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# Alpha-bisabolol Promotes Glioma Cell Death by Modulating the Adenosinergic System

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**Abstract.** *Background/Aim: Glioblastoma multiforme is the most malignant type of glioma. Alpha-bisabolol is an essential oil reported as a potent cell death agent. In the present work, we evaluated the effect of alpha-bisabolol on ecto-5'-nucleotidase/CD73, the most well-characterized enzymatic source of adenosine, present in lipid rafts. Materials and Methods: Glioma cells were treated with alpha-bisabolol and, in some experiments, pre-treated with an $A_3$ antagonist. MTT assay (viability), malachite green method (ecto-5'-nucleotidase/CD73 activity) and quantitative polymerase chain reaction (qPCR) ($A_3$ mRNA) were carried out. Results: Alpha-bisabolol led to a decrease in C6 and U138-MG glioma cells viability, accompanied by an increase in ecto-5'-NT/CD73 activity. Pre-treatment with an $A_3$ antagonist reverted the effect of α-bisabolol with an increase of mRNA expression of this receptor. Conclusion: Our data indicated the participation of ecto-5'-nucleotidase/CD73 and $A_3$ receptor in the anti-proliferative effect of α-bisabolol on glioma cells.*

Glioblastoma multiforme is the most aggressive brain tumor, with a median survival of patients normally not exceeding 15 months. Despite recent advances, this brain tumor remains a challenging disease to treat as prognosis is still poor (1). Hence, the development of alternative treatments and the discovery of new therapies targets are needed.

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Chamomile (*Matricaria recutita L., Chamomilla recutita L., Matricaria chamomilla*) is one of the oldest and most well-documented medicinal plants (2). The main component of its essential oil is alpha-bisabolol (α-bisabolol), a small and non-toxic sesquiterpene. Recently, studies have explored biological activities of this molecule, which include depigmenting, anti-mutagenic, anti-inflammatory, anti-fungal, and cytotoxic effects on some cancer cell lines, including glioma and other cancer cells (3-5). Previous studies also reported that this compound rapidly accumulates in the brain (3).

Adenosine, the final product of adenosine triphosphate hydrolysis, represents an important extracellular signaling molecule that mediates diverse biological effects in both normal and tumor cells (6). Adenosine can act on the P1 receptors, which are subdivided into $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$, all of them coupled to G proteins. Among these receptors, the $A_3$ adenosine receptor has been described as a cell death modulator in cardiomyocytes, brain astrocytes and glioma cells (6, 7). The most well-characterized enzymatic source of extracellular adenosine is the ecto-5'-nucleotidase (ecto-5'-NT/CD73). This enzyme catalyzes the de-phosphorylation of nucleotide monophosphates, leading to the respective nucleoside. Previous studies have suggested a role for ecto-5'-NT/CD73 in the modulation of cell growth, differentiation and cell-cell and cell-matrix interactions (8-10). In some pathological conditions, such as glioblastoma multiforme, the activity and expression of this enzyme are increased (6), revealing an important correlation between this enzyme and tumor progression (10).

Given that (i) α-bisabolol can be incorporated into lipid rafts (11) rich in glycosyl phosphatidylinositol-anchored proteins (12) and (ii) ecto-5'-NT/CD73 is anchored on the cell surface *via* this lipid anchor (9), the aim of the present study is to evaluate the effect of α-bisabolol on the activity of ecto-5'-NT/CD73 and investigate a possible involvement of adenosine on the glioma cell death.

## Materials and Methods

*Cell lines and maintenance*. U138-MG human and C6 rat glioma cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 0.5 U/ml penicillin/streptomycin and supplemented with 5% fetal bovine serum (FBS), (all from Gibco; Grand Island, NY, USA). Cells were kept at 37˚C, at a minimum relative humidity of 95% in an atmosphere of 5% $CO_2$.

