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ORIGINAL ARTICLE



MRGPRX2 is critical for clozapine induced pseudo-allergic reactions

Di Wei, Tian Hu, Ya-jing Hou, Xiang-jun Wang, Jia-yu Lu, Shuai Ge, Cheng Wang and Huai-zhen He

School of Pharmacy, Xi'an Jiaotong University, Xi'an, China

ABSTRACT

Background: Clozapine is one of the most widely used second-generation antipsychotics in clinic. However, allergy-like symptoms such as rash and angioedema have been reported frequently, and the mechanism is still not clear. Mas-related G protein-coupled receptor X2 (MRGPRX2) expressed on mast cells is a crucial receptor for drug induced pseudo-allergic reactions. Therefore, we explored whether the symptoms induced by clozapine were associated with allergic reaction through MRGPRX2.

Methods: The effects of clozapine on pseudo-allergic reactions were evaluated by mast cells degranulation and calcium mobilization assay *in vitro*, and mice hindpaw swelling, serum histamine detection, avidin and H&E staining assay *in vivo*. The overexpressed MRGPRX2 cells membrane chromatography (MRGPRX2-HEK293/CMC), MRGPRX2-HEK293 cells calcium mobilization assay and molecular docking were applied to research the correlation between clozapine and MRGPRX2.

Results: The study showed that clozapine induced the release of β -hexosaminidase, histamine and monocyte chemoattractant protein-1 (MCP-1), and triggered calcium mobilization in mast cells. *In vivo*, clozapine induced paw swelling, degranulation and vasodilation. Furthermore, clozapine could activate the calcium mobilization obviously in MRGPRX2-HEK293 cells, not in NC-HEK293 cells. Clozapine also had a good retention characteristic on MRGPRX2-HEK293/CMC column and the K_D value is $(2.33 \pm 0.21) \times 10^{-11}$ M.

Conclusions: Our findings demonstrated that clozapine could induce pseudo-allergic reactions and MRGPRX2 might be the critical receptor for it.

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Clozapine; pseudo-allergic reactions; MRGPRX2; calcium mobilization; degranulation

Introduction

Clozapine, a second-generation antipsychotic drug, exhibits strong antipsychotic and sedative effects by acting on dopamine receptors [1–3]. It is one of the most commonly used antipsychotic in clinic. However, several adverse reactions of clozapine have been reported, including extrapyramidal symptom, angioedema, rash, etc. [4–7]. Among them, rash and angioedema are cutaneous symptoms of allergy. Some patients experience these symptoms during the first use of clozapine. Although these symptoms are mild in most cases, the potential harm cannot be ignored. At present, the cause of allergy-like symptoms is still not clear.

Pseudo-allergic reaction is a non-immune-mediated allergic reaction. Different from the traditional allergic reactions, pseudo-allergic reactions can be induced directly by the antigen stimulating mast cells or basophils for the first time [8,9]. Activated mast cells or basophils could degranulation and release histamine [10–13]. The released histamine then activates the histamine H_1 receptor, which expressed on multiple cell types such as smooth muscle cells and endothelial cells, causing vasodilation and increased vascular permeability [14–16]. These changes might eventually lead to local skin redness, swelling, itching, and other symptoms [17]. Therefore, these cutaneous symptoms can be used as the land markers for allergic reactions. The human G-protein-

coupled receptor MRGPRX2 was confirmed as one of crucial receptors for the activation of mast cells in pseudo-allergic reaction [18]. Meanwhile, several researchers found that some small molecule drugs, such as morphine, sulfamethoxazole, mivacurium, could activate mast cells *via* MRGPRX2 in a dose-dependent manner [19,20]. The study on MRGPRX2 may provide a potential therapeutic target for drug-induced allergic reactions.

Since clozapine could cause such skin symptoms when used for the first time, we suspected that these effects might be pseudo-allergic reactions. In this study, we tried to explore whether the symptoms induced by clozapine are pseudo-allergic reactions and verified its correlation with MRGPRX2. Furthermore, the binding ability of clozapine to MRGPRX2 was also examined.

Materials and methods

Reagents

Compound 48/80 (C48/80) and Evans blue reagent were purchased from Sigma-Aldrich (St. Louis, MO). Clozapine ($\geq 98\%$) was purchased from Meilunbio Co., Ltd. (Dalian, China). Sinomenine ($\geq 98\%$) was obtained from Chengdu Pufei De BiotechCo., Ltd. (Chengdu, China). Fluo-3 AM and Pluronic F-127 were obtained from Biotium (Fremont, CA). Silica gel

(ZEX-II, 5 μ m, 100 Å) was purchased from Qingdao Meigao Chemical (Qingdao, China).

Cell culture

The Laboratory of Allergic Disease 2 (LAD2) human mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, Bethesda, MD) and were cultured in StemPro-34 medium supplemented with StemPro nutrient supplement, 100 U penicillin–streptomycin, 2 mmol/L L-glutamine and 100 ng/mL human stem cell factor in a 37 °C incubator with 5% CO₂. MRGPRX2-HEK293 cells (highly expressed MRGPRX2) were provided by Pro. Xinzhong Dong (Johns Hopkins University, Baltimore, MD), and cultured in DMEM medium supplemented with 10% FBS, 100 U penicillin–streptomycin.

