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ORIGINAL ARTICLE

In Vitro Physiological Effects of Betahistine on Cell Lines of Various Origins

Betahistinin Farklı Orijinlere Sahip Hücreler Üzerindeki *İn Vitro* Fizyolojik Etkileri

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ABSTRACT

Objectives: Betahistine is a histamine analog commonly prescribed for symptomatic treatment of vertiginous symptoms. *In vitro* studies have shown that betahistine was not toxic at the prescribed doses in a nasal epithelial cell line. However, the effect of betahistine on other cell types has not been studied. In this study, we aimed to investigate some of the physiological effects of betahistine on L929 fibroblast, A549 lung cancer, human umbilical vein endothelial (HUVEC), and Ishikawa endometrial cell lines.

Materials and Methods: Cellular proliferation was assed assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, apoptosis was evaluated by acridine orange-ethidium bromide staining, and cellular migration was assed assessed by scratch assay.

Results: Betahistine treatment (0.1-0.5 mg/mL, 24 hours) can inhibit cell proliferation and induce apoptosis in HUVEC, A549, Ishikawa, and L929 cell lines. Betahistine (≥0.1 mg/mL) significantly increased the number of apoptotic cells (HUVEC: 26.3%, A549: 17.3%, L929: 8.6%, and Ishikawa: 2.3%). Betahistine at doses over 0.1 mg/mL significantly suppressed the cell migration rate in all of the cell lines. In contrast, exposure to a low dose of betahistine (0.025 mg/mL) induced migration rates of HUVEC and Ishikawa cells by 81% and 48%, respectively.

Conclusion: Betahistine may alter the processes of cellular proliferation, apoptosis, and cellular migration in a cell line- and dose-dependent manner. In this sense, proliferative and metastatic properties of certain cancer cells can potentially be altered in response to betahistine treatment.

Key words: Betahistine, cellular-migration, apoptotsis, proliferation, cancer

ÖZ

Amaç: Betahistin vertigo semptomlarının semptomatik tedavisi amacıyla sıklıkla reçete edilen bir histamin analoğudur. *İn vitro* çalışmalar, betahistinin reçete edilen dozlarda nazal epiteliyal hücreler üzerinde toksik etkisi olmadığını göstermiştir. Ancak, betahistinin diğer hücre hatlarındaki üzerindeki etkileri hakkında çalışma bulunmamaktadır. Bu çalışmada, betahistinin L929 fibroblast, A549 akciğer kanseri, insan umbilikal ven endoteliyal (HUVEC) ve Ishikawa endometriyal hücre hatları üzerindeki bazı fizyolojik etkilerini araştırmayı amaçladık.

Gereç ve Yöntemler: Hücre proliferasyonu 3-(4,5-dimetiltiyazol-2-yl)-2,5-difeniltetrazolyum-bromür yöntemiyle, apoptoz akridin turuncusu-etidyum ikili boyama yöntemiyle ve hücre göçü hızı çizik deneyi ile araştırılmıştır.

Bulgular: Betahistin uygulamasının (0,1-0,5 mg/mL, 24 saat), HUVEC, A549, Ishikawa ve L929 hücre hatlarında hücre proliferasyonunu baskılayabileceği ve apoptozu indükleyebileceği belirlenmiştir. Betahistin'nin (≥0,1 mg/mL) apoptotik hücrelerin sayısında önemli bir artışa neden olmuştur (HUVEC: %26,3, A549: %17,3, L929: %8,6 ve Ishikawa: %2,3). Betahistin 0,1 mg/mL dozun üzerinde tüm hücre hatlarında hücre göçü hızını önemli derecede baskılamıştır. Buna karşılık, düşük dozlarda betahistin uygulaması (0,025 mg/mL) HUVEC ve Ishikawa hücre hatlarında hücre göçü hızını sırasıyla %81 ve %48 oranlarında artırmıştır.

