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Research Article

Typical antimicrobials induce mast cell degranulation and anaphylactoid reactions via MRGPRX2 and its murine homologue MRGPRB2

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Mast cells are unique immune cells that function as sentinels in host defence reactions, including immediate hypersensitivity responses and allergic responses. The mast cell-specific receptor named MAS-related G protein-coupled receptor X2 (MRGPRX2) triggers mast-cell degranulation, a key process in anaphylactoid reactions. It is widely observed that antimicrobials can induce pseudo-allergic reactions (i.e. IgE-independent mechanism) with symptoms ranging from skin inflammation to life-threatening systemic anaphylaxis. However, their direct involvement and the mechanisms underlying anaphylactoid reactions caused by antimicrobials have not been demonstrated. Structurally different antimicrobials were screened by Ca^{2+} imaging using MRGPRX2 overexpressing HEK293 cells. MRGPRX2 related anaphylactoid reactions induced by these components were investigated by body temperature drop and mast cell degranulation assays. We showed that MRGPRX2 is involved in allergic-like reactions to three types of antimicrobials in a dose-dependent manner. However, mast cells lacking the receptor show reduced degranulation. Furthermore, mice without MAS-related G protein-coupled receptor B2 (the orthologous gene of *MRGPRX2*) exhibited reduced substance-induced inflammation. Interestingly, β-lactam and antiviral nucleoside analogues did not induce anaphylactic reactions, which were also observed in vitro. These results should alarm many clinicians that such drugs might induce anaphylactoid reactions and provide guidance on safe dosage of these drugs.

Keywords: Anaphylactoid reaction · Antimicrobials · Degranulation · Mast Cells · Mrgprx2

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Introduction

Drug-induced allergies can be triggered by direct drug stimulation of mast cells or basophils, leading to the degranulation of mast cells and the release of active inflammatory mediators, such as histamine [1, 2]. Drug allergy reactions may occur in first-time users, resulting in one or more adverse clinical outcomes, which in some cases may be life-threatening [3, 4]. These

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reactions can also significantly increase the treatment cost for the patient [5].

Antimicrobials are used to treat infections caused by pathogenic microorganisms [6, 7], with specific types of antimicrobials used depending on the type of pathogenic microorganism [8]. For example, acrylamide antimicrobials are antifungal drugs [9], aminoglycosides are especially effective against infections with members of the *Enterobacteriaceae* and *Staphylococcus* aureus [10] and sulfonamides are broad-spectrum antimicrobials [11]. A large number of reports on clinical adverse reactions indicate that acrylamides, represented by terbinafine hydrochloride, can cause serious cutaneous side effects, such as pityriasis rosea [12]. Aminoglycosides, such as sisomicin hydrochloride can lead to systemic contact dermatitis [13], while sulfa drugs, such as sulfamethoxazole, have been shown to cause hypersensitivity reactions in the treatment of children with brucellosis [14]. The number of anaphylactic reactions caused by antimicrobials, especially those administered by injection, has increased in recent years [15]. However, the molecular mechanisms of allergy induction, especially the targets that trigger allergy-like reactions, have not been elucidated [16].

Recently, Dong and colleagues found that *MrgprB2* in mouse mast cells is homologous to human *MRGPRX2* [17]. Upon direct binding of drugs to the MrgprB2 receptor, the Ca^{2+} concentration in mast cells increases, causing degranulation and leading to allergic reactions. This study confirmed for the first time that MRGPRX2 on human mast cells is a membrane receptor that specifically triggers drug-induced allergy-like reactions [17]. It has now been confirmed that drugs that often induce anaphylaxis in clinical practice [18], such as anesthetics [19] and fluoroquinolones [20] cause allergy-like reactions by MRGPRX2-induced activation of mast cells. Our current study investigated whether antimicrobials induce allergic reactions through MRGPRX2, and clarified the mechanism by which these antimicrobials induce allergy-like reactions. Knowledge of these mechanisms would help build a strong foundation for better clinical use of antimicrobials.

Results

Clinically relevant antimicrobials interact with the MRGPRX2 receptor inducing mast cell response

In this study, we selected thirty-one clinical used antimicrobials from five classes: We found that terbinafine hydrochloride, amorolfine hydrochloride, ketoconazole, sisomicin sulfate, gentamicin sulfate, micronomicin sulfate, sulfamethoxazole, mafenide acetate, and sulfadoxine which belong to three classes antimicrobials increased intracellular calcium ion (Ca^{2+}) concentration in MRGPRX2-HEK293 cells (Fig. 1; Supporting

