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CELL-BASED PHENOTYPIC DRUG SCREENING

HUMAN EOSINOPHILS ADHESION TO ENDOTHELIUM

1H-Indole like derivatives

Ligand	Eosinophils adhesion in the presence of histamine	Eosinophils adhesion in the absence of histamine
JNJ7777120	↓↓	0
MWJ-3	↓	0
JNJ10191584	↓	0

1,3,5 – triazine derivatives

Ligand	Eosinophils adhesion in the presence of histamine	Eosinophils adhesion in the absence of histamine
JN-25	↓↓	↓
KP-9D	↓↓	0
TR-AF-45	↓↓	↓
TR-AF-49	↓	0
JN-35	↓	↓
TR-DL-20	↓/0	0
TR-18	↑	↑
TR-7	↑	↑

IMMUNOPHARMACOLOGY AND INFLAMMATION

TITLE

Eosinophils adhesion assay as a tool for phenotypic drug screening - the pharmacology of 1,3,5 – triazine and 1*h*-indole like derivatives against the human histamine H_4 receptor.

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ABSTRACT

Histamine is a pleiotropic biogenic amine, having affinity towards four distinct histamine receptors. The existing pharmacological studies suggest the usefulness of histamine H_4 receptor ligands in the treatment of many inflammatory and immunomodulatory diseases, including allergic rhinitis, asthma, atopic dermatitis, colitis or pruritus. Up to date, several potent histamine H_4 receptor ligands were developed, none of which was registered as a drug yet. In this study, a series of potent indole-like and triazine derivatives were tested, in radioligand displacement and functional assays at histamine H_4 receptor, as well as in human eosinophils adhesion assay to endothelium. For selected compounds permeability, cytotoxicity, metabolic and *in vivo* studies were conducted. Adhesion assay differentiates the activity of different groups of compounds with a known affinity towards the histamine H_4 receptor. Most of the tested compounds downregulated the number of adherent cells. However, adhesion assay revealed additional properties of tested compounds that had not been detected previously in radioligand displacement and aequorin-based functional assays that make use of the artificial recombinant cell lines. Furthermore, for some tested compounds, these abnormal effects were confirmed during the *in vivo* studies. In conclusion, eosinophils adhesion assay uncovered pharmacological activity of histamine H_4 receptor ligands that has been later confirmed *in vivo*, underscoring the value of well-suited cell-based phenotypic screening approach in drug discovery.

KEYWORDS

Histamine, Histamine receptors, Eosinophils, Endothelium, Adhesion, 1*H*-Indole like derivatives, 1,3,5 – Triazine derivatives.

CHEMICAL COMPOUNDS STUDIED IN THIS ARTICLE

1*H*-Indole like derivatives

JNJ10191584 (5-chloro-1*H*-benzo[*d*]imidazol-2-yl)(4-methylpiperazin-1-yl)methanone), Pub- Chem CID: 10446295),

JNJ7777120 (5-chloro-1*H*-indol-2-yl)(4-methylpiperazin-1-yl)methanone), Pub- Chem CID: 4908365),

MWJ-3 (5-chloro-7-nitro-1*H*-indol-2-yl)(4-methylpiperazin-1-yl)methanone, Pub- Chem CID: 70692530),

1,3,5 – Triazine derivatives

TR-7 (4-(4-chlorophenyl)-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-amine),

TR-18 (4-(4-bromophenyl)-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-amine),

TR-AF-45 (4-(4-methylpiperazin-1-yl)-6-neopentyl-1,3,5-triazin-2-amine),

TR-AF-49 (4-(cyclohexylmethyl)-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-amine),

TR-DL-20 (4-(1-cyclohexenylmethyl)-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-amine),

JN-35 (4-(4-methylpiperazin-1-yl)-6-(3-phenylpropyl)-1,3,5-triazin-2-amine),

JN-25 (4-[(*E*)-2-(3-chlorophenyl)ethenyl]-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-amine),

KP-9D (2-(4-chlorophenyl)-4-(4-methylpiperazin-1-yl)-1,3,5-triazine),

1. INTRODUCTION

The human histamine H_4 receptor is the fourth and most recent histamine receptor identified at the turn of the millennium (Cogé et al., 2001; Liu et al., 2001; Morse et al., 2001; Nakamura et al., 2000; Nguyen et al., 2001; Oda et al., 2000; Zhu et al., 2001). Up to date, its physiological function has been associated mainly with histamine-mediated immunological reactions and their further maintenance (Thurmond et al., 2004; Zhang et al., 2007), however, additional properties were further investigated, including histamine H_4 receptor expression and function on the nervous system (Schneider et al., 2014; Strakhova et al., 2009), its role on brain endothelial cells (Karlstedt et al., 2013), or anti-proliferative activity on several human cancer cells (Massari et al., 2017, 2011; Panula et al., 2015). The existing pharmacological studies suggest the usefulness of histamine H_4 receptor ligands in the treatment of the inflammatory and immunomodulatory diseases, e.g., allergic rhinitis, asthma, atopic dermatitis, colitis and pruritus (de Esch et al., 2005; Thurmond et al., 2008, 2004; Zampeli and Tiligada, 2009; Zhang et al., 2007). Among the histamine H_4 receptor antagonists/inverse agonists several structurally different compounds have been developed, some of which have already made their way into the clinical trials (de Esch et al., 2005; Lażewska et al., 2016). Among them, indole like and triazine moieties are one of the most promising ones (Lażewska et al., 2016; Panula et al., 2015).

However, despite ongoing work, no histamine H_4 receptor ligands have been approved as a drug yet. Therefore, further studies are needed to develop new compounds with improved pharmacological profile, as well as newer analytical methods, some of which would be in line with reemerging phenotypic-based drug discovery, that would allow to measure the activity of the developed ligands more accurately than existing methods (Moffat et al., 2017; Zheng et al., 2013).

In this study, a series of indole-like and triazine derivatives were tested, in the newly developed assay of human eosinophils adhesion to endothelium (Grosicki et al., 2016). The pharmacological profile of tested compounds' was also examined in classical biological assays including [^{3}H] histamine binding experiments and cellular aequorin-based functional assay at the recombinant cellular model (Table 1). Furthermore, we aimed to evaluate the effects of some ligands *in vivo* to characterize their different pharmacological properties on croton oil – induced ear edema and pruritus in CD-1 mice.

2. MATERIALS AND METHODS

2.1 MATERIALS

For the eosinophils isolation: dextran from Leuconostoc spp. 500,000 (Sigma-Aldrich, St. Louis, MO, USA), Ficoll-Paque Plus d=1.077 g/ml (GE Healthcare, Boston, MA, USA), and eosinophil isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) were used. Mouse anti-human CD16 (clone 3G8), fluorescein isothiocyanate (FITC) conjugated antibody (Life Technologies, Carlsbad, CA, USA) was used for eosinophils purity estimation. Cells were incubated in RPMI 1640, HAM's F12 and DMEM cell culture medium supplemented with L- glutamine (Gibco, Carlsbad, CA USA), fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), HAT supplement (Sigma-Aldrich, St. Louis, MO, USA) and penicillin-streptomycin (10,000 U/mL) (Gibco, Carlsbad, CA, USA). For the tests histamine dihydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was used. All of the tested compounds were synthesized at Jagiellonian University, Medical College, Faculty of Pharmacy, Department of Technology and Biotechnology of Drugs and showed LC-MS purity > 98%: JNJ10191584 (5-chloro-1*H*-benzo[*d*]imidazol-2-yl)(4-methylpiperazin-1-yl)methanone) (Terzioglu et al., 2004; Venable et al., 2005) JNJ7777120 (5-chloro-1*H*-indol-2-yl)(4-methylpiperazin-1-yl)methanone) (Jablonowski et al., 2003; Venable et al., 2005); MWJ-3 (5-chloro-7-nitro-1*H*-indol-2-yl)(4-methylpiperazin-1-yl)methanone) (Engelhardt et al., 2012; Nijmeijer et al., 2013); TR-7 (4-(4-chlorophenyl)-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-amine) (Łażewska et al., 2014); TR-18 (4-(4-bromophenyl)-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-amine) (Łażewska et al., 2014); TR-AF-45 (4-(4-methylpiperazin-1-yl)-6-neopentyl-1,3,5-triazin-2-amine) (Łażewska et al., 2019); TR-AF-49 (4-(cyclohexylmethyl)-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-amine) (Łażewska et al., 2019); TR-DL-20 (4-(1-cyclohexenylmethyl)-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-amine) (unpublished); JN-35(4-(4-methylpiperazin-1-yl)-6-(3-phenylpropyl)-1,3,5-triazin-2-amine) (unpublished); JN-25 (4-[(*E*)-2-(3-chlorophenyl)ethenyl]-6-(4-methylpiperazin-1-yl)-1,3,5-triazin-2-amine) (Kamińska et al., 2015); KP-9D (2-(4-chlorophenyl)-4-(4-methylpiperazin-1-yl)-1,3,5-triazine) (Gaul et al., 2009). Compounds stock solutions (10 mM) were prepared in dimethylsulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA). Further dilutions were done in RPMI 1640. In the adhesion assay, cellular nuclei

were stained with Hoechst 33342 (20 mM) solution (Thermofisher Scientific, USA). Functional tests were conducted with the use of the AequoScreen assay (Perkin Elmer, Waltham, MA, USA), and EZ4U Non-radioactive cell proliferation and cytotoxicity assay kit, (Biomedica, Vienna, Austria). For metabolic studies mouse liver microsomes (Sigma-Aldrich, St. Louis, MO, USA) were used. Caffeine, doxorubicin, croton oil and other chemicals were purchased from (Sigma-Aldrich, St. Louis, MO, USA).

