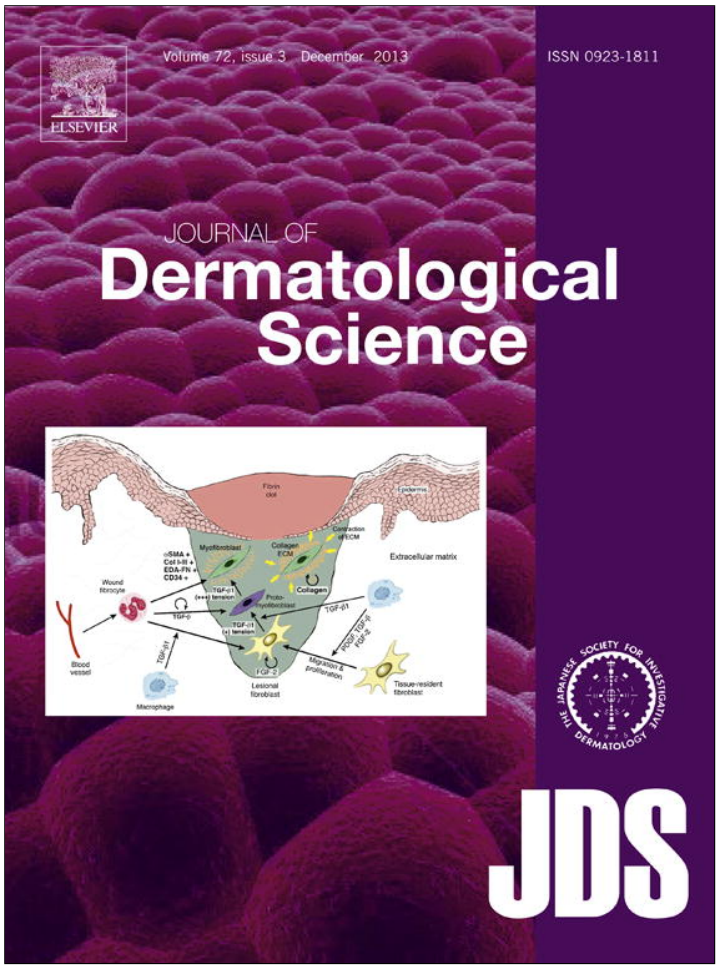

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Antitumor activity of histamine and clozapine in a mouse experimental model of human melanoma

Noelia A. Massari [a], Vanina A. Medina [a,b,*], Graciela P. Cricco [a], Diego J. Martinel Lamas [a], Lorena Sambuco [c], Romina Pagotto [d], Clara Ventura [a], Pablo J. Ciraolo [a], Omar Pignataro [d], Rosa M. Bergoc [a,b], Elena S. Rivera [a,**]

[a] Laboratory of Radioisotopes, School of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires 1113, Argentina
[b] National Scientific and Technical Research Council (Conicet), Buenos Aires, Argentina
[c] Institute of Immunooncology, Buenos Aires, Argentina
[d] Laboratory of Molecular Endocrinology and Signal Transduction, Institute of Biology and Experimental Medicine-Conicet, Buenos Aires 1428, Argentina

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We dedicate this work to the memory of our friend, colleague and excellent pathologist Maximo Croci, MD.

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ABSTRACT

Background: Functional presence of histamine H_4 receptor (H_4R) was demonstrated in human melanoma cell lines and biopsies.
Objective: The purposes of this work were to investigate signal transduction pathways and biological responses triggered by the activation of H_4R in human primary (WM35) and metastatic (M1/15) melanoma cell lines and to evaluate the *in vivo* antitumor activity of histamine (HA) and clozapine (CLZ) on human M1/15 melanoma xenografts.
Methods: Clonogenic assay, incorporation of BrdU, cell cycle distribution, phosphorylation levels of ERK1/2 and cAMP production were evaluated *in vitro*. An experimental human melanoma model was developed into athymic nude mice. Tumor growth, survival and histochemical studies were performed in order to investigate the expression levels of H_4R, HA, PCNA, mitotic index (MI), and angiogenesis.
Results: The results indicate that H_4R agonists inhibited forskolin-induced cAMP levels only in M1/15 cells while increased phosphorylation levels of ERK1/2 and decreased proliferation in both cell types. *In vivo* studies show that HA and CLZ (1 mg kg^{-1}, *sc*) significantly increased median survival and decreased tumor volume. These effects were associated to a reduction in MI, in the expression of proliferation marker and in intratumoral neovascularization.
Conclusions: We conclude that HA and CLZ exhibit an antitumoral effect *in vitro* and *in vivo* on human melanoma, suggesting the therapeutic potential of these compounds for the treatment of malignant melanoma.

1. Introduction

Melanoma accounts for less than 5% of skin cancer cases but causes a large majority of skin cancer deaths. Therefore, new therapeutic targets are urgently needed to improve the development of an effective systemic therapy.

Histamine (HA) has been implicated as one of the mediators involved in regulation of proliferation in both normal and neoplastic tissues. Melanoma cells but not normal melanocytes contain large amounts of HA that has been found to accelerate malignant growth [1]. HA 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, and H_4R), resulting in the activation of different signal transduction pathways [2–4]. It has been previously reported the expression of H_1R, H_2R and H_3R in human melanoma cell lines [5]. In human primary WM35 melanoma cells, HA acting through the H_1R decreases cell proliferation, whereas it enhances growth when acting through the H_2R [6], while stimulation of the H_3R in human melanoma cells did not show mitogenic signaling [5]. On the other hand, in highly metastatic M1/15 human melanoma cells HA produces a significant decrease in cell proliferation, effect that was mimicked by an H_1R agonist [6].

Numerous *in vivo* studies employing animal models bearing syngenic or xenogenic melanoma grafts demonstrated that both endogenous and exogenous histamine have the ability to stimulate tumor growth while H_2R antagonists inhibit this effect [1,7,8]. In

* Corresponding author at: Laboratorio de Radioisótopos, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, Buenos Aires 1113, Argentina.
** Corresponding author at: Laboratorio de Radioisótopos, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, Buenos Aires 1113, Argentina. Tel.: +54 11 4964 8277/8202; fax: +54 11 4964 8277/8202x31.
E-mail addresses: vmedina@ffyb.uba.ar, vaninamedina78@yahoo.com.ar (V.A. Medina), erivera@ffyb.uba.ar (E.S. Rivera).

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agreement with these results, H_2R antagonists stimulated melanogenesis and inhibited proliferation in B16-C3 mouse melanoma cells [9]. It was also found that melanoma tumor growth was not modulated by *in vivo* HA treatment while treatment with terfenadine, an H_1R antagonist, significantly inhibited tumor growth *in vitro* and *in vivo* in murine melanoma models [10].

The latest identified member of the G protein-coupled histamine receptor subfamily was the H_4R with potential functional implications in inflammatory diseases and cancer [4,11,12]. H_4R describes distinct isoforms [13,14] and oligomeric structures resulting in diverse signaling pathways [2,14]. In recombinant systems activation of $G_{i/0}$ proteins resulted in a decrease in adenylyl cyclase activity and consequently reduction in levels of cAMP within the cell [15,16]. However, in some cell-types, such as mouse mast cells, the endogenous H_4R has been shown to couple to Ca^{2+} mobilization in a pertussis toxin-sensitive manner, but not to cAMP [17]. Furthermore, there is growing evidence that where multiple pathways are activated by the same receptor, certain agonists are able to preferentially activate one pathway over another, displaying collateral efficacy [18].