Sonuç: Betahistinin hücre proliferasyonu, apoptoz ve hücre göçü hızını hücre tipi ve doza bağımlı şekilde etkileyebilir. Bu kapsamda, betahistin uygulamasına yanıt olarak bazı kanser hücrelerinin proliferatif ve metastatik özellikleri de potansiyel olarak değişebilir.

Anahtar kelimeler: Betahistin, hücre göçü, apoptoz, proliferasyon, kanser

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INTRODUCTION

Betahistine is a histamine analog used for symptomatic treatment of vertiginous symptoms related to Ménière's disease.[1] It is considered to exert its effects partially by acting as an antagonist of H3 receptors. Betahistine can enhance the release of histamine in the central nervous system.[2] Histamine is a neuromodulatory transmitter that regulates important cerebral activities, including vestibular functions.[3] Additionally, betahistine is reported to improve the microcirculation of the inner ear.

In vitro cell culture models are valuable tools for biocompatibility and drug toxicity studies prior to the use of animal models.[4] For example, the commercially available nasal epithelial cell line RPMI 2650 has been widely studied in drug toxicology tests.[5] There is a limited number of reports on the possible physiological effects of betahistine in cell culture models. Pilicheva et al.[6] showed that betahistine was not toxic at prescribed doses in a nasal epithelial cell line. Toxicity was observed only at very high concentrations (>50 mg/mL), which are not achievable under *in vivo* conditions. However, systemic administration of betahistine can have effects on many other cell types. Although *in vitro* cell culture tests suggest that use of betahistine is safe for nasal epithelial cells, the possible effect of this drug on other cell types, such as cancer, endothelial, or fibroblast cell lines, has not been extensively studied. Therefore, we aimed to investigate the effect of betahistine on L929 fibroblast, A549 lung cancer, human umbilical vein endothelial (HUVEC) endothelial, and Ishikawa endometrial cell lines. For this purpose, viability, the changes in the ratio of apoptotic cells, and cell migration rates were compared.

MATERIALS AND METHODS

Cell culture and chemicals

HUVEC, human Asian endometrial adenocarcinoma (Ishikawa), pulmonary adenocarcinoma human alveolar epithelial (A549), and murine fibroblast (L929) cell lines were cultured in high-glucose Dulbecco's Modified Eagle's medium (DMEM) (Sigma, 5546) supplemented with P/S (50 U/mL penicillin and 50 µg/mL streptomycin; Biological Industries, 03-031-1B), 1% 2 mM L-glutamine (Biological Industries, BI03-020-1B), and 10% FBS (Biowest, S1810-500). Cells, $1.5x10^6$ from each cell line, were seeded in 10 cm plates and split after 72 hours. Commercially available betahistine tablets (Betaserc®, Abbott Healthcare SAS, Châtillon-sur-Chalaronne, France) were ground (~100 mg) in a muller and then dissolved in a 100 mL volumetric flask containing 50 mL 0.1N HCl (pH: 1.2). The flasks were immersed in a water bath, maintained at 75°C for 2-3 min, and shaken until all the pellets were completely melted. The flasks were cooled for 1 hour (room temperature), and 0.1N HCl was added to bring the volume to 100 mL. The resulting suspensions were filtered through a 0.45 µm syringe filter.[7] Betahistine stock solutions (24 mg/mL) were kept at -20°C. A non-treated (NT) control group (vehicle) was prepared by diluting 8.28 mL of HCl (Sigma 320331) in 1000 mL DMEM.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

A 12 mM (5 mg/mL) stock solution of MTT (Neofrox 3580 MTT) was prepared as described by Mosmann.[8] Approximately 10^4 cells were seeded in each well of a 96-well plate in a volume of 100 µL. The MTT assay was carried out as follows: 10 µL from the 12 mM MTT stock solution was added to each well and incubated at 37°C for 4 hours (final concentration in incubation medium was 0.5 mg/mL). Medium alone (100 µL) was included as a negative control. After 4 hours of incubation with MTT, 75 µL of medium was removed from the wells, and then formazan crystals were dissolved with 50 µL of dimethyl sulfoxide by mixing thoroughly with a pipette. Following an additional incubation at 37°C for 10 minutes, the samples were mixed again briefly, and the absorbance at 540 nm was recorded.