2.2. METHODS

2.2.1. *IN VITRO* STUDIES

2.2.1.1. HUMAN HISTAMINE H_1 RECEPTOR RADIOLIGAND BINDING ASSAY

Radioligand binding assay was performed using membranes from CHO-K1 cells stably transfected with the human histamine H_1 receptor (Perkin Elmer, Waltham, MA, USA). All experiments were carried out in duplicates. Briefly, 50 µl working solution of the tested compounds, 50 µl [^{3}H]-pyrilamine (final concentration 1.5 nM) and 150 µl diluted membranes (5 µg protein per well) prepared in assay buffer (50 mM Tris, pH 7.4, 5 mM $MgCl_2$) were transferred to polypropylene 96-well microplate using 96-wells pipetting station Rainin Liquidator (Mettler Toledo, Columbus, OH, USA). Mepyramine (10 µM) was used to define nonspecific binding. The microplate was covered with sealing tape, mixed and incubated for 60 min at 27°C. The reaction was terminated by rapid filtration through GF/B filter mate presoaked with 0.5% polyethyleneimine for 30 min. Ten rapid washes with 200 µl 50 mM Tris buffer (4°C, pH 7.4) were performed using 96-well FilterMate harvester (Perkin Elmer, Waltham, MA, USA). The filter mates were dried at 37°C in a forced air fan incubator and then solid scintillator MeltiLex was melted on filter mates at 90°C for 4 min. The radioactivity on the filter was measured in MicroBetaTriLux 1450 scintillation counter (Perkin Elmer, Waltham, MA, USA). Competition binding data were analyzed according to the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

2.2.1.2. HUMAN HISTAMINE H_4 RECEPTOR RADIOLIGAND BINDING ASSAY

Radioligand binding assay at human histamine H_4 receptor was performed according to the methods described previously (Łażewska et al., 2014). Briefly, cell membranes, prepared from Sf9 cells transiently expressing human histamine H_4 receptor, co-expressed with G-protein $G_{\alpha i2}$and $G_{\beta 1\gamma 2}$ subunits, were sedimented through centrifugation (16 000×g, 4°C, 10 min) and resuspended in binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA and 75 mM Tris/HCl, pH=7.4). Competition binding was carried out by incubating membranes, 35-40 µg/well in a final volume of 200 µl containing binding buffer and [^{3}H]-histamine (10 nM) in presence of serial dilutions of test ligands (ranging from 0.1 nM to 100 µM) prepared in triplicates. The test was run for 60 min at room temperature and shaking at 250 rpm. Nonspecific binding was studied in the presence of 10 µM unlabeled JNJ7777120. Bound radioligands were separated by rapid filtration through GF/B filters pretreated with 0.3% (mass/vol) polyethylenimine (PEI) solution and washed three times with 300 µl of ice-cold binding buffer. The amount of radioligand collected on the filter was determined by liquid scintillation counting. Competition binding data were analyzed using a non-linear least squares fit (GraphPad Prism software). K_i values were calculated from the IC_{50} values according to the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

2.2.1.3. HISTAMINE H_4 RECEPTOR FUNCTIONAL ASSAY

Compounds were dissolved in DMSO at a concentration of 1 mM. Serial dilutions were prepared in 96-well microplate in assay buffer. A cellular aequorin-based functional assay was performed with recombinant CHO-K1 cells expressing mitochondrially targeted aequorin, human GPCR and the promiscuous G protein α16 for histamine H_4 receptor. The assay was executed according to the standard protocol provided by the manufacturer (PerkinElmer, Waltham, MA, USA). After thawing, cells were transferred to assay buffer (DMEM/HAM's F12 with 0.1% protease-free BSA) and centrifuged. The cell pellet was resuspended in assay buffer and coelenterazine h (Promega, Madison, WI, USA) was added at final concentrations of 5 µM. The cell suspension was incubated at 16°C, protected from light with constant agitation for 16 h and then diluted with assay buffer to the concentration of 200,000 cells/ml. After 1 h of incubation, 50 µl of the cells suspension was dispensed using automatic injectors built into the radiometric and luminescence plate counter MicroBeta2

LumiJET (Perkin Elmer, Waltham, MA, USA) into white opaque 96-well microplates preloaded with test compounds. Immediate light emission generated following calcium mobilization was recorded for 30 s. In antagonist mode, after 30 min of incubation, the reference agonist was added to the above assay mix and light emission was recorded again. The final concentration of the reference agonist was equal to EC_{80} (900 nM histamine).

2.2.1.4. HUMAN HISTAMINE H_3 RECEPTOR RADIOLIGAND BINDING ASSAY

JN-35 was evaluated for potential affinity towards human histamine H_3 receptor, using [^{3}H]-N^{α}-methylhistamine displacement from receptors in membrane preparations of stably transfected HEK-293 cells. A previously described method for this assay was followed without modifications (Kottke et al., 2011). Data were acquired by four independent experiments performed in triplicate and were analyzed as described in section 2.2.1.2.

2.2.1.5. JN-35 CYTOTOXICITY STUDY

In vitro antiproliferative assay was conducted against two distinct cell lines: Neuroblastoma, IMR-32 (kindly provided by the Department of Oncogenomics, AcademischMedisch Centrum, Amsterdam, Netherlands, ATCC CCL-127) and HEK-293 (kindly donated by Prof. Dr Christa E. Muller, Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn, Germany, ATCC CRL-1573). IMR-32 and HEK-293 cells were cultured as described by (Latacz et al., 2016). The cytotoxicity study was conducted as follows. The cells were seeded in 96-well plates at a concentration of 2×10^4 cells/well (IMR-32) or 1.5×10^4 cells/well (HEK-293) in 200 µl culture medium and routinely cultured for 24 h to reach 60% confluence. Next, tested compounds in different concentrations were added into the microplate. After 48 h, 20 µl of EZ4U labeling mixture (EZ4U Non-radioactive cell proliferation and cytotoxicity assay) was added to each well and the cells were further incubated for 5 h. The absorbance of the samples was measured using a microplate reader (EnSpire, Perkin Elmer, Waltham, MA, USA) at 492 nm. Doxorubicin was used as a reference drug in this study.

2.2.1.6. JN-35 PERMEABILITY STUDY

The permeability study for the JN-35 compound was tested based on the Caco-2 assay, described in detail by (Lubelska et al., 2019). The Caco-2 (ATCC® HTB-37TM) cell line was cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Carlsbad, CA, USA). Cells at a density of 2×10^4 were seeded on the Corning 3413 Transwell® 6.5 mm polycarbonate membrane inserts with 0.4 µm pores (Sigma-Aldrich, St. Louis, MO, USA). The plate was incubated at 37°C for 14 h, and the non-adherent cells were removed. The proper monolayer integrity was determined according to the TEER (transepithelial electrical resistance) measurements, 20 days after cell with the Millicell ERS-2 Volt-Ohm Meter (Merck Millipore, Burlington, MA, USA). Afterward, the monolayer was rinsed with HBSS (Hank's balanced salt solution) and tested compounds were added. The compounds' concentrations in apical and basolateral wells were analyzed using the UPLC-MS method with internal standard. To confirm the membrane integrity, the fluorescence of lucifer yellow was measured in the basolateral compartment by EnSpire microplate reader (Perkin Elmer, Waltham, MA, USA). The apparent permeability P_{app} was calculated from two experiments according to the formula (Di and Kerns, 2016): P_{app}= dc/dt × V/(A × C0), where: dc/dt—the change in concentration in the receiving compartment over time V—volume of the solution in the receiving compartment (µl) A—surface area of the membrane (cm^2) C0—the initial concentration in the donor compartment (µM).