The Ras/Raf/MEK/ERK (MAPK) pathway is among the most commonly deregulated pathways identified in tumors and there is no doubt that it is critical for melanoma development and progression, and a primary therapeutic target [19,20]. It was described that H_4R stimulation results in the pertussis-toxin-sensitive activation of downstream activated protein kinase pathways [2]. However, little is known about the signal transduction pathways associated to this receptor in cancer cells.

We have previously explored the expression of H_4R and some associated biological responses in human malignant melanoma cell lines (WM35 and M1/15). Results demonstrated that melanoma cells express H_4R at the mRNA and protein level. By using histamine agonists (VUF 8430 and clobenpropit), antagonists (JNJ7777120) and genetic tools (siRNA H_4R) it was shown that the inhibitory effect of HA on proliferation was in part mediated through the stimulation of the H_4R. Treatment with a specific H_4R antagonist and the use of siRNA specific for H_4R mRNA blocked the decrease in proliferation triggered by the H_4R agonists. Furthermore, the decrease in proliferation exerted by H_4R agonists was associated with a two-fold induction of cell senescence and an increase in melanogenesis that is a differentiation marker on these cells [21]. In addition, the H_4R was expressed in human melanoma biopsies, confirming that the H_4R was present not only in these cell lines but also in human melanoma tissue. H_4R was detected in 42% (8/19) of melanoma biopsies with different histopathological types, including superficial spreading, nodular and acral-lentiginous types and exhibited cytoplasmic localization [21].

Clozapine (CLZ) is an atypical antipsychotic drug primarily prescribed to patients who are unresponsive to or intolerant of conventional neuroleptics. CLZ has been shown to fully activate the H_4R, although it displays a moderate (submicromolar) affinity and several works support the idea to use CLZ as H_4R agonist *in vitro* and *in vivo* [16,22–25].

Based on the presented evidences, the aims of this work were to investigate the action of H_4R agonists *in vitro* on signal transduction pathways and cell proliferation, and to evaluate the *in vivo* effect of HA and CLZ on tumor growth, levels of cell proliferation markers and vascularization and also survival of human melanoma xenografts developed in nude mice.

2. Materials and methods

2.1. Chemicals

Histamine (HA), 2-(1H-imidazol-4-yl)ethanamine (Sigma Chemical Co., Missouri, USA); H_4R agonists: VUF 8430 (VUF), 2-[(aminoiminomethyl)amino]ethyl carbamimidothioic acid ester dihydrobromide (Tocris Bioscience, Ellisville, Missouri, USA); clozapine (CLZ), 3-chloro-6-(4-methylpiperazin-1-yl)-5H-benzo[c][1,5]benzodiazepine (kindly provided by Fabra Laboratories S.A, Buenos Aires, Argentina); H_4R antagonist: JNJ7777120 (JNJ77), 1-[(5-chloro-1H-indol-2-yl)carbonyl]-4-methylpiperazine (Johnson & Johnson Pharmaceutical Research and Development, USA).

2.2. In vitro *studies*

2.2.1. *Cell culture*

The human melanoma cell lines WM35 (primary melanoma, radial growth phase) and M1/15 (derived from liver metastasis, radial and vertical growth phase) were kindly provided by Professor A. Falus (Budapest, Hungary). WM35 cells are non-tumorigenic in nude mice [26], while M1/15 cells are able to induce tumors in nude mice when are injected subcutaneously [7].

Cells were cultured in RPMI 1640 supplemented with 10% (v/v), FBS, 0.3 g l^{-1} glutamine, and 0.04 g l^{-1} gentamicin (all from Gibco BRL, Grand Island, NY, USA). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2.2. *Cell proliferation assays*

For clonogenic assay, WM35 and M1/15 cells were seeded in six-well plates (1000 cells per well). Cells were treated with 0.01 to 10 μM of CLZ (diluted in 0.5% ethanol final concentration) and/or 10 μM of JNJ77. Cells were incubated for 7 days and then fixed with 10% (v/v) formaldehyde in PBS (Sigma Chemical Co., Missouri, USA) 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 BrdU (Sigma Chemical Co., Missouri, USA) incorporation. Cells were seeded into 12-well plates in culture medium (25 000 cells per well), and treated with 10 μM of CLZ and/or 10 μM of JNJ77 for 48 h. After that, BrdU (30 μM) was added into culture medium for 2 h. 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., Missouri, 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 in the ratio of 1:100 in 1% bovine seroalbumine (w/v) in PBS. Cells were washed with PBS and further incubated for 30 min with 1:100 fluorescein isothiocyanate-conjugated antimouse IgG (Sigma Chemical Co., Missouri, USA) and 4-6-diamidino-2-phenylindole (Sigma Chemical Co.) at room temperature. Coverslips were mounted with Fluor-Save™ Reagent (Calbiochem, Darmstadt, Germany) 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.

2.2.3. *Senescence-associated β-galactosidase staining*

Cells were seeded into 12-well plates in culture medium (25 000 cells per well) and were left untreated or treated with 10 μM CLZ and/or 10 μM JNJ77 for 48 h. Senescence-associated β-galactosidase-positive cells were detected using the method described by Dimri et al. [27] and also previously by us [21]. Briefly, cells were fixed and incubated at 37 °C for 8 h with 1 mg ml^{-1} 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 total cells were scored for each determination. All photographs were taken at 630× magnification using a Canon PowerShot G5 camera (Tokyo, Japan).

2.2.4. Cell cycle analysis

Cells were plated, cultured for 24 h and serum-starved for an additional 24 h. Synchronized cells were left untreated or treated with 10 μM HA, 10 μM VUF, or 10 μM of CLZ, 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 propidium iodide (PI) staining solution (50 μg ml^{-1} PI in PBS containing 0.2 mg ml^{-1} of DNase-free RNase A; Sigma Chemical Co., Missouri, USA). After incubation for 30 min at 37 °C, samples were evaluated by flow cytometry. Cell cycle distribution was analyzed using Cylchred version 1.0.2 software (Cardiff University, UK).

2.2.5. Determination of cyclic adenosine monophosphate (cAMP)

Intracellular cAMP was measured in cell monolayers at 70–90% confluence. Cells were washed with saline solution and exposed to 1 mM isobutylmethylxanthine (IMBX) in culture medium for 3 min at 37 °C and then treated with HA (0.01–10 μM), VUF (0.01–10 μM) and or Forskolin (FK) (10 μM) (Sigma Chemical Co., Missouri, USA) for 15 min at 37 °C as it was previously described [28]. Cells were then washed and reaction was stopped by ethanol (Merck, Argentina) addition followed by centrifugation at 1500 × *g* for 15 min. The ethanol phase was then dried, and the residue was resuspended in 150 mM sodium acetate buffer (pH 6.0). The cAMP produced was determined by radioimmunoassay. Unknown samples and standards were acetylated and assayed by RIA using the method described previously [29]. The interassay and intraassay variations of coefficients were lower than 10%.

2.2.6. Western blot analysis

Western blot analysis was performed as previously described [21]. The cells were collected in ice-cold PBS, and the cell extracts were prepared in RIPA buffer with appropriate proteinase and phophatases inhibitors. Cell lysates were boiled with loading buffer for 10 min. Equal amounts of proteins (30 μg) were fractionated on SDS-polyacrylamide gels (12%) and transferred electrophoretically onto poly-(vinylidine difluoride) (PDVF) membranes. Membranes were blocked and probed overnight with primary rabbit anti-phospho-ERK1/2 (p-ERK1/2) (Cell Signaling, Beverly, MA, USA). Antibodies recognizing p-ERK1/2 (1:500) and total ERK1/2 (ERK1/2) (Cell Signaling, Beverly, MA, USA) (1:500) were used sequentially in the same membrane after treatment of the blots with stripping buffer (62.5 mM Tris, pH 6.8, 100 mM 2-mercaptoethanol and 2% SDS) for 30 min at 60 °C. Immunoreactivity was detected by using horseradish peroxidase-conjugated anti-rabbit IgG (1:1000) (Sigma Chemical Co., MO, USA) and visualized by enhanced chemiluminescence (Amersham Biosciences, USA). Densitometric analyses were performed using the software Image J 1.32J (NIH, USA).

