Molecular Immunology 111 (2019) 118–127

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Phenothiazine antipsychotics exhibit dual properties in pseudo-allergic reactions: Activating MRGPRX2 and inhibiting the H_1 receptor

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ARTICLE INFO

Keywords:
Phenothiazine antipsychotics
Pseudo-allergic reactions
MRGPRX2
H_1 receptor antagonist
Mast cell

ABSTRACT

Phenothiazines are a class of antipsychotics that share the same tricyclic structure and are widely used in clinical settings. Adverse reactions from these drugs, however, have been regularly reported, with allergic skin reactions noted in some cases. Nevertheless, the mechanisms underlying anaphylaxis by these drugs have not been described. In the present study, we found that phenothiazine antipsychotics increased calcium mobilization and activated mast cells to release β-hexosaminidase, histamine, and tumor necrosis factor-α via Mas-related G-protein-coupled receptor member X2 (MRGPRX2) *in vitro*. In addition, they induced histamine release in serum *via* Mrgprb2 in C57BL/6 mice without Evans blue extravasation or paw swell. Further experiments indicated these drugs had good interaction with the histamine H_1 receptor (H1R) and show an anti-calcium mobilization effect on H_1R-HEK293 cells, which confirmed a potential antagonist effect of these drugs on the H_1R. The molecular docking and activity experiments indicated that the N-methyl substitution on the side chain of these drugs played a significant role in activating MRGPRX2, while the phenothiazine tricyclic ring was associated with the inhibiting effect on the H_1R. Therefore, due to their dual properties of increasing histamine levels without obvious allergic symptoms, clinicians should be highly vigilant for damage from histamine accumulation and long-term inflammatory reactions during the clinical use of phenothiazine antipsychotics.

1. Introduction

Phenothiazine antipsychotics refer to a group of drugs that share the tricyclic structure with phenothiazine, but with different side chains, and are divided into three classes: aliphatic, piperidine, and piperazine (Meltzer, 2013). As dopamine receptor antagonists, they have been widely used in clinical settings as tranquilizers and antipsychotics since the 1950s (Daniel, 2003; Takeuchi et al., 2012; Motohashi et al., 2000). Moreover, they were reported to block histamine, acetylcholine, and serotonin receptors in receptor-binding assays (Miyamoto et al., 2008). Despite their therapeutic benefits and long history of clinical use, adverse reactions, primarily nervous system damage (Barnes and Mcphillips, 1996; Güzey et al., 2007; Raja, 1998), hepatotoxicity (Selim and Neil, 1999; Ishak and Irey, 1972), and gastrointestinal stimulation (Nurnberg and Greenwald, 1981), have also been regularly reported. Allergic skin reactions, such as urticaria, erythema multiforme, and exfoliative dermatitis, have also been noted in some cases (Boyer, 1978; Hollister, 1957). These allergic symptoms often occur after intravenous injection of phenothiazine antipsychotics and appear quickly on first-time use. The allergic reactions caused by phenothiazine antipsychotics not only lead to significant discomfort in patients, but also increase the risks associated with further clinical use.

Unlike the immunoglobulin E-dependent anaphylactic reaction mediated by FcεRI (Ishizaka and Ishizaka, 1984; Wiecek et al., 2016; Coleman et al., 1994), pseudo-allergic reactions occur in first-time users and in a significantly dose-dependent manner (Pichler and Hausmann, 2016; Demoly et al., 2014). This is referred to as non-immune-mediated allergic reaction with direct stimulation of mast cells or basophils (Meng et al., 2016; Luskin and Luskin, 1996; Ring and Behrendt, 1999), leading to mast cell degranulation and histamine release. In allergic reaction, histamine exerts its effects through the activation of the H_1 receptor (H_1R), which is expressed in various human tissues, including the airway and intestinal and vascular smooth muscle, and can induce many types of allergic symptoms (Jones and Kearns, 2011). Cutaneous

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https://doi.org/10.1016/j.molimm.2019.04.008
Received 13 February 2019; Received in revised form 10 April 2019; Accepted 22 April 2019
Available online 30 April 2019
0161-5890/

anaphylaxis is a typical symptom that occurs after the activation of H_1R, resulting in skin irritation, itching, and other allergic symptoms (Lieberman and Garvey, 2016). Thus, histamine and H_1R play an important role in pseudo-allergic reactions. H_1R antagonists are used frequently in clinic to prevent the development of allergic diseases. Pseudo-allergic reactions are transient and immediate-type. Histamine is a primary medium which can induce pseudo-allergic reactions. In most cases, drugs-induced pseudo-allergic reactions are reversible after discontinuation of the drugs which may cause anaphylaxis. To some extent, H_1R antagonists have obvious and rapid therapeutic effects on pseudo-allergic reactions.

Mas-related G-protein-coupled receptor member X2 (MRGPRX2) was recently confirmed to be crucial for direct activation of mast cells and drug-induced pseudo-allergic reactions. Many types of drugs, including muscle relaxants (Mcneil et al., 2015), quinolones (Zhang et al., 2017) and opioid receptor analgesics (Liu et al., 2017; Lansu et al., 2017), have been reported to cause acute pseudo-allergic reaction mediated by MRGPRX2. This finding filled an important knowledge gap in research investigating drugs that induce pseudo-allergic reactions (Mcneil et al., 2015).

In this study, chlorpromazine (CPZ), thioridazine (TDZ), and trifluoperazine (TFZ) were selected as representative drugs of the three different structural types of phenothiazine antipsychotics. *in vivo* and *in vitro* experiments were performed to explore the mechanisms underlying pseudo-allergic reactions by these drugs.

2. Material and method

2.1. Drugs and reagents

Drug substances: chlorpromazine (CPZ), trifluoperazine (TFZ), thioridazine (TDZ) promethazine and ciprofloxacin were from Meilunbio Co., Ltd (Dalian, China) and purified to ≥98%. Compound 48/80 (C48/80) and histamine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluo-3, AM ester and Pluronic F-127 were from Biotium (California, USA). Tyrode´s solution was prepared fresh on the day of use (6.954 g/l NaCl, 0.353 g/l KCl, 0.282 g/l CaCl2, 0.143 g/l MgSO4, 0.162 g/l KH2PO4, 2.383 g/l HEPES, 0.991 g/l glucose, and 1 g/l BSA, pH 7). 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, pH = 7.4) was prepared before the experiments. The p-nitrophenyl N-acetyl-β-D-glucosamide and Triton X-100 were from Sigma Aldrich Co., LLC. (Shanghai, China) and prepared to the proper concentration before used. Stop buffer was made up of 0.1 M sodium carbonate: 0.1 M sodium bicarbonate = 9:1. All aqueous solutions were prepared using ultrapure water produced by MK-459 Millipore Milli-Q Plus ultra-pure water system.

