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Role of histamine H_4 receptor in breast cancer cell proliferation

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1. ABSTRACT

In order to better understand the role of histamine H4 (H4R) receptor in breast cancer, we studied the receptor expression pattern, associated signal transduction pathway and biological responses, in breast cancer cell lines with different malignant characteristics. A different pattern of protein expression was observed in MDA-MB-231 compared to MCF-7 cells determined by western blot, exhibiting the presence of a diverse range of molecular weight species of the H4R. H4R agonist reduced cyclic adenosine monophosphate (cAMP) formation induced by forskolin only in MCF-7 cells. In MDA-MB-231 cells, H4R agonists significantly decreased cell

roliferation, augmented the Annexin-V and TdT-mediated UTP-biotin Nick End labelling (TUNEL) positive cells and produced a 2.5-fold increase in cell senescence. In MCF-7 cells, H4R agonists inhibited proliferation by 50%, increasing the exponential doubling time. This effect was associated to an augment in Annexin-V and TUNEL positive cells, and a 2-fold increase in cell senescence. We conclude that H4R is functionally expressed in human breast cancer cell lines, exhibiting a key role in histamine-mediated biological processes such as cell proliferation, senescence and apoptosis.

2. INTRODUCTION

Breast cancer is the most common neoplastic disease in women, and continues to rise in incidence (1,2). About 30% of patients with early-stage breast cancer have recurrent disease, which is metastatic in most cases and whose cure is very limited showing a 5-year survival rate of 20% (3). Therefore, the elucidation of molecules and biochemical pathways involved in breast carcinogenesis are of utmost importance for the development of novel therapeutic approaches that can contribute to offer increased efficacy and low toxicity.

A large body of literature has been reported indicating that histamine [2-(4-imidazolyl)-ethylamine] can modulate proliferation of different normal and malignant cells (4-8). Histamine exerts its actions through the interaction with four histamine receptor subtypes, H1, H2, H3 and H4 (H1R, H2R, H3R, H4R). All these receptors belong to the family of heptahelical G-protein coupled receptors (GPCR) (9-11).

Histamine is involved in the pathologic and also physiologic aspects of the mammary gland including growth regulation, differentiation and functioning during development, pregnancy and lactation (12-15). Numerous studies support the role of histamine in breast cancer development. We have reported that histamine becomes an autocrine growth factor capable of regulating cell proliferation via H1R and H2R in experimental mammary carcinomas, and the in vivo treatment with H2R antagonists produced the complete remission of 70% of experimental tumours (6,16-18). Although many reports indicate the presence of H1R and H2R in normal and malignant tissues as well as in different cell lines derived from human mammary gland (19-21), the clinical trials carried out with H2R antagonists in cancer patients demonstrated controversial results for breast cancer (22,23).

The H3R has initially been identified in both central and peripheral nervous system as a presynaptic receptor and therefore, it has gained pharmaceutical interest as a potential drug target for the treatment of various important brain disorders (24-26). On the other hand, the identification of the human H4R in medullary and peripheral hematopoietic cells by several groups, has converted the H4R in a novel therapeutic target for inflammatory and immune disorders (27-30). H4R is coupled to Gαi/o proteins and isoforms and dimeric structures that include homo- and hetero-oligomer formation and post-translational changes have been described for the H4R (9,10,29,31,32).

Recently, we have demonstrated that H3R and H4R are expressed in cell

lines derived from human mammary gland (33,34). Histamine modulated the proliferation of MDA-MB-231 breast cancer cells in a dose-dependent manner producing a significant decrease at 10 μmol.L-1 concentration whereas at lower concentrations it increased proliferation moderately, and no effect on proliferation or expression of oncogenes related to cell growth is observed in non-tumorigenic HBL-100 cells (33,35). The negative effect on proliferation is in part mediated through the H4R (34). In agreement with this, recent data indicate that H3R and H4R are expressed in human biopsies of benign lesions and breast carcinomas, being the level of their expression significantly higher in carcinomas. Furthermore, 50% of malignant lesions expressed H4R, all of them corresponding to metastases or high invasive tumours (34).

The identification of H4R and the elucidation of its role in the development and growth of human mammary carcinomas may represent an essential clue for advances in breast cancer treatment.

Thus, in order to better understand the role of H4R in breast cancer, we have, in the present work compared the receptor expression pattern, and the biological responses triggered by histamine through this receptor in two breast cancer cell lines with different malignant characteristics.

3. MATERIALS AND METHODS

3.1. Cell culture

The human breast cancer cell lines MDA-MB-231 and MCF-7 (American Type Tissue Culture Collection, VA, USA) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 0.3 g L-1 glutamine, and 0.04 g L-1 gentamicin (Gibco BRL, NY, USA). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2.

3.2. RT-PCR

The retrotranscription reaction was performed with 2 μg of RNA that was isolated using TRIZOL reagent, according to the manufacturer's instructions (Invitrogen, USA). H4R primers were, H4R-F: GGG GTC TTG AAG ATT GTTA C; H4R-R: GCA GTT CAA CAT GTT CCC; 512 bp, 35 cycles of 45 s at 94°C; 45 s at 58°C; 50 s at 72°C (33). Negative controls were performed with water instead of cDNA. Fragments identity was corroborated by sequenciation (Macrogen, Korea). β-actin was used as load control, β-actin-F: ACC TCA TGA AGA TCC TGA C, β-actin-R: ACT CCT GCT TGC TGA TCC; 521 pb, 25 cycles of 30 s at 95ºC, 30 s at 58ºC, 60 s at 72ºC. PCR products were subjected to gel electrophoresis and photographed using a Sony Cyber-Shot DSC-S75 camera.

3.3. Western blot analysis

Total extracts were obtained as previously described (33). Briefly, cells were washed twice with phosphate-buffered saline (PBS) and scraped into a lysis buffer (100 mM Tris/HCl buffer, pH 8, containing 1% (v/v) Triton X-100 and protease inhibitors) and incubated for 20 min on ice. After centrifugation at 3000 g for 10 min, the supernatants were termed the total extracts. Protein concentration in the extracts was determined according to Bradford assay (36). Equal amounts of proteins were fractionated by SDS-PAGE. After blotting to nitrocellulose membrane and

blocking of nonspecific binding [5% (w/v) dry milk in PBS / 0.05% Tween 20; 2 hour at room temperature], membranes were incubated overnight at 4°C with the anti-H4R primary antibody (Alpha Diagnostic International, TX, USA, and diluted 1:500 in PBS). After being washed and incubated with secondary peroxidase-coupled anti-rabbit antibody (BioRAD, CA, USA, and diluted 1:1000 in PBS), proteins were visualized by autoradiography using enhanced chemiluminescence (Amersham Biosciences, USA). Densitometric analyses were performed using Image J 1.32J software (NIH, USA).

