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Research Paper

The role of histamine in human mammary carcinogenesis

H3 and H4 receptors as potential therapeutic targets for breast cancer treatment

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Abbreviations: HR, histamine receptor; Rα-MeH, R-(-)-α-Methylhistamine; HDC, histidine decarboxylase; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate biotin nick end labeling; PI, propidium iodide; PBS, phosphate-buffered saline; FBS, fetal bovine serum; BrdU, 5-bromo-2'-deoxyuridine

Key words: histamine, human breast cancer, histamine H3 receptor, histamine H4 receptor, cell proliferation, apoptosis, therapeutic targets

There is increasing evidence that describes a histamine role in normal and cancer cell proliferation. To better understand the importance of histamine in breast cancer development, the expression of histamine H3 (H3R) and H4 (H4R) receptors and their association with proliferating cell nuclear antigen (PCNA), histidine decarboxylase (HDC) and histamine content were explored in mammary biopsies. Additionally, we investigated whether H3R and H4R were implicated in the biological responses triggered by histamine in MDA-MB-231 breast cancer cells. The expression levels of H3R, H4R, PCNA, HDC and histamine content were determined by immunohistochemistry in 40 benign and malignant lesions. MDA-MB-231 cells proliferation (clonogenic assay and BrdU incorporation) and cell cycle distribution (flow cytometry) were evaluated upon treatment with histamine, H3R and H4R agonists and antagonists. Apoptosis was determined by Annexin staining and TUNEL assay. Cell migration was assessed by transwell system. Results indicate that H3R was detected in 67% (10/15) of benign lesions and in almost all carcinomas (24/25), being the level of its expression significantly higher in carcinomas ($p = 0.0016$). The non-tumoral breast tissue surrounding carcinomas revealed a lower H3R expression compared to the tumor cells. Only 13% (2/15) of the benign lesions expressed H4R compared to 44% (11/25) of the carcinomas. Interestingly, H3R expression was correlated in carcinomas with the expression of HDC and PCNA ($p < 0.0001$), and also histamine content ($p = 0.0229$). Accordingly, histamine increased MDA-MB-231 cells proliferation and also migration via H3R. In contrast, activation of H4R inhibited proliferation and this effect was associated with an arrest in the G_0/G_1 phase of the cell cycle and an induction of apoptosis. Present findings demonstrate the presence of H3R and H4R in human mammary tissue and suggest that H3R may be involved in the regulation of breast cancer growth and progression representing a novel molecular target for new therapeutic approach.

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Introduction

Breast cancer is the most common neoplastic disease in women, accounting for over one-fifth of the estimated annual 4.7 million cancer diagnoses in females and continues to rise in incidence.[1,2] Despite much research directed at understanding and controlling this disease, it persists as a major health burden.[1] Thus, the identification of genes and biochemical pathways involved in breast carcinogenesis are of utmost importance for the development of rational molecularly-based preventive and therapeutic approaches.

Considerable evidence has been accumulated indicating that histamine can modulate proliferation of different normal and malignant cells.[3-5] In mammary gland, histamine plays a critical role in growth regulation, differentiation and functioning during development, pregnancy and lactation.[6-8]

Histamine is a biogenic amine involved in the regulation of different physiological processes. It is synthesized from L-histidine by a specific enzyme, L-histidine decarboxylase (HDC, EC 4.1.1.22). Histamine exerts its functions through binding to G-protein-associated histamine H1, H2, H3, H4 receptors (H1R, H2R, H3R, H4R), resulting in activation of different signal transduction pathways.[9-12]

High histamine biosynthesis and content has been reported in different human neoplasias including breast cancer, as well as in experimental tumors induced in rodents.[5,13-15] We have previously reported that H1R and H2R are present in different normal and malignant cell lines and benign lesions and tumors derived from human mammary gland.[16-20] Furthermore, in experimental mammary carcinomas, histamine becomes an autocrine growth factor capable of regulating cell proliferation via H1R and H2R.[16,21,22]

H3R was found to be primarily expressed in the central nervous system where it modulates neurotransmitter release either as an auto- or hetero-receptor.[23-27] On the other hand, H4R is predominantly expressed in bone marrow, eosinophils and mast cells.[10-12,28,29]

Recently, we have demonstrated that H3R and H4R are expressed in cell lines derived from human mammary gland. In addition, histamine is capable of modulating cell proliferation exclusively in malignant cells while no effect is observed in non-tumorigenic cells.[30]

At present there are no reports about the expression of these histamine receptor subtypes in normal or malignant human mammary tissue.

In order to better understand the importance of histamine in tumor development, we explored the expression of H3R and H4R and their association with HDC, proliferating cell nuclear antigen (PCNA) and histamine content, in human mammary tissue biopsies. Additionally, we investigated whether H3R and H4R were implicated in the biological responses triggered by histamine in human breast cancer cells.

Materials and Methods

Cell culture. Experiments were done on the MDA-MB-231 human breast cancer cell line (American Type Tissue Culture Collection, VA, USA). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 0.3 g/l glutamine and 0.04 g/l gentamicin (Gibco BRL, NY, USA). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and subcultured with 0.05% trypsin and 0.02% EDTA (Gibco BRL, NY, USA).