Mouse model

C57BL/6 mice were purchased from the Experimental Animal Center of Xi'an Jiaotong University. The mast cell-deficient *W-sash c-kit* mutant Kit^{W-sh/W-sh} mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China). All mice were housed in the Experimental Animal Center of Xi'an Jiaotong University in individual cages with free access to water and food. The adult male mice weighed about 20 g were used in this experiment.

Ethics statement

The study was carried out strictly in accordance with the recommendations stated in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. The experimental protocols for the use of mice were approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2011-0045).

Cell viability assay

Cell viability was tested by Cell Counting Kit-8 (Monmouth Junction, NJ). LAD2 cells were seeded into a 96-well plate at 5×10^3 cells/well and incubated overnight at 37 °C. The different concentrations of clozapine were added to cells and incubated for 8 h. After that, 10 μ L of CCK-8 solution was added to each well followed by incubation for 4 h. Finally, the samples were tested under 450 nm by microplate reader. (Bio-Rad, Carlsbad, CA). Cell survival rate was calculated by the following formula: $[(OD_{\text{Treated}} - OD_{\text{Blank}})/(OD_{\text{Control}} - OD_{\text{Blank}})] \times 100\%$.

β -hexosaminidase release assay

LAD2 cells were seeded into 96-well plate at 2×10^5 cells/well and incubated overnight. After that, the culture medium was removed. The drug prepared by Tyrode's solution was added to each well and the cells were incubated for 30 min. Then, 96-well plate was centrifuged at 2000 g for 5 min to

get 50 μ L supernatant. To obtain the total β -hexosaminidase content, the cells in each well were lysed with 0.1% Triton X-100 and centrifuged again to obtain supernatant. Finally, the supernatant reacted with p-nitrophenyl *N*-acetyl- β -D-glucosamine in 0.1 M citric acid/sodium citrate (pH = 4.5) for 90 min at 37 °C. The reaction was halted by stop buffer (0.1 M sodium carbonate/sodium bicarbonate, pH 11.0), and the sample was measured at 405 nm using microplate spectrophotometer.

Histamine release assay

The histamine from mast cells was obtained as the method of β -hexosaminidase assay. Serum histamine was got from C57BL/6 mice administrated with clozapine. The detection method was according to previous studies [21]. In brief, histamine was separated with a HILIC column and detected by LC-ESI-MS/MS. d4-HA was used as an internal standard. Mobile phase consisted by an isocratic elution with mixed solution of acetonitrile-water added with 0.1% formic acid and 20 mM ammonium formate (82:18, v/v) at a flow rate of 0.3 ml/min.

MCP-1 release assay

LAD2 cells were seeded into 96-well plate at 2×10^5 cells/well and incubated overnight at 37 °C with 5% CO₂. After that, the culture medium was removed. The drug prepared by Tyrode's solution was added to each well and the cells were incubated for 6 h at 37 °C. Finally, 96-well plate was centrifuged at 2000 g for 5 min at 4 °C to get 100 μ L supernatant and then detected by MCP-1 ELISA kit.

Intracellular calcium mobilization assay

Cells were seeded into 96-well plate and incubated overnight. The cells were washed twice with calcium imaging buffer (CIB: 125 mM NaCl, 0.6 mM MgCl₂, 2.5 mM CaCl₂, 3 mM KCl, 10 mM HEPES(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 20 mM glucose, 1.2 mM NaHCO₃, 20 mM sucrose) followed by incubation for 30 min in incubation buffer (1 μ L Fluo-3, 3 μ L Pluronic F-127 and 996 μ L CIB). For imaging, the cells were washed twice again and added 100 μ L drug dissolved in CIB. Finally, pictures were taken at a rate of 2 s for 120 s.

Hindpaw swelling and extravasation assay

The adult mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). After anesthesia, the mice were injected intravenously with 200 μ L 0.4% Evans blue in saline and a Vernier caliper was used to measure the thickness of the left paw. Five minutes later, the drug (5 μ L) was administered by subcutaneous injection in left paw. In addition, 30 μ g/mL C48/80 was used as a positive control. Fifteen minutes later, the mice were euthanized by cervical dislocation and the thickness of the paw was measured again. Then, the paw tissue was collected, dried at 50 °C and

weighted. To extract Evans blue, tissue was soaked in 500 μ L acetone-saline (7:3) for 12 h and then cut into pieces. Fragmented tissue was ultrasound for 30 min followed by centrifugation at 9000 g for 20 min. Finally, 100 μ L supernatant were measured at 620 nm using a spectrophotometer.

Avidin and H&E staining assay

The skin of mice paw was collected after administration, and then fixed in 4% formaldehyde for 48 h. The fixed tissue was embed by paraffin and sliced. The tissue for avidin staining was processed as literature reported [22]. Tissue sections were incubated successively with primary and secondary antibodies. The photos were taken under fluorescence microscope. In addition, the tissue was stained with hematoxylin and eosin, respectively, for H&E staining. Then the sections were dehydrated through different concentrations of ethanol and xylene. Finally, the images were photographed under confocal scanning laser microscope.

