



BODIPY[®] FL Histamine as a New Modality for Quantitative Detection of Histamine Receptor Upregulation Upon IgE Sensitization in Murine Bone Marrow-Derived Mast Cells

Arash Mirzahosseini,^{1,2*} Marianna Kovács,¹ Károly Kánai,¹ Péter Csutora,¹ Balázs Dalmadi¹

¹Sanofi Research and Development, Tó utca 1-5, H-1045, Budapest, Hungary

²Faculty of Pharmacy, Semmelweis University, Hőgyes Endre utca 9, H-1092, Budapest, Hungary.

Received 11 March 2014; Revised 17 June 2014; Accepted 22 August 2014

Grant sponsor: Sanofi (Budapest, Hungary)

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Arash Mirzahosseini, Department of Pharmaceutical Chemistry, Semmelweis University, H-1092 Budapest, Hőgyes Endre utca 9, Hungary. E-mail: mirzahosseini.arash@pharma.semmelweis-univ.hu

This work was supported by Sanofi (Budapest, Hungary). The authors report no conflict of interest.

Published online 00 Month 2014 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.22566

© 2014 International Society for Advancement of Cytometry

• Abstract

Flow cytometry is one of the most widely used methods for the qualitative and quantitative analysis of cell surface expressed proteins by making use of fluorescent specific antibodies. Lacking an antibody validated for flow cytometry, an alternative approach for labeling cell surface receptors is the use of fluorescently tagged ligands. In this study, histamine H4 receptor transfected Chinese hamster ovary cells and murine bone marrow-derived mast cells (mBMMCs) were selected for studying the possibility of staining individual histamine receptors using BODIPY[®] FL histamine and selective antagonists. Flow cytometric measurements and supporting calculations showed that BODIPY FL histamine is suitable tool for quantitating cell surface histamine receptors. The binding, and competitive inhibition of this fluorescent ligand were characterized, which were in good agreement with a semi-empirical model constructed from fundamental protein-binding relationships. Using this method it was shown for the first time that even though mature mBMMCs express H2R and H4R to the same extent, immunoglobulin E sensitization results in H4R upregulation only, while the surface expression of H2R remains unchanged. © 2014 International Society for Advancement of Cytometry

• Key terms

mast cell; histamine H4 receptor; fluorescent ligand; flow cytometry; IgE sensitization

INTRODUCTION

HISTAMINE is a multifunctional messenger molecule that is released from tissue mast cells and basophils and plays an important role in inducing inflammation. Four histamine receptors are known to mediate the effects of histamine: H1 receptors have been extensively studied for their effects on allergic reactions, whereas H2 and H4 receptors have also been demonstrated to have immunoregulatory effects, while H3 receptors are known for their role in the nervous system (1). The most recently described histamine receptor, histamine receptor H4, is expressed mainly on cells from hematopoietic lineage, particularly mast cells, basophils and eosinophils (2–6). Histamine receptor H4 is a G_zi/o coupled receptor (7), which plays a key role in the chemotaxis of eosinophils (8), monocyte-derived dendritic cells (9), and mast cells as well as in the regulation of T cells and dendritic cells (10,11).

One of the most robust methods for the quantitative measurement of cell surface expressed receptors is flow cytometry. The convenience of this method relies on the use of fluorescent specific antibodies. The binding of these fluorescent antibodies to their targets on the cell surface allows for the detection of fluorescence intensity of stained cells, which in turn correlates with relative receptor expression. However, the quantification of the recently discovered H4 receptor is not entirely attainable, since

there are no unanimously accepted commercially available labeled H4R antibodies (murine or human, at the time of preparation of the article) that have been validated for use in flow cytometry. Although antibodies raised against histamine receptors are used in some studies (12,13), a viable alternative method for labeling receptors is the use of fluorescent dye–ligand conjugates. There are strengths and limitations in using a ligand-based vs. antibody assay. Other than the difference in valency, monovalent vs. bivalent binding of ligand and antibody, there is a difference in binding affinity and selectivity. Antibody binding is paradigmatically tighter and specific, while its limitation is effortful manufacture. For the ligand-based assay of histamine receptors, first a fluorescein–histamine conjugate was proposed (14), followed by a more recent dye, BODIPY® FL histamine, that has been successfully used in flow cytometry in several studies (9,12,15). Several other classes of receptors have also been measured with fluorescently tagged ligands. The measurement of adenosine A1 receptor expression is also possible with ligand-based assays (16). Furthermore, a recent study has investigated the influence of different fluorophores and linker composition on the binding affinity, thus usefulness of various fluorescent adenosine A1 receptor dyes (17). Naturally, quantification of receptors is possible without flow cytometry, using traditional binding assays employing filtration and scintillation proximity assays (18).

In the present study, the possibility of measuring histamine receptors quantitatively with a fluorescent ligand using flow cytometry was explored. Murine bone marrow-derived mast cells (mBMMCs) were selected as a suitable model to investigate histamine receptors using a fluorescently tagged ligand. Mast cells are bone marrow-derived cells that participate in innate immunity and reside in tissues in proximity to surfaces that interface the external environment. They play an important role in inflammation and hypersensitivity reaction type I. During sensitization, mast cells bind immunoglobulin E (IgE) through the high affinity IgE receptors (FcεRI). Subsequently, IgE binds specific antigens, and the FcεRI–IgE–antigen complex forms cross-links and larger aggregates (19–21), resulting in the release of mediators stored in mast cell granules such as histamine and proteases, and de novo synthesis of mediators such as prostaglandins and leukotrienes (22). A number of complex signal transduction pathways are at work during the sensitization phase of mast cell activation. One regulatory function of IgE is the upregulation of FcεRI expression (23–25). In a recent work, we reported the relationship between histamine H4 activity and mast cell degranulation/FcεRI receptor upregulation (26). In the present study, we sought to explore the existence of regulatory effects of IgE on histamine receptors present on mast cells. There is evidence that human skin mast cells express H2 and H4 receptors (12), however the complex regulatory functions of these histamine receptors on mast cells, particularly in IgE-mediated sensitization processes is yet to be fully understood.

In the present study, CHO cells expressing human H4 receptor and mast cells differentiated from murine bone marrow stem cells were used as models. The fluorescent labeling

of histamine receptors with BODIPY FL histamine was compared between H4R transfected cells and parental CHO cells not expressing H4R. By inhibiting the fluorescent ligand binding with unlabeled histamine or selective histamine receptor antagonists directed toward the histamine binding site, a method for the quantitative analysis of H4R using flow cytometry was constructed, supported by a series of calculations. Using this method the determination of mH2R and mH4R levels on mBMMC was made possible, together with the examination of the regulatory effects of IgE sensitization on mast cell histamine receptor expression.

MATERIALS AND METHODS

Materials

BODIPY FL histamine, Ham's F-12 nutrient mixture, Dulbecco's phosphate buffered saline (DPBS), and GlutaMAX were purchased from Life Technologies (Carlsbad, CA). Cimetidine, Accutase solution, Tris-EDTA buffer, RPMI-1640 medium, and penicillin–streptomycin solution stabilized were purchased from Sigma-Aldrich Co. (St. Louis, MO). Rat anti-mouse IgE FITC, rat anti-mouse CD117 PE, rat anti-mouse CD16/CD 32 (Mouse Fc Block), and BD Pharmingen Stain Buffer were purchased from BD Biosciences (Erembodegem, Belgium). Recombinant mouse IL-3 and recombinant mouse SCF were purchased from R&D Systems (Minneapolis, MN). FBS was purchased from Thermo Fisher Scientific (Waltham, MA). ACK lysis buffer was purchased from Quality Biological (Gaithersburg, MD). UR-63325 (Palau Pharma S.A., Barcelona, Spain), a furo[3,2-d]pyrimidine derivative is a selective histamine receptor H4 antagonist/inverse agonist (27–29), and was synthesized as a reference compound using the synthesis method described in the patent WO2009056551 (A1)—Furo[3,2-d]pyrimidine derivatives (Palau Pharma S.A., Carceller Gonzalez E, Medina Fuentes EM, Martina Via J, Virgili Bernado M, 2009). The purity of the samples was assessed with high-performance liquid chromatography–mass spectrometry and ¹H-NMR.

