Activity of LaSOM 65, a Monastrol-derived Compound, Against Glioblastoma Multiforme Cell Lines

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Abstract. *Background/Aim: Despite recent progress in glioblastoma treatment, prognosis is still poor. Monastrol is a kinesin spindle protein (KSP) inhibitor and anticancer effects for this molecule have been reported. Here we describe the effect of LaSOM 65, a monastrol derivated compound, against glioma cell lines. Materials and Methods: Cell counting, viability assay, lactate dehydrogenase (LDH) activity, cell-cycle analysis, immunofluorescence and organotypic hippocampal slice cultures were performed. Results: LaSOM 65 reduced cell number and cell viability of gliomas cells, but did not cause arrest in the cell cycle at the G_2/M phase. Measurement of LDH activity showed that LaSOM 65 induces necrosis after 48 h of treatment. Conclusion: LaSOM 65 appears to a be promising new molecule to treat glioblastoma since it promotes a decrease of cell growth and cell viability of glioma cells in vitro and does not induces the neurotoxic characteristics of the anti-mitotic drugs currently used.*

Gliomas are the most common primary cancer in the central nervous system (CNS) and are classified by the World Health Organization (WHO) into four levels (I-IV) according to their degree of malignancy (1). Glioblastoma multiforme IV is considered the most aggressive and lethal form of glioma (2). Despite recent progress in the treatment of glioblastoma, median patient survival still remains at 15 months, and the majority of patients die within two years of diagnosis (3).

Kinesin spindle protein (KSP) has been reported as a potential target for cancer therapy. KSP is required early in mitosis to separate the centrosomes of the emerging spindle poles, thus driving establishment of a bipolar mitotic spindle (4). Besides being extremely important for cell division, KSP is present in a variety of solid tumors and leukemias and is associated with a high mitotic rate (5). Little or no expression of this kinesin is detected in non-proliferating cells (6). Since the discovery of the first specific KSP inhibitor, monastrol (7), interest in the development of other structural analogs has increased. Considering this information, the objective of the present study was to investigate the effect of LaSOM 65 (5-ethoxycarbonyl-6-methyl-4-(3-nitrophenyl)-3,4-dihydropyrimidin-2-(1-H)-thione), a monastrol-derivative against glioma cell lines in culture.

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Key Words: Glioma, KSP inhibitors, LaSOM 65, antitumor activity.

Materials and Methods

Maintenance of cell lines. The glioma C6 (rat) cells and human U138 cells were obtained from the American Type Culture Collection (Rockville, Maryland, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 2.5 mg/ml Fungizone, 100 U/l garamicin and supplemented with fetal bovine serum (FBS), all from Gibco (Gibco BRL, Carlsbad, CA, USA). Cells were kept at 37˚C, at minimum relative humidity of 95% and in an atmosphere of 5% CO_2 in air.

Drug exposure. LaSOM 65 and monastrol (Figure 1) were synthesized employing the Biginelli reaction (8). The compounds were dissolved in dimethylsulfoxide (DMSO) Sigma (St. Louis, MO, USA). Glioma cells were seeded according to each experiment and upon reaching semi-confluence, the cultures were exposed for 24 or 48 hours to 15, 30, 45, 75 and 100 μM LaSOM 65. As a vehicle control, cells were treated with 0.5% DMSO.

Cell counting. At the end of treatment, C6 and U138 cells were detached with 100 μl of 0.05% trypsin/EDTA solution, followed by the addition of 400 μl of DMEM. Cells were counted in a hemocytometer.

Cell viability. The cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. At the end of treatment, the medium was removed and cells were washed with phosphate-buffered saline (PBS). Subsequently,

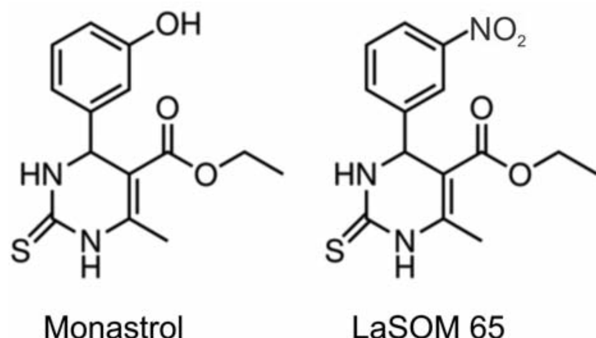

Figure 1. *Chemical structure of LaSOM 65 and monastrol. The original hydroxyl group of monastrol was replaced by a NO_2 group on the LaSOM 65 molecule 5-ethoxycarbonyl-6-methyl-4-(3-nitrophenyl)-3,4-dihydropyrimidin-2-(1-H)-thione.*