MRGPRX2-HEK293/cell membrane chromatography

MRGPRX2-HEK293/cell membrane chromatography (MRGPRX2-HEK293/CMC) column was prepared according to previous literature [23]. In brief, the harvested MRGPRX2-HEK293 cells were washed with physiological saline and lysed by ultrasonic in ice bath for 30 min. After that, the supernatant was collected by centrifugation at 1000 g , 4°C for 10 min, and followed by centrifugation at 12,000 g to obtain cell membrane. Then cell membrane dissolved in physiological saline was mixed with silica (50 mg, activated at 105°C for 30 min). The mixture was stirred at 4°C for 30 min and left to stand overnight. Finally, MRGPRX2-HEK293 cell membrane stationary phase was packed into a CMC column (10 mm $\times$ 2.0 mm). Before injection, the CMC column was pre-equilibrated for 2 h. Then, clozapine was analyzed on MRGPRX2-HEK293/CMC column.

Competitive binding study

MRGPRX2-HEK293/CMC column was prepared as above. Competitive binding study was carried out by using sinomenine as site-specific probe. The concentrations of drug to be analyzed ranged from 2×10^{-8} to 1×10^{-6} M. Before injection of sinomenine, CMC column was balanced with corresponding concentration of clozapine. Next, clozapine solution was flowed through the column to eluate sinomenine. Processing of experimental data was according to previous literature [24].

Molecular docking

Molecular docking studies were conducted using the Sulflex-Dock Mode of Sybyl-X program package. The structure of MRGPRX2 complex with ZINC-3573 was according to the work of Bryan L Roth's group, who calculated and predicted the model of MRGPRX2 based on homologous Modeling [25]. Clozapine was constructed with Sybyl/Sketch module

and optimized by Powell's method. Then protein structure was optimized by adding hydrogens, repairing sidechains, etc. Finally, clozapine was docked with the protein in optimal energy mode referencing the docking mode of (R)-ZINC-3573 interaction with MRGPRX2.

Statistical analysis

Experiments in this study were repeated three times. Group data are expressed as the mean $\pm$ SD. Two-tailed tests were used for two-group comparisons. Differences were considered significant at * $p < .05$, ** $p < .01$, *** $p < .001$.

Results

Clozapine induced degranulation and calcium mobilization in mast cells

In this study, LAD2 cells were used as cell models. Firstly, we assessed the toxicity of clozapine on LAD2 cells. No obvious toxicity of clozapine on LAD2 cells was found up to 200 μ M (Figure 1(A)). Therefore, clozapine concentrations of 50 μ M, 100 μ M, and 200 μ M were used in the following studies.

Degranulation is an important marker for mast cells activation. Clozapine induced LAD2 cells to release β -hexosaminidase, histamine, and MCP-1 in a dose-dependent manner (Figure 1(B–D)). Compared with control group, clozapine hardly lead to degranulation at 50 μ M. However, clozapine at 100 μ M and 200 μ M could stimulate the degranulation process significantly. Calcium ions play a significant role in the activation of mast cells as an important second messenger. Here, we found that C48/80 and clozapine can both cause the increase of calcium ions concentrations in LAD2 cells, the calcium ions concentrations increased significantly with increased clozapine concentrations (Figure 1(E)). However, CIB, the negative control, could not induce calcium mobilization.

Clozapine induced mice hindpaw inflammation and degranulation

Clozapine has been verified to induce mast cells degranulation and calcium mobilization *in vitro*. Further, we investigated the related effects of clozapine *in vivo*. Compared with saline group, clozapine could lead a significant Evans blue exudation at 1.0 mg/mL, but no obvious increase of Evans blue exudation can be observed at 0.2 mg/mL and 0.5 mg/mL *in vivo*. Clozapine could also increase the paw thickness at 0.5 mg/mL and 1.0 mg/mL (Figure 2(A)). As shown in Figure 2(B), clozapine promoted the release of serum histamine in a dose-dependent manner. There is a statistical difference between clozapine group at 5 mg/kg and saline group. Avidin and H&E staining assay showed that clozapine stimulated the mast cells in the hindpaw skin of mice to release a large number of fluorescent particles and induced vasodilation at 1.0 mg/mL, compared with the control group (Figure 2(C, D)).