Acridine orange/ethidium bromide (AO/EtBr) double staining

The AO/EtBr dual staining technique was performed as described by Liu et al.[9] Briefly, cells were seeded in a 96-well plate at a density of approximately 10^4 cells/well. Following incubation with betahistine for 48 hours, cells were trypsinized, and 10-25 µL cell suspensions were transferred onto glass slides. One microliter of AO/EtBr staining solution (a mixture of dyes containing 100 µg/mL AO and 100 µg/mL EtBr) was added to cell suspensions, and then the samples were covered with a coverslip. The cell morphology was examined under a fluorescent microscope (Carl-Zeiss/Axio observer 3., Zen 2.3 Blue Edition software) within 20 minutes after addition of the Ao/EtBr stain. For statistical analysis, at least 200 cells were counted, and the results were expressed as mean values obtained from at least three independent experiments. In the asay, both live and dead cells are stained with AO, while EtBr stains only dead cells that have lost membrane integrity. Live cells appear uniformly green, whereas early apoptotic cells show green dots in their nuclei. Late apoptotic cells stain orange and show condensed and/or often fragmented nuclei. Necrotic cells stain orange, with a nuclear morphology resembling that of viable cells, but without condensed chromatin.[9]

In vitro scratch assay

For the evaluation of cell migration rates, an *in vitro* scratch assay was carried out according to the protocol described by Liang et al.[10] Cell lines were seeded at $1x10^5$ cells/well into 6-well plates in growth medium overnight. Briefly, a scratch on the surface of the well was made with a 10 µL sterile pipette tip in 6-well plates. Following gentle washing (to remove the detached cells) with culture medium, photos of the scratch were taken at different time points (0-24 hours) under a microscope at 10x magnification (Carl-Zeiss/Axio observer 3). The gap size was analyzed using ImageJ software, and the rate of cell migration was calculated by comparing the cell-free areas of the scratches at 24-hour post-wounding and the area of the scratches at 0 hour. The percent changes in the migration rates were compared against the vehicle (NT control group). The results were expressed as means of triplicate experiments.

Statistical analysis

Statistical analysis was performed using GraphPad (Prism 5) software. Multiple comparisons were made using Tukey's procedure. $P<0.05$ was considered to indicate statistical significance. Analysis of variance was used for significant differences in the apoptotic index among groups.

RESULTS

Firstly, we investigated the effects of betahistine on cell viability by comparing the changes in proliferation rates of A549 (human pulmonary adenocarcinoma basal epithelial), HUVEC, Ishikawa (human endometrial adenocarcinoma), and L929 (murine fibroblast) cell lines by MTT assay. For this purpose, several doses (25, 50, 100, 250, and 500 µg/mL for 24 hours) were tested. Analysis of data obtained from proliferation assay studies showed that low levels of betahistine treatment (25 µg/mL, 24 hours) slightly induced proliferation rates in all of the cell lines tested (HUVEC by 109.8%, A549 by 107.7%, L929 by 116.2%, and Ishikawa by 153.5%) (Figure 1a-d). Betahistine (100 µg/mL) inhibited the proliferation rate by 35% in the endothelial HUVEC cell line at (Figure 1c), while A549, L929, and Ishikawa cell lines seemed to be more resistant to 100 µg/mL betahistine treatment (Figure 1a, b, d).

Betahistine treatment (250 µg/mL) resulted in a significant inhibition in A549 (56.86%), L929 (56.88%), and Ishikawa (43.21%), cell lines (Figure 1c). As seen in Figure 1c, HUVEC cell line seemed to be more resistant to 250 µg/mL betahistine when compared with the other cell lines (33.5% inhibition). On the other hand, 500 µg/mL betahistine treatment resulted in an almost 50% reduction in the proliferation rates of all of the cell lines tested.