90 µl DMEM and 10 µl MTT (5 mg/ml) were added to each well. Cells were incubated for 3 h and the solution was removed from the precipitate. A total of 100 µl of DMSO were added to the wells and the absorbance was read in an ELISA plate reader at 490 nm.

Lactate dehydrogenase (LDH) activity. The degree of cellular integrity was evaluated by measuring the activity of LDH (EC 1.1.1.27). Briefly, after 48 h treatment with LaSOM 65 the culture medium was collected and LDH activity was evaluated according to the procedure described by Whitaker *et al.* (9).

Organotypic hippocampal slice cultures and quantification of cellular death. Organotypic hippocampal slice cultures were prepared as described by Stoppini and collaborators, with modifications (10, 11). The experiments were carried out after 14 days in culture, when cells were treated with 100 µM LaSOM 65, 100 µM monastrol or DMSO (control) for 48 h. After treatment, cultures were incubated with 5 µM of propidium iodide (PI) for two hours. Cultures were observed with an inverted microscope (Nikon Eclipse TE3005) using a standard rhodamine filter set. Images were captured and then analyzed using Scion Image software.

Immunofluorescence. To access the formation of monopolar arrest, C6 glioma cells were left to adhere for at least 24 h on coverslips in 24-well plates before treatment. Following exposure for 6 h to 100 µM LaSOM 65 or 100 µM monastrol, cells were washed with PBS, pH 7,4, fixed with 95% acetone and 5% formalin (10% paraformaldehyde-PBS) at 4˚C, for 5 min. After two washes and blocking with 7% goat serum, 0.2% Tween 20 and PBS, cells were incubated with anti-alpha-tubulin (ab52866, Abcam Ltd., Cambridge, UK) for 90 min, followed by a Fluorescein(FITC)-conjugated goat anti-rabbit (Invitrogen, Life Technologies Ltd, Paisley, UK) secondary antibody incubation for 1 h. Cells were washed with PBS and incubated with 4',6-diamino-2-phenylindole (DAPI), a nuclear marker. Images were collected using an Olympus Scanning Confocal Microscope.

Cell-cycle analysis. At the end of treatment (45, 75 or 100 µM of LaSOM 65 for 24 h), the medium was removed, cells were washed with PBS and 200 µl of 0.05% trypsin/EDTA solution was added to detach the cells. The cell suspension was centrifuged at 400 ×*g* for 10 min. A quantity of 1×10^6 cells were fixed with 500 µl PBS and 4.5 ml ethanol 70%-PBS and maintained in a freezer. On the day of analysis, cells were centrifuged, washed with PBS and incubated with PI and RNAse for 30 min. The cells were analyzed using flow cytometry (FACS Calibur cytometry system, FACS Calibur; BD Bioscience, Mountain View, CA, USA). The data obtained were analyzed by FLOWJO® software.

Statistical analysis. All experiments were carried out at least three times in triplicate. Data were analyzed by analysis of variance (ANOVA) followed by post-hoc comparisons (Tukey's test).

Results

Effect of LaSOM 65 on glioma cell growth. As shown in Figure 2, different concentrations of LaSOM 65 caused a decrease in cell number in C6 cells 21%, 40%, 55% and 73% for 30, 45, 75 and 100 µM, respectively. For the U138 cell line, only the concentration of 100 µM was potent to reduce the cell number (24.3%).

LaSOM 65 reduces glioma cells viability. After 48 h of treatment, LaSOM 65 significantly reduced C6 cell viability at a concentration of 75 and 100 µM (reduced by 28% and 46%, respectively) and U138 viability at 100 µM (17% of reduction) (Figure 3). Cell viability was also evaluated by measuring the LDH activity in cell culture medium. For C6 cells treatment with 75 and 100 µM LaSOM 65 for 48 h increased in 40% and 34% LDH activity in relation to control, respectively, indicating cell lysis (Figure 4). However, there was no difference in the LDH levels between the control and the treated groups of U138 cells (Figure 4). Taken together, these results indicate that C6 cells are more sensitive to LaSOM 65 than U138 glioma cells.