Human breast tissues. Forty breast tissue surgical, formalin-fixed and paraffin-embedded specimens were selected from the files of the Pathology department tumor bank of the Institute of Immunooncology, Buenos Aires. They comprised two groups of patients who did not receive any treatment before surgery: those with invasive breast carcinoma (25), and those with benign lesions including fibroadenoma, epitheliosis and adenosis (15). Characteristics of breast cancer patients and tumors are shown in Table 1.[31]

Immunohistochemical staining. Tissue morphology was examined on tissue sections after hematoxylin-eosin staining. For the immunodetection of proteins, paraffin sections after deparaffinization were placed in citrate buffer (10 mM, pH 6.0) and heated in a microwave oven twice for 2 minutes at boiling temperature for antigen retrieval. Endogenous peroxidase activity was blocked with 3% H_2O_2 in distilled water. Specimens were then incubated overnight in a humidified chamber at 4°C with primary rabbit anti H3R and H4R (1:100, Alpha Diagnostic International, TX, USA), rabbit anti-histamine (1:100, Sigma Chemical Co., MO, USA), mouse anti-PCNA (1:100, DakoCytomation, Denmark), guinea pig anti HDC (1:100, Euro-Diagnostica AB, Sweden) antibodies, as stated. Immunoreactivity was detected by using horseradish peroxidase-conjugated anti-mouse, anti-rabbit or anti-guinea pig IgG, as appropriate, and visualized by 3,3-diaminobenzidine staining (Sigma Chemical Co., MO, USA). Finally, the specimens were counter-stained by immersion in hematoxylin. Light microscopy was performed on an Axiolab Karl Zeiss microscope (Göttingen, Germany). All photographs were taken at 1000X magnification using a Canon PowerShot G_5 camera (Tokyo, Japan). To control the signal specificity, serial sections were made from five selected positive cases which were subjected to the same staining procedure, with either a normal mouse or rabbit IgG or phosphate-buffered saline (PBS) to replace the first antibody. This control staining did not give rise to a signal. To confirm the specificity of these antibodies, lysates prepared from different human mammary cells were evaluated by immunoblot analysis (data not shown). The immunostaining assessment was performed blind to the clinical data in all tests by consensus agreement of two observers (Medina V, Croci M). An overall examination of staining was carried out at 10X magnification, and representative area of breast specimen was then viewed at 1000X magnification. For H3R, H4R, histamine content and HDC, the scores used based on the intensity of positive homogeneous staining were the following: 0 (undetectable), 1 (very low), 2 (low), 3 (medium), 4 (high), 5 (very high) immunoreactivity. For PCNA, a percentage score was used based on the number of stained cells: 0 (undetectable), 1 (1–20%), 2 (21–40%), 3 (41–60%), 4 (61–80%) and 5 (81–100%). These scoring systems were published elsewhere.[32,33] Determinations were made in normal and invasive epithelial cells within each breast specimen and at least 10 fields were examined.

Cell growth assays. For clonogenic assay, cells were seeded in 6-well plates (1.5 x 10^3 cells/well) and incubated in the presence of treatment for 8 days. Cells were treated with histamine (0.01 and 10 μM), 0.001 μM Imetit and 0.01 μM R(-)-α-methylhistamine (Rα-MeH, both H3R agonist and less efficient H4R agonist), 10 μM Thioperamide (H3R and H4R antagonist), 10 μM Clobenpropit (H3R antagonist and H4R agonist), 10 μM Anthamine and Dimaprit (both H2R agonists) (Tocris, UK), 10 μM JNJ5207852 (H3R antagonist) (Johnson & Johnson Pharmaceutical Research and Development, CA, USA) or 10 μM 2-(3-(trifluoromethyl)phenyl)histamine (H1R agonist, kindly provided by Prof. W. Schunack, Freie Universat Berlin, Institut fur Pharmazie, Germany). Cells were fixed with 10% formaldehyde in PBS and stained with 1% toluidine blue in 70% ethanol, and the clonogenic proliferation was evaluated by counting the colonies containing 50 cells or more and was expressed as percentage values of the untreated wells.

Cells were also incubated in 6-well plates (7 x 10^4 cells/well) and treated with 0.001 μM Imetit and/or 10 μM JNJ5207852 for up to 48 hours. Cells were trypsinized and counted on a hemocytometer using trypan blue exclusion to differentiate dead from live cells.

Table 1 **Characteristics of breast cancer patients and tumors**

Characteristic	N	% of patients
Total patients	25	NA
Age at diagnosis, yr		NA
Mean	54	NA
Range	43–65	NA
Histopathology		
Infiltrating ductal cancer	16	64
Infiltrating lobular cancer	7	28
Others	2	8
Stage		
I	11	44
II	10	40
III/IV	3	12
Not classified	1	4
Lymph nodes		
Positive	10	40
Negative	15	60

NA, not applicable.

Quantification of cellular DNA synthesis was performed on cells by the addition of 30 μM 5-bromo-2'-deoxyuridine (BrdU, Sigma Chemical Co., MO, USA) for 2 hours. Cells were then trypsinized, washed with PBS and fixed with cold 70% ethanol. To denature the DNA into single-stranded molecules, cells were incubated with 2N HCl for 30 minutes at room temperature. After centrifugation, cells were washed in 1 ml of 0.1 M $Na_2B_4O_7$, pH 8.5 to neutralize the acid (Sigma Chemical Co., MO, USA). Cells were then incubated for 30 minutes at room temperature with anti-BrdU mouse monoclonal antibody diluted 1:100 in 0.5% Tween 20/1% bovine seroalbumine/PBS (Sigma Chemical Co., MO, USA). Cells were washed with PBS and incubated for an additional 30 minutes with 1:400 Alexa Fluor 488-conjugated anti-mouse IgG (Invitrogen, Argentina). After centrifugation, cells were resuspended in 0.5 ml of PBS containing 5 μg/ml of propidium iodide (PI, Sigma Chemical Co., MO, USA) and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, CA, USA).