H4R Transfected Cells

CHO cells transfected with the coding sequence of the human histamine receptor H4 were purchased from Euroscreen (Gosselies, Belgium). Cells were grown at 37°C and 5% CO₂ in Ham's F-12 nutrient mixture supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. During each passage adherent cells were washed with DPBS (without Ca²⁺ and Mg²⁺), treated with 5 ml Accutase for 5 minutes, centrifuged (1,200 rpm, 10 min), and finally cells plated at 3 × 10⁵ cell/flask. In the last passage, before the flow cytometric measurements, adherent cells were nonenzymatically dissociated using EDTA solution. Parental CHO cells were grown in RPMI-1640 medium supplemented with GlutaMAX, 100 U/ml penicillin, and 100 µg/ml streptomycin; all other procedures were same as above.

Bone Marrow Mast Cell Culture

The method of Singh et al. (30) was used to prepare mBMMC with slight modifications. Briefly, the femurs of

BALB/c male mice (8–12 weeks) were surgically removed and flushed with cold DMEM. Cells were collected, filtered through a 70- μ m cell strainer and centrifuged (1,200 rpm, 10 min). After treatment with ACK Lysis Buffer to lyse red blood cells, the cells were washed twice with DMEM and plated at 2×10^6 cell/ml in BM12 medium (DMEM with 2% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin) containing 50 ng/ml recombinant murine SCF and 10 ng/ml recombinant murine IL-3. Twice every week nonadherent cells were removed, centrifuged (1,200 rpm, 10 min), fresh media added and transferred to a new dish for further culture. Cells were analyzed twice every week by flow cytometry for CD117 and Fc ϵ RI expression. Mast cells were considered mature from usually 4–5 weeks, when more than 90% of cells were positive for both CD117 and Fc ϵ RI compared to isotype control, and cell cultures not older than 15 weeks were used in further experiments. Handling and animal experiments were conducted according to 86/608/EEC 2010-63-EU directive. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Sanofi.

FACS Analysis of H4R Surface Expression

Cells (10^5 cell/tube) were incubated in Stain Buffer (end volume: 100 μ l/tube) with BODIPY FL histamine (or specific antigens against CD117 and Fc ϵ RI with Fc Block) and optionally co-incubated with unlabeled ligands (histamine, UR-63325, cimetidine) for 30 min at +4°C. Samples were then centrifuged (1,300 rpm, 5 min), the supernatant was completely removed promptly, and the samples were resuspended in 100 μ l stain buffer. Flow cytometric analysis was conducted using FACS-Canto II (BD Biosciences, Erembodegem, Belgium), with at least 10,000 events recorded during each measurement. Data were analyzed using FACSDiva (BD Biosciences, Erembodegem, Belgium) software. In every case autofluorescence of unstained samples was subtracted from measured values.

UV/Vis Spectroscopic Measurement of BODIPY FL Histamine

Following the incubation of H4 transfected cells with BODIPY FL histamine in stain buffer, cells were centrifuged (1,300 rpm, 5 min) and a sample was taken from the supernatant. 50 μ l of supernatant was pipetted onto a 96-well plate along with BODIPY FL histamine solutions of known concentration for calibration purposes and blank stain buffer solutions. Absorbance was measured (at $\lambda_{\text{max}} = 504$ nm, provided by the manufacturer) using SpectraMax Plus384 Microplate Reader (Molecular Devices, Sunnyvale, CA).

Statistical and Mathematical Analysis

Experimental data are presented as mean $\pm$ standard deviation (S.D.; unless stated otherwise) from the number of independent samples. Statistical significance was determined using Student's *t*-test for two groups or analysis of variance (ANOVA) with Dunnett's test for multiple groups using Statistica v9 (StatSoft, Tulsa, OK). Normality of the data was inspected with the Shapiro–Wilk test. In all analyses $P < 0.05$ was considered significant. Competitive binding curves and ligand binding vs. nonspecific binding curves were created

using GraphPad Prism 5 software v5.04 (GraphPad Software, La Jolla, CA). Solutions to the equations in the mathematical model were determined using MATLAB v2010b (MathWorks, Natick, MA).

RESULTS

Fluorescent Labeling of Histamine Receptors With BODIPY FL Histamine for Flow Cytometry

The cell-surface expression of histamine receptor H4 was measured on CHO transfected cells using flow cytometry. Parental CHO and H4 transfected CHO cells were labeled with BODIPY FL histamine, and subsequently analyzed by flow cytometry using the FITC channel. Increasing BODIPY FL histamine concentration during incubation resulted in increasing fluorescence intensity values for both parental and H4 transfected cells (Fig. 1). However, at a certain BODIPY FL histamine concentration during incubation, the measured fluorescence intensity value of parental cells appeared to be always roughly half of that of H4 transfected cells. The concentration dependent binding of BODIPY FL histamine (receptor saturation assay) was evaluated using ordinary fit for total binding/nonspecific binding nonlinear regression analysis (31). Next, this fluorescence labeling of H4 transfected cells was quenched with excess unlabeled histamine (10^{-3} mol/l). For each labeling experiment with various BODIPY FL histamine concentrations, excess histamine was used to inhibit labeling of H4 transfected cells, and in every case the inhibition percentage of histamine was approximately the same: $45 \pm 10\%$, mean $\pm$ S.D. (Fig. 2). These data are in agreement with nonspecific binding on parental cells causing roughly half the fluorescence intensity value relative to that of H4 transfected cells. On the other hand, the fluorescence labeling of parental cells was not affected significantly by excess histamine (Fig. 2). BODIPY FL histamine labeling was also inhibited by selective H4 and H2 antagonists (UR-63325 and cimetidine, respectively). Excess UR-63325 (10^{-4} mol/l) demonstrated $65 \pm 9\%$ inhibition, while excess cimetidine (10^{-4} mol/l) only $50 \pm 10\%$ on H4 transfected cells; on the other hand the fluorescence intensity of parental cells was diminished by $42 \pm 8\%$ and $20 \pm 10\%$ due to excess UR-63325 (10^{-4} mol/l) and cimetidine (10^{-4} mol/l), respectively (data are in mean $\pm$ S.D.). These data imply that UR-63325 and cimetidine, contrary to histamine, also inhibit the nonspecific binding of BODIPY FL histamine to cells. The detailed inhibition percentages of competitive ligands (histamine, UR-63325, cimetidine) on BODIPY FL histamine binding for the various labeled samples (BODIPY FL histamine incubation concentrations: 10^{-5} mol/l, 10^{-6} mol/l, 10^{-7} mol/l, and 10^{-8} mol/l) are shown in Figure 2. Finally, we sought to reveal the nature of BODIPY FL histamine fluorescent labeling, as a well-described receptor–ligand binding. Since BODIPY FL histamine is a conjugate of the intrinsic ligand of H4 receptors; competitive inhibition assays were performed, akin to radioligand binding assays. The competitive binding curve of histamine and UR-63325 were achieved by coincubating fluorescently labeled (10^{-6} mol/l BODIPY FL histamine) H4

