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MRGPRX2 activation in mast cells by neuromuscular blocking agents and other agonists: Modulation by sugammadex

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i. Title page

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ii. Abstract

Background: Neuromuscular blocking agents (NMBAs) can cause both IgE-dependent and independent anaphylactic reactions, with activation of the mast cell receptor MRGPRX2 being

important to the latter. Sugammadex, a reversal agent for certain aminosteroid NMBAs, has been proposed as an antidote for these anaphylactic events with conflicting outcomes.

Objective: We further characterise the involvement of MRGPRX2 in NMBA-induced mast cell activation and determine how this is influenced by sugammadex. We then apply these *in vitro* results to infer the possible utility of sugammadex in the acute management of non-IgE dependent anaphylaxis.

Methods: The LAD2 human mast cell line and a MRGPRX2 knock-down derivative were used to validate the involvement of MRGPRX2 and to test the effect of sugammadex on mast cell activation by NMBAs and other MRGPRX2 agonists.

Results: All MRGPRX2 agonists tested were shown to induce MRGPRX2-dependent LAD2 mast cell calcium mobilization and cytokine release and all, apart from rocuronium, induced degranulation. Co-treatment of mast cells with sugammadex and some MRGPRX2 agonists significantly reduced cell activation, but if sugammadex was administered a few minutes following stimulation, degranulation was not attenuated. However, addition of sugammadex up to 180 minutes following LAD2 MRGPRX2 stimulation, significantly reduced CCL2 mRNA and protein induction.

Conclusions and clinical relevance: We show that sugammadex, known to reverse muscle blockade by certain NMBAs, is also able to reduce MRGPRX2 activation by NMBAs and other, but not all, MRGPRX2 agonists. As sugammadex was ineffective in attenuating mast cell degranulation when added rapidly post MRGPRX2 activation, this suggests against the agent having efficacy in controlling acute symptoms of anaphylaxis to NMBAs caused by MRGPRX2 activation. Interestingly however, sugammadex did impair MRGPRX2-induced CCL2 release, suggesting that it may have some benefit in perhaps dampening less well-defined adverse effects of MRGPRX2-dependent anaphylaxis associated with the more slowly elaborated mast cell mediators.

Key words:
MRGPRX2, sugammadex, anaphylaxis, mast cells, drug hypersensitivity, anaphylactoid, IgE-independent drug reactions

iii. Introduction

Drug-induced anaphylaxis can have devastating consequences, with anaphylaxis to neuromuscular blocking agents (NMBAs), such as rocuronium and atracurium, being a serious complication during anaesthesia[1]. The recorded incidences of perioperative anaphylaxis vary between geographical locations with rates of 1 in 10,000 – 20,000 surgical patients reported in Australia and UK but as high as 1 in 3,500 in France[2-4]. Symptoms of anaphylaxis are attributed to the release of mediators from activated mast cells[5]. This activation is generally considered to be antibody-mediated largely by drug-reactive IgE[5, 6]. However, not all events of anaphylaxis following an adverse reaction to NMBAs have been associated with drug-specific IgE[7]. Whilst a role for drug-specific IgG has recently been suggested[8], new research has also identified that the G protein-coupled receptor (GPCR) MRGPRX2 (Mas-related GPCR X2) can trigger IgE-independent activation of mast cells[9]. Ligands of this receptor are commonly basic molecules including drugs like NMBAs, opioids, neuropeptides such as substance P, antimicrobial peptides such as LL-37, and laboratory research tools such as the long investigated compound 48/80 and the more recently identified ZINC-3573(R)[10, 11].

Following mast cell activation, pre-formed mediators such as histamine, tryptase and a range of other proteases are released in the process of degranulation that is initiated 1-2 minutes following drug exposure[12, 13]. This almost instantaneous process initiates the immediate symptoms of anaphylaxis including hypotension (caused by vasodilatation), increased heart rate, bronchospasm and urticaria[14, 15]. Following the immediate release of mediators via degranulation, activated mast cells also release other mediators, such as eicosanoids and cytokines, that over a period of minutes-hours can further contribute to the symptoms of anaphylaxis[5].

In the event of anaphylaxis during surgery, depending on the severity, immediate treatment is necessary to protect the patient and halt potential injury or even death. Adrenaline and resuscitation fluids are used to stabilise blood pressure, and additional supportive treatments may be considered including anti-histamines and steroids[14, 15]. In addition, some case studies have reported the off-label use of sugammadex in the treatment of anaphylaxis induced by certain NMBAs, with conflicting reports of efficacy[16-19].

Sugammadex is a γ-cyclodextrin drug that encapsulates aminosteroid NMBAs such as rocuronium, preventing interaction with the nicotinic receptor, and thereby reversing

neuromuscular blockade[20, 21]. However, whether sugammadex is able to shield these NMBAs from activating MRGPRX2 remains unknown.

In this study, we aimed to further investigate the role of MRGPRX2 in NMBA-mediated mast cell activation and determine if the chemical antagonism of NMBAs, and perhaps other MRGPRX2 agonists, by sugammadex can inhibit MRGPRX2-dependent activation of human mast cells.

iv. Methods

Materials

The following products were purchased from Sigma Aldrich (Castle Hill, New South Wales, Australia); rocuronium bromide, ZINC-3573(R), ZINC-3573(S), Triton X, Hank's buffered salts, sodium bicarbonate, HEPES, glucose, bovine serum albumin (BSA), $MgSO_4$, $CaCl_2$, probenecid and glycine. Atracurium besylate and Fura-2AM were purchased from Abcam (Melbourne, Australia). Compound 48/80 was obtained from Enzo Life Sciences (Farmingdale, New York, United States). Sugammadex (Bridion®) was obtained from the Royal Melbourne Hospital Pharmacy (Melbourne, Victoria, Australia). NIP-BSA (4-hydroxy-3-iodo-5-nitrophenylacetyl bovine serum albumin) was purchased from Biosearch Technologies (Novato, California, United States). Jw8-IgE with specificity for the hapten NIP was generated by culturing the Jw8/5/13 cell line (ECACC hybridoma collection). Substance P (provided by Ms. Haoyue Zhang and Dr. Susan Northfield (Department of Pharmacology and Therapeutics, University of Melbourne, Victoria, Australia)) was produced by standard solid phase peptide synthesis methods and purified to >95% homogeneity by preparative HPLC[22]. The following products were purchased from ThermoFisher Scientific (Scoresby, Victoria, Australia); Iscove's Modified Dulbecco's Media (IMDM), GlutMAX™ (100X) supplement, phosphate buffered saline (PBS), StemPro™-34 media, high capacity cDNA reverse transcriptase kit, TaqMan™ primers, RNAiMAX lipofectamine, penicillin (5000 unit/mL)/streptomycin (5000 µg/mL), and CyQUANT™ lactate dehydrogenase (LDH) cytotoxicity assay. ELISA high binding 96 well plates were purchased from Greiner Bio-One (Kremsmünster, Austria). Stem cell factor (SCF) was purchased from Peprotech (Rocky Hill, New Jersey, USA). Illustra RNA mini spin kit was purchased from GE Healthcare, Life sciences (Chicago, Illinois, United States). SSA Advanced™ universal probes supermix was obtained from Bio-Rad (Hercules, California, United States). Mouse anti-human CD63-PE, TMB substrate (3,3',5,5'-Tetramethylbenzidine) and CCL2 (MCP-