Cell cycle analysis. Cells were plated, cultured for 24 hours and serum-starved for an additional 24 hours. Synchronized cells were left untreated or were treated with Clobenpropit (10 μM) immediately after release from the block and harvested at indicated time points. Cells were collected by trypsinization, fixed with ice-cold methanol, centrifuged and resuspended in 0.5 ml of PI staining solution (50 μg/ml PI in PBS containing 0.2 mg/ml of DNase-free RNase A; Sigma Chemical Co., MO, USA). After incubation for 30 minutes at 37°C, samples were evaluated by flow cytometry (FACSCalibur flow cytometer, Becton Dickinson, CA, USA). Cell cycle distribution was analyzed using Cylchred version 1.0.2 software (Cardiff University, UK).

Determination of apoptosis. Apoptosis was evaluated in MDA-MB-231 cells after treatment with 10 μM Clobenpropit, and/or JNJ7777120 (H4R antagonist, Johnson & Johnson Pharmaceutical Research and Development, CA, USA) for 48 hours as we have previously described (ref. 30). Shortly, phosphatidylserine exposure on the surface of apoptotic cells was detected by flow cytometry after staining with Annexin V-FITC (BD biosciences, USA), and PI (50 μg/ml). Apoptotic cells were also determined by TUNEL (TdT-mediated UTP-biotin Nick End labeling) assay. Cells grown on glass coverslips were washed, fixed and the fragmented DNA was detected using Apoptag^{TM} 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 (Göttingen, Germany) and photographs were taken at 1000X magnification using a Canon PowerShot G_5 camera (Tokyo, Japan). At least 200 cells were scored for each determination.

Variations of the mitochondrial transmembrane potential ($\Delta\psi_m$) were studied using 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6$; Sigma Chemical Co., MO, USA).[34] 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. Flow cytometry data were analyzed using WinMDI 2.8 software (Scripps Institute, CA, USA).

Migration assays. To asses the migration-promoting capability of H3R and H4R agonist, cells were seeded and left untreated or were treated with 0.01 μM histamine, 0.01 μM Imetit or 10 μM Clobenpropit for 48 hours. Medium was removed and replaced with complete medium containing 5 μM calcein-AM (Invitrogen, Spain) and after incubation for two hours at 37°C, cells were detached and resuspended in FBS free medium containing 0.1% bovine seroalbumin and treated at a density of 10^5 cells/ml. The fluorescence blocking PET membrane inserts (pore size 8 μm) were placed in a 24-well plate (Falcon HTS FluoroBlock, BD, CA, USA). Six-hundred microliters of 10% FBS medium, used as a chemoattractant, were added to the lower well while 500 μl of cell suspension were added to the inside of each insert. The number of cells migrated through the membrane after 3 h were evaluated by determining the calcein fluorescence (excitation at 485 nm and emission at 530 nM) using a fluorescence microplate reader in the bottom-read mode and KC4 software (FL600FA, BIO-TEK Instruments, Winooski, VT) and values were calculated from a standard curve using known numbers of cells.

Statistical analysis. Mann-Whitney non-parametric test was used to compare average scores of staining intensity or percentage. For determination of the association between H3R and PCNA expression, H3R and HDC expression and H3R expression and histamine content, Spearman's rho correlation coefficients and two-tailed significances were determined.

For proliferation and migration assays, apoptosis determination and cell cycle analysis, determinations were repeated at least three times with duplicate and triplicate measurements for each condition. Representative results are presented as means ± SEM (standard error of the mean). Statistical evaluations were made by one-way analysis of variance (ANOVA), which was followed by Tuckey's Multiple Comparison Test.

A two-sided $p < 0.05$ was considered statistically significant. All statistical analyses were performed with GraphPad Prism Version 4.00 software (CA, USA).

Results

Histamine H3 and H4 receptor expression in benign lesions and carcinomas of human mammary gland epithelium. The immunohistochemical analysis showed that H3R was detected in 67% (10/15) of benign lesions and in almost all carcinomas studied (24/25), being the level of its expression significantly higher in carcinomas ($p = 0.0016$; two-sided Mann Whitney's Test) (Fig. 1A). Figure 1B shows low to very low H3R expression in benign lesions, while moderate to high expression was observed in malignant lesions.

In this context it is worth noting that the non-tumoral breast tissue surrounding carcinomas revealed a lower or negative expression compared to high expression of H3R in tumor cells of the same patient. Figure 1C displays a low H3R expression in non-tumoral tissue, whereas a high expression was observed in the infiltrating carcinoma area of the same patient.

We further observed that only 13% (2/15) of the benign lesions studied expressed H4R protein while nearly half (11/25) of the carcinomas expressed it. However, we found no significant difference in the staining intensity between both groups (Fig. 1A). As it can be observed, H4R was undetectable in almost all benign lesions investigated, while malignant lesions presented low or undetectable expression of H4R (Fig. 1B).

The specificity of these two antibodies was verified by Western blots and confirmed by reverse transcriptase-mediated polymerase chain reaction in previous studies, (refs. 30 and 35). Furthermore, as shown in Figure 1B (arrows), lymphoid cells expressed H4R as it was previously described (refs. 9–11, 28, 29 and 36).

