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Development of novel cellular model for affinity studies of histamine H_4 receptor ligands*

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The G protein-coupled histamine H_4 receptor (H_4R) is the last member of histamine receptors family discovered so far. Its expression pattern, together with postulated involvement in a wide variety of immunological and inflammatory processes make histamine H_4 receptor an interesting target for drug development. Potential H_4R ligands may provide an innovative therapies for different immuno-based diseases, including allergy, asthma, pruritus associated with allergy or autoimmune skin conditions, rheumatoid arthritis and pain. However, none of successfully developed selective and potent histamine H_4 receptor ligands have been introduced to the market up to date. For that reason there is still a strong demand for pharmacological models to be used in studies on potent H_4R ligands. In current work we present the development of novel mammalian cell line, stably expressing human histamine H_4 receptor, with use of retroviral transduction approach. Obtained cell line was pharmacologically characterized in radioligand binding studies and its utility for affinity testing of potent receptor ligands was confirmed in comparative studies with the use of relevant insect cells expression model. Obtained results allow for statement that developed cellular model may be successfully employed in search for new compounds active at histamine H_4 receptor.

Key words: retroviral transduction system, histamine H_4 receptor, radioligand binding studies, recombinant proteins

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INTRODUCTION

Human histamine H_4 receptor, being a biological target of presented studies is, by far, the last histamine receptor discovered. Its gene has been cloned ca. 2000, independently by several groups (Leurs *et al.*, 2009). Histamine H_4 receptor expression on basophils, eosinophils, mast cells and dendritic T cells suggests its role in immunological answer (Nguyen *et al.*, 2001). Therefore its ligands could provide promising drugs for immunobased diseases (Jablonowski *et* al., 2004; Tiligada *et al.*, 2009; Zampeli & Tiligada, 2009), however none of the potent ligands was brought to the market so far. Thus, histamine H_4 receptor remains an important target in drug development (Smits *et al.*, 2009) and for that reason development of new experimental models for pharmacological studies of receptor ligands is still a scientific issue of high importance.

Recombinant receptors systems are pharmacological tools widely utilized in search for novel ligands of G-protein coupled receptors (GPCRs) (Kenakin, 1996). Contrary to historically used native receptors systems, they can be involved in studies on poorly characterized receptors, for which selective ligands are not known and low expression levels are observed in native cells (Sarramegna *et al.*, 2003). On the other hand, main limitations of recombinant receptors experiments include: the risk of measuring artifacts, related to disrupted proportions of receptors and G proteins molecules (Kenakin, 1997) as well as influence of artificial environment of host cells devoid of natural allosteric modulators (e.g. other receptors) (Haack & McCarty, 2011) or functional specialization. Despite mentioned disadvantages heterologous expression of GPCRs is dominantly used for development of models for molecular pharmacology studies on metabotropic receptors.

Recombinant proteins can be obtained in various expression systems (Baldi *et al.*, 2007). In studies on GPCRs, mammalian cell lines are predominantly used. These models guarantee satisfactory grade of posttranslational protein modifications and enable cellular membrane localization of recombinant proteins. Moreover, mammalian cells contain whole range of necessary intracellular signaling pathway elements (Hermans, 2004; Siehler, 2008). In the group of cells most frequently used in recombinant receptors production — CHO (Chinese hamster ovary) cell line is playing a dominant role, due to easiness of cell culture and transfection, lack of significant expression of many investigated receptor in wild-type cells and confirmed in numerous experiments usefulness of mentioned cell line for studies on G-protein coupled receptors pharmacology (Gazi *et al.*, 1999; Nash *et al.*, 2001).

