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Title: Role of H4 receptor in histamine-mediated responses in human melanoma

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Corresponding Author: Elena E Rivera, Ph.D

Corresponding Author's Institution:

First Author: Noelia A Massari, Biochemist

Order of Authors: Noelia A Massari, Biochemist;Vanina A Medina, Ph.D;Diego J Martinel, Biotechnologist;Graciela P Cricco, Ph.D;Maximo Croci, Dr;Lorena Sambuco, Biochemist;Rosa M Bergoc, Ph.D;Elena E Rivera, Ph.D

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Abstract: We have previously reported that histamine at micromolar concentrations reduces the proliferation of melanoma cell lines. It is also known that melanoma cells express histamine H1, H2 and H3 receptors. The aim of this work was to investigate the presence of histamine H4 receptor (H4R) in human melanoma cells and its associated biological processes. In order to better understand the importance of histamine in tumor development, we explored the expression of H4R in human melanoma tissue biopsies. The expression of H4R in WM35 and M1/15 cells was analyzed by RT-PCR, Western blot and immunocytochemistry. To characterize the biological responses we evaluated cell proliferation by clonogenic assay and BrdU incorporation. In addition, cell senescence and differentiation were determined by β-galactosidase enzyme assay and dopa oxidase activity, respectively. The expression levels of H4R were determined by immunohistochemistry in 19 samples of human malignant lesions. Results indicate that melanoma cells express H4R at the mRNA and protein level. By using histamine agonists, antagonists and H4 siRNA we showed that the inhibitory effect of histamine on proliferation was in part mediated through the stimulation of the H4R. The decrease in proliferation was associated with an induction of cell senescence and an increase in melanogenesis that is a differentiation marker of these cells. Furthermore, H4R was expressed in 42% of human melanoma biopses. To our knowledge this is the first report that describes the presence of the H4R in melanoma cells and tissue suggesting a potential therapeutic application of H4R ligands.

Point by point response

Reviewer Comments:

Reviewer #1:

In this work the H4 histamine receptor expression pattern in two human melanoma cell lines were analyzed using RT-PCR, Western Blot and immunocytochemistry. Using specific H4R agonist the authors found a down regulation of cell proliferation and differentiation. The results could be potentially considered interesting however there are many deficiencies in this manuscript to be corrected. Moreover, in reading the manuscript, it seems to be a preliminary study. The author should explore the mechanisms involved and discuss the results more deeply.

Figure 1 show the Western blot analysis of the H4 histamine receptor expression in two melanoma cell lines. The results shown in the Western Blot are not very convincing and a positive control would be included, for example protein lysates from MCF-7 human breast cancer cells. In the same way in Figure 1C a positive control would be included. I believe that the adequate controls will considerably improve the impact of the manuscript.

We have included positive controls in figure 1.

In the legend of Figure 2 there are mistakes about the clonogenic assay; on the left cells were treated with clobenpropit and on the right cells were treated with VUF 8430. Again in the legend of Figure 2B there is a mistake: cells have been treated with VUF or Clobenpropit?

We have corrected the legend of figure 2.

It is very surprising that histamine 10 µM down regulates cell proliferation very significantly and induces senescence almost equal to the H4R agonist. These results should be confirmed using others approach i.e. interference experiments.

In both cell lines the histamine inhibitory effect on proliferation is mediated not only by the H4R but also through the H1R. That is why we observed a higher inhibitory effect on proliferation with histamine than with the H4R agonists. On the other hand, apparently the only receptor involved in histamine-mediated cell senescence is the H4R in both cell lines.

Furthermore, we have confirmed the results of cell proliferation not only by a pharmacological approach employing the specific antagonist JNJ7777120 but also by means of genetic tools using siRNA of the H4R.

Figure 3: The author should better explain their method to measure senescence in the M&M. What does "at least 500 cells were scored for each determination" mean?

We have clarified the method employed to measure cell senescence in M&M section.

Reviewer #2:

Massari et al. have evaluated the expression and potential biologic significance of H4R in human melanoma cell lines in vitro. They suggest that H4R is expressed by two melanoma cell lines and the proliferation of these cell lines can be inhibited by micromolar concentrations of histamine via a mechanism that is at least partially dependent upon (negative) signaling via H4R. Decreased proliferation was associated with enhanced melanogenesis in vitro. The main strength of the paper is a focus on H4R which remains understudied (in comparison to H1R-H3R) in the melanoma setting. While there is clear merit to this report, it has a number of issues that need to be addressed to improve overall quality:

1. In Fig. 1B, the western blot data for WM35 is not convincing. If one expects an approximate 66 kDa band (as shown for WM1/15), there is no credible band of this size in the WM35 blot. At a minimum, the authors should provide control blots (either control isotype pulldowns or beta-actin pulldowns) so that differences in the 66 kDa region can be appreciated for WM35.

These cell lines have different malignant characteristics since the WM35 and the M1/15 deriving from a primary melanoma and from a metastasis, respectively. We considered that these cells express a different prolife of molecular weight species that possibly includes H_4R isoforms as well as robust dimeric structures. This could explain why that band is missing in the WM35. In the same way, we have previously reported the expression of different isoforms in two breast cancer cell lines with different aggressive phenotypes that also exhibited different intracellular signaling associated to this receptor (Medina V. in press 2011). It is important to highlight that both cell lines expressed a ~45 kDa molecular weight specie as it is indicated in Results section.

2. Fig. 1C similarly needs specificity validation. Can anti-H4R staining be ablated by inclusion of histamine in the staining procedure? Is there a blocking peptide (to which the Ab was raised)? If not (and less preferable), can IHC for an H4Rneg melanoma (or alternate human tumor) be provided?

As we have mentioned previously, we have included positive controls. In addition, this H4R antibody does not recognized histamine (we have verified that in human melanoma and breast carcinoma biopsies which express HDC and have intracellular histamine but do not express H4R.

Regarding the specificity of the antibody, it has been previously employed for the detection of H_4R in breast cancer biopsies, and in different cells lines in which the expression of the H_4R was confirmed by RT-PCR followed by the sequence of the

fragments (Medina et al. 2006, 2008). It is important to point out that the different molecular weight species that are observed in the profiles of both cell lines are in coincidence with various molecular weight species previously reported by other authors as it is indicated in the discussion.

Finally, we have included a figure showing results in human melanoma samples that demonstrate positive and negative immunoreactivity for the H4R.

3. In Fig. 2, the strongest data for H4R-specific effects is the comparison of untreated versus HA treated versus HA + JNJ77 treated, given the reported high degree of discrimination for JNJ77 for H4R versus H1R-H3R. Data using of Clobenpropit and VUF 8430 that are reported to also affect H3R (albeit to a lesser extent) and potentially alternate signaling pathways in melanoma are suspect. However, despite the high degree of selectivity of JNJ77 among the HRs, this does not preclude off target effects that could modulate the proliferation assay results. The authors should state such reasons for caution in their Discussion. Fig. 2 would be further strengthened if pictures were also provided for melanoma cells treated with nil, HA, JNJ77 and HA + JNJ77.