1) cytokine ELISA kit were purchased from BD Biosciences (Scoresby, Victoria, Australia). pNAG (p-nitrophenyl-N-acetyl-β-D-glucosaminide) was purchased from Glycosynth (Winwick Quay, Warrington, United Kingdom). Alt-R CRISPR-Cas9 kit along with tailor-made guide RNA for the gene interruption of MRGPRX2 was purchased from Integrated DNA Technologies (Singapore Science Park II, Singapore). Human anti-MRGPRX2-PE (clone K125H4), anti-FcεRIα-APC (clone Cra-1), along with appropriate isotype controls, were purchased from BioLegend (San Diego, CA, United States). Anti-CXCR4-PE (clone 12G5) was from R&D systems (Minneapolis, MN, USA). Cortistatin-14 (trifluoroacetate salt) and LL-37 (trifluoroacetate salt) were purchased from Cayman Chemical (Ann Arbor, Michigan, United States).

Culture of LAD2 mast cells

LAD2 cells[23] were provided by Dr. Arnold Kirshenbaum (National Institute of Health, Bethesda, United States). The cells were cultured in Stem-pro34 medium supplemented with SCF (100 ng/mL), GlutMAX™ (1X), penicillin (50 Units/mL) and streptomycin (50 μg/mL) and maintained in a humidified incubator (5% CO_2, 37˚C).

CRISPR-Cas9 gene disruption of MRGPRX2 in LAD2 cells

CRISPR-Cas9 technology was used to knock out the MRGPRX2 gene from wild type LAD2 cells. CRISPR guide RNA (crRNA) *GAGCATGGATCCAACCACC* was designed (by Dr. Li Eon Kuek, Department of Pharmacology and Therapeutics, University of Melbourne, Victoria, Australia), to specifically target the human MRGPRX2 gene early in the coding region. A transfection control (no CRISPR-Cas9 target sequence) and a scramble control sequence (with no known target in the genome) were used as controls. Transfection was performed in accordance with the manufacturer's protocol. In brief, the crRNA-tracRNA complex was mixed with lipofectamine RNAiMAX to form a transfection complex. This complex was then mixed with cells and seeded on a 12-well plate at a density of 4.5 x 10^5 cells/mL in antibiotic free Stem-pro34 growth medium.

48 hours following transfection, MRGPRX2 knock-out cells were selected using a functional screen based on CD63 surface levels. CD63 is a vesicular marker expressed on the mast cell surface upon degranulation and the basis of the screen was that a loss of MRGPRX2 expression would impair CD63 surface expression upon stimulation with receptor agonists. Briefly, cells were stimulated with MRGPRX2 agonist, compound 48/80 (10 μg/mL), for 15

minutes in the presence of mouse anti-human-CD63-PE. As is shown in supplementary ***Figure S1A,*** upon stimulation with compound 48/80, MRGPRX2 gene-targeted cells (red trace) had a much greater $CD63^{low}$ population compared to the control targeted cells (blue and grey traces). $CD63^{low}$ cells were sorted using flow cytometry (BD FACS ARIA III; BD Biosciences), expanded, and then re-stimulated and sorted again as above to enrich further for functional MRGPRX2 knockouts (***Figure S1B***). As a phenotypic screen was used, targeting of pathways unrelated to MRGPRX2 expression might also produce this result. We thus tested if the IgE-pathway of mast cell activation was interrupted in these cells. As shown in ***Figure S1C***, IgE-dependent mast cell activation by antigen was little changed by MRGPRX2-targeting. Following two rounds of sorting, there remained a remnant population of MRGPRX2-targeted cells that still responded to compound 48/80, likely reflecting non-targeted cells or single-allelic knockouts. Given that LAD2 cells are slow growing and, in our hands, do not expand readily from clones, this mixed population of cells termed MRGPRX2 knock down (KD) cells were used in all further experiments. The cells were then expanded and tested for surface expression of MRGPRX2 along with another GPCR, CXCR4 and the high-affinity IgE receptor, FcεRIα, via flow cytometry. Confirming the selectivity of the phenotypic CD63 expression assay, sorted cells had reduced levels of MRGPRX2 whilst, compared to WT cells, expression of FcεRIα and CXCR4 was essentially unchanged (***Figure S1 D-F***).

Quantification of LAD2 cell calcium mobilization

IgE-sensitized cells were incubated with FURA2-AM (2 μM) in Hank's buffered saline solution (HBSS) (1X Hank's buffered salts, sodium bicarbonate (0.14%), HEPES (10 μM), glucose (5.5 mM), BSA (0.05%), $MgSO_4$ (0.7 mM), $CaCl_2$ (1.8 mM)) supplemented with probenecid (2.5 mM) at a density of 5 x 10^6 cells/mL for 90 minutes at 37˚C. Cells were washed and seeded into 96-well plates (5 x 10^4 cells/well) and calcium mobilization was measured ratiometrically (excitation 340/380 nm; emission 510 nm) using a Flex station III (Molecular Devices, San Jose, California, USA). LAD2 mast cell stimulating agents (in the absence of or in complex with sugammadex) were added at 30 seconds. In some experiments, sugammadex was added 40 seconds following stimulus addition

Quantification of LAD2 mast cell degranulation

β-hexosaminidase release is a commonly used measure of mast cell degranulation. LAD2 cells were sensitized with human IgE (1 μg/mL; conditioned media from Jw8/5/13 cells) for a

period of 24-48 hours. The cells were then washed and resuspended in serum free IMDM (+ 0.1% BSA) or HBSS and seeded (5 x 10^4 cells/well) into 96-well plates. Stimuli were added to wells and incubated for 45 minutes (37˚C). Where applicable, cells were either co-treated with sugammadex and stimulus or sugammadex was added 2 minutes following stimulus addition. Cell supernatant (25 µL) was then transferred to a fresh plate where pNAG substrate was added (75 µL; 2 mM pNAG; pH=4.5 in phosphate-citrate buffer). The plate was incubated for 120 minutes at 37°C in an oscillating incubator (70 RPM). The reaction was stopped by the addition of glycine (100 µL; 0.4 M; pH=10.7), and an absorbance reading was taken at 405 nm (Multiskan Ascent; ThermoFisher Scientific). The absorbance readings were used to calculate β-hexosaminidase release as a percentage of the total cellular β-hexosaminidase content (quantified using TritonX100 (0.1%)-induced cell lysis). The percentage spontaneous release of β-hexosaminidase was subtracted from all responses.