3.4. Histamine H_4 receptor immunostaining

Cells were cultured on glass coverslips and then fixed with 4% (v/v) formaldehyde in PBS and permeabilized with 0.25% (v/v) triton X-100 in PBS. The endogenous peroxidase activity was blocked with 3% hydrogen peroxide in distilled water. After blocking in 1% (w/v) bovine seroalbumine (BSA) in PBS, cells were incubated overnight at 4°C in a humidified chamber with 1:100 rabbit anti-histamine H4R antibodies diluted in 1% (w/v) BSA in PBS (Alpha Diagnostic International, TX, USA). Cells were washed with PBS and incubated for 2 hours with a goat peroxidase-conjugated secondary antibody diluted in 1% (w/v) BSA in PBS (1:100, Sigma Chemical Co., MO, USA) and visualized by 3,3-diaminobenzidine staining (Sigma Chemical Co., MO, USA) and haematoxylin counterstaining. Light microscopy was performed on an Axiolab Karl Zeiss microscope (Göttingen, Germany). Photographs were taken at 630X or 1000X magnification using a Canon PowerShot G5 camera (Tokyo, Japan). No immunostaining was observed when the primary or secondary antibodies were omitted. Cell nuclei haematoxylin staining was used to confirm the presence of cells.

3.5. Determination of cyclic adenosine monophosphate (cAMP)

Intracellular cAMP was measured in cell monolayers at 70-90% confluence after the stimulation with histamine (0.01 μM – 10 μM), VUF8430 (0.001 μM – 10 μM) and/or Forskolin (10 μM) for 15 min at 37°C. The cAMP produced was extracted with ethanol (Merck, Argentina) and determined by radioimmunoassay as we have previously described (37).

3.6. Cell proliferation assays

For clonogenic assay, cells were seeded in six-well plates (1200 cells/well) and treated with 0.001 to 10 μM VUF8430 (H4R agonist), 10 μM Anthamine (H2R agonist), 10 μM 2-(3-(trifluoromethyl)phenyl)histamine (H1R agonist) (Tocris Bioscience, USA), 0.001 to 10 μM clobenpropit (H3R antagonist and H4R agonist), 0.01 μM Imetit and 0.01 μM R(-)-α-methylhistamine (H3R agonists) (Sigma Chemical Co., USA), or 0.001 to 10 μM histamine (Fluka, USA). The cells were incubated for 7 days and then fixed with 10% (v/v) formaldehyde in PBS and stained with 1% (w/v) toluidine blue in 70% (v/v) ethanol. The clonogenic proliferation was evaluated by counting the colonies containing 50 cells or more and was expressed as a percentage of the untreated wells.

Quantification of cellular DNA synthesis was performed by 5-bromo-2'-deoxyuridine (BrdU, Sigma Chemical Co., USA) incorporation. Cells were

seeded on glass coverslips into 12-well plates in culture medium (50000 cells/well), and treated with 10 µM Histamine (Fluka, USA), 10 µM VUF8430, 10 µM clobenpropit, and/or 10 µM JNJ7777120 (H4R antagonist, Johnson & Johnson Pharmaceutical Research and Development, USA) for 48 h. After that, BrdU (30 µM) was added into culture medium for 2 h. The cells were then washed twice and fixed for 15 min in 4% (v/v) formaldehyde in PBS. To denature the DNA into single-stranded molecules, cells were incubated with 3 N HCl, 1% Triton X-100 (v/v) in PBS for 15 min at room temperature. Cells were washed in 1 ml of 0.1 M Na2B4O7 (Sigma Chemical Co., USA), 1% Triton X-100 (v/v) in PBS, pH 8.5 to neutralize the acid. After blocking with 5% FBS (v/v) in PBS, cells were then incubated with anti-BrdU mouse monoclonal antibody diluted 1:100 in 1% bovine seroalbumine (w/v) in PBS (Sigma Chemical Co., USA). Cells were washed with PBS and further incubated for 30 min with 1:100 fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG and 4'-6-diamidino-2-phenylindole (Dapi) (Sigma Chemical Co., USA) at room temperature. Coverslips were mounted with FluorSaveTM Reagent (Calbiochem, USA) and fluorescence was observed by epifluorescence using an Olympus BX50 microscope. Photography was carried out with a CoolSnap digital camera. At least 500 cells were scored for each determination. Pictures were taken at a 400X-fold magnification.

Cells were also incubated overnight in 6-well plates (100000 cells/well), and then incubated with 10 µM histamine, 10 µM VUF8430, 10 µM clobenpropit or were left untreated for up to 72 hours. Cells were trypsinized at 0, 1, 2, 3 days and counted using a hemocytometer. The equation for exponential growth was Nt=N0xe(kxt), where N0 was the initial number of cells that increased exponentially with a rate constant, k. The doubling time was calculated as 0.69/k. All experiments were performed at least three times with duplicate and triplicate measurements for each condition.

3.7. Determination of apoptosis

Phosphatidylserine exposure on the surface of apoptotic cells was detected by flow cytometry after staining with Annexin V-FITC (BD biosciences, USA), and propidium iodide (50 µg/ml). Data were analyzed using WinMDI 2.8 software (Scripps Institute, CA, USA).

Apoptotic cells were determined by TUNEL (TdT-mediated UTP-biotin Nick End labeling) assay. Cells were cultured on glass coverslips into 12-well plates for 24 hours and then incubated with 10 µM histamine, 10 µM VUF8430, 10 µM clobenpropit for 72 hours. Cells were washed, fixed and the fragmented DNA was detected using ApoptagTM plus peroxidase in situ apoptosis Detection Kit (CHEMICON International, CA, USA) according to the manufacturer's instructions. Cells were visualized using Axiolab Karl Zeiss microscope (Germany). At least 500 cells were scored for each determination.