2.3. In vivo *studies*

2.3.1. Treatments and animals

HA was diluted in saline solution. CLZ was diluted in 0.1 N HCl, neutralized with 4 N NaOH and diluted with saline solution.

Specific pathogen-free athymic male nude (NIH nu/nu) mice were purchased from the Division of Laboratory Animal Production, School of Veterinary Sciences, University of La Plata, Buenos Aires (Argentina), and maintained in sterile isolated conditions. Mice were kept 5–10 per cage and maintained in our animal health care facility at 22–24 °C and 50–60% humidity on a 12 h light/dark cycle with food and water available *ad libitum*. Animals with an age of 8–10 weeks and an average weight of 25–30 g were used. All animal protocols were supervised and managed by qualified trained personnel according to international guidelines for animal care.

Animal procedures were in accordance with recommendations from the Guide for the Care and Use of Laboratory Animals of the National Research Council [30], and protocols were approved by the Ethical Committee for the Use and Care of Laboratory Animals of the School of Pharmacy and Biochemistry and also by Ethical and Educational Committee of the Institute of Immunooncology.

M1/15 cells (3×10^6) were collected by centrifugation and resuspended in 100 μL RPMI-1640 (GIBCO, Grand Island, New York, USA). Human melanoma tumors were originally induced by subcutaneous (*sc*) injection of human melanoma cells into the right flank of two male athymic nude mice and were subsequently maintained by *sc* transplantations into male athymic nude mice. The animals were randomly separated into three groups and received a subcutaneous daily injection of saline solution (control group, $n = 14$), 1 mg kg^{-1} histamine (HA, $n = 8$) or 1 mg kg^{-1} clozapine (CLZ, $n = 13$).

2.3.2. Tumor growth

The length and width of the subcutaneous tumors were measured using a caliper three times a week. The tumor size was calculated as sphere volume according to the following formula: Tumor volume [cm^3] = $4/3\pi \times r$ [cm]^3. The tumor growth curve was constructed based on tumor size. Treatments lasted 70 d and tumors were excised and *ex vivo* histochemical studies were performed. Two-way ANOVA and Bonferroni post test of tumor growth data were carried out by GraphPad Prism version 5.00™.

2.3.3. Survival

Survival was evaluated in mice bearing xenografts until spontaneous death. Kaplan–Meier survival curves, median survival time of each group and *P*-value were obtained using GraphPad Prism 5™.

2.4. Ex vivo *studies*

2.4.1. Histochemistry and immunostaining

Tumors were excised, fixed in 40 g l^{-1} formaldehyde in PBS (formalin), paraffin embedded and sliced into 3-μm thick sections to evaluate the expression levels of H_4R (1:75, Alpha Diagnostic International, TX, USA) and HA (1:100, Sigma Chemical Co., MO, USA).

Cell growth was assessed by determining the proliferating cell nuclear antigen (PCNA) (1/100, Dako Cytomation, Denmark) expression and by mitotic index (MI), as the number of cells with visible chromosomes in 400× magnification fields.

The appropriate secondary HRP-conjugated antiserum was employed in each case. DAB tablets were used for staining and hematoxylin for counterstaining.

Finally, vascularization was determined using Massons trichromic staining by screening trichrome stained sections at 50× magnification to identify the largest vascular areas around the tumor. In these hot spots, intratumoral vascularity was evaluated by counting vessels inside the tumor at 200× magnification in 10 random fields.

Microscopic observations were done in 10 random fields and performed using an Axiolab Karl Zeiss (Göttingen, Germany) microscope by two independent observers. Photographs were taken with a Canon Power Shot G5 (Tokyo, Japan) digital camera and processed with Remote Capture 2.7 software.