Kinetics of CCL2 expression and release by LAD2 mast cells

IgE-sensitized LAD2 cells were activated with various stimuli in serum free IMDM for qPCR measurement (2 x 10^6 cells/mL; 4-hour stimulation) or CCL2 immunoassay (3.5 x 10^5 cells/mL; 24-hour stimulation). As above, where applicable, cells were either co-treated with stimuli and sugammadex or sugammadex was added at various time points following stimuli. The plates were then centrifuged (233 xg), supernatants removed and stored at -20˚C until further use and cell pellets processed for RNA isolation. CCL2 levels were measured using a sandwich ELISA in accordance with manufacturer's instructions. The plates were developed using TMB substrate the reaction halted using sulfuric acid (0.2 M) and absorbance measured at 450 nm (Multiskan Ascent; ThermoFisher). Absorbance readings were translated to absolute concentrations of CCL2 using a standard curve. Absolute readings were then normalised to respective basal release of CCL2 (percentage of basal CCL2 levels).

RNA concentration and purity were quantified using a nanodrop spectrophotometer (Nanodrop 1000; Applied Biosystems). Diluted RNA (5 µL, containing 200 ng) was mixed with a reverse transcriptase master mix (5 µL) prepared according to manufacturer's instructions (ThermoFisher). The samples were incubated (37 °C; 2 hours) followed by reverse transcriptase denaturation (85 °C for 5 minutes; Mastercycler® nexus gradient; Eppendorf). The resultant cDNA was further diluted 1:10 with RNase free water, and mixed (2 µL) with SSA Advanced™ universal probes supermix (3 µL) containing TaqMan assays CCL2

(Hs01871556_s1), GAPDH (Hs02758991_g1) or UBC (Hs01871556_s1) in a 384 well thermocycler plate. Real-time quantitative PCR was carried out with 40 cycles of 95˚C for 15 seconds followed by 60˚C for 60 seconds (Quant Studio 6; Applied Biosystems). ΔCT was calculated by the subtraction of the sample cycle numbers from the average of the cycle number of two house-keeper genes used (UBC and GAPDH). ΔΔCT was calculated by the subtraction of resting expression levels of CCL2 from each sample.

Data Presentation and Statistical analysis

Flow cytometry data was analysed and represented in overlayed histograms using FlowJo_V10.0 . Other graphical representations (expressed as n=x ±SEM) were plotted using Prism (version 8.02, GraphPad, San Diego, California, USA). Statistical significance was determined using either a one-way ANOVA with Dunnett's multiple comparison post hoc test or a two-way ANOVA with Bonferroni multiple comparison post hoc test (version 8.02, GraphPad PRISM), with levels of significance shown (where relevant) in figure legends.

v. Results

MRGPRX2 is required for NMBA-induced mast cell activation

All drugs produced calcium mobilization in the WT LAD2 cells with varying maximal effects (***Figure 1 A-D***). The calcium mobilization induced by rocuronium, atracurium and compound 48/80 was virtually eliminated in the LAD2 MRGPRX2 KD cells. MRGPRX2-dependency was also observed with cell degranulation where the MRGPRX2 KD cells stimulated with compound 48/80, substance P, ZINC-3573(R), atracurium, LL-37 and cortistatin-14 had significantly reduced degranulation compared to WT cells (***Figure 1E***). However, not all stimuli induced WT LAD2 degranulation. Rocuronium, whilst causing an induction of calcium mobilization, failed to trigger degranulation. Whilst ZINC-3573(R) triggered LAD2 cell degranulation, consistent with previous findings[11], its enantiomer ZINC-3573(S) did not (data not shown). As ZINC-3573(R) interfered with the FURA-2AM assay, we were unable to quantify the calcium responses to this ligand.

To investigate the effect of MRGPRX2 KD, if any, on IgE-mediated mast cell activation, the cells were incubated with NIP- specific IgE for 24-48 hours and then challenged with antigen (NIP-BSA). There was no decrease in equivalent IgE-mediated responses in LAD2 MRGPRX2 KD cells (***Figure 1D, E***).

Sugammadex-mediated reversal of drug-induced LAD2 activation

Having established MRGPRX2-dependence in NMBA and other agonist-induced mast cell activation, we next aimed to determine if sugammadex could modulate these responses. We examined this by either co-treating cells with sugammadex (500 μM) and the MRGPRX2 agonists or, to better mimic the possible clinical use of sugammadex in treating NMBA-induced anaphylaxis, adding it to the cells 2 minutes following the MRGPRX2 agonist. Calcium mobilisation was measured and supernatants from other experiments harvested for β-hexosaminidase and CCL2 quantification. In separate studies, sugammadex (500 μM) was shown not to mediate any direct cytotoxicity of LAD2 cells as measured by LDH release assays (data not shown).

Compared to agonist alone, co-treatment of cells with sugammadex significantly reduced rocuronium and compound 48/80 calcium mobilization responses (***Figure 2A, C***). Interestingly, although sugammadex is not known to reverse atracurium-induced nicotinic receptor block[24], it was able to impair atracurium-induced MRGPRX2- calcium mobilization but not as comprehensively as the rocuronium response (***Figure 2B***). Addition of sugammadex 40 seconds following LAD2 stimulation, also reduced the rocuronium, atracurium and compound 48/80-induced sustained calcium mobilization response (***Figure 2A-C***).

Degranulation induced by atracurium, compound 48/80 and ZINC-3573(R) was significantly diminished when the cells were co-treated with sugammadex (500 μM) (***Figure 3A-C)***. The inhibitory effects of sugammadex on degranulation induced by these stimuli was concentration-dependent with compound 48/80 being particularly sensitive to inhibition by sugammadex (***Figure S2A-C***). However, perhaps unsurprisingly given the rapidity of mast cell degranulation, there was no change in stimulus-induced degranulation when sugammadex was added 2 minutes post drug stimulation (***Figure 3A-C***).

Whilst sugammadex (500 μM) produced a profound inhibition of atracurium and compound 48/80-induced degranulation, regardless of the MRGPRX2 agonist concentration tested, inhibition of the ZINC-3573(R) response was surmountable by addition of more agonist (***Figure S3A-C***). This suggests a likely lower affinity interaction between sugammadex and ZINC-3573(R). Surmountability was harder to test for atracurium, given its lower potency.

However, evidence from experiments where the concentration of sugammadex was varied (Fig S2A), also suggest a lower affinity interaction between atracurium and sugammadex.