Variations of the mitochondrial transmembrane potential ($\Delta\psi m$) were studied using 3,3'-dihexyloxacarbocyanine iodide (DiOC6; Sigma Chemical Co., MO, USA) (38). Cells were plated and after 24 hours treated with 10 µM histamine, 10 µM VUF8430, 10 µM clobenpropit for 72

hours. The diluted dye at a final concentration of 40 nM in PBS was applied to cells for 15 min at 37°C. Cells were then washed, harvested and analyzed by flow cytometry. Data were analyzed using WinMDI 2.8 software (Scripps Institute, CA, USA).

3.8. Senescence-associated β-galactosidase staining

Cells were seeded on glass coverslips into 12-well plates in culture medium (50000 cells/well) and treated with 10 μM histamine, 10 μM VUF8430, 10 μM clobenpropit, and/or 10 μM JNJ7777120 for 48 h. Senescence-associated β-galactosidase-positive cells were detected using the method described by Dimri et al. (39) and also previously by us (40). Briefly, cells were fixed and incubated at 37°C for 8 hours with 1 mg/ml 5-bromo-4-chloro-indolyl-β-galactoside (USB Corp., USA) in an appropriate buffer. After incubation, cells were washed twice with PBS and counterstained with hematoxylin and the percentage of β-galactosidase-positive cells was assessed under light microscopy (Axiolab Karl Zeiss, Göttingen, Germany). At least 500 cells were scored for each determination. All photographs were taken at 630X magnification using a Canon PowerShot G5 camera (Tokyo, Japan).

4. RESULTS

4.1. Histamine H_4 receptor expression in breast cancer cells

We first evaluated the H4R expression by RT-PCR and results showed that MCF-7 cells and, as we have previously described, MDA-MB-231 cells express H4R at the mRNA level (33) (Figure 1A).

Western blot analysis of protein extracts displayed the presence of a diverse range of molecular weight species of the H4R, exhibiting a different pattern when MDA-MB-231 cells are compared with MCF-7 cells. In MDA-MB-231 cells, a high molecular weight specie (Mw ~75 kDa) was observed which is consistent with the recombinant dimeric human H4R and native dimeric species detected in human lymphocytes and brain (31,32,41). An additional band (Mw ~ 45 kDa) was observed, which could represent a proteolytic fragment (31). On the other hand, in MCF-7 cells we detected the putative monomer at Mw 31 kDa and two higher molecular weight species, which could correspond to dimeric structures. One of these dimeric forms (Mw ~75 kDa) was also observed in MDA-MB-231 cells (29,32). In addition, a shorter isoform can be differentiated only in MCF-7 cells (Figure 1B).

We further investigated the H4R by immunocytochemistry employing specific polyclonal antibodies and peroxidase conjugated secondary antibodies. Results confirmed the H4R expression in MDA-MB-231 and MCF-7 cells (Figure 1C).

4.2. Role of H_4R in cAMP production in breast cancer cells

We additionally investigated whether histamine could modulate cAMP production in breast cancer cells. In MDA-MB-231 cells histamine at any dose tested was unable to modify cAMP production (Figure 2A). On the contrary, histamine at 10 μM concentration produced a 2-fold increase in cAMP levels in MCF-7 cells (Figure 2B). Furthermore, we studied if H4R agonist could inhibit the increase in cAMP levels triggered by forskolin. Results demonstrated that VUF8430 was not able to reduce the forskolin-induced cAMP accumulation in MDA-MB-231 cells (Figure 2C). Conversely, in MCF-7 cells treatment with VUF8430 (10 μM) produced a maximal inhibition of cAMP of approximately 30% (Figure 2D).

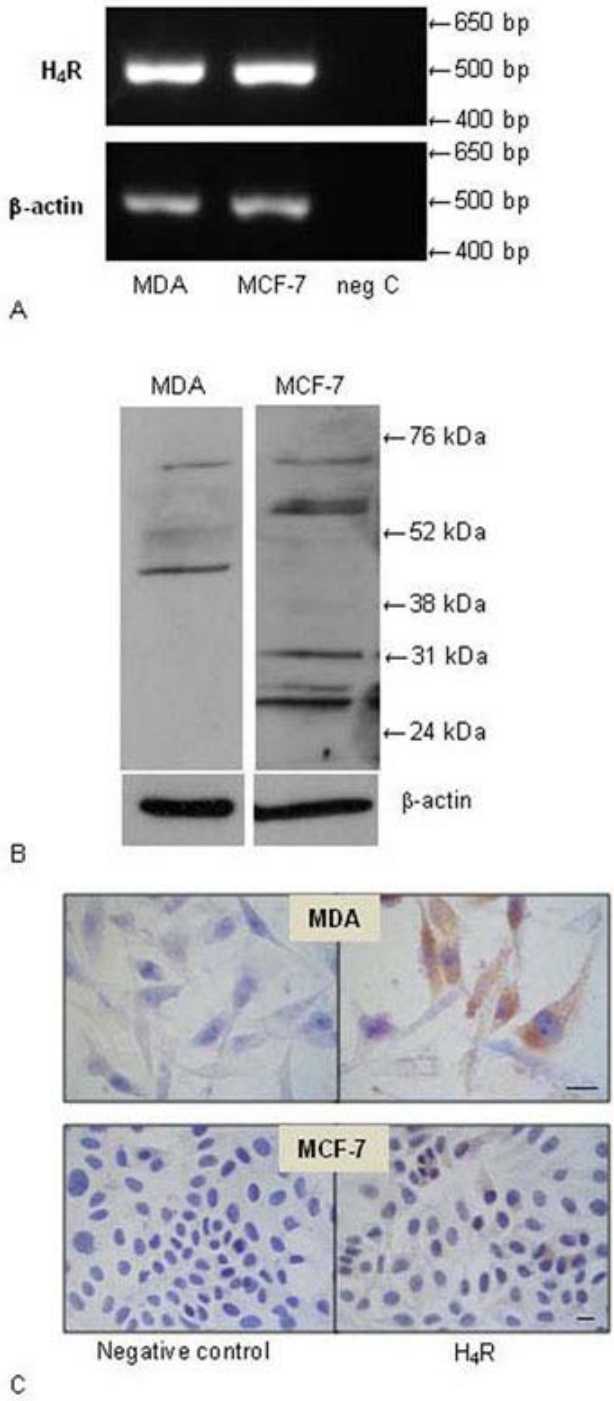

Figure 1. Histamine H_4R expression in breast cancer cell lines. A) RT-PCR analysis of H_4R mRNA expression (512 bp). Lanes: 1, MDA-MB-231 cells; 2, MCF-7 cells; 3, negative control. B) Western blot analysis of MDA-MB-231 (lane 1, MDA) and MCF-7 (lane 2) cells were carried out with 35 μg and 75 μg of total cellular proteins, respectively, and H_4R were detected by incubation with specific antibodies. C) Immunocytochemical detection of H_4R. Pictures were taken at a 630x-fold and 1000x-fold magnification. Scale bar= 20 μm. Data are representative of three independent experiments. β-actin was used as load control.

