Am J Cancer Res 2023;13(4):1377-1386
www.ajcr.us /ISSN:2156-6976/ajcr0149418

Original Article

Effect of sunitinib derivatives on glioblastoma single-cell migration and 3D cell cultures

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Received January 30, 2023; Accepted March 14, 2023; Epub April 15, 2023; Published April 30, 2023

Abstract: This study aimed to evaluate the anticancer activity of 16 new sunitinib derivatives in brain cancer cells (2D model) and spheroids (3D model). The effect on cell viability was determined by the MTT assay. Single-cell migration assay was performed to examine the effect of selected compounds on individual cell migration. The activity of compounds in 3D cell cultures was examined by measuring the size change of spheroids formed using the Hanging drop method. The viability of brain cancer (U-87MG and A-172) cells was most reduced by compound EMAC4001. EMAC4001 showed the strongest effect on U-87MG cell migration, and EMAC4007 was the most active in the A-172 cell line. Only sunitinib had a statistically significant impact on spheroid growth at 100 nM and 500 nM concentrations in the U87-MG cell line and EMAC4007 had a statistically significant impact on A-172 spheroid growth at 100 nM and 500 nM concentrations, similarly to sunitinib.

Keywords: Sunitinib analogs, glioblastoma, tyrosine kinase inhibitors, spheroids, cell migration

Introduction

Glioblastoma (GBM), also called grade IV astrocytoma, is the most common and most malignant cancer of the central nervous system in adults [1]. The GBM and low-grade gliomas mainly differ in growth rate, angiogenesis, and necrosis [2]. The GBM prognosis is associated with poor quality of life: necrosis-induced changes in the nervous tissue cause behavioral, emotional, and cognitive disorders, which lead to patients' mental disability and lack of independence [3]. The median survival time for GBM patients is about 14 to 16 months, and there are still extremely limited opportunities for conventional therapy [4]. Therefore, to this day, GBM is considered an incurable type of cancer.

Astrocytoma develops from irregular, star-shaped glial cells called astrocytes, characterized by the ability to form pseudopodia, which allow GBM cells to spread deeper into healthy brain tissues while complicating the determination of the exact border between normal and malignant brain sites, making complete tumor resection impossible and GBM recurrence inevitable [5, 6]. There are various commercially available glioma cell lines. However, more elongated cells like U-87MG and A-172 are characterized by a higher invasiveness level [7]. Infiltrative brain tumors are associated with the disintegration of the normal astrocytic-endothelial links in the brain-blood barrier, but increased permeability of such barrier causes an increase of expression of various membrane transporters and pumps that limit drugs entry to tumor parenchyma making GBM hardly reachable for therapeutics. For instance, temozolomide (TMZ), the first-line agent for GBM treatment, has shown limited efficacy in GBM patients due to developed resistance related to overexpression of ATP-binding cassette drug efflux transporters and it was found that maximum TMZ concentration in the brain reaches only up to 20% of serum concentration, suggesting that research for new methods capable

of improving GBM therapy and prognosis is crucial [8-10].

A relatively new approach to treat GBM is targeted therapy that involves small molecular drugs targeting cell surface receptors called receptor tyrosine kinases (RTKs), which play a significant role in most cellular processes by initiating intracellular signal transduction through two major pathways Ras/MAPK/ERK and Ras/PI3K/AKT [11]. Studies have shown that invasive and angiogenic tumor behavior is related to the activation of RTKs and growth factors, which are overexpressed in GBM as a result of various genetic alterations [12]. Small molecular inhibitors have been developed to block the active site of the kinase and thereby prevent phosphorylation resulting in inhibition of migration and apoptosis of cancer cells [13]. In GBM increased kinase activity is commonly detected in EGFR, PDGFR and VEGFR families based on tumor subtype and region. EGFR is known to be more often present in the classical subtype of GBM and is usually associated with highly proliferative properties [14, 15]. A few inhibitors targeting EGFR, such as erlotinib and lapatinib, were tested in clinical trials, unfortunately, compounds did not have significant effect for GBM patients suggesting that single-target or dual agents might not be sufficient in blocking tumor intracellular pathways and overcoming drug resistance [16, 17]. Other important targets for kinase inhibitors are PDGFR and VEGFR, as they promote invasive growth and angiogenesis of GBM [12, 18]. It was found that PDGFRs and their ligands are co-expressed in high-grade gliomas proposing an autocrine loop, not common for normal brain tissue, thereby making specific targeting possible [19]. VEGF is considered to be a critical mediator of new blood vessels formation in GBM and is known to be released as a response to hypoxia [20]. Additionally, upregulation of its receptors is common in mesenchymal subtype of GBM, which is associated with the lowest survival rates of all four subtypes [14]. Studies have demonstrated VEGFR and its ligands involvement not only in endothelial cells but also in GBM cells chemotaxis by activation of ERK1/2 pathway [21]. Moreover, sunitinib, a multi-targeted kinase inhibitor, reduced U87 and M059K glioma cells migration by inhibiting VEGFR-integrinβ(3) complex formation *in vitro* [22]. After encouraging results of *in vitro* and *in vivo* research, sunitinib has been tested as a potent antiangiogenic agent for GBM patients in clinical trial, however, regardless the high activity in renal cell carcinoma and gastrointestinal stromal tumors, compound did not have sufficient effect in GBM treatment [23-25].

