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**Article** *in* Inflammation & Allergy - Drug Targets (Formerly ?Current Drug Targets - Inflammation & Allergy) · July 2010

DOI: 10.2174/187152810792231869



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# Histamine and Histamine Receptor Antagonists in Cancer Biology

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**Abstract:** Histamine has been demonstrated to be involved in cell proliferation, embryonic development, and tumour growth. These various biological effects are mediated through the activation of specific histamine receptors (H1, H2, H3, and H4) that differ in their tissue expression patterns and functions. Although many *in vitro* and *in vivo* studies of the modulatory roles of histamine in tumour development and metastasis have been reported, the effect of histamine in the progression of some types of tumours remains controversial; however, recent findings on the role of histamine in the immune system have shed new light on this question. This review focuses on the recent advances in understanding the roles of histamine and its receptors in tumour biology. We report our recent observations of the anti-tumoural effect of H1 histamine antagonists on experimental and human melanomas. We have found that in spite of exogenous histamine stimulated human melanoma cell proliferation, clonogenic ability and migration activity in a dose-dependent manner, the melanoma tumour growth was not modulated by *in vivo* histamine treatment. On the contrary, terfenadine-treatment *in vitro* induced melanoma cell death by apoptosis and *in vivo* terfenadine treatment significantly inhibited tumour growth in murine models. These observations increase our understanding of cancer biology and may inspire novel anticancer therapeutic strategies.

**Keywords:** Antihistamines, cancer, histamine, histamine receptors, melanoma.

## 1. INTRODUCTION: HISTAMINE AND ITS RECEPTORS

### 1.1. Synthesis, Storage, and Metabolism of Histamine

Histamine, a vasoactive amine, was isolated from the mould ergot by Dale and Laidlaw almost 100 years ago [1, 2]. It is located in most body tissues but is highly concentrated in the lungs, skin, and gastrointestinal tract. Histamine is synthesized from decarboxylation of L-histidine by the enzyme L-histidine decarboxylase (HDC), the only enzyme in mammalian tissue that catalyses histamine formation [3]; this enzymatic reaction takes place in the Golgi apparatus of mast cells and basophils [4]. Once it is synthesized, histamine is stored in mast cells and basophils, where it is localised primarily in the cytosol and detained in the intracellular granules in a complex with an acidic protein and macroheparin [5]. Platelets [6], lymphocytes [7], condrocytes [8], neurons [9], gastric enterochromaffin-like cells [10], and even tumour cells [11] also serve as cellular sources of histamine. Histamine is involved in a wide range of physiological and pathological processes, including allergic skin reactions, septic inflammation, various gastrointestinal events, and neurotransmission [12].

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Approximately 3-8 pg histamine/cell is found in mast cells isolated from human lung, skin, lymphoid tissue, and the small intestine; this histamine is released to the circulation upon cell activation. Released histamine is rapidly metabolised by oxidative deamination to imidazoleacetic acid (ImAA) or by methylation of the imidazole ring to methylhistamine, which subsequently is deaminated and oxidized to tele-methylimidazoleacetic acid (MeImAA). Approximately 70% of all histamine produced in humans is metabolised by methylation. Histamine degradation takes place mainly in the liver and the kidneys, but in the gastrointestinal mucosa and possibly in the vascular endothelium. The metabolites are directly excreted in the urine. It is possible to determine *in vivo* histamine turnover simply by measuring the urinary excretion of histamine and its metabolite MeImAA, which has no other known precursors [13, 14].

### 1.2. Histamine Receptors

The effects of histamine on target cells are mediated by specific surface receptors of the "histamine receptor" G-protein family. These receptors are divided into the H1, H2, H3, and H4 subtypes, which were first identified on the basis of their clinical pharmacological properties, and later characterised in terms of their DNA sequences [15, 16]. Recently, another class of intracellular histamine binding sites has been discovered in the nucleus; these sites are known as Hic (intracellular Histamine receptors) [17].

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**H1 histamine receptors** are expressed in a wide variety of tissues including mammalian brain, bronchial smooth muscles, gastrointestinal cells, genitourinary cells, cardiovascular cells, the adrenal medulla, endothelial cells, and lymphocytes [18].

The H1 receptor was cloned in 1991 from bovine adrenal medulla by expression cloning in the *Xenopus* oocyte system [19]. Based on the deduced amino acid sequence, the protein consists of 491 amino acids with a calculated molecular weight of 56 kD. The protein has the seven putative transmembrane domains characteristic of a G-protein coupled receptor and possesses N-terminal glycosylation sites. Strikingly, the proposed structure has a very large third intracellular loop (212 amino acids) and a relatively short (17 amino acids) intracellular C terminal tail. The availability of the bovine sequence and its lack of introns enabled cloning of the H1-receptor from several species, including rat, guinea pig, mouse, and human [20]. The human histamine H1 receptor gene has now been localized to chromosome 3p14-p21 [21].

The primary mechanism by which histamine H1 receptors produce functional cellular responses is the activation of phospholipase C (PLC) *via* the G protein Gq/11 [18, 22]. Once activated, PLC breaks down phosphoinositol-2-phosphate ($PIP_2$) to inositol-3-phosphate ($IP_3$) and diacylglycerol (DAG). $IP_3$ acts on specific receptors in the membrane of the endoplasmic reticulum (ER) to mobilise $Ca^{2+}$ from ER stores, activating certain proteins involved in cellular proliferation and cell survival [20, 23]. However, in some tissues, histamine can stimulate inositol phospholipid hydrolysis independently of H1 receptors. For example, in the longitudinal smooth muscle of the guinea pig ileum and the neonatal rat brain, a histamine-responsive component was identified that was resistant to inhibition by H1-receptor antagonists [20].

In addition to the major signalling pathway through PLC, H1 receptor activation may link several other signalling pathways, many of which appear to be secondary to changes in the intracellular calcium concentration or in the activation of protein kinase C. Thus, histamine can stimulate nitric oxide synthesis activity (*via* a $Ca^{2+}$/calmodulin-dependent pathway) and subsequent activation of soluble guanylyl cyclase in a variety of cell types [24, 25]. Arachidonic acid release and the synthesis of arachidonic acid metabolites such as prostacyclin and thromboxane A2 can also be enhanced by H1-receptor stimulation [19].