2.2.1.7. *IN SILICO* AND *IN VITRO* METABOLISM OF JN-35

The JN-35, *in silico* and *in vitro* metabolic stability investigation was performed as described previously (Lubelska et al., 2019). The UPLC/MS spectra necessary for compounds concentrations determination in Caco-2 assay and analysis of the reaction mixtures from metabolic stability assay were obtained by Waters ACQUITY™ TQD system with the TQ Detector (Waters, Milford, USA). Mouse liver microsomes were purchased from Sigma-Aldrich.

2.2.1.8. THE HUMAN EOSINOPHILS ADHESION TO ENDOTHELIUM ASSAY

2.2.1.8.1. HUMAN EOSINOPHILS ISOLATION

Eosinophils were isolated from human peripheral blood using the immunomagnetic cell separation method, described previously in detail (Grosicki et al., 2016). Briefly, residual erythrocytes from the collected blood samples were sediment in the 1% dextran solution centrifugation (50×g for 7 min, at room temperature, without brake). Remaining white blood cells (in upper layer after first centrifugation) were carefully layered over Ficoll-Paque density gradient, to separate peripheral blood mononuclear cells (PBMC) from the polymorphonuclear leukocytes (PMN) (centrifugation at 400×g for 12 min, at room temperature, without brake). After centrifugation, blood plasma, PBMC, and Ficoll were discarded. Remaining erythrocytes were removed by hypotonic shock lysis. Eosinophils were purified, using human eosinophil isolation kit (Miltenyi Biotec) according to the manufacturer's instruction. Isolated eosinophils were suspended in RPMI 1640 cell culture medium supplemented with 10% (v/v) fetal bovine serum (FBS), counted and used immediately after isolation procedure. The purity of isolated eosinophils was evaluated during the flow-cytometry analysis based on anti-CD16 staining, and it was estimated between 90-98% (data not provided).

2.2.1.8.2. HUMAN ENDOTHELIAL CELL CULTURE

For the adhesion assays human endothelial cell line, EA.hy926 was used (ATCC CRL-2922). Cells were cultured in DMEM cell culture medium, supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 1×HAT and penicillin-streptomycin solution. For the experiments, endothelial cells were seeded on the 24 well-plates at the density of 1×10^5 cells per well. Cells were incubated at 37°C, 5% CO_2 for 48 h until reached complete confluence.

2.2.1.8.3. HUMAN EOSINOPHILS ADHESION ASSAY

The human eosinophils adhesion to endothelium assay was performed in static conditions as described previously (Grosicki et al., 2016). Briefly, immediately after eosinophils isolation, confluent endothelial cells were washed with DPBS (Dulbecco's phosphate-buffered saline). After washing, the titrations of tested compounds (diluted in 10% (v/v) FBS/RPMI 1640) were added to appropriate microplate wells, followed by the eosinophils addition at the density of 4×10^4 cells per well. The activity of compounds was tested in the presence and absence of 1 μM histamine. Eosinophils were allowed to adhere to the endothelial monolayer for 30 min at 37°C, 5% CO_2. After incubation, cells

were washed gently twice with DPBS to eliminate nonadherent cells. Remaining cells were stained against their nuclei with Hoechst 33342. Microscopic images were acquired using an Olympus Scan^R automated fluorescent microscope. Images were analyzed in Columbus 2.4.2 (Perkin Elmer, Waltham, MA, USA), using nuclei morphological properties determination. Eosinophils were identified as cells with small, irregular nuclei (nuclei area of 60–90 μm^2) in contrast to endothelial cells with single-shape, round and big nuclei (nuclei area of 200-320 μm^2). Due to donor to donor data variability, results were presented as percent (%) of control, calculated for each experiment separately. Control (100%) was defined as number of adherent eosinophils to endothelium not treated with tested ligands. 100% control was calculated separately for tests in presence/absence of 1 µM histamine.

2.2.2. *IN VIVO* STUDIES

2.2.2.1. ANIMALS

Unfasted male CD-1 mice, aged 10-12 weeks (28-32 g body weight), were purchased from a commercial source (Envigo, Udine, Italy). After 1-week acclimatization, mice were housed in groups of five mice per cage at 23 ± 1°C, 45 ± 5% humidity, and a 12 h light/dark cycle. Food and water were provided *ad libitum*. The experimental protocol was carried out after approval by the Animal Care and Research Ethics Committee of the University of Parma (Italy), under license from the Italian Department of Health (authorization n° 93/2014-B). All experiments were carried out between 10 a.m. and 3 p.m., to avoid the influence of circadian variations in corticosteroid levels in the inflammatory responses.

2.2.2.2. DRUG ADMINISTRATION

Equivalent volumes (3 ml/kg) of test drugs or vehicle were administered subcutaneously (s.c.) in separate groups of mice. Single administrations of the compound were given immediately before the topical application of croton oil. JNJ7777120, JN-25, JN-35, TR-7, and TR-18 were dissolved in a vehicle containing 20% DMSO and 80% 2-hydroxypropyl-β-cyclodextrin. Croton oil was dissolved in 100% acetone. Each agent was prepared immediately before use.

2.2.2.3. EAR EDEMA EVALUATION

For this study, the method described by (Coruzzi et al., 2012) was followed. Cutaneous inflammation was induced in conscious CD-1 mice by topical application of croton oil (2.5% in acetone). The irritant agent was applied with a micropipette (20 µl/ear) to the inner surface of the right ear (inflamed ear). Acetone was applied to the left ear, which served as a control (uninflamed ear). Two or four h after croton oil application, mice were killed; both left (acetone) and right (croton oil in acetone) ears were removed, by cutting horizontally across the indentation at the base of the ear. For each mouse, the extent of the edema was expressed as the difference in weight (Δ mg) between right (inflamed) and left (uninflamed) ear.

2.2.2.4. EAR PRURITUS EVALUATION

Pruritus was induced in conscious CD-1 mice by the application of croton oil to the inner surface of the right ear (see the above protocol). The itch was measured by counting the number of bouts of scratching immediately following the application of croton oil. A bout of scratching was defined as two or more rapid individual scratch movements with the hind paw to the area around the ear. Mice were directly observed for 1 h immediately after the croton oil application. Likewise to the evaluation of edema, for each mouse the extent of the pruritus was expressed as a difference in counting the scratching bouts (Δ scratching bouts) between right (inflamed) and left (uninflamed) ear.

2.2.3. STATISTICS

Results were expressed as means ± S.E.M. from (n) number of experiments. During the adhesion assays, the dose-dependent statistical significance was calculated by parametric one-way analysis of variance (ANOVA), preceded by normality and equality of variances tests evaluation using StatSoft, Inc. STATISTICA 12. Post hoc comparisons were performed with Bonferroni's multiple comparison tests. Differences between values were considered significant when $P < 0.05$. The EC_{50}/IC_{50} values were calculated by nonlinear regression analysis using GraphPad Prism 5.00 (GraphPad Software, San Diego California USA). During the *in vivo* studies, comparisons between two groups were made by using Student's *t*-test for unpaired data. A value of $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. *IN VITRO* STUDIES

3.1.1. COMPOUNDS AFFINITY AND POTENCY AGAINST HISTAMINE RECEPTORS

Tested compounds consisted of a group of eleven structurally different histamine H_4 receptor ligands, that belong to the groups of 1,3,5 – triazine and 1*H*-indole like derivatives. They displayed different affinities at the histamine H_4 receptor. Ligands MWJ-3, JNJ10191584, JNJ7777120, TR-7, TR-18, TR-AF-45, TR-AF-49, JN-25, and KP-9D were characterized by high affinity towards the histamine H_4 receptor, with JNJ7777120, JNJ10191584 and KP-9D exerting their activity at low nanomolar concentration range. Among the tested ligands JN-35 and TR-DL-20 exhibited low affinities at the receptor tested (Table 1). All of the tested compounds were classified as the antagonists of the histamine H_4 receptor, on the cellular aequorin-based functional assay. The most active compounds were ($IC_{50} \leq 20$ nM) JNJ7777120, JNJ10191584, TR-AF-45, KP-9D, and TR-18.