2.2. Mice model

C57/BL6 mice were purchased from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China). Adult male mice weighing 18–22 g were included in the study. Mast cell deficient $kit^{W-sh/W-sh}$ mice on C57BL/6 background and $H_1R^{-/-}$ mice on C57BL/6 background were purchased by Model Animal Research Center of Nanjing University (Nanjing, China). $Mrgprb2^{-/-}$ mice on C57BL/6 background were provided as a kind gift from the Xinzhong Dong Laboratory in Johns Hopkins University. 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 fed a standard dry food twice a day. The breeding environment was 20˜25℃, with a relative humidity of 40% and a day-night cycle of 12/12 h. The mice were randomly divided into the following groups (n = 5): control, compound 48/80, and the experimental group. All experiments involving equal treatments in animals were conducted by experimenters blind to the conditions.

2.3. 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.

2.4. Cell lines

The Laboratory of Allergic Disease 2 (LAD2) human mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, USA). Cells were cultured 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 week 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, USA). H_1R-expressing HEK293 cells were built by the preliminary work of our group. Cells were cultured in DMEM medium supplemented with 10% FCS, 100 U of penicillin and streptomycin.

2.5. EC_{50} assay

Human MRGPRX2-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 purchased from Molecular Devices, LLC. (Sunnyvale, California, USA) and all the steps were finished strictly following the instructions. The EC_{50} value was analysis by FlexStation 3 (Molecular Devices, LLC. Sunnyvale, California, USA).

2.6. β-hexosaminidase assay

This assay was carried out in a 96-well plate. LAD2 cells which were kept at a density of 2×10^5 cells/well and incubated overnight at 37℃ with 5% CO_2. The cell concentration in each well was rigorously counted. And all the drugs were preparation by Tyrode's solution. 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℃ with 5% CO_2. The 96-well plate was then centrifuged at 2000 *g* for 5 min at 4 °C. In order to analyze the total β-hexosaminidase content, the cells in each well 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℃. The reaction was stopped by the addition of stop buffer and measured at 405 nm using microplate spectrophotometer

2.7. Histamine release assay

Histamine 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 (Waltham, MA, USA). Grade for mass spectrometry 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 mixed solution of acetonitrile–water added with 0.1% formic acid and 20 mM ammonium formate (77:23, v/v) at a flow rate of 0.3 ml/min.

2.8. 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 6 h at 37 °C with 5% CO_2. Human TNF-α Array Kit was purchased from Excell BIOTECH (BEIJING) CO., LTD. (Beijing, China). All the steps were finished strictly according to the instruction.

2.9. siRNA transfection of LAD2 cells

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'- GUACAACAGUGAAUGGAA ATT-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.

2.10. Intracellular Ca^{2+} mobilization assay

All drug substances were diluted to the required concentration in calcium imaging buffer (CIB). The incubation buffer consisted of 0.8 μL Fluo-3, 3 μL Pluronic F-127 and 996.2 μL CIB. MRGPRX2-HEK293 cells or H_1R-HEK293 cells were plated at 1×10^4 cells per well in a 96-well plate and incubated overnight at 37 °C with 5% CO_2 and washed twice in CIB. Cells were incubated in incubation buffer for 30 min and washed twice more. 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.

2.11. Hind paw swelling and extravasation

Adult C57/BL6 male mice weighing 18–22 g were anaesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Fifteen minutes after the induction of anesthesia, each mice was injected intravenously with 0.2 ml of 0.15% Evans blue in saline. A vernier caliper was used to measure the thickness of the paw before any injection. Five minutes 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. 30 μg/mL C48/80 was used as a positive control. Fifteen minutes later, paw thickness was measured again and recorded. Mice were then sacrificed by decapitation, and a photo of each paw was taken. Paw tissue was collected, dried for 24 h at 50℃, and weighed. Tissues were incubation in 500 μL acetone-saline (7:3) at 37℃ for 12 h and then cut into pieces for 10 min in an ultrasonic machine followed by centrifuged for 20 min at 9000 rpm. The supernatant was equally distributed in 150 μL aliquots into 96 well cell culture plates, and the OD was read at 620 nm using a spectrophotometer for detecting Evans blue content.

2.12. H_1R-HEK293/ cell membrane chromatography (H_1R-HEK293/ CMC)

H_1R-HEK293/CMC columns was prepared using H_1R-HEK293 cells. According to the published procedure (Hou et al., 2009), the CMC assay was performed via LC-20 A high-performance liquid chromatography. The CMC column was pre-equilibrated for 1 h before sample injection. Then, phenothiazine antipsychotics were analysed with the CMC columns, respectively.

2.13. Surface plasmon resonance (SPR)

For the analysis of SPR, H_1 protein (50 μg/mL) was fixed on the COOH sensor chip by capture-coupling, the interaction of H_1R with the small molecules fixed was detected by OpenSPRTM (Nicoya Lifesciences, Waterloo, Canada) at 25 °C. The binding time and dissociation time were both 250 s, the flow rate was 20 μl/s. A one to one diffusion corrected model was fitted to the wavelength shifts corresponding to the varied drug concentration. The data was retrieved and analyzed with TraceDrawer software.

2.14. Molecular docking

Molecular docking studies were conducted using the Sulflex-Dock Mode of Sybyl-X program package (New Tripos International, St. Louis, USA). The docking model of MRGPRX2 is according to the work of Bryan L Roth's group, who calculated and predicted the model of MRGPRX2 using homology model construction method (Lansu et al., 2017). The docking model of H_1R is download from PDB.Bank, and the PDB code is 3RZE (Shimamura et al., 2011).

2.15. Synthesis of CPZ-1

2-Chlorophenothiazine (1.30 g) was dissolved in methylbenzene (30 ml), and NaOH (0.93 g) was added. The reaction mixture was stirred at 120 °C, N-(3-Chloropropyl) morpholine (2.00 g) was dripped in slowly, and then reflux for 8 h. After cooling, the reaction mixture was washed by water(2×50 ml)and saturated NaCl(2×50 ml)successively, dried over Na_2SO_4, and purified by column chromatography after concentrated. Finally, the product was verified by HPLC and mass. The HPLC method was using a C18 column (SunFire C18, 2.1 mm × 150 mm, 3.5 μm,), and an isocratic elution with methanol and water (80:20, v/v) at a flow rate of 0.3 mL/min. The optimal mass spectrometer parameters were: N_2 nebulizing gas, 1.5 L/min, and drying gas, 109 kPa, Temperature of desolvation line and heat block set at 200℃, CID gas was Ar, voltage of the interface and detector set at 4.5 and 1.57 kV, respectively. All gases had purity > 99.999%. The mass range was set at m/z 100–600 in positive and negative ionization scan mode.

