Effect of the Fexofenadine on the expression of HRH-1 and HRH-4 receptor in Peripheral Blood Mononuclear Cell isolated from children with diagnosed allergy – *in vitro* study *Short communication*

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ABSTRACT - Purpose: Fexofenadine (FXF) is the active metabolite of terfenadine with selective peripheral H_1 receptor antagonist activity. FXF is a third-generation antihistamine, non-sedating, rapid and very long acting used in symptoms associated with allergic diseases such as allergic rhinitis, asthma and dermatitis. The pleiotropic effects of histamine are mediated by four types of receptors that belong to the G-protein-coupled receptor family: histamine H_1 receptor (HRH-1), histamine H_2 receptor, histamine H_3 receptor, and histamine H_4 receptor. Our hypothesis is that HRH-4 opens new possibility in treatment in allergy diseases and FXF could be the antagonist of both HRH-1 and HRH-4. **Methods:** We isolated a peripheral blood mononuclear cell (PBMC) from children with diagnosed allergies and healthy – control group and measured the *HRH-1* and *HRH-4* mRNA gene expression using Quantitive Real-Time PCR. We obtained the results from basal gene expression and after FXF and histamine stimulation. **Results:** *HRH-1* mRNA basal gene expression shows significantly higher, and *HRH-4* shows significantly lower expression in allergy group compared to control. In both groups *HRH-1* mRNA gene expression was observed as statistically significant increased after histamine stimulation compared to cells not treated, while in HRH-4 only in allergy group we observed statistical increase. FXF successively blocked histamine affinity in HRH-1 mRNA gene expression but not in HRH-4, where we not observed any reaction. **Conclusions**: Results clearly overturned our hypothesis about the possibility of using FXF to block over-expression HRH-4 and open new way of treatment in allergy diseases.

INTRODUCTION

Fexofenadine (FXF) is the active metabolite of terfenadine with selective peripheral H_1 receptor antagonist activity. It inhibits antigen-induced bronchospasm in sensitized guinea pigs and also histamine release from peritoneal mast cells in rats. FXF is a third-generation antihistamine, non-sedating, rapid and very long acting used in symptoms associated with allergic diseases such as allergic rhinitis, asthma and dermatitis. It is not metabolized by the liver and has no effect on cytochrome P450. In addition, human clinical and animal studies have shown that FXF is free of cardiotoxicity and nervous system effects, such as those witnessed with other non-sedating anti-histamines [1 – 4].

The pleiotropic effects of histamine are mediated by four types of receptors that belong to the G-protein-coupled receptor family: histamine H1 receptor (HRH-1), histamine H2 receptor, histamine H3 receptor, and histamine H4 receptor. H1 receptors are expressed on multiple cell types including endothelial cells and smooth muscle cells, where they mediate vasodilation and bronchoconstriction. Antagonists of H1 receptors, such as FXF have been used for many years in the treatment of allergic inflammatory responses [5]. Histamine H4 receptor activation promotes the accumulation of inflammatory cells at sites of allergic inflammation. It was recently reported that the histamine H1-/H2-receptor antagonist doxepin and the histamine H1 receptor antagonist cinnarizine and promethazine exhibit high affinity binding to the histamine H4 receptor [6 – 7]. The data regarding the potential impact of FXF on HRH-1 and HRH-4 mRNA gene expression in peripheral blood mononuclear cells (PBMCs) are not available. The role for HRH-4 in many cells associated with asthma and allergy suggests that it may be involved, but as of yet, no trials with HRH-4 antagonists have been reported [5]. Our hypothesis is that HRH-4 opens new possibility in treatment in allergy diseases and FXF could be the antagonist of both HRH-1 and HRH-4.

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MATERIALS AND METHODS

The study was approved by the Local Bioethics Committee (No. 19/2016; 18/5/2016). The patients were recruited by specialists in the Regional Specialized Children's Hospital in Olsztyn, Poland, and informed consent was obtained from all children's parents and the control group comprised 28 healthy children with no history of behavioural disorders and the study group consisted of 30 children with diagnosed allergies. Demographic and clinical characteristic of the study population are shown in Table 1.

Chemicals

Fexofenadine (FXF; PubChem CID: 63002), osthole (PubChem CID: 10228) and histamine (PubChem CID: 774) were obtained from Sigma-Aldrich, ST. Lois, MO, USA. The FXF was dissolved in 8% dimethylsulfoxide (DMSO, Sigma-Aldrich, ST. Lois, MO, USA) to final concentration below 0.1%; thus not affecting cell viability. All solutions were sterilized through a 0.22 µg/ml filter and stored at 4° C as stock solutions for later dilution.