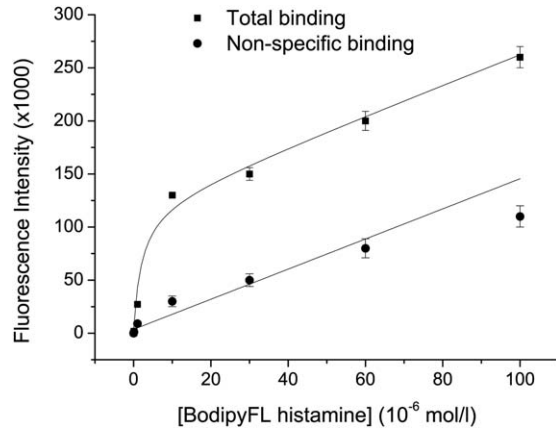


Figure 1. H4 transfected cells (Total binding) and parental CHO cells (Nonspecific binding) were labeled with increasing concentrations of BODIPY[®] FL histamine. The saturation curve of Specific binding = (Total binding) – (Nonspecific binding) is not depicted. Fluorescence intensity values are presented in mean $\pm$ S.D., $n = 5$. The figure is representative of three independent experiments. Best-fit values: $B_{\max} = 1.2 \times 10^5 \pm 2 \times 10^4$, $K_d = 2.1 \times 10^{-6} \pm 2 \times 10^{-5}$, $R^2 = 0.9716$.

transfected cells with increasing concentrations of the unlabeled ligands that inhibited binding of the fluorescent dye, and eventually performing flow cytometric analysis (Fig. 2). Note that the difference between lower plateau values of plot A and plot B (Fig. 2) is due to the inhibitory effect of UR-63325 on nonspecific binding of BODIPY FL histamine; this is in agreement with the results of CHO parental and H4 transfected cell labeling using BODIPY FL histamine.

Supporting the Fluorescent Labeling of H4 Transfected Cells With Calculations

In order to further confirm BODIPY FL histamine labeling as an acceptable modality for the quantification of H4 receptor, we attempted to support the measured data with a series of calculations. It seemed straightforward to calculate predictive fluorescence intensity values for the BODIPY FL histamine staining experiments of H4 transfected cells, and comparing these values with the experimental data. However, before the calculation could be carried out an initial auxiliary data had to be measured. The unbound BODIPY FL histamine equilibrium concentration over H4 transfected cells was needed in order to determine the histamine receptor content of the H4 transfected cells. For this purpose, 10^7 cells were incubated in 100 μ l stain buffer with 10^{-5} mol/l BODIPY FL histamine for 2 h. Next, the cells were centrifuged and a 10- μ l sample was taken from the supernatant in order to determine unbound BODIPY FL histamine content by measuring absorbance. The measured absorbance was 0.1783 ± 0.0004 (mean $\pm$ S.D.); absorbance was measured in triplicates in five separate experiments (from different batches of transfected cells). Using the calibration curve [Absorbance = $15,970 \times$ concentration (mol/l), $R^2 = 0.9942$] and also considering blank sample absorbance (0.0390 ± 0.0004) the calculated unbound BODIPY FL histamine concentration in this experimental setting was found to be $8.73 \times 10^{-6} \pm 3 \times 10^{-8}$ mol/l ($P < 0.05$, Student's t -test).

The foundation of the mathematical model is comprised of the laws of mass action for respective equilibria of fluorescent ligand binding (BODIPY FL histamine (B) and unlabeled ligands (L)):



Further equilibria present in the system are those of membrane binding according to log P values, where “m” denotes membrane-bound state:



The resulting equations are

$$K_B = \frac{[B][H4]}{[BH4]} \quad (5)$$

$$K_L = \frac{[L][H4]}{[LH4]} \quad (6)$$

$$c_B = [B] + [BH4] + \alpha[B_m] \quad (7)$$

$$c_L = [L] + [LH4] + \alpha[L_m] \quad (8)$$

$$[H4_{TOT}] = [H4] + [BH4] + [LH4] \quad (9)$$

$$P_B = \frac{[B_m]}{[B]} \quad (10)$$

$$P_L = \frac{[L_m]}{[L]} \quad (11)$$

where square brackets denote equilibrium concentration in mol/l, c denotes total concentration of a species in mol/l, and K_B , K_L , are the K_i values. α is the ratio of total cell membrane volume with respect to sample volume, since the expression of P contains the concentration of membrane bound species with respect to the volume of the lipophilic phase. In our experiments $\alpha = 6.6 \times 10^{-6} \pm 4 \times 10^{-7}$ was calculated as:

$$\alpha = \frac{4\pi(\frac{d}{2})^2 h N}{V} \quad (12)$$

where π is the mathematical constant, d is average cell diameter and was considered to be $14.5 \pm 0.3 \mu\text{m}$ (32), h is average membrane thickness and was considered to be $10 \pm 3 \text{ nm}$ (33), N is cell number in our samples and was $1.0 \times 10^5 \pm 2 \times 10^4$ cells in the flow cytometric experiments (determined by Bürker cell counting chamber), and V is sample volume which was $100 \pm 5 \mu\text{l}$. During the calculation of α , cells were assumed to be spherical, and the volume of the cell membrane was simply calculated as area of the sphere times membrane thickness, since the thickness of the membrane is orders of magnitude smaller (1/1,000 times) than the cell diameter. The pK_i of BODIPY FL histamine at H4R was assumed to be the same as that of histamine, $pK_i = 7.8 \pm 0.1$ (34). The pK_i of cimetidine at H4R was assumed to be 5 (34). Log P values for all substances were predicted using ACD/I-lab lab v2.0 (Advanced Chemistry Development, Toronto, ON). For UR-