Gene transfer to the considered cells in recombinant protein production procedure can be achieved by means of transfection or viral transduction. Retroviral transduction system is an example of second method. It is based on the coordinated design of packaging cell lines and retroviral expression vectors (Morgenstern & Land, 1990; Cepko & Pear, 2001). To develop a packaging cell line, the viral *gag*, *pol* and *env* genes are stably integrated into the genome of the packaging cell line (Markovitz *et al.*, 1988). The separate introduction and integration of the structural genes minimizes the chances of producing replication-competent virus due to recombination events during cell proliferation. Retroviral expression vectors provide the packaging signal Psi^+, transcription and pro-

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Abbreviations: CHO, Chinese hamster ovary; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GPCR, G-protein coupled receptor; H_4R, histamine H_4 receptor; PBS, phosphate buffered saline; PCR, polymerase chain reaction

cessing elements and a target gene. Once a packaging cell line is transfected with a retroviral expression vector that contains a packaging signal, the viral genomic transcript containing the target gene and a selectable marker are packaged into infectious viruses within 48–72 h. Produced virus can infect target cells and transmit target genes; however, it cannot replicate within target cells because the viral structural genes are absent.

Described technique was used in current study to express human histamine H_4 receptor in CHO cells. As the obtained cell line is supposed to be used in affinity testing of potent receptor ligands it was basically characterized in various radioligand binding assays.

MATERIALS AND METHODS

Reagents. The pcDNA 3.1 plasmid, containing human histamine H_4 receptor cDNA sequence (pcDNA3.1-*h*H4R), consistent with GenBank database entry No. AY136745 (Benson *et al*., 2013) was obtained from Missouri S&T cDNA Resource Center, Rolla, MO, U.S. Packaging cell line GP+envAM12 (Markovitz *et* al., 1988) was a kind gift from Prof. Dr. C.E. Müller (Pharma-Zentrum, Bonn, Germany). Baculoviruses encoding the human histamine H_4 receptor were prepared as described recently (Schneider *et al*., 2009). Baculoviruses encoding the G proteins $G\alpha_{i2}$ and $G\beta_1\gamma_2$ subunits together with Sf9 cells were kindly provided by Prof. Dr. Holger Stark (Institute for Pharmaceutical and Medicinal Chemistry, Heinrich-Heine-University Duesseldorf, Germany). 4-methylpiperazino-amide derivatives (H4-21, H4-23, DB-5-DB-7) were synthesized in the Department of Technology and Biotechnology of Drugs, Medical College, Jagiellonian University, Kraków as described previously (Karcz *et al*., 2010). Unlabelled histamine was from Sigma-Aldrich, St. Luis, MO, U.S. Radioactively labelled [^{3}H]histamine (specific activity 10.6 Ci/mmol) and reference membrane preparations from CHO cells stably expressing *h*H_4R were obtained from PerkinElmer, Waltham, MA, U.S. Stock solutions (10 mM) of test compounds were prepared in DMSO.

Molecular cloning. Human histamine H_4 receptor cDNA was obtained by PCR amplification from pcDNA3.1-*h*H4R plasmid using forward primer introducing BamHI recognition sequence and reverse primer carrying NotI restriction site. The entire cDNA sequence was then introduced into pQCXIN retroviral vector (Takara Bio Europe/Clontech Inc., Saint-Germain-en-Laye, France) with BamHI/NotI restriction enzymes, creating pQCXIN-*h*H4R vector. The result of molecular cloning experiment was confirmed by means of restriction analysis and DNA sequencing.