*Alpha-bisabolol treatment*. Alpha-bisabolol was prepared in dimethylsulphoxide (DMSO) (Sigma; St. Louis, MO, USA). The final concentration of DMSO in each well was 0.5%; the same concentration of DMSO was used for the vehicle control. The glioma cells were seeded on 6-, 24- or 48-well plates, according to the experimental setting employed. For the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide)) assay, cells were treated with 30, 45, 60, 75, 100 or 115 μM α-bisabolol for 48 h. For other experiments, concentrations of 35, 45 or 55 μM α-bisabolol were used for C6 glioma cells and 55, 65 or 75 μM α-bisabolol for U138-MG glioma cells. In experiments performed with MRS1220 (9-Chloro-2-(2-furanyl)-5-((phenylacetyl) amino)-(1,2,4)triazolo(1,5-c)quinazoline) (Tocris; Bristol, UK), an $A_3$ adenosine receptor antagonist, this agent was added to the cells at a concentration of 1.0 μM, 30 min before exposure to α-bisabolol treatments.

*Cell viability assay.* C6 or U138-MG glioma cells were seeded onto a 48-well plate and, after achieving semi-confluence, treated with α-bisabolol for 48 h. After treatment, MTT was added to each well. The level of absorbance was read by an ELISA plate reader at 492 nm.

*Ecto-5'-NT/CD73 assay.* After treatment with α-bisabolol, 24-well plates containing C6 or U138-MG glioma cells were washed with incubation medium (2 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM Hepes, pH 7.4). The enzymatic reaction was started by the addition of 200 μl of the same incubation medium containing 2 mM AMP, at 37˚C. After 10 min, 150 μl of the incubation medium was collected and transferred to Eppendorf tubes containing trichloroacetic acid (5% final concentration) previously placed on ice. The green malachite method was used to measure the inorganic phosphate (Pi) release (13). The protein was measured by the Coomassie Blue method (14). Specific activity was expressed as nmol Pi released/min/mg of protein.

*$A_3$ mRNA expression analysis*. Total RNA of C6 cells was isolated with TRIzol Reagent (Invitrogen; Carlsbad, CA, USA). Total RNA (0.5 μg) was added to each cDNA synthesis reaction using the SuperScript-III RT pre-amplification system (Invitrogen). Real-time polymerase chain reactions (PCRs) were carried out in the Applied-Biosystem Step One Plus cycler using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). The real time PCR reactions were performed using the following temperature protocols: 2 min at 50˚C, 2 min at 95˚C, followed by 40 rounds of 95˚C for 15 s, annealing at primer specific temperature for 30s. The same program was used for the amplification of the reference gene (β-actin). All results were analyzed by the $2^{-\Delta/\Delta CT}$ method (15).

The following sequences of primers were used: *$ADORA_3$* receptor: forward: 5'-CTG CGA GTC AAG CTG AC-3' and reverse: 5'-GTC CCA CCA GAA AGG ACA-3'. *ACTB* (β-actin): forward: 5'-GGT CAT CAC TAT CGG CAA T-3' reverse: 5'-GAA TGT AGT TTC ATG GAT GC-3'.

*Cell number assay*. After 48 h of treatment with different concentrations of α-bisabolol, as described above, the medium was removed, cells were washed twice with phosphate buffered saline (PBS), detached with 0.05% trypsin/EDTA and immediately counted in a hemocytometer.

*Statistical analysis*. Data, expressed as mean±standard deviation (S.D.), were analyzed for statistic significance either by One Way analysis of Variance (ANOVA), followed by *post-hoc* comparisons (Tukey test) or Two Way ANOVA using GraphPad Prism Software (La Jolla, CA, USA). Differences were considered significant in relation to control when $p<0.05$.