As cytokines are released from LAD2 cells with slower kinetics, we examined if the addition of sugammadex following addition of stimulus affected CCL2 release. Unlike degranulation, rocuronium was able to trigger CCL2 release from LAD2 cells (***Figure 4A***). Interestingly, rocuronium, atracurium and compound 48/80-induced CCL2 levels were significantly reduced both when cells were co-treated with agonist and sugammadex, and when sugammadex was added 2 minutes following activation (***Figure 4A-C***). ZINC-3573(R)-mediated CCL2 release was significantly reduced when LAD2 cells were co-treated with sugammadex but whilst inhibited, did not reach statistical significance when sugammadex was added 2 minutes after the stimulus (***Figure 4D***).

Interestingly, while sugammadex significantly impaired MRGPRX2 activation induced by the endogenous peptide agonist LL-37 (***Figure S3G***), it did not impair the actions of substance P and cortistatin-14 (***Figure 2D, 3D, 4E, S2D, S3D, S3F***). This suggests that sugammadex may be capable of selectively influencing the activity of certain endogenous MRGPRX2 peptide agonists.

In calcium mobilisation studies (***Figure 2D***), the co-treatment of cells with sugammadex and substance P appeared to increase responses. However, peak Ca^{2+} levels were not statistically different to substance P alone and this enhancing effect was not seen when examining the actions of sugammadex on substance P-induced degranulation and MCP-1 release (***Figure 3D, 4E***).

Throughout all our studies, as expected, sugammadex had no effect on IgE-dependent responses (***Figure 2E, 3E, 4F, S2E, S3E***).

Determining the kinetics of the sugammadex-induced reduction in MRGPRX2 agonist-induced CCL2 release.

Having established an effect on cytokine release by the addition of sugammadex 2 minutes post LAD2 cell stimulation, we next aimed to determine how long the addition of sugammadex could be delayed and still produce an inhibition of mast cell CCL2 release.

In these studies, compound 48/80 (10 µg/mL) was used as the prototypic MRGPRX2 activator. Even 3 hours following stimulation with compound 48/80, addition of sugammadex (500 µM) significantly reduced CCL2 levels in LAD2 cell supernatants (***Figure 5***). To better understand the mechanism behind this effect, qPCR studies measuring CCL2 transcript levels were conducted. Stimulation with compound 48/80 resulted in an approximate 5-fold induction of LAD2 cell CCL2 transcript levels. This was significantly reduced by co-treating cells with compound 48/80 and sugammadex or by addition of sugammadex up to 3 hours following cell stimulation (***Figure 5***).

vi. Discussion

We have successfully developed a polyclonal LAD2 mast cell model where the MRGPRX2 gene was stably knocked down using CRISPR-Cas9 gene editing. Consistent with, but more pronounced in comparison to existing research using siRNA MRGPRX2 KD[9,11], the CRISPR-Cas9 knock-down of the receptor significantly reduced the ability of rocuronium, atracurium, compound 48/80,ZINC-3573(R), substance P, LL-37 and cortistatin-14 to activate human LAD2 mast cells across a variety of measures. However, the MRGPRX2 gene disruption had no effect on FcεRI expression, the IgE/FcεRI pathway of mast cell activation nor on expression of another mast cell GPCR, CXCR4. Consistent with previous research[9,25], the NMBA's rocuronium and atracurium produced MRGPRX2-dependent LAD2 cell calcium mobilisation, although the former NMBA did not trigger degranulation.

It is unclear as to why rocuronium is not degranulation-competent in LAD2 cells but this observation has been made by several groups[9,25,26]. The calcium flux it induces in LAD2 cells is certainly more transient than is triggered by other MRGPRX2 activators or through the IgE/FcεRI pathway. In addition, calcium-independent contributors to degranulation, albeit with IgE-directed activation, have been identified in mast cells[27]. Perhaps these additional pathways are poorly activated by rocuronium. Further work on MRGPRX2 signalling in response to rocuronium is required to better understand this. Such efforts might perhaps also provide an explanation as to why some rare individuals respond so dramatically and adversely to NMBAs.

Sugammadex is a negatively charged cyclodextrin drug designed to encapsulate the positively charged aminosteroid NMBAs such as rocuronium, thus preventing it from antagonising nicotinic receptors[24]. However, whether this interaction prevents drug-induced activation of MRGPRX2 in mast cells has not been examined. This is of potential clinical significance as some case studies report the use of sugammadex in events of anaphylaxis, but with mixed outcomes[16-19]. We used an *in vitro* model to gain insight into the possible efficacy of sugammadex in treating MRGPRX2-dependent anaphylaxis. Firstly, the possible interaction of sugammadex with the known MRGPRX2 agonists (rocuronium, atracurium, compound 48/80, substance P, ZINC-3573(R), LL-37, cortistatin-14) was tested by co-treating the cells with agonist and sugammadex. Secondly, the more clinically relevant model of anaphylaxis treatment during surgery was mimicked by the addition of sugammadex post agonist stimulation.

Sugammadex when co-administered with rocuronium, impaired the ability of the NMBA to induce LAD2 cell calcium mobilisation and CCL2 production. This presumably occurs through sugammadex encapsulating and thus shielding the positive charge on the NMBA that is necessary for MRGPRX2 activation[21]. Interestingly, this action is extended to other known MRGPRX2 agonists including compound 48/80, ZINC-3573(R) and the endogenous peptide agonist LL-37, but not to substance P or cortistatin-14. It is intriguing to consider sugammadex as a potential selective regulator of certain endogenous MRGPRX2 agonists, although the basis for this selectivity remains unclear. As expected, sugammadex had essentially no effect on IgE/FcεRI-dependent responses. Some recent clinical reports have suggested that a complex of rocuronium and sugammadex can itself induce anaphylaxis[28, 29]. Our data indicate that this is unlikely mediated by MRGPRX2 activation and is most likely an IgE-dependent mechanism as has been suggested[28,29].

Even when given promptly (2 minutes) after MRGPRX2 stimulation, sugammadex was however ineffective in inhibiting mast cell degranulation, the primary cause of acute anaphylactic symptoms. This is not unexpected given the kinetics of degranulation that takes place within a few minutes post stimulus exposure. Therefore, assuming rapid distribution of a MRGPRX2-activating NMBA in the body, with consequent uniform activation of mast cells, it would seem unlikely that sugammadex administration would influence a clinically manifested MRGPRX2-dependent acute anaphylactic response.

Despite this, sustained calcium responses and the more delayed mast cell functional responses (*e.g.* cytokine release) were inhibited by the later addition of sugammadex. Whilst most patients suffer a rapid onset "uniphasic" anaphylactic event, others are seen to have a slower or "biphasic" reaction[30-32]. Biphasic reactions are characterized by symptoms (i.e. worsening of previous symptoms or new symptoms) that recur within 2 – 36 hours after the initial reaction[31]. These later stage symptoms could perhaps be partly mediated by the observed late phase release of mediators from mast cells[30]. The delayed release of mediators (cytokine CCL2) in response to rocuronium, atracurium and compound 48/80 in LAD2 cells was significantly reduced by the addition of sugammadex following drug stimulation. For compound 48/80, inhibition of CCL2 release by sugammadex was observed even up to 3 hours post MRGPRX2 activation demonstrating that unlike degranulation, CCL2 release requires sustained MRGPRX2 stimulation. Whilst speculative, this suggests that sugammadex administration may indeed have some benefit in reducing more slowly emerging deleterious responses that result from delayed mast cell cytokine release by certain MRGPRX2 agonists.