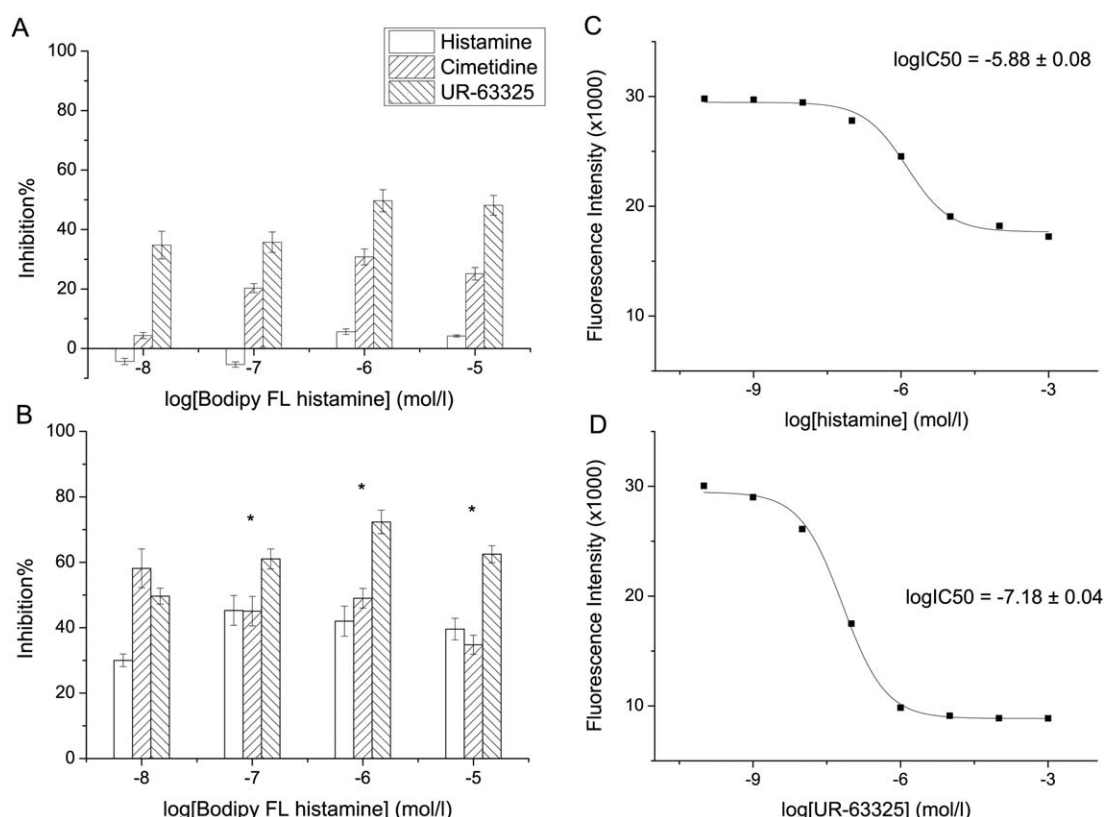


Figure 2. Left side: inhibition of BODIPY[®] FL histamine receptor binding by agonist and antagonists on (A) parental cells and (B) H4 transfected cells were labeled with increasing concentrations of BODIPY FL histamine, and fluorescent labeling was inhibited with histamine (10^{-3} mol/l), UR-63325 (10^{-4} mol/l), and cimetidine (10^{-4} mol/l). Fluorescence intensity values are presented in mean $\pm$ S.D., $n = 5$ (* $P < 0.05$, ANOVA and Dunnett's test were performed). The figure is representative of three independent experiments. Right side: The competitive binding curve of histamine (C) and UR-63325 (D) on H4 transfected cells labeled with BODIPY FL histamine (10^{-6} mol/l). Data are presented in mean $\pm$ SEM, $n = 3$. The figures are representative of three independent experiments. R^2 values are 0.9941 and 0.9983, respectively.

63325 and cimetidine the log P values were 1.85 and 0.52, respectively, the predicted log P value of BODIPY FL histamine was 2.63; it was assumed that histamine being a very hydrophilic molecule does not significantly compete for non-specific binding. First, total H4R content was determined in the samples using the UV/Vis spectroscopic data. By incubating 10^7 H4 transfected cells with 10^{-5} mol/l BODIPY FL histamine in 100 μ l sample volume and determining the [B] (free BODIPY FL histamine in solution) by measuring absorbance from the supernatant, the calculation of $[H4_{TOT}]$ was possible, using Eqs. (5), (7), (9), and (10). According to calculations

Table 1. The determined log IC_{50} values of histamine and UR-63325 on H4R from competitive inhibition assays on H4 transfected cells labeled with BODIPY[®] FL histamine

	HISTAMINE	UR-63325
log IC_{50}	-5.88 ± 0.08	-7.18 ± 0.04
pK_i (calc)	7.7 ± 0.1	9.1 ± 0.1
pK_i (32)	7.8 ± 0.1	

Calculated pK_i value of histamine on H4R from measured log IC_{50} is depicted, along with the measured pK_i value determined by Lim et al. (34) using [^3H]histamine radioligand binding assay.

H4R content on H4 transfected CHO cells in our experiments was $1.6 \times 10^{-14} \pm 3 \times 10^{-15}$ mol/cell. Therefore, for all of our flow cytometric experiments $[H4_{TOT}] = 1.58 \times 10^{-9}$ mol/l was considered. Next it was assumed that in very dilute solutions, such as in our experiments, fluorescence intensity is in a linear relationship with the effective labeled fluorescent species in the solution:

$$FI = k([BH4] + \alpha[B_m]) \quad (13)$$

where k is the linearity constant. Using the fluorescence intensity values of the fluorescence saturation experiment of H4 transfected cells using only BODIPY FL histamine (Fig. 1) and with calculated $[BH4]$ and $[B_m]$ values, k was calculated for the different samples, and the mean value was determined to be $7.7 \times 10^{12} \pm 3 \times 10^{11}$.

Eventually, comparison of the calculations with the experimental and literature data was performed. With the calculated $[H4_{TOT}]$ and IC_{50} values from the competitive binding experiment (Fig. 2) K_d values were calculated [using Eqs. (5), (7), (9), (10) and the Cheng-Prusoff Eq. (35)], and pK_i values for both UR-63325 and histamine at H4R were determined; values are presented in Table 1. The pK_i value of histamine is in good agreement with literature data, which supports our calculation hypothesis and the described BODIPY FL histamine flow

Table 2. Calculated and measured fluorescence intensity values of competitive inhibition assays performed with histamine, UR-63325, and cimetidine on H4 transfected cells labeled with BODIPY[®] FL histamine are depicted

LOG[BODIPY] (MOL/L)	HISTAMINE INHIBITION		UR-63325 INHIBITION		CIMETIDINE INHIBITION	
	CALCULATED	MEASURED	CALCULATED	MEASURED	CALCULATED	MEASURED
−5	102,891	87,284	63,268	54,203	93,795	94,233
−6	20,433	14,785	91,65	7,553	18,290	9,195
−7	1,078	975	917	695	503	979
−8	436	113	92	71	70	59

Values are presented in mean, $n = 5$.

cytometric method. Furthermore, the fluorescence intensity values of the fluorescence saturation experiments of H4 transfected cells inhibited with excess histamine, UR-63325 and cimetidine (Fig. 2) were calculated and are compared to the measured fluorescence intensity values (Table 2), which also show good agreement. In samples with relatively high fluorescent ligand concentration, in order to more accurately emulate the competition for nonspecific binding sites, it was assumed that the upper limit of the concentration of membrane bound species is determined by the fluorescence intensity value of the corresponding parental cell sample, since this fluorescence value represents the maximum amount of nonspecific binding. This correction was performed as follows:

$$c_{\max} = \frac{FI_{\text{parental}}}{k} \quad (14)$$

where $c_{\max}$ is the concentration of membrane-bound species when the membrane is saturated. The equations for the corrected concentrations of membrane-bound species are as follows:

$$[B_m] = \frac{c_{\max} c_B P_B}{c_B P_B + c_L P_L} \quad (15)$$

$$[L_m] = \frac{c_{\max} c_L P_L}{c_B P_B + c_L P_L} \quad (16)$$

(the membrane bound species will be distributed among B_m and L_m with proportion to their total concentration and P

value). Finally, the competitive binding curves of histamine and UR-63325 on BODIPY FL histamine labeled H4 transfected cells (Fig. 2) were simulated using the mathematical model [Eqs. (1)–(13)]; the simulated curves superposed on the measured curves and corresponding parameters are presented in Figure 3.

IgE Sensitization Increases mH4R Expression on mBMMC

Since the fluorescent labeling of H4 receptors for flow cytometry seemed promising as described above, we sought to label cells that naturally express histamine receptors. For this purpose mBMBCs were cultivated and labeled with BODIPY FL histamine for FACS analysis. First, an adequate window of detection was optimized for the BODIPY FL histamine concentration. The concentration dependent labeling of mBMMC with BODIPY FL histamine resulted in a similar result to that of H4 transfected CHO cells (Fig. 4). The optimal BODIPY FL histamine concentration was determined to be 10^{-6} mol/l, since choosing a concentration where the relationship between fluorescent dye concentration and fluorescence intensity remains linear is crucial for the reproducibility of results. Furthermore, at this BODIPY FL histamine concentration the fluorescence intensity of samples is in the order of 100 thousand counts, optimal for flow cytometry measurements. The fluorescent labeling of mBMMC was next inhibited with excess

