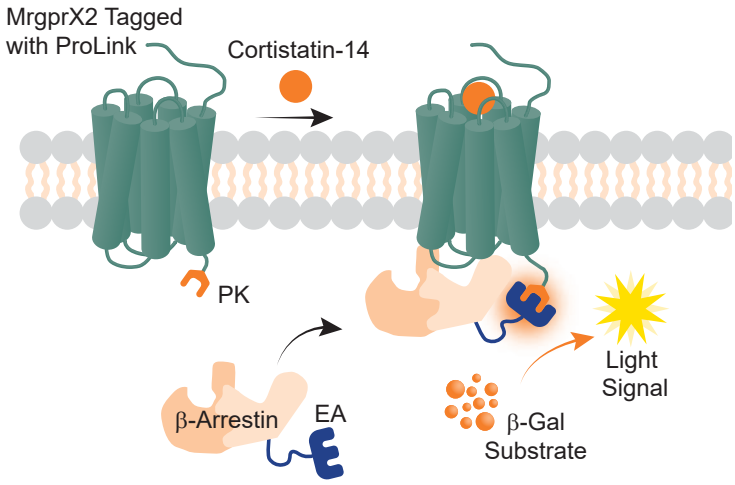
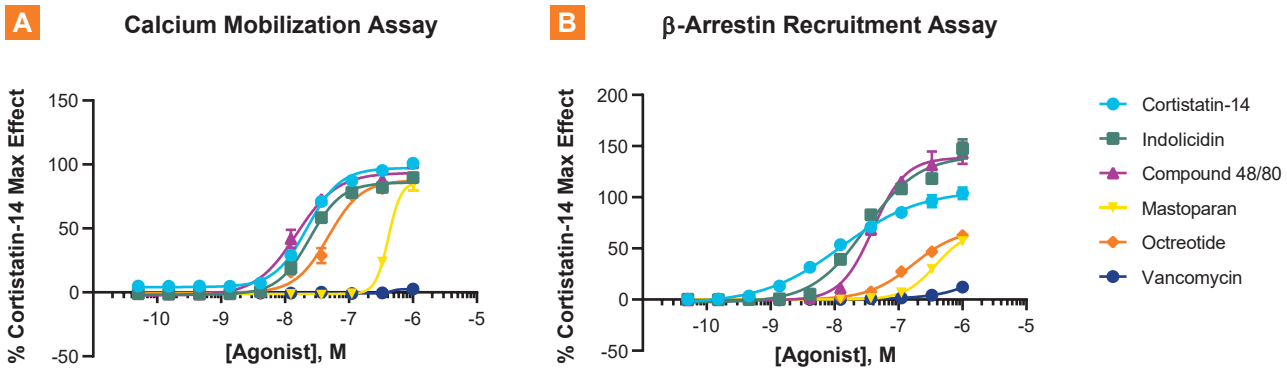


Figure 3. Eurofins DiscoverX's EFC-based β-arrestin recruitment assay principle. The PathHunter MrgprX2 β-arrestin recruitment assay principle is based on the EFC platform. Binding of cortistatin-14 results in MrgprX2 activation thereby stimulating recruitment of β-arrestin-EA fusion protein to MrgprX2-PK. The resulting complementation of the two enzyme fragments results in an active β-gal enzyme whose activity can be measured using chemiluminescent PathHunter detection reagents.

Published studies using a wide range of chemotypes were previously characterized using calcium release and β-arrestin recruitment assays for MrgprX2. The studies adopted the same clone used in this application note and have been useful in ascribing the role of receptor activation to its purported effect of non-immune mediated mast cell activation (Grimes *et al.* 2019 and Lafleur *et al.* 2020). Results herein are in good agreement with those previously published with respect to rank orders of potency agonists (Grimes *et al.* 2019) maintained relative to cortistatin-14 used as reference agonist for both assays (Figure 4.). Vancomycin activity is negligible with the concentration range used in these assays, but consistent with its expected low potency. These studies also highlight the potential for the β-arrestin assay to report higher levels of efficacy relative to cortistatin-14 that was not observed in the calcium mobilization assay. Our recent data set is limited, but these apparent differences in efficacy may warrant further consideration.



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	Calcium Mobilization		β-Arrestin Recruitment	
	EC_{50} μM	% Max Cortistatin-14	EC_{50} μM	% Max Cortistatin-14
Cortistatin-14	0.22911	100	0.11446	100
Indolicidin	0.2614	92	0.30684	139.1
Compound 48/80	1.6901	100	3.8828	136
Mastoparan	4.3427	89	3.2843	60
Octreotide	4.8441	90	15.005	65
Vancomycin	>100	3	>100	12

Figure 4. Dose response and rank order potency for calcium mobilization and β-arrestin recruitment assays. Readouts showing select agonists effect on (A.) calcium mobilization of intracellular calcium and on (B.) β-arrestin recruitment upon binding to MrgprX2 receptors. Both dose response curves are the mean of two replicates and show normalized data calculated from the maximum effect obtained with cortistatin-14. C. Table showing potency and efficacy of agonist relative to cortistatin-14.

CONCLUSION

Several targets are given consideration to represent pharmacological panels in safety liability screens as it is crucial to identify any specific molecular interactions at profiling stages that may cause ADRs in humans. Hence, an improved understanding of the pharmacological relevance of targets, including establishment of in vitro testing methodologies that can show the impact of a particular target (and its mechanism-of-action) on the safety liability of the drug at a much earlier stage in the discovery process is needed.

In an effort to support the characterization and possible role of MrgprX2 in non-classical mast cell-mediated anaphylactoid reactions, Eurofins DiscoverX collaborated with Andrew Brown (GSK) and helped identify compounds/peptides that activated MrgprX2 via calcium mobilization and β-arrestin recruitment assays (Grimes *et al.* 2019). Further, assessing MrgprX2 activation in an engineered cell model can provide value as a rapid, high-throughput, economical mechanism-based screening tool for early mast cell degranulation or hazard identification during preclinical safety evaluation of therapeutics.

The importance and role of MrgprX2 as an emerging activator of non-IgE mediated MCD underscores its value in being a top panelist in safety pharmacology. Besides, its potential as a drug target for conditions/diseases related to mast cell function and regulation, including anaphylaxis, attests to its position as central to safety liability testing. Overall, MrgprX2 antagonism promotes a fresh interest as an emerging therapeutic target for treating pseudo-allergic reactions, and Eurofins DiscoverX qualified assays are ideal for supporting research programs centered on MrgprX2 activation and mast cell regulation.

For more information, visit discoverx.com/gpcrs.

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