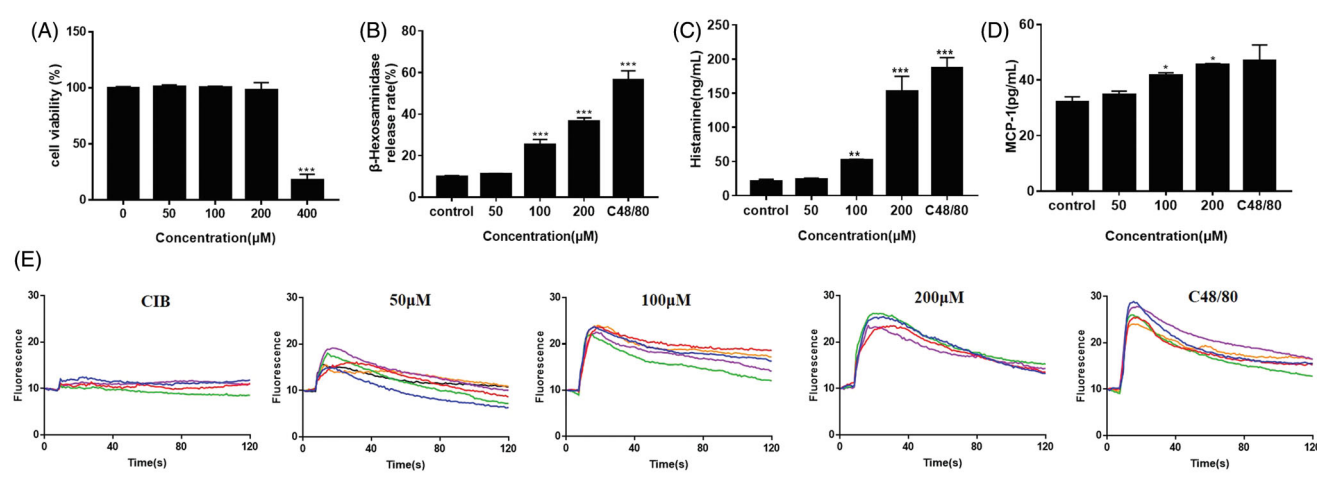


Figure 1. Clozapine induced degranulation and calcium mobilization in mast cells. (A) Clozapine showed no cytotoxicity at the concentrations below 200 μM on LAD2 cells. (B–D) Clozapine stimulated LAD2 cells to release β -hexosaminidase, histamine, and MCP-1. (E) Clozapine induced calcium mobilization in LAD2 cells. Each trace of color curve represents a cell's response. Data are expressed as mean $\pm$ SEM ($n = 3$). Statistical significance was accepted at $p < .05$ (* $p < .05$, ** $p < .01$, *** $p < .001$).