HDC expression and histamine content in benign lesions and carcinomas of human mammary gland epithelium. We also evaluated the histamine intracellular content and observed that most of the samples were positive and that no significant difference was observed between the two groups studied. However, HDC was detected in 60% (9/15) of benign lesions and in 92% (23/25) of carcinomas in which HDC expression was significantly increased ($p = 0.0012$, two-sided Mann Whitney's Test) (Fig. 2A). The specificity of these two antibodies was previously established by other methodologies elsewhere[37] and we confirmed it by the positive staining of infiltrating mast cells within breast specimen (data not shown).

Interestingly, we observed a highly significant correlation between H3R and HDC expression scores in breast carcinomas with a $p < 0.0001$ significant level for the Spearman's rank correlation (correlation coefficient, r: 0.7704). Additionally, we found a correlation between H3R expression and histamine content scores ($p = 0.0229$, r: 0.4625). The correlations obtained for each tumor investigated are depicted in the scatter diagrams of Figure 2B.

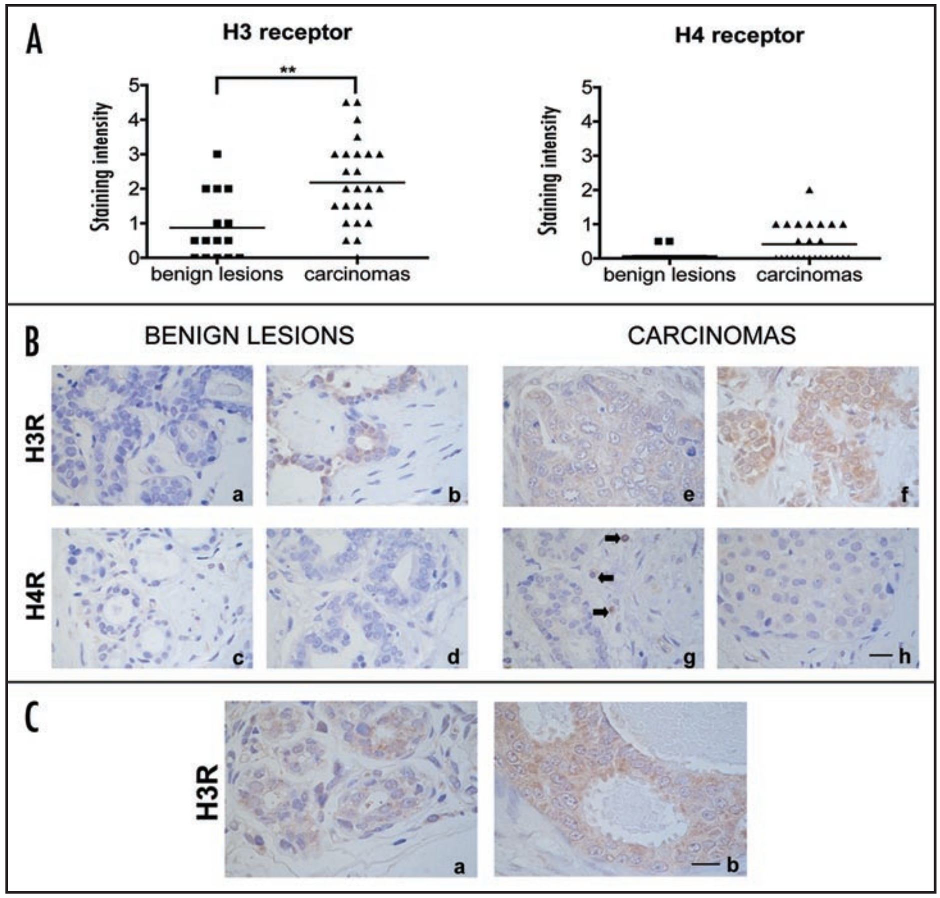

Figure 1. Immunohistochemical detection of H3R and H4R in benign and malignant lesions derived from human mammary gland. (A) H3R and H4R immunostaining scores in benign lesions and carcinomas. Solid line indicates the mean. $^{**}p = 0.0016$ vs. benign lesions; two-sided Mann Whitney's Test. [a] 0 undetectable, 1 very low, 2 low, 3 medium, 4 high, 5 very high. (B) Examples of immunostaining for H3R and H4R protein in human breast specimens. a) Ductal hyperplasia showing negative immunoreactivity for H3R, b) epithelial components of pericanalicular fibroadenoma positive for H3R, c) and d) adenosis and ductal hyperplasia in dysplasic breast tissue negative for H4R. e) Infiltrating ductal breast carcinoma with moderate expression of H3R, f) ductal carcinoma showing very high expression of H3R, g) ductal carcinoma negative for H4R. Arrows indicate lymphoid cells positively stained, and h) lobular carcinoma with low positivity for H4R. (C) H3R expression in non-tumoral breast tissue adjacent to carcinomas. a) Adenosis focus in breast tissue surrounding malignant lesion with low immunoreactivity for H3R, b) very high expression of H3R in comedo-carcinoma of the same patient. Pictures were taken at a 1000X-fold magnification. Scale bar: 20 μm.

Histamine H3 receptor expression correlates with proliferation in breast carcinomas. We additionally evaluated PCNA immunostaining as an indicator of active proliferation.[38] Results indicated that most of the samples expressed PCNA, being the level of its nuclear expression significantly higher in carcinomas ($p = 0.0002$, two-sided Mann Whitney's Test) (Fig. 3A).