We have included pictures of melanoma cells treated with nil, HA, JNJ77 and HA + JNJ77 in fig. 2. In addition, we have incorporated a comment of the compounds used in the discussion.

4. Flow cytometry data should be provided to discern whether all melanoma cells are H4R+ for WM35 and WM1/15. If not all tumor cells are positive, these should be sorted into positives and negatives and comparative analyses performed against the 2 sub-populations.

As it is evidenced in the immunocytochemistry of fig 1C, all cells (WM35 and M1/15) are H4R positive.

5. Further evidence for the role of H4R should be provided via specific siRNA knockdown of H4R in the melanoma cell lines.

As we have indicated previously, we have included results of proliferation using siRNA knockdown of H4R in the melanoma cell lines in order to strength our manuscript.

6. Senescence and differentiation results should be reported for the treatment cohorts nil, HA, JNJ77 and HA + JNJ77.

We have included cell senescence and differentiation results in both cell lines treated with nil, HA, JNJ77 and HA + JNJ77.

7. The potential clinical relevance of H4R should be supported by IHC of primary melanoma tissues from patients demonstrating the prevalence of H4R expression in situ. The authors indicate that this is being done for a prospective paper, but this information belongs in the current report.

As you recommended, we have reported in the present work the immunohistochemical study of the expression of H4R en human melanoma biopsies in a new figure.

8. Figure legend titles should provide summative information of what is depicted, not merely state the analysis under study.

We have changed the figure legend titles for more appropriate ones.

Role of H_4 receptor in histamine-mediated responses in human melanoma

Noelia A Massari[a], Vanina A Medina[a,b], Diego J Martinel Lamas[a], Graciela P Cricco[a], Máximo Croci[c], Lorena Sambuco[c], Rosa M Bergoc[a,b], Elena S Rivera[a]*.

a Laboratorio de Radioisótopos, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires 1113, Argentina.

b Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina.

c Instituto de Inmunooncología; Buenos Aires, Argentina.

*Correspondence to:

Prof. Elena S Rivera, Ph.D., Laboratorio de Radioisótopos, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, Buenos Aires, ARGENTINA (1113). e-mail: erivera@ffyb.uba.ar. Phone: +54-11-4964-8277/8202. Fax: +54-11-4964-8277/8202 ext: 31.

Short title: H_4R in human melanoma

ABSTRACT

We have previously reported that histamine at micromolar concentrations reduces the proliferation of melanoma cell lines. It is also known that melanoma cells express histamine H_1, H_2 and H_3 receptors. The aim of this work was to investigate the presence of histamine H_4 receptor (H_4R) in human melanoma cells and its associated biological processes. In order to better understand the importance of histamine in tumor development, we explored the expression of H_4R in human melanoma tissue biopsies. The expression of H_4R in WM35 and M1/15 cells was analyzed by RT-PCR, Western blot and immunocytochemistry. To characterize the biological responses we evaluated cell proliferation by clonogenic assay and BrdU incorporation. In addition, cell senescence and differentiation were determined by β-galactosidase enzyme assay and dopa oxidase activity, respectively. The expression levels of H_4R were determined by immunohistochemistry in 19 samples of human malignant lesions. Results indicate that melanoma cells express H_4R at the mRNA and protein level. By using histamine agonists, antagonists and H4 siRNA we showed that the inhibitory effect of histamine on proliferation was in part mediated through the stimulation of the H_4R. The decrease in proliferation was associated with an induction of cell senescence and an increase in melanogenesis that is a differentiation marker of these cells. Furthermore, H_4R was expressed in 42% of human melanoma biopses. To our knowledge this is the first report that describes the presence of the H_4R in melanoma cells and tissue suggesting a potential therapeutic application of H_4R ligands.

Key words: histamine, human melanoma, histamine H_4 receptor, cell proliferation, cell senescence, therapeutic targets.

INTRODUCTION

Malignant melanoma is an aggressive malignancy of melanocytes with high therapy resistance. Its incidence has been progressively increasing worldwide which makes it a central public health issue. Paradoxically, melanocytes are injured and transformed after exposure to ultraviolet radiation (UVR), the same agent from wich they are programmed to defend our organism. Other important risk factors are fair skin, dysplastic nevi syndrome and a family history of melanoma. Early detection is vital for long-term survival, given that there is a direct correlation between tumor thickness and mortality [1, 2].

Histamine has been implicated as one of the mediators involved in regulation of proliferation in both normal and neoplastic tissues. Histamine levels are regulated by histidine decarboxylase, (EC 4.1.1.22), that is the enzyme that catalyzes the formation of histamine from L-histidine [3]. Melanoma cells but not normal melanocytes contain large amounts of histamine that has been found to accelerate malignant growth [4].

Histamine exerts its functions through binding to G protein-associated histamine H_1, H_2, H_3, H_4 receptors (H_1R, H_2R, H_3R, H_4R) resulting in the activation of different signal transduction pathways [5-8].

It has been previously reported the expression of H_1R, H_2R and H_3R in melanoma cell lines [9]. In addition, it was described that in melanoma cells histamine acting through the H_1R decreases cell proliferation, whereas it enhances growth when acting through the H_2R [10]. Furthermore, there is no evidence of mitogenic signaling through the H_3R in human melanoma [9].

H_4R is the latest of the four histamine receptor subtype identified. Several organs and immune tissues, such as the spleen, thymus, bone marrow and leukocytes express H_4R [11-13]. Recently, we have demonstrated that H_3R and H_4R are expressed in cell lines and biopsies 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 [14, 15]. Additionally, we demonstrated that histamine decreases proliferation at least in part through the H_4R in human breast cancer cells and this effect was related to the induction of apoptosis [15].

In order to better understand the involvement of histamine in melanoma progression, we explored the expression of H_4R and its associated biological responses in human malignant melanoma cell lines and also we investigated its expression in human melanoma tissue.

MATERIALS AND METHODS

Cell culture

The human melanoma cell lines WM35 (primary melanoma) and M1/15 (derived from liver metastasis) were kindly provided by Professor A. Falus (Budapest, Hungary). Cells were cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 0.3 g/L glutamine, and 0.04 g/L gentamicin (Gibco BRL, Grand Island, NY, USA). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Cell growth assays

For clonogenic assay, cells were seeded in 6-well plates (1000 cells/well) and treated with 0.01 to 10 µM VUF 8430 (H4R agonist) (Tocris Bioscience, USA) or 0.01 to 10 µM Clobenpropit (H_3R antagonist and H_4R agonist) (Sigma Chemical Co., USA). The cells were incubated for 7 days and then fixed with 10% (v/v) formaldehyde in phosphate-buffered saline (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 into 12-well plates in culture medium (25000 cells/well), and treated with 10 µM Histamine

(Fluka, USA), 10 µM VUF 8430, 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 with PBS 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 with 1 ml of 0.1 M $Na_2B_4O_7$ (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 (Sigma Chemical Co., USA) 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 300 cells were scored for each determination. Pictures were taken at a 400X-fold magnification.