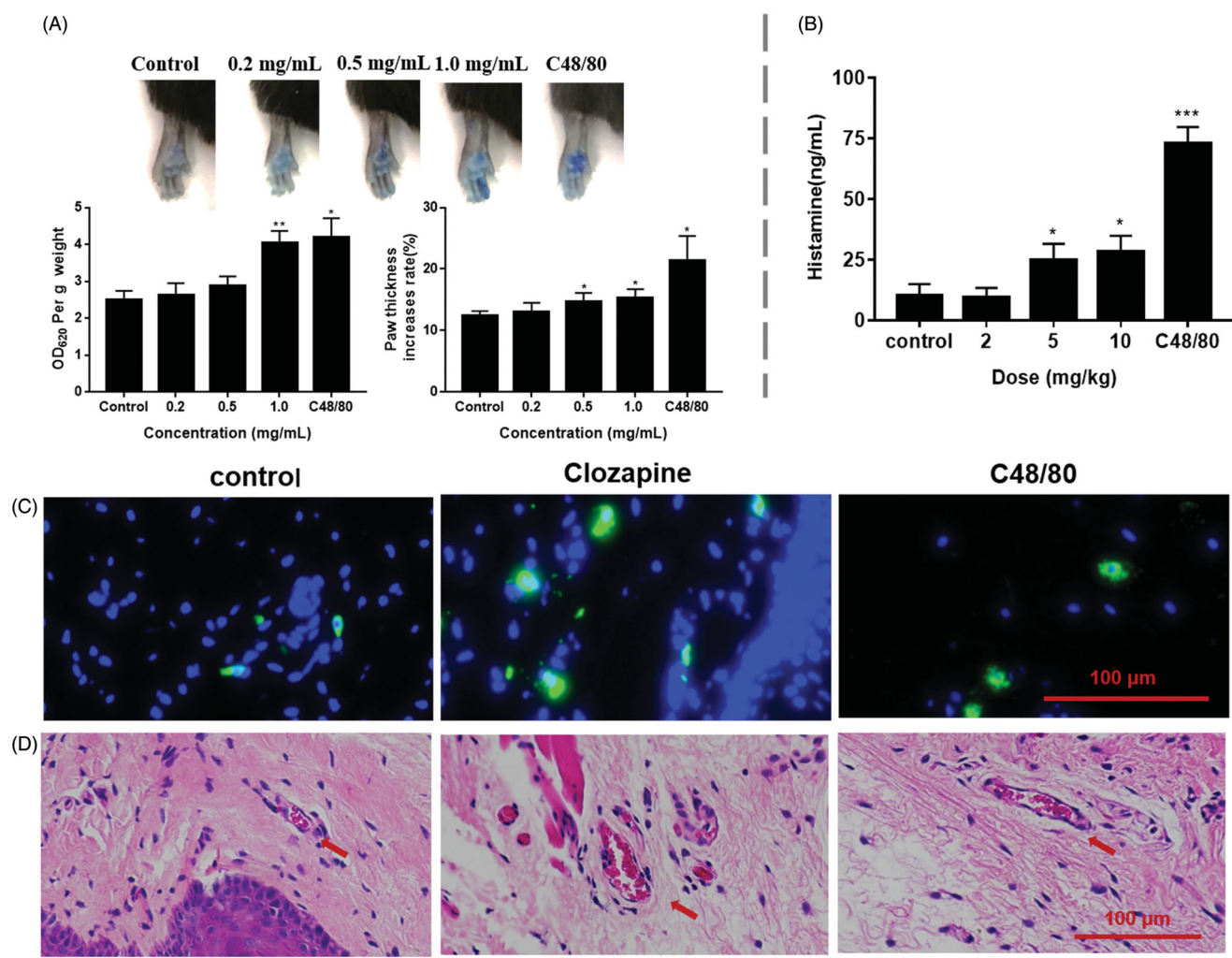


Figure 2. Clozapine induced mice hindpaw inflammation and degranulation. (A) Clozapine increased Evans blue extravasation and paw thickness. (B) Clozapine increased serum histamine concentration in mice. (C, D) Clozapine induced mast cells degranulation and vasodilation of mice skin at 1.0 mg/mL. C48/80 at 30 $\mu\text{g/mL}$ has been used for positive control. Data are expressed as mean $\pm$ SEM ($n = 5$). Statistical significance was accepted at $p < .05$ (* $p < .05$, ** $p < .01$, *** $p < .001$).

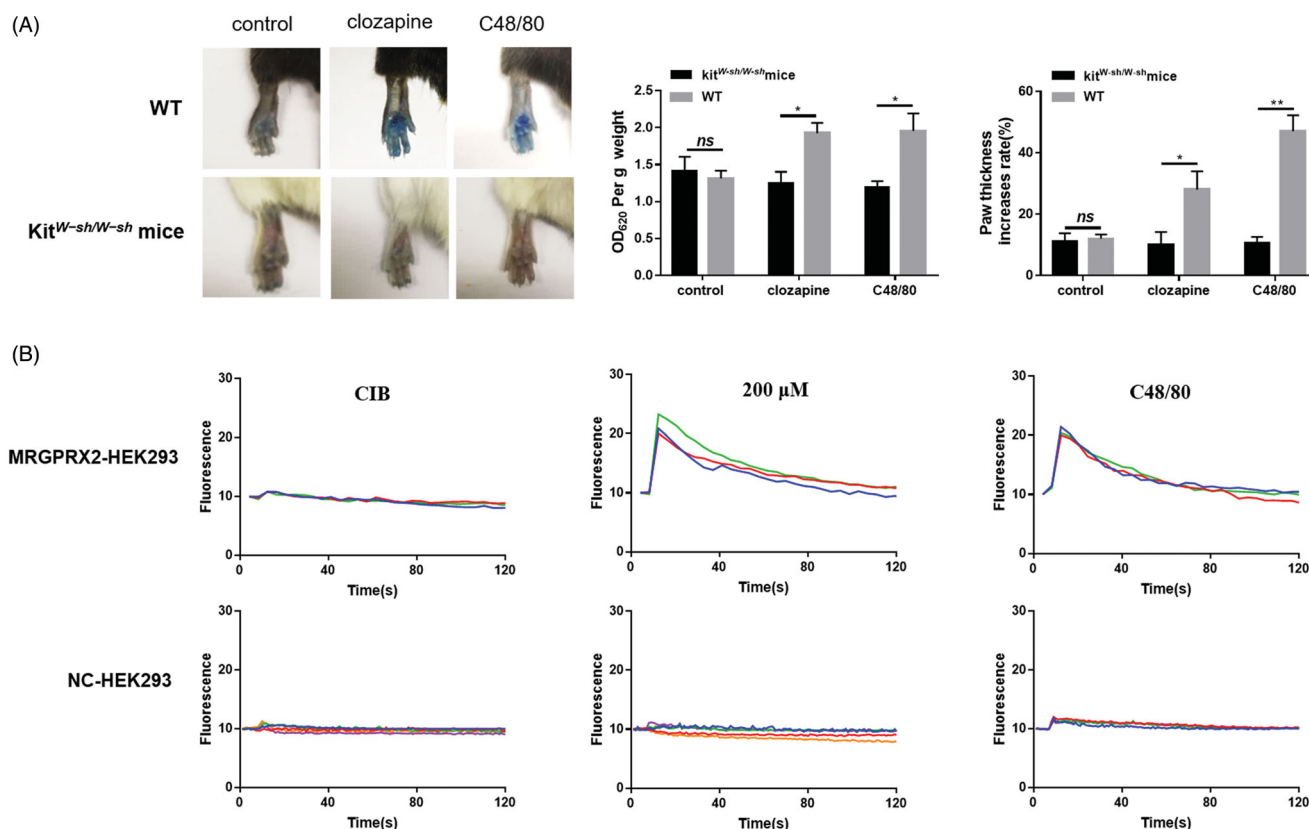


Figure 3. Clozapine increased Evans blue extravasation and paw swelling via mast cells, and activated calcium mobilization via MRGPRX2. (A) Clozapine increased Evans blue exudation and paw swelling at 1.0 mg/mL in WT mice, but not in Kit^{W-sh/W-sh} mice. (B) Clozapine activated calcium mobilization in MRGPRX2-HEK293, but failed in NC-HEK293. Data are expressed as mean $\pm$ SEM ($n = 5$). Statistical significance was accepted at $p < .05$ (* $p < .05$, ** $p < .01$).