It is interesting to observe that H3R expression is strongly correlated with PCNA expression exclusively in breast carcinomas ($p < 0.0001$, r: 0.7333) (Fig. 3B).

Histamine modulates the proliferation of breast cancer cells differentially through the activation of H3 and H4 receptors. We then investigated whether the H3R and H4R were implicated in the biological responses triggered by histamine. In MDA-MB-231 breast cancer cells, histamine regulated the proliferation in a dose-dependent manner with an IC_{50} value of 0.56 ± 0.05 μM. In agreement with our previous study (ref. 30) histamine at 10 μM significantly decreased proliferation resulting in a 14.3 ± 4.3 of cell survival whereas lower concentrations (0.01 μM) increased proliferation moderately ($137.7 \pm 9.3\%$). In order to explore the involvement of H3R in cell growth, we examined the clonogenic proliferation after treatment with specific H3R agonists and antagonists. As shown in Figure 4A, 10 μM of the H3R and H4R antagonist, Thioperamide completely inhibited the histamine-induced breast cancer cells proliferation. Furthermore, the H3R agonists, Imetit and Rα-MeH, mimicked the effect of histamine at low concentration. The compounds used to address the H3R role in proliferation also bind to the H4R, therefore, despite they present a different rank order of affinity and potency, they do not allow the complete discrimination of the two receptors.[10-12,26,28,29,39] To allow the dissection of H3R and H4R signaling, a specific H3R antagonist, JNJ5207852, was used.[26] JNJ5207852 treatment blocked the proliferation increase triggered by 0.01 μM histamine, Imetit and Rα-MeH. Accordingly, Imetit at 0.001 μM augmented the MDA-MB-231 cell number and this effect was reverted by JNJ5207852 treatment (Fig. 4A). Thus, histamine-induced breast cancer cells proliferation appeared to be mediated by the H3R.

On the other hand, histamine at 10 μM remarkably decreased cell growth and this outcome was mimicked by H1R agonist 2-[3-(trifluoromethyl)phenyl]histamine, the H2R agonists Anthamine or

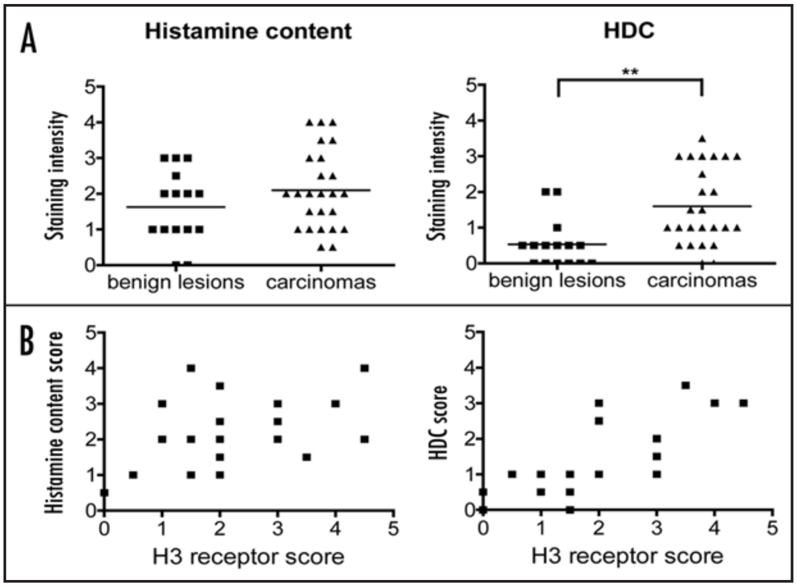

Figure 2. Immunohistochemical detection of intracellular histamine and HDC in benign and malignant lesions derived from human mammary gland. (A) Histamine and HDC immunostaining scores in benign lesions and carcinomas. Solid line indicates the mean. **p = 0.0012 vs. benign lesions; two-sided Mann Whitney's Test. ^{a}0 undetectable, 1 very low, 2 low, 3 medium, 4 high, 5 very high. (B) Spearman's correlation between H3R protein and histamine content (p = 0.0229, correlation coefficient, r = 0.4625) or HDC protein (p < 0.0001, r = 0.7704) immunostaining in carcinomas' group.

Dimaprit, and the H4R agonist Clobenpropit, indicating that the histamine inhibitory effect on proliferation was exerted through the H1R, H2R and H4R (Fig. 4B).

Consistent with these observations, histamine modulated the active DNA synthesis as it was evaluated by BrdU incorporation (Fig. 4C).

Histamine induces cell cycle arrest and apoptosis through the H4 receptor. In addition, we evaluated the effect of the H4R agonist on the cell cycle distribution and apoptosis of MDA-MB-231 cells. Clobenpropit treatment produced an accumulation of cells in the G_0/G_1 phase of the cell cycle starting at 24 hours and continuing over the time with the highest effect after 72 hours of treatment (Fig. 5).

To determine if the decrease in proliferation exerted by Clobenpropit could be due to an apoptotic effect, we assessed apoptosis by three different methodologies and we showed that Clobenpropit after 48 hours of treatment increased the number of apoptotic cells determined by Annexin-V staining and this effect was blocked by the specific H4R antagonist JNJ7777120 (Fig. 6A). This result was confirmed by TUNEL assay (Fig. 6B). In accordance to this, Clobenpropit produced the disruption of the mitochondrial transmembrane potential that is associated with apoptosis (Fig. 6C).