## Results

*Treatment with α-bisabolol decreases cell viability and increases ecto-5'-NT/CD73 activity*. The MTT assay was used to investigate the effect of α-bisabolol on cell viability. Increasing concentrations of this compound led to increasingly significant decreases in the percentages of viable cells, when compared to DMSO-treated cells. For the C6 cells, the half-maximal inhibitory concentration ($IC_{50}$) calculated was approximately 45 μM (Figure 1A). It was also possible to observe a decrease in cell viability of U138-MG glioma treated with α-bisabolol (Figure 1B). It is also interesting to note a difference of sensitivity to this drug between the two cell lines studied.

To evaluate the effect of α-bisabolol on ecto-5'-NT/CD73 activity, the C6 and U138-MG cells were treated with α-bisabolol for 48 h. There was a significant increase in enzyme activity at all concentrations tested in the C6 cells, while the intermediate concentration of α-bisabolol tested (45 μM) was able to induce a more significant increase in AMP hydrolysis (Figure 1C). For the U138-MG cells, α-bisabolol increased ecto-5'-NT/CD73 activity only at 55 and 65 μM (Figure 1D).

*The α-bisabolol-induced decrease in cell number is partly mediated by the $A_3$ receptor.* Given that ecto-5'-NT/CD73 stimulation can increase the levels of adenosine in the extracellular medium with a possible involvement of the $A_3$ adenosine receptor in cell death (23, 31), the participation of this receptor was also investigated. For this purpose, C6 cells were pre-treated with 1.0 μM MRS1220, an $A_3$ receptor antagonist, for 30 min before α-bisabolol exposure for 48 h