Retroviral transduction system. The packing cell line (GP+envAM12) was splitted one day before transfection (1.0×10^6 in 25 cm^2 flask) in DMEM medium, supplemented with 10% (v/v) fetal bovine serum. Cells were transfected by using TurboFect reagent (Thermo Fisher Scientific Inc., Waltham, MA, U.S.). Briefly, 18 μg of DNA (11.25 μg of pQCXIN-*h*H4R plasmid and 6.75 μg of pcDNA3.1-VSV-G — encoding G Protein of Vesicular Stomatitis Virus) was diluted to final volume of 600 μl with DMEM (without FBS or antibiotics); then, 24 μl of TurboFect reagent was added to the diluted DNA and obtained mixture was incubated for 15 minutes before adding to the GP+envAM12 cells (80% confluent; 25 cm^2 flask). 14 hours after transfection of packaging cells culture medium was changed with 3 ml of fresh medium (DMEM, 10% (v/v) FBS, 100 U/ml penicillin G, 100 μg/ml) and 30 μl of sodium butyrate (500 mM in H_2O) was added. The cells were further cultured at 32°C for 48 h. Finally, 3 ml of virus supernatant were collected and filtered through 45 μm filter. The sterile virus supernatant together with 2.4 μl of polybrene solution (10 mg/ml in H_2O) was transferred into CHO cells culture (80% confluent, 25 cm^2 flask) for 2 hours and after that time medium was exchanged for a new one. 48 hours post-transduction cells were subcultured in growth medium containing 800 μg/ml geneticin and the selection of the positive clones was continued for two weeks. Resultant cell line: CHO-*h*H_4R, expressing human histamine H_4 receptor was used in further experiments.

Cell culture and membranes preparation of CHO-*h*H_4R cells. The cells were grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 supplemented with 10% (v/v) fetal bovine serum, and 200 μg/ml geneticin in an atmosphere of 5% CO_2 at 37°C. Cells were grown until reaching 80–90% confluence. After medium removal cells were washed with sterile PBS buffer and frozen at –80°C until further preparation.

Thawed cells were collected in 5 mM Tris/HCl and 2 mM EDTA, pH 7.4 (Tris/EDTA buffer) and were homogenized using Ultra-Turrax homogenizer. The resulting suspension was centrifuged at $1000\times g$ for 10 min to remove unbroken cells and nuclei. The supernatant was homogenized again with Ultra-Turrax and subsequently centrifuged at $48\,000\times g$ for 60 min. The resulting pellets ware resuspended in 50 mM Tris/HCl buffer, pH 7.4. Determination of membrane protein concentration was carried out by the method of Lowry and obtained membranes were stored at –80°C until required.

Cell culture and membrane preparation of Sf9 cells expressing the human histamine H_4 receptor. Sf9 cells were cultured in 250 ml Spinner flasks at 28°C under rotation at 100 rpm in SF 900 II medium supplemented with 5% (v/v) fetal bovine serum and penicillin (50 U/ml)/streptomycin (50 μg/ml) under normal atmosphere. Cells were grown at a density of 3×10^6 cells/ml and were infected with a combination of baculovirus stock solutions (1:100), each encoding for the human histamine H_4 receptor and G protein $G\alpha_{i2}$ and $G\beta_1\gamma_2$ subunits. 48 h after infection cells were harvested. Sf9 membranes were prepared as described previously (Schneider *et al*., 2009) and were stored in binding buffer (12.5 mM $MgCl_2$, 1 mM EDTA and 75mM Tris/HCl, pH 7.4) at –80°C till needed.

Radioligand binding assays at membranes preparations from recombinant CHO cells. Binding experiments were carried out as follows: membranes (15 μg protein per well for reference preparation from commercial source and 100 μg protein per well for preparation from retrovirally transduced CHO cells) were incubated with 10 nM [^{3}H]histamine solution and different concentrations of the respective test ligand, spanning a range of at least three log units, in total volume of 0.35 ml of assay buffer (50 mM TRIS/HCl, 5mM EDTA, pH 7.4). Differently, in case of saturation binding experiments CHO-*h*H4R cells membranes were incubated with 11 different concentrations of radioligand ranging from 0.5 to 350 nM. In all experiment incubations were performed for 60 min at 25°C with shaking and shaking at 250 rpm and non-specific binding was determined in the presence of 100 μM unlabelled histamine. Bound radioligand was separated from free radioligand by filtration through GF/B filters pretreated with 0.3% (m/v) polyethyleneimine and washed eight times with 0.2 ml of ice-cold washing buffer (50 mM Tris/HCl, pH 7.4). The amount of radioactivity collected on the filter was de-

termined by liquid scintillation counting. Determination of affinity values (K_i) was carried out using GraphPad Prism program (GraphPad Software Inc., La Jolla, CA, U.S.) according to Cheng-Prusoff equation (Cheng & Prushoff, 1973).