2.16. Experiments repetition and statistical analysis

All the experiments in vitro and in vivo were repeated at least 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 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Results

3.1. Phenothiazine antipsychotics activate calcium mobilization and induce degranulation in mast cells

In this study, LAD2 cells were used as a model to detect whether phenothiazine antipsychotics induced mast cell calcium mobilization and degranulation. Calcium imaging experiments revealed that these drugs increased intracellular calcium ion (Ca^{2+}) concentration in LAD2 cells, while the incubation buffer CIB did not (Fig. 1A). Further experiments indicated that they also activated LAD2 cells to release β-hexosaminidase and histamine in a dose-dependent manner; among these phenothiazine antipsychotics, CPZ caused the greatest degree of degranulation at the lowest concentration (Fig. 1B, C).

Tumor necrosis factor-alpha (TNF-α) is a key cytokine released by mast cells in degranulation and is involved in systemic inflammatory

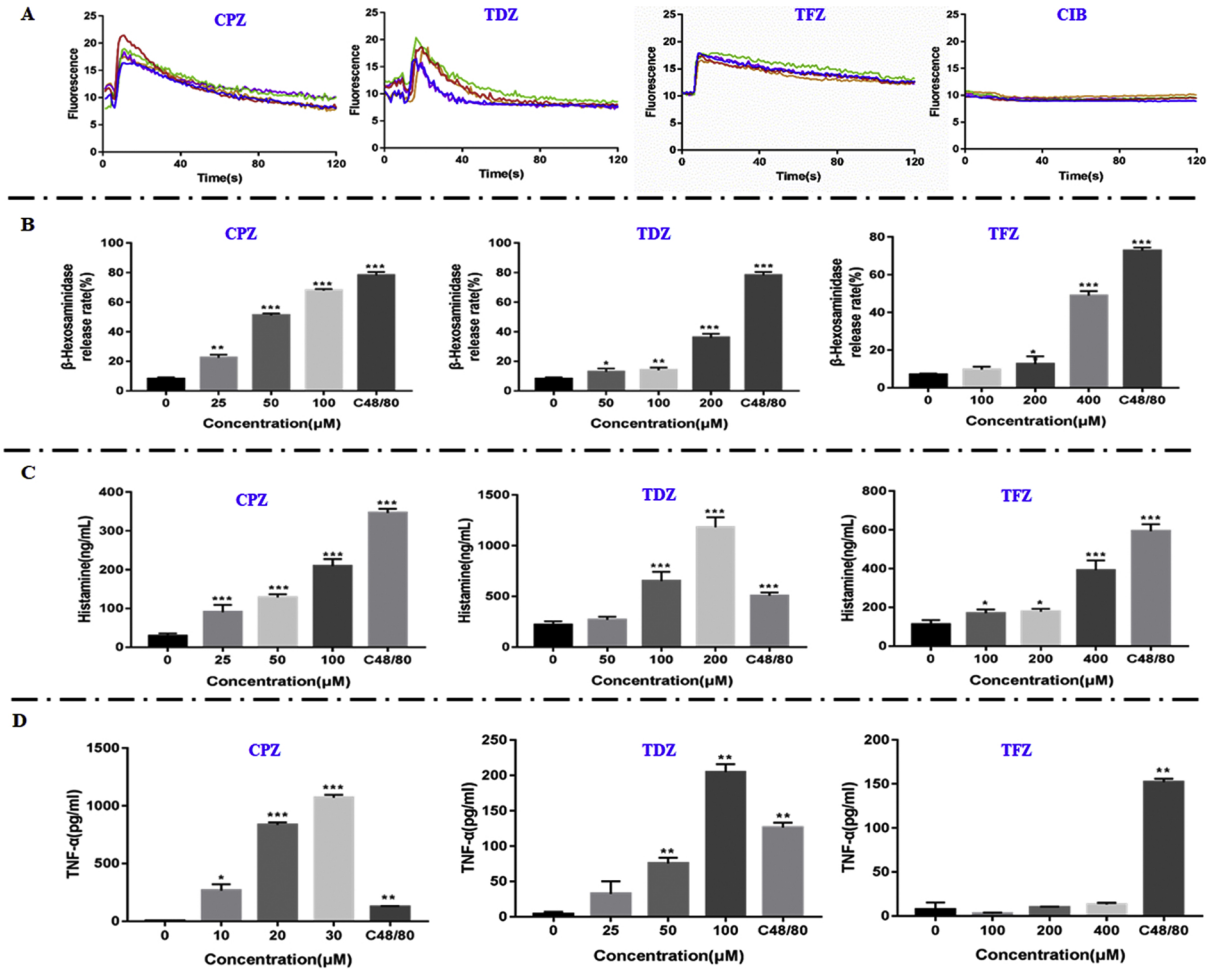

Fig. 1. Phenothiazine antipsychotics activated calcium mobilization and induced degranulation in a dose-dependent manner in mast cells. **(A)** Phenothiazine antipsychotics induce calcium mobilization in LAD2 cells. (**B**) β-hexosaminidase release of LAD2 cells induced by phenothiazine antipsychotics. (**C**) Histamine release of LAD2 cells induced by phenothiazine antipsychotics. **(D)** TNF-α release of LAD2 cells induced by phenothiazine antipsychotics. 10 μg/mL C48/80 was used for a positive control. Each trace of the colored line was a response to a unique cell. 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 statistical significance was accepted at $p < 0.05$(*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

responses (Leonardi, 2002). This study measured TNF-α release by LAD2 cells; results revealed that CPZ and TDZ induced TNF-α release while TFZ did not. CPZ, however, caused the most dramatic increase in TNF-α when used at a concentration of 30 μM, with an increase to 1070.75 ± 17.36 pg/ml (Fig. 1D).