RT-PCR

The retrotranscription reaction was performed with 2 µg of RNA that was isolated using TRIZOL reagent, according to the manufacturer instructions (Invitrogen,

USA). H_4R primers were, H4R-F: GGT GAT GCA CAT GAT CAG TAG C; H_4R-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 [14]. Negative controls were performed with water instead of cDNA and MCF-7, an estrogen receptor α positive breast cancer cell line, (American Type Tissue Culture Collection, USA) was used as positive control to compare the expression of H4R.

Primers for tyrosinase were TYR-F: TTG GCA GAT TGT CTG TAG CC; TYR-R: AGG CAT TGT GCA TGC TGC TT; 284 bp, 35 cycles of 1 min at 95ºC; 1 min at 55ºC; 1 min at 72ºC [16-18]. Water instead of cDNA and MCF-7 cell line were used as negative controls.

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 [14]. PCR products were subjected to gel electrophoresis and photographed using a Sony Cyber-Shot DSC-S75 camera.

Fragments identity was corroborated by sequenciation (Macrogen, Korea).

Western blot Analysis

Western blot analysis was performed as previously described [14]. Equal amounts of proteins (100 µg) were fractionated on SDS-polyacrylamide gels (12%) and transferred electrophoretically onto nitrocellulose membranes (Sigma Chemical Co., USA). Membranes were blocked and probed overnight with primary rabbit anti H4R (1:500) (Alpha Diagnostic International, USA). Immunoreactivity was detected

by using horseradish peroxidase-conjugated anti-rabbit IgG (1:1000) (Bio-rad Laboratories, USA) and visualized by enhanced chemiluminescence (Amersham Biosciences, USA). MCF-7 cells were used as positive control to compare the expression of H_4R. Densitometric analyses were performed using the software Image J 1.32J (NIH, USA).

Immunostaining

Cells were seeded into 12-well plates in culture medium (25000 cells/well). The cells were washed twice with PBS and fixed for 15 min in 4% (v/v) formaldehyde in PBS. Endogenous peroxidase activity was blocked with 3% H_2O_2 (v/v) in distilled water. The cells were then incubated overnight in a humidified chamber at 4°C with primary rabbit anti H_4R (1:100) (Alpha Diagnostic International, USA). After washing, cells were incubated with peroxidase-conjugated anti-rabbit (1:100) (Bio-rad Laboratories, USA) and developed with diaminobenzidine (Sigma Chemical Co., USA). MCF-7 cells were used as positive control to compare the expression of H4R. Finally, the cells were counter-stained by immersion in hematoxylin. Light microscopy was performed on an Axiolab Karl Zeiss microscope (Göttingen, Germany). All photographs were taken at 630X magnification using a Canon Power-Shot G5 camera (Tokyo, Japan). To control the signal specificity, cells were subjected to the same staining procedure, with either a normal rabbit IgG or PBS to replace the first antibody. This control staining did not give rise to a signal.

Senescence-associated β-galactosidase staining

Cells were seeded into 12-well plates in culture medium (25000 cells/well) and treated with 10 µM Histamine, 10 µM VUF 8430, 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. [19] and also previously by us [20]. 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).

L-dopa Staining

Staining with L-dopa was performed using the method described by Hamoen et al. [21]. Cells were seeded into 12-well plates in culture medium (780 cells/well). Cells were untreated or treated for 7 days with 10 µM Histamine, 10 µM VUF 8430, 10 µM Clobenpropit and/or 10 µM JNJ7777120. As TPA (12-O-Tetradecanoylphorbol 13-acetate) inhibits cell growth and induces differentiation of human melanoma cells, it was used as positive control of pigment formation and morphological changes in a final concentration of 16 nM [22-24]. The cells were further fixed, washed with PBS containing 1 mM $MgCl_2$ and then incubated in 16 mM Sorensen's

phosphate buffer containing 10 mM L-dopa (Sigma Chemical Co., USA), pH 7.4. After incubation for 4 h at 37ºC, the L-dopa was removed and cultures were washed with 70% ethanol (v/v) for 2 h at room temperature to remove unreacted dopa. The location of dopa oxidase (tyrosinase) was indicated by the presence of an insoluble brown/black precipitate. All photographs were taken at 630X magnification using a Canon PowerShot G5 camera (Tokyo, Japan).

Human melanoma tissues

Nineteen formalin-fixed and paraffin-embedded human melanoma specimens, superficial spreading, nodular, acral-lentiginous types and different types of metastases, were selected from the files of the Pathology Department tumor bank of the José María Penna Hospital, Buenos Aires. The study was in accordance to the latest version of the Declaration of Helsinki and was approved by the ethics committee of the School of Pharmacy and Biochemistry, University of Buenos Aires.

Immunohistochemistry

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% H2O2 in distilled water. Specimens were then incubated overnight in a humidified chamber at 4°C with primary rabbit anti H_4R (1:100, Alpha Diagnostic International, TX, USA). Immunoreactivity was detected by using horseradish peroxidase-conjugated anti-rabbit IgG 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 630X magnification using a Canon PowerShot G5 camera (Tokyo, Japan). To control the signal specificity, serial sections were made from two selected positive cases which were subjected to the same staining procedure, with either a normal rabbit IgG or phosphate-buffered saline (PBS) to replace the first antibody. This control staining did not give rise to a signal. The immunostaining assessment was performed blind to the clinical data by consensus agreement of two observers (Massari N, Croci M). An overall examination of staining was carried out at 10X magnification, and representative area of breast specimen was then viewed at 630X magnification.

Small interfering RNA (siRNA) H_4R silencing

Cells were seeded in 6-well plates (150000 cells/well) or 24-well plates (50000 cells/well) and cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum and 0.3 g/L glutamine. Cells were transfected according to the manufacturer's instructions using Lipofectamine 2000 (Invitrogen), 8µl (80 pmol) of

human H_4R siRNA (sc-40025) pools of three to five target-specific 19-25 nucleotides siRNAs designed to knockdown H4R gene expression, 8µl (80 pmol) of scrambled unconjugated control siRNA-A (sc-37007) negative control that consists of a scrambled sequence that will not lead to the specific degradation of any cellular message (shared no homology to the human genome), (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Transfection was performed during 6 h and then cells were washed with PBS and fresh medium was added. Cells were then treated with 10 µM HA, 10 µM VUF 8430 or were left untreated for 18 h. The extinction of H4R expression was ascertained by immunocytochemistry analysis and RT-PCR (data not shown). Quantification of cellular DNA synthesis was performed by BrdU incorporation, as it was described previously.

Statistical analysis

All determinations were repeated at least three times. Representative results are presented as means +/- standard error of the mean (SEM). Statistical evaluations were made by analysis of variance (ANOVA) that was followed by Dunnet test and/or Newman-Keuls Multiple Comparison Test. All statistical analyses were performed with GraphPad Prism Version 5.00 software (CA, USA).