Radioligand binding assays at membranes preparations from recombinant Sf9 cells. Experiments were performed similarly to binding assays with use of CHO cells-based membranes preparations, however some minor modifications of protocol were included as follows. Assays were carried out in total volume of 0.2 ml of Sf9 binding buffer, containing 12.5 mM $MgCl_2$, 1 mM EDTA and 75 mM Tris/HCl, pH 7.4. 35 μg of protein was used per single sample and GF/B filters washing step included three repeats with 0.5 ml of Sf9 binding buffer. K_D constant for [^{3}H]histamine, determined in former experiments (Kottke *et al.*, 2011) and used for calculations of affinity values (K_i) was 11.66 nM.

RESULTS AND DISCUSSION

The aim of the study was to develop a novel cellular model suitable for affinity studies of histamine H_4 receptor ligands. Retroviral transduction system was employed in order to obtain stable expression of recombinant human histamine H_4 receptor protein in CHO cell line. Major advantage of proposed technique is achievement of stable and efficient integration of few copies of viral genes into a host chromosome, which is not always available in case of transfection methods (Cepko & Pear, 1996).

Following the molecular biology experiments, which led to obtaining of pQCXIN-*h*H4R retroviral vector, containing human histamine H_4 receptor cDNA sequence, transduction of target CHO cell line was performed with use of retroviral particles produced in GPenv+AM12 packaging cells. Transduced cells were subject to selection procedure with use of selective antibiotic geneticin in order to isolate positive clones stably co-expressing receptor gene together with antibiotic resistance gene from bicistronic transcript.

Resultant cell line was tested for its utility in radioligand binding assays. First, recombinant receptor was characterized in saturation binding assay with use of [^{3}H] histamine as a radioligand. Obtained results confirmed receptor expression in developed cell line. We were able to observe specific and saturable binding of radioligand in case of membranes preparations from developed cell line (Fig. 1). Basing on saturation binding experiment results K_D constant value was determined for proposed assay conditions, which was later used for calculations of affinity values (K_i) from IC_{50} values in competition studies. [^{3}H]histamine K_D value determined in saturation binding experiment equaled 16.1 nM and was in accordance to literature data (Jablonowski *et al.*, 2004; Kottke *et al.*, 2011).

Homological histamine competition binding assay was employed for comparison of obtained cell line's properties with reference membranes preparation from commercial source (PerkinElmer, Waltham, MA, U.S). Comparative binding experiments were performed simultaneously for both sources of cell membranes. Obtained results confirmed expected pharmacological properties of developed experimental model (Fig. 2). Determined IC_{50} values were in the same range for both membranes preparations, however expression level was significantly lower in case of prepared cell line, because higher amount of protein had to be used in order to obtain level of specific binding similar to commercial membranes (100 μg per sample *vs.* 15 μg per sample). Yet, usage of increased protein amount in assay resulted in elevated level of nonspecific binding (compare lower plateaus of curves on Fig. 2). Moderate receptor expression level in developed cell line may be due to erroneous folding or improper trafficking of recombinant protein to cellular membrane and its retention in subcellular compartments.