**H2 histamine receptors** are also G-protein coupled receptors, with expression occurring mainly on the cell surface of tissues such as the brain, the stomach, and the heart. The most important function of H2 receptors is the production of hydrochloric acid in the stomach, but smooth muscle relaxation has also been documented in airway, uterus, and vascular tissue. Finally, histamine H2 receptors can inhibit a variety of immune functions; there is increasing evidence that H2 receptors on lymphocytes can inhibit antibody synthesis, T-cell proliferation, cell-mediated cytolysis, and cytokine production [19, 21, 26].

The H2 receptor was first cloned through amplification of a partial-length H2 receptor sequence from canine gastric parietal cDNA using degenerate oligonucleotide primers [27]. Rapid cloning of the rat, human, guinea pig, and mouse H2 receptors followed [28-30]. These DNA sequences encode a 359 (canine, human, guinea pig) or 358 (rat) amino acid protein with the general characteristics of a G-protein coupled receptor. The most significant differences between the structure of the cloned H2 and H1 receptors are the much shorter third intracellular loop of the H2 receptor and the longer H2 receptor C-terminus. Recent chromosomal mapping studies have assigned the H2 receptor gene to human chromosome 5 [31].

Signal transduction by H2 receptors couples to adenylyl cyclase *via* the GTP-binding protein Gs. Histamine is a potent stimulant of cyclic adenosine monophosphate (cAMP) accumulation in many cell types, particularly cells of central nervous system origin [21]. Thus, H2 receptor–mediated effects on cAMP accumulation have been observed in brain tissue, gastric mucosa, fat cells, cardiac myocytes, vascular smooth muscle, basophils, and neutrophils [19]. Although the H2 receptor was traditionally thought to couple to the adenylate cyclase pathway, recent work with the cloned receptor indicates that it can also activate the phosphoinositide signalling cascade through an independent G protein-coupled receptor mechanism [32]. The novel observation that histamine may stimulate c-*fos* gene expression lends further support to the possible role of this receptor in the regulation of cell growth and differentiation in gastric parietal cells [33].

**H3 histamine receptors** were discovered by Arrang *et al* in 1983 [34]. This histamine receptor subtype is expressed on the cells of the nervous system, mainly in the brain. H3 receptors regulate the synthesis and release of histamine as well as other important neurotransmitters such as acetylcholine, noradrenaline, dopamine, serotonin, and γ-aminobutyric acid (GABA). The H3 receptor is coupled to the Gi/o G-proteins and negatively regulates adenylyl cyclase function, leading to decreased cAMP levels and resulting in reduction of PKA activation and CREB-induced gene expression [35]. It has been also shown that H3 receptor stimulation can lead to PLA2 activation and arachidonic acid release [36]. On the other hand, H3 receptor can activate the mitogen-activated protein kinase (MAPK) and PI3K pathways [37].

The H3 histamine receptor gene was cloned in 1999 by Lovenberg *et al.* [38] and the human gene has been mapped to chromosome 20. Using the polymerase chain reaction, six alternative splice variants of the H3 receptor were cloned from human thalamus; these variants were coexpressed in the human brain, but their relative distributions varied in a region-specific manner. The existence of multiple H3 isoforms with different signal transduction capabilities suggests that H3-mediated biological functions may be tightly regulated through alternative splicing mechanisms [39, 40].

The **H4 histamine receptor** subtype was first identified by Oda *et al.* [41], and the H4 receptor gene was mapped through radiation hybrid experiments to human chromosome 18q11.2 [42]. The H4 receptor is highly expressed in bone marrow and peripheral hematopoietic cells, including eosinophils [43], dendritic cells and natural killer (NK) cells [44], T cells [45, 46], B cells [47], basophils, and mast cells [48]. Moreover, it is also expressed in fibroblasts [49],

endocrine cells in the gastrointestinal tract [50], and in the central nervous system [51].

Similar to the H3 histamine receptor, the H4 receptor is linked to the Gi/o protein to inhibit adenylate cyclase activity, suppressing the production of cAMP. The H4 receptor induces the migration and accumulation of inflammatory cells at sites of inflammation by up-regulation of adhesion molecules such as Mac-1 and intracellular adhesion molecule-1 (ICAM-1) [43, 52].

## 2. ANTIHISTAMINES

### 2.1. H1 Histamine Receptor Antagonists

Bovet and Staub discovered the first H1 receptor antagonist (thymol ether 929F) in 1937, marking the first generation of antihistamines to treat allergic diseases [53]. Although antagonists have been used in a widespread manner, major central nervous system adverse effects such as sedation and performance deficits, as well as anticholinergic activities, pushed scientists to search for a new generation of antihistamines with fewer side effects. The second generation of antihistamines, developed in the late 1980s, showed fewer sedating side effects [54].

H1 histamine antagonists are among the most widely used drugs in the world. They are indicated for the treatment of allergic rhinitis, allergic conjunctivitis, allergic dermatological conditions (contact dermatitis), urticaria, angioedema, pruritus (atopic dermatitis, insect bites), anaphylactic reactions, nausea, vomiting, and insomnia. H1 histamine antagonists also provide symptomatic relief for motion sickness and the common cold [54]. Antihistamines suppress the wheal-and-flare reaction by blocking the binding of histamine to its receptors on nerves, vascular smooth muscle, glandular cells, endothelium, and mast cells, effectively exerting competitive antagonism of histamine for H1 receptors. They have also been used in the treatment of certain types of headaches, Crohn's disease, acute multiple sclerosis, and some stomach secretary conditions. Antihistamines can be applied locally or systemically, according to the nature of the allergic condition. However, topical administration is no longer recommended due to the high incidence of sensitisation reported in the literature [55]. Antihistamines are absorbed from the gut and metabolised primarily in the liver, and their effect lasts for approximately four to six hours (first-generation antihistamines).