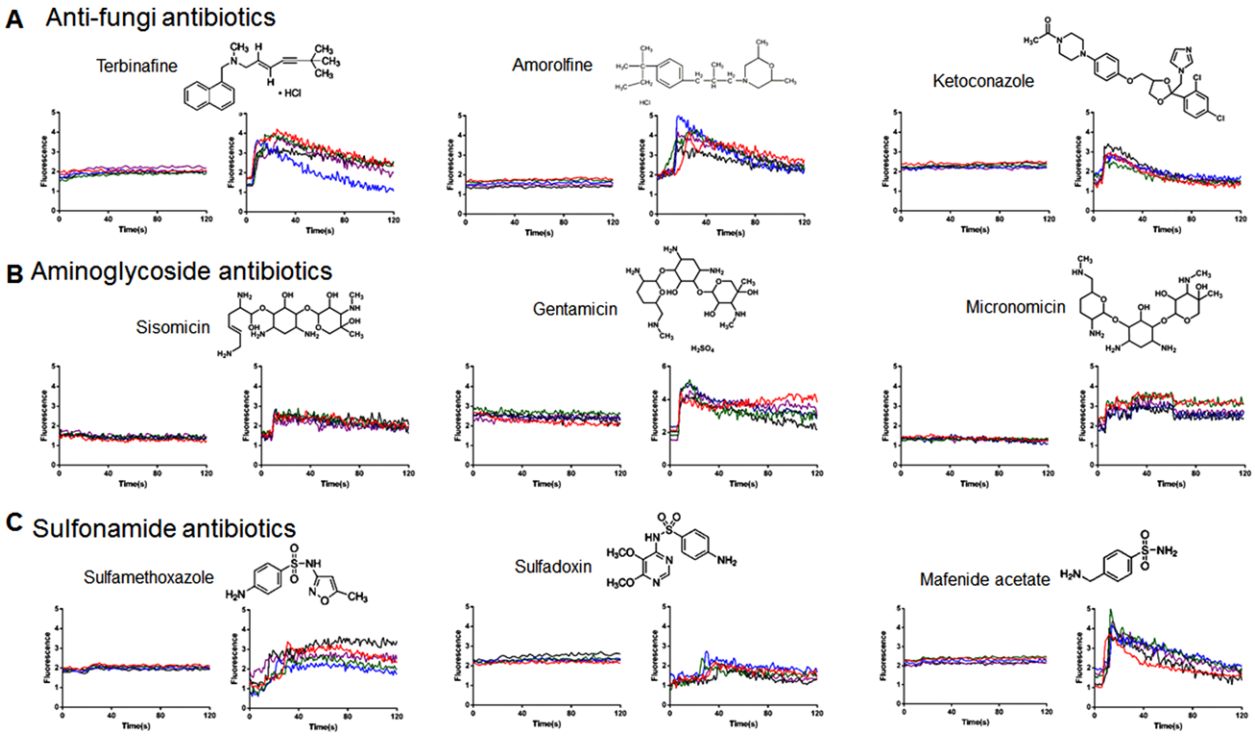

Figure 1. Substances belonging to three types of antimicrobials induce cell calcium influx via MRGPRX2 signaling. Calcium imaging was performed using MRGPRX2 over-expressing HEK293 cells by calcium imaging assay. Different colored lines correspond to fluorescence intensity change of different cell measured under the same experimental conditions. (Left: NC-HEK293 cell (tranfected with empty plasmid), Right: MRGPRX2-HEK293 cell (tranfected with pcDNA-MRGPRX2 plasmid). Upper: name and structure of antimicrobial agents, below: calcium imaging).
(A) Antifungal agents. (B) Aminoglycosides. (C) Sulfonamides. Data shown is representative of three independent experiments with five cells per experiment.

Information Fig. 1A) at the same concentration of 100 μg/mL, but did not alter the intracellular Ca^{2+} concentration in HEK293 cells transfected with an empty plasmid (Fig. 1; Supporting Information Fig. 1). Both β-lactam antimicrobials and antiviral nucleoside analogs did not alter the intracellular Ca^{2+} concentration in HEK293 cells overexpressing MRGPRX2 (Supporting Information Fig. 1 C, D). Therefore, the antifungals, aminoglycosides, and sulfonamide antimicrobials interact with the MRGPRX2 receptor and trigger a mast cell response.

Determination of half-maximal effective concentration and antimicrobial agent pharmokinetics

MRGPRX2-HEK293 cells were used to determine the half-maximal effective concentration (EC_{50}) of the nine drugs tested (Fig. 2). The EC_{50} values of terbinafine hydrochloride, amorolfine hydrochloride, and ketoconazole were 109.9 ± 13.4, 161.0 ± 37.4, and 84.4 ± 10.7 μg/mL, respectively (Fig. 2), whereas those of sisomicin sulfate, gentamicin sulfate and micronomicin sulfate were 191.7 ± 35.9, 703.9 ± 53.9, and 808.4 ± 41.2 μg/mL, respectively (Fig. 2). The EC_{50} values of sulfamethoxazole, mafenide acetate and sulfadoxine were 391.3 ± 44.2, 643.2 ± 93.4 and 408.7 ± 57.3 μg/mL, respectively (Fig. 2). Terbinafine hydrochloride was selected in the fact that it is the most widely used antifungal of the three tested. Sisomicin sulfate had the lowest EC_{50} among the three aminoglycoside antimicrobials while sulfamethoxazole had the lowest EC_{50} among the sulfonamide antimicrobials. Of the three classes of antimicrobials, terbinafine hydrochloride, sisomicin sulfate, and sulfamethoxazole interacted with MRGPRX2 to a greater degree.

$kit^{W\text{-}sh/W\text{-}sh}$ deficient mice show resistance to antimicrobial triggered local inflammatory reactions

Terbinafine hydrochloride, sisomicin sulfate, and sulfamethoxazole have been found to cause human mast cell (LAD2)