Clozapine induced pseudo-allergic reactions via MRGPRX2

Mast cell-deficient *W-sash c-kit* mutant Kit^{W-sh/W-sh} mice were used for local pseudo-allergic reaction test. The results showed that clozapine induced the increase of the Evans blue extravasation and paw thickness at 1.0 mg/mL in wild type (WT) mice, but not in mast cell-deficient *W-sash c-kit* mutant Kit^{W-sh/W-sh} mice, as same as the C48/80 group (Figure 3(A)). In order to investigate the possible relevant receptor of clozapine in pseudo-allergic reactions, MRGPRX2-HEK293 and NC-HEK293 cells were applied separately in calcium imaging experiment. CIB did not cause the calcium fluctuation in both MRGPRX2-HEK293 and NC-HEK293 cells, which could eliminate the influence of solvent on the experiment. Clozapine activated the calcium mobilization obviously in MRGPRX2-HEK293 cells at 200 μ M, but not in NC-HEK293 cells, which was consistent with the positive control (C48/80) group (Figure 3(B)).

Cell membrane chromatography (CMC) is a bioaffinity chromatography, which can be used to determine the interaction between membrane receptors and small molecules [26]. In this study, the results showed that clozapine had a good retention characteristic on MRGPRX2-HEK293/CMC (Figure 4(A)). Further, we carried out the competition study to explore the interaction strength between clozapine and MRGPRX2. Sinomenine was used as a probe here to verify the competition between clozapine and sinomenine on binding MRGPRX2, which has been proved to interact with

MRGPRX2 [27]. As showed in Figure 4(B), a decrease k value was observed with the increase of clozapine concentrations. The fitting line for clozapine is $Y = 0.026X + 0.060$ ($R^2 = 0.9704$, $p < .01$) (Figure 4(C)). The dissociation equilibrium constant for clozapine was $(2.33 \pm 0.21) \times 10^{-6}$ M.

Molecular docking analysis

In order to explore the active binding site of clozapine and MRGPRX2, molecular docking was performed by Sybyl/Surflex-dock. (R)-ZINC-3573, a potent MRGPRX2 selective agonist, was used as a chemical probe for MRGPRX2 [25]. As shown in Figure 5(B), clozapine was well docked at the active pocket. The nitrogen atom in the piperazine structure of clozapine had the same binding position as the nitrogen atom in the nitrogen methyl group of (R)-ZINC-3573. Meanwhile, we found the docking position changed obviously, when the nitrogen atom in piperazine was replaced by a carbon atom, such as compound 1. We considered that the nitrogen atom might play an important role in the binding between clozapine and MRGPRX2.

Discussion

Pseudo-allergic reaction is an IgE-independent allergic reaction. MRGPRX2 expressed on mast cells had been verified to be a crucial receptor for drug-induced pseudo-allergic reactions. In this study, we tried to explore the pseudo-allergic