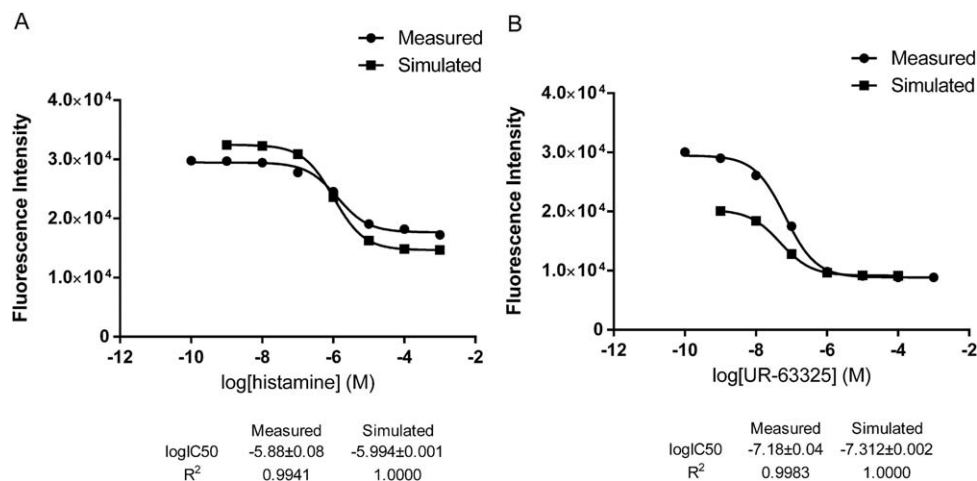


Figure 3. Competitive inhibition curves of BODIPY[®] FL histamine labeled H4 transfected cells with histamine and UR-63325 was simulated. Simulated curves are calculated using the mathematical analysis method described in the calculation section. Measured data are from the competitive inhibition studies on H4 transfected cells. Measured data are represented in mean ± SEM, $n = 3$.

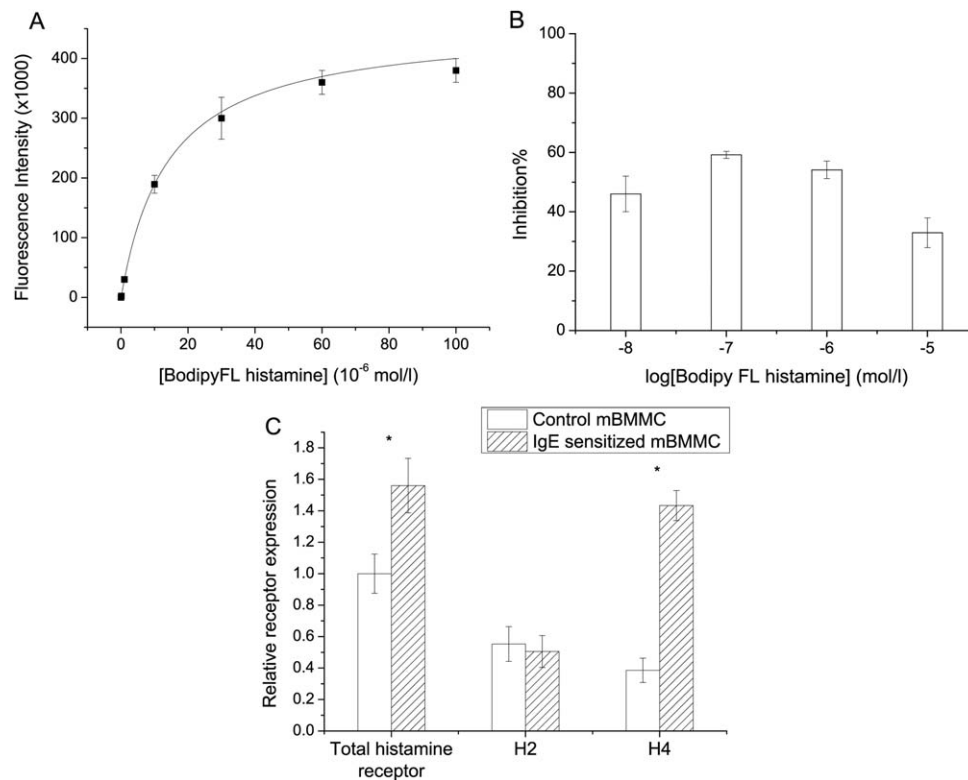


Figure 4. (A) The binding curve of BODIPY[®] FL histamine on mBMMC. Fluorescence intensity values are presented in mean $\pm$ S.D., $n = 5$. The figure is representative of three independent experiments. Best-fit values: $B_{\max} = 4.6 \times 10^5 \pm 2 \times 10^4$, $K_d = 1.41 \times 10^{-5} \pm 8 \times 10^{-7}$, $R^2 = 0.9956$. (B) Competitive inhibition of BODIPY FL histamine binding by histamine on mBMMC. Mast cells were labeled with BODIPY FL histamine and coincubated with increasing concentrations of histamine. The inhibition percentage of histamine at various concentrations compared to the control is depicted. Values are presented in mean $\pm$ S.D., $n = 5$. The figure is representative of three independent experiments from different cell batches. (C) The H2 and H4 receptor expression was quantitated on BODIPY FL histamine labeled mBMMC using competitive binding assays. IgE sensitization resulted in only H4 receptor upregulation. Values are presented in mean $\pm$ S.D. (* $P < 0.05$, Student's t -test). The figure is representative of three independent experiments.

histamine (10^{-3} mol/l) for all the various BODIPY FL histamine concentrations; roughly about half of the fluorescence intensity was diminished (Fig. 4), which is also in agreement with the histamine inhibition results of CHO parental and H4 transfected cells. Therefore, roughly half of the fluorescence intensity of stained mBMMC cells stems from nonspecific binding of BODIPY FL histamine. Next, the selective quantification of mH2R and mH4R expression was attempted, by selectively inhibiting BODIPY FL histamine binding to these receptors with cimetidine and UR-63325, respectively. In these selective inhibition experiments the H2 and H4 selective antagonists will inhibit some portion of nonspecific binding as well, apart from competing for their respective histamine receptors. In order to take this phenomenon into account, the fluorescence intensity values of these experiments were corrected with the following method. In principle, since the inhibition percentage of cimetidine and UR-63325 on labeled CHO parental cells is known, this value can be implemented on the selective inhibition experiments of mBMMC by simply subtracting the “left over” amount of nonspecific labeling from the intensity values. For this purpose, mBMMC and CHO cells were separately labeled with 10^{-6} mol/l BODIPY FL histamine and co-incubated with either histamine (10^{-3}

mol/l), cimetidine (10^{-6} mol/l), or UR-63325 (10^{-7} mol/l). For the evaluation of the results first, the nonspecific binding of BODIPY FL histamine to parental CHO cells (CHO cells labeled with BODIPY FL histamine) was normalized to the nonspecific binding to mBMMC (mBMMC labeled with BODIPY FL histamine, inhibited with histamine). Next, fluorescence intensity values of selective inhibition experiments (mBMMC labeled with BODIPY FL histamine, inhibited with cimetidine and UR-63325, respectively) were corrected using the nonspecific binding of cimetidine and UR-63325 (CHO cells labeled with BODIPY FL histamine, inhibited with cimetidine and UR-63325, respectively). These corrected fluorescence intensity values for the cimetidine and UR-63325 selective inhibition experiments are depicted as relative H4 and H2 receptor expression, respectively (Fig. 4). This could be made possible because of the fact that the binding of BODIPY FL histamine occurs similarly on CHO cells and mBMMC cells (due to the mutual agreement of previous results) and nonspecific binding can be presumed to function by the same principles on different cells.

Since in an earlier work, we found that H4 receptors regulate IgE mediated processes (26), it was compelling to investigate the histamine receptor expression of mBMMC in

different conditions, namely native and IgE sensitized mBMMC. The relative histamine receptor expression on mast cells was monitored with respect to IgE sensitization. For this purpose, mBMMC were incubated overnight with 1 μ g/ml anti-DNP IgE after which cells were stained with BODIPY FL histamine and relative histamine receptor expression was quantitated using the same procedure as above. The results revealed that IgE sensitization increases overall histamine receptor expression, only by affecting H4 receptor expression, but leaving H2 receptor levels unaffected. In our experiments H4 receptor expression of mBMMC increased due to IgE sensitization roughly by fourfold (Fig. 4).