RESULTS

Expression of histamine H_4 receptor in human melanoma cells

In order to determine the expression of H_4R in WM35 and M1/15 malignant melanoma cells, mRNA was analyzed by RT-PCR as well as the protein by means of Western blot analysis and immunoassay. Figure 1 shows that both cell lines expressed the mRNA and are positive for the presence of the H4R protein. The fragment obtained by RT-PCR (Fig 1 A) presented an expected molecular weight (512 bp) for the H_4R that was additionally confirmed by sequenciation.

The bands obtained by Western blot are in agreement with previous reports for the H4R [15, 25-27] (Fig. 1 B). Western blot analysis demonstrated the presence of a diverse range of molecular weight species of the H_4R, exhibiting a different pattern when WM35 cells are compared with M1-15 cells. In the latter, a high molecular weight band (Mw~75 kDa) was observed which is consistent with native dimeric species detected in human lymphocytes and brain, and the recombinant dimeric human H4R [28-30]. An additional band (Mw~45 kDa) was observed, which could represent a proteolytic fragment [29]. That is also observed in WM35 cells. In these cells a higher molecular weight specie was detected (Mw~90 kDa) that could correspond to robust dimeric structures reported in human leukemic mast cells (HMC-1) [27]. As a positive control we employed breast cancer cell line MCF-7 [31], which exhibited a different expression pattern, displaying the presence of the putative monomer at Mw 31 kDa and two higher molecular weight species, which could correspond to dimeric structures [26].In addition, H_4R protein expression was demonstrated by immunocytochemistry (Fig. 1 C).

Histamine modulates melanoma cell proliferation

To investigate the role of H_4R in melanoma cell growth, we evaluated proliferation by the clonogenic assay using H_4R agonists (Clobenpropit and VUF 8430). Results indicate that the negative effect of histamine on proliferation was at least partially mediated via H_4R (Fig 2 A) (Clobenpropit IC_{50}=1.7 μM; VUF 8430 IC_{50}=1.66 μM for WM35 and Clobenpropit IC_{50}=4.7 μM; VUF 8430 IC_{50}=4.8 μM for M1/15).

Additionally, the inhibitory effect of H_4R on proliferation was confirmed by assessing the incorporation of BrdU, a thymidine analog. Both agonists significantly reduced the incorporation of BrdU in WM35 and M1/15 cells. Treatment with a specific H_4R antagonist, JNJ7777120, added 30 minutes before any other treatment, partially blocked the decrease in proliferation triggered by histamine and Clobenpropit while completely reversed the effect of VUF 8430 in WM35 cells. In M1/15 cells, JNJ7777120 completely blocked the histamine and the H_4R agonists inhibitory effect on proliferation (Fig 2 B).

To further explore the role of H_4R in cell proliferation, siRNA specific for H4R mRNA was used to knock down its expression in melanoma cells. Transfection optimization was performed by the evaluation of the H_4R protein and mRNA, as it is described in materials and methods. Results demonstrated that histamine and H_4R agonist reduced the incorporation of BrdU, effects that were not observed in both cell lines transfected with H_4R siRNA (Table 1).

Histamine induces cell senescence and differentiation

We additionally investigated whether histamine-mediated inhibition of proliferation could be associated with an induction of cell senescence and/or differentiation. Results demonstrated that histamine and H_4R agonists significantly increased the percentage of senescent cells evidenced by an enhanced activity of senescence associated β-galactosidase (Fig. 3).

Moreover, we evaluated the melanogenesis as a differentiation marker of melanoma cells [21, 23, 24, 32, 33]. Taking into account that tyrosinase (monophenol monooxygenase, EC 1.14.18.1) is the rate-limiting enzyme for production of melanin [28, 29], we first evaluated the expression of tyrosinase at mRNA level and we confirmed its presence in both cell lines by RT-PCR (Fig 4 A). The fragment obtained presented a molecular weight as expected (284 bp). The expression of tyrosinase seemed not be significantly modified by histamine or H_4R agonist treatment. In accordance with that, we performed the staining with L-dopa and we observed that the treatment with histamine and H_4R agonists increased the synthesis of melanin, detected as a brown pigmentation in cells under light microscopy, indicating that they lead to the stimulation of melanogenesis in vitro. It is important to point out that the combined treatment with the specific H_4R antagonist, JNJ7777120, blocked histamine effect on cell senescence and differentiation (Fig 3 and Fig 4).

Histamine H_4 receptor expression in human melanoma lesions

The immunohistochemical analysis showed that the H_4R was detected in 42% (8/19) of melanoma biopsies, showing cytoplasmic localization. H_4R was detected in different histopathological types, including superficial spreading, nodular, acral-lentiginous types (Fig. 5).

DISCUSSION

The presented results provide novel information about the role of histamine in the biological responses of human melanoma cells. A large body of literature indicates that histamine can modulate proliferation of different normal and malignant cells by acting through its specific membrane receptors [34-36]. High histamine concentrations have been reported in a wide number of human and experimental tumors [37-39] and also histamine receptors are expressed in different malignant cell types where they can be associated to multiple signaling pathways [9,14,15, 25,40-42]. In addition, different human tumors such as melanoma, colon and breast carcinomas showed higher histamine concentration compared to surrounding normal tissues [43-46].

In the present study we demonstrated for the first time the presence of the fourth histamine receptor subtype in two human melanoma cell lines. Accordingly our studies indicate that the H_4R is expressed in human melanoma biopsies, confirming that the H_4R is present not only in these cell lines but also in human melanoma tissue.

Western blot analysis showed the presence of a diverse range of molecular weight species of the H_4R. In M1-15 cells, a high molecular weight specie (Mw~75 kDa) was observed, which is consistent with native dimeric species detected in human lymphocytes and brain, and the recombinant dimeric human H_4R [28-30], and also an additional band (Mw~45 kDa) was detected [29]. This band is also observed in WM35 cells, and a higher molecular weight specie (Mw~90 kDa) was also

evidenced that could correspond to robust dimeric structures detected in human leukemic mast cells (HMC-1) [27].

In line with these observations, H_4R isoforms [29] and oligomeric structures [30], potentially resulting in signaling pathways diversity were described. Furthermore, distinct sizes detected in immunoblotting studies could be due to tissue-specific complements of H_4R isoforms and differential tissue-specific post-translational modifications [26].

In order to investigate some functional characteristics of the H_4R we employed pharmacological tools such as high affinity H_4R agonists (VUF 8430 and Clobenpropit) and the selective neutral antagonist JNJ7777120. Although H_4R agonists are reported to also affect the H_3R, they exhibit a higher potency for the H_4R, particularly the compound VUF 8430 that has 30-fold lower affinity for the H_3R [47]. On the other hand, JNJ7777120 compound shows more than a thousand fold selective over other histamine receptor subtypes and is considered an H_4R reference antagonist [26].