Ligand	**Molecular mass [g/mol]**	**LC-MS purity**	**Affinity K_i [nM] ± S.E.M.** Human histamine H_1 receptor	Human histamine H_3 receptor	Human histamine H_4 receptor	**pIC_{50}± S.E.M.** (antagonistic activity)
1H-indole like derivatives						
JNJ10191584[a]	278.7	100%	nt	14.1×10^3	26 ± 8	8.46 ± 0.020
JNJ7777120[b,c]	277.9	100%	$>10\times10^3$	5.1×10^3	4 ± 1	7.87 ± 0.100
MWJ-3[d]	322.7	99,01 %	nt	nt	6.4 ± 0.1	6.23 ± 0.207
1,3,5 – triazine derivatives						
TR-AF-45	264.4	100%	nt	nt	247*	9.81 ± 0.029
TR-DL-20	288.4	100%	nt	nt	4700	8.44 ±0.083

KP-9D[e]	289.9	98,60 %	nt	nt	289 ± 133	8.13 ± 0.127
TR-18[f]	349.2	100%	nt	15×10^3	524 ± 159	8.04 ± 0.063
TR-AF-49	290.4	100%	$>5\times10^3$	nt	160[*]	7.50 ± 0.054
TR-7[f]	304.9	100%	$>5\times10^3$	13.2×10^3	203 ± 37.5	7.16 ± 0.017
JN-35	298.4	100%	$>2\times10^3$	6.1×10^3	5995	6.51 ± 0.045
JN-25[g]	330.9	100%	nt	$<30\times10^3$	253 ± 75	6.43 ± 0.020

Table 1. The pharmacological properties of the tested compounds. Tested ligands possessed different affinity towards histamine H_4 receptor (9 compounds with an affinity at nM range). All of the tested ligands were classified as antagonists at histamine H_4 receptor, based on the recombinant aequorin-based functional assay. The most active compounds were ($IC_{50} \leq 20$ nM) JNJ7777120, JNJ10191584, TR-AF-45, KP-9D, and TR-18.

[*]TR-AF-45 K_i 95% CI = 84.6 – 719 nM, n=3

[*]TR-AF-49 K_i 95% CI = 66.6 – 385 nM, n=4

The affinity data from: [a](Venable et al., 2005); [b](Jablonowski et al., 2003) ,[c](Thurmond et al., 2004); [d](Engelhardt et al., 2012); [e](Gaul et al., 2009); [f](Łażewska et al., 2014); [g](Kamińska et al., 2015)

3.1.2. EFFECTS OF TESTED COMPOUNDS ON HUMAN EOSINOPHILS ADHESION TO ENDOTHELIAL CELLS

3.1.2.1. HISTAMINE EFFECT ON EOSINOPHILS ADHESION

Histamine increased the number of adherent eosinophils to endothelial cells in a dose-dependent manner, with an EC_{50} of 336 nM (95% CI = 73 – 1551 nM, n=3). At the highest tested histamine concentration (100 µM), the 3-fold increase in adherent cells was observed in comparison to non-activated cells (Fig. 1).

3.1.2.2. 1*H*-INDOLE LIKE DERIVATIVES EFFECT ON EOSINOPHILS ADHESION

The tested 1*H*-indole derivatives and their benzimidazole analogues downregulated the number of adherent eosinophils, in the presence of 1 μM histamine (Fig. 2). The maximal eosinophils response was observed at the highest tested compounds concentration (50 μM). MWJ-3 and JNJ10191584 inhibited eosinophils adhesion to endothelium in presence of 1 μM histamine with IC_{50} of 399.9 nM (95% CI = 42.95 – 3724 nM, n=6) and 948.2 nM (95% CI = 263.2 – 3416 nM, n=6), respectively. Both compounds exhibited lower potency in comparison to JNJ7777120 (IC_{50} = 5.57 nM (95% CI = 1.93 – 16.08 nM), n=12). In contrast, no statistically significant effect on eosinophils adhesion of tested compounds was observed in the absence of histamine, however, the weak intrinsic effect of JNJ10191584 was detected.

3.1.2.3. 1,3,5 – TRIAZINE DERIVATIVES EFFECT ON EOSINOPHILS ADHESION

In the presence of 1 μM histamine compounds: JN-25, KP-9D, TR-AF-45, TR-AF-49, TR-DL-20 and JN-35 downregulated the number of adherent eosinophils with IC_{50} of 2.31 nM (95% CI = 0.2763 - 19.27 nM, n=6), 2.42 nM (95% CI = 0.58 - 10.08 nM, n=6), 14.82 nM (95% CI = 0.33 - 663 nM, n=6), 138.4 nM (95% CI = 12.2 - 570 nM, n=6), ≥1000 nM (ambiguous data due to incomplete concentration–response curve, n=6), and 1119 nM (95% CI = 466.1 - 2689 nM, n=6), respectively (Fig. 3). Among the tested ligands only KP-9D, TR-AF-49, and TR-DL-20 did not have a statistically significant effect on eosinophils adhesion to endothelium in the absence of histamine. In contrast, compounds JN-25, TR-AF-45, and JN-35 downregulated the number of adherent eosinophils even in the absence of histamine with IC_{50} values of 2814 nM (95% CI = 209 - 37830 nM, n=4), 4240 nM (95% CI = 16 – 1084000 nM, n=4), and 440 nM (95% CI = 122 - 1588 nM, n=4), respectively. Since unexpectedly, JN-35 exhibited high activity at the human eosinophils adhesion assay to endothelium, (despite its low affinity towards the human histamine H_4 receptor), its pharmacological properties were further studied in details (see section 3.1.3 to 3.1.5). Interestingly, two compounds among the tested 1,3,5 – triazine derivatives exhibited paradoxical agonistic activity during the adhesion assay in presence and absence of 1 μM histamine. The agonistic effect was most pronounced at the highest concentration of tested compounds (50 μM). In presence of 1 μM histamine, compounds TR-7 and TR-18 upregulated the number of adherent eosinophils with EC_{50} values of 11750 nM (95% CI = 5 -

28660000 nM, n=6) and 403 nM (95% CI = 76 - 2135 nM, n=6), respectively. In the absence of histamine, both TR-7 and TR-18 upregulated the number of adherent eosinophils with EC_{50} of 689 nM (95% CI = 126 - 3775 nM, n=4) and 185 nM (95% CI = 7 - 5154 nM, n=4), respectively.

3.1.3. JN-35 CYTOTOXICITY STUDY

Human embryonic kidney HEK-293 and neuroblastoma IMR-32 cell lines were used for preliminary cytotoxicity and neurotoxicity evaluation of JN-35. As shown in Fig. 4, the effects of JN-35 on cell viability was only significant at the highest dose used (250 μM), that indicates lack of cytotoxicity.

3.1.4. JN-35 PERMEABILITY STUDY

The calculated JN-35 permeability coefficient (P_{app}) based on the Caco-2 assay was 13.18×10^{-6} cm/s. In comparison, caffeine, a reference compound with good permeability, exhibited the permeability coefficient (P_{app}) at 22.04×10^{-6} cm/s. According to the guidelines found in the literature (Di and Kerns, 2016), the calculated in Caco-2 model permeability coefficient $P_{app} < 2.0 \times 10^{-6}$ cm/s indicates low permeability, P_{app} from 2.0×10^{-6} to 20×10^{-6} cm/s moderate permeability, whereas compounds with $P_{app} > 20 \times 10^{-6}$ cm/s are considered as high permeable. Based on the obtained results, JN-35 was estimated as compound with moderate ability to cross the cell line barrier in the intestine.

3.1.5. *IN SILICO* AND *IN VITRO* METABOLISM OF JN-35

The MetaSite 6.0.1. software simulation predicted the methyl substituent at the *N*-methylpiperazine moiety as the most probably site of JN-35 metabolism (Fig. 5.1). The benzene ring was also indicated as a very susceptible for metabolism (Fig. 5.1). Indeed, the incubation of JN-35 with mouse liver microsomes (MLMs) for 120 min, and subsequent UPLC/MS analysis resulted in identification of two metabolites, of which the main metabolite M1 (m/z = 329.30) was found as an effect of hydroxylation (Fig. 5.2.A, 5.2.B). Based on *in silico* and *in vitro* data, the most probable site of hydroxylation was proposed at the benzene moiety as shown at Fig. 5.3. The metabolite M2 was identified as an effect of further metabolism of M1 by hydroxylation and *N*-demethylation reactions (Fig. 5.2.C, 5.3). It should be pointed out, that a peak corresponding to the parent compound JN-35 (t_R=3.39) was not observed in

the reaction mixture (Fig. 5.2.A), indicating very low JN-35 metabolic stability and almost complete metabolisation.