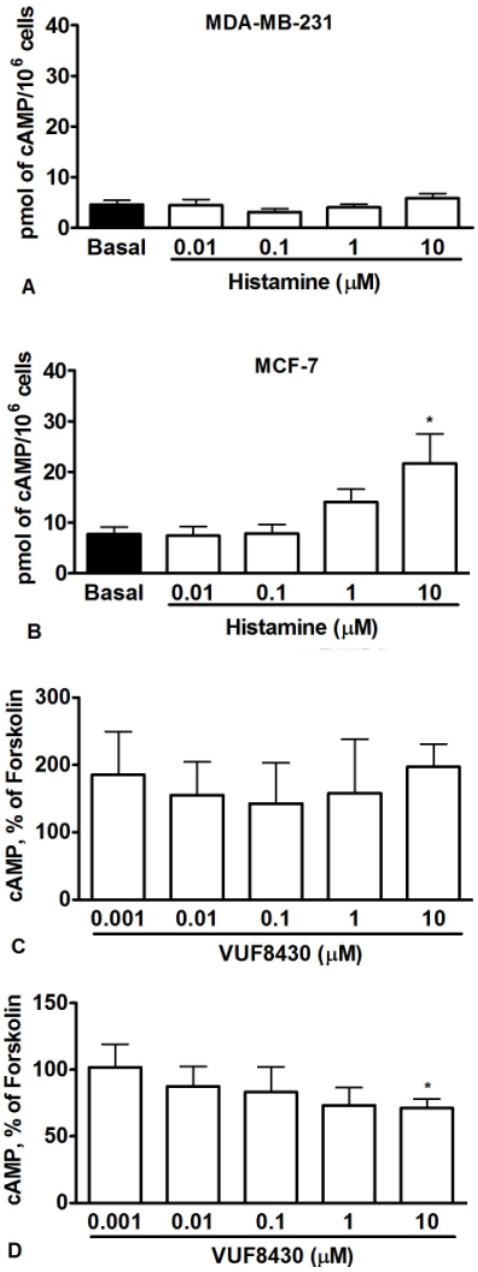

Figure 2. Effect of histamine and VUF8430 on cAMP production. cAMP levels in A) MDA-MB-231 and B) MCF-7 cells were determined after the treatment with histamine (0.01-10 μM) for 15 minutes. Data represent the means ± SEM (*P<0.05 vs. Basal; ANOVA and Tukey's Multiple Comparison Test). Forskolin-induced cAMP accumulation in C) MDA-MB-231 and D) MCF-7 cells. Cells were incubated with forskolin (10 μM) and when required VUF8430 (0.001-10 μM) for 15 minutes. Results are expressed as the percent change of the forskolin-evoked response which represented 25.2 pmol and 1396.5 pmol per 10^6 MDA-MB-231 and MCF-7 cells, respectively. Data represent the means ± SEM (*P<0.05 vs. Forskolin; ANOVA and Tukey's Multiple Comparison Test).

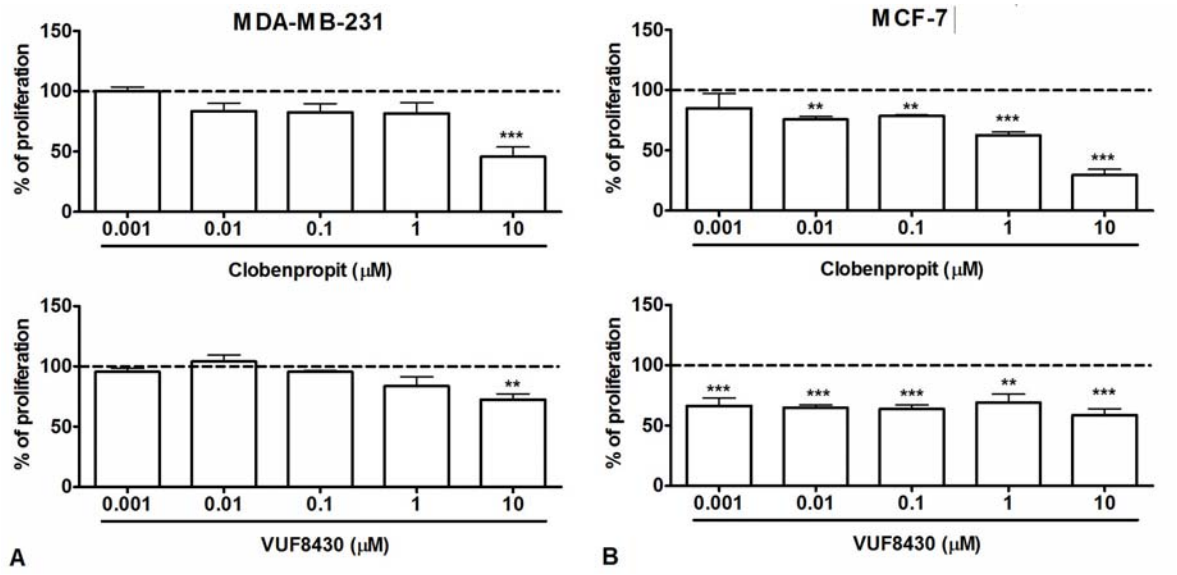

Figure 3. Effect of H_4R agonists on breast cancer cell proliferation. Proliferation was determined by the clonogenic assay. A) MDA-MB-231 and B) MCF-7 cells were left untreated or were treated with clobenpropit or VUF8430 (0.001-10 μM). Results are expressed as percentage of control values. Error bars represent the means ± SEM. (**P<0.01, ***P<0.001 vs. Control; ANOVA and Tukey's Multiple Comparison Test).