Our findings show that the activation of H_4R using specific agonists such as Clobenpropit and VUF 8430 led to a decreased in WM35 and M1/15 cell proliferation. Furthermore, the inhibitory effect on proliferation induced by histamine and H_4R agonists was blocked by the combined treatment with the specific H_4R antagonist JNJ7777120, confirming the involvement of H_4R. The reduced in the amount of H_4R protein expression by H_4R siRNA transfection completely prevented

the decreased in proliferation, reinforcing the role of H_4R in proliferation in both cell lines.

Previous reports also support the role of H_4R as a modulator of cell growth. In MDA-MB-231 and MCF-7 breast cancer cells H_4R mediated the inhibition of proliferation and this effect was associated to an induction of apoptosis [15, 31]. In agreement with this, in Panc-1, a cell line derived from a human ductal pancreatic carcinoma, the H_4R agonist Clobenpropit negatively regulates cell growth [40]. In contrast, histamine exerts both a proproliferative and a proangiogenic effect via H_2R/H_4R activation in colon cancer cells [48].

The reduction in proliferation produced by the H_4R agonists in this malignant melanoma cell lines was mediated at least in part by an induction of premature or accelerated cell senescence, a biological program of terminal growth arrest characterized by disruption of lysosomal function through enhanced activity of senescence associated β-galactosidase [49]. In accordance with this, we have previously reported that histamine induced senescence in WM35 cells [20].

Cancer cells are characterized by uncontrolled replication and blockade of differentiation processes. Cell differentiation is under a complex regulatory control by multiple agents such as α-melanocyte stimulating hormone, adrenocorticotropic hormone, UV radiation, betalipotropin, beta-adrenergic agonists and so forth [50-52]. It is characterized by the acquisition of differentiation markers, such as extension of elongated dendrite-like cellular protrusions and diminished cell proliferation. Nevertheless, the production of melanin in specialized organelles, melanosomes, is the key feature of melanocyte differentiation [24].

In our study, we demonstrated an increase in melanogenesis when both cell lines were treated with histamine or H4R specific agonists.

The combined treatment with JNJ7777120 blocked the histamine induced cell senescence and differentiation, supporting the role of H_4R in these responses.

As far as we know, this is the first report that describes the functional presence of H_4R in human malignant melanoma cells and its role as a modulator of cell proliferation, senescence and differentiation which are key processes in melanoma progression. Further studies are needed to corroborate its importance as potential target for new drug development for the treatment of this disease.

ACKNOWLEDGEMENTS

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Figure Legends

Fig.1: Expression of H_4R in human melanoma cells.

(A) H4R expression was determined by RT-PCR (512 bp)**.** Lanes: M, DNA ladder molecular size marker; 1, positive control (MCF-7 human breast cancer cells); 2, negative control (without cDNA); 3, WM35 cells; 4, M1/15 cells. β-actin (521bp) was used as load control.

(B) Western blot analysis. β-actin (42 kDa) was used as load control. MCF-7 cells were used as positive control.

(C) Immunostaining of the H_4R in melanoma cells. MCF-7 cells were used as positive control. Picture was taken at 630X-fold magnification.

Fig. 2: Effect of histamine and H4R ligands on human melanoma cell proliferation.

(A) Clonogenic assay. Histamine decreases WM35 and M1/15 cells proliferation via H_4R. On the left: Cells were left untreated (control) or treated with Clobenpropit in concentrations ranging 0.01-10 µM. On the right: Cells were left untreated (control) or treated with VUF 8430 in concentrations ranging 0.01-10 µM. Error bars represent the means ± SEM. (Dunnet test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Control).

(B) Incorporation of BrdU. Histamine modulates active DNA synthesis evaluated by the BrdU incorporation. WM35 and M1/15 cells were left untreated or treated with 10 µM histamine, 10µM VUF 8430, 10µM Clobenpropit and/or 10 µM JNJ7777120 for 48 h. Error bars represent the means ± SEM. Dunnet test *$p < 0.05$, **$p < 0.01$,

***p < 0.001 vs. Control. Newman-Keuls Multiple Comparison Test #p < 0.05, ##p < 0.01, ###p < 0.001. Pictures were taken at a 400X-fold magnification. Scale bar: 20 µm.

Fig. 3: Effect of histamine and H_4R ligands on cell senescence–associated β-galactosidase staining.

The cells were treated with 10 µM histamine, 10 µM VUF 8430, 10 µM Clobenpropit and/or 10 µM JNJ7777120 for 48 h. Arrows indicate senescent cells. Pictures were taken at 630X original magnification. 10 fields were analyzed for each determination. Error bars represent the means ± SEM. *p<0.05, **p<0.01, ***p<0.001 vs. Control. Results are representative of three independent experiments.

Fig. 4: Effect of histamine and H_4R ligands on cell differentiation.

(A) Tyrosinase expression was evaluated by RT-PCR (284 bp). Lanes: M, DNA ladder molecular size marker; 1, Negative control (without cDNA); 2: Negative control (MCF-7 human breast cancer cells), 3, 4 and 5: WM35 cells were untreated (Control) or treated with 10 µM histamine, 10 µM VUF 8430 for 48 h before RNA extraction; 6, 7 and 8: M1/15 cells were untreated (Control) or treated with 10 µM histamine, 10 µM VUF 8430 for 48 h before RNA extraction. β-actin (521bp) was used as load control.

(B) Cell differentiation was evaluated by the staining with L-Dopa. The cells were untreated or treated with 10 µM histamine, 10 µM VUF 8430, 10 µM Clobenpropit and/or 10 µM JNJ7777120 for 7 days and then incubated with 10 mM L-Dopa. The

localization of dopa-oxidase was indicated by the presence of an insoluble brown/black precipitate. Pictures were taken at 630 X-fold magnifications (WM35) and 400X-fold magnifications (M1/15). Positive control: 16 nM TPA + L-DOPA; Negative control: without L-DOPA; Control: L-DOPA only.

<u>Fig.5:</u> H_4R expression in human melanoma biopsies by immunohistochemical staining.

(A) Negative Control staining for H_4R protein. (B) Nodular malignant melanoma, showing positive immunoreactivity for H_4R. (C) Brain metastasis, displaying negative immunoreactivity for H_4R. Pictures were taken at a 630X-fold magnification. Scale bar: 20 µm.

Buenos Aires, March 18th, 2011

Editor-in-Chief; Ferdy J. Lejeune
Editorial Office, Melanoma Research
Professor Emeritus of Oncology
Division of Experimental Oncology
CePO-University of Lausanne
Chemin du Levant 98
CH-1005 Lausanne
Switzerland

Dear Editor,

We are submitting the revised version of our manuscript entitled: "ROLE OF H_4 RECEPTOR IN HISTAMINE-MEDIATED RESPONSES IN HUMAN MELANOMA" by Massari et al. In our study we demonstrate for the first time the presence of the histamine H_4 receptor in human malignant melanoma cells and tissue and its role as an inductor of cell senescence and differentiation. Our findings suggest that the histamine H_4 receptor may be involved in the regulation of melanoma growth and progression, representing a novel molecular target for a new therapeutic approach. The identification of H_4R and the elucidation of its role in the development and growth of human malignant melanoma may represent an essential clue for advances in the treatment of this disease.