PBMC isolation, incubation, RNA preparation

Fresh PBMC's were then prepared as previously described by Kordulewska [1 – 3]. PBMC's were counted by Scepter automatic cell counter and seeded for up to 3 days in 24-well plates in 1×106 /0.5 ml of RPMI- 1640 containing 1% gentamicin, 1% human AB serum, and 0.25% phytohaemagglutinin; at 37 °C in humidified 5% CO2. PBMC's were in medium alone or with histamine, FXF in concentrations of 150 ng/ml histamine and 300 ng/ml FXF and mixture of them. These concentrations were chosen because this is the FXF human serum level following its administration; They are therapeutically relevant in human serum. The mixture of histamine and FXF where used to first of all induce the allergic inflammation after histamine stimulation and then FXF was added to check how the drug affect our cultured PBMC's. The incubated cells suspension was then centrifuged at 800g and 20 °C for 5 min and the cell residue was rinsed twice with Dulbecco's phosphate-buffered saline. The supernatant and plasma were collected and stored at −80 °C for further analysis. Cells for the RNA isolation were collected by centrifugation at 800 × g for 10 min at 20° C (Eppendorf Centrifuge 5804R, Germany) and lyzed in 1 ml TRIzol Reagent (Life Technologies, UK) as described by Kordulewska et al. 2017 [3].

Reverse transcription

Purified RNA (50–200 ng) was reverse transcribed by High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, UK) according to manufacturer instructions and templates were store at −20° C in RNase-free water for further gene expressions made by quantitative real-time RT-PCR.

Quantitative real-time RT-PCR

Conditions for all RT-PCR analysis were optimized at 55–65°C melting point and primer concentrations before commencing relevant experiments. Two genes: HRH-1, HRH-4 and the housekeeping human-actin gene (ACTB) were examined; with ACTB used as reference gene to normalize differences in total RNA amounts in each sample. Oligonucleotide primers specific to each gene were designed with Primer-BLAST: HRH-1(*NM_001098213.1*) F 5'GCCGAGAGGACAAGTGTGA3', R 5'GGAGACTCCTTCCCTGGTTT3' 63°C; HRH-4 (*NM_021624.3*) F 5'ACTCTGATGGTGGCCGTTT3', R 5'TCAGTCCAGGATGGCTTTG3' 63°C and ACTB (*NM-001101.3*) F 5'TCCCTGGAGAAGAGCTACGA3', 5'AGCACTGTGTTGGCGTACAG3' 60°C. RT-

Table 1. Demographic data and clinical characteristics of the study population.

Characteristics	Control	Allergy
Patient's samples, no.	28	30
Age (y), mean ± SD	7.60 ± 1.93	4 ± 1.75
Female sex, no. (%)	10 (35,7%)	8 (26,6%)
Diagnosed Allergy, no. (%)	0 (0%)	30 (100%)
Moderate/severe asthma, no. (%)	0 (0%)	15 (50%)
Skin prick test positivity, no. (%)	0 (0%)	30 (100%)
cIgE [IU/mL] mean ± SD	90.13 ± 43.93 ****	307.7 ± 130.0
a-sIgE [IU/mL] class	Negative predictive value, class 0	Positive predictive value, class 4 - 6
Blood eosinophilia (mean % of eosinophils in the blood smear) mean ± SD	3.668 ± 1.104 ****	23.92 ± 5.587

Data are presented as the **mean ± SD.** **** $p < 0.0001$ control group vs. allergy group.

PCR was performed in the LightCycler 96 Real-Time PCR System with the FastStart Essential DNA Green Master Kit (both Roche Diagnostics, Switzerland) as previously described Kordulewska et al. 2017 [3].

STATISTICAL ANALYSIS

All statistical analyses were performed in triplicate by GraphPad Prism version 6.0 (GraphPad Software, Inc., USA). The statistical significance of difference between mean values ± standard error was determined by analysis of variance (ANOVA); with $p<0.05$ (95% confidence interval).

RESULTS

Basal expression of HRH-1 and HRH-4 receptors

After 3 days incubation, we detected that *HRH-1* receptor shows significantly higher and *HRH-4* receptor shows significantly lower expression in allergy group compared to control (Figs. 1A, B).

HRH-1 gene expression

Cultured cells were incubated with and without histamine (150 ng/ml), FXF (300 ng/ml) and histamine/FXF 1:2 (v/v) for 72 hours to analyze their effect on *HRH-1* mRNA expression and this was measured by real-time PCR (Fig. 2A). In control group histamine 2.6-fold increased expression of *HRH-1* mRNA compared to cells without stimulation. In allergy group histamine increased 2.8-fold. We noted that FXF in both group significantly decreased expression of *HRH-1* mRNA compared to histamine stimulation. What is more, in cells after stimulation of mixture histamine/FXF 1:2 (v/v) we do not observe any significant differences compared to cells treated only FXF in allergy group.