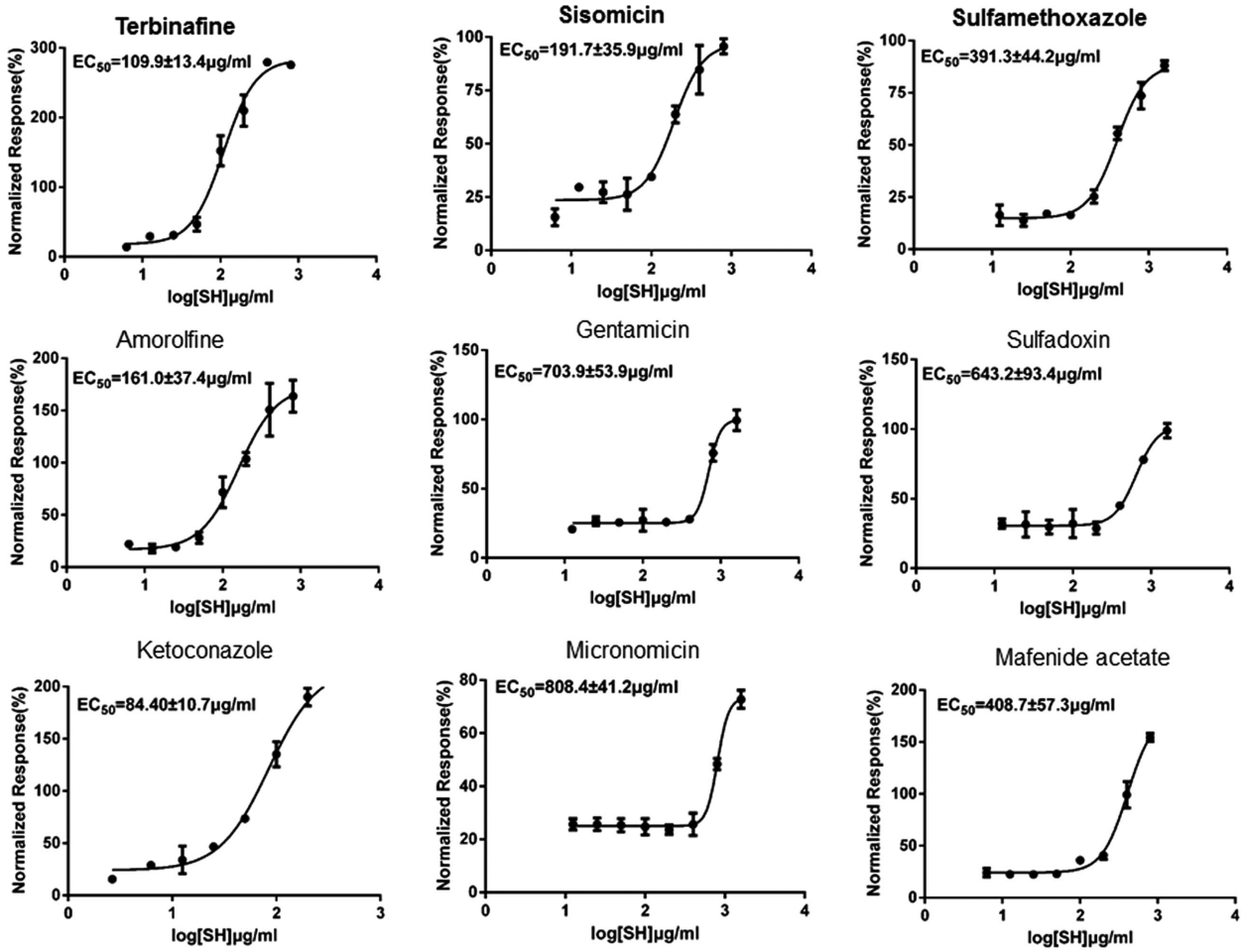

Figure 2. Analysis of binding affinity between antimicrobial agents and MRGRPX2. Binding strength of nine antimicrobial agents with MRGRPX2 was determined by half-maximum effective concentration (EC_{50}) assay.
Data are presented as mean ± S.E.M and are representative of three independent experiments with six mice per experiment. Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

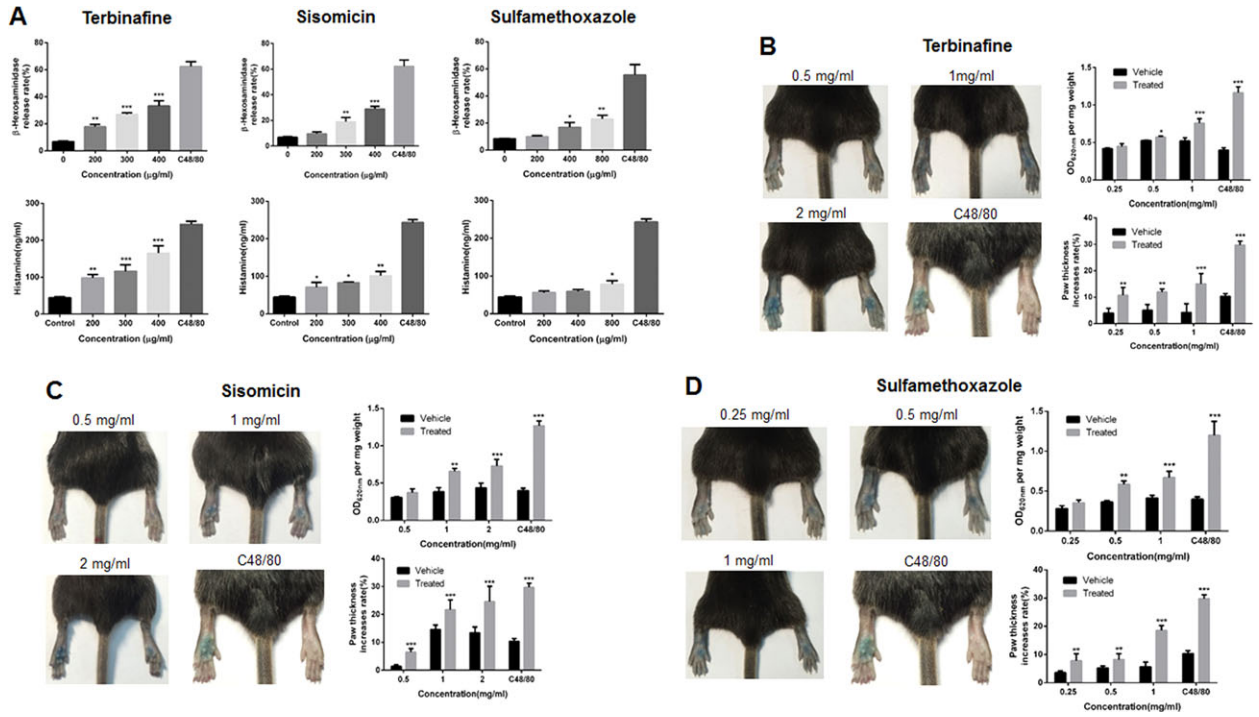

Figure 3. Antifungals, sulfonamides, and aminoglycosides induce mast cell degranulation and anaphylactoid reactions.
(A) Terbinafine hydrochloride, sisomicin sulfate, and sulfamethoxazole degranulation effect on LAD2 mast cells.
(B, C, D) Anaphylactoid reactions of mice treated with different concentrations of terbinafine hydrochloride (B), Sisomicin sulfate (C), and Sulfamethoxazole (D). Left, representative images of three independent experiments with six mice per experiment showing Evans blue dye extravasation after intraplantar injection of different concentrations of antimicrobial agents (left) or vehicle (saline: right). Right, quantification of Evans blue leakage into the paw and paw thickness increase after 15 min. ($n = 6$).
Data are presented as mean ± S.E.M and are representative of three independent experiments with six mice per experiment. Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