Although compound 48/80 and ZINC-3573(R) have similar efficacy at the MRGPRX2 receptor, as measured by LAD2 degranulation, sugammadex was less able to inhibit the response of ZINC-3573(R). From this we suggest that the complex formed between sugammadex and ZINC-3573(R), is more transient and as such, may be less able to mediate the sustained block of MRGPRX2 activation necessary for significant CCL2 production. However, binding studies between these drugs are needed to establish this conclusively.

Interestingly, although sugammadex is not clinically used to reverse the neuromuscular block induced by atracurium[24], we have shown that it does significantly reduce atracurium-induced MRGPRX2-mediated mast cell activation. This suggests that a molecular interaction takes place between sugammadex and atracurium, that whilst not affecting the NMBA's ability to block nicotinic receptors, is able to impair its activation of MRGPRX2. Interestingly, compared to degranulation and cytokine release, sugammadex was less able to inhibit the atracurium-induced Ca^{2+} response. The reason for this is as yet unclear, but could relate to the complex of atracurium and sugammadex showing biased ligand activity at MRGPRX2, retaining Ca^{2+} mobilisation activity but being deficient in triggering other signalling pathways necessary to initiate mast cell functional responses. The concept of biased agonism is now well established with clinically relevant exemplars existing for multiple GPCRs including opioid

receptors[33]. Interestingly, given their sequence similarity, a molecular model of MRGPRX2 has been derived from crystallography data of the κ opioid receptor[11]. Signalling studies using atracurium and the putative atracurium/sugammadex complexes would need to be carried out to validate the relevance of biased agonism at MRGPRX2.

The ability of sugammadex to influence ligands beyond its designed aminosteroid NMBA complement, is clearly demonstrated by its ability to neutralise the polybasic research tool compound 48/80. However, there is clear specificity in the actions of sugammadex, as it was inactive in preventing substance P-induced MRGPRX2 activation. Extended modelling, like that carried out for rocuronium[19], on the interaction between sugammadex and these various MRGPRX2 agonists would be useful to help identify the structural basis for the differential effects of the γ-cyclodextrin.

Beyond the clinical implications of this study, the inhibition by sugammadex of ongoing MRGPRX2 responses to some agonists, suggests that it could also be a useful experimental tool. Analogous to the use of free hapten to terminate hapten-specific IgE-mediated FcεRI responses[34], sugammadex, through the rapid neutralisation of some MRGPRX2 agonists, could be used to examine MRGPRX2 receptor activity, signalling and desensitisation.

In summary, we have examined the effect of the aminosteroid NMBA-reversing agent sugammadex on MRGPRX2-dependent activation of mast cells. We have shown that sugammadex has no effect on mast cell degranulation when given after exposure to MRGPRX2 agonists including NMBAs, suggesting against its use in the acute management of non-IgE dependent anaphylaxis. However, sugammadex was able to inhibit release of the more slowly elaborated mast cell mediator CCL2, even with delayed administration. Thus, sugammadex may be of some potential benefit in preventing less well-defined symptoms of drug hypersensitivity that are associated with the MRGPRX2-driven, delayed release of certain mast cell mediators.

vii. Acknowledgements

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viii. Conflict of Interest

There is no conflict of interest to declare .

viii. Reference List

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ix. Tables

N/A

x. Figure Legends

Figure 1: Rocuronium, atracurium, compound 48/80, ZINC-3573(R), LL-37, cortistatin-14 and substance P-induced LAD2 cell stimulation is MRGPRX2 dependent.

Calcium mobilization in response to a variety of MRGPRX2 agonists was quantified (Figure 1A-D) using the Ca^{2+} indicator Fura2. Addition of rocuronium (200 µM; 1A), atracurium (150 µM; 1B), compound 48/80 (10 µg/mL; 1C) and NIP-BSA (100 ng/mL; 1D) all produced an increase in intracellular Ca^{2+} levels with all responses (except for NIP-BSA) being significantly reduced in the MRGPRX2 KD cells. A similar effect of MRGPRX2 KD was observed in degranulation

studies with compound 48/80 (10 µg/mL), substance P (10 µM), ZINC-3573(R) (10 µM), atracurium (150 µM), LL-37 (5 µM), cortistatin-14 (5 µM) (Figure 1E), although rocuronium lacked a measurable degranulation response. Results shown are N≥5, means ± SEM. For statistical analysis (Figure 1E), a two-way ANOVA was conducted with Bonferroni multiple comparison post hoc test. Each MRGPRX2 KD response was compared to the respective response in the LAD2 WT cells. * $p < 0.01$

Figure 2: Sugammadex inhibits calcium mobilization induced by selected MRGPRX2 agonists in LAD2 cells.

Calcium mobilization of LAD2 cells was monitored in real time using Fura2. Where relevant, cells were either co-treated with sugammadex and agonist (t= 30 seconds) or sugammadex was added 40 seconds following agonist drug addition (t= 70 seconds). Sugammadex addition significantly inhibited (*$p < 0.01$) calcium flux induced by rocuronium (100 µM; 2A), compound 48/80 (1 µg/mL; 2C) with a less pronounced effect on atracurium (150 µM; 2B). There was no effect of sugammadex on substance P (10 µM; 2D) or NIP-BSA (100 ng/ml; 2E) induced responses. Results shown are N≥5. Mean responses are shown with error bars removed to improve clarity. A two-way ANOVA was conducted, on the peak Ca^{2+} response, with Bonferroni multiple comparison post hoc test.

Figure 3: Sugammadex inhibits degranulation induced by selected MRGPRX2 agonists in LAD2 cells.

Mast cell degranulation was quantified by the release of β-hexosaminidase. Where relevant, cells were either co-treated with sugammadex and MRGPRX2 agonist or sugammadex was added 2 minutes following agonist drug addition. Sugammadex, but only when added with agonist, inhibited atracurium (150 µM; 3A), compound 48/80 (10 µg/mL; 3B) and ZINC-3573(R) (10 µM; 3C) induced degranulation. The drug had no effect on substance P (10 µM; 3D) and antigen (100 ng/ml; 3E) induced degranulation. Results shown are N≥6, mean ±SEM. A one-way ANOVA was conducted with Dunnett's multiple comparison post hoc test. Each condition was compared to the respective response in the absence of sugammadex. * $p < 0.01$

Figure 4: Sugammadex inhibits CCL2 release induced by selected MRGPRX2 agonists in LAD2 cells.