Next, we compared the changes in the ratio of apoptotic cells using the "AO/EtBr" double staining protocol. For this purpose, the changes in the ratio of apoptotic and necrotic cells upon exposure to 25, 50, 100, 250, and 500 µg/mL (24 hours) of betahistine were evaluated. Our findings clearly suggested that lower doses of betahistine (25 µg/mL, 24 hours) did not significantly induce apoptosis in any of the cell lines tested (Figure 2a-d). However, as seen in Figure 2a-d, 100 µg/mL or higher concentrations of betahistine significantly increased the number of apoptotic and necrotic cells (HUVEC: Apoptotic cell 26.33%, A549: Apoptotic cells 17.33%, L929: Apoptotic cells 8.6%, and Ishikawa: Apoptotic cells 2.3%). L929 fibroblast and endothelial HUVEC cells were among the most sensitive cell lines (HUVEC: Apoptotic cells 26.33%, A549: 17.33% apoptotic cells), while endometrial Ishikawa cells seemed to be more resistant to betahistine (Ishikawa: 2.3% apoptotic cells) (Figure 2a-d). This result suggests that stromal and endothelial tissues may be at higher risk when betahistine is used at high concentrations. Representative microscopy images from AO/EtBr-stained samples are presented in Figure 3a-h.

Then, we investigated whether or not betahistine treatment could induce changes in cell migration rates. For this purpose, we tested the effect of 25, 50, 100, 250, and 500 µg/mL of betahistine (24 hours) treatment on cell migration rates in L929, A549, HUVEC, and Ishikawa cell lines using the *in vitro* scratch assay technique (Figure 4, 5). Our findings indicated that low-

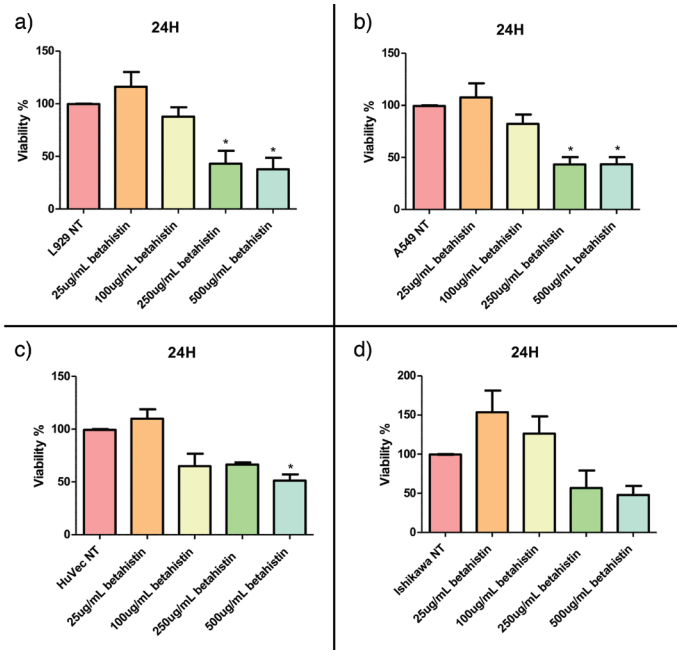

Figure 1. The effect of betahistine on cell proliferation. a) L929 fibroblast, b) A549 lung cancer, c) HUVEC endothelial, and d) Ishikawa endometrial cell lines were treated with 25-500 µg/mL betahistine for 24 hours in an incubator. MTT assays were performed 24 hours after treatment with the indicated doses of betahistine. Relative % changes in proliferation rates were compared against the vehicle (NT) and statistical significance was tested using One-Way ANOVA followed by Tukey's multiple comparison test

*: $p<0.05$, n=3, HUVEC: Human umbilical vein endothelial, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, NT: Non-treated