DISCUSSION

Flow cytometric analysis of cell surface expressed proteins is a powerful method for attaining a qualitative and quantitative description of cell phenotypes. Specific antibodies that have been covalently conjugated to fluorophore molecules are used to specifically label cell surface proteins and measure fluorescence intensity. Alternately, a different labeling method is possible for cell surface receptors, namely fluorescent ligands. In this study, our goal was to validate the flow cytometric measurement of H4 receptor expression using BODIPY FL histamine.

We have shown that H4 transfected cells could be labeled with BODIPY FL histamine in a concentration-dependent manner. The nonspecific binding of the fluorescent ligand could be determined by staining the parental CHO cells with BODIPY FL histamine, or inhibiting the fluorescent labeling of H4 transfected cells with excess histamine. Both approaches lead to the same conclusion: as it can be inferred from Figure 1, roughly half of BODIPY FL histamine binds nonspecifically to H4 transfected cells. This result is also supported by the fact that the same excess histamine did not inhibit the fluorescence labeling of parental cells, which implies that mostly the lipophilic BODIPY FL moiety is responsible for nonspecific binding, probably due to non-polar interactions with the cell membrane.

The inhibition of fluorescent labeling using selective histamine receptor antagonists was also examined. In our study cimetidine was used as a selective H2 antagonist, and the reference compound UR-63325 as a selective H4 antagonist/inverse agonist [IC_{50} human = 24 nM, IC_{50} murine = 38 nM, >200-fold selectivity vs. H1R, H2R, and H3R (28)], because the efficacy has been proven both in vitro on human and mouse receptors, in vivo in mouse pharmacology models, and also in human clinical trials. The selective histamine receptor antagonists also inhibited fluorescent labeling on the H4 transfected cells (to a greater extent compared to histamine), as well as on the parental CHO cells. This implies that cimetidine and UR-63325 (predicted log P values 1.85 and 0.52) not only competitively inhibit BODIPY FL histamine binding to histamine receptors, but also inhibit nonspecific binding, presumably due to their lipophilicity. Although, nonspecific binding is thought of as nondisplacable, it is not uncommon for nonspecific binding to be inhibited by competing ligands (36–38). The reversibility of BODIPY FL-histamine binding is

poor when inhibited with histamine (45% nonspecific binding seems too high), however reversibility is almost 80% with UR-63325. Usually endogenous ligands or antibodies are studied widely in the literature; these substances by virtue are hydrophilic and thus demonstrate a low degree of nonspecific binding [usually by other mechanisms than membrane partitioning, e.g., protein–protein interactions (39)]. However, xenobiotics, especially fluorescent dyes with highly lipophilic conjugated ring systems are partitioned into biological membranes producing substantial nonspecific binding.

Since protein binding and lipophilic partitioning are conspicuous physico-chemical processes, we investigated the possibility of providing theoretical proof to our method by comparing the observed results with a series of calculations. The mathematical model was constructed from the most basic physical chemistry relationships, perhaps used in this setting (verification of a flow cytometric method) for the first time. Calculations were performed using the fundamental laws of mass action describing protein–ligand binding and nonspecific binding, with the data of parental cell labeling as input data. The output of the calculations: the predicted values of competitive inhibition experiments on H4 transfected cells, and the competitive-binding curves of histamine and UR-63325 are in good agreement with the experimental data, attributing our study consistency and credibility. Perhaps the most compelling evidence of the veracity of our method is the calculated pK_i of histamine at H4R which shows excellent agreement with literature data.

Mast cells are essential participants of anaphylactic reactions and other inflammatory processes. It is suggested that human mast cells express H2R and H4R receptors (12); and several immunoregulatory functions of histamine have been discovered, particularly mediated via H4R. The selective quantification of H2 and H4 receptors, if achieved, would be a useful tool in studying cellular immunological processes. Using BODIPY FL histamine, histamine receptors were stained for flow cytometry, and by inhibiting the fluorescent labeling with selective antagonists this analysis was made possible. In this study it was shown that the fluorescent ligand BODIPY FL histamine can be used in order to selectively quantitate histamine receptor H2 and H4 surface expression on mBMBCs. First, an appropriate window of measurement regarding the BODIPY FL histamine concentration was determined, followed by the careful design of selective inhibition experiments. Ultimately relative H2 and H4 receptor levels were calculated by correcting for the nonspecific binding of cimetidine and UR-63325, using the preliminary experiments with CHO cells. It can be concluded that there was no significant difference between H2 and H4 receptor expression levels on native mast cells, however IgE-sensitization stimulated H4R expression on mast cells, but not H2R expression. This implies that during the early sensitization phase of inflammatory diseases downregulation of immune response cascades should be sought mostly in H4-mediated processes. Together with the useful tool of fluorescent ligand flow cytometry, these findings may contribute to a conceptive design in immunopharmacological research and enable novel approaches in chronic inflammation and autoimmune disease therapy.

ACKNOWLEDGMENTS

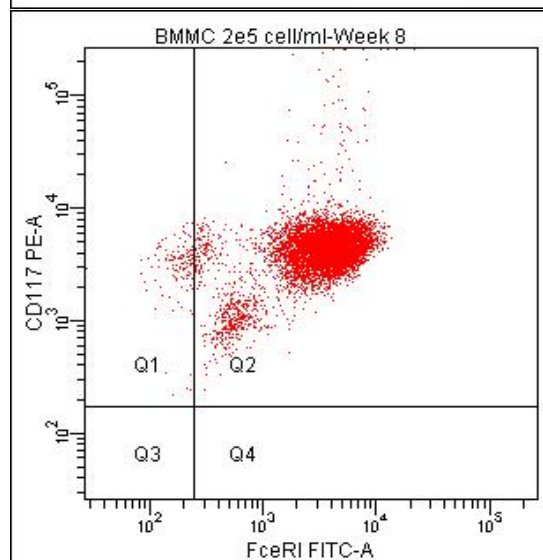
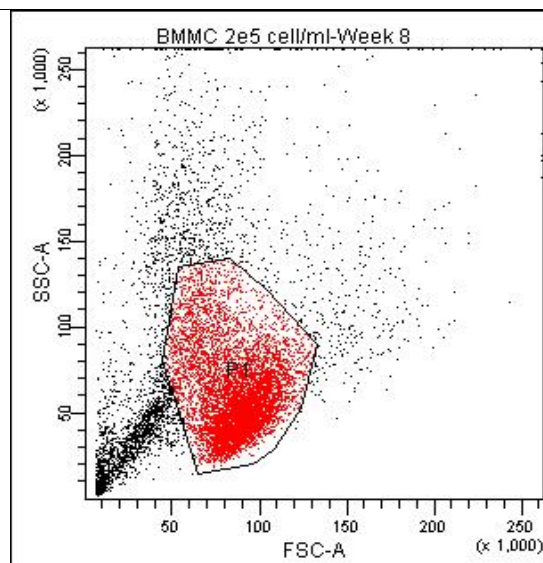
The authors wish to thank Szabone Dr. Zsuzsanna Cserjes (Sanofi, Budapest, Hungary) for her assistance in the biostatistical analyses. We also thank Ms. Marianna Toth for her excellent technical assistance.