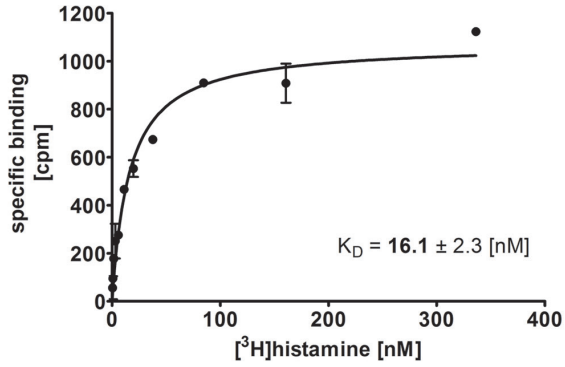


Figure 1. Saturation of [^{3}H]histamine binding to histamine H_4 receptors at CHO-*h*H_4R cells.
Saturation binding experiment was performed in triplicates with use of membranes preparations from CHO cells transduced with retroviral particles bearing cDNA sequence of human histamine H_4 receptor. Specific binding was determined as a difference between total binding measured in absence of unlabelled receptor ligand at particular [^{3}H]histamine concentrations and nonspecific binding measured for respective radioligand dilutions in presence of histamine (100 μM).

In order to finally confirm novel cell line usefulness for affinity studies and validate proposed assay platform, competition binding experiments for estimation of unknown K_i values of tested compounds were performed. Developed assay was validated against the insect cell line-related system, used for the same purposes by our scientific collaborators. Series of potent histamine

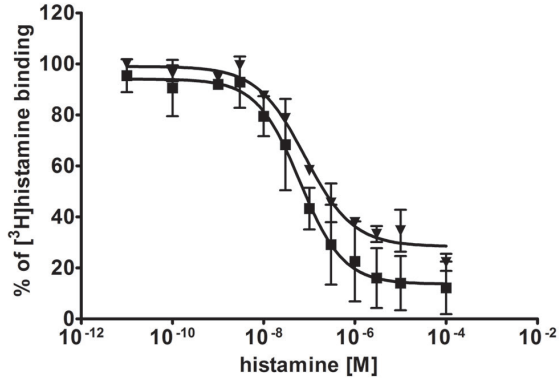
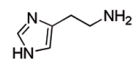
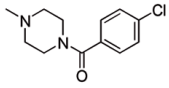
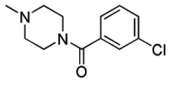
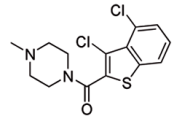
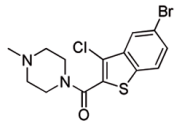
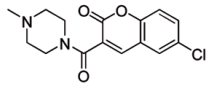


Figure 2. Homologous competition binding of [^{3}H]histamine to various membranes preparations from cells stably expressing histamine H_4 receptor.
Homologous competition binding experiments were performed with use of membranes preparation from: novel cell line obtained with use of retroviral transduction system (▼) and reference membrane preparation from commercial source (■). Respective IC_{50} values determined in homologous competition binding assay were as follows: retroviral transduction system — IC_{50} = 80.2 ± 19.6 [nM] and commercial membranes preparation — IC_{50} = 62.2 ± 21.9 [nM]. Data points represent means ± S.E.M. of two independent experiments, performed in triplicates.

Table 1. Comparison of K_i values of potent histamine H_4 receptor ligands, estimated in affinity assay with use of different receptor expression models.
Competition binding experiments were performed at membranes preparations from: CHO cells stably expressing human histamine H_4 receptor, obtained with the use of retroviral transduction system (RTS) and insect cells infected with baculovirus encoding for the human histamine H_4 receptor (Sf9).