HRH-4 gene expression

Our results showed 4-fold increased induction of *HRH-4* gene expression in response to histamine in children with diagnosed allergies (Fig. 2B). Incubation PBMC with FXF showed no differences compared to control, what is more the same situation we noted in cells treated with histamine and mixture of histamine/FXF. That's mean that FXF do not have any blocked HRH-4 receptor in PBMCs.

DISCUSSION

The ability of histamine to produce allergic responses in humans is incontrovertible. However, its involvement in various allergic diseases has been debated, largely because of the ineffectiveness of existing HRH-1 antagonists, like FXF in the clinic [8]. However, it is now known that the diverse biological effects of histamine are mediated through four different histamine receptors, including histamine HRH-4 receptor [5]. The FXF that are currently used in the clinic have big affinity for the HRH-1 receptor and this receptor has been shown to function in inflammatory responses *in vitro* and *in vivo*, what we also confirmed in this research.

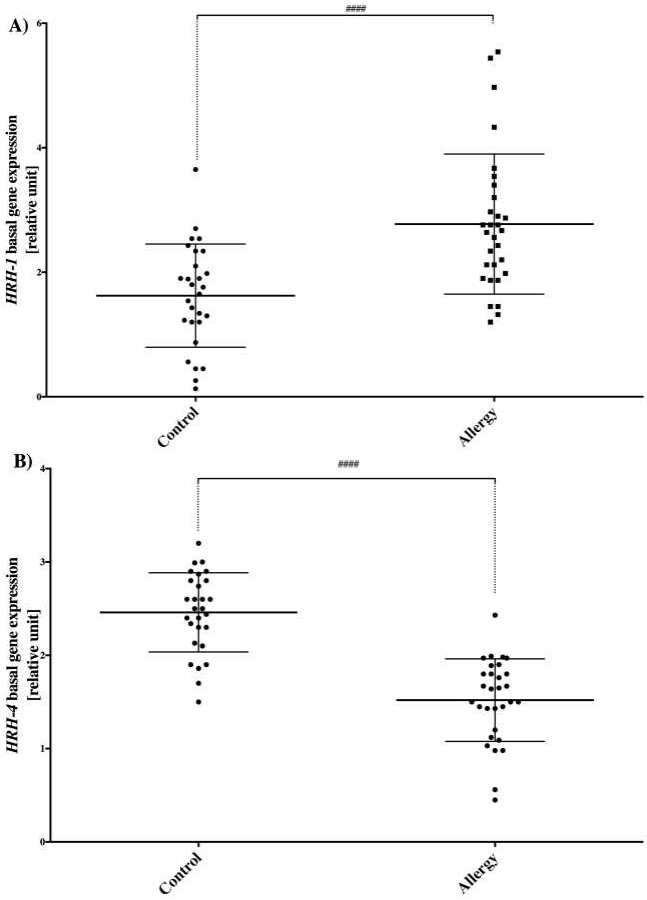

Figure 1: Basal expression of **A)** *HRH-1 receptor*, **B)** *HRH-4 receptor* in cultured PBMC from control and allergy group. Statistically significant differences between the control and tested samples are directly above the error bar: **** $p<0.0001$.

FXF is an antihistamine pharmaceutical drug used in the treatment of allergy symptoms. Therapeutically is a selective peripheral HRH-1-blocker. Blockage prevents the activation of the HRH-1 receptors by histamine, preventing the symptoms associated with allergies from occurring [9]. In our research we confirmed that FXF blocked HRH-1 receptor and significantly decreased histamine action in PBMC isolated from children.