LaSOM 65 has no cytotoxic effects on organotypic hippocampal slice cultures. We used organotypic hippocampal slice cultures as a model to evaluate the effects of LaSOM 65 on normal neural cells. After 14 days in culture, the organotypic cultures were treated for 48 h with 100 µM LaSOM 65 and monastrol and cell death was analyzed by PI uptake. Neither of the compounds caused organotypic hippocampal culture damage (Figure 5).

Morphological observation of nuclei. To test whether LaSOM 65 causes monopolar arrest in glioma cells, C6 glioma cells treated with 100 µM LaSOM 65 for six hours were immunostained for nuclei and α-tubulin. Cells treated with monastrol were used as a positive control. As shown in Figure 6, monastrol caused cellular arrest and monopolar spindle formation in C6 cells. The treatment with LaSOM 65 did not cause the monopolar spindle effect in the same proportions as monastrol. These data indicate that the main mechanism, which explains the observed decrease on cell number, is the

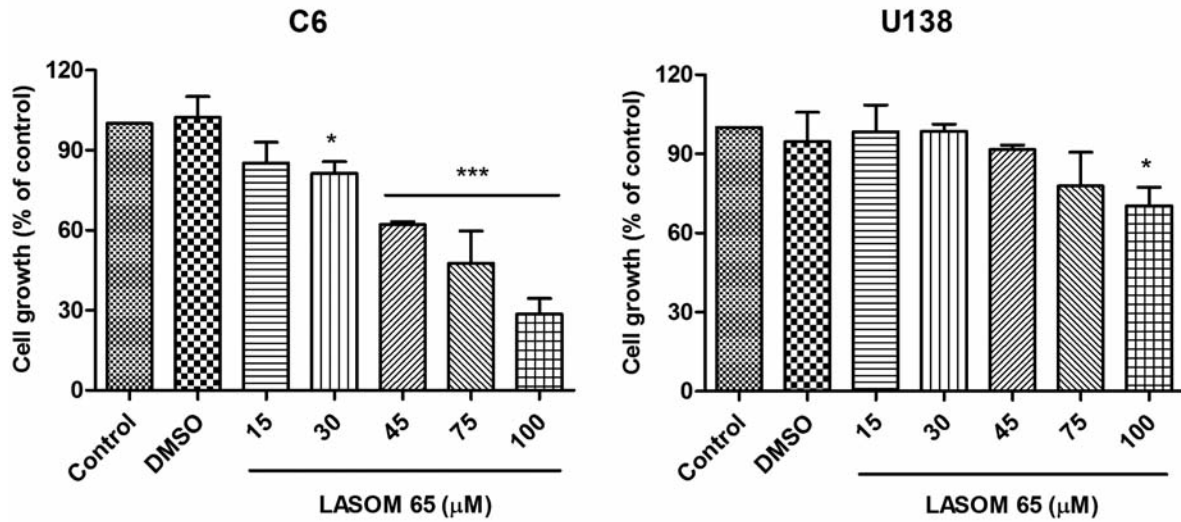

Figure 2. *Effect of LaSOM 65 on C6 and U138 cell growth. C6 and U138 glioma cells were treated with different concentrations of LaSOM 65 for 48 h. Cells were detached and counted. Values represent as the mean±SD of three independent experiments in triplicate. Data was analyzed by one-way ANOVA followed by Tukey post test. Significantly different from the DMSO-treated group at *p<0.05 and ***p<0.001.*