First-generation H1 antihistamines (for example, dexchlorpheniramine and hydroxyzine) block the effects of naturally occurring histamine in the body. The most common side effect is sedation. Other common side effects are dizziness, tinnitus, blurred vision, euphoria, uncoordination, anxiety, insomnia, tremor, constipation, dry mouth, and dry cough. These H1 antihistamines can also cause urinary retention, palpitation, hypotension, headache, hallucination, and psychosis [54, 55], and therefore should be administered cautiously in patients with glaucoma or prostate enlargement.

The second generation of H1 antihistamines are new selective drugs for peripheral H1 receptors rather than central nervous system histaminergic receptors. This selectivity is related to the inability to pass the blood-brain barrier easily, and reduces the occurrence of most side effects. This group of anithistimaines includes alkylamines (acrivastine, piperidines (astemizole, loratadine, mizolastine, terfenadine), piperazine (cetirizine) and miscellaneous (azelastine, emedastine, and epinastine) groups [55].

Terfenadine and astemizol are almost free from central nervous system side effects such as sedation and drowsiness because these molecules cannot pass the blood-brain barrier. Nevertheless, they may prolong the QT interval in electrocardiograms, possibly causing ventricular arrhythmia such as ventricular tachycardia. These compounds are metabolised *via* the cytochrome system, a system known to be involved in drug metabolism in humans. Concurrent administration of drugs that use the same hepatic metabolic route (ketoconazol, erithromycin) may reduce the degradation rate for terfenadine and astemizol, increasing plasma concentrations [56]. It is not yet clear yet whether these drugs are harmful during pregnancy and lactation [54].

Terfenadine and astemizol were withdrawn from the market in many countries due to potential cardiotoxicity. However, fexofenadine, the active metabolite of terfenadine, is now in clinical use as it is free from serious side effects as a result of minimal metabolism. Fexofenadine and other new agents (desloratadine and levocetirizine) belong to the third generation of H1 antagonists.

In addition to its antihistamine activity, terfenadine has been shown to block voltage-dependent ion channels and to reverse drug resistance in a variety of cell types *via* its interaction with P glycoprotein [57]. Terfenadine, ebastine, and carebastine compounds (which possess a similar chemical structure) inhibited the secretion of the Th2-type cytokines IL-4 and IL-5 in T helper cells and inhibited the lymphocyte proliferative response; however, production of Th1-type cytokines IL-2 and IFN-γ was unaffected [58]. These additional pharmacological properties are independent of the action of the compounds on the H1 receptor [59-61].

### 2.2. H2 Histamine Receptor Antagonists

H2 histamine receptor antagonists are a group of drugs used to block histamine action on target cells such as parietal cells in the stomach, leading to decreased acid production by these cells. These drugs, including cimetidine, ranitidine, famotidine and nizatidine, are therefore used to treat dyspepsia and peptic ulcer.

Like the H1 antagonists, H2 antagonists are inverse agonists rather than true receptor antagonists. The H1 and H2 receptors engage in agonist-independent signal transduction, and are constitutively active even in the absence of histamine. Therefore, it is believed that the H1 and H2 antihistamines inhibit this constitutive signal and stabilise the receptors' inactive configuration, acting as inverse agonists and not as antagonists [62, 63].

H2 antagonists are competitive inhibitors of histamine at the parietal cell H2 receptor, suppressing constitutive and meal-stimulated secretion of acid by parietal cells. Histamine released in the stomach is blocked from binding to parietal cell H2 receptors, which stimulate acid secretion. Other substances that promote acid secretion (such as gastrin and acetylcholine) have a reduced effect on parietal cells when

the H2 receptors are blocked. While H2 antagonists are generally well tolerated, adverse drug reactions may occur, including hypotension, headache, tiredness, dizziness, confusion, diarrhoea, constipation, rash, and gynaecomastia.

### 2.3. H3 and H4 Histamine Receptor Antagonists

H3 and H4 histamine receptor antagonists are still considered experimental agents and are not in clinical use. Initially the imidazole ring was considered essential for receptor affinity, and the first reported antagonists contain this aromatic heterocyclic core [64]. Compounds like thioperamide, ciproxifan, and clobenpropit are now mainly used as reference structures; the basic ring causes potential problematic issues (risk of cytochrome P450 interactions and low levels of brain penetration), possibly excluding them from development as therapeutic agents. H3 receptor antagonists are potential therapeutics for the treatment of various central nervous system disorders accompanied by histaminergic dysregulation; non-imidazole H3 receptor inverse agonists with drug-like properties (tiprolisant, ABT-239, GSK189254, and JNJ-10181457) showed distinct pharmacological profiles in preclinical animal models and clinical studies in narcoleptic and epileptic patients, implicating these H3 receptor ligands in the treatment of sleep-wake disorders, dementia, epilepsy, schizophrenia, depression, and pain [65].

Bayer Healthcare AG has recently patented aminopyrimidines as H4 receptor antagonists. Since this disclosure, other companies have claimed distinct pyrimidines as H4 receptor ligands. The H4 receptor antagonist A-943931, developed by Abbott Laboratories, is reported to exhibit antagonistic activities *in vitro* and *in vivo* across multiple species, while also possessing excellent metabolic stability and good oral bioavailability. A-943931 is a good anti-inflammatory agent in mice and also displays good efficacy in rat pain models. Further exploration of aminopyrimidines resulted in the recently described H4 receptor antagonist A-987306, a potent and selective compound with an excellent pharmacokinetic profile. A-987306 revealed anti-inflammatory activity in a peritonitis model and was efficacious as an analgesic in a murine pain model [66].

## 3. HISTAMINE AND ANTIHISTAMINES IN CANCER

Numerous studies have explored the effects of histamine and its receptor antagonists on cancer cell proliferation [67-73]. For example, histamine has been reported to act as a mitogen in human carcinoma (HeLa and A431), melanoma, and astrocytoma cells [74]. On the other hand, other studies have indicated that histamine inhibits cell proliferation in human pancreatic and melanoma cell lines [75, 76]. These results suggest that the effect of exogenous histamine on *in vitro* tumour growth seems to vary according to the specific tumour cell line and the drug concentration. Indeed, histamine reduction of viability or enhancement of cell proliferation was dose-dependent in tumour breast cells [77]. To confirm these observations, we have studied the effect of histamine on the viability of four melanoma cell lines. We observed that histamine concentrations of 100 nM to 100 µM significantly increased the proliferation rate in three human melanoma cell lines (A375, HT144, and Hs294T) ($p<0.05$), but did not modulate the proliferation of B16F10 mouse melanoma cells (Fig. **1**). Moreover, no cytotoxic effect was observed at any dose of histamine.