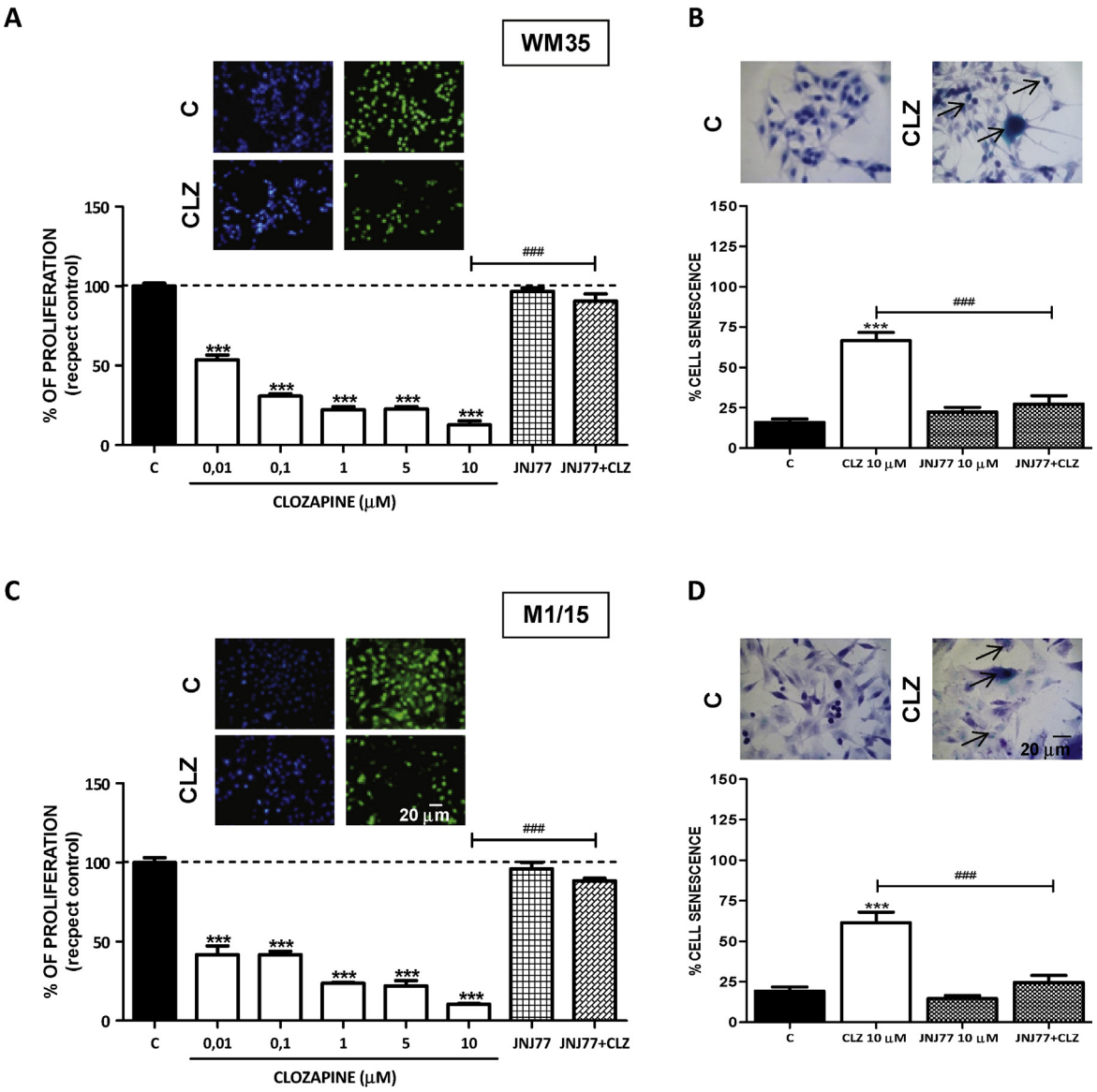

Fig. 1. Effect of clozapine as H_4R agonist on human melanoma cells proliferation and cell senescence-associated β-galactosidase staining. Clonogenic assay (A and C). Clozapine decreased WM35 and M1/15 cells proliferation *via* H_4R. Cells were left untreated (control) or treated with clozapine in concentrations ranging 0.01–10 μM and/or 10 μM JNJ7777120 (JNJ77). Error bars represent the means ± SEM (Dunnet's test, $^{***}P < 0.001$ *vs.* control; Newman–Keuls Multiple Comparison Test, $^{###}P < 0.001$, 10 μM CLZ *vs.* JNJ77 10 μM + 10 μM CLZ). *Inset: Incorporation of BrdU.* WM35 and M1/15 cells were left untreated (C) or treated with 10 μM clozapine (CLZ) for 48 h. Pictures were taken at a 400× fold magnification. Scale bar: 20 μm. Senescence-associated β-galactosidase staining (B and D). Clozapine significantly increased the percentage of senescent cells evidenced by an enhanced activity of senescence associated β-galactosidase. The combined treatment with the specific H_4R antagonist, JNJ77, blocked CLZ effect on cell senescence. 10 fields were analyzed for each determination. Error bars represent the means ± SEM (Dunnet's test, $^{***}P < 0.001$ *vs.* control; Newman–Keuls Multiple Comparison Test, $^{###}P < 0.001$, 10 μM CLZ *vs.* JNJ77 10 μM + 10 μM CLZ). *Inset: Senescent cells.* Cells were left untreated (C) or treated with 10 μM CLZ for 48 h. Arrows indicate senescent cells. Pictures were taken at 630× original magnification. Results are representative of three independent experiments.

PCNA expression was determined considering positive nuclei staining as positive cells. For HA and H_4R level evaluation, staining diffuse positive cytoplasms were considered as positive cells.

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.

2.4.2. Statistical analysis

Representative results are presented as means ± standard error of the mean (SEM). Statistical evaluations were made by unpaired *t*-test or analysis of variance (ANOVA) that was followed by Dunnet's test, Newman–Keuls Multiple Comparison Test, and Bonferroni test. All statistical analyses were performed with GraphPad Prism version 5.00™ (CA, USA).

3. Results

3.1. In vitro *studies*

3.1.1. Role of H_4R agonists in human melanoma cell proliferation

In the present study we investigated the behavior of CLZ as an H_4R agonist in human melanoma cell proliferation. Results demonstrate that CLZ treatment significantly decreased proliferation of human melanoma cells (CLZ IC_{50} = 0.18 μM; for WM35 cells and CLZ IC_{50} = 0.27 μM for M1/15 cells), effect that was completely blocked with the combined treatment with JNJ77 (Fig. 1A and C). Accordingly, CLZ significantly reduced the incorporation of BrdU

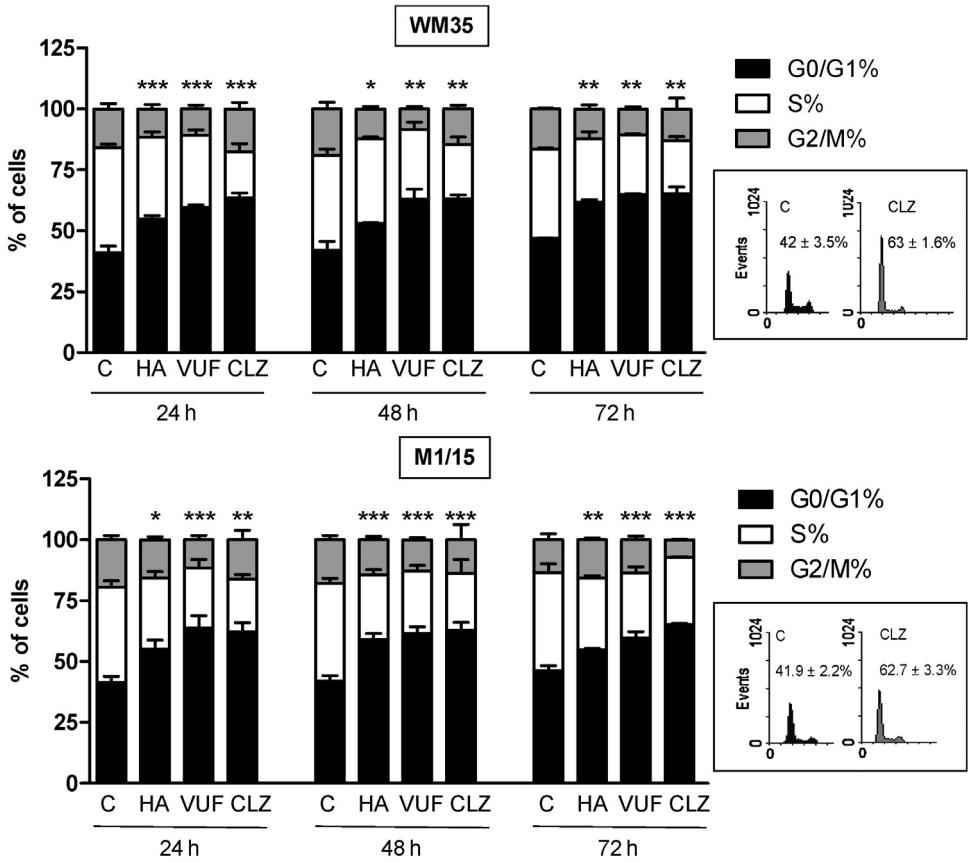

Fig. 2. Activation of the H_4R results in an accumulation of WM35 and M1/15 cells in G_0/G_1 phase of the cell cycle. Cells were synchronized and treated with 10 μM histamine (HA), 10 μM VUF 8430 (VUF), 10 μM clozapine (CLZ) or left untreated (C) for 24, 48 or 72 h. Percentage of cells in different phases of the cell cycle was monitored as a function of time using flow cytometry. Results represent the mean value of three independent experiments ± SEM. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs.* control; ANOVA and Dunnett's Multiple Comparison Test). Insets show data of C and CLZ at 48 h in WM35 and M1/15 cells.

and treatment with the H_4R antagonist, JNJ77, reversed its inhibitory effect on proliferation (Table 1), (Fig. 1 inset).

We additionally investigated whether CLZ-mediated inhibition of proliferation could be associated with an induction of cell senescence. CLZ significantly increased the percentage of senescent cells (66.4 ± 5.2% *vs.* 15.9 ± 2.0% in WM35 cells; 61.3 ± 6.6% *vs.* 19.2 ± 2.7% in M1/15 cells) evidenced by an enhanced activity of senescence associated β-galactosidase (Fig. 1B and C). The combined treatment with the specific H_4R antagonist, JNJ77, blocked CLZ effect on cell senescence.

We further evaluated the effect of HA and the H_4R agonists on cell cycle distribution of WM35 and M1/15 cells. Treatment with HA (10 μM), VUF (10 μM) or CLZ (10 μM) produced an accumulation of cells in the G0/G1 phase of the cell cycle. The effect was clearly observed at 24 h and continued up to 72 h of treatment (Fig. 2).

Table 1
Incorporation of BrdU in human melanoma cells. Clozapine modulates active DNA synthesis evaluated by the BrdU incorporation.