4.3. Role of H_4R in breast cancer cell proliferation

We have previously reported that histamine modulated the proliferation of MDA-MB-231 breast cancer cells in a dose-dependent manner producing a significant decrease at micromolar concentration, whereas at lower concentrations it increased proliferation moderately through the H3R (33). Present results demonstrate that clobenpropit and VUF8430 treatments at 10 μM concentration significantly decreased proliferation to 45.5±14.8% and to 76.7±5.3%, respectively, while lower concentrations were unable to modify clonogenic proliferation (Figure 3A). Accordingly, both agonists significantly reduced the incorporation of BrdU and treatment with the H4R antagonist, JNJ7777120, reversed the histamine inhibitory effect on proliferation (Table 1).

We further investigated the biological responses triggered by histamine in a more differentiated breast cancer cell line, MCF-7. We showed that histamine at all doses tested, decreased the proliferation of MCF-7 cells (Figure 4A), and increased doubling time (Table 2). This negative effect on proliferation was mimicked by the treatment with H1R, H2R, H3R and H4R agonists (Figure 3B and 4B). Accordingly, histamine reduced BrdU incorporation, effect that was partially blocked by the combined treatment with JNJ7777120 (Table 1). Furthermore, treatment with clobenpropit exhibited a dose response relationship starting at 0.01 μM. On the other hand, all concentrations evaluated of VUF8430 inhibited similarly proliferation (Figure 3B). Also, both agonists significantly diminished BrdU incorporation (Table 1). In agreement with these results, clobenpropit and VUF8430 treatments increased the exponential doubling time from 32.6 h to 47.2 h and 44.1 h in treated cells, respectively (Table 2).

Table 1. Incorporation of BrdU in breast cancer cells

Treatment	MDA-MB-231 (% of BrdU positive cells)	MCF-7 (% of BrdU positive cells)
Control	43.1 ± 1.0	34.6 ± 1.4
Histamine	33.0 ± 3.0[1]	25.8 ± 1.8[1]
JNJ7777120	49.1 ± 8.0	33.1 ± 2.3
Histamine +JNJ7777120	58.4 ± 6.9[1,2]	30.7 ± 2.0
Clobenpropit	34.3 ± 2.3[1]	22.2 ± 2.2[1]
VUF8430	32.2 ± 2.1[1]	27.0 ± 2.5[1]

Cells were left untreated or treated with clobenpropit, VUF8430, Histamine (10 μM) and/or JNJ7777120 (10 μM) for 48 h. Error bars represent the means ± SEM. [1]P<0.05 vs. Control, [2]P<0.05 vs. Histamine; ANOVA and Tukey's Multiple Comparison Test.

Table 2. Effect of histamine and H_4R agonists on the exponential growth of MCF-7 cells

Treatment	Doubling time (h)
Control	32.6 ± 1.3
Histamine	43.8 ± 2.8[1]
VUF8430	44.1 ± 3.1[1]
Clobenpropit	47.2 ± 0.8[1]

MCF-7 cells were left untreated or were treated with clobenpropit, VUF8430 or Histamine (10 μM) for up to 72 h and doubling time (0.69/k) was calculated according to the equation for exponential growth, $N_t = N_0 x e^{(kxt)}$, where N_0 was the initial number of cells that increased exponentially with a rate constant, k. Data represent the means ± SEM. [1]P<0.05 vs. Control; ANOVA and Tukey's Multiple Comparison Test.

4.4. Effect of H_4R agonists on breast cancer cell apoptosis

We have previously demonstrated that treatment with histamine or clobenpropit elicited an apoptotic response in MDA-MB-231 cells determined by an increase in Annexin-V and TUNEL positive cells (33,34). We also showed a marked enhancement of the TUNEL positive cells after VUF8430 treatment (12.5±0.5 vs. 3.1±0.6 in untreated, P<0.01) (Table 3). Similarly, the inhibitory effect on proliferation exerted through the H4R in MCF-7 cells was also associated to an induction of apoptosis assessed after 72 hours of treatment. Histamine, clobenpropit and VUF8430 treatments produced a 3-fold increase in the percentage of apoptotic cells determined by Annexin-V staining (Figure 5A). In accordance to this, all treatments produced the disruption of the mitochondrial transmembrane potential ($\Delta\psi m$) (Figure 5B). These results were further confirmed by TUNEL assay that showed an increased in the number of TUNEL positive cells after the challenge (Figure 5C and D).

4.5. Role of H_4R in breast cancer cell senescence

Cellular senescence is a tumour-suppressive mechanism characterized by a biological program of terminal growth arrest associated to an enhanced activity of senescence associated β-galactosidase (42). Present results indicate that the negative effect on proliferation exerted by histamine in MDA-MB-231 cells was elated to a 2-fold increase in cell senescence. In addition, this effect was reversed by JNJ7777120 treatment and H4R agonists mimicked histamine effect, suggesting the involvement of H4R in histamine-induced cell senescence

Table 3. Role of histamine and H_4R agonists on MDA-MB-231 cell apoptosis

Treatment	TUNEL positive cells (%)
Control	3.1 ± 0.6
Histamine	19.6 ± 1.6[1]
VUF8430	12.5 ± 0.5[1]
Clobenpropit	14.5 ± 0.5[1]

MDA-MB-231 cells were left untreated or were treated with clobenpropit, VUF8430 or Histamine (10 μM) for 48 h and the percentage of apoptosis was determined by the TUNEL assay. Data represent the means ± SEM. [1]P<0.01 vs. Control; ANOVA and Tukey's Multiple Comparison Test.