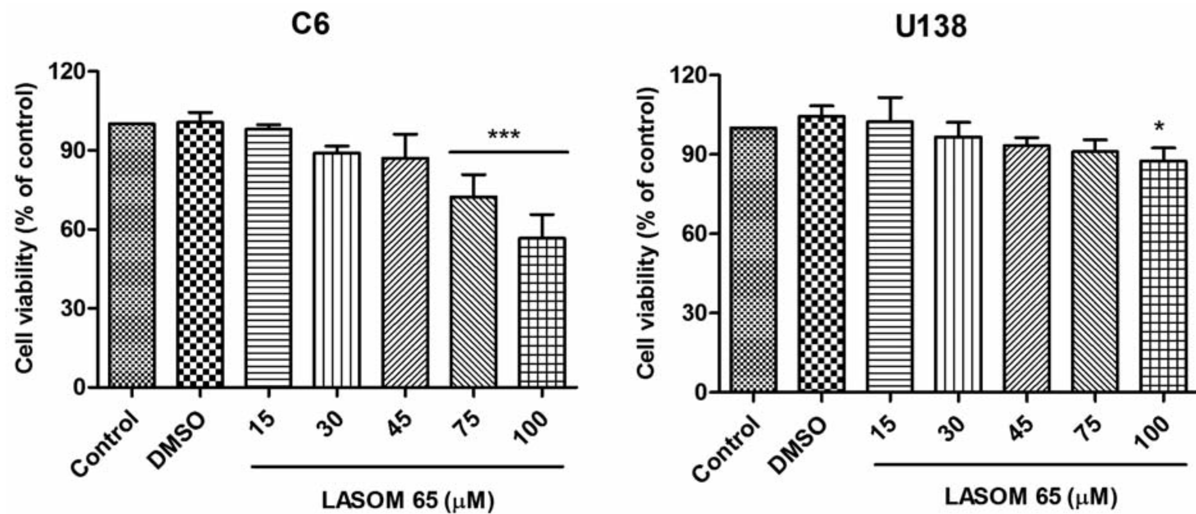

Figure 3. *LaSOM 65 reduces C6 and U138 cell viability. C6 and U138 glioma cells were treated with different concentrations of LaSOM 65 for 48 h. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Values represent means±SD of three independent experiments in triplicate. Data were analyzed by one-way ANOVA followed by Tukey's post-test. Significantly different from the DMSO-treated group at *p<0.05 and ***p<0.001.*

ability of LASOM 65 to induce necrosis on C6 glioma cells, witnessed by increase of LDH activity (Figure 4).

LaSOM 65 does not induce cell-cycle arrest in G_2/M phase. C6 glioma cells were treated with 100 μM LaSOM 65 for 24 and 48 h. Monastrol (100 μM) was used as a positive control. Flow cytometric analyses showed that LaSOM 65 did not affect the cell cycle, neither at 24 h (Figure 7) nor at 48 h (data not shown). Of all the LaSOM 65-treated cells, only 6.75% were G_2/M phase, while 82.1% were in the G_1 phase (data similar to those observed with control and DMSO-treated groups). However, the monastrol-treated cells showed a significant increase in G_2/M phase (67.35%) and a subsequent decrease in G_1 phase (5.27% cells). Similar results were obtained with 45 μM and 75 μM LaSOM 65 (data not shown).

