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

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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

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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