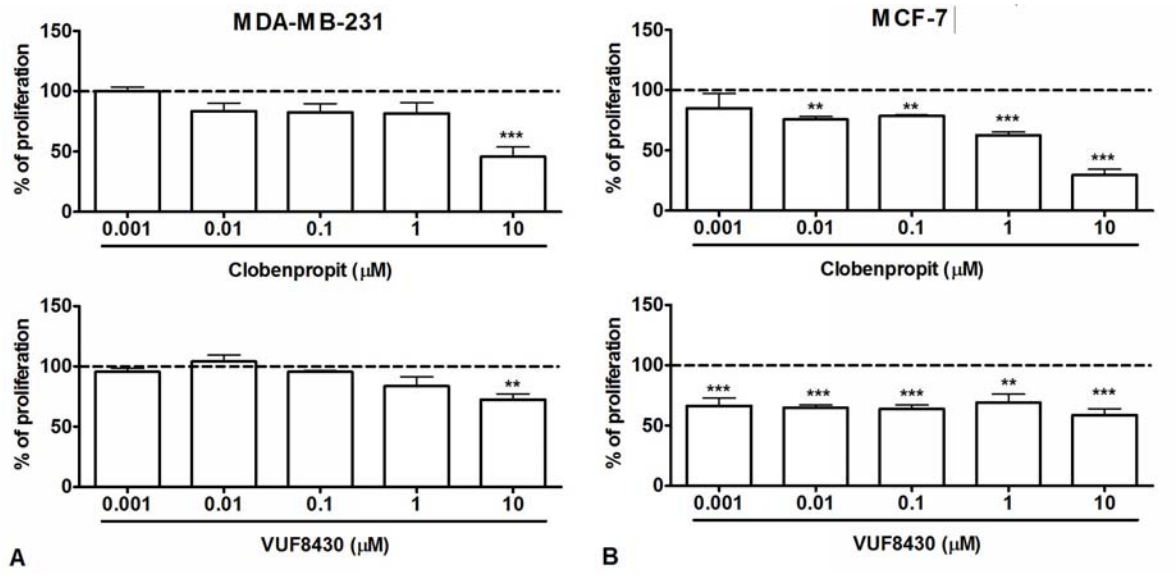

Figure 3. Effect of H_4R agonists on breast cancer cell proliferation. Proliferation was determined by the clonogenic assay. A) MDA-MB-231 and B) MCF-7 cells were left untreated or were treated with clobenpropit or VUF8430 (0.001-10 μM). Results are expressed as percentage of control values. Error bars represent the means ± SEM. (**P<0.01, ***P<0.001 vs. Control; ANOVA and Tukey's Multiple Comparison Test).

(Figure 6A). In the same way, histamine was able to augment the percentage of MCF-7 senescent cells (15.7±1.3 vs. 7.8±0.4 in untreated, P<0.001), which was partially blocked by the treatment with JNJ7777120. Moreover, H4R agonists elicited a similar response, increasing cell senescence (Figure 6B).

5. DISCUSSION

The identification of the human H4R by genomics-based approach has helped refine our understanding of histamine roles. It appeared to have a selective expression pattern restricted to hematopoietic cells. However, H4R was reported to be present on other cell types including central nervous system, nerves of nasal mucosa, enteric neurons, intestinal epithelium, spleen, lung, stomach, and interestingly in cancer cells (29,33,41,43-50). The significance of the H4R presence in various human tissues remains to be elucidated and therefore, new roles of H4R are still unrevealed (28,29).

The human H4R gene that mapped to chromosome 18 encodes a protein of 390 amino acids (9,10,29,48,51). Although the genes for many GPCRs lack introns

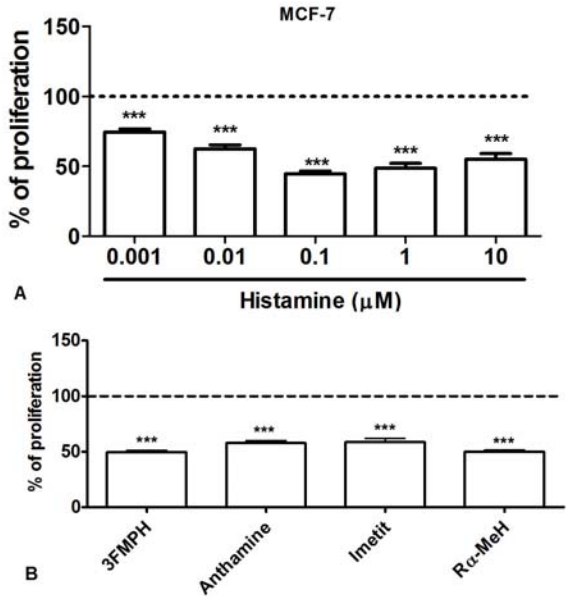

Figure 4. Effect of histamine and its agonists on MCF-7 breast cancer cell proliferation. Proliferation was determined by the clonogenic assay. A) Cells were left untreated or were treated with histamine (0.001-10 μM). B) Cells were left untreated or treated with 2-(3-(trifluoromethyl)phenyl)histamine (3FMPH, 10 μM), Anthamine (10 μM), Imetit (0.01 μM) or R-αMethylhistamine (R-αMeH, 0.01 μM). Results are expressed as percentage of control values. Error bars represent the means ± SEM. (***P<0.001 vs. Control; ANOVA and Tukey's Multiple Comparison Test).

analysis of the H4R gene has revealed the presence of two large introns, indicating that alternative splicing may result in the generation of H4R isoforms. Isoforms have been described for the H4R which have different ligand binding and signalling characteristics. H4R splice variants [H4R (67) and H4R (302)] have a dominant negative effect on H4R (390) functionality, being able to retain it intracellularly and to inactivate a population of H4R (390) presumably via hetero-oligomerization (29,32). In addition, H4R dimeric structures that include homo- and hetero-oligomer formation and post-translational changes of the receptor might contribute to added pharmacological complexity for H4R ligands (29,31,32).

In the present work, we demonstrated the functional expression of H4R in two breast cancer cell lines with different malignant characteristics. A different pattern of protein expression was observed in MDA-MB-231 and MCF-7 cells. MCF-7 cells exhibited the presence of a diverse range of molecular weight species of the H4R, which are compatible with the putative monomer at Mw 31 kDa, and shorter molecular weight specie that might correspond to an H4R isoform. Additionally, two higher molecular weight species were observed that well-matched robust dimeric structures (29,32). The molecular weight specie of ~75 kDa was also detected in MDA-MB-231 cells and in other human cancer cells such as Panc-1 cells derived from a ductal pancreatic carcinoma (52), and M1/15 cells derived from a liver metastasis of malignant melanoma (Massari et al., unpublished data). In addition, it is consistent with the recombinant dimeric human H4R and the native dimeric specie detected in human lymphocytes and brain (31,32,41). In MDA-MB-231 cells another band (Mw ~ 45 kDa) was detected, which might represent a proteolytic fragment (31) or, as previously reported, the recombinant H4R expressed in COS-7 (45). Furthermore, results indicate that a specific antibody reacting with the H4R demonstrated cytoplasmic staining and a higher intensity in anti-H4R reactivity in MDA-MB-231 cells compared to MCF-7 cells.