CCL2 was quantified using a commercial sandwich ELISA. Where relevant, cells were either co-treated with sugammadex and stimuli or sugammadex was added 2 minutes following stimulus addition. Sugammadex addition in both protocols, inhibited rocuronium (200 µM; 4A), atracurium (150 µM; 4B) and compound 48/80 (10 µg/mL; 4C) induced increase in CCL2 levels. The effect on ZINC-3573(R) (10 µM; 4D) was only observed when cells were co-treated with sugammadex and agonist. The drug had no effect on substance P (10 µM; 4E) and antigen (100 ng/ml; 4F) -induced degranulation. Results shown are N≥6, mean ±SEM. A One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test. Each condition was compared to the respective response in the absence of sugammadex. * $p < 0.01$

Figure 5. Sugammadex inhibits compound 48/80-induced CCL2 release even when added up to 3 hours following LAD2 cell stimulation.

LAD2 cells were stimulated with compound 48/80 (C48/80; 10 µg/mL) and where applicable, either co-treated with sugammadex or sugammadex was added at various time points following compound 48/80 addition. Supernatant for CCL2 release quantification was obtained 24 hours following stimulation. Cell lysates for CCL2 mRNA expression quantification were harvested 4 hours following compound 48/80 addition. The inhibitory effects of sugammadex were dependent upon its time of addition, but were still manifest when it was added up to 3 hours following compound 48/80 addition. The effect of sugammadex on CCL2 release was paralleled by a concomitant inhibition of CCL2 transcript level. Results shown are N=5, with mean ±SEM. One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test. Each time point was compared to the compound 48/80 response in the absence of sugammadex. * $p < 0.01$

Figure S1: Fluorescence activated cell sorting (FACS) of CRISPR-Cas9 MRGPRX2 KD LAD2 cells.

CRISPR-Cas9 gene editing was used to selectively disrupt the MRGPRX2 gene. Gene targeted cells were stimulated with compound 48/80 (10 μg/mL) (or NIP-BSA (antigen) (100 ng/mL)) for 15 minutes and MRGPRX2 low responding cells sorted using flow cytometry based on cell surface CD63 expression (Figure S1A). A polyclonal population of MRGPRX2 knock down cells were successfully established (Figure S1B). IgE-mediated responses were minimally affected by MRGPRX2 gene-targeting (Figure S1C). While cell surface expression of MRGPRX2 was significantly reduced in the LAD2 MRGPRX2 KD model (Figure S1D), the expression of FcεRIα (Figure S1E) and CXCR4 (Figure S1F) was unaffected.

Figure S2: Concentration-dependent inhibition of atracurium, compound 48/80 and ZINC-3573(R)-induced LAD2 degranulation by sugammadex.

LAD2 cells were co-treated with various concentrations of sugammadex and a fixed concentration of stimulus. Degranulation was quantified by the release of β-hexosaminidase. Baseline (BL) degranulation induced by the MRGPRX2 agonist was also measured in the absence of sugammadex. There was a concentration-dependent inhibition by sugammadex of LAD2 mast cell degranulation induced by compound 48/80 (10 μg/mL; S3A) and to a lesser extent ZINC-3573(R) (10 μM; S3B) and atracurium (150 μM; S3C). Sugammadex had no effect on substance P (10 μM; S3D) or NIP-BSA (100 ng/mL; S3E) -induced degranulation. Results shown are N≥5, mean ±SEM. One-way ANOVA was conducted with Dunnett's multiple comparison post hoc test with each measurement compared to that of BL; * $p < 0.05$, ** $p < 0.01$.

Figure S3: Sugammadex has varying activity on mast cell activation induced by different MRGPRX2 agonists.

LAD2 degranulation was quantified by the release of β-hexosaminidase. The cells were co-treated with sugammadex (500 μM) and a concentration range of stimuli. Sugammadex inhibited degranulation induced by atracurium (Figure S2A), compound 48/80 (Figure S2B), ZINC-3573(R) (Figure S2C) and LL-37 (Figure S2G), but with varying potencies. Sugammadex

had no effect on substance P (Figure S2D), cortistatin-14 (Figure S2F) or NIP-BSA (Figure S2E) -induced degranulation. Results shown are N≥5, mean ±SEM.