Compound	RTS K_i ± SEM	Sf9 K_i ± SEM
 histamine	**29.7** ± 4.2 [nM]	**36.9** ± 1.5 [nM]
 H4-21	**14.1** ± 1.8 [μM]	**7.55** ± 4.22 [μM]
 H4-23	**3.12** ± 0.80 [μM]	**3.88** ± 2.34 [μM]

Compound	RTS K_i ± SEM	Sf9 K_i ± SEM
 DB-6	**12.9** ± 3.2 [μM]	**21.3** ± 10.5 3 [μM]
 DB-7	**8.36** ± 0.43 [μM]	**17.3** ± 5.9 [μM]
 DB-5	**> 30** [μM]	**> 30** [μM]

receptor ligands were tested in competition binding assays *vs.* [^{3}H]histamine. Considered structures represented 4-methylpiperazino-amide derivatives and histamine was included as reference. Determined affinities were in a micromoloar range (Table 1). Histamine K_i value was in accordance with literature data (Leurs *et al.*, 2009). Estimated affinities correlated well with the results obtained in formerly used insect cells model for all investigated compounds (Fig. 3). The most active compound in present series was (3-chlorophenyl)carbonyl-4-methylpiperazine (H4-23), showing respective K_i values of 3.12 μM for CHO cells stably expressing human histamine H_4 receptor, obtained with use of retroviral transduction system and 3.88 μM for insect cells infected with baculovirus encoding for the human histamine H_4 receptor (Fig. 4).

To conclude, we obtained a novel mammalian cell line expressing functional human histamine H_4 receptor with use of retroviral transduction system, which was confirmed in a variety of radioligand studies. Developed cellular model is suitable for investigation of potent histamine H_4 receptor ligands affinities.

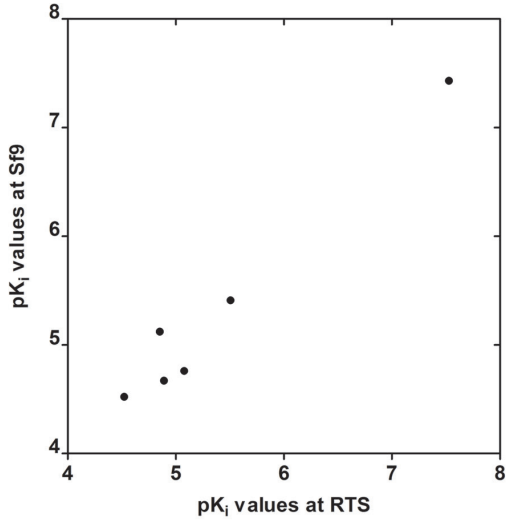


Figure 3. Correlation between pK_i values obtained for considered histamine H_4 receptor ligands in two compared receptor expression systems.
pK_i values determined for tested compounds (histamine, H4-21, H4-23, DB-5, DB-6 and DB-7) with use of membranes preparations from recombinant cell line obtained with the use of retroviral transduction system (RTS) were compared with relevant results developed basing on membranes from genetically modified *Spodoptera frugiperda* cells (Sf9).

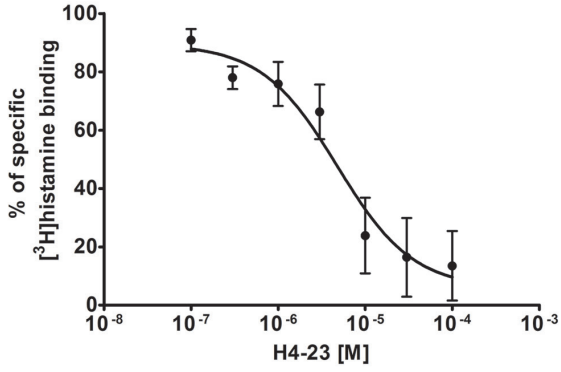


Figure 4. Competition binding curve for compound H4-23 *vs.* [^{3}H]histamine, obtained at membranes preparation of retrovirally transduced CHO cells stably expressing human histamine H_4 receptor.
Presented competition binding experiment was performed for H4-23, a most potent *h*H_4R ligand of investigated series of 4-methylpiperazino-amide derivatives. Determined IC_{50} parameter was used, according to Cheng-Prusoff equation, to calculate K_i value (3.12 ± 0.80 μM). Data points represent means ± S.E.M. of two independent experiments, performed in duplicates.

Acknowledgements

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