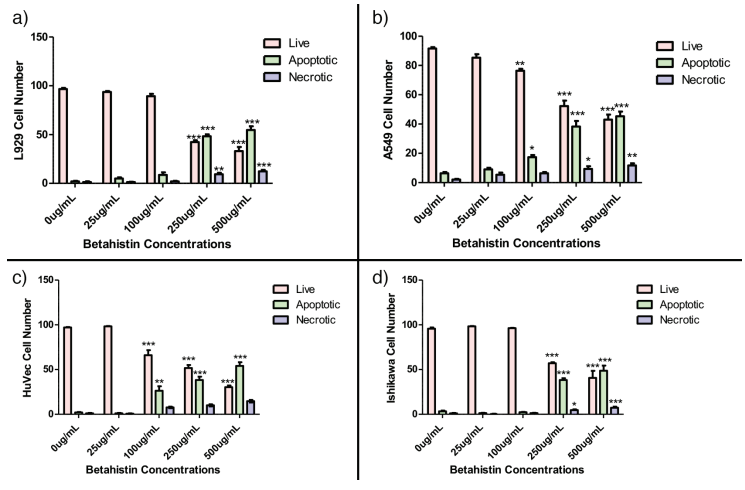

Figure 2. Betahistine induces apoptosis at high concentrations. a) L929 fibroblast, b) A549 lung cancer, c) HUVEC endothelial, and d) Ishikawa endometrial cell lines were treated with 25-500 µg/mL betahistine for 24 hours in an incubator. AO/EtBr double staining was performed 24 hours after treatment with the indicated doses of betahistine. Percentage changes in the ratio of apoptotic cells were compared against the vehicle (NT control) and statistical significance was tested using One-Way ANOVA followed by Tukey's multiple comparison test

*, **, ***: $p<0.05$, n=3, HUVEC: Human umbilical vein endothelial, AO/EtBr: Acridine orange/ethidium bromide, NT: Non-treated

dose betahistine treatment (25 µg/mL, 24 hours) induced cell migration rates in both HUVEC (by 81%) and Ishikawa cell lines (by 48%) (Figure 4c, d). In contrast, the cell migration rate was reduced by 67% in the L929 fibroblast cell line, while A549 cells did not seem to be affected by treatment with 25 µg/mL of betahistine for 24 hours (Figure 4a, b). However, as seen in Figure 4a-d, 100 µg/mL or higher doses (24 hours) of betahistine exposure significantly suppressed the cell migration rate in all of the cell lines tested. Representative microscopy images from scratch assay experiments are presented in Figure 5a-l.

DISCUSSION

Betahistine is a commonly prescribed drug for the treatment of vertiginous symptoms related to Ménière's disease.[11] Betahistine is a structural analog of histamine that acts as a weak partial postsynaptic histamine H1 receptor agonist and presynaptic H3 receptor antagonist, with no effect on postsynaptic H2 receptors.[12] The proposed mode of action of betahistine in Menière's disease involves increased blood flow to the inner ear, which in turn shifts the balance of production and re-absorption of endolymph toward absorption.[13] Indeed, Ihler et al.[14] demonstrated that betahistine exerted a dose-dependent effect on the increase in blood flow in cochlear capillaries in Guinea pigs.

In some clinical cases, increasing doses of betahistine are administered for relatively long periods of time up to a year.[15] Although long-term betahistine treatment at high doses is reported to be clinically safe,[16] very little is known about its *in vitro* cytotoxic effects. Betahistine, which has been in clinical use for over 40 years, has shown an excellent safety profile within the dose range of 8-48 mg daily.[17] Only a total of three cases of neoplasm have been reported in relation with the use of betahistine.[18] For example, although histamine is shown to be involved in the regulation of cancer-associated biological processes during cancer development,[19] no data are available for the histamine analog betahistine.