3.2. *IN VIVO* STUDIES

3.2.1. ANTI-INFLAMMATORY ACTIVITIES OF SELECTED COMPOUNDS

3.2.1.1. CROTON OIL-INDUCED EAR EDEMA AND PRURITUS

The topical application of croton oil caused a time-dependent increase in the right ear weight when compared to the left ear that received only vehicle (acetone). The difference (Δ mg) in ear weight ranged from 22.73 ± 3.18 up to 29.70 ± 3.11 for 2 h and 28.83 ± 3.03 for 4 h after croton oil application, respectively. TR-7, TR-18, JN-25, JN-35, JNJ7777120 decreased ear swelling 2 h after croton oil application (Fig. 6). The difference in ear weight (Δ mg/2 h) for tested compounds were: TR-7 (50 mg/kg) 9.48 ± 2.74 (n=9), TR-18 (50 mg/kg) 10.70 ± 4.70 (n=8), JN-25 (50 mg/kg) 7.45 ± 2.76 (n=6), JN-35 (50 mg/kg) 4.74 ± 2.39 (n=7), JNJ7777120 (30 mg/kg) 12.18 ± 1.88 (n=11). Interestingly, the effect of tested compounds on ear edema was not significant 4 h after the croton oil application (Fig. 6). The difference in ear weight (Δ mg/4 h) for tested compounds were: TR-7 (50 mg/kg) 36.69 ± 2.35 (n=7), TR-18 (50 mg/kg) 36.30 ± 2.96 (n=7), JN-25 (50 mg/kg) 36.00 ± 3.05 (n=6), JN-35 (50 mg/kg) 23.48 ± 5.40 (n=6), JNJ7777120 (30 mg/kg) 32.14 ± 5.09 (n=5).

The majority of mice did not show spontaneous scratching behavior (data not shown). Ear application of croton oil induced scratching in all treated mice, the maximal effect being evaluated after 20-30 min. The averaged difference in scratching bouts (Δ bouts/1 h) between inflamed and uninflamed ear ranged from 114.40 ± 21.15 up to 141.80 ± 26.90 after croton oil application (Fig. 7). No statistically significant effect on ear pruritus was detected for TR-7, TR-18, JN-25, and JNJ7777120 (Fig. 7). The difference in scratching bouts (Δ bouts/1 h) for these compounds were 119.20 ± 11.32 (n=10), 122.00 ± 6.87 (n=6), 109.50 ± 9.57 (n=6), 79.00 ± 18.23 (n=9), respectively. In contrast, among the tested compounds JN-35 significantly inhibited croton oil induced ear scratching, (Δ bouts/1 h: 14.40 ± 5.34, n=5, $P < 0.01$; Fig. 7).

4. DISCUSSION

Previously, we demonstrated that histamine upregulated the number human eosinophils to endothelium (Fig. 1), through the activation of histamine H_4 receptor (Grosicki et al., 2016). Here, we took advantage of the eosinophils adhesion assay to profile the anti-inflammatory activity of the selected 1*H*-indole like, and 1,3,5-triazine derivatives with known affinity towards histamine H_4 receptor (Table 1). The developed assay allowed us to characterize tested ligands based on their activity and potency with regards to eosinophils adhesion. Importantly, using eosinophils assay to endothelium we revealed additional properties of tested compounds that were not detected in radioligand displacement and aequorin-based functional assays using artificial recombinant cell lines.

In this study, all of the tested compounds had an effect on eosinophils adhesion to the endothelium (Fig. 2 and 3). Differently from TR-7 and TR-18, all ligands downregulated the number of adherent eosinophils at the highest tested concentration. The summary of obtained pharmacological effects of tested ligands against *in vitro* and *in vivo* studies were shown in Table 2.

***IN VITRO* DATA**					***IN VIVO* DATA**				
Ligand	**Eosinophils adhesion in the <u>presence</u> of histamine**		**Eosinophils adhesion in the <u>absence</u> of histamine**		**Dose** *mg/kg s.c.*	**Ear edema[a]** *Δ mg/2 h*		**Ear pruritus[a]** *Δ scratching bouts/1 h*	
1H-indole like derivatives									
JNJ7777120	↓↓	IC_{50} = 5.57 nM	0	-	30	↓	12.18 ± 1.88	0	79.00 ± 18.23
MWJ-3	↓	IC_{50} = 399.9 nM	0	-	-	-	*n.d.*	-	*n.d.*
JNJ10191584	↓	IC_{50} = 948.2 nM	0	-	-	-	*n.d.*	-	*n.d.*
1,3,5 – triazine derivatives									
JN-25	↓↓	IC_{50} = 2.31 nM	↓	IC_{50} = 2814 nM	50	↓	7.45 ± 2.76	0	109.50 ± 9.57
KP-9D	↓↓	IC_{50} = 2.42 nM	0	-	-	-	*n.d.*	-	*n.d.*
TR-AF-45	↓↓	IC_{50} = 14.82 nM	↓	IC_{50} = 4240 nM	-	-	*n.d.*	-	*n.d.*
TR-AF-49	↓	IC_{50} = 138.4 nM	0	-	-		*n.d.*	-	*n.d.*
JN-35	↓	IC_{50} = 1119 nM	↓	IC_{50} = 440 nM	50	↓	4.74 ± 2.39	↓	14.40 ± 5.34
TR-DL-20	↓/0	$IC_{50} \geq$ 1000 nM	0	-	-	-	*n.d.*	-	*n.d.*
TR-18	↑	EC_{50} = 403 nM	↑↑	IC_{50} = 185 nM	50	↓	10.70 ± 4.70	0	122.00 ± 6.87
TR-7	↑	EC_{50} = 11750 nM	↑	IC_{50} = 689 nM	50	↓	9.48 ± 2.74	0	119.20 ± 11.32

Table 2. Summary of pharmacological effects of tested ligands against *in vitro* and *in vivo* studies. The effects of these histamine H_4 receptor antagonists were characterized based on human eosinophils adhesion to endothelium assay as well as on croton oil-induced ear edema and pruritus in CD-1 mice. 0 = no effect; ↓ = decrease; ↑ = increase; *n.d.* = not determined.

[a]croton oil-induced ear edema and pruritus in CD-1 mice, s.c. – subcutaneously.

1*H*-indole derivatives exhibited similar effects. Compounds MWJ-3, JNJ10191584, and JNJ7777120 inhibited eosinophils adhesion in the presence of 1 μM histamine (Fig. 2) and were inactive (or weakly active) in absence of histamine. Among the studied compounds, JNJ7777120 was the most active one. These observations were in line with receptor affinity data and aequorin-based functional assay. JNJ7777120 was used as a reference compound in this study since it is a first highly selective and potent histamine H_4 receptor antagonist with proven anti-inflammatory properties (Jablonowski et al., 2003; Thurmond et al., 2004). Furthermore, it fully antagonized effects of histamine through histamine H_4 receptor in the presence of histamine, not affecting eosinophils adhesion when administrated independently (Grosicki et al., 2016).

A more diverse response was observed in the group of 1,3,5 – triazine derivatives. In this group of compounds three structurally different ligands (JN-25, KP-9D, and TR-AF-45) exhibited higher or similar activity as reference compound JNJ7777120. Interestingly, almost all 1,3,5 – triazine derivatives (except of KP-9D, TR-AF-49 and TR-DL-20) had a statistically significant effect on eosinophils adhesion, in the presence and the absence of histamine, indicating their intrinsic activity. Furthermore, two tested compounds TR-7 and TR-18 exhibited paradoxical and unexpected agonistic activity during the human eosinophils adhesion assay, both in the presence and without histamine (Fig. 3). During the pharmacological examination of triazine derivatives, some discrepancies between the human eosinophils functional test, the histamine H_4 receptor binding assay and the aequorin-based functional study were detected. For example, while the triazine derivative TR-DL-20 showed similarity between eosinophils adhesion inhibition potencies, and affinity towards human histamine H_4 receptor a difference to the potencies estimated from the aequorin-based functional assay was observed. We attribute the discrepancies between radioligand displacement and functional assays at

histamine H_4 receptor to tissue-dependent differences arising from use of different cell lines (Sf9 insect cells vs. CHO-K1 cells) in combination to differences in e.g. lipophilicity. Furthermore, previous test on TR-7 and TR-18, based on aequorin functional assay as well as forskolin-induced cAMP accumulation assay (Łażewska et al., 2014) indicated their antagonistic activity, rather than agonistic one (detected by eosinophils adhesion assay).

This differences requires further investigation, however, obtained results suggests that eosinophils assay might be an interesting testing platform that allows detecting additional pharmacological properties of the tested compounds, before their introduction into the clinical trials. Furthermore, these observations are in line with newly reemerging concept of cell-based phenotypic drug discovery, as the tested compounds did not shown clear target association activity referring to histamine H_4 receptor binding and functionality, but rather involve numerous other potential targets on human eosinophils. Such phenotypic approach might bypass the incomplete understood drug target mechanism and rather help in addressing full disease complexity with promising novel drugs (Moffat et al., 2017; Zheng et al., 2013).