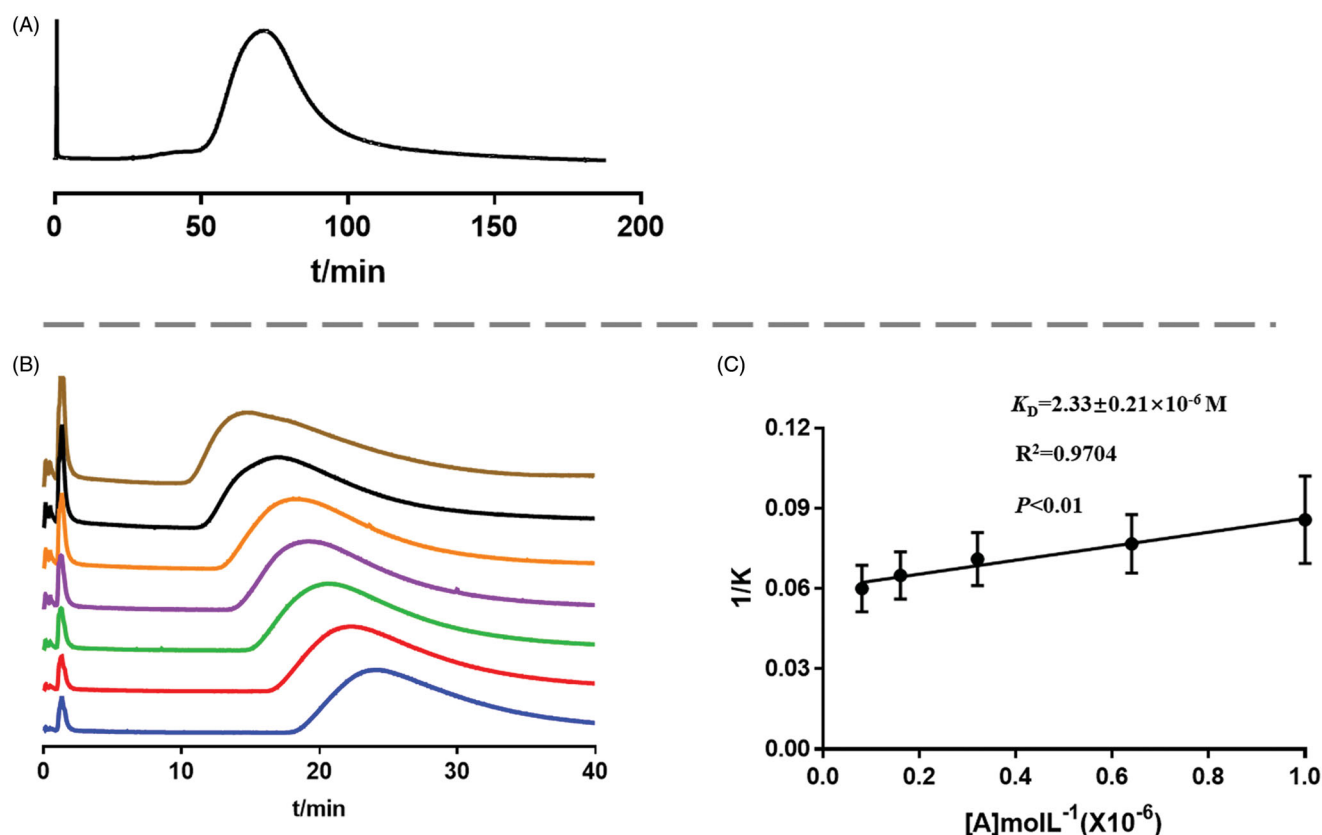


Figure 4. The interaction between clozapine and MRGPRX2. (A) The retention characteristic of clozapine on MRGPRX2-HEK293/CMC ($n = 3$). (B) Elution profiles of sinomenine on MRGPRX2-HEK293/CMC with different concentrations of clozapine (2×10^{-8} , 4×10^{-8} , 8×10^{-8} , 1.6×10^{-7} , 3.2×10^{-7} , 6.4×10^{-7} , and 1×10^{-6} M from bottom to up), ($n = 3$). Each trace of color curve represents an elution profile of sinomenine on MrgrprX2-HEK293/CMC at a certain concentration of clozapine. (C) Regression curves achieved by plotting $1/k$ versus $[A]$.

reaction induced by clozapine and the receptor it interacted with both *in vivo* and *in vitro*.

In pseudo-allergic reactions, mast cells can be directly stimulated by allergens, inducing degranulation and calcium mobilization. We found out that clozapine could promote the release of β -hexosaminidase, histamine and MCP-1 in mast cells, and increase the intracellular calcium ions concentrations. The results of calcium imaging experiment were consistent with the degranulation process, suggesting that increased calcium ions concentrations might promote degranulation. Clozapine was then explored to increase the Evans blue extravasation and paw thickness, and stimulate the release of serum histamine in mice. It was further found that clozapine induced mast cells degranulation and vasodilatation in handpaw skin. Acting as an inflammatory factor, histamine released from mast cells can directly interact with histamine receptor, leading to increased vasodilation and vascular permeability. So, the increased hindpaw inflammation may be related to the increased histamine levels in serum. Based on the above results, we concluded that clozapine could induce pseudo-allergic reactions.

Next, the receptor of clozapine induced pseudo-allergic reactions was explored. Compared with WT mice, clozapine could not increase the Evans blue extravasation and paw thickness in mast cell-deficient *W-sash c-kit* mutant $\text{Kit}^{W-sh/W-sh}$ mice, suggesting that clozapine might induce related effects by acting on mast cells. MRGPRX2 expressed on mast cells has been reported as a critical receptor in pseudo-

allergic reaction. Therefore, we tried to confirm that clozapine triggered pseudo-allergic reactions through MRGPRX2. According to the calcium imaging experiment performed on MRGPRX2-HEK293 and NC-HEK293 cells, we found clozapine could selectively induce intracellular calcium ions release in MRGPRX2-HEK293 cells, not in NC-HEK293 cells, which indicated that clozapine might activate mast cells through MRGPRX2. Cell membrane chromatography further showed that clozapine had a good affinity ability on MRGPRX2 and competition with sinomenine for the binding of MRGPRX2. According to the docking results, clozapine and ZINC-3573 possessed the same docking site with their *N*-methyl structure. However, when the *N*-methyl was replaced by C-methyl, the docking site changed obviously, which is consistent with the literatures. Therefore, we found that the nitrogen atom in the piperazine structure of clozapine might play a critical role in the interaction between clozapine and MRGPRX2. This needs further verification.