Only a single *in vitro* study suggested that betahistine was not toxic at prescribed doses, and toxicity was observed only at extremely high concentrations (>50 mg/mL) in a nasal epithelial cell line.[6] However, systemic administration of betahistine may also affect other cell types. For example, the possible effect of this drug on other cell types, such as cancer, endothelial, or

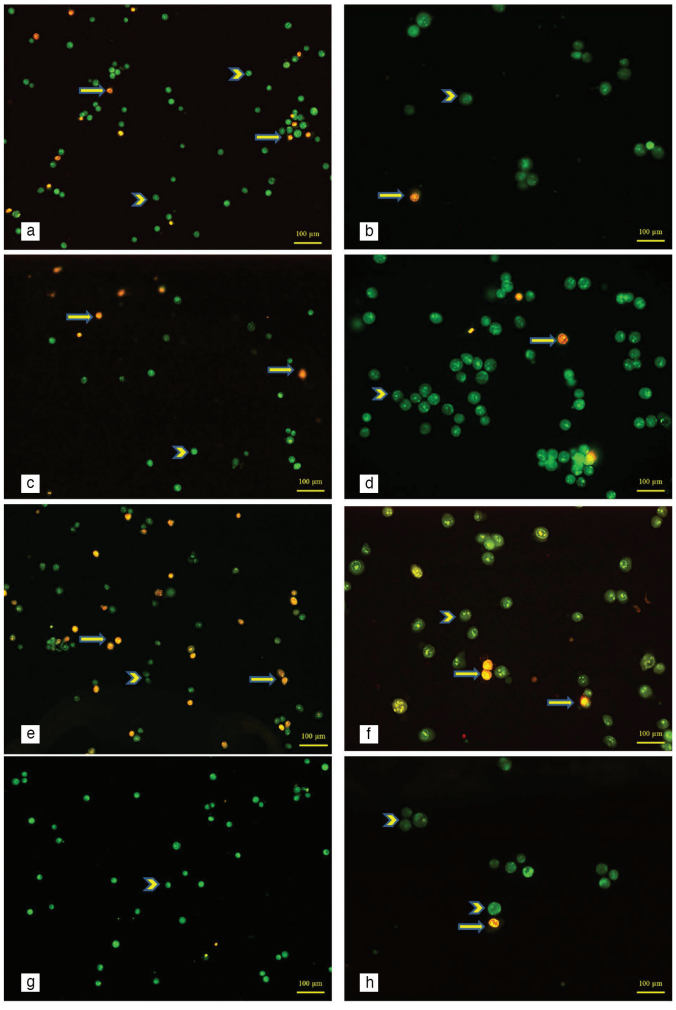

Figure 3. Representative microscope images from AO/EtBr double staining. A) Magnification: 10x L929 fibroblast cells, b) magnification: 40x L929 fibroblast cells, c) magnification: 10x A549 cells, d) magnification: 40x A549 cells, e) magnification: 10x HUVEC cells, f) magnification: 40x HUVEC cells, g) magnification: 10x Ishikawa cells, and h) magnification: 40x Ishikawa cells (100 µg/mL betahistine). Arrows point to apoptotic cells, and arrow heads point to live cells

AO/EtBr: Acridine orange/ethidium bromide, HUVEC: Human umbilical vein endothelial