Histamine induces migration of breast cancer cells through the activation of H3 receptor. Migration of tumoral cells toward histamine was investigated using Transwell system. Histamine at 0.01 μM induced MDA-MB-231 cell migration through the H3R as this effect was mimicked by the H3R agonist Imetit, while the H3R antagonist and H4R agonist Clobenpropit decreased migration (Fig. 7). These results were further confirmed by wound-induced migration assay (data not shown).

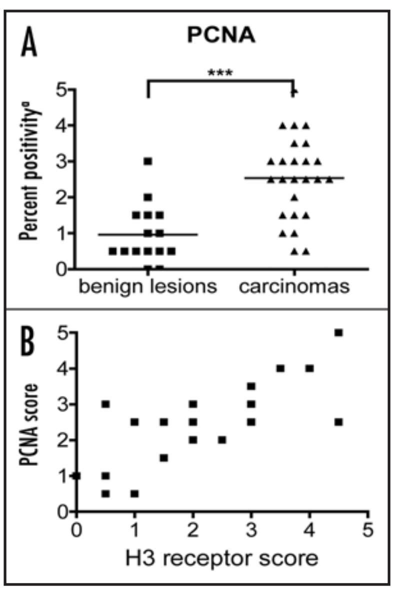

Figure 3. Immunohistochemical detection of PCNA in benign and malignant lesions derived from human mammary gland. (A) PCNA immunostaining scores in benign lesions and carcinomas. Solid line indicates the mean. ***p = 0.0002 vs. benign lesions; two-sided Mann Whitney's Test. ^{a}0 (undetectable), 1 (1–20%), 2 (21–40%), 3 (41–60%), 4 (61–80%) and, 5 (81–100%). (B) Spearman's correlation between H3R and PCNA protein immunostaining in carcinomas' group (p < 0.0001, r = 0.7333).

Discussion

Since the discovery of the H3R, accumulated information in the literature suggests that it is restricted mainly to neurons while the most recently discovered H4R is expressed preferentially in hematopoietic cells.[10-12,23-29] In the present study, we showed that both H3R and H4R are expressed in human mammary lesions. In agreement with this, we have recently reported that H3R and H4R are expressed, at the protein and mRNA level, by cell lines derived from normal and transformed human mammary gland.[30] There is increasing evidence to demonstrate that histamine plays a significant role in breast cancer since functional histamine receptors and HDC activity is demonstrated in breast tissue. In this line, Vesuna and Raman, on the basis of the reported evidence, suggested the potential role of histamine as a novel therapeutic agent for breast cancer.[40]

To elucidate the role of H3R and H4R in breast oncogenesis, we compared the expression of these receptors in benign and malignant lesions of the human mammary gland. We showed that H3R was detected in 67% of benign lesions while almost all carcinomas studied expressed it, being the level of its expression significantly higher than in benign lesions. Furthermore, carcinomas have substantially more H3R protein expression than adjacent nontumor-bearing mammary gland.

Although not significant, a slight increase in the level of expression of H4R was observed in malignant with respect to benign lesions. A