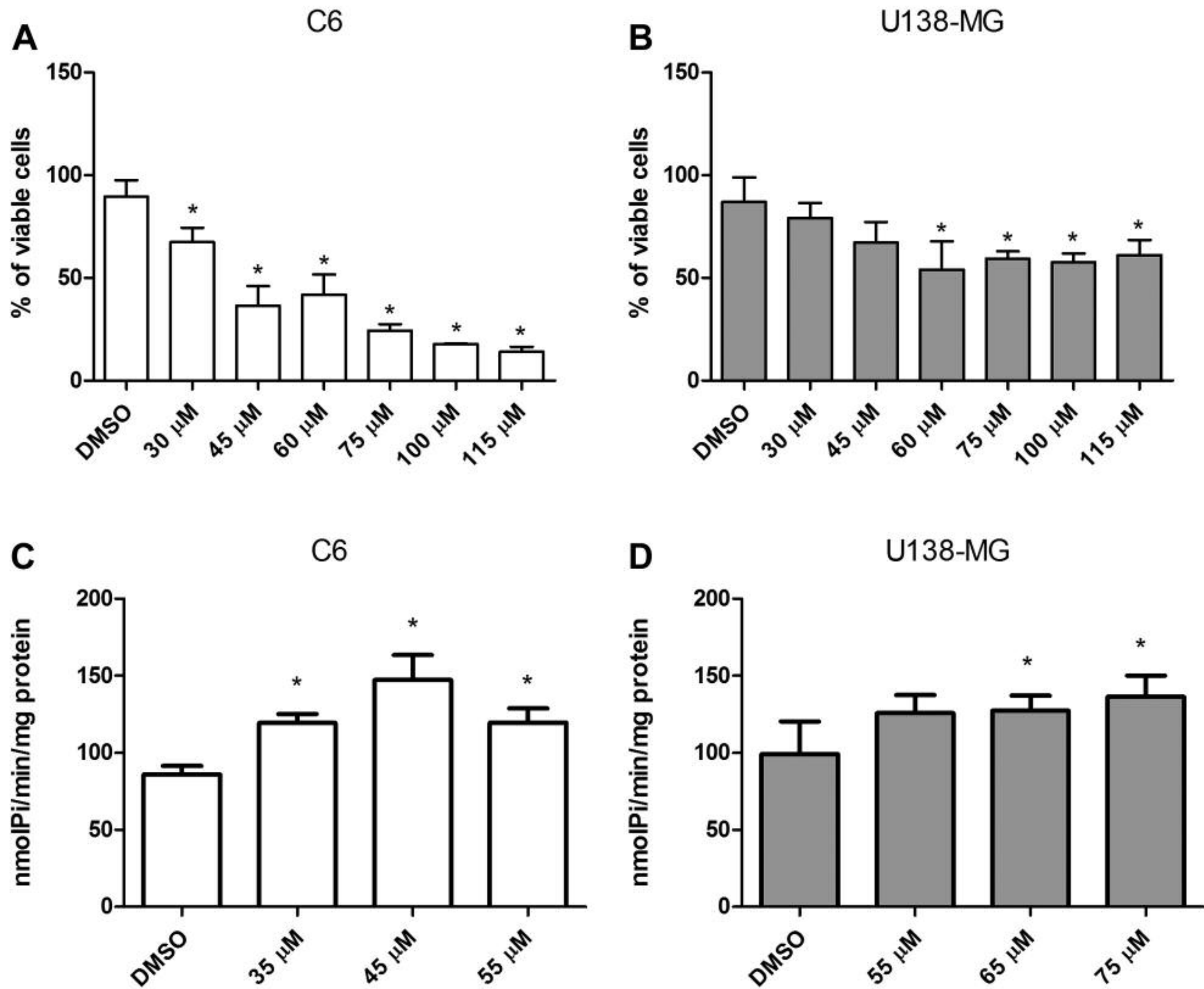


Figure 1. *Alpha-bisabolol decreases glioma cell viability and stimulates glioma cell ecto-5'-NT/CD73 activity. Glioma cells were treated with the indicated concentrations of α-bisabolol for 48 h. Cell viability was accessed by the MTT assay, as described in Materials and Methods. (A) C6 rat glioma cells. (B) U138-MG human glioma cells. After α-bisabolol treatments, cells were incubated with 2 mM AMP. (C) and (D) Specific activities expressed as nmol Pi/min/mg protein for C6 and U138-MG cells, respectively. Values represent means±S.D. Data were analyzed by one-way ANOVA, followed by post-hoc comparisons (Tukey). *Significantly different from the DMSO group (p<0.05).*

in culture. Figure 2A shows that the decrease in cell number caused by 45 and 55 μM α-bisabolol was significantly reversed by the treatment with the $A_3$ adenosine receptor antagonist. We also evaluated whether the treatment with α-bisabolol modulates the expression of the gene encoding the $A_3$ receptor. At all concentrations tested, α-bisabolol increased $A_3$ adenosine receptor mRNA levels (Figure 2B).

## Discussion

In the present study, we showed that treatment with α-bisabolol decreases cell viability in two glioma cell lines. The C6 rat glioma cell line is more sensitive than the U138-MG human glioma cell line. The difference of response could be explained by different genetic characteristics for each cell type; while U138-MG is $PTEN^{null}$ and $p53^{mut}$, C6 has both functional proteins (16-18).

When ecto-5'-NT/CD73 activity was assessed, a significant activation of this enzyme was observed for the three concentrations tested. Interestingly, a peak of activity was found for the intermediate concentrations tested in C6 glioma cells, although previous studies from our laboratory demonstrated a linear dose-response decrease or increase in ecto-5'-NT/CD73 activity after different treatments (8, 19). This apparent discrepancy may be due to α-bisabolol treatment that leads to decreased cell confluence and