We have tried to address to all the comments and requests suggested by the reviewers as it is described at point by point response file.

We hope you find the revised version suitable for publication in the Melanoma Research.

In regard to the authors of our work, we would like to state that all of them qualify for authorship and have read and approved the manuscript.

We declare no conflict of interest.

If you consider necessary we can send by courier a CD with a higher resolution copy of the Figures saved as TIF format.

Yours sincerely,

Prof. Dr. Elena Rivera
Facultad de Farmacia y Bioquímica
Universidad de Buenos Aires
Junín 956 PB, 1113, Buenos Aires
Phone +54-11-4964-8277
Fax: +54-11-4962-8202, ext.31
E-mail: erivera@ffyb.uba.ar

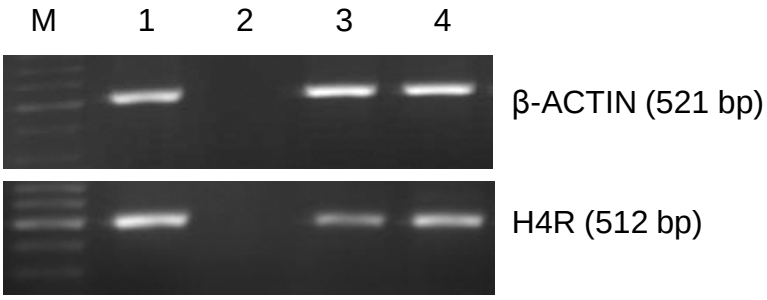
Table 1: Incorporation of BrdU in transfected cells

	Control	HA	VUF	scramble siRNA	H_4 siRNA	H_4 siRNA + HA	H_4 siRNA + VUF
WM35	51,0 ± 2,9	40,6 ± 1,4 *	37,4 ± 2,1**	56,2 ± 3,3	54,0 ± 1,5	50,6 ± 1,3$^{\#}$	54,8 ± 3,5$^{∫∫}$
M1/15	61,2 ± 4,5	41,0 ± 2,9***	36,1 ± 1,7***	58,4 ± 3,4	57,4 ± 2,8	61,1 ± 0,8$^{\#\#\#}$	60,8 ± 0,6$^{∫∫∫}$

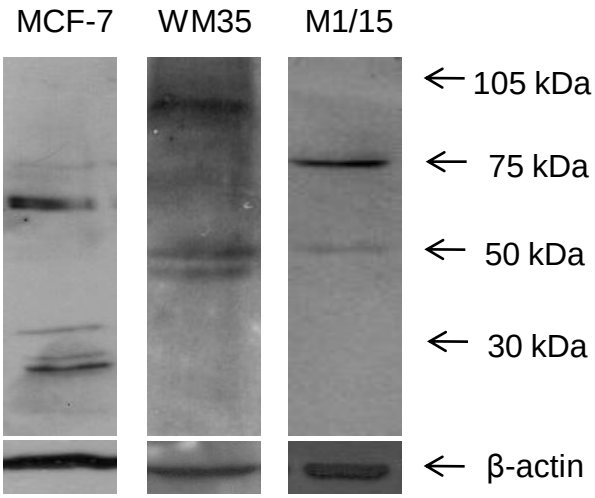
Control: untreated cells; HA: cells treated with 10 µM Histamine for 18 h; VUF: cells treated with 10 µM VUF8430 for 18 h; scrambled siRNA (negative control): cells transfected with scrambled siRNA; H_4 siRNA: cells transfected with specific sequences siRNA designed to knockdown H_4R gene expression; H_4 siRNA + HA: transfected cells with H_4 siRNA and treated with 10 µM Histamine for 18 h; H_4 siRNA +VUF: transfected with H_4 siRNA cells and treated with 10 µM VUF8430 for 18 h. Error bars represent the means ± SEM. Dunnet test *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. Control. Newman-Keuls Multiple Comparison Test #$p < 0.05$, ###$p < 0.001$ vs HA. Newman-Keuls Multiple Comparison Test ∫∫$p < 0.01$, ∫∫∫$p < 0.001$ vs VUF.

Figure 1

A


M
1
2
3
4
β-ACTIN (521 bp)
H4R (512 bp)