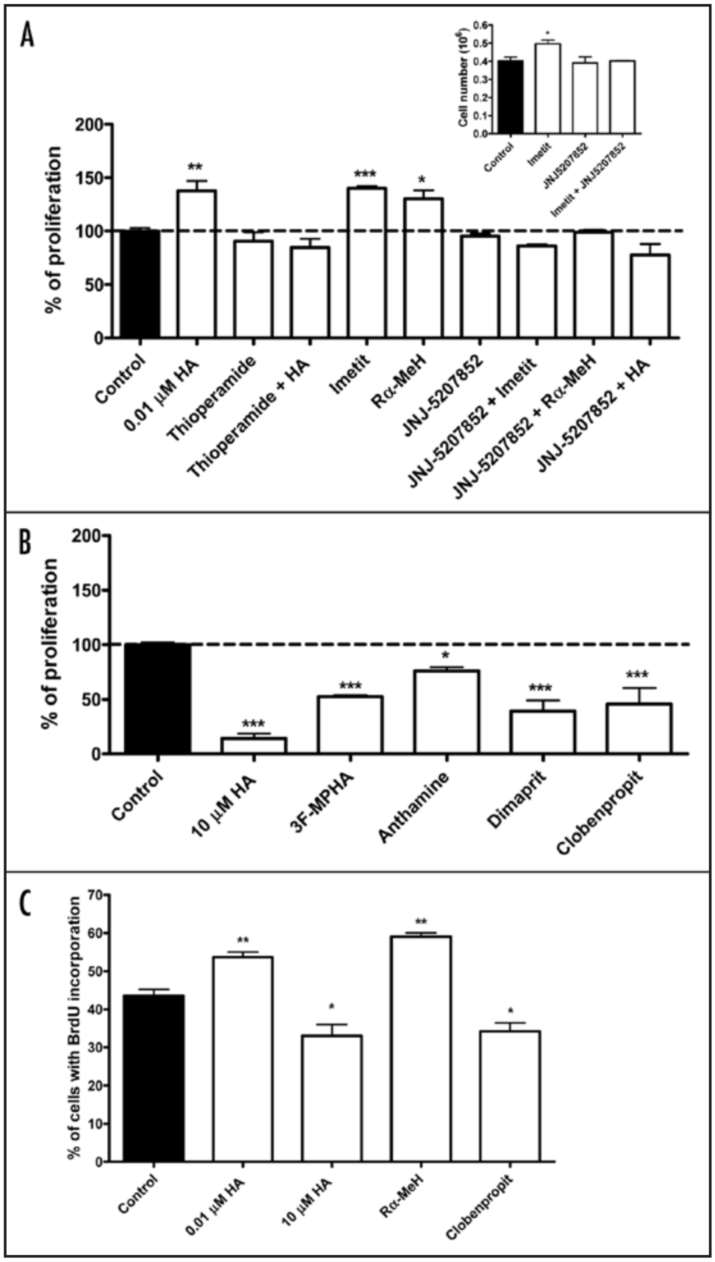

Figure 4. Histamine modulates cell proliferation of breast cancer cells. (A) Histamine-induced MDA-MB-231 cells proliferation is mediated via the H3R. Cells were left untreated or were treated with 0.01 μM histamine (HA), Thioperamide (10 μM), Imetit (0.001 μM), R-αMeH (0.01 μM), JNJ5207852 (10 μM) and proliferation was evaluated by the clonogenic assay. Inset: MDA-MB-231 cells were treated with Imetit and/or JNJ5207852, harvested and counted at 48 hours after treatment. (B) Histamine decreased MDA-MB-231 cells proliferation via H1R, H2R, and H4R. Cells were left untreated or were treated with 10 μM of histamine, 2-(3-(trifluoromethyl)phe nyl)histamine (3F-MPHA), Anthamine, Dimaprit, Clobenpropit, and proliferation was evaluated by the clonogenic assay. (C) Histamine modulated active DNA synthesis evaluated by the BrdU incorporation. MDA-MB-231 were left untreated or were treated with histamine (0.01 and 10 μM), R-αMeH (0.01 μM) or Clobenpropit (10 μM) for 48 hours. Error bars represent the means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Control.

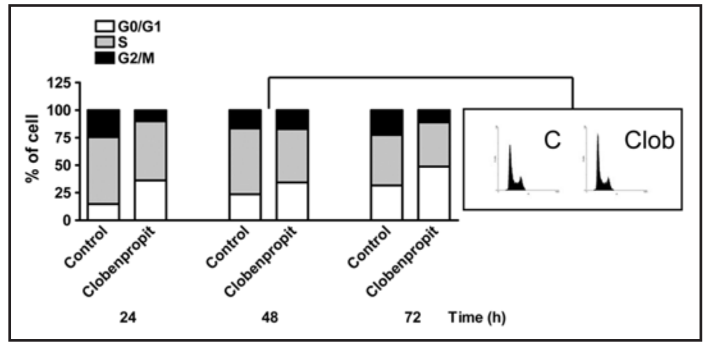

Figure 5. Activation of the H4R results in an accumulation of MDA-MB-231 in G_0/G_1 phase of the cell cycle. Cells were synchronized and treated with 10 μM Clobenpropit (Clob) or left untreated (C). Percentage of cells in different phases of the cell cycle was monitored as a function of time using flow cytometry. Results represent the mean value of three independent experiments. Inset shows the data of C and Clob at 48 hours.

more accurate estimate of the function of H4R in primary mammary carcinoma and the elucidation of its possible role as a prognostic marker of breast cancer disease would require the investigation of a higher number of tumor samples and patient follow up.

In accordance with previous reports demonstrating higher HDC activity in breast cancer in comparison to normal tissue,[13-15] HDC protein expression was significantly augmented in breast carcinomas. Nevertheless, the increase in histamine content observed in carcinomas was not significant. In our study, we demonstrated a significant direct correlation between H3R and HDC expression, as well as with the histamine content exclusively in breast carcinomas. Our results are in line with data reported that H3R mRNAs are expressed together with those of the histamine-synthesizing enzyme in the development of rapidly growing liver and adipose tissues, and in various epithelia including that from the skin, lung, stomach, and intestine.[41]

Moreover, we found an important positive correlation in breast carcinomas between H3R and PCNA expression, a well-known marker of proliferation. Accordingly, Héron et al demonstrated that histamine increased proliferation of epithelial stem cells of adult intestinal crypts through the H3R.[41] Furthermore, no correlation was observed between H3R or H4R and hormone receptors (data not shown). This is in agreement with previous reports, in which we found that biopsies of human breast tumors express H1R and H2R, but they were not correlated with either estrogen or progesterone receptors.[16,18] Furthermore, in experimental mammary adenocarcinomas induced in rats, the regulation of growth exerted by histamine seems to be independent of the hormone responsiveness of the tumors. In these tumors, histamine behaves as an autocrine growth factor inducing proliferation via H2R and the in vivo treatment with H2R antagonists resulted in a very significant tumor regression.[5,22] However, the clinical trials performed with histamine H2R antagonists exhibited no encouraging results for breast cancer patients.[42]

In the present study, we demonstrate a highly significant correlation between H3R expression and proliferation and, furthermore, its association with histamine production, reflecting the importance of H3R in the development of breast carcinoma. This was additionally evidenced by the considerably increased H3R expression in tumoral compared to non-tumoral tissue. Therefore, we postulate that the