3.2. Phenothiazine antipsychotics increase serum histamine via Mrgprb2 without Evans blue extravasation or paw swell

As a key mediator secreted by mast cells, histamine plays an important role in allergic reactions (Lieberman and Garvey, 2016). Therefore, serum histamine levels in C57/BL6 mice treated with phenothiazines were analyzed in vivo. The results revealed that serum histamine levels increased significantly in a dose-dependent manner after tail vein injection with phenothiazine antipsychotics (Fig. 2A). To further investigate whether the serum histamine concentration increased due to phenothiazine antipsychotics was related to mast cells and Mrgprb2, which is a receptor homologous to human MRGPRX2 (Mcneil et al., 2015), serum histamine levels in wild-type, $kit^{W-sh/W-sh}$, and $Mrgprb2^{-/-}$ (MUT) mice were analyzed for comparison. It was clear that the histamine concentration in $kit^{W-sh/W-sh}$ mice and $Mrgprb2^{-/-}$ (MUT) mice were only slight different, while wild-type mice exhibited significantly increased levels (Fig. 2B). Then, to explore phenothiazine-induced local anaphylaxis, a mouse model of passive cutaneous anaphylaxis (PCA) was established (Mcneil et al., 2015). The results were surprising in that these drugs induced little Evans blue extravasation and paw swell (Fig. 2C). These results indicated that phenothiazine antipsychotics activated mast cells via Mrgprb2 to release histamine, but the increased histamine didn't increase Evans blue extravasation or paw swell.

3.3. Phenothiazine antipsychotics could interact with H1R and exhibit as antagonists

It is reported that phenothiazine antipsychotics also can act on the histamine receptor (Miyamoto et al., 2008). In order to investigate the interaction of these drugs with the H_1R, H_1R-HEK293/CMC was conducted. The results showed that all of them could retain on the H_1R-HEK293/CMC column, and the retention time of TDZ was the longest, which indicated a better binding with H_1R (Fig. 3A). To further confirm whether they can bind H_1R, SPR assay was conducted. The results showed that they all could bind the H_1 protein and the KD values for CPZ, TDZ, and TFZ calculated by TraceDrawer™ were 3.44 ± 0.23e-8 mol/L, 2.52 ± 0.16e-8 mol/L and 5.87 ± 0.28e-8 mol/L (Fig. 3B). The obtained data from two methods indicated there was good interaction between these drugs and H_1 protein. Next, H_1R-HEK293 cells were incubated with 25 μM phenothiazines for 30 min and then treated with histamine to active calcium mobilization. Promethazine, a widely used H_1R antagonist, was

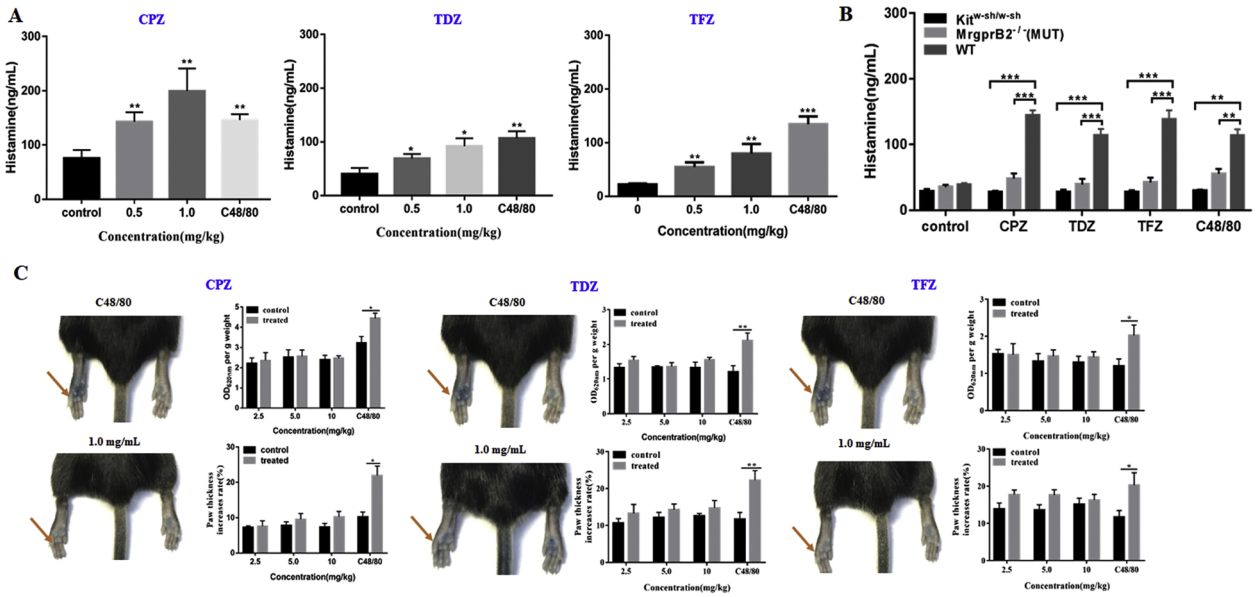

Fig. 2. Phenothiazine antipsychotics increase serum histamine via Mrgprb2 without Evans blue extravasation or paw swell. 30 μg/mL C48/80 was used as a positive control. **(A)** Histamine content in the serum of wide-type mice treated with different concentration of CPZ, TDZ, TFZ. **(B)** Histamine content in the serum of wide-type mice, $kit^{W-sh/W-sh}$ mice and $Mrgprb2^{-/-}$ (MUT) mice (n = 5) treated with 1.0 mg/kg CPZ, TDZ and TFZ. **(C)** Evans blue extravasation in the paw of wide-type mice treated with different concentration of CPZ, TDZ and TFZ. Left, representative images showing Evans blue dye extravasation after intraplantar injection of 5 μL different concentrations of phenothiazines (left) or saline (right). Right, quantification of Evans blue leakage into the paw and paw thickness increase after 15 min. Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < 0.05$(*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) *(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).*

used as a positive control. The results revealed that both of promethazine and these drugs demonstrated significant inhibition (Fig. 3C). All of these results revealed that phenothiazine antipsychotics could interact with H_1R to exhibit as antagonists.