B


MCF-7
WM35
M1/15
105 kDa
75 kDa
50 kDa
30 kDa
β-actin

C

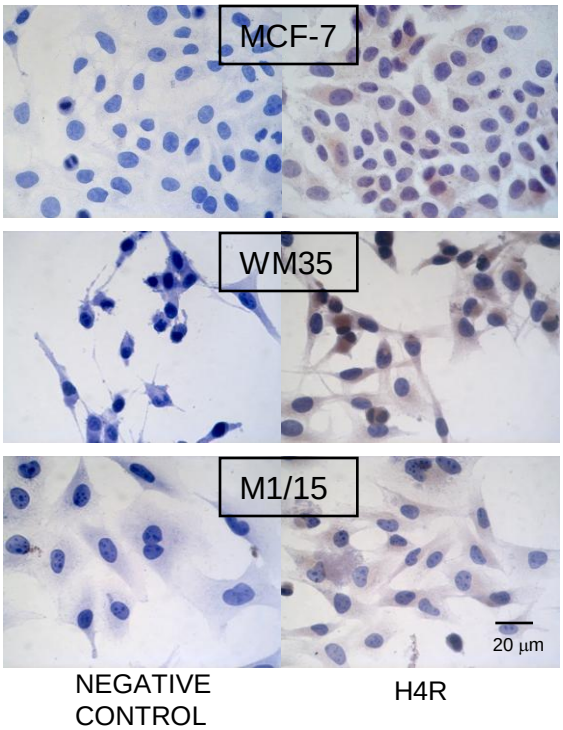

MCF-7
WM35
M1/15
20 μm
NEGATIVE CONTROL
H4R

Figure 2

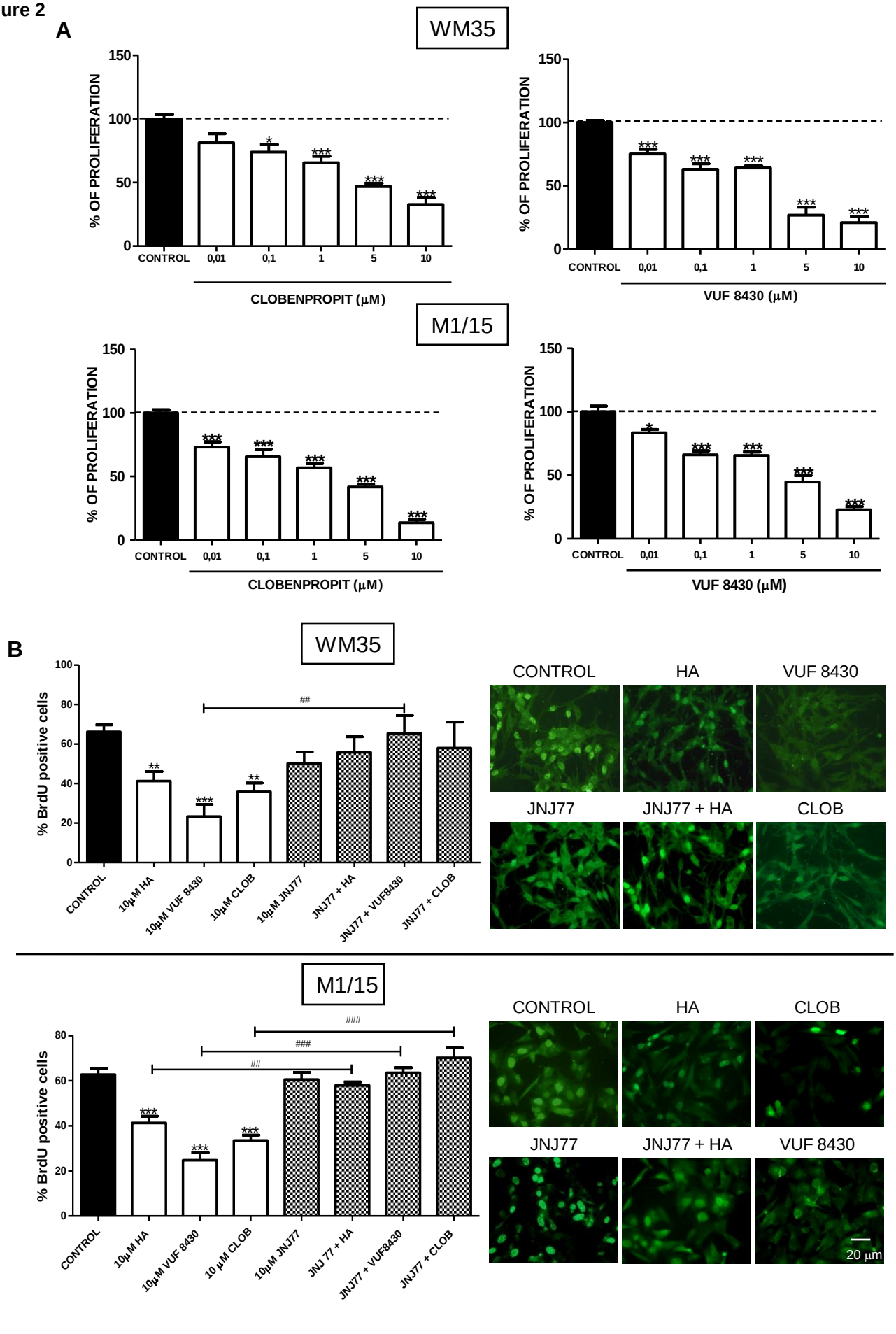

A
WM35
% OF PROLIFERATION
CONTROL
0,01
0,1
1
5
10
CLOBENPROPIT (µM)
VUF 8430 (µM)
M1/15
B
WM35
% BrdU positive cells
10µM HA
10µM VUF 8430
10µM CLOB
10µM JNJ77
JNJ77 + HA
JNJ77 + VUF8430
JNJ77 + CLOB
CONTROL
HA
VUF 8430
JNJ77
JNJ77 + HA
CLOB
M1/15
10 µM CLOB
20 µm

Figure 3

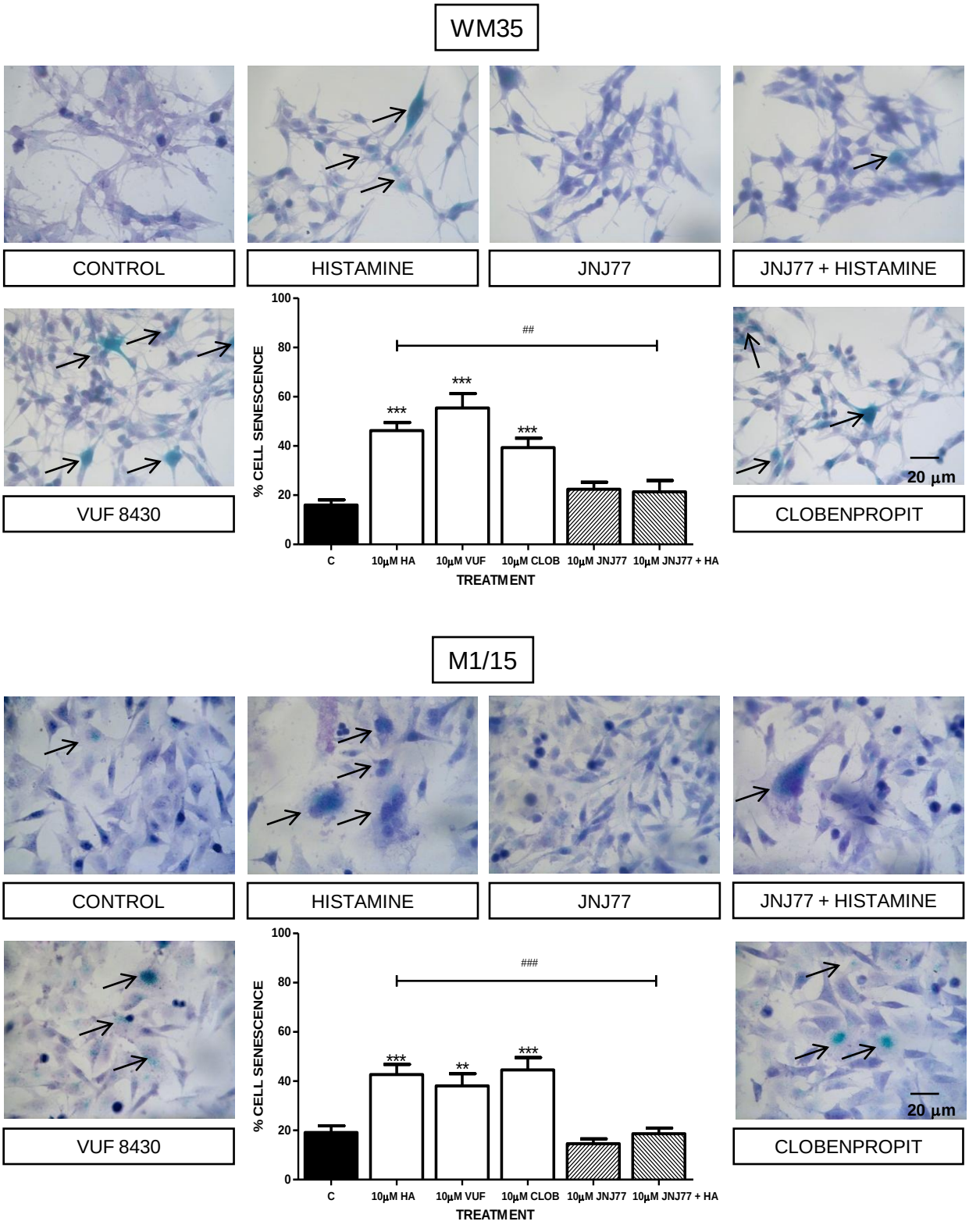

WM35
CONTROL
HISTAMINE
JNJ77
JNJ77 + HISTAMINE
VUF 8430
CLOBENPROPIT
20 µm
% CELL SENESCENCE
TREATMENT
C
10µM HA
10µM VUF
10µM CLOB
10µM JNJ77
10µM JNJ77 + HA
##
***
M1/15
CONTROL
HISTAMINE
JNJ77
JNJ77 + HISTAMINE
VUF 8430
CLOBENPROPIT
20 µm
% CELL SENESCENCE
TREATMENT
###
**
***