	Control	CLZ	JNJ77	JNJ77+ CLZ
WM35	62.1 ± 2.9	42.7 ± 1.7***	54.9 ± 4.6	56.4 ± 1.9###
M1/15	70.9 ± 3.3	39.8 ± 2.1***	69.1 ± 3.8	69.7 ± 2.0###

Clozapine treatment inhibited the incorporation of BrdU, 5-bromo-2′-deoxyuridine. Control, cells treated with 0.5% ethanol final concentration; CLZ, cells treated with 10 μM of clozapine for 48 h; JNJ77, cells treated with 10 μM of JNJ7777120 for 48 h; JNJ77 + CLZ, cells treated with 10 μM of JNJ7777120 and 10 μM of CLZ for 48 h. Results represent the mean ± SEM.
*** ANOVA and Dunnett's Multiple Comparison Test: $P < 0.001$ *vs.* Control
ANOVA and Newman–Keuls Multiple Comparison Test: $P < 0.001$ *vs.* CLZ.

3.1.2. Role of H_4R agonists in cAMP production of human melanoma cells

The HA-induced cAMP modulation was previously reported [6,31]. In this study we investigated whether a potent and specific H_4R agonist could modulate cAMP production in human melanoma cell lines. In both cell types, HA produced a significant increase in cAMP levels at high doses (Fig. 3A and B). In contrast, VUF at any dose tested was unable to modify cAMP production or to reduce the forskolin-induced cAMP accumulation in WM35 cells (Fig. 3A). On the other hand, treatment of M1/15 cells with VUF (10 μM) produced a maximal inhibition of cAMP levels (approximately 34%) compared to basal values and treatment with this H_4R agonist partially reversed the increase in cAMP levels triggered by forskolin. The maximal inhibition of forskolin-induced cAMP accumulation was of 57% in this cell line (Fig. 3B).

3.1.3. Effect of histamine and H_4R agonists' treatment on ERK1/2 phosphorylation

ERK1/2 phosphorylation was assessed by western blot. Results indicate that the phosphorylated form of ERK1/2 was induced in both cell types when FBS was added to the culture medium and treatment with HA and with H_4R agonists resulted in an increase in ERK1/2 phosphorylation in a time-dependent manner in both cell lines (Fig. 4). The magnitude of ERK1/2 phosphorylation was similar for all compounds and showed the same time courses. HA,

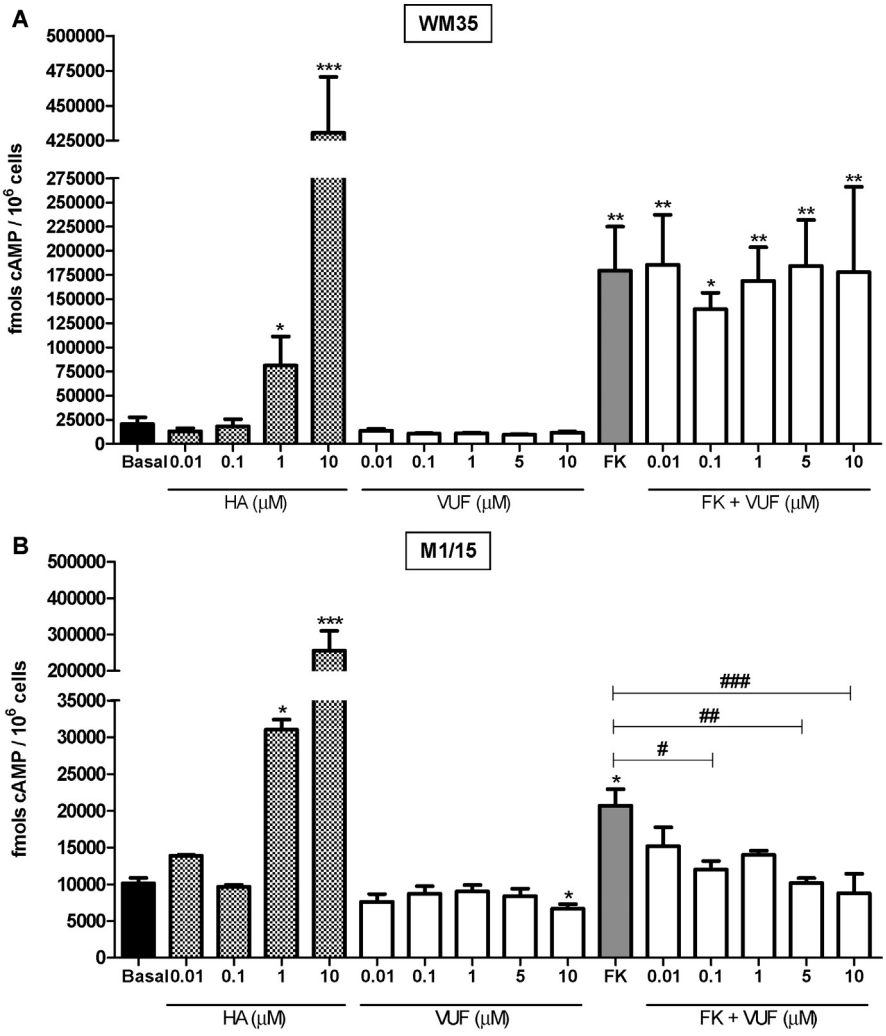

Fig. 3. Effect of histamine and VUF 8430 on cAMP production in human melanoma cells. cAMP levels in (A) WM35 and (B) M1/15 cells were determined after the treatment with histamine (0.01–10 μM) or VUF8430 (0.01–10 μM) for 15 min ($^{*}P < 0.05$, $^{***}P < 0.001$ *vs.* Basal; ANOVA and Dunnett's Multiple Comparison Test). Forskolin-induced cAMP accumulation in (A) WM35 and (B) M1/15 cells ($^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* Basal; ANOVA and Dunnett's Multiple Comparison Test). Cells were incubated with forskolin (10 μM) and/or with VUF8430 (0.01–10 μM) for 15 min ($^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* Basal; ANOVA and Dunnett's Multiple Comparison Test); ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* forskolin; ANOVA and Dunnett's Multiple Comparison Test). Results are expressed as fmol cAMP/10^6 cells. Data represent the means ± SEM.

VUF, and CLZ induced a sustained phosphorylation of ERK1/2 that peaked at 10–15 min and remained increased until 60 min (Fig. 4). WM35 cells showed phosphorylated ERK1/2 even in the absence of serum in culture medium. In order to evaluate the specificity of H_4R agonist treatments on the ability to induce ERK1/2 phosphorylation we employed an H_4R antagonist, JNJ77. Treatment with the H_4R antagonist reversed ERK1/2 phosphorylation for all agonists in the maximum point of phosphorylation (Fig. 4), indicating that the effect observed was mediated by the activation of H_4R.

3.2. In vivo *studies*

3.2.1. *Effect of histamine and clozapine on human melanoma tumors developed in nude mice*

Orthotopic xenografted tumors of the invasive human melanoma cell line M1/15 were developed in immune deficient nude mice.

HA and CLZ treatments significantly diminished the tumor volume starting at day 30th compared to control group, increasing the tumor volume doubling time (Fig. 5A and Table 2).

In addition, median survival (i.e. the time at which survival percent equals 50%) of animals treated with HA (61 days), or CLZ (61 days) was significantly higher than that of control mice (38 days), (Fig. 5B).

3.3. Ex vivo *studies*

3.3.1. *Immunohistological and histochemical characteristics of xenografted tumors*

Results indicate that tumors were highly undifferentiated, exhibiting marked anisokaryosis and anisocytosis and appreciable intracellular histamine levels. These characteristics were not significantly modified by HA or CLZ treatments. Non significant differences in the expression levels of H_4R were observed between control, HA and CLZ treated groups (Table 3), (Fig. 6).