LITERATURE CITED

- Hill SJ, Ganellin CR, Timmermann H, Schwartz JC, Shnakley NP, Young JM, Schunack W, Levi R, Haas HL. International Union of Pharmacology. XIII. Classification of histamine receptors. *Pharmacol Rev* 1997;49:253–278.
- Oda T, Morikawa N, Saito Y, Masuho Y, Matsumoto S. Molecular cloning and characterization of a novel type of histamine receptor preferentially expressed in leukocytes. *J Biol Chem* 2000;275:36781–36786.
- Morse KL, Behan J, Laz TM, West RE Jr, Greenfeder SA, Anthes JC, Umland S, Wan Y, Hipkin RW, Gonsiorek W, et al. Cloning and characterization of a novel human histamine receptor. *J Pharmacol Exp Ther* 2001;296:1058–1066.
- Liu C, Ma X, Jiang X, Wilson JS, Hofstra CL, Blevitt J, Pyati J, Li X, Chai W, Carruthers N, et al. Cloning and pharmacological characterization of a fourth histamine receptor (H4) expressed in bone marrow. *Mol Pharmacol* 2001;59:420–426.
- Zhu Y, Michalovich D, Wu H, Tan KB, Dytko GM, Mannan JJ, Boyce R, Alston J, Tierney LA, Li X, et al. Cloning, expression and pharmacological characterization of a novel human histamine receptor. *Mol Pharmacol* 2001;59:434–441.
- Nguyen T, Shapiro DA, George SR, Setola V, Lee DK, Cheng R, Rauser L, Lee SP, Lynch KR, Roth BL, et al. Discovery of a novel member of the histamine receptor family. *Mol Pharmacol* 2001;59:427–433.
- Hofstra CL, Desai PJ, Thurmond RL, Fung-Leung WP. Histamine H4 receptor mediates chemotaxis and calcium mobilization of mast cells. *J Pharmacol Exp Ther* 2003;305:1212–1221.
- Ling P, Ngo K, Nguyen S, Thurmond RL, Edwards JP, Karlsson L, Fung-Leung WP. Histamine H4 receptor stimulation suppresses IL-12p70 production and mediates chemotaxis in human monocyte-derived dendritic cells. *J Immunol* 2005;174:5224–5232.
- del Rio R, Noubade R, Saligrama N, Wall EH, Kremmentsov DN, Poynter ME, Zachary JF, Thurmond RL, Teuscher C. Histamine receptor H4 optimizes T regulatory cell frequency and facilitates anti-inflammatory responses within the central nervous system. *J Immunol* 2012;188:541–547.
- Simon T, László V, Láng O, Buzás E, Falus A. Histamine regulates relevant murine dendritic cell functions via H4 receptor. *Front Biosci (Elite Ed)* 2011;3:1414–1424.
- Lippert U, Artuc M, Grützkau A, Babina M, Guhl S, Haase I, Blaschke V, Zachmann K, Knosalla M, Middel P, et al. Human skin mast cells express H2 and H4, but not H3 receptors. *J Invest Dermatol* 2004;123:116–123.
- Damaj BB, Becerra CB, Esber HJ, Wen Y, Maghazachi AA. Functional expression of H4 histamine receptor in human natural killer cells, monocytes, and dendritic cells. *J Immunol* 2007;179:7507–7515.
- Harris JN, Hutchinson IV. A novel fluorescein-histamine reagent to quantitate histamine receptor expression on leukocytes. *Int J Immunopharmacol* 1994;16:633–640.
- van Rijn RM, Chazot PL, Shenton FC, Sansuk K, Bakker RA, Leurs R. Oligomerization of recombinant and endogenously expressed human histamine H(4) receptors. *Mol Pharmacol* 2006;70:604–615.
- Middleton RJ, Briddon SJ, Cordeaux Y, Yates AS, Dale CL, George MW, Baker JG, Hill SJ, Kellam B. New fluorescent adenosine A1-receptor agonists that allow quantification of ligand-receptor interactions in microdomains of single living cells. *J Med Chem* 2007;50:782–793.
- Baker JG, Middleton R, Adams L, May LT, Briddon SJ, Kellam B, Hill SJ. Influence of fluorophore and linker composition on the pharmacology of fluorescent adenosine A1 receptor ligands. *Br J Pharmacol* 2010;159:772–786.
- Paoletta S, Kosh DK, Finley A, Gizewski ET, Moss SM, Gao Z-G, Auchampach JA, Salvemini D, Jacobson KA. Rational design of sulfonated A3 adenosine receptor-selective nucleosides as pharmacological tools to study chronic neuropathic pain. *J Med Chem* 2013;56:5949–5963.
- Zheng Y, Shopes B, Holowka D, Baird B. Conformations of IgE bound to its receptor FcεRI and in solution. *Biochemistry* 1991;30:9125–9132.
- Ishizaka T, Ishizaka K. Triggering of histamine release from rat mast cells by divalent antibodies against IgE receptors. *J Immunol* 1978;120:800–805.
- Segal DM, Taurog JD, Metzger H. Dimeric immunoglobulin E serves as a unit signal for mast cell degranulation. *Proc Natl Acad Sci USA* 1977;74:2993–2997.
- Metcalfe DD, Baram D, Mekori YA. Mast cells. *Physiol Rev* 1997;77:1033–1079.
- Hsu C, MacGlashan D Jr. IgE Antibody up-regulates high affinity IgE binding on murine bone marrow derived mast cells. *Immunol Lett* 1996;52:129–134.
- Yamaguchi M, Lantz CS, Oettgen HC, Katona IM, Fleming T, Miyajima I, Kinet JP, Galli SJ. IgE enhances mouse mast cell FcεRI expression in vitro and in vivo: Evidence for a novel amplification mechanism in IgE-dependent reactions. *J Exp Med* 1997;185:663–672.
- MacGlashan D Jr. IgE receptor and signal transduction in mast cells and basophils. *Curr Opin Immunol* 2008;20:717–723.
- Mirzahasineini A, Dalmadi B, Csutora P. Histamine receptor H4 regulates mast cell degranulation and IgE induced FcεRI upregulation in murine bone marrow-derived mast cells. *Cell Immunol* 2013;283:38–44.
- Alfon J, Sanchez-Gomez S, Salcedo C, Fernandez A, Gil-Torregrosa B, Ardanaz N, Balsa D, Gomez L, Bartoli J, Merlos M. Efficacy of Ur-63325, a new histamine H4 receptor antagonist, in house dust mite-induced mouse asthma models. *Am J Respir Crit Care Med* 2011;183: A1302.
- Alfon J, Sanchez-Gomez S, Fernandez A, Gil-Torregrosa B, Ardanaz N, Gomez-Valades AG, Mascaro C, Carceller E, Gomez LI, Balsa D, et al. UR-63325, a novel H4 receptor antagonist that shows good efficacy in an ovalbumin-induced mouse asthma-model. In: 29th EAACI; 2010. p 1511. DOI: 10.3252/psa.eu.29EAACI.2010.
- Lazewska D, Kiec-Kononowicz K. Azines as histamine H4 receptor antagonists. *Front Biosci (Schol Ed)* 2012;4:967–987.
- Singh R, Gupta PP, Singh PP. Cultivation of mouse bone marrow-derived mast cells using concavalin-A stimulated splenocyte supernatant. *Indian J Pharmacol* 1998;30: 195–198.
- Van Zoelen EJJ. Receptor–ligand interaction: A new method for determining binding parameters without a priori assumptions on non-specific binding. *Biochem J* 1989; 262:549–556.
- Han Y, Liu X-M, Liu H, Li S-C, Wu B-C, Ye L-L, Wang Q-W, Chen Z-L. Cultivation of recombinant Chinese hamster ovary cells grown as suspended aggregates in stirred vessels. *J Biosci Bioeng* 2006;102:430–435.
- Curtis H, Barnes NS. *Biology*. 5th ed. New York: Worth publishers; 1989. p 104.
- Lim HD, van Rijn RM, Ling P, Bakker RA, Thurmond RL, Leurs R. Evaluation of histamine H1-, H2-, and H3-receptor ligands at the human histamine H4 receptor: Identification of 4-methylhistamine as the first potent and selective H4 receptor agonist. *J Pharmacol Exp Ther* 2005;314:1310–1321.
- Cheng Y, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* 1973;22:3099–3108.
- Almon RR. Cholinergic sites in skeletal muscle. I. Denervation effects. *Biochemistry* 1976;15:3662–3667.
- Mendel CM, Almon RR. Association of [3H]dihydroalprenolol with biological membranes. *Gen Pharmacol* 1979;10:31–40.
- Mendel CM, Mendel DB. 'Non-specific' binding. The problem, and a solution. *Biochem J* 1985;228:269–272.
- James LC, Tawfik DS. The specificity of cross-reactivity: Promiscuous antibody binding involves specific hydrogen bonds rather than nonspecific hydrophobic stickiness. *Protein Sci* 2003;12:2183–2193.