Figure 4

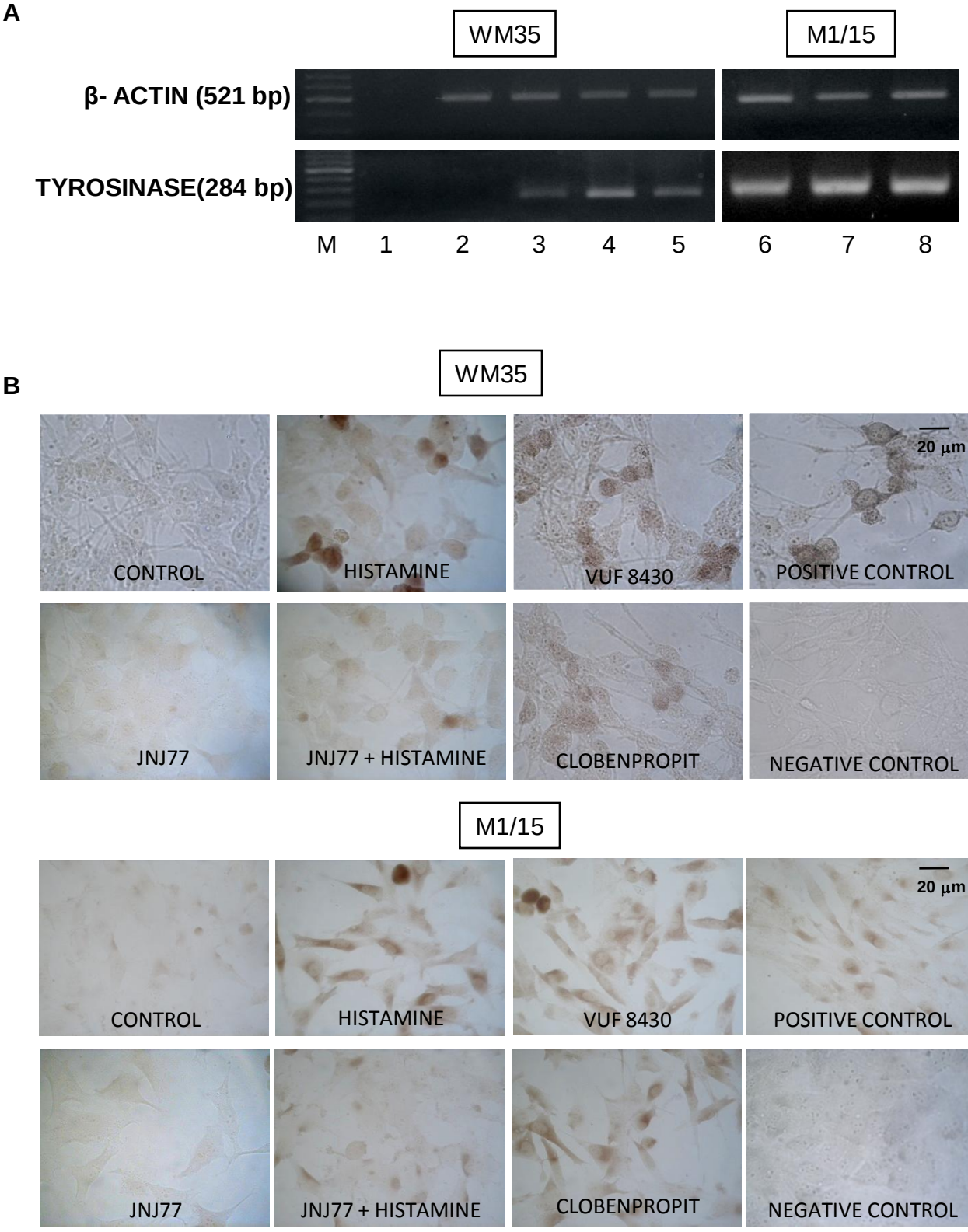
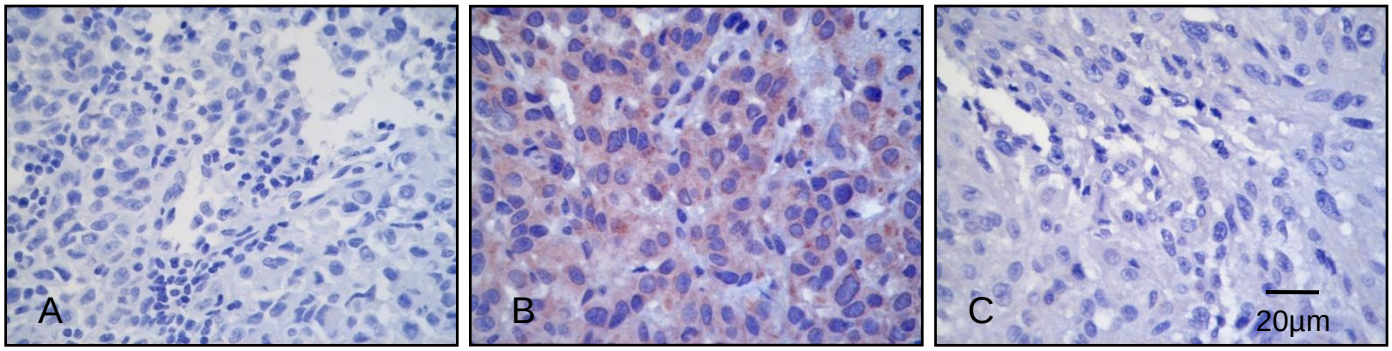

A
WM35
M1/15
β- ACTIN (521 bp)
TYROSINASE(284 bp)
M 1 2 3 4 5 6 7 8
B
WM35
CONTROL
HISTAMINE
VUF 8430
20 µm
POSITIVE CONTROL
JNJ77
JNJ77 + HISTAMINE
CLOBENPROPIT
NEGATIVE CONTROL
M1/15
CONTROL
HISTAMINE
VUF 8430
20 µm
POSITIVE CONTROL
JNJ77
JNJ77 + HISTAMINE
CLOBENPROPIT
NEGATIVE CONTROL

Figure 5



Melanoma Research

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Corresponding Author: ELENA SUSANA RIVERA, PhD -
Mailing Address and Telephone/Fax Numbers: JUNIN 956, BUENOS AIRES CITY. ARGENTINA (1113)
+54-11-4964-8277 / Fax: +54-11-4964-8277 ext 31

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