Proliferation of tumor cells evaluated by PCNA expression and mitotic index were diminished in HA and CLZ treated mice. Also, intratumoral neovascularization was reduced upon HA and CLZ treatments. Vascularity showed large and medium size vessels predominantly present in intratumoral areas of tumors of control mice, while capillaries and medium size vessels were observed in HA or CLZ treated tumors (Fig. 6).

4. Discussion

In this work, we evaluated the signal transduction pathways triggered by the activation of H_4R in two human melanoma cells

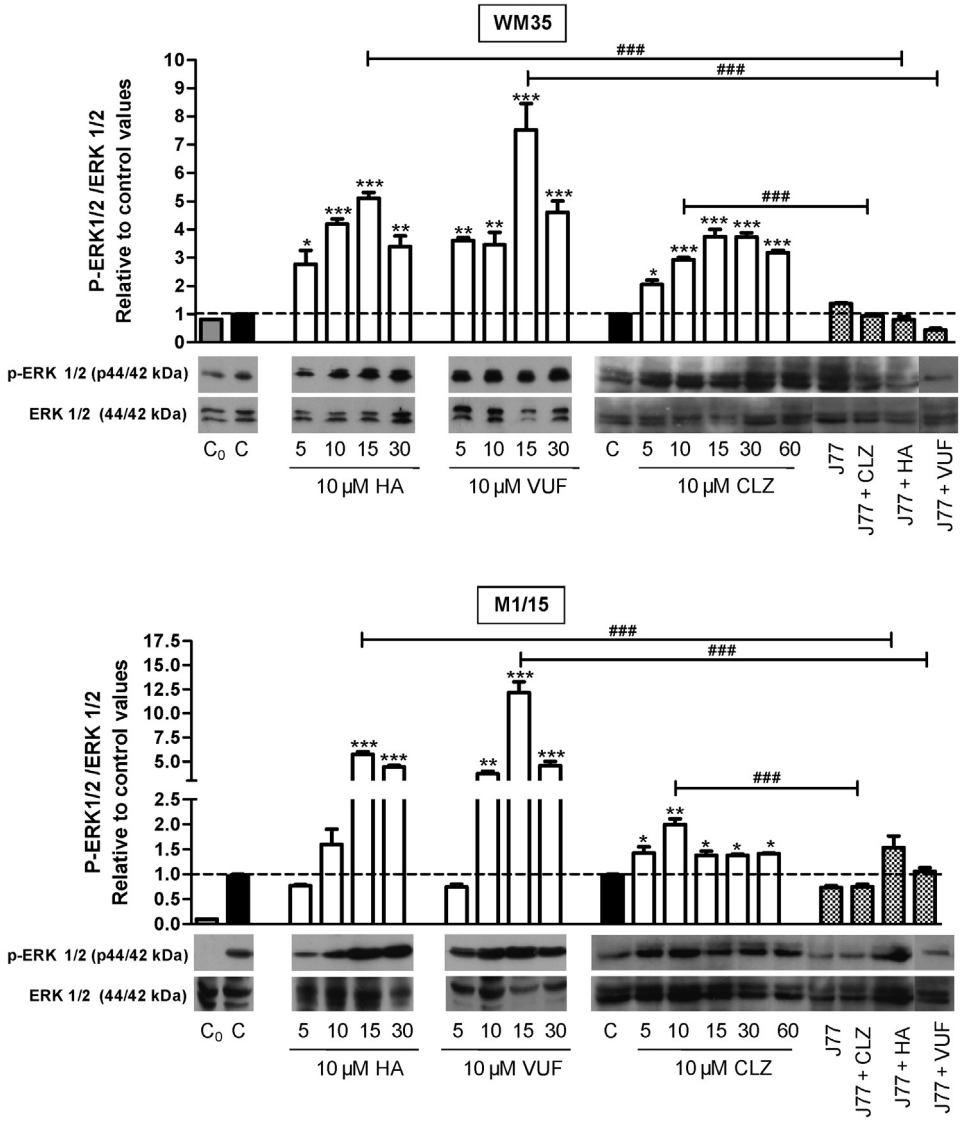

Fig. 4. ERK1/2 phosphorylation after histamine or H_4R agonists' treatment. Cells were plated, cultured for 24 h and serum-starved for an additional 24 h. Synchronized cells were left untreated (C) or treated with 10 μM histamine (HA), 10 μM VUF 8430 (VUF), for 5, 10, 15 or 30 min. C_0 represent cells in serum free culture condition. The same procedure was carried out in order to evaluate clozapine (CLZ) treatment on ERK1/2 phosphorylation. Cells were left untreated (C) or treated for 5, 10, 15, 30 or 60 min. JNJ77, cells treated with 10 μM of JNJ7777120 for 15 min; JNJ77 + CLZ, cells treated with 10 μM of JNJ7777120 for 15 min and 10 μM of clozapine for 10 min; JNJ77 + HA, cells treated with 10 μM of JNJ7777120 and 10 μM of histamine for 15 min, JNJ77 + VUF, cells treated with 10 μM of JNJ7777120 and 10 μM of VUF 8430 for 15 min. JNJ7777120, reversed the phosphorylation triggered by CLZ, HA and VUF. Cell lysates (30 μg of protein) were subjected to SDS-PAGE and probed with specific antibodies against p-ERK1/2. Blots were stripped and reprobed with an antibody against total ERK1/2 to confirm equal protein loading and for normalization ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* Basal; ANOVA and Dunnett's Multiple Comparison Test); ($^{\#\#\#}P < 0.001$ CLZ *vs.* JNJ77 + CLZ, $^{\#\#\#}P < 0.001$ HA *vs.* JNJ77 + HA, $^{\#\#\#}P < 0.001$ VUF *vs.* JNJ77 + VUF; ANOVA and Newman–Keuls Multiple Comparison Test).

lines, as well as the *in vivo* antitumoral activity displayed by HA or CLZ, on M1/15 human melanoma xenografts.

Among agonists, the atypical neuroleptic CLZ has been shown to fully activate the H_4R, although it displays a moderate (sub-micromolar) affinity [16,22–25]. In SK-N-MC/hH_4, CLZ binds with moderate potency to the hH_4R and exerts full agonistic activity at the hH_4R. The antipsychotic effects are thought to be mediated principally by 5-$HT_{2A/2C}$ and dopamine receptor blockade [32,33]. As it is well known, neurons (i.e. dopaminergic) and melanocytes share the same ectodermal origin and are both able to produce melanin. However, it was reported that human melanoma cells did not express dopamine D1 and D2 receptors [34,35] or 5-$HT_{2A/2C}$ receptors [36]. Several reports support the idea to use CLZ as H_4R agonist *in vitro* and *in vivo* [16,22–25,37–40]. Anyway, in order to evaluate CLZ behavior as an H_4R agonist and in order to investigate some functional characteristics of the H_4R in these studies, we employed pharmacological tools such as the high affinity H_4R agonist VUF 8430, and JNJ77 which shows more than a thousand fold selective over other histamine receptor subtypes and is considered an H_4R reference antagonist [41].