degranulation in vitro, which leads to the dose-dependent release of β-hexosaminidase and histamine (Fig. 3A). We also carried out a degranulation assay using several other antimicrobials and found that they all lead to LAD2 cell degranulation in a dose-dependent manner (Supporting Information Fig. 2). In vivo analysis showed that the three types of antimicrobials cannot trigger local inflammatory reactions in mast cell deficient $kit^{W\text{-}sh/W\text{-}sh}$ mice (Fig. 4). Furthermore, the degree of swelling and Evans blue dye (EBD) exudation in the paws of C57 wild-type mice injected with these three drugs were higher than in the hindpaws, which were injected with physiological saline solution (Fig. 3B-D) and that these effects were directly proportional to drug concentrations (Fig. 3B-D). Several other antimicrobials were also found to provoke the same allergic inflammatory response (Supporting Information Fig. 3). The phenomenon indicated that mast cells are responsible for allergic inflammatory reaction induced by the substances.

Defective mast cell degranulation and secretions following MRGPRX2 knockdown in LAD2 cells

MRGPRX2 in LAD2 human mast cells was down-regulated using small interfering RNA (siRNA) technology, while LAD2 cells transfected with non-functional siRNA were used as a control (Supporting Information Fig. 4). The rate of β-hexosaminidase release (Fig. 5A) and the amount of histamine released (Fig. 5B) by LAD2 mast cells were determined 30 min after drug administration. The results showed that all nine antimicrobials induced LAD2 cell degranulation in a dose-dependent manner (Fig. 5). At the same drug concentrations, the amounts of β-hexosaminidase and histamine released by MRGPRX2-knockdown LAD2 cells were lower than that from NC LAD2 cells, with a significant decrease in drug-induced degranulation (Fig. 5). A further experiment confirmed that the amount of TNF-α released from LAD2 mast cells induced by these three antimicrobials was significantly higher than that from MRGPRX2-knockdown LAD2 mast cells (Fig. 5C).

Mouse deficient in *MrgprB2* the murine homologue for MRGPRX2 recapitulate the LAD2 cell phenotype

Since the human MRGPRX2 gene is homologous to the mouse *MrgprB2* gene (17), a comparison between wild-type C57 mice and MrgprB2-knockout mice was performed to further elucidate the target of drug-induced allergic reactions. Terbinafine hydrochloride, sisomicin sulfate, and sulfamethoxazole, the three drugs with the highest risks of allergic reactions, were selected from the three classes of antimicrobials for in vivo animal experiments (Fig. 6A).

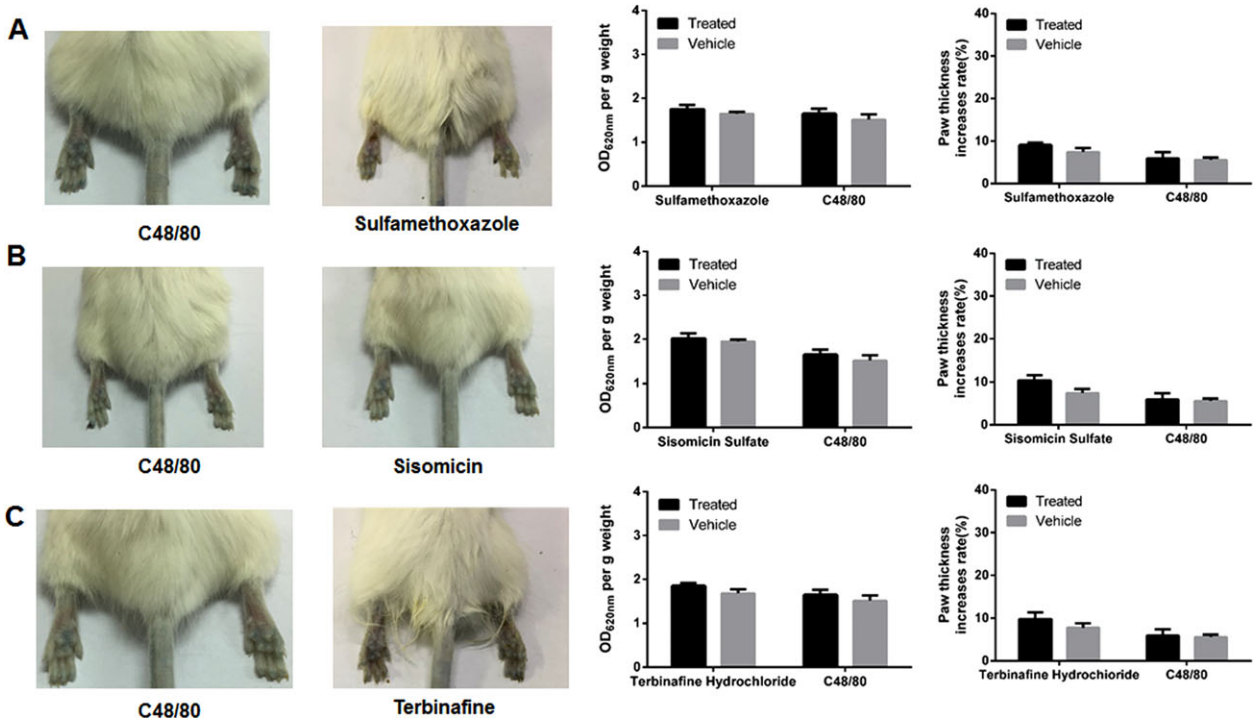

Figure 4. Evans blue extravasation in the paw of kit$^{W\text{-}sh/W\text{-}sh}$ mice. (A) Representative image of three independent experiments with six mice per experiment showing Evans blue dye extravasation after intraplantar injection of Terbinafine hydrochloride, Sulfamethoxazole, Sisomicin sulfate, and C48/80. ($n = 6$). (B) Quantification of Evans blue leakage into the paw after treatment.
Data are presented as mean ± S.E.M and are representative of three independent experiments with six mice per experiment. Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