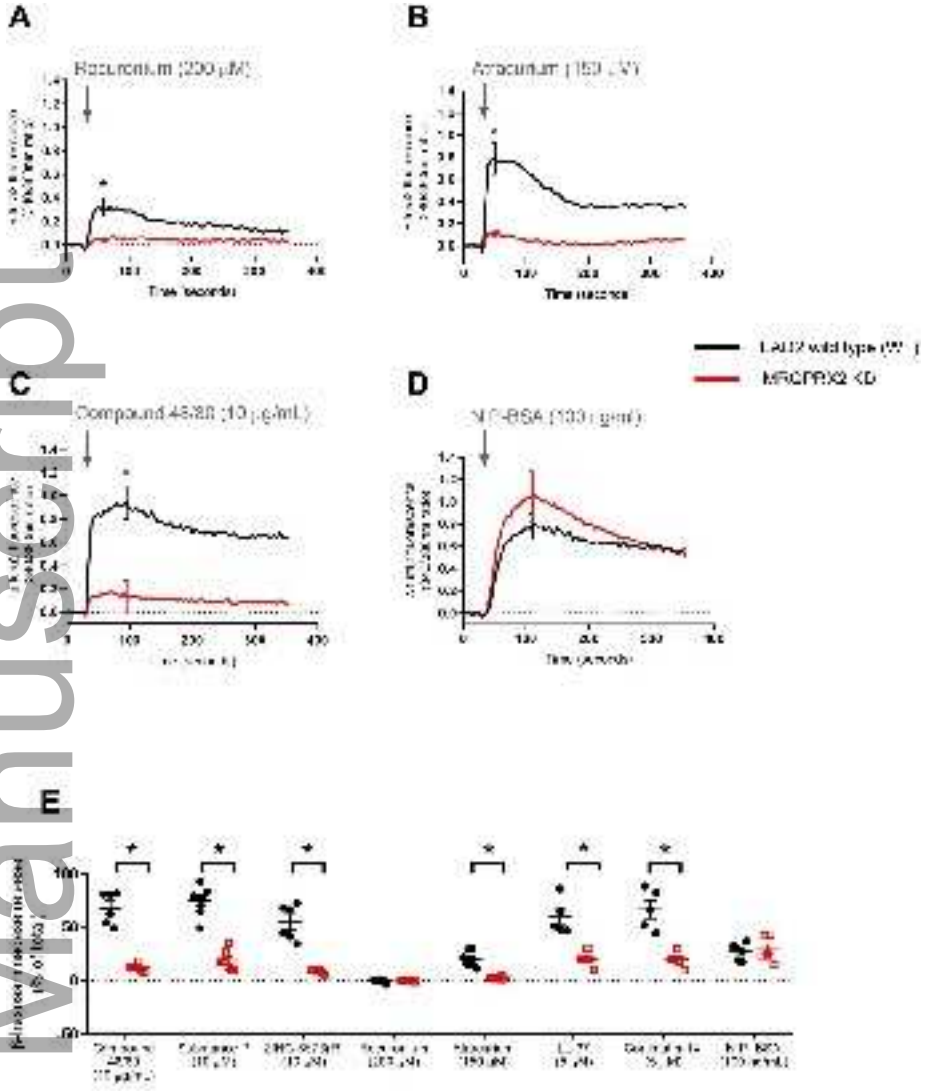

Figure 1

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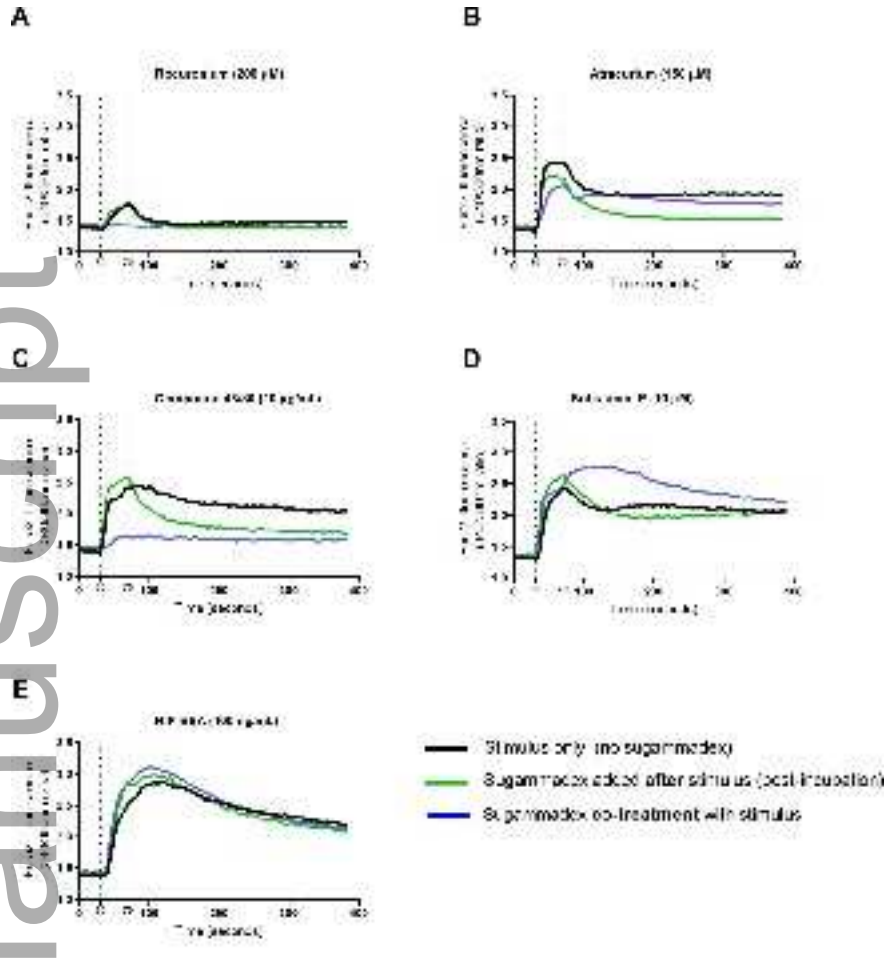

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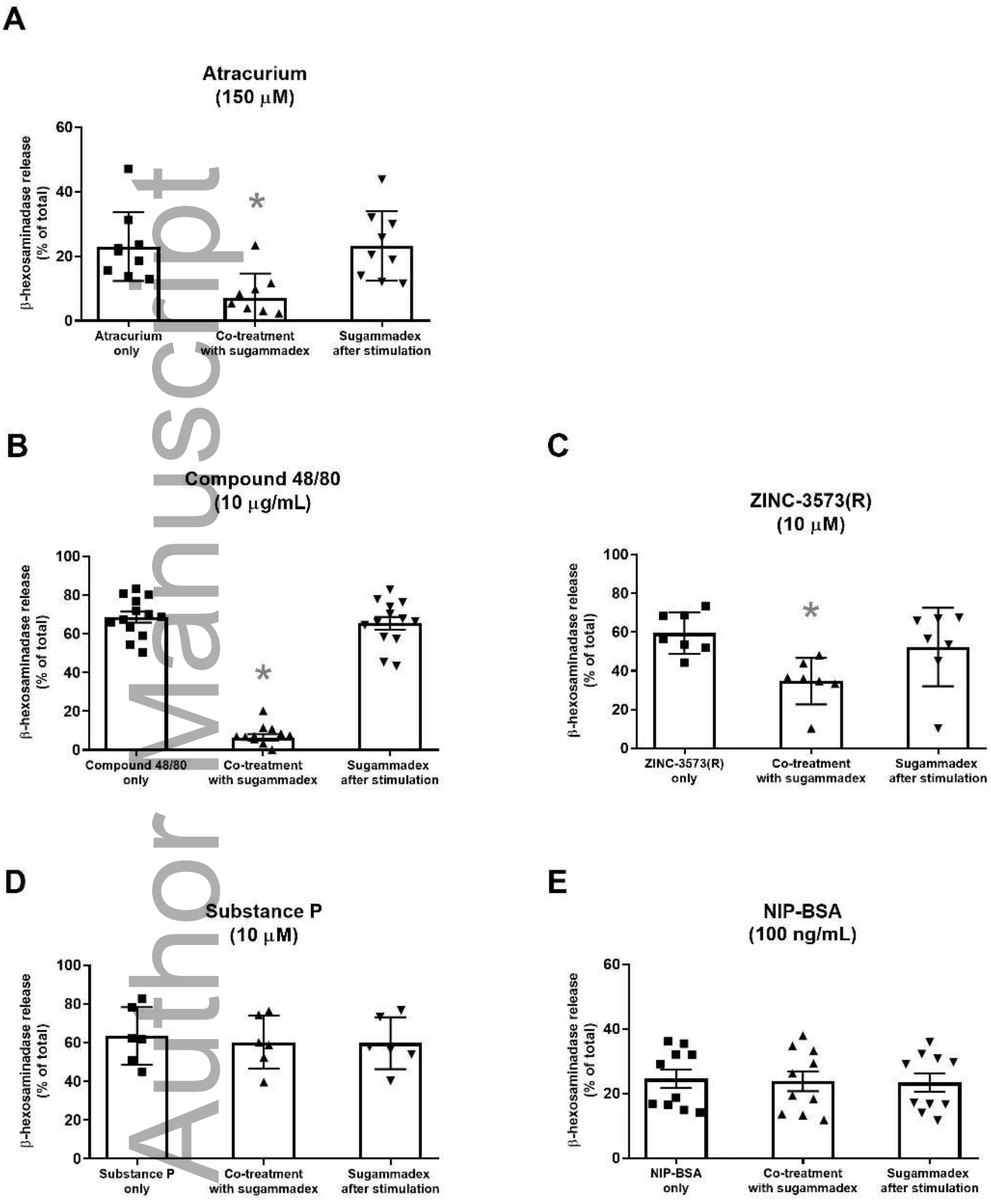