3.4. MRGPRX2 is essential for the degranulation of mast cells triggered by phenothiazine antipsychotics

MRGPRX2-transfected HEK293 cells and NC-HEK293 cells were

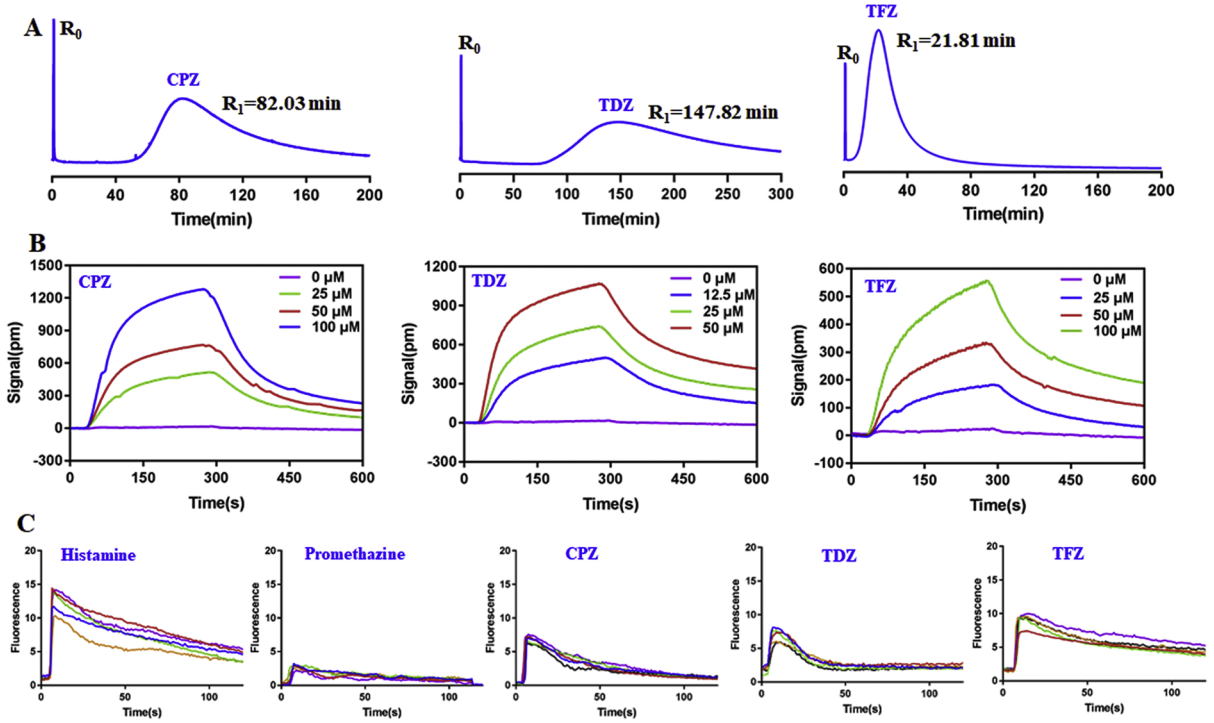

Fig. 3. Phenothiazine antipsychotics could interact with H_1R and exhibit as antagonists. **(A)** Chromatogram of Phenothiazine antipsychotics on the H_1R-HEK293/CMC model. **(B)** The binding curves of Phenothiazine antipsychotics on the H_1 – COOH sensor chip by SPR **(C)** Representative imaging traces and the quantification of H_1R-HEK293 cells showing changes in $[Ca^{2+}]i$ induced by histamine. Each trace of the colored line represented a response from a unique cell. Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < 0.05$(*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