We first investigated *in vitro* the role of CLZ on cell proliferation. Our findings showed that CLZ treatment markedly decreased proliferation of both WM35 and M1/15 cell lines. The inhibitory effect on proliferation induced by CLZ was blocked by the combined treatment with the specific H_4R antagonist JNJ77, confirming that CLZ behaves as an H_4R agonist in this model and showing the same potency of other H_4R agonists evaluated previously in these cell lines [21]. Accordingly, we have previously reported that the reduction in the amount of H_4R protein expression by H_4R siRNA transfection completely prevented the

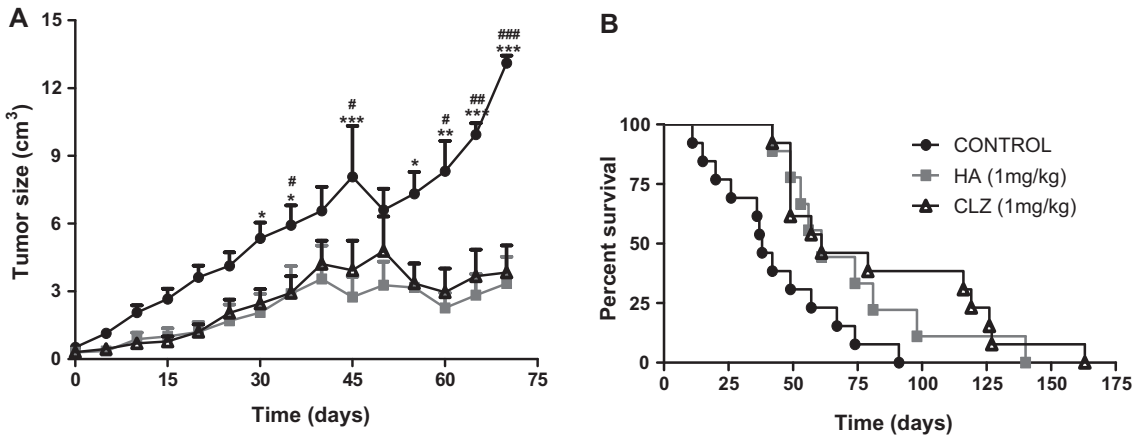

Fig. 5. Antitumor effect of histamine and clozapine in M1/15 xenografted mice. (A) Histamine and clozapine treatment significantly diminished the tumor growth volume starting at day 30th *vs.* control group ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for HA *vs.* control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ CLZ *vs.* control group, two way ANOVA and Bonferroni post test). (B) Time of spontaneous death of mice was determined. Kaplan–Meier survival curves were plotted and median survival was calculated [$P < 0.05$ HA (61 days) *vs.* control (38 days), and $P < 0.01$ CLZ (61 days) *vs.* control (38 days), Log-rank (Mantel–Cox) test].

decreased in proliferation triggered by histamine, VUF and clobenpropit, reinforcing the role of H_4R in proliferation in melanoma cell lines with different malignant capacity [21].

The decrease on proliferation produced by the H_4R agonists was associated to an accumulation of cells in the G_0/G_1 phase of the cell cycle. These results were in accordance with previous reports in which activation of the H_4R resulted in an accumulation of pancreatic carcinoma Panc-1 cells and MDA-MB-231 breast cancer cells in G_0/G_1 phase of the cell cycle [43] and also mediated cell cycle arrest in growth factor-induced murine and human hematopoietic progenitor cells [44].

Senescence is a biological program of terminal growth arrest characterized by disruption of lysosomal function through enhanced activity of senescence associated β-galactosidase [42].

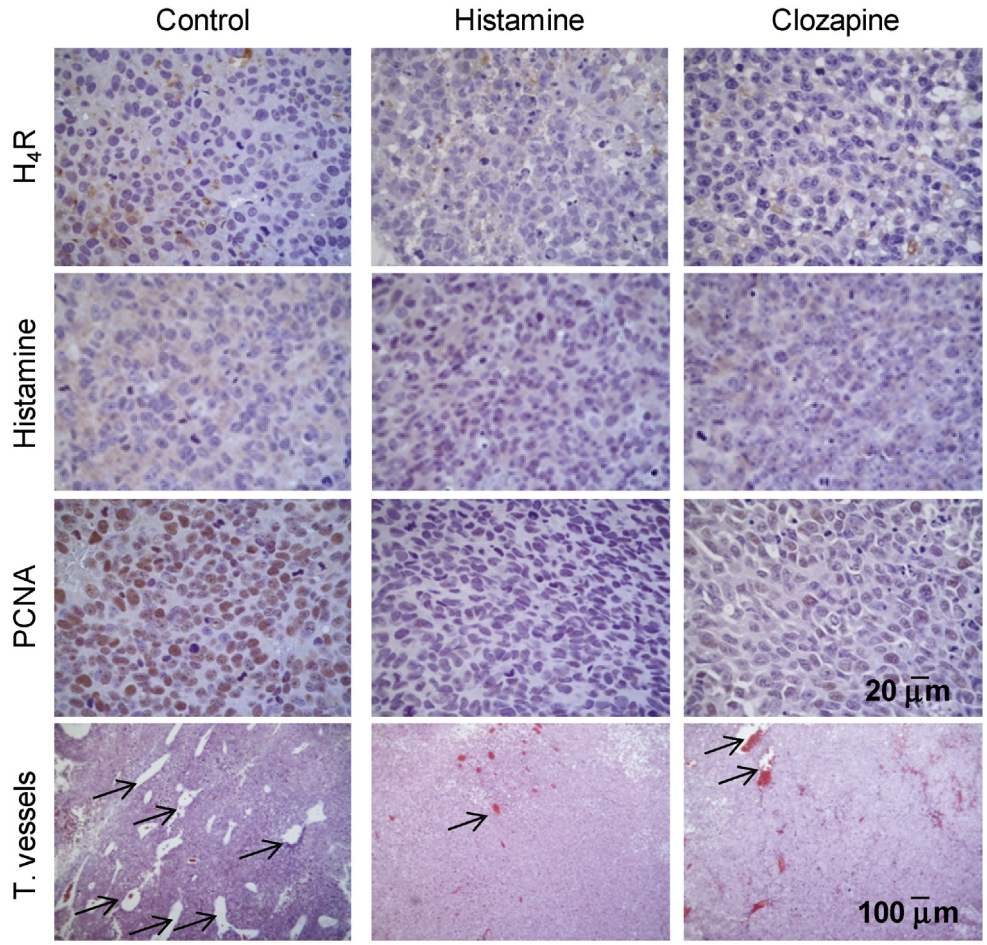

Fig. 6. Immunohistochemistry of M1/15 xenografted mice. Formalin fixed paraffin embedded tissue sections of control, histamine (HA) and clozapine (CLZ) mice were stained against several antigens in order to evaluate histamine levels, H_4R expression, proliferation and vasculogenesis. Pictures were taken at a 630× fold magnification for immunostaining (Scale bar: 20 μm) and 50× fold magnification for tricromic staining (Scale bar: 100 μm).

Table 2
Histamine and clozapine treatment increased tumor doubling time.

	Control	Histamine	Clozapine
Tumor doubling time (days)	5.0 ± 1.1	12.8 ± 4.3*	13.8 ± 4.3*

Tumor doubling time was enlarged in mice treated with histamine or clozapine *vs.* control group. Results were expressed as mean ± SEM.
* $P < 0.05$ (unpaired *t*-test).

The negative effect on proliferation exerted by CLZ in WM35 and M1/15 cells was related to a 4-fold and a 3-fold increase in premature or accelerated cell senescence, respectively. CLZ mimicked histamine and others H_4R agonists effect observed previously, and also the induction of cell senescence was completely reversed by JNJ77 treatment, suggesting the involvement of H_4R in histamine-induced cell senescence [21]. As it was previously described in human melanoma cell lines, HA acting through the H_1R decreases cell proliferation, whereas it enhances growth when acting through the H_2R, meanwhile the stimulation of the H_3R did not show mitogenic signaling [5,6]. However, considering the data presented, the main receptor involved in the antitumoral effect of histamine on these melanoma cell lines seems to be the H_4R.