In multiple malignant cell types, histamine receptors can be associated to multiple signalling pathways. The regulation of receptor density at cell surface can strongly affect the receptor ability to functionally couple and regulate different signal transduction pathways (17,53). Results showed that histamine was not able to modulate cAMP levels in MDA-MB-231 cells while it produced an increase in cAMP levels in MCF-7 cells at 10 μM concentration that was completely blocked

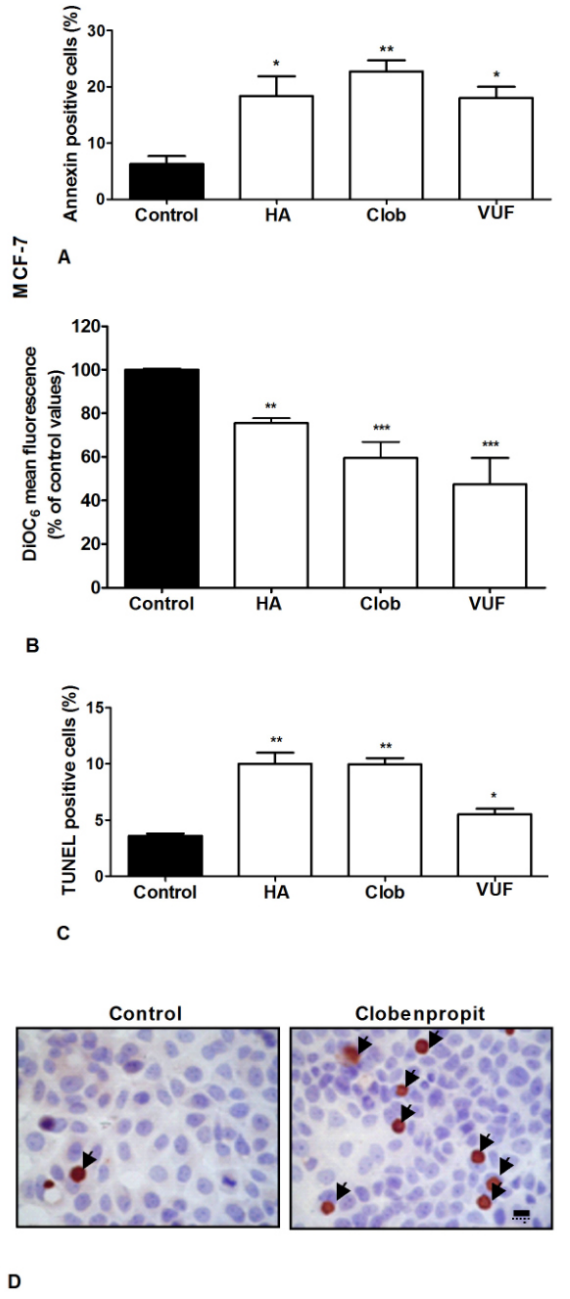

Figure 5. Effect of histamine and H_4R agonists on cell death. A) Apoptotic cells were determined by Annexin V-FITC staining and FACS analysis. B) Disruption of mitochondrial transmembrane potential ($\Delta\psi m$) was evaluated by flow cytometry using $DiOC_6$ staining. C) Apoptosis was determined by the TUNEL assay. At least 500 cells were scored for each determination. D) Apoptotic cells are indicated with arrows. Cells were left untreated or were treated with 10 μM histamine (HA), 10 μM clobenpropit (Clob), or 10 μM VUF8430 (VUF) for 72 h. Error bars represent the means ± SEM. (*P<0.05, **P<0.01, ***P<0.001 vs. Control; ANOVA and Tukey's Multiple Comparison Test).

by the treatment with the H2R antagonist famotidine (data not shown), and an H2R agonist significantly increased cAMP levels (54). Like the recombinant H3R (10,25,26), the recombinant H4R has been described to be coupled to Gi/0 proteins, leading to inhibition of cAMP formation (9,10,29). Therefore, we further investigated the modulation of cAMP production by the H4R putative agonist VUF8430. Results demonstrated that the H4R agonist decreased the cAMP formation induced by forskolin, indicating that the H4R is coupled to the inhibition of adenylate cyclase in MCF-7 cells. H4R agonist produced a maximal inhibition of forskolin-induced cAMP accumulation of 30% in MCF-7 cells. On the other hand, H4R agonist was not able to decrease the cAMP level induced by forskolin in MDA-MB-231 cells, suggesting that in these cells the H4R is doubtable coupled to the Gαi/0. Additionally, in both cell lines, treatment with VUF8430 did not significantly modify the cAMP basal levels (data not shown). In agreement with our results, a similar maximal inhibitory response of around 30% was previously reported for human H4R expressed in HEK cells when treated with H4R agonists (55), and also in other studies direct coupling of the H4R to inhibition of adenylate cyclase could not be detected (43) or was also found to be weak (44). These results might suggest that the reduction of cAMP by the inhibition of adenylate cyclase is not a primary transduction pathway of the H4R in these cells. Current studies are aimed to fully explore the signal transduction pathway associated to the H4R as well as the H4R isoforms in breast cancer cell lines.

Several lines of evidence indicate that carcinogenesis is a multistep process that drives the progressive transformation of normal cells into highly malignant derivatives. These steps reflect genetic alterations that lead to physiologic changes including self-