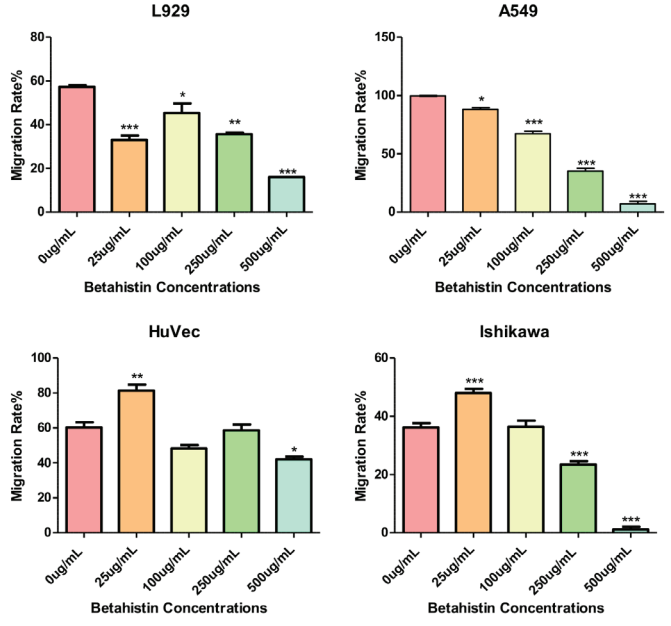

Figure 4. Betahistine reduces the cell migration rate at high concentrations. a) L929 fibroblast, b) A549 lung cancer, c) HUVEC endothelial, and d) Ishikawa endometrial cell lines were treated with 25-500 µg/mL betahistine for 24 hours in an incubator. A scratch assay was performed 24 hours after treatment with the indicated doses of betahistine. The rate of migration (how soon the gap was closed) in 24 hours was calculated by measuring the gap at 0 and 24 hours after scratcing the plates. % changes in the migration rates were compared against the vehicle (NT control) and statistical significance was tested using One-Way ANOVA followed by Tukey's multiple comparison test

*, **, ***: $p<0.05$, n=3, HUVEC: Human umbilical vein endothelial, NT: Non-treated

fibroblast cell lines, has not been extensively studied. In this study, we investigated some physiological effects of betahistine on L929 fibroblasts, A549 lung cancer, HUVEC endothelial, and Ishikawa endometrial cell lines.

Previously, Pilicheva et al.[6] reported betahistine toxicity at very high concentrations (>50 mg/mL) in the RPMI 2650 nasal epithelial cell line. In contrast with this previous report, we found that 500 µg/mL (0.5 mg/mL) betahistine treatment resulted in an almost 50% reduction in proliferation rates in all of the cell lines tested (Figure 1). Our findings clearly show that betahistine treatment at 0.1-0.5 mg/mL can inhibit cell proliferation in a cell-type-dependent fashion. These observations imply that betahistine administration may impact the process of wound healing. Interestingly, however, 25 µg/mL betahistine seemed to increase the proliferation rate of Ishikawa cells significantly (Figure 1).

Data from the AO/EtBr double staining protocol suggested that treatment with betahistine (25 µg/mL, for 24 hours) did not induce apoptosis in A549, L929, HUVEC, or Ishikawa cell lines (Figure 2). However, at concentrations of 100 µg/mL or higher, betahistine significantly increased the number of apoptotic and necrotic cells in all of the cell lines tested, and L929 fibroblast and endothelial HUVEC cells were found to be the most sensitive cell lines (Figure 2a-d), suggesting that primarily stromal and endothelial tissues may be affected when betahistine is used at high concentrations. This subject has yet to be investigated,