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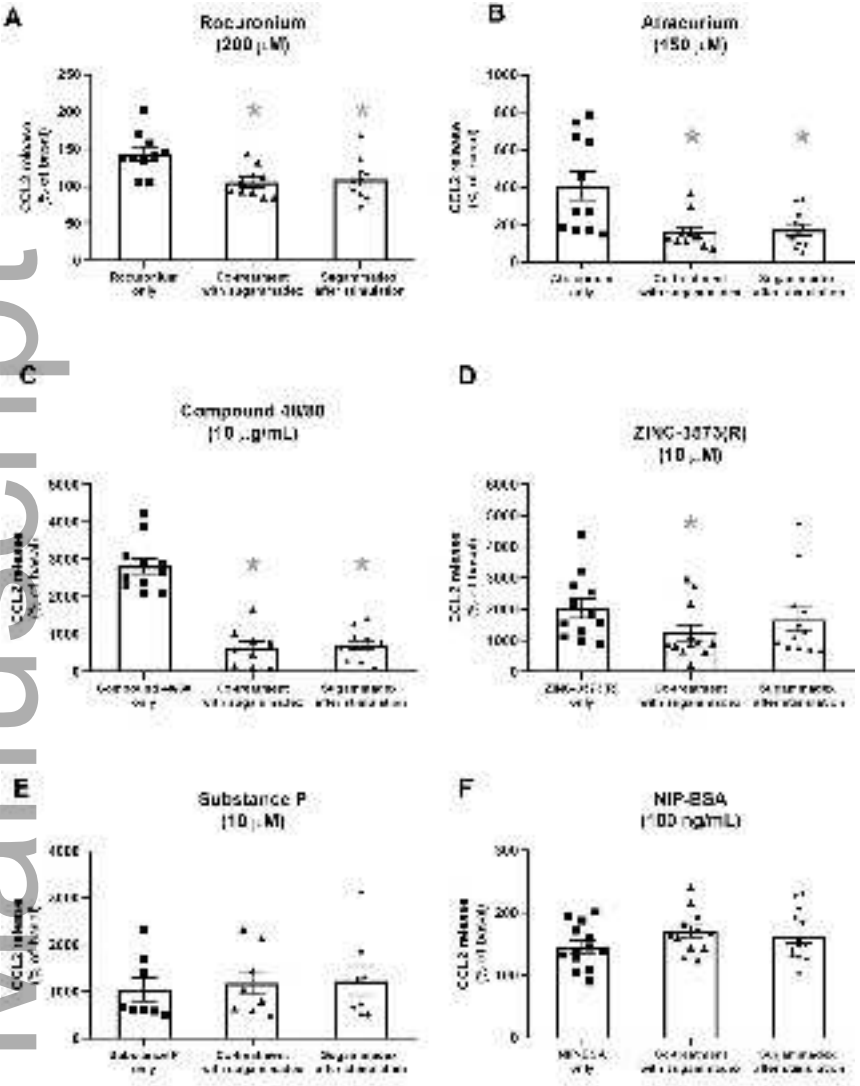

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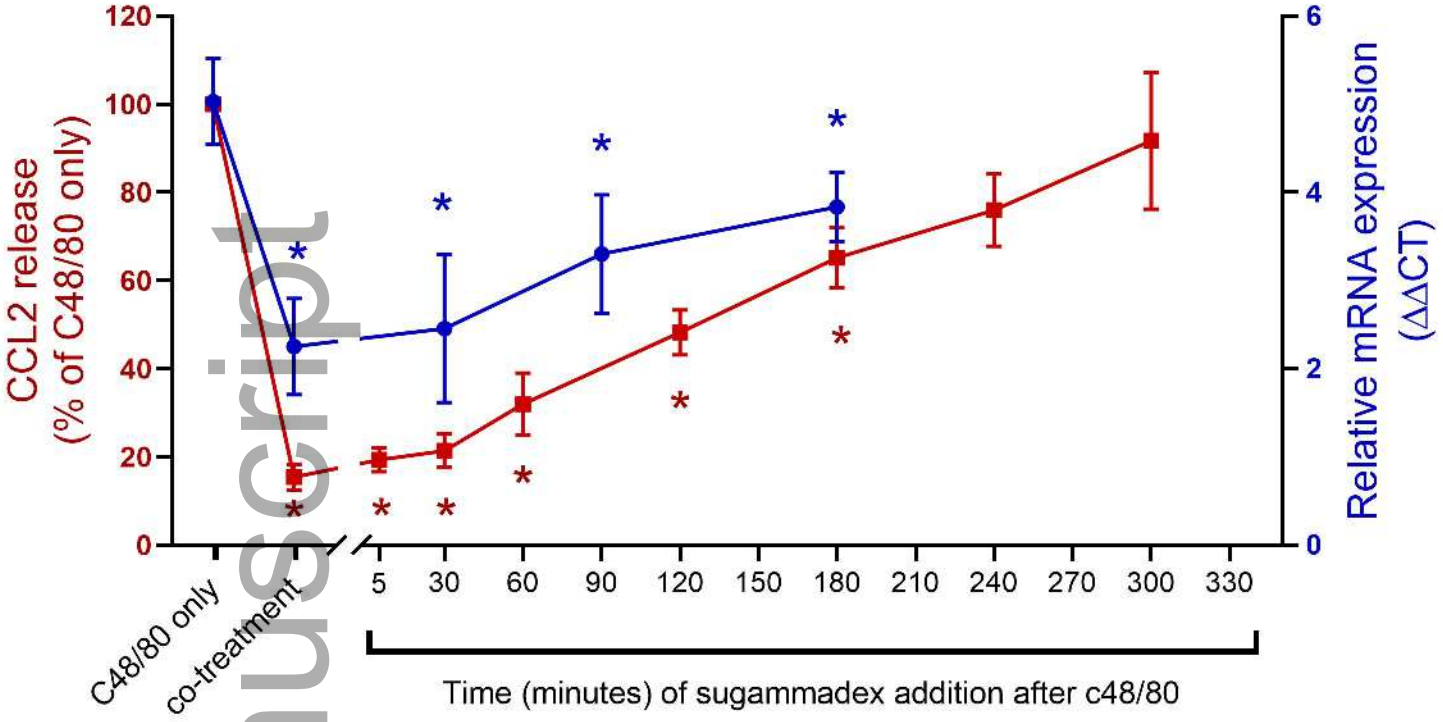

Figure 5

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