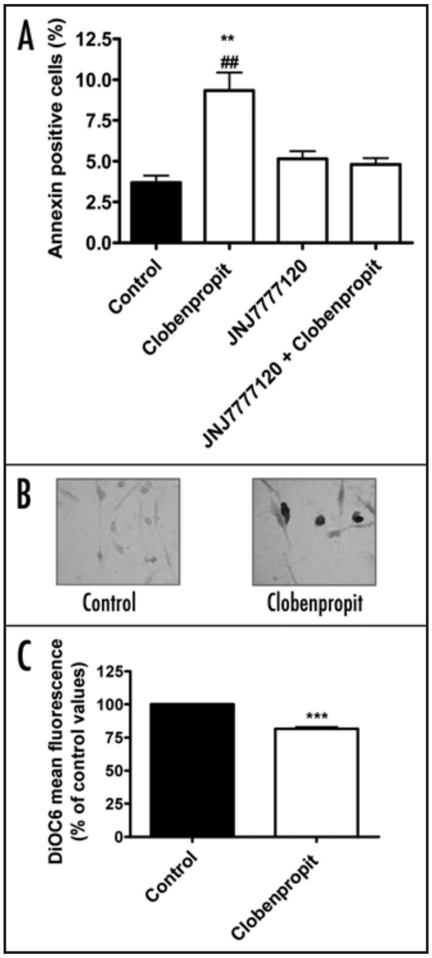

Figure 6. Histamine induces apoptosis of MDA-MB-231 cells via H4R. (A) Apoptotic cells were determined by Annexin V-FITC staining and FACS analysis. (B) Apoptosis was demonstrated by in situ TUNEL staining. (C) Disruption of mitochondrial transmembrane potential was evaluated by flow cytometry using DiOC6 staining. Cells were treated with 10 μM Clobenpropit, and/or 10 μM JNJ7777120 or were left untreated for 48 hours. Data represent the mean fluorescence intensity in percentage compared with control values. Error bars represent the means ± SEM. $^{**}p < 0.01$ vs. Control, $^{\#\#}p < 0.01$ vs. Clobenpropit + JNJ7777120.

blockade of the H3R may represent a novel therapeutic target for breast cancer treatment.

In order to better understand the role of H3R and H4R in carcinogenesis, we evaluated their function in breast cancer cell proliferation and migration, which are essential processes in tumor progression. We have previously reported that histamine modulates proliferation of MDA-MB-231 breast cancer cells in a dose-dependent manner increasing cell growth at lower concentrations while decreasing it at higher ones. The latter effect was associated with an induction of cell cycle arrest, differentiation and apoptosis.[30] Here, by using specific histamine receptor agonists and antagonists, we demonstrated that the positive effect on proliferation is exerted through the activation of the H3R. These results are consistent with previous findings that disclose a primary role of H3R ligands in enhancing cell proliferation and migration in rat fundic mucosa epithelium.[43] Conversely, the decrease in proliferation is mediated in part via the H4R and this effect was related to the accumulation of cells in the G_0/G_1 phase of the cell cycle and the induction of apoptosis.

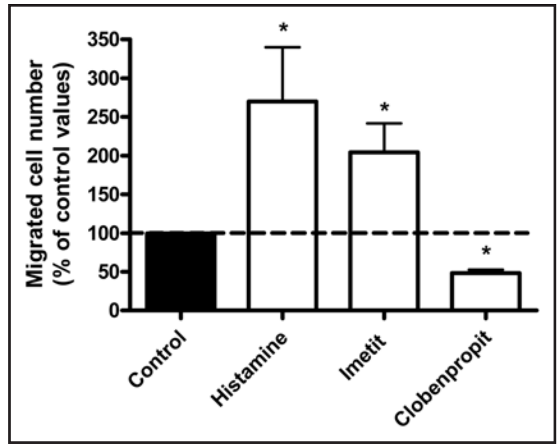

Figure 7. Histamine stimulates MDA-MB-231 cells migration via the H3R while inhibited it via H4R. Cells were left untreated (control) or were treated with histamine (0.01 μM), Imetit (0.01 μM), Clobenpropit (10 μM), and migrated cells were evaluated by transwell system. Data are expressed as percentage of migrated cells in untreated Boyden chambers ± SEM. $^{*}p < 0.05$ vs. Control.

Imetit is known to be an agonist for both H3R and H4R, although with less affinity to the latter, whereas Clobenpropit behaves as an agonist for the H4R, but as an antagonist for the H3R.[10-12,26,28,29,39] In the present study, we showed that histamine promotes migration of breast cancer cells and this effect was mimicked by Imetit while was inhibited by Clobenpropit, suggesting that histamine could stimulate breast cancer cell migration via H3R, playing an important role in invasion and metastasis of malignant tumors. In accordance to this, we have recently described that histamine induces migration while it decreases adhesion of PANC-1 pancreatic carcinoma cells.[44] Coincidently, current studies indicate that histamine is capable of modulating the expression of Ets-1 in MDA-MB-231 cells (data not shown), known as a transcription factor that regulates cell motility.[45]

Further studies are needed to elucidate the H3R-associated signal transduction pathways and the H3R isoforms that are expressed in breast tissue. Several H3R isoforms varying in the length of their third intracellular loops have been identified,[23-26,46] and due to the fact that this molecular domain is thought to be responsible for coupling to G proteins, it is likely that the different H3R isoforms have differences in their signaling pathways.

To our knowledge, our report is the first to describe the presence of H3R and H4R in breast lesions and suggests a main role for H3R in the regulation of cell growth, development and progression of human breast cancer offering novel therapeutic potentials for H3R ligands. Further investigation of additional human breast tumors, at precisely defined grades and stages, and follow up studies, will contribute to more fully elucidate the biological, therapeutic and prognostic importance of H3R and also H4R expression in breast cancer. Our findings contribute to the identification of molecules involved in breast carcinogenesis that may represent potential targets for the development of rational molecularly based preventive and therapeutic approaches.

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