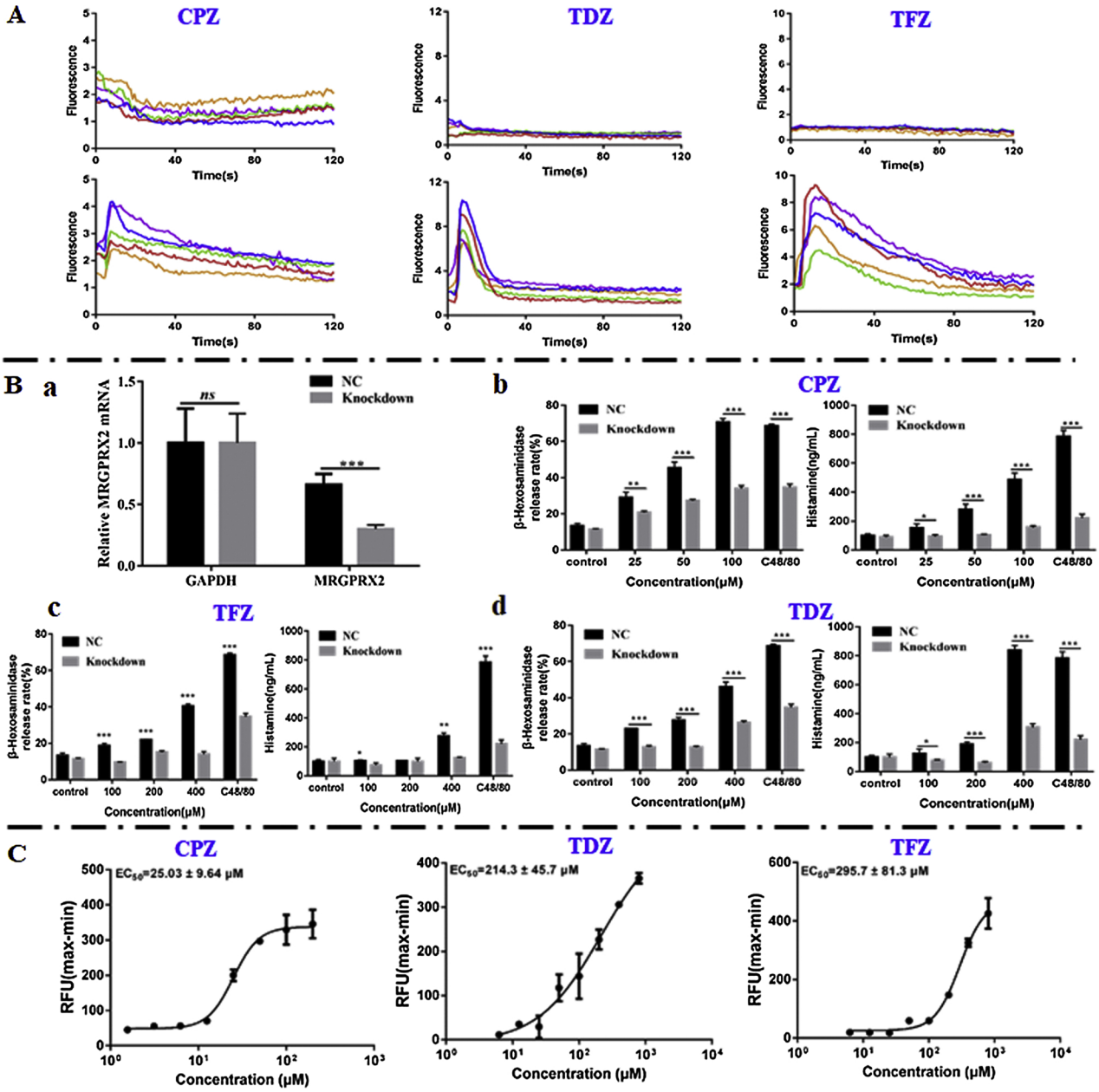

Fig. 4. MRGPRX2 is essential for the degranulation of mast cells triggered by phenothiazine antipsychotics. **(A)** Phenothiazine antipsychotics induced calcium mobilization in MRGPRX2-HEK293 cells (down) while NC-HEK293 cells (up) didn't. **(B)** The comparison of CPZ(b), TDZ(c), TFZ(d) induced β-hexosaminidase release and histamine release between NC-LAD2 cells and MRGPRX2 knockdown LAD2 cells. a: The knockdown efficiency of MRGPRX2 in LAD2 cells. **(C)** EC_{50} of Phenothiazine antipsychotics in MRGPRX2-HEK293 cells. Each trace of the colored line was a response from a unique cell. Data are presented as mean ± S.E.M and are representative of three independent experiment with three samples per experiment. Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < 0.05$(*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

used to confirm that calcium mobilization of mast cells induced by phenothiazines was via MRGPRX2. Results demonstrated that they all increased intracellular Ca^{2+} concentration in MRGPRX2-HEK293 cells, but did not alter that in NC-HEK293 cells (Fig. 4A). On the other hand, MRGPRX2 in LAD2 cells was downregulated using small interfering RNA (siRNA) technology, while non-functional siRNAs were transfected into LAD2 cells as a control. As expected, β-hexosaminidase and histamine released by MRGPRX2-knockdown LAD2 cells were significantly lower than NC-LAD2 cells (Fig. 4B). Further experiments indicated that the EC_{50} values of CPZ, TFZ and TDZ on MRGPRX2 were 25.03 ± 9.64 μM, 295.7 ± 81.3 μM and 214.3 ± 45.7 μM, respectively (Fig. 4C). CPZ had the lowest EC_{50} among these phenothiazine antipsychotics, which indicated more sensitive binding to MRGPRX2.

3.5. Dual properties of phenothiazine antipsychotics in pseudo-allergic reactions rely on structural specificity

To explain the dual properties of phenothiazines in pseudo-allergic reactions, chemical structure was considered. ZINC-9232 and promethazine were used as the positive control in binding to MRGPRX2 and H_1R, respectively (Lansu et al., 2017). As shown in Figs. 5A and 6A, these phenothiazines all possess an N-methyl scaffold similar to the structure of ZINC-9232 (Fig. 5A), and the same phenothiazine ring as promethazine (Fig. 6A). The docking results demonstrated that the three phenothiazines all fit the binding site of MRGPRX2 well, and the N-methyl moiety in the side chain of phenothiazines exhibit virtually the same spatial stretch direction as ZINC-9232 (Fig. 5B). However, the

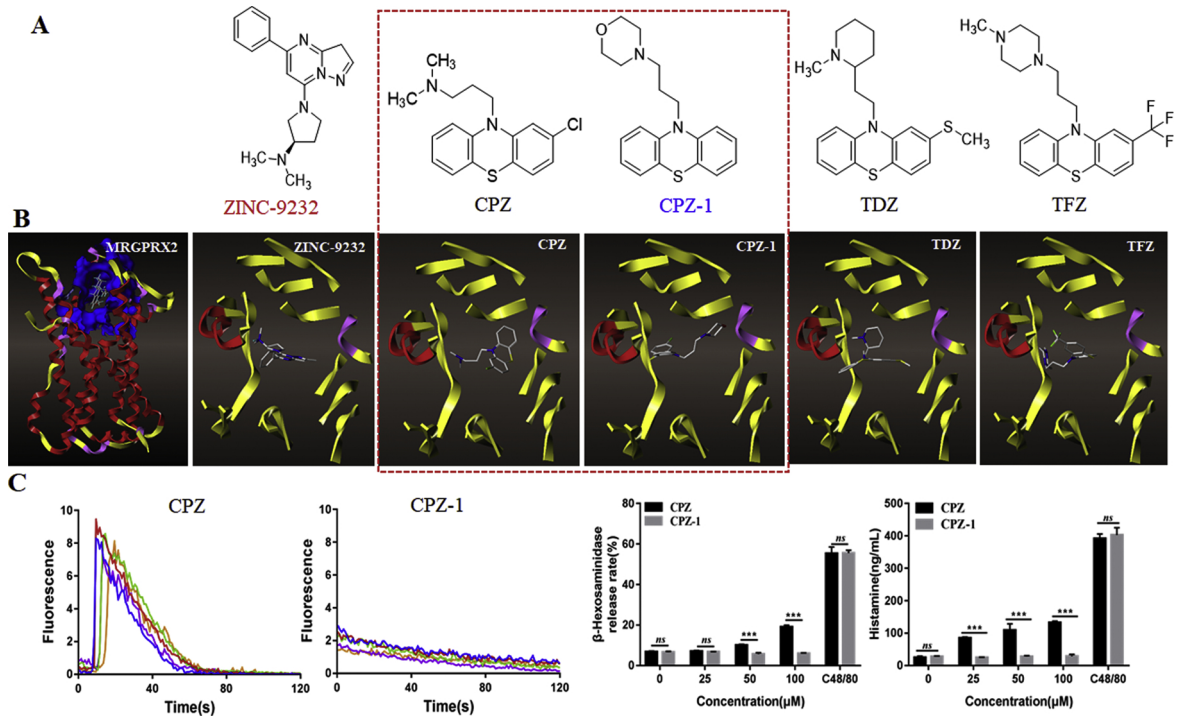

Fig. 5. N-methyl substituted of the side chain in phenothiazine antipsychotics played a significant role in activating MRGPRX2. **(A)** The chemical structure of docking compounds. **(B)** Binding mode of phenothiazine antipsychotics with MRGPRX2. **(C)** Comparison of the activation ability of MRGPRX2 between CPZ and CPZ-1 in LAD2 cells. Data are presented as mean ± S.E.M and are representative of three independent experiment with three samples per experiment. Each trace of the colored line was a response to a unique cell. Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < 0.05$(*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