The H_4R has previously been shown to couple to multiple signaling pathways, dependent upon cell background. Besides, the regulation of receptor density at cell surface can strongly affect this receptor ability to functionally couple and to regulate different transduction mechanisms. Therefore, we further investigated the modulation of cAMP production in these cell lines. Results demonstrated that HA produced a significant increase in cAMP levels in both cell types at micromolar concentrations. In agreement with these results, it was previously reported that histamine and an H_2R agonist were able to increase cAMP production in human primary melanoma cells (WM35) and those responses were blocked by famotidine, indicating that they were exerted through H_2R (coupled to G_s protein). On the other hand, neither histamine nor H_1R or H_2R agonists were able to stimulate cAMP in a highly metastatic human melanoma cell line (HT168) [31]. In addition, VUF decreased the second messenger formation at high doses tested compared to basal levels and was able to reduce the cAMP accumulation induced by forskolin. The maximal inhibition of forskolin-induced cAMP accumulation in M1/15 cells was of 57%, indicating that in these cells the H_4R is coupled to the inhibition of adenylate cyclase. On the other hand, results showed that the H_4R agonist was unable to modify cAMP production or reduce the forskolin-induced cAMP accumulation in WM35 primary melanoma cells. In accord with our results, a similar maximal inhibitory response was previously reported for human H_4R expressed in HEK cells when treated with H_4R agonists [45] and for MCF-7 breast cancer cells [28], and also in other studies a direct coupling of the H_4R to inhibition of adenylate cyclase could not be detected [28,46] or was found to be weak [15].

Table 3
Histopathology and immunohistochemistry of M1/15 xenografted tumor into nude mice.

	Control	Histamine	Clozapine
H_4R[a]	15.7 ± 6.9	43.3 ± 10.8	16.7 ± 9.6
HA[a]	28.9 ± 5.9	23.3 ± 3.3	14.3 ± 3.7
PCNA[b]	89.0 ± 3.0	32.0 ± 12.0***	63.0 ± 12.0*
Mitotic Index[c]	17.0 ± 1.0	9.0 ± 3.0**	10.0 ± 2.0*
No. of tumoral vessels[d]	40.0 ± 5.0	16.0 ± 3.0**	18.0 ± 4.0**

[a] Percentage of stained cells.
[b] Percentage of positive nuclei.
[c] Number of cells with visible chromosomes at 400× magnification in 10 random fields.
[d] Vessels inside the tumor at 200× magnification in 10 random fields. Results were expressed as mean ± SEM.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$ (unpaired *t*-test).

In addition, stimulation of H_4R led to activation of MAPK *via* pertussis toxin-sensitive pathway [40]. Depending on the duration, the magnitude and its subcellular localization, ERK activation controls various cell responses, such as proliferation, migration, differentiation, senescence and death [19,47,48]. Sustained cytoplasmic ERK activity might promote senescence or autophagy, whereas sustained nuclear sequestration of ERK activity might trigger apoptosis [19]. The ERK pathway exerts a critical role in melanoma, with ERK being constitutively activated in up to 90% of melanomas [20]. We additionally investigated this signaling pathway in human melanoma cells by western blot. Treatment with histamine and H_4R agonists induced a sustained phosphorylation of ERK1/2, which was reversed when cells were co-treated with the H_4R antagonist, indicating that the effect observed was mediated through the activation of the H_4R. Besides, phosphorylated ERK1/2 persisted up to 48 h after treatments preferentially in the cytoplasmic protein fraction (data not shown). In agreement with these results, time-dependent increase in phosphorylated ERK levels were observed in U2OS-H_4 transfected cells (stably expressing the H_4R) after treatment with a maximal concentration (100 μM) of HA for between 0 and 60 min [49]. These observations might correlate with our previous reports in which activation of H_4R induces cell senescence and differentiation [21].

In addition we evaluated the *in vivo* effect of H_4R ligands on a human melanoma experimental orthotopic model, which was developed by subcutaneous injection of M1/15 cells into athymic male nude mice. Mice receiving HA or CLZ showed an increased median survival. Accordingly, HA and CLZ treatments significantly diminished tumor growth, and augmented tumor volume doubling time in comparison to the control group. These results are in agreement with recent data obtained with the xenografted tumors of the highly invasive and metastatic human melanoma cell line 1205Lu developed into immune deficient nude mice. In this experimental model, treatment with JNJ28610244 (H_4R agonist), HA and CLZ (1 mg kg^{-1}, *sc*) decreased tumor growth and reduced nodular, spleen and lung metastases compared to control group (data not shown). Accordingly, in xenografts tumors of the highly invasive human breast cancer cell line MDA-MB-231 model, the H_4R was the major histamine receptor expressed and *in vivo* treatment with CLZ or HA significantly decreased tumor growth and HA also enhanced survival of bearing tumor mice [50]. On the other hand, numerous *in vivo* studies employing animal models with melanoma grafts demonstrated that both endogenous and exogenous HA have the ability to stimulate tumor growth while H_2R antagonists (e.g. cimetidine, famotidine, and roxatidine) inhibited this effect [1,7,8]. It was also reported in murine models that melanoma tumor growth was not modulated *in vivo* by histamine treatment, while terfenadine, an H_1R antagonist, induced *in vitro* melanoma cell death by apoptosis and *in vivo* significant inhibition of tumor growth [10]. The ability to produce and degrade HA could explain the different sensitivities of melanoma cell types to exogenous HA treatment [51]. In agreement with our results, it was demonstrated the clinical benefit of histamine (Ceplene) as an adjuvant to immunotherapy with IL-2 in several phase II and III clinical trials in metastatic melanoma [52].

Ex vivo studies show that appreciable intracellular HA content and H_4R protein expression levels were observed in melanoma tumors and non significant differences upon treatments were observed. In addition, tumoral PCNA expression and mitotic index were diminished in treated animals. The mitotic index is an important prognostic factor predicting both overall survival and

response to chemotherapy in most types of cancer cells. Analysis of the American Joint Committee on Cancer melanoma staging database demonstrated a significant inverse correlation between primary tumor mitotic rate and survival. Among the independent predictors of melanoma-specific survival, mitotic rate was the strongest prognostic factor after tumor thickness [53]. Interestingly, an increased p-ERK1/2 staining was observed in tumors of HA and CLZ-treated mice (data not shown).

Furthermore, HA and CLZ were able to reduce intratumoral neovascularization. Capillaries and medium size vessels were predominantly observed in HA or CLZ treated tumors while large and medium size vessels were observed in intratumoral areas of control tumors. In agreement with our observations, in an orthotopic rat glioma model study, treatment with histamine and IL-2 in combination significantly reduced the density of tumor microvessels. Besides, mean vessel area, perimeter and diameter all decreased significantly in the HA group [54]. Anyway, the molecular mechanisms behind the decreased angiogenesis remain unclear and further studies are needed.

As it is well known CLZ treatment has a wide range of adverse effects. CLZ-induced agranulocytosis is a potentially life-threatening pharmacological adverse drug reaction, which limits the application of this drug [55]. This side effect was evaluated by microscopic observation of bone marrow and necropsy of most important organs (lung, kidneys, liver, etc.) of mice treated with these H_4R agonists. No aberrant effects in population elements of bone marrow or other side toxic effects in organs were observed (data not shown).

On the basis of the presented evidence, we conclude that HA and CLZ exhibit an antitumoral effect *in vitro* and *in vivo* on human melanoma, suggesting the therapeutic potential of these compounds for the treatment of malignant melanoma and further clinical trials are warranted.

Acknowledgements

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