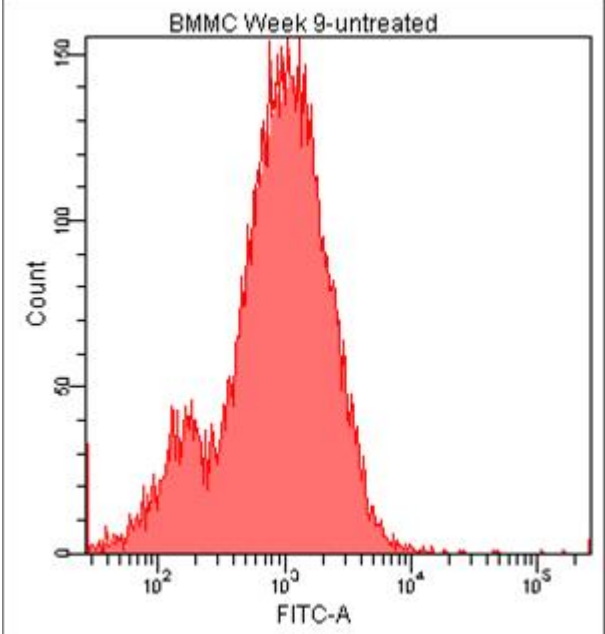
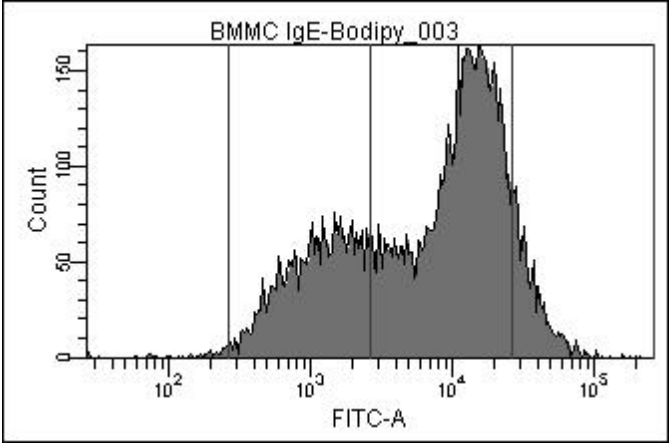
Cytometry Part A
Author Checklist: MIFlowCyt-Compliant Items

Requirement	Please Include Requested Information
1.1. Purpose	The purpose of the experiments presented was to test whether histamine receptors can be analyzed by staining with BODIPY® FL Histamine, a fluorescent ligand, using flow cytometry.
1.2. Keywords	histamine H4 receptor, BODIPY® FL Histamine, mast cells
1.3. Experiment variables	We examined H4 transfected CHO cells and murine bone marrow-derived mast cells, expressing CD117 and FcεRI, by labeling with BODIPY® FL Histamine/competitive inhibition with histamine/cimetidine/UR-63325 and measuring fluorescence intensity.
1.4. Organization name and address	Sanofi Research and Development, Hungary, Budapest H-1045, Tó utca 1-5.
1.5. Primary contact name and email address	Arash Mirzahosseini, mirzahosseini.arash@pharma.semmelweis-univ.hu
1.6. Date or time period of experiment	January-June 2012
1.7. Conclusions	BODIPY® FL Histamine was found to stain H4 transfected cells and mast cells in a concentration-dependent manner; this staining was competitively inhibited with unlabeled ligands. Furthermore, IgE sensitization was found to increase H4 receptor expression in mast cells.
1.8. Quality control measures	The cell phenotype (CF117, FcεRI) and immunocytostaining controls are described in the Materials and Methods.
2.1.1.1. (2.1.2.1., 2.1.3.1.) Sample description	CHO and BMMC cells were incubated with Stain Buffer with specific antigens (anti-CD117-PE, anti-FcεRI-FITC)/BODIPY® FL Histamine and Fc Block for 30 min at +4 °C.0
2.1.1.2. Biological sample source description	CHO cell line purchased from Euroscreen (Gosselies, Belgium), and murine bone marrow-derived mast cells.
2.1.1.3. Biological sample source organism description	8-12 week old male BALB/c mice
2.1.2.2. Environmental sample location	not applicable
2.3. Sample treatment description	CHO and BMMC cells were grown in RPMI-1640 or BMMC medium (see Materials and Methods) and treated with 100 U/ml penicillin and 100 µg/ml streptomycin. BMMC cell cultures were also treated with 50 ng/ml recombinant SCF and 10 ng/ml recombinant murine IL-3.
2.4. Fluorescence reagent(s) description	Rat anti-mouse IgE FITC, rat anti-mouse CD117 PE from BD Biosciences (Erembodegem, Belgium). BODIPY® FL Histamine (detectable with FITC channel) from Life Technologies (Carlsbad, CA).
3.1. Instrument manufacturer	BD Biosciences (Erembodegem, Belgium)
3.2. Instrument model	FACSCanto II
3.3. Instrument configuration and settings	All samples were measured with the same protocol-Standard setting 1000V FSC/1000V SSC. All instrument settings were the calibrated configurations of the manufacturer. Fluorescent emission was captured for FITC conjugates and BODIPY® FL Histamine using 525/40 nm Band Pass filter and for PE conjugates using 575/25 nm Band Pass filter.
4.1. List-mode data files	Since all flow cytometry experiments were performed in an identical fashion one example of FACS data files are presented: BMMC cells were gated according to SSCx FSC dot plot to identify cellular events. P1 population was found to be double positive for CD117 and FcεRI based on PE and FITC staining.



Population	#Events	%Parent	FSC-A Mean	SSC-A Mean	FcεRI FITC-A Mean	CD117 PE-A Mean
All Events	10,000	###	75,483	69,754	3,364	5,085
P1	7,034	70.3	85,398	61,831	3,832	5,187
Q1	130	1.8	78,437	50,135	179	3,371
Q2	6,904	98.2	85,529	62,051	3,901	5,222
Q3	0	0.0	###	###	###	###
Q4	0	0.0	###	###	###	###

BMMC stained with BODIPY® FL Histamine were measured. Below a typical plot of untreated and IgE-treated BMMC histogram for FITC is presented.

	  For original list-mode data, please visit the repository identifier: http://flowrepository.org/id/FR-FCM-ZZEL For additional data contact the corresponding author.
4.2. Compensation description	Relevant flow cytometry assays were single color (no compensation).
4.3. Data transformation details	All data were analyzed using the FACSDiva software suggested by the manufacturer BD Biosciences (Erembodegem, Belgium)
4.4.1. Gate description	For all analytical flow cytometry experiments, a primary gate was placed on SSCxFSC dot plot to identify cellular events, as presented in List-mode data files figures.
4.4.2. Gate statistics	See: List-mode data files.
4.4.3. Gate boundaries	See: List-mode data files. For additional data contact the corresponding author.