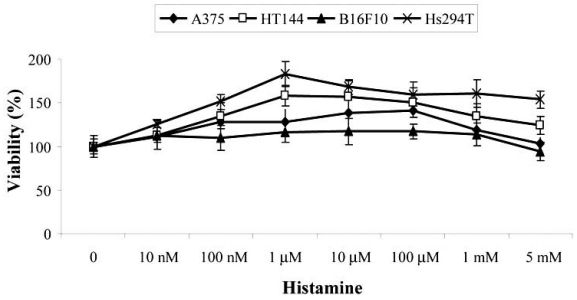


**Fig. (1).** Effect of histamine on cell viability of melanoma cells. Cells ($10 \times 10^4$) were seeded in DMEM culture medium and incubated overnight to promote cell adherence to the surface of 96-well microplates. Cells were treated with histamine (Sigma Chemical Co., St Louis, MO, USA) for 48 hours, and viability was estimated *via* metabolic activity using the XTT assay (Roche Molecular Biochemicals, Indianapolis, IN, USA). Histamine concentrations between 100 nM and 100 µM significantly increased the proliferation rates of the three human melanoma cell lines (A375, HT144, and Hs294T) ($p<0.05$). However, histamine did not modulate the proliferation of B16F10 mouse melanoma cell line. The level of statistical significance between sample means was determined using the Student t-test; $P<0.05$ was considered to be statistically significant. Data are presented as percentage of untreated control cells ± the standard deviation of at least three independent experiments performed in quadruplicate.

The autocrine pro-tumoural effect of histamine has been established through studies that highlight a direct relationship between HDC activity and tumour growth [67, 78-81]. An analysis of colorectal carcinomas revealed that higher histamine content and HDC activity in tumours correlated with the presence of lymph and/or distant metastasis [82]. Although the quantification of protein expression in tissues specimens is complicated, we have also observed differential HDC expression between melanoma samples obtained from patients with good prognoses and tumours from patients that developed metastasis during follow-up. Melanocytes from benign and atypical nevi express HDC (Fig. **2a**, **b**), but higher expression levels were detected in biopsies from patients who developed metastasis during the two first years of follow-up (Fig. **2e**, **f**) compared with melanoma samples from patients who remained disease-free over ten years (Fig. **2c**, **d**).

According with our results, B16F10 experimental melanoma cells were modified to produce different histamine concentrations (HDC up-regulated, un-modified, or down-regulated). These cells grafted in syngenic mice to produce skin primary tumours and lung metastases, leading to an increase of tumour growth and lung metastatic colonies in the case of local histamine production increased. In addition, the authors looked for molecular progression markers affected by melanoma histamine secretion and found a positive correlation between histamine production, tumour histamine H2 receptor and rho-C-expression [83]. These results suggest that histamine may be involved in the metastatic progression of melanoma.