Further, we preliminarily assessed the possible clinical harm of clozapine by analyzing the relationship between the effective dose of clozapine-induced pseudo-allergic reactions and clinical dose. The clinical dosage of clozapine is ranged from 200 to 400 mg/day. With an oral bioavailability between 60% and 70% [28], the final dose into the blood circulation is about 2.00–4.67 mg/kg in patients. However, in this study, mice exhibited allergic symptoms after administration of 5 mg/kg clozapine, i.e. 0.55 mg/kg in human, which was lower than its clinical dosage. In fact, not everyone who

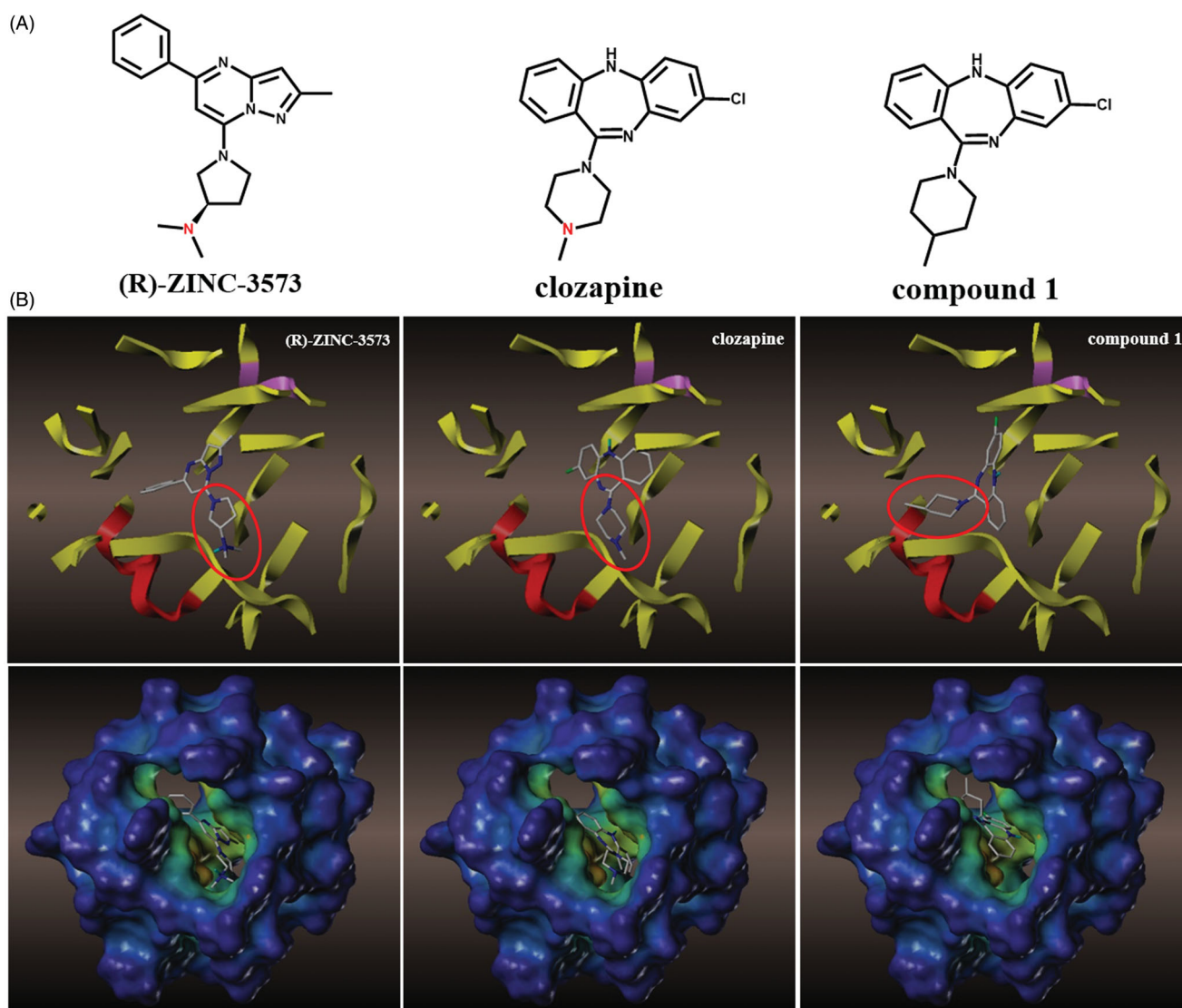


Figure 5. Molecular docking analysis between clozapine and MRGPRX2. (A) The chemical structures of docking compounds. (B) The possible binding mode of compounds with MRGPRX2.

used clozapine suffers from pseudo-allergic reactions. This may be related to the differences in MRGPRX2 expression between people, which finally lead to the differences in human sensitivity to clozapine [29]. In addition, patients with mental disorders are usually less sensitive to allergic symptoms and it is hard for them to name the symptoms, especially when the allergic symptoms are mild. This may be another reason for clinical neglect of these cases. Therefore, the clinical use of clozapine should be given more attention.

Conclusion

In this study, we found out that clozapine could induce pseudo-allergic reactions *via* MRGPRX2. The interaction strength between clozapine and MRGPRX2 was $(2.33 \pm 0.21) \times 10^{-6}$ M. Meanwhile, the nitrogen atom in the piperazine structure of clozapine might play a key role in the interaction. Besides, in view of the dosage of clozapine and the particularity of the patient, the clinical application of clozapine should attract much more attention.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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