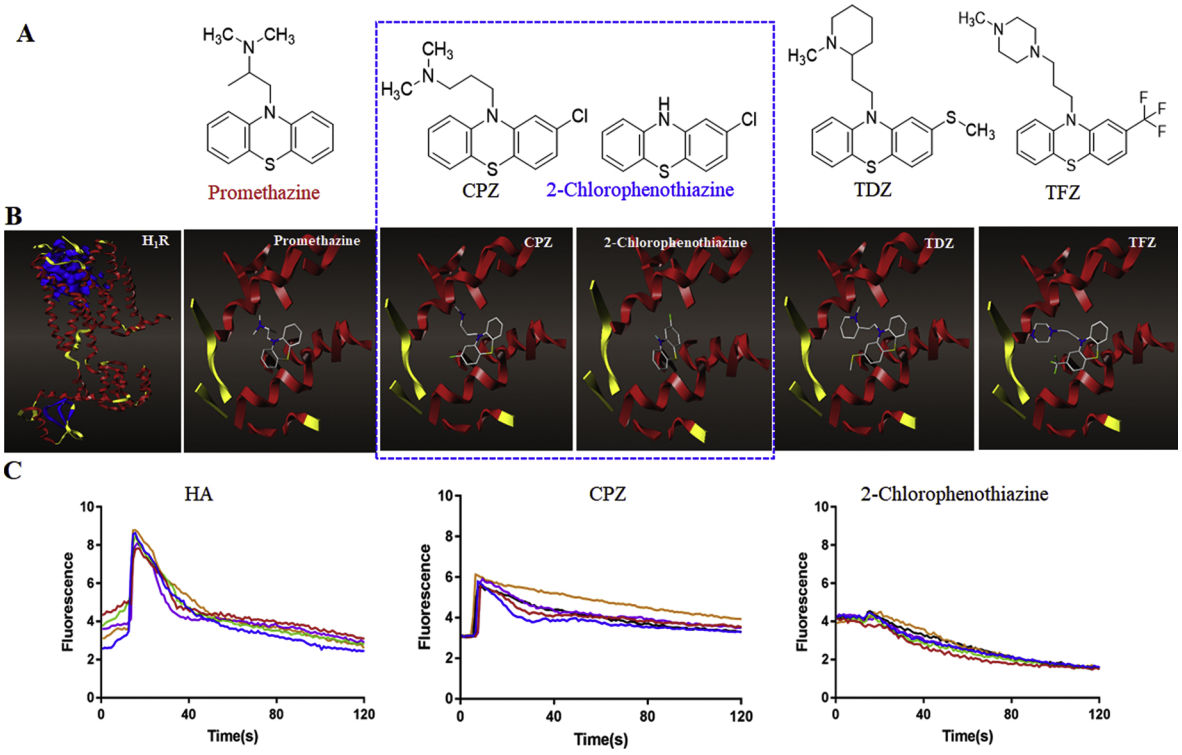

Fig. 6. Phenothiazine tricyclic ring is associated with the antagonistic effect of phenothiazine antipsychotics on H_1R. **(A)** The chemical structure of docking compounds. **(B)**. Binding mode of phenothiazine antipsychotics with H_1R. **(C)** 2-Chlorophenothiazine and CPZ reduced histamine-induced calcium flux in H_1R-HEK293 cells. Each trace of the colored line represented a response to a unique cell. Data are presented as mean ± S.E.M and are representative of three independent experiment with three samples per experiment. Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < 0.05$(*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

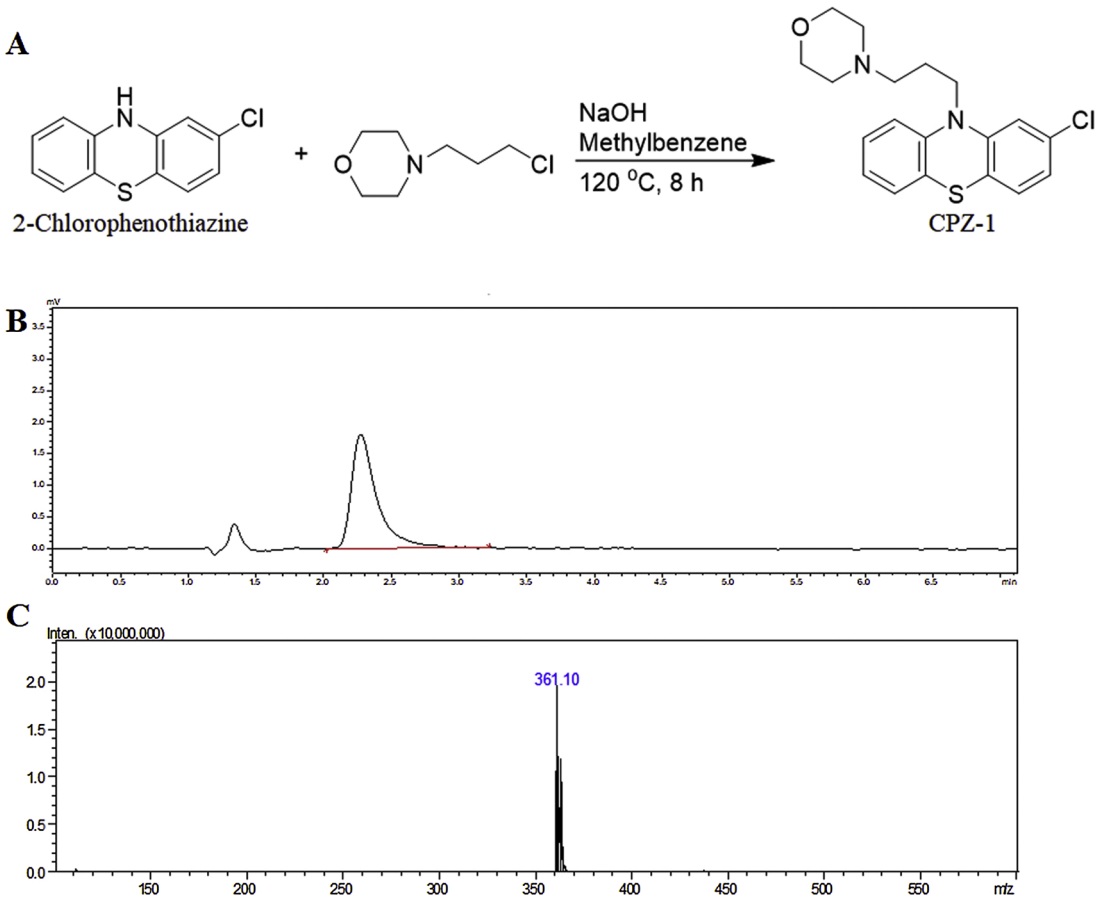

Fig. 7. Synthesis of CPZ-1. (A) The synthesis route of CPZ-1. (B) The HPLC chromatogram of synthetic product. (C) The MS chromatogram of synthetic product.

spatial stretch direction of CPZ-1 was significantly different from CPZ (Fig. 5B). Subsequent experiments then indicated that CPZ-1 could not induce calcium mobilization in MRGPRX2-HEK293 cells as well as the degranulation of mast cells at the same concentration of CPZ (Fig. 5C).