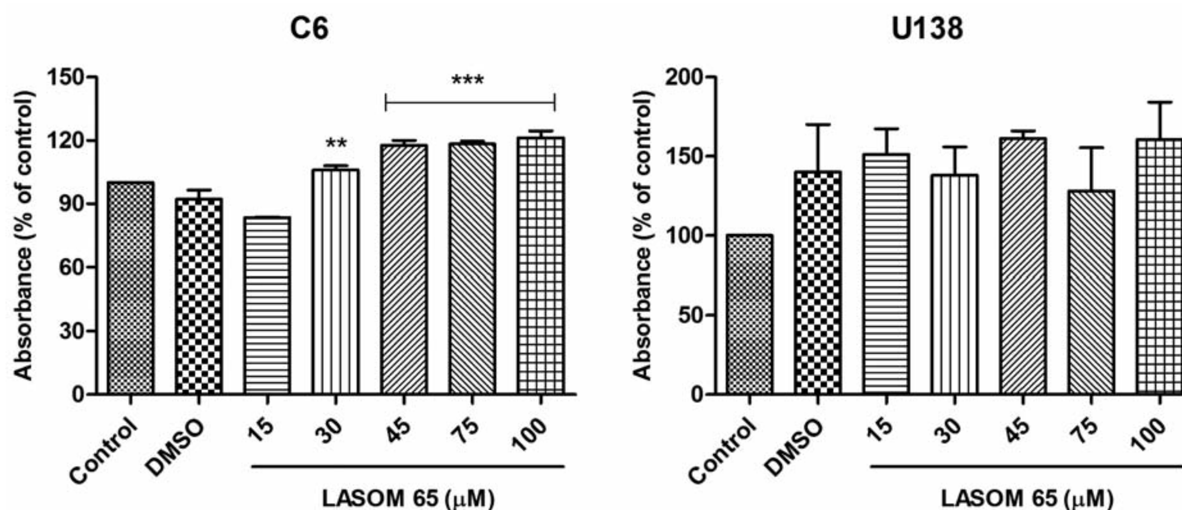

Figure 4. *Lactate dehydrogenase (LDH) activity of LaSOM 65-treated cells. C6 and U138 glioma cells were treated with different concentrations of LaSOM 65 for 48 h. The cell culture medium was then removed and the LDH activity was measured in a spectrophotometer at 570 nm. Values represent the mean±SD of three independent experiments in triplicate. Data were analyzed by one-way ANOVA followed by Tukey's post-test. *Significantly different from the DMSO-treated group at $p<0.05$.*

Discussion

Monastrol is the first known anti-mitotic compound that blocks mitosis by inhibiting the kinesin motor protein kinesin-5 (Eg5) (7). There is great interest in developing Eg5 inhibitors due to their potential to reduce side-effect profiles caused by taxanes and other anti-mitotic drugs. In the current study, we investigated the effect of LaSOM 65 (Figure 1), a monastrol analog, against glioma cell lines. Rat glioma cells (C6) exposed to LaSOM 65 for 48 h exhibited a significant decrease of cell growth, reducing cell number by 72% and cell viability by 46% at 100 μM. Even a concentration as low as 35 μM is sufficient to promote a decrease in cell number. Human U138 glioma cells were more resistant to the treatment, with cell viability and cell number decreasing by only 17% and 24%, respectively, and only at the highest concentration tested. The differential effect of LaSOM 65 against C6 rat and human U138 glioma cells could be the result of different signaling pathways affected by this molecule, as previously shown for monastrol (12). In the neurotoxicity experiments carried out against organotypic hippocampal slice cultures, LaSOM 65 was found to be non-toxic to normal cells of the brain as was monastrol and this makes the investigation of other monastrol derivatives promising (13). Furthermore, recently, Torres *et al.*, have published the non-acute toxicity in rats after *i.v.* and oral LaSOM 65 administration (14).

In order to investigate if cells undergo necrosis due to treatment with LaSOM 65, we measured the intracellular enzyme LDH in cell culture medium. The C6 glioma cells treated with 100 μM LaSOM 65 for 48 h showed an increase of 34% LDH in supernatants compared to control, indicating cell disruption and leakage of this intracellular enzyme into the culture medium.

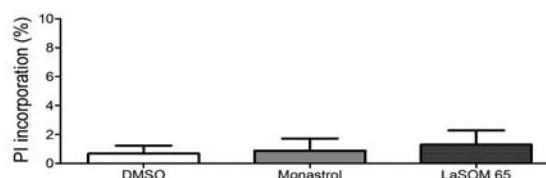

Figure 5. *Effect of LaSOM 65 on organotypic hippocampal slice cultures. Quantitative DMSO, LaSOM 65 and monastrol 100 μM analysis of hippocampal ccll damage after treatment. Cell death was analyzed by propidium iodide (PI) incorporation, which was visualized using a Nikon inverted microscope (at ×40 magnification) and then analyzed using the Scion Image software. Data represent the means±SEM of three independent experiments performed in duplicate. Data were analyzed by ANOVA followed by Tukey's post-test. There was not statistical difference between treatments.*

Next, we investigated if this molecule acts by the same mechanism as monastrol. Many researchers have shown that monastrol inhibits human Eg5, which is required for the formation of a bipolar spindle, causing mitotic arrest and leading cells to monopolar spindle (15). To investigate if LaSOM 65 causes this cell event, we stained for DNA and α-tubulin of C6 glioma cells, treated with 100 μM LaSOM 65 for 6 h. The images obtained show some cells with a monopolar spindle, but the effect was not as evident as that observed in cells treated with monastrol.