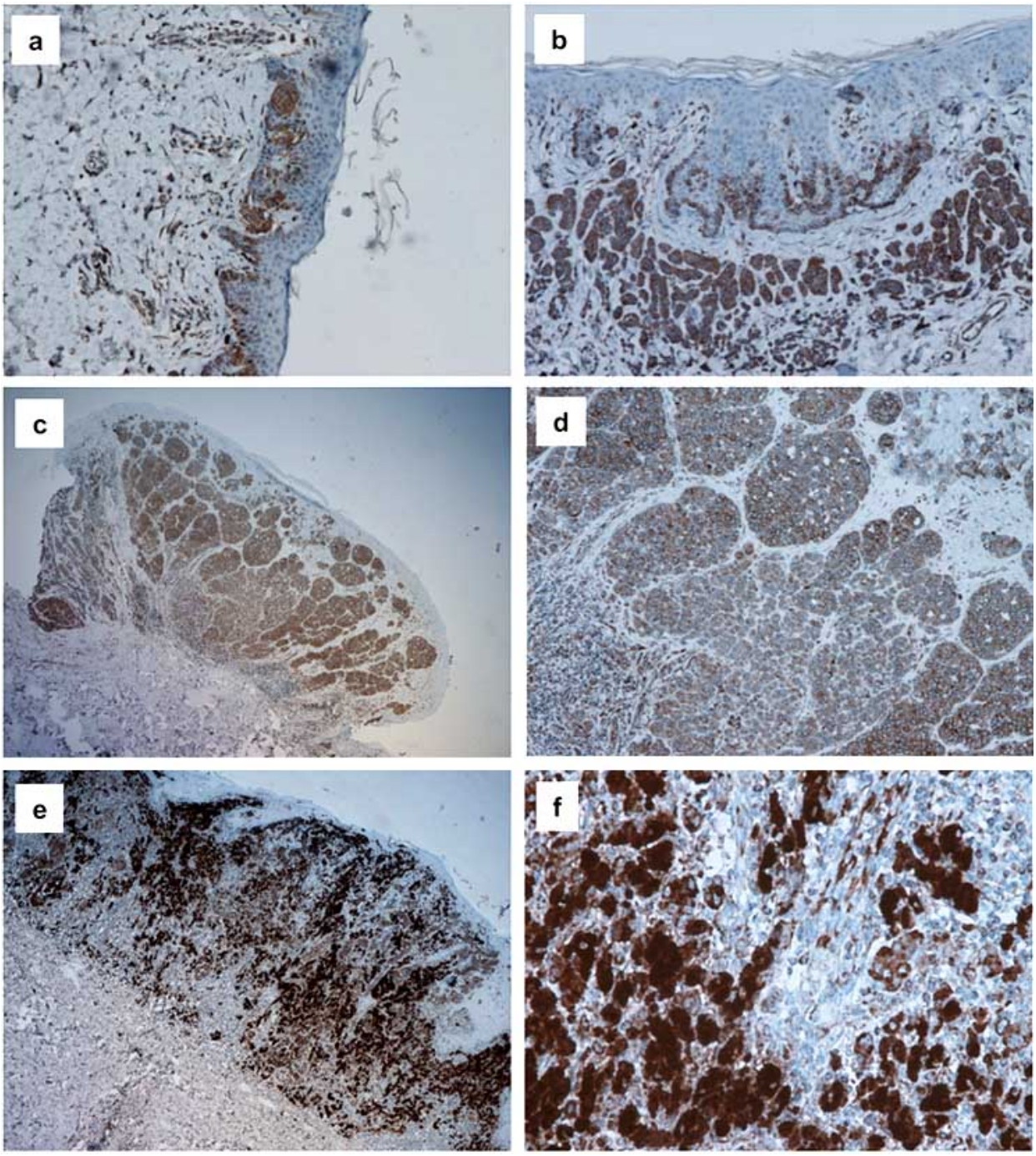


**Fig. (2).** HDC expression in tissue sections of melanocytic junctional nevus (**a**), dysplastic nevus (**b**), good-prognosis primary melanoma (**c**, **d**), and bad-prognosis primary melanoma (**e**, **f**). Tissue samples were probed with an antibody to HDC and counterstained with hematoxylin. Tissue specimens were obtained from five patients with junctional nevus, five patients with dysplastic nevus, and ten patients with a clinical history of melanoma (stage II as classification of the American Joint Committee of Cancer, AJCC) who had not undergone systemic treatment for their disease before or after surgical excision of the lesions. During the follow-up period, five melanoma patients remained disease-free over at least ten years (good prognosis), while five patients developed metastasis during the first two years of follow-up (bad prognosis). All tissue samples were obtained in compliance with the approved clinical and institutional review board protocols. Patient consent for experiments was not required because the materials were obtained from the pathology archive. Standard immunohistochemistry was performed as described [81], using a mouse mAb to HDC (Biosource International, Camarillo, CA, USA). Acquisition of images was performed by an ProgRes CT3 (Jenoptik) camera connected to an Nikon microscope. Images were white balanced such that the average background colour in each image is the same. Scale bar = 50 μm.

**Table 1. Effect of Histamine on the Clonogenic Capacity of Melanoma Cells**

| Histamine | A375 | HT144 | Hs294T | B16F10 |
|---|---|---|---|---|
| **0** | 18.5 ± 4.5 | 17.5 ± 4.5 | 6.0 ± 0.5 | 32.1 ± 6.3 |
| **100 µM** | 168.5 ± 11.5* | 173.0 ± 18.0* | 136.5 ± 14.5* | 40.5 ± 8.12 |
| **100 nM** | 208.5 ± 15.5* | 77.0 ± 14.0* | 197.0 ± 40.0* | 36.5 ± 20.2 |

Cells were seeded at limiting dilutions in DMEM culture medium and incubated overnight to promote the adherence of cells to the 24-well macroplates. The cells were exposed to the indicated concentrations of histamine for seven days, after which the cultures were washed with phosphate-buffered saline and the cells were fixed in 70% ethanol and stained with crystal violet. Experiments were repeated three times in triplicate. Colonies >50 cells were counted under microscope. Data are presented as the mean ± standard deviation of the number of colonies of three independent experiments. *Significant differences as determined by Student's t-test ($p < 0.001$).

Independent of the autocrine growth loop, higher levels of endogenous histamine may be released to the extracellular medium and interact with membrane histamine receptors, triggering a biological response through the regulation of different signal transduction pathways. Therefore, we wished to investigate the effect of histamine in malignant melanoma progression by studying the effect of histamine on clonogenic and migration activity in three human melanoma cell lines. We found that the presence of histamine in the culture medium significantly increased the clonogenic ability of the three human melanoma cell lines, but did not modulate the number of colonies in B16F10 experimental melanoma cells (Table **1**). In addition, the migration capacity of experimental and human melanoma cells increased significantly only in the presence of 100 µM histamine (Fig. **3**). From these results we deduce that, under tumour-specific conditions, the presence of histamine in the microenvironment could modulate cellular properties involved in cancer progression. In this context, histamine has recently been implicated in the modification of the invasive phenotype in MDA-MB-231 breast cancer cells by decreasing cell adhesion and by altering the balance between MMP-9 and TIMP-2 [84]. Histamine has also been shown to modulate cell survival and invasiveness in the human pancreatic adenocarcinoma cell line PANC-1 [85, 86].

On the other hand, histamine may indirectly affect tumour growth through its effect on the immune system. Histamine inhibits the formation of reactive oxygen species in monocytes *via* histamine H2 receptors, protecting NK and T cells from oxidative damage [87]. Histamine optimises cytokine-induced activation of T cells and NK cells; therefore, the addition of histamine to IL-2 therapy improves responses rates and disease-free survival, especially in metastatic melanoma and acute myeloid leukaemia [88]. However, less promising results have been reported in renal cell carcinomas [89]. Furthermore, histamine may also disrupt the balance among Th1, Th2, and Treg lymphocytes in neoplastic tissues [83]. Accordingly, systemic histamine treatment decreased Th-2-secreted IL-10 in murine colorectal tumour implants, resulting in tumour growth [90, 91]. Conversely, histamine was observed to induce an imbalance of antioxidant enzymes, resulting in the elevation of intracellular hydrogen peroxide levels and leading to the inhibition of WM35 human melanoma cell proliferation [92]. These discrepancies suggest that the selective or controversial effect of histamine is dependent on cell type, histamine concentration and histamine receptor expression on cell surfaces.