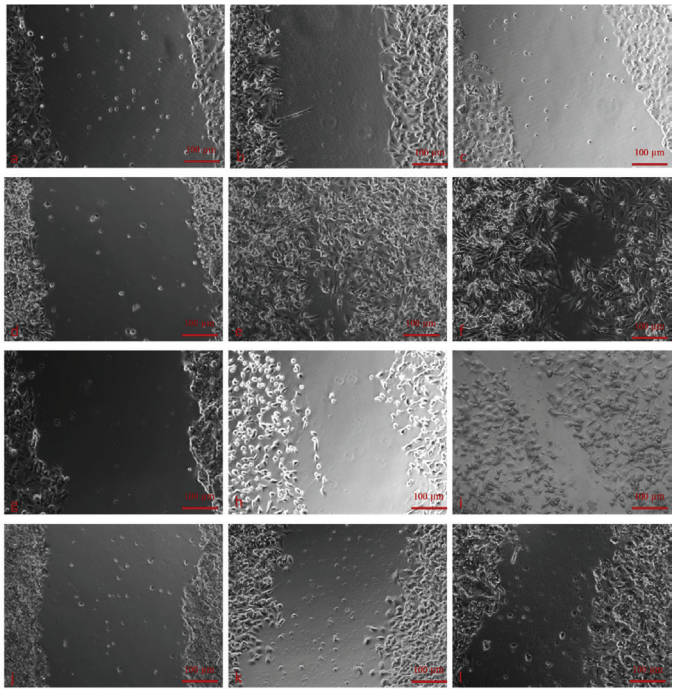

Figure 5. Representative microscope images from the *in vitro* scratch assay. a) 0 hour L929 fibroblast cells, b) 24 hour 0 µg/mL betahistine L929 fibroblast cells, c) 24 hour 25 µg/mL betahistine L929 fibroblast cells, d) 0 hour A549 cells, e) 24 hour 0 µg/mL betahistine A549 cells, f) 24 hour 25 µg/mL betahistine A549 cells, g) 0 hour HUVEC cells, h) 24 hour 0 µg/mL betahistine HUVEC cells, i) 24 hour 25 µg/mL betahistine HUVEC cells, j) 0 hour Ishikawa cells, k) 24 hour 0 µg/mL betahistine Ishikawa cells, and l) 24 hour 25 µg/mL betahistine Ishikawa cells (magnification: 10x)

HUVEC: Human umbilical vein endothelial

and clarification of molecular mechanisms underlying this observation can have important clinical and pharmacological implications.

We also demonstrated that betahistine at ≥100 µg/mL can exert inhibitory effects on the migration rate of all of the cell lines tested in this study (Figure 4). Especially, fibroblasts were among the most sensitive to betahistine treatment. This finding suggests that betahistine administration may have implications in cellular migratory processes such as wound healing or cellular extravasation. Although the underlying molecular mechanisms remains elusive, Tang et al.[20] showed that betahistine suppressed Th17 expansion in lymph nodes of collagen-induced arthritis mice. A betahistine-induced decrease in cell migration rates might be due to the reduced proliferative capacity upon betahistine treatment. Thus, further tests should be performed to clarify whether or not the effect of betahistine on cellular migration is independent of the proliferation rate.

Intriguingly, we found that treatment with 25 µg/mL betahistine-induced cell migration in endothelial (HUVEC) and endometrial (Ishikawa) cell lines (Figure 4). Similarly, the proliferation rates of these cell lines were also increased upon treatment with 25 µg/mL betahistine. These observations point to the possibility that betahistine treatment at low doses can induce proliferation and cell migration rates in certain types of cancer cells. Thus, betahistine treatment can pose a risk for cancer patients (especially for tumors with endometrial origin). However, we could not find any reports investigating the possible link between cancer and betahistine treatment. Thus, further experimental and clinical studies are required to investigate this hypothesis.

Study limitations

In this study, we found that betahistine administration may alter the processes of cellular proliferation, apoptosis, and migration in a cell line- and dose-dependent manner, suggesting that betahistine can potentially affect cellular processes such as wound healing or proliferative properties in certain cell types. However, we were not able to provide any molecular information on the underlying mechanisms that might have affected apoptosis, cellular migration, or proliferation in the cell lines tested. Moreover, animal experiments should also be performed to further test the validity of our *in vitro* observations in living systems.

CONCLUSION

Our findings demonstrate that high doses of betahistine seem to inhibit proliferation and cellular migration and induce apoptosis in HUVEC, human Asian endometrial adenocarcinoma (Ishikawa), pulmonary adenocarcinoma human alveolar epithelial (A549), and murine fibroblast (L929) cell lines. Our preliminary *in vitro* findings suggest that betahistine administration may alter cellular migration and therefore can potentially impact the metastatic properties of some cancer cells in a cell type- and dose-dependent manner. Animal models can be useful for understanding the molecular mechanisms underlying betahistine-induced physiological changes.

Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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