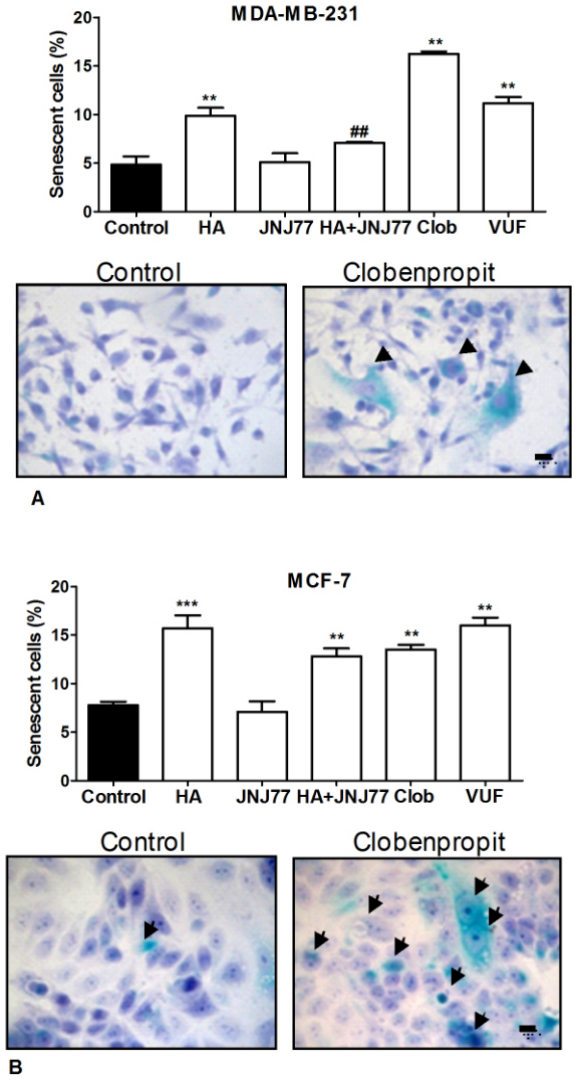

Figure 6. Senescence-associated β-galactosidase staining. A) MDA-MB-231 and B) MCF-7 cells were left untreated or were treated with 10 μM histamine (HA), 10 μM clobenpropit (Clob), 10 μM VUF8430 (VUF) and/or 10 μM JNJ7777120 (JNJ77) for 48 h. At least 500 cells were scored for each determination. Arrows indicate senescent cells. Error bars represent the means ± SEM. (**P<0.01, ***P<0.001 vs. Control; ##P<0.01 vs. HA; ANOVA and Tukey's Multiple Comparison Test).

sufficiency in growth signals, insensitivity to programmed cell death (apoptosis) and limitless replicative potential, which collectively dictate malignant growth (56,57).

In order to investigate the role of H4R in breast carcinogenesis we first evaluated its involvement in breast cancer cell proliferation. In both cell lines histamine has the ability to modulate cell proliferation. In MDA-MB-231 breast cancer cells it produced a dose-dependent effect, eliciting a significant decrease at micromolar concentration, whereas at lower concentrations it increased proliferation moderately through the H3R (33). In contrast, in MCF-7 cells histamine decreased proliferation at all doses tested and the combined treatment with JNJ7777120 reverted in part the reduced BrdU incorporation induced by histamine. In this line, the 4 histamine receptor subtype agonists reduced proliferation, indicating that the inhibitory response elicited by histamine is not only mediated by the H4R. Interestingly, histamine was incapable of inducing proliferation via the H3R in these cells as compared with the more undifferentiated MDA-MB-231 breast cancer cells, suggesting that a different isoform expression, protein-protein interaction, or signalling pathways could be responsible for the variation in histamine responses. Furthermore, H4R agonists inhibited proliferation and reduced BrdU incorporation of both cell lines; however, in MDA-MB-231 cells this effect was only achieved at 10 μM concentration while decrease in MCF-7 cell proliferation was observed at lower concentrations. Thus, the H4R agonists evoked a response at low concentrations in MCF-7 cells, suggesting a different potency to inhibit proliferation that may be associated to the dissimilar molecular weight species/isoforms of the H4R detected, and also a consequence of the different signal transduction pathway triggered through this receptor in these cell lines. In addition, in both cell lines clobenpropit treatment elicited a higher response in terms of proliferation than the putative H4R agonist VUF8430, suggesting that part of the observed effect

could not be mediated by the H4R. The involvement of H4R in cancer cell proliferation was also reported in Panc-1 cancer cells (52) and melanoma cells (Massari et al., unpublished data) in which H4R agonists reduced proliferation (58). Furthermore, the expression of H4R was reported not only in cell lines but also in tissue derived from colon carcinoma (49,59). Interestingly, the H4R antagonist, JNJ7777120, prevented the cell growth-promoting activity of histamine in three colon cancer cell lines without affecting the basal growth of the cells (49). Therefore, the precise role of H4R in cell proliferation seems to be cancer type dependent.

In order to determine whether the reduced proliferation exerted by the H4R agonists could be associated to an induction of programmed cell death, we investigated their effect on cell apoptosis. As we have previously showed, histamine and H4R agonists evoked an apoptotic response in MDA-MB-231 cells, producing a 4-fold increase in the number of TUNEL positive cells (33,34). Here we demonstrated that histamine and H4R agonists also induced apoptosis in MCF-7 cells, producing a 3-fold increase in the percentage of apoptotic cells determined by Annexin-V staining and TUNEL assay. In agreement, all treatments produced the disruption of $\Delta\psi m$, which constitutes an obligate irreversible step of apoptosis (38). It was recently described that stimulation of the H4R is involved in the development of sepsis-induced splenic apoptosis through counteraction of the antiapoptotic action of NF-kappaB (60). To our knowledge, these findings are the first to describe the involvement of the H4R in cancer cell apoptosis.

Cell senescence, characterized by disruption of lysosomal function through enhanced activity of senescence-associated β-galactosidase, is another biological process associated with permanent growth arrest and lack of proliferative activity and, therefore, may influence the overall tumour response to anticancer therapy (42,61). In light of this, we evaluated the effect of histamine and H4R agonists on cell senescence. Results indicate that both treatments assessed were able to induce premature or accelerated cellular senescence, increasing the activity of senescence-associated β-galactosidase and producing morphological changes that include enlarged and flattened cell shape (42). Accordingly, we have previously described that the inhibition of WM35 melanoma cell proliferation produced by histamine was mediated at least in part by an induction of cell senescence (40).

On the basis of the present findings we conclude that H4R is functionally expressed in human breast cancer cell lines, exhibiting a key role in histamine-mediated biological processes such as cell proliferation, senescence and apoptosis. The presented data in addition to previous reports that demonstrate the expression of H4R in human breast lesions, suggest a novel and complex role of H4R in breast carcinogenesis that might represent a new molecular target and avenue potentially useful for the design of more specific and effective therapies for breast cancer, though further investigation is needed to fully understand its function in the diverse types of tumours.

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Abbreviations: H_4R: Histamine H_4 receptor; ER: estrogen receptor; TUNEL: TdT-mediated UTP-biotin Nick End labelling; cAMP: cyclic adenosine monophosphate; BrdU: 5-bromo-2'-deoxyuridine; GPCR: G-protein coupled receptors; H_1R: Histamine H_1 receptor; H_2R: Histamine H_2 receptor; H_3R: Histamine H_3 receptor; PBS: phosphate-buffered saline; BSA: bovine seroalbumine; FITC: fluorescein isothiocyanate; $\Delta\psi m$: mitochondrial transmembrane potential.

Key Words: Human Breast Cancer, Histamine H_4 Receptor, Apoptosis, Cell Senescence, Proliferation

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