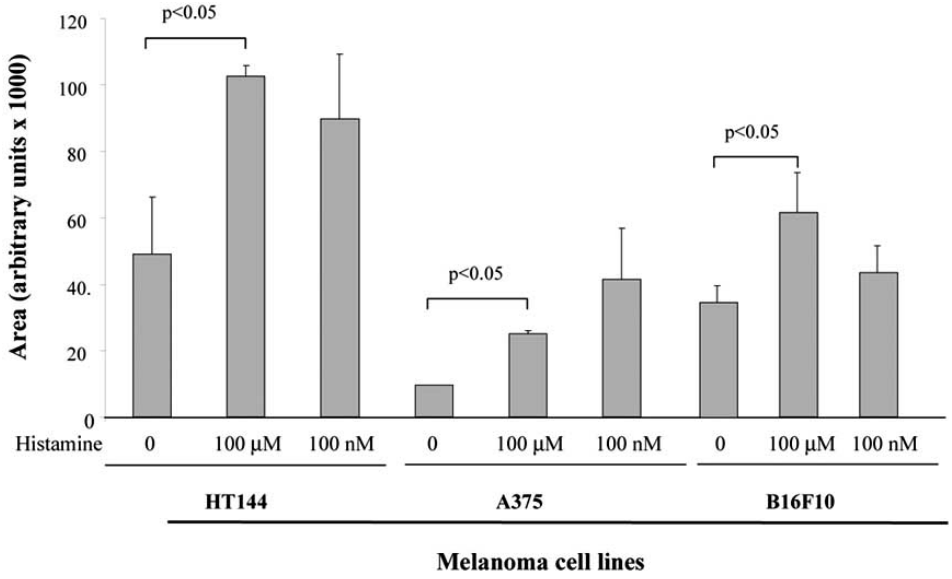


**Fig. (3).** Effect of histamine on migration ability of melanoma cells. Cells ($5x10^3$) were seeded in the upper compartment of transwell cell culture chambers (Falcon Cell Culture Inserts, Becton-Dickinson Laboratories, Orangeburg, NY, USA) in 24-well macroplates and cultured in 1% FCS medium with 100 µM or 100 nM histamine. After 48 hours, melanoma cells migrated to the filter (8 µm diameter pores) and adhered to the lower surface of the 24-well macroplate. Cells were fixed in 70% ethanol and stained with crystal violet. Areas from stained cells were measured using a Nykon-microscope AZ 100 with digital camera Sight DS-5Mg. Positive areas for crystal violet were analysed using Image J software. The level of statistical significance between sample means was determined using the Student t-test; $P<0.05$ was considered to be statistically significant. Values are shown as the cell-stained areas in arbitrary units ± standard deviations of at least three independent experiments performed in quadruplicate.

The effect of histamine on tumour growth depends on the expression of the functional histamine receptors directly on the surface of tumour cells or in tumour-surrounding cells. Hence, histamine antagonists could also regulate cell proliferation. For example, in hepatocellular carcinoma cells, some doses of histamine induced proliferation mediated by the H2 receptor, while other concentrations decreased proliferation through the H1 receptor [93]. In both cases these effects were reversed by treatment with the H2 antagonist ranitidine and the H1 antagonist terfenadine, respectively. Furthermore, histamine stimulated proliferative and pro-angiogenic effects through the H2 and H4 receptors in colon cancer cells [80]. In patients with colorectal cancer, preoperative treatment with the antagonist famotidine (40 mg/day) for one week before resection increased lymphocytic inflammation in the tumours, decreased recurrences, and augmented the survival of the patient group [94], suggesting a potential role for antihistamines in cancer therapy.

In recent years we have been very interested in the anti-tumoural effects of H1 histamine receptor antagonists. We demonstrated that diphenhydramine (DPH), an H1 histamine receptor antagonist, induced Bcl-2-dependent apoptosis mainly through the mitochondrial pathway in two acute lymphoblastic leukaemia cell lines (CEM and Yurkat) [95]. We extended this study to human melanoma cell lines, finding that four H1 histamine receptor antagonists (DPH, triprolidine, astemizol, and terfenadine) induced apoptosis in four human melanoma cell lines but not in normal melanocytes or mouse embryonic fibroblasts. We found that the corresponding process of apoptosis is related with DNA damage and caspase-2 activation and proceeds *via* the