For example, eosinophils assay detected robust anti-inflammatory activity of the JN-35 compound, although this ligand showed low affinity at histamine H_4 receptor and exhibited only mediocre receptor-mediated potency (Table 1). Interestingly, the JN-35 mediated reduction of eosinophils adhesion was independent from presence of histamine. JN-35 not only significantly inhibited eosinophils adhesion to endothelium but was also active during the *in vivo* examination. During the anti-inflammatory examination, JN-35 was the only tested compound that potently inhibited both the croton oil-induced ear edema and pruritus in CD-1 mice. At the same time, JN-35 exhibited low affinity towards histamine H_1 and H_3 receptors (Table 1), was not toxic against the tested cells and was classified as a ligand with moderate permeability across the biological barriers, according literature-based classification for Caco-2 permeability assays (Di and Kerns, 2016). The anti-inflammatory activity of JN-35 could be clearly demonstrated within our experiments, however, the observed effects seem to be independent of histamine receptors. This phenomenon could involve the release of other pro-inflammatory mediators such as leukotrienes and cytokines, that are inhibited by tested triazine

derivative and therefore, influencing leukocytes adhesion through activation of endothelial cells (Gimbrone et al., 1984; Jawień et al., 2005; Kita, 2011; Mogilski et al., 2017). Furthermore, some triazine derivatives were shown to modulate the activity of protein kinases e.g. during the anti-breast cancer treatment, which could explain observed JN-35 activity (Nie et al., 2007; Weixin et al., 2018). Another explanation might arise from the JN-35 metabolic study. JN-35 have weak metabolic stability (Fig. 5), that was in line with previous observations for the similar *N*-methyl piperazine triazine derivative TR-7 (Popiolek-Barczyk et al., 2018). Despite of its susceptibility for extensive biotransformation, TR-7 showed excellent effects *in vivo* (Popiolek-Barczyk et al., 2018). Thus, the metabolites of *N*-methyl piperazine triazines require more comprehensive studies and further scientific considerations due to their potential influence on observed *in vivo* effects.

Interesting results were also obtained for the two tested 1,3,5 – triazine derivatives, TR-7 and TR-18. These two compounds exhibited paradoxical agonistic activity during the human eosinophils adhesion assay. However, this was not in line with results from aequorin-based functional assay (Table 1), forskolin-induced cAMP accumulation assay (Łazewska et al., 2014) and with the *in vivo* examination, where both TR-7 and TR-18 compounds significantly inhibited croton oil-induced ear edema 2 h after drug administration. Moreover, in different studies, anti-inflammatory activities of TR-7 and TR-18 were further confirmed during the *in vivo* mouse studies, where both compounds reduced paw edema, mechanical and thermal hyperalgesia in the carrageenan-induced acute inflammation (Mogilski et al., 2017). Additionally, TR-7 and TR-18 directly influence process of cellular adhesion as they could decrease total granulocyte influx in the zymosan-induced mouse peritonitis model (Mogilski et al., 2017). Observed confusing effects could indicate unclear pharmacological profile of TR-7 and TR-18. One possible explanation for the obtained results involves inter-species differences in histamine H_4 receptor structure and function (Leurs et al., 2009; Liu et al., 2001; Oda et al., 2000). Secondly, observed agonist effect of the tested compounds was detected both in presence and absence of histamine. This suggests that compounds activity in adhesion assay might not be directly associated with histamine receptors but rather on off-target effects, unknown at this time.

It is important to be aware of the limitations of eosinophils adhesion assays, that might have direct effects on obtained results. For example, the limited number of eosinophils isolated from the human peripheral blood results in a small number of measurement points for analysis. In many cases, due to small number of measurements points, the saturation of concentration-response curves was not fully obtained. Furthermore, due to significant data variability, dependent on the health condition of blood donors, the calculated inhibitory potencies of tested compounds should be treated more like an approximation, rather than the accurate values. However, such data discrepancy obtained from the native human cells was also reported by other authors. For example, there exists a significant difference in the calculated potency of histamine itself, ranging from 8 nM to 100 nM, based on human eosinophils chemotaxis assays (Ling et al., 2004; O'Reilly et al., 2002; Reher et al., 2012).

5. CONCLUSIONS

Taking advantage of the human eosinophils adhesion assay to endothelium we demonstrated a distinct profile of pharmacological activities of 1*H*-indole-, and 1,3,5 – triazine based derivatives, in terms of their activity and potency. In particular, we revealed additional properties of tested compounds that could not be detected in previous assays employing artificial recombinant cell systems. Therefore, we believe that the human eosinophils-based adhesion assay to endothelium is a suitable model for further ligand research especially in the context of the renewed interest of cell-based phenotypic screening in drug discovery.

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Conflict of interest

The authors state no conflict of interest.

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Fig. 1. Histamine effect on human eosinophils adhesion to endothelial cells. Histamine increased the number of adherent eosinophils in a dose-dependent manner, with the EC_{50} of 336 nM. Data represented as mean ± S.E.M. [%] of n=3. ***) $P < 0.001$ compared with untreated cells (one-way ANOVA with Bonferroni's post-test).

Fig. 2. The effect of tested 1*H*-indole like derivatives on eosinophils adhesion to endothelial cells. Tested indole-like compounds downregulated the number of adherent eosinophils in presence of 1 µM histamine. This effect was not statistically significant in the absence of histamine. Data represented as mean ± S.E.M. [%] of n=6. *) $P < 0.05$, **) $P < 0.01$, ***) $P < 0.001$ compared with control (one-way ANOVA with Bonferroni's post-test). Control was defined as spontaneous eosinophils adhesion to endothelium in presence/absence of 1 µM histamine. Due to the donor to donor data variability, data were calculated against the control (0 or 1 µM histamine) separately for each experimental data set.

Fig. 3. The effect of tested triazine derivatives on eosinophils adhesion to endothelial cells. All of the tested compounds affected eosinophils adhesion when histamine was present in the assay. Ligands KP-9D, TR-AF-49 and TR-DL-20 were inactive when histamine was absent. Compounds JN-25, KP-9D, TR-AF-45, TR-AF-49, TR-DL-20, and JN-35 downregulated the number of adherent eosinophils, whereas TR-7 and TR-18 increased the number of adherent cells. Data represented as mean ± S.E.M. [%] of n=4 - 6. *) $P < 0.05$, **) $P < 0.01$, ***) $P < 0.001$ compared with control for samples in presence of 1 µM histamine. #) $P < 0.05$, ##) $P < 0.01$, ###) $P < 0.001$ compared with control for samples in absence of histamine (one-way ANOVA with Bonferroni's post-test). Control was defined as spontaneous eosinophils adhesion to endothelium in presence/absence of 1 µM histamine. Due to the donor to donor data variability, data were calculated against the control (0 or 1 µM histamine) separately for each experimental data set.

Fig. 4. The cytotoxic effect of the JN-35 compound against the HEK-293 and IMR-32 cell lines. The effect of compound JN-35 and the reference drug doxorubicin (DX) on HEK-293 (A) and IMR-32 (B) and cell lines viability after 48 h of incubation. Data represented as mean ± SD [%] of n=4. ***) $P < 0.001$ compared with control (control – cells not treated, one-way ANOVA with Bonferroni's post-test).

Fig. 5.1. The MetaSite 6.0.1. software prediction of the most probably sites of JN-35 metabolism. The darker red color - the higher probability to be involved in the metabolism pathway. The blue circle marked the site of compound with the highest probability of metabolic bioconversion.

Fig. 5.2. UPLC (A) and MS (B, C) spectra of JN-35 reaction with mouse liver microsomesin TRIS buffer pH = 7.4, 120 min, 37°C.Two metabolites found. No substrate observed.

Fig. 5.3. The most probable structures of JN-35 metabolite M1 (m/z = 329.30) and M2 (m/z = 331.23) determined according to in silico and in vitro data.