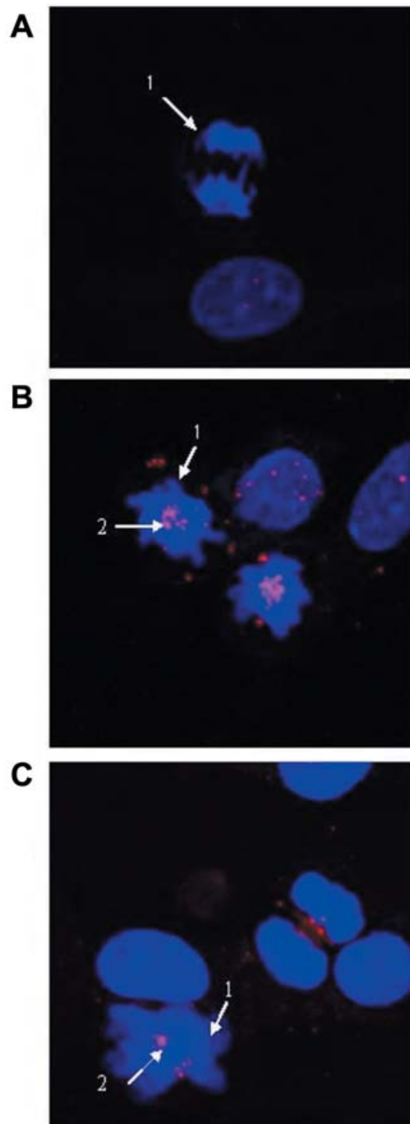

Figure 6. *Immunofluorescence microscopy. Fixed C6 cells were stained for nuclei (1) and α-tubulin (2). A: Control cells. B: Cells treated with 100 μM monastrol for six hours. C: Cells treated with 100 μM LaSOM 65 for 6 h.*

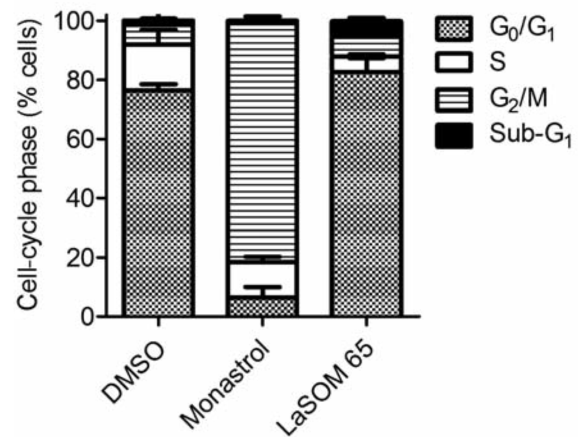

Figure 7. *Cell-cycle analysis. Effect of LaSOM 65 on cell-cycle progression of C6 glioma cells. Cells were treated with 100 μM monastrol or LaSOM 65 for 24 h. After treatment, cells were stained with propidium iodide and analyzed by flow cytometry. LaSOM 65 did not induce arrest of the cell cycle in G_2/M phase, in contrast to monastrol.*

Monopolar spindle formation caused by monastrol induces a blockade of the cell-cycle in the G_2/M phase. In most cases, mitotic arrest induces apoptosis through mitochondrial membrane depolarization and caspase-3 activation (15, 16). Therefore, we performed experiments to determine if LaSOM 65 causes that arrest in the G_2/M phase. Surprisingly, after 24 and 48 hours of treatment, the cells usually passed through the complete cell cycle. This result is in agreement with the images obtained from immunocytochemistry of cells treated with LaSOM 65 that show few cells with the monoastral phenotype, reinforcing that this compound acts by a mechanism other than Eg5 inhibition.

Much remains to be understood in order to improve anti-mitotic cancer chemotherapy. LaSOM 65 appears to be promising since promotes a decrease of cell growth and cell viability of glioma cells *in vitro* and did not cause the neurotoxicity characteristic of currently used anti-mitotic drugs. Besides, LaSOM 65 has shown both, non-toxicity to rats after acute treatment and good pharmacokinetic parameters (14). However, more studies are necessary to understand the mechanisms by which LaSOM 65 exerts its cytotoxic effects. The results presented herein have encouraged us to investigate the effect of other new monastrol analogs on the proliferation of glioma cell lines.

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