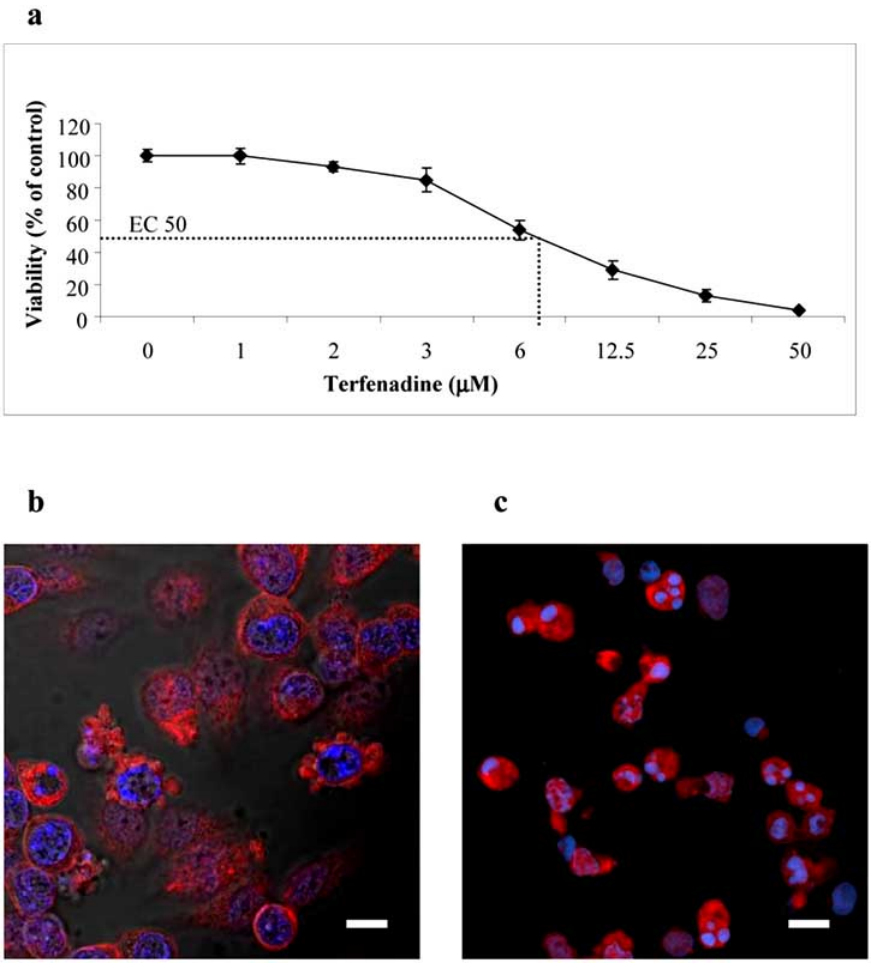


**Fig. (4).** Cytotoxic effect of terfenadine on melanoma cells. Cells (10 x $10^4$) were seeded in DMEM culture medium and incubated overnight to promote adherence to the surface of 96-well microplates. Cells were treated with terfenadine (Sigma) for 24 hours and viability was estimated *via* metabolic activity using the XTT assay. (**a**) Terfenadine induced a dose-dependent cytotoxic effect on all melanoma cell lines. Values are shown as percentage of untreated control cells ± standard deviation of at least three independent experiments performed in quadruplicate. Non-linear regression analysis was performed using GraphPad Prism version 3 for Windows (GraphPad Software, San Diego, CA, USA) to calculate the $IC_{50}$ for tested products. Morphological changes were characteristic of apoptosis induced by terfenadine in A375 human melanoma cells. Cells were treated with 10 μM terfenadine for 18 hours, fixed with 70% ethanol, and incubated with (**b**) anti-actin antibody (Abcam, Cambridge, UK) or (**c**) caspase-3 rabbit polyclonal antibody (Cell Signalling Technology, Danvers, MA, USA). Both samples were counterstained with DAPI (Sigma) for observation of the nucleus. Images show typical cellular changes associated with apoptosis: cell shrinkage and rounding, blebbing, and nuclear condensation and fragmentation in response to treatment. Bar = 20 μm.

mitochondrial pathway [96]. Moreover, apoptosis induced by terfenadine was independent of the H1 receptor signalling pathway in the A375 melanoma cell line, since knocking down the H1 receptor with small interfering RNAs did not protect melanoma cells from terfenadine-induced apoptosis [97]. We discovered that terfenadine induced DNA damage and apoptosis through the modulation of $Ca^{2+}$ homeostasis and tyrosine kinase activity [96]. In agreement with our results, several studies have recently demonstrated that most second-generation H1 receptor antagonists exhibit additional pharmacological properties independent of their H1 receptor functions [98].

Finally, accumulated data suggest a potential use of histamine receptor antagonists in the treatment of cancer. H1 and H2 receptor antagonists have exhibited anti-tumoural effects on leukaemia, myeloma, mantle cell lymphoma, colon cancer, melanoma, and gastric cancer through apoptosis induction [99-101].

In this context we have evaluated the anti-melanoma activity of terfenadine in a mouse model of melanoma xenografts using the B16F10 mouse experimental cell line. Similar to the case in human melanomas, *in vitro* terfenadine treatment also decreased proliferation and induced apoptosis in a dose-dependent manner (Fig. **4**). B16F10 cells were subcutaneously inoculated in the syngenic C57BL/6 mice, and the mice were subdivided in groups of ten to receive treatment: histamine (5 mg/mouse/day), terfenadine (5 mg/kg/day), and the corresponding solvent solutions (PBS and DMSO respectively) by intraperitoneal injection. Tumour size was monitored during the experiment and tumour weights were determined after sacrifice. Importantly, the administration of terfenadine dramatically reduced the growth of melanoma tumours in mice; however, no significant differences were detected when C57BL/6 mice were treated with histamine (Fig. **5**). Moreover, terfenadine did not appear to induce toxicity, as the body weight (data not shown) and the appearance of mice were not different from controls.

To validate the anti-tumoural activity of terfenadine, A375 human melanoma cells were inoculated in nude mice and the mice were treated with terfenadine using osmotic pumps to dispense the drug (Fig. **6**). In this assay terfenadine also demonstrated anti-tumoural activity when compared with the control group, indicating that terfenadine has a strong anti-melanoma activity *in vivo.*

In summary, we have not found that *in vivo* histamine treatment modulates melanoma tumour growth; however, terfenadine treatment significantly inhibited tumour growth in murine models. In addition, *in vitro* terfenadine treatment induced melanoma cell death by apoptosis, independent of

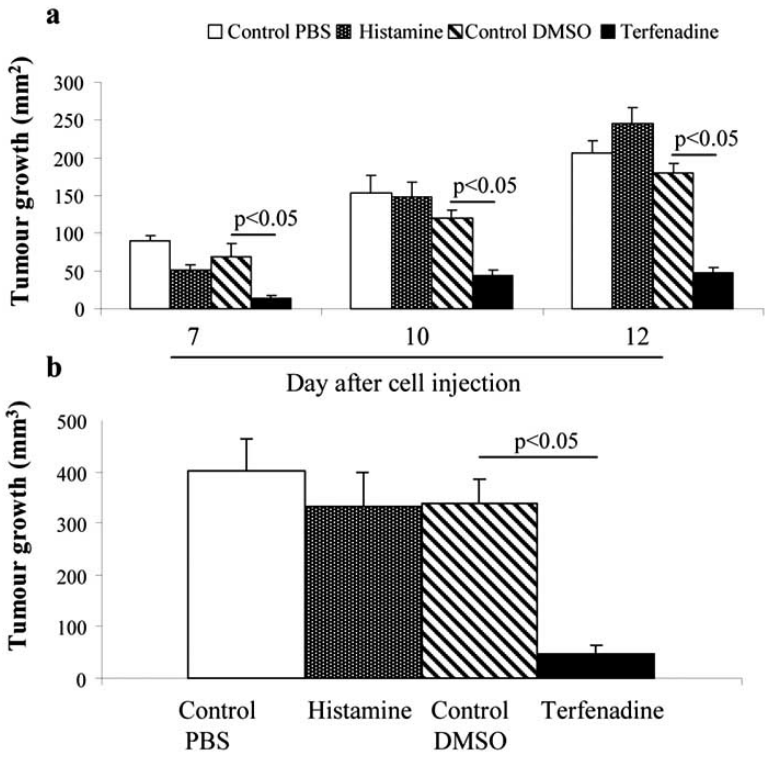


**Fig. (5).** Effect of terfenadine on B16F10 melanomas in mice. Mice were inoculated subcutaneously with B16F10 melanoma cells (5 x $10^5$), and after five days animals (n = 10 in each group) were treated with terfenadine (5 mg/kg/day), histamine (5 mg/day), or the solvents PBS and DMSO as controls over 13 days. This experiment was repeated twice and similar results were obtained; data from one trial appear in the figure. (**a**) Tumour growth curves were determined by measuring length x width ($mm^2$) tumours using a calliper. (**b**) Mice were sacrificed and tumours were weighed. Values are expressed as $mm^3$ (length x width x weigh). All mice developed tumours and additional growth was observed in the untreated control groups throughout the study. Animals treated with terfenadine exhibited lower tumoural growth rates than controls. The level of statistical significance between sample means was determined using the Student t-test; $P<0.05$ was considered to be statistically significant.