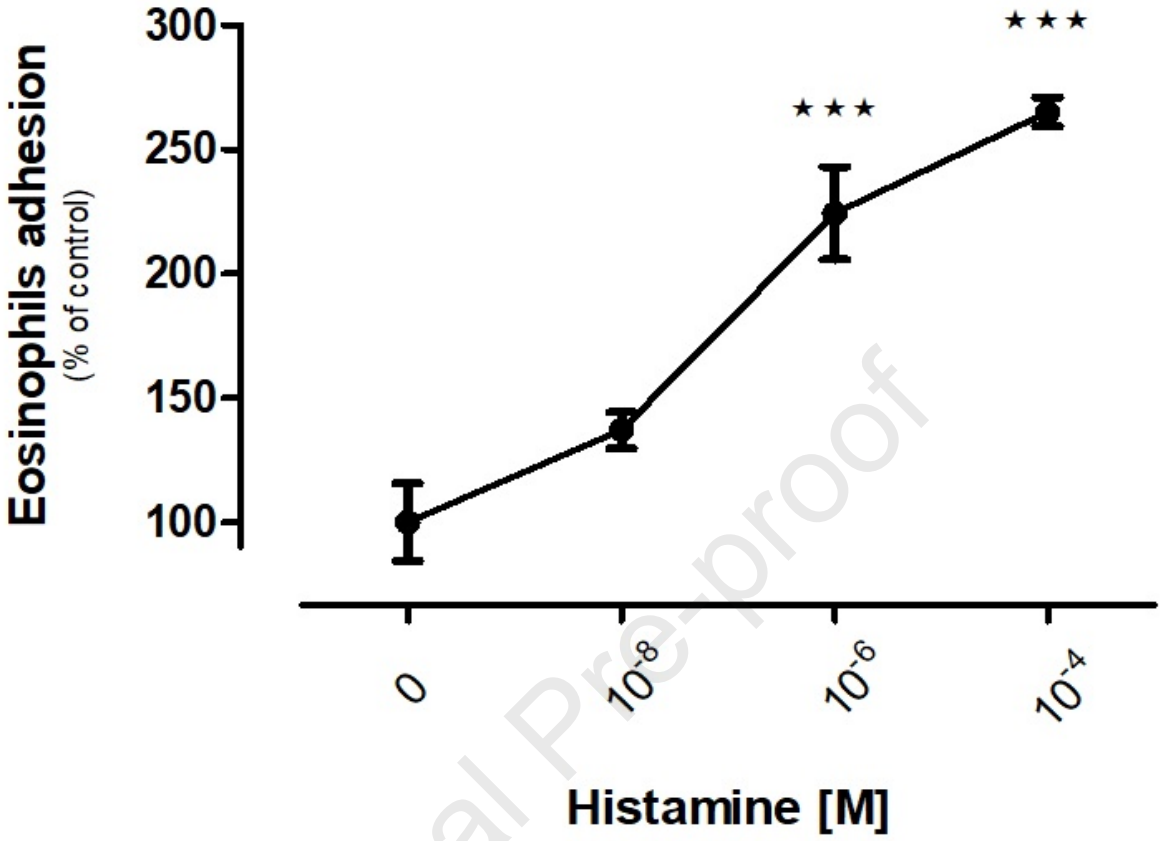
Fig. 5. The JN-35 metabolic stability studies.

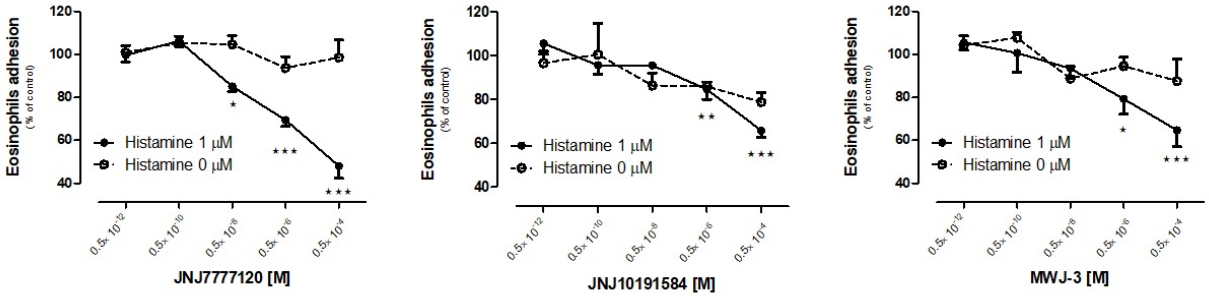
Fig. 6. Effects of tested ligands on croton oil-induced ear edema in CD-1 mice. Single s.c. administrations of tested ligands were given immediately before the topical application of croton oil. On the ordinate differences of ear weight (Δ mg) between right (inflamed) and left (uninflamed) ears at 2 or 4 h. Mean ± S.E.M. of n= 5-10 mice for each group. **) $P < 0.01$ compared with vehicle-treated animals (Student's *t*-test).

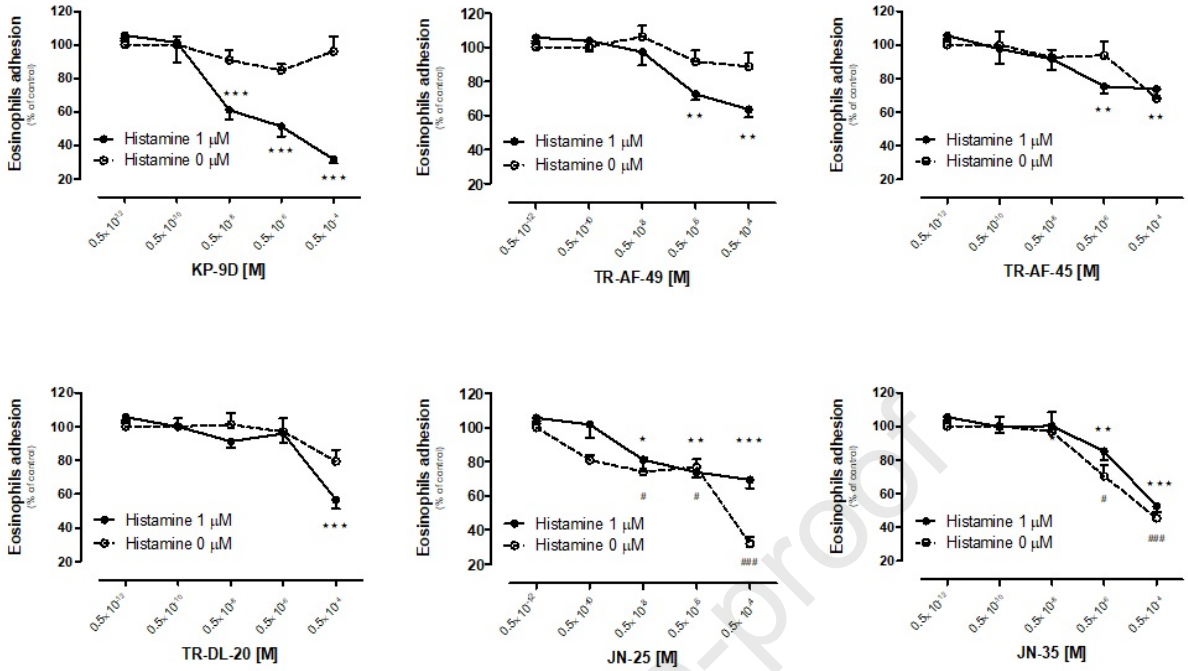
Fig. 7. Effects of tested ligands on croton oil-induced ear pruritus in CD-1 mice. Single s.c. administrations of tested ligands were given immediately before the topical application of croton oil. On the ordinate difference in counting the total scratching bouts between right (inflamed) and left (uninflamed) ear at 1 h (Δ scratching bouts/1 h). Mean ± S.E.M. of n=5-10 mice for each group. ***P*<0.01 compared with vehicle-treated animals (Student's *t*-test).