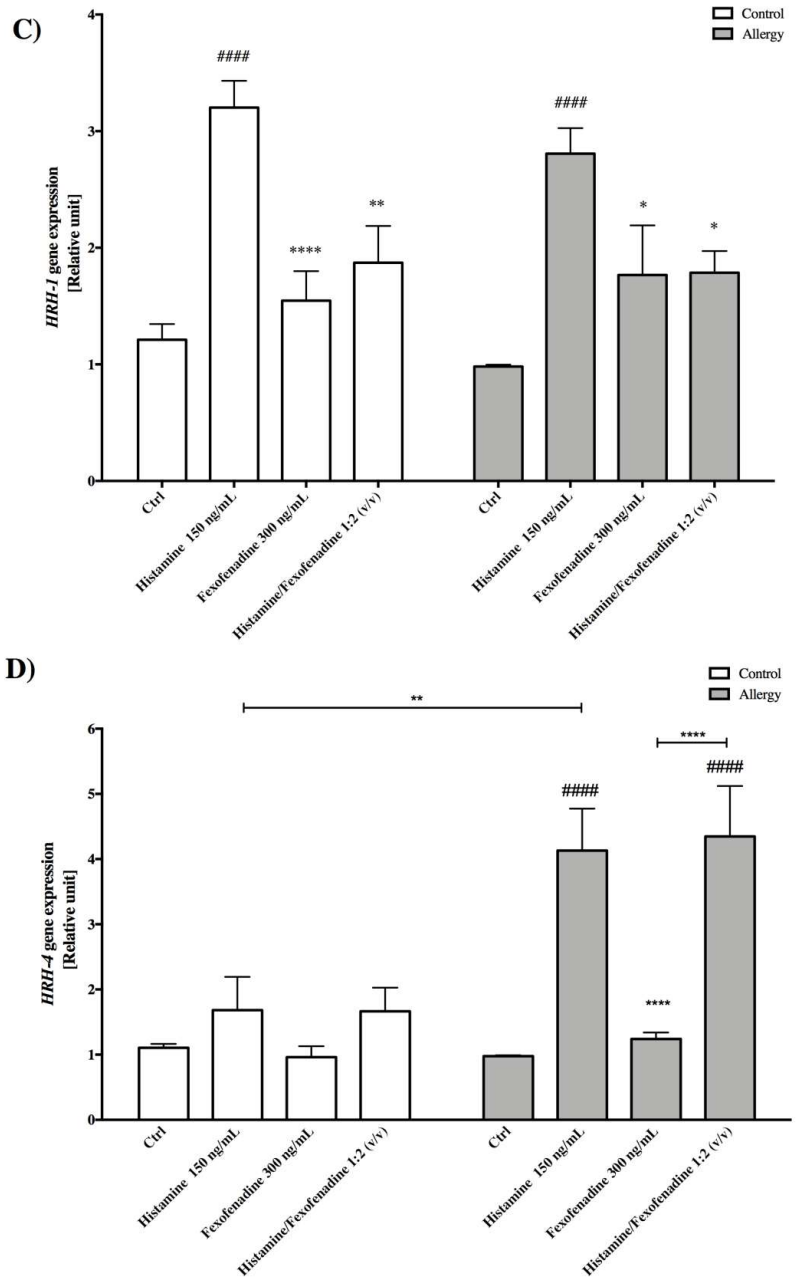

Figure 2: Comparison of mRNA gene expression changes in control and allergy group PBMC's under the influence of histamine, FXF and histamine/FXF between control and allergy group. **A)** *HRH-1 receptor*, **B)** *HRH-4 receptor*. The ctrl is gene expression in native cells; presented as 1. Statistically significant differences between the control and tested samples are directly above the error bar: Data are presented as the mean ± S.E.M. ### $p<0.001$, #### $p<0.0001$ vs. control; *$p<0.05$, ** $p<0.01$, **** $p<0.0001$ vs. treated histamine cells.

What is more, we demonstrated that children with diagnosed allergies have increased basal gene expression of *HRH-1* compared to control. The significantly higher expression of *HRH-1* observed in the allergy group, can be caused by higher levels of histamine and IgE in serum, which could affect the reaction of HRH-1.

Literature data states [10] that expression of the HRH-4 has been difficult to identify conclusively which cell types express the HRH-4 because this receptor is expressed at low levels in most tissues. Furthermore, expression seems to be controlled by inflammatory stimuli [9], what we also confirmed. After histamine stimulation, we noted 4-times fold increased HRH-4 gene expression compared to cells not treated. However, there are a few reports of the expression of the HRH-4 receptors: Northern-blot data show that the

receptor is present in the bone marrow and spleen, and on eosinophils and mast cells in human. Additionally, expression has been determined in human mast cells and in basophils by several groups [8]. In our research we confirmed that expression of *HRH-1* and *HRH-4* is observed in PBMC isolated from children blood. The same results were obtained by GANTNER ET AL., (2002) in CD8+ cells, which express the HRH-4 receptor by RT–PCR and this is confirmed by HRH-4-dependent bioactivity [11].

Currently, little is known about the FXF (ligand-binding site) – of the HRH-4 receptor. The binding pocket for histamine has been identified in a pocket formed by transmembrane domain 3 (TM3), TM5 and TM6 [12]. We noted that FXF had no effect on *HRH-4* mRNA expression. Furthermore, FXF did not block the action of histamine. Our results clearly overturned our hypothesis about the possibility of using FXF to block over-expression HRH-4.

Obtained results indicated also that, in allergy group expression mRNA of HRH-4 is significantly lower than in control group. This may justify the atopic symptoms of allergic inflammation resulting from the lack of suppression of the immune response in which HRH-4 seems to play an important role.

Conflict of interest: None of the authors reported financial interest or potential conflict of interest, nor have financial relationship with commercial entities who have interest in the subject matter of this manuscript.

Ethical approval: All procedures performed in our studies with human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments and comparable ethical standards.

Informed consent: Informed consent was obtained for all individual study participants.

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