These experiments showed that all three antimicrobials could induce local allergic reactions in wild-type mice. However, the feet of knockout mice injected with these three antimicrobials did not swell, and showed no obvious EBD exudation or local allergic reaction. The antimicrobials were then administered systemically to the mice via tail vein injections and changes in their body temperature were monitored (Fig. 6B). The results showed that all three antimicrobials significantly reduced the body temperature of the mice, but the reduction in body temperature was insignificant with no allergic reaction in knockout mice compared to that in the wild-type mice. The primary peritoneal mast cells from wild-type and *MrgprB2*-knockout mice were treated with the nine substances. The results showed elevated intracellular Ca^{2+} concentration in more than 80% of primary peritoneal mast cells from wild-type mice, whereas approximately 10% of primary peritoneal mast cells from *MrgprB2*-knockout mice showed elevated Ca^{2+} levels. The difference between wild-type and *MrgprB2*-knockout mice was significant (Fig. 6C). The histamine release assay showed that mice peritoneal mast cells from wild-type mice released higher levels of histamine than the *MrgprB2*-deficient mice (Fig. 6D).

Discussion

In this study, we screened and analyzed the effect of thirty-one antimicrobials on changes in calcium mobilization in MRGPRX2-HEK293 cells and found that all nine antimicrobials, including antifungals, aminoglycosides, and sulfonamides induce changes in intracellular Ca^{2+} concentrations in MRGPRX2-HEK293 cells, but are unable to alter calcium flow in normal HEK293 cells. This indicates that these nine antimicrobials (from three different classes) may potentially induce anaphylactoid reactions via the MRGPRX2 receptor.

The antimicrobials investigated are often intravenously or intramuscularly administered in high doses, and thus are more likely to achieve high blood concentrations and rapid tissue distribution. Thus they are more likely to activate mast cells located around vasculature and skin [21, 22]. Therefore, our in vivo assay confirmed that terbinafine hydrochloride, sisomicin sulfate, and Sulfamethoxazole, specifically activate MRGPRX2, induce mice skin flushing, such as footpad swelling and vascular permeability, in a dose-dependent manner compared with the negative control groups. Similarly, the other six drugs also could trigger same inflammatory reactions. However, we found that three types of antimicrobials cannot trigger local inflammatory reactions without mast cell participation. Patients are administered high dose of such drugs and thus are more likely to activate mast cell located around vasculature leading to induced anaphylactoid reaction(s). In vitro assays also confirmed that antimicrobials, including terbinafine hydrochloride, sisomicin sulfate, and sulfamethoxazole caused LAD2 human mast cell degranulation, leading to β-hexosaminidase and histamine release in a dose-dependent

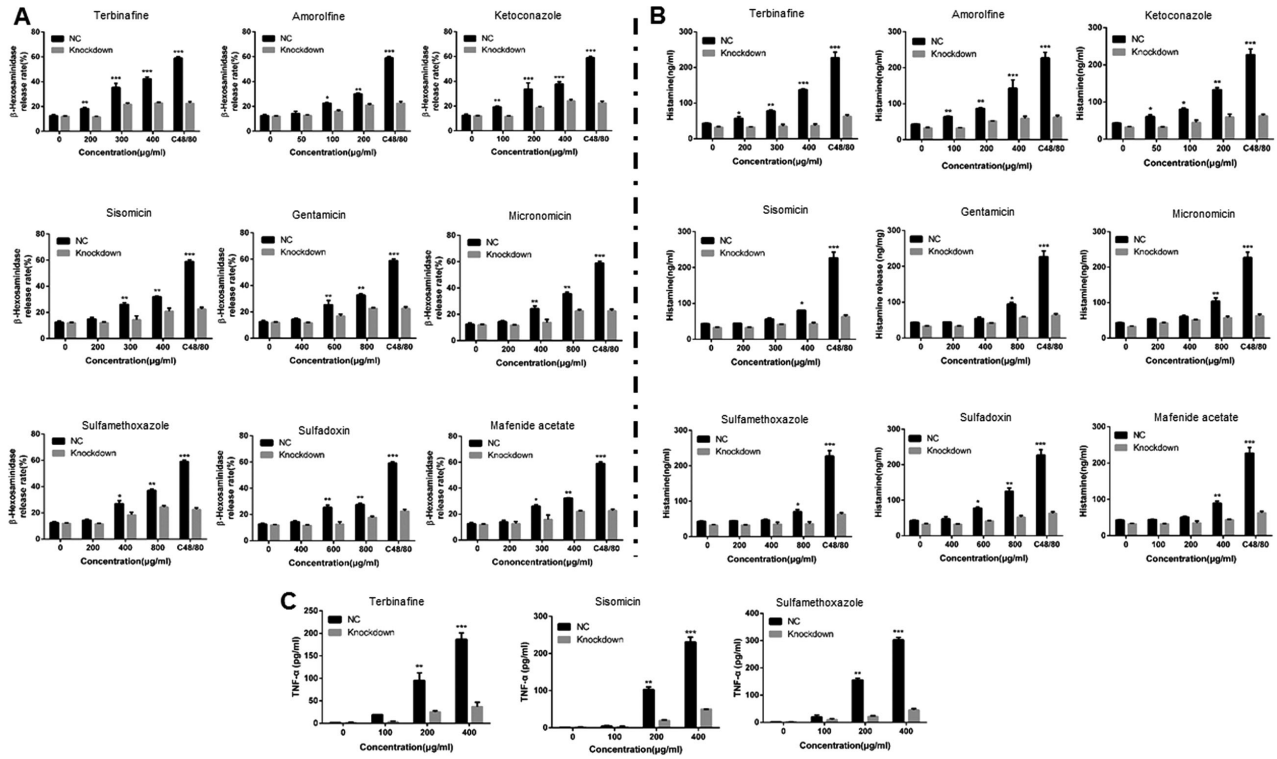

Figure 5. MRGPRX2 mediated LAD2 mast cell responsiveness of antimicrobials. Serial concentration of substances were incubated with LAD2 (NC) and MRGPRX2 knockdown mast cells for 15 min.
(A) β-hexosaminidase release, (B) Histamine release, and (C) TNF-α release were determined by spectrophotometry (A, B) and ELISA (C) respectively. Data are presented as mean + S.E.M and are representative of three independent experiments with three samples per experiment. Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and, ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

manner. Based on the in vitro and in vivo results, we conclude that antifungal agents, aminoglycosides, and sulfonamides induce mast cell degranulation to trigger anaphylactoid reaction. Therefore, drug-induced allergy-like reactions should be considered as a possibility when using these types of antimicrobials clinically.