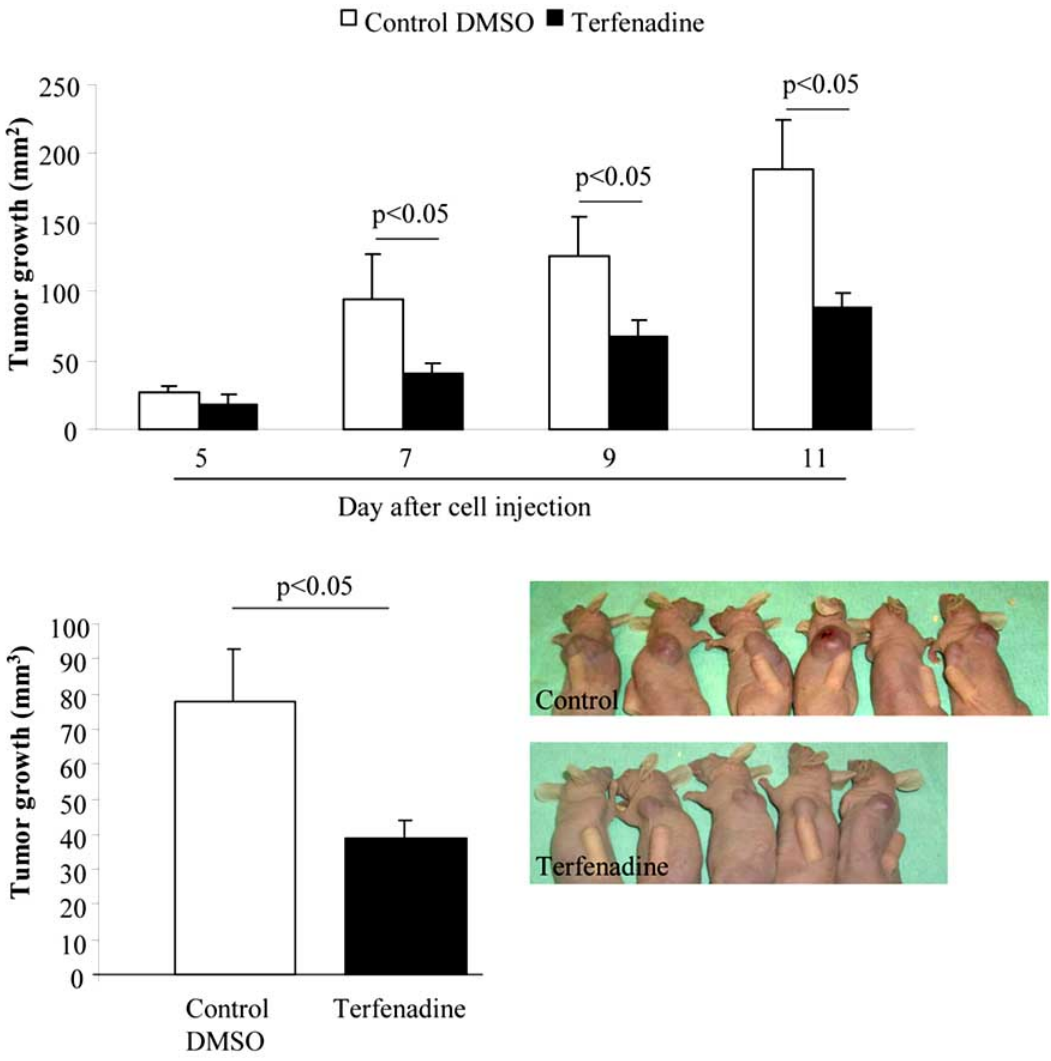


**Fig. (6).** Effect of terfenadine on A375 human melanoma growth in nude mice. Mice were operated on to introduce subcutaneous osmotic pumps (ALZET, Durect Corporation, Cupertino, CA, USA) delivering terfenadine (5mg/Kg/day) or DMSO after A375 human melanoma cells (5 x $10^5$) were inoculated close to the osmotic pumps. (**a**) Tumour sizes were determined by measuring length x width ($mm^2$) using a calliper. (**b**) Mice were sacrificed and tumours were weighed. Values are expressed in $mm^3$ (length x width x weigh). All mice developed clinically palpable tumours, and more tumour growth was observed in the untreated control group throughout the study. Animals treated with terfenadine showed significantly smaller tumours that weighed less than control mice. These data clearly demonstrate that terfenadine has anti-melanoma activity *in vivo*. The level of statistical significance between sample means was determined using the Student t-test; P<0.05 was considered to be statistically significant.

H1 histamine receptor expression. These data suggest that it will be critical to initiate detailed molecular and preclinical studies with terfenadine and other H1 histamine receptor antagonists to evaluate their potential as new anti-melanoma drugs, a disease that currently possesses few treatment options.

## ACKNOWLEDGEMENTS

This study was supported by grants from the Heath Department of the Government of the Basque Country (Exp: 2006111036) and the University of the Basque Country (GIC-IT-423-07). Microscopic photographs were taken in the General Service of Analytical and High Resolution Microscopy of the University of the Basque Country. Animal experimentation was performed in the General Service of Animal House of the University of the Basque Country.

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**Received: June 28, 2010** **Revised: June 29, 2010** **Accepted: June 29, 2010**