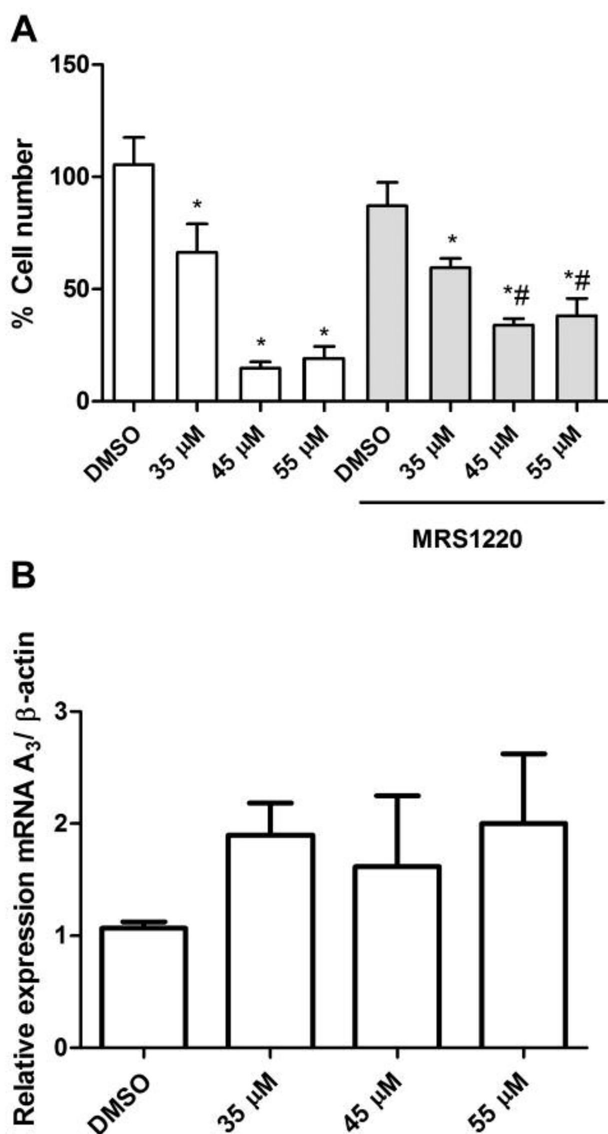


Figure 2. *The α-bisabolol-induced decrease in cell number is partly mediated by the A3 receptor. Cells were treated with the indicated concentrations of α-bisabolol and maintained in culture for 48 h. (A) Cells were pre-treated with 1 µM MRS1220, a selective $A_3$ antagonist, 30 min before the 48-h treatment with α-bisabolol. Following treatment, the cells were immediately detached and counted in a hemocytometer. Data represent mean±S.D. Data were analyzed by two-way ANOVA. *Significantly different from the respective DMSO group (p<0.001). #Significantly different from α-bisabolol alone (p<0.05). (B) After 48 h of treatment, A3 receptor mRNA was isolated and cDNA was synthesized. Using specific primers, qPCR was performed. Values represent mean±S.D. Data were analyzed by one-way ANOVA followed by post-hoc comparisons (Tukey).*

increased cell death (20), thus influencing ecto-5'-NT/CD73 activity, which results in a decrease in the enzyme's activity at higher concentrations tested.

Considering that C6 cells were shown to be more sensitive to α-bisabolol, to better investigate the potential participation of adenosine in cell death we subsequently investigated the role of adenosine, the product of ecto-5'-NT/CD73 activity. In the presence of increased levels of adenosine, the activation of the $A_3$ adenosine receptor may induce cell death (8) in a large variety of cancer cell types, including glioma cell lines (19, 21). To evaluate the participation of this receptor, we tested the effect of MRS1220, an $A_3$ adenosine receptor antagonist, on the cell death induced by α-bisabolol. It was observed that pre-treatment of C6 cells with MRS1220 reverted the effect of α-bisabolol alone, indicating that this receptor is, at least partially, involved in the cell death induced by this molecule. Thus, we sought to determine whether α-bisabolol alters receptor levels and found that cells treated with α-bisabolol present augmented transcriptional levels of the A3 receptor.

In conclusion, the present study establishes, for the first time, the cytotoxic effect of α-bisabolol on ecto-5'-NT/CD73, showing an interesting correlation between ecto-5'-NT/CD73 activity and A3 adenosine receptor. Although further studies are necessary, the present data indicate that α-bisabolol can be a strategy as an adjuvant therapy for glioblastoma treatment.

## Conflicts of Interest

The Authors declare that these are no conflicts of interest associated with this study.

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