The structural relationship between phenothiazine antipsychotics and H_1R was also investigated. The spatial position of the phenothiazine rings in these drugs is basically the same as promethazine in docking with H_1R (Fig. 6B). Furthermore, the phenothiazine ring of CPZ and 2-chlorophenothiazine also exhibit a similar spatial position in the H_1R active site (Fig. 6B). Moreover, 2-chlorophenothiazine even demonstrated a better inhibitory effect than CPZ on Ca^{2+} mobilization in H_1R-HEK293 cells induced by histamine (Fig. 6C).

4. Discussion

MRGPRX2 is a novel receptor expressed on mast cells and is associated with the adverse reactions caused by several drugs. To our knowledge, the present study was the first to demonstrate that phenothiazine antipsychotics induced degranulation of mast cells via MRGPRX2. Three phenothiazine antipsychotics, CPZ, TDZ and TFZ, (i.e., representing the different structural types) increased intracellular Ca^{2+} concentration in LAD2 cells, and induced mast cells degranulation of β-hexosaminidase and histamine in a dose-dependent manner. The degree of degranulation was significantly decreased in MRGPRX2-knockdown LAD2 cells. These results suggest that phenothiazine antipsychotics induce mast cell degranulation via MRGPRX2 *in vitro*. In addition, our results indicated that only CPZ and TDZ induced the release of TNF-α while TFZ didn't. It was proofed that the mast cell secretion low amounts of cytokines in pseudo-allergic reactions than in IgE-dependent allergic reaction (Gaudenzio et al., 2016). Moreover, our results indicated that TFZ has the weakness interaction with MRGPRX2 among the three tested drugs. Therefore, we inferred TFZ released TNF-α amount is too low to reach the detection limit of the ELISA kit, so it could not be detected. So TFZ being the safest among the three types of phenothiazine antipsychotics in clinical use. One particular phenomenon in our study, however, was noteworthy in that all of these drugs could increase serum histamine concentration in a dose-dependent manner *in vivo*; however, the typical anaphylactic reaction, such as increases in Evans blue dye extravasation and thickness, were not obvious. Consequently, an important question emerged: why did phenothiazine antipsychotics cause the degranulation of mast cells and increase in histamine concentration but did not lead to obvious classic symptoms?

To answer this question, the anti-histamine effect of phenothiazine antipsychotics was initially considered. These phenothiazine antipsychotics were modified based on promethazine, a classical histamine receptor antagonist wisely used in the clinic. Thus, they may also act as potential H_1 receptor antagonists to reduce symptoms of allergic reactions. Our actual experimental results also indicated that these drugs had good interaction with H_1 protein, and could inhibite intracellular Ca^{2+} concentration to varying degrees in H_1R-HEK293 cells induced by histamine. Therefore, the reason why these drugs did not induce anaphylaxis was because they acted as H_1R antagonists to reduce typical anaphylaxis symptoms, such as hemangiectasis and edema, caused by histamine, even though they induced histamine release. However, excessive production of endogenous histamine may result in the incomplete degradation and accumulation of histamine in the body, which leads to histamine intolerance and a negative effect in patients (Maintz and Novak, 2007; Bartholeyns and Fozard, 1985). Moreover, it was also reported that histamine was related to the growth and metastasis of some gastrointestinal tumors (Bartholeyns, 1985; Merwid-Lad and Szelag, 2005; Panula et al., 2000). Some clinical cases reported

phenothiazine-treated patients signed with adverse gastrointestinal diseases, but nearly no attention has been paid to whether this was induced by allergic reaction. This further implied that the potential accumulation of histamine may be significantly more dangerous and should be carefully monitored. Drugs, such as phenothiazine antipsychotics, resulted in little anaphylaxis but induced pseudo-allergic reactions. Thus, accumulation of histamine should raise awareness in clinical applications.

Recently, several studies have reported that the compounds with N-methyl scaffolding are crucial for the activation of MRGPRX2 (Zhang et al., 2017; Lansu et al., 2017). Some studies have shown that MRGPRX2 is rich in anionic residues, which are easily activated by cationic compounds (Lansu et al., 2017; Reddy et al., 2017). Anionic residues tend to bind protons, and methylation can make the nitrogen more electronegative to accept a proton. For this purpose, CPZ-1, a nitrogen non-methylation substituted compound was prepared (Fig. 7). The difference in docking mode and decreasing bioactivity confirmed that the N-methyl moiety makes the compound easy to activate MRGPRX2, and compounds with more N-methyl are easier to active MRGPRX2 at low concentration (EC_{50} value: ZINC-9232 < CPZ < TDZ < TFZ < CPZ-1). On the other hand, our actual results also demonstrated that the tricyclic structure in phenothiazine antipsychotics is crucial to their antihistamine effect. Collectively, these findings suggest that the structural specificity of these drugs determine their dual properties when acting on MRGPRX2 and H_1R.

Even though phenothiazine antipsychotics could activate MCs via MRGPRX2, but not all patients occurred pseudo-allergic reactions. Due to drugs in the same class worked in different ways in activating MRGPRX2 and the activation was positively correlated with concentration (Nasser and Ewan, 2001). In addition, skin MCs from different people showed different degree of response to C48/80 or substance P, which may be related to the expression quantity of MRGPRX2. In addition, human SCF could inhibite pseudo-allergic degranulation of skin mast (Babina et al., 2018). It has already been established that naturally occurring SNPs in the MRGPRX2 gene can influence the responsiveness to MRGPRX2 ligands (Alkanfari et al., 2018). All the reports showed that there were individual differences in MRGPRX2-mediated MCs response.

In conclusion, phenothiazine antipsychotics induced mast cell degranulation via MRGPRX2 and inhibited H_1R at the same time. This double-target effect may lead to increased histamine levels in the body, but do not necessarily result in obvious allergic symptoms that would draw attention or lead to serious concerns. Considering long-term use, clinicians should pay attention to histamine accumulation and the possible inflammatory response caused by phenothiazine antipsychotics, and should be vigilant.

Conflict of interest

The authors declare no commercial or financial conflict of interest.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant Number: 81230079 and 81227802). We thanked Prof. Langchong He for practical guidance during the course of the project and the revise of this paper.

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