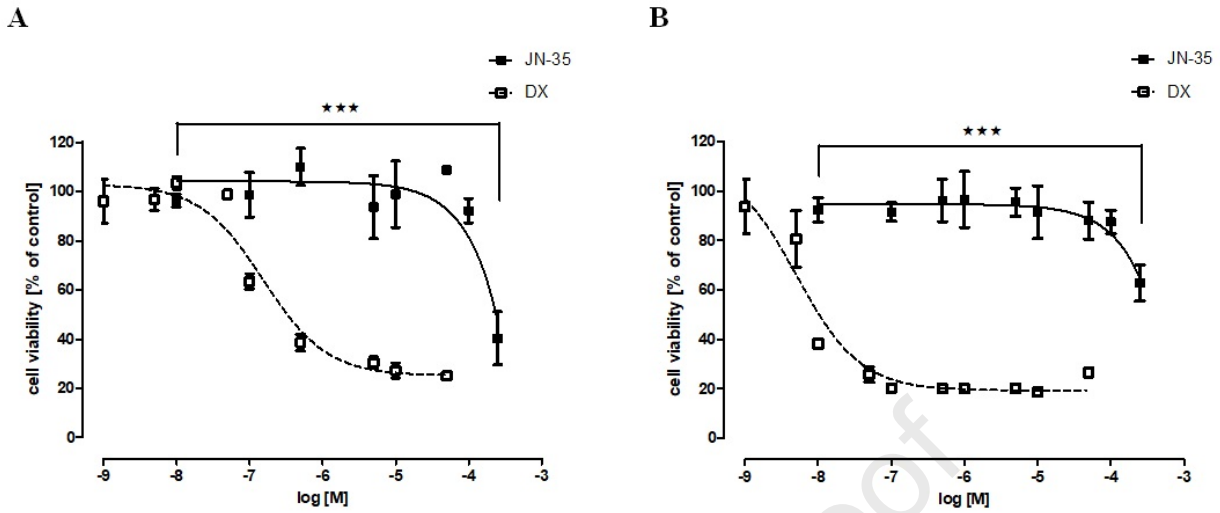
Ligand	Molecular mass [g/mol]	LC-MS purity	Affinity K_i [nM] ± SEM			pIC_{50}± SEM
			Human histamine H_1 receptor	Human histamine H_3 receptor	Human histamine H_4 receptor	(antagonistic activity)
1H-indole like derivatives						
JNJ10191584[a]	278.7	100%	nt	14.1×10^3	26 ± 8	8.46 ± 0.020
JNJ7777120[b,c]	277.9	100%	$>10\times10^3$	5.1×10^3	4 ± 1	7.87 ± 0.100
MWJ-3[d]	322.7	99,01 %	nt	nt	6.4 ± 0.1	6.23 ± 0.207
1,3,5 – triazine derivatives						
TR-AF-45	264.4	100%	nt	nt	247*	9.81 ± 0.029
TR-DL-20	288.4	100%	nt	nt	4700	8.44 ±0.083
KP-9D[e]	289.9	98,60 %	nt	nt	289 ± 133	8.13 ± 0.127
TR-18[f]	349.2	100%	nt	15×10^3	524 ± 159	8.04 ± 0.063
TR-AF-49	290.4	100%	$>5\times10^3$	nt	160*	7.50 ± 0.054
TR-7[f]	304.9	100%	$>5\times10^3$	13.2×10^3	203 ± 37.5	7.16 ± 0.017
JN-35	298.4	100%	$>2\times10^3$	6.1×10^3	5995	6.51 ± 0.045
JN-25[g]	330.9	100%	nt	$<30\times10^3$	253 ± 75	6.43 ± 0.020

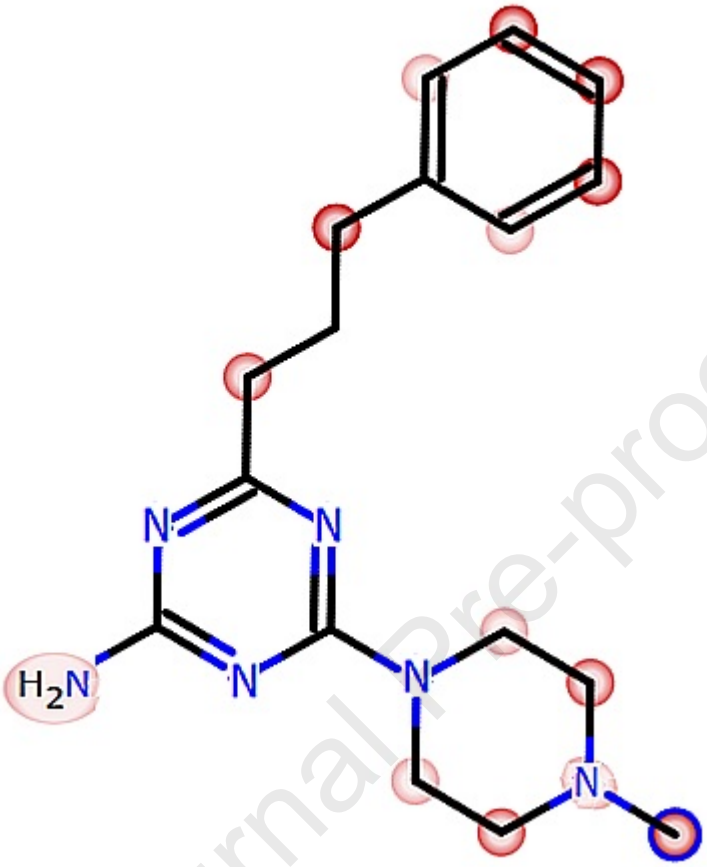
	***IN VITRO* DATA**				***IN VIVO* DATA**				
Ligand	**Eosinophils adhesion in the <u>presence</u> of histamine**		**Eosinophils adhesion in the <u>absence</u> of histamine**		**Dose** *mg/kg s.c.*	**Ear edema[a]** *Δ mg/2 hrs*		**Ear pruritus[a]** *Δ scratching bouts/1 hr*	
1H-indole like derivatives									
JNJ7777120	↓↓	IC_{50} = 5.57 nM	0	-	30	↓	12.18 ± 1.88	0	79.00 ± 18.23
MWJ-3	↓	IC_{50} = 399.9 nM	0	-	-	-	*n.d.*	-	*n.d.*
JNJ10191584	↓	IC_{50} = 948.2 nM	0	-	-	-	*n.d.*	-	*n.d.*
1,3,5 – triazine derivatives									
JN-25	↓↓	IC_{50} = 2.31 nM	↓	IC_{50} = 2814 nM	50	↓	7.45 ± 2.76	0	109.50 ± 9.57
KP-9D	↓↓	IC_{50} = 2.42 nM	0	-	-	-	*n.d.*	-	*n.d.*
TR-AF-45	↓↓	IC_{50} = 14.82 nM	↓	IC_{50} = 4240 nM	-	-	*n.d.*	-	*n.d.*
TR-AF-49	↓	IC_{50} = 138.4 nM	0	-	-		*n.d.*	-	*n.d.*
JN-35	↓	IC_{50} = 1119 nM	↓	IC_{50} = 440 nM	50	↓	4.74 ± 2.39	↓	14.40 ± 5.34
TR-DL-20	↓/0	$IC_{50} \geq$ 1000 nM	0	-	-	-	*n.d.*	-	*n.d.*
TR-18	↑	EC_{50} = 403 nM	↑↑	IC_{50} = 185 nM	50	↓	10.70 ± 4.70	0	122.00 ± 6.87
TR-7	↑	EC_{50} = 11750 nM	↑	IC_{50} = 689 nM	50	↓	9.48 ± 2.74	0	119.20 ± 11.32

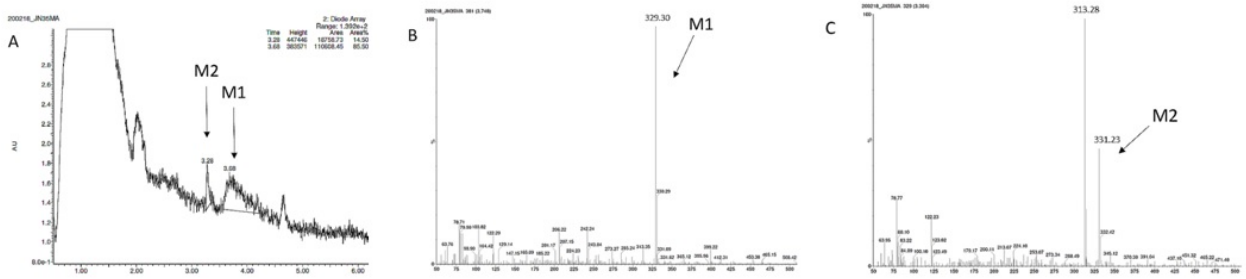




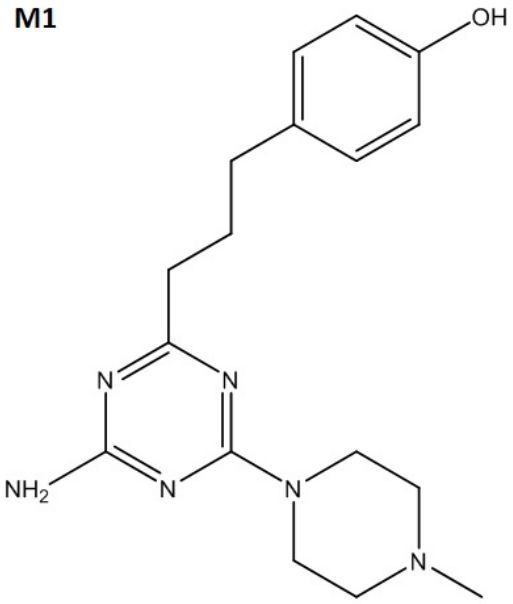








M1



M2