Several speculations were made based on the theory of peptidergic pathway of mast cell activation according to which

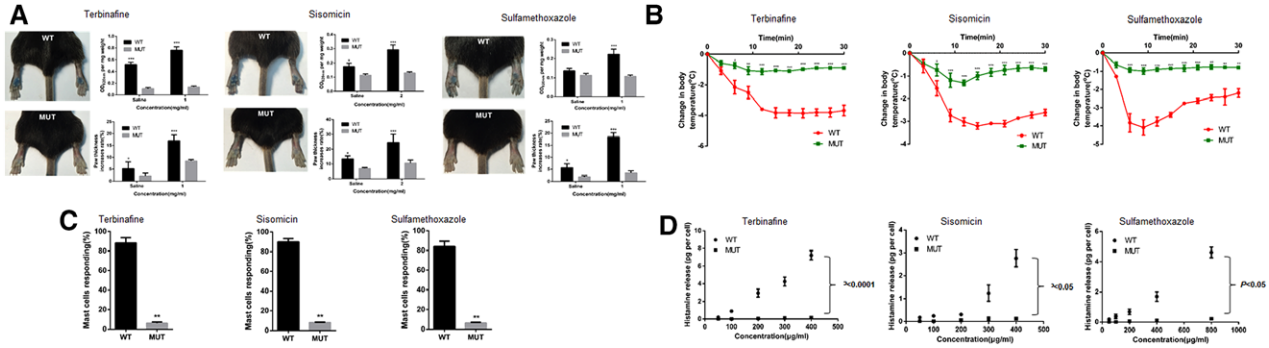

Figure 6. MrgprB2 mediated mast-cell responsiveness and anaphylactoid reaction of antimicrobials.
(A) Evans blue extravasation in the paw of wild-type and Mrgprb2$^{-/-}$ (MUT) mice. Left, one representative image of three independent experiments with five mice showing extravasation of Evans blue dye after intraplantar injection of three types of antimicrobials into individual mice. Right, quantification of Evans blue leakage into the paw. ($n = 5$).
(B) Body temperature changes from wild-type and Mrgprb2$^{-/-}$ (MUT) mice after injecting intravenously with antimicrobial agents. ($n = 5$).
(C) Percentage of responding cells from wild-type (WT) and MrgprB2$^{-/-}$ (MUT) peritoneal mast cells after drug application, assayed using Fluo-3 imaging.
(D) Histamine release from wild-type and MrgprB2$^{-/-}$ (MUT) mice after incubation with antimicrobial agents.
Data are presented as mean ± S.E.M obtained from three independent experiments with five mice per genotype per experiment. Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons,($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

degranulation may occur in a receptor independent manner mediated by interaction of positively charged compounds with the cell surface and then with the $G\alpha_i$ proteins [23]. Peptidergic pathway was widely accepted as the mechanism of mast cell activation by a range of cationic substances, collectively called basic secretagouges. In a major study, a single receptor, MRGPRX2, was identified and reported to mediate basic secretagouge activation of mast cells [24]. Notably, the study reported that Drug Administration (FDA)-approved small-molecule drugs, such as fluoroquinolones and neuromuscular blocking drugs, induced pseudoallergic reaction via MRGPRX2 [17].

We used MRGPRX2-knockdown LAD2 mast cells as a model system to further confirm that these antimicrobials induce mast cell degranulation through MRGPRX2. We found that their degranulation ability decreased significantly after knocking down the MRGPRX2 receptor. We also used MrgprB2-knockout mice to compare the local inflammatory reactions induced by terbinafine hydrochloride, sisomicin sulfate, and sulfamethoxazole. We found that MrgprB2-knockout mice had a lower intensity of local cutaneous flare reactions compared to the wild-type mice. We found that primary peritoneal mast cells from wild-type mice had more than 80% responsiveness to these three antimicrobials, whereas MrgprB2-knockout mice did not respond at all. A decrease in body temperature is usually used as the criterion for detecting drug-induced allergic-like reactions in mice [25]. Based on dose conversion between mice and human, drug dose applied in mice body temperature drop is relate to human clinic doses. However, these drugs administrated rapidly caused a quick drop in body temperature that was very slow to recover, while MrgprB2-knockout mice showed a much smaller temperature drop. Therefore, anaphylactoid reaction may be triggered if high dose of such drugs administrated rapidly by patient with overexpressing MRGPRX2 [26].

It is found that antifungal agents, aminoglycosides, and sulfonamides have large amounts of tertiary amine and secondary amine groups which have large number of nitrogen atoms easily protonated and positively charged. However, the antiviral nucleosides and β-lactam compounds have little amine group. Recently, McNeil et al reported that ligand with charged nitrogen is critical for MRGPRX2 activation to induce anaphylactoid reaction [17]. Therefore, we proposed that nitrogen atom easily protonated is the key characteristics for MRGPRX2 mediated drug anaphylactoid reaction.

In this study, we found that the three types of antimicrobials used significantly decreased the body temperature of wild-type mice. Furthermore, the release of histamine increased significantly compared to MrgprB2-knockout mice. And in vitro cytokine release, such as TNF-α, display same trend compared versus in vivo assays. This indicates that these three classes of antimicrobials, represented by terbinafine hydrochloride, sisomicin sulfate, and sulfamethoxazole, could induce allergy-like reactions via MRGPRX2. Therefore, targeting human MRGPRX2 is a promising way to prevent antimicrobials induced drug anaphylactoid reaction.

Materials and methods

Drugs and reagents

Compound 48/80 (C48/80) was purchased from Sigma-Aldrich. Drug substances: Terbinafine, amorolfine, ketoconazole, sisomicin, gentamicin, micronomicin, sulfamethoxazole, sulfadoxin, mafenide acetate, amoxicillin, ampicillin sodium salt, oxacillin sodium, flucloxacillin, mezlocillin sodium, piperacillin, penicillin G potassium, penicillin V potassium, ticarcillin, cefpirome sulfate, cefuroxime sodium, cefixime, cefoperazone, ceftriaxone sodium, cefalotin, cefotaxime sodium, cytarabine, acyclovir, idoxuridine, famciclovir, ganciclovir, valaciclovir hydrochloride were from Meilunbio Co., Ltd (Dalian, China) and purified to ≥ 98%. Fluo-3, AM ester and Pluronic F-127 were from Biotium. TM buffer (6.954 g/L NaCl, 0.353 g/L KCl, 0.282 g/L $CaCl_2$,0.143 g/L $MgSO_4$, 0.162 g/L KH_2PO_4, 2.383 g/L HEPES, 0.991 g/L glucose and 1 g/L BSA, pH = 7). was prepared on the day of experiments. The p-nitrophenyl N-acetyl-β-D-glucosamide and Triton X-100 were from sigma Aldrich Co., LLC. (Shanghai, China) and should be prepared to the proper concentration before used. Stop buffer was made up of 0.1 M sodium carbonate and sodium bicarbonate, pH = 11. All aqueous solutions were prepared using ultrapure water produced by MK-459 Millipore Milli-Q Plus ultra-pure water system.

Mouse model

Mice were purchased from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China). Adult male mice weighing 25–30 g were included in the study. Mast cell deficient $kit^{W\text{-}sh/W\text{-}sh}$ mice on a C5Bl/6 background were provided by National Resource Center of Model Mice (NRCMM). MrgprB2 knockout mice on a C5Bl/6 background were provided as by Professor Xinzhong Dong, Johns Hopkins University, Baltimore. All mice were housed in the Experimental Animal Center of Xi'an Jiaotong University and housed in individual cages in a large colony room, with free access to water, and were fed a standard dry food twice a day. The breeding environment was 20∼25°C, with a relative humidity of 40% and a day-night cycle of 12/12 h. The mice were randomly divided into the following groups (5 mice in each group): control, compound 48/80, and the experimental group. No randomization was used for MrgprB2 knockout mice as it was not applicable for these studies. All experiments requiring equal handling of the animals were conducted by experimenters blinded to the conditions.

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. The experimental protocols for using the mice were approved by the Animal Ethics Committee

at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2011-0045). All animals were operated on under chloral hydrate anesthesia.

Cell lines

The Laboratory of Allergic Disease 2 (LAD2) human mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, USA) [27]. Cells were maintained in StemPro-34 medium supplemented with 10 mL/L StemPro nutrient supplement, 1:100 penicillin- streptomycin, 2 mmol/L L-glutamine and 100 ng/mL human stem cell factor in an atmosphere containing 5% CO_2 at 37°C. Culture medium was replaced every 1 weeks and the cells were kept at a density of 2×10^6 cells/mL. Human MRGPRX2-expressing HEK293 cells were provided by Professor Xinzhong Dong, Johns Hopkins University, Baltimore. Cells were cultured in DMEM medium supplemented with 10% FCS, 100 U of penicillin, and 100 μg/mL of streptomycin (Hyclone, UT).

Peritoneal mast cell purification

Adult male and female mice 2–5 months of age were sacrificed through CO_2 inhalation. A total of 12 mL ice-cold mast cell dissociation media was used to make two to three sequential peritoneal lavages, in which MCDM and cells were mixed and then centrifuged at 10 min, 200 g at 4°C. The cells from each mouse were resuspended in 2 mL MCDM, layered over 4 mL of an isotonic 70% Percoll suspension and centrifuged for 20 min, 500 g at 4°C. Mast cells were collected from the pellet. The cells were the transferred to a new centrifuge tube and were diluted with MCDM in twice the volume of the suspensions. This solution was well mixed and then centrifuged at 10 min, 200 g, 4°C. Mast cells were collected in the pellet and their purity was >95%, as assayed by morphology. Mast cells were resuspended at 5×10^5 to 1×10^6 cells/mL in DMEM with 0.05% recombinant mouse stem cell factor (SCF) and seeded into 96 well plates.

Hindpaw swelling and extravasation

Adult male mice weighing 25–30 g were anaesthetized with an intraperitoneal injection of 0.2 mL 0.35% chloral hydrate. Fifteen min after the induction of anesthesia, each mouse was injected intravenously (i.v.) with 0.2 mL of 0.15% Evans blue in saline for 15 min. A vernier caliper was used to measure the thickness of the paw before any injection. Five min later, 5 μL of the test drug was administered by a microinjector in the left paw and saline was administered in the right paw as a negative control. Fifteen min later, hindpaw thickness was measured again and recorded. Mice were then killed by decapitation, and a photo of each paw was taken.

Paw tissue was collected, dried for 24 h at 50°C, and weighed. Evans blue was extracted by a 24 h incubation in 500 μL acetone-saline (7:3) at 37°C.

Tissues were cut into pieces, and placed for 10min in an ultrasonic machine, centrifuged for 20 min at 3000 rpm. The supernatant was equally distributed in 200 μL aliquots into 96 well cell culture plates, and the OD was read at 620 nm using a spectrophotometer. For studies using substances, mice were injected with 5 μL of a 30 μg/ml solution of compound 48/80 as a positive control.

Body temperature decrease

Adult male mice weighing 25–30g were anaesthetized with an intraperitoneal injection of 0.2 mL 0.35% chloral hydrate. And mice were injected intravenously (i.v.) with the drugs in saline (n = 5). Then the temperature transducers and BL-410 biological functional system (Chengdu Techman Software Co., Ltd., Chengdu, China) were used for analysis.

β-hexosaminidase assay

This assay was carried out in a 96-well plate. The cell concentration of each well was strictly counted for normalized cells. LAD2 cells which were kept at a density of 2×10^5 cells/well were incubated overnight at 37°C with 5% CO_2. Following incubation, the culture medium was removed and substances drug substances were added at the indicated concentrations and the cells were incubated for 30 min at 37°C with 5% CO_2. The 96-well plate was centrifuged. For analysis of total β-hexosaminidase content, the negative control cells were lysed with 0.1% Triton X-100 in TM buffer. The β-hexosaminidase released into the supernatants and in cell lysates was quantified by hydrolysis of p-nitrophenyl N-acetyl-β-D-glucosamide in 0.1 M citric acid/sodium citrate buffer (pH = 4.5) for 90 min at 37°C. The reaction was stopped by the addition of stop buffer. The percentage of β-hexosaminidase release was assessed by measuring samples at 405 nm using microplate spectrophotometer and calculated as follows: absorbance of culture supernatant at 405 nm × 100/absorbance of total cell lysate supernatant at 405 nm. 30 μg/mL compound 48/80 was used as positive control.

Histamine release assay

This assay was carried out in a 96-well plate. LAD2 cells which were kept at a density of 1×10^6 cells/well were incubated overnight at 37°C with 5% CO_2. Cell count method were applied for normalize each samples. Histamine (HA) was purchased from Sigma, Histamine•2HCl (A, A, B, B-D4, 98%) was obtained from Cambridge Isotope Laboratories, Inc. (MA, USA), HPLC-grade methanol and acetonitrile were purchased from Thermo Fisher Scientific (Pittsburgh, USA). Mass spectrometry grade formic acid was from Sigma. In the applied LC-ESI-MS/MS method, an LCMS 8040 mass spectrometer (Shimadzu Corporation, Kyoto, Japan) was used. Histamine was evaluated on the system employing

a HILIC column (Venusil HILIC, 2.1 mm × 150 mm, 3 μm, Agela Technologies, Tianjin, China), and an isocratic elution with acetonitrile–water containing 0.1% formic acid and 20 mM ammonium formate (77:23, v/v) at a flow rate of 0.3 mL/min.

Intracellular Ca^{2+} mobilization assay

All drug substances were diluted to the required concentration in calcium imaging buffer (CIB; NaCl 125 mM, KCl_3 mM, $CaCl_2$ 2.5 mM, $MgCl_2$ 0.6 mM, HEPES 10 mM, glucose 20, mM, $NaHCO_3$ 1.2 mM, sucrose 20 mM, brought to pH 7.4 with NaOH). The incubation buffer consisted of 0.8 μL Fluo-3, 3 μL Pluronic F-127 and 996.2 μL CIB. MRGPRX2-HEK293 cells or NC-HEK293 cells were plated at 1×10^4 cells per well in 96-well plates and washed twice in 100 μL CIB. Cells incubated for 30 min and washed twice again. The cells were used immediately for imaging. For calcium imaging, the cells were magnified 200 times and one photo per second was taken under the blue light. Cells were identified as responding if the $[Ca^{2+}]i$ rose by at least 50% after injected the substances.

For peritoneal mast cell response, mice peritoneal mast cells were plated at 1×10^4 cells per well in 96-well plates in DMEM with 0.05% recombinant mouse stem cell factor and seeded for 2 h. After the drugs were added into well, the cells fluorescence changes were read by fluorescence microscope following the method described in HEK293 cell calcium influx.

siRNA transfection of LAD2 cells

RNA interference. Specific knockdown was achieved using small interfering (si)RNAs targeting MRGPRX2 or a control siRNA. A smart pool of double–stranded siRNAs targeting MRGPRX2 as well as non–specific siRNAs were obtained from Shanghai GenePharma Co., Ltd. The siRNA sequences were as follows: Forward, 5'– GUACAACAGUGAAUGGAAATT –3′, and reverse, 5′–UUUCCAUUCACUGUUGUACTT–3′ for MRGPRX2; and forward, 5′–UUCUCCGAACGUGUCACGUTT–3′, and reverse, 5′–ACGUGACACGUUCGGAGAATT–3′ for the control. For transfection, siRNA was delivered at a final concentration of 80 nM using Lipofectamine® 2000 reagent according to the manufacturer's instructions. The cells were incubated for 48 h to allow knockdown of MRGPRX2. These cells were then used for the β-hexosaminidase assay and histamine release assay.

TNF-α release assay

LAD2 cells were incubated in a 96-well plate overnight at 37°C with 5% CO_2 and the cell density was 1×10^6 per well. The culture medium was removed and drug substance was added at the indicated concentrations and the cells were incubated for 12 h at 37°C with 5% CO_2. Human TNF-α Array Kit was from ExCell Biology, Inc. (Shanghai, China). All the steps were executed strictly according to the manufacturers' instruction.

EC_{50} assay

Human MRGPRX2-expressing HEK293 cells were incubated in a 96-well plate overnight at 37°C with 5% CO_2 and the cell density was 2×10^4 per well. FLIPR Calcium 5 Assay KIT was from Molecular Devices (Sunnyvale, California). And the EC_{50} Value was analysis by FlexStation 3.

Experiments repetition and statistical analysis

All the experiments in vitro and in vivo were repeated at last 3 times. Group data are expressed as mean ± S.E.M. Independent sample variance analysis was used to determine significance in statistical comparisons using SPSS.

Differences were considered significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

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Abbreviations: **MRGPRX2**: MAS-related G protein-coupled receptor X2 · **MrgprB2**: MAS-related G protein-coupled receptor B2 · **EC_{50}**: half-maximal effective concentration · **EBD**: Evans blue dye · **LAD2**: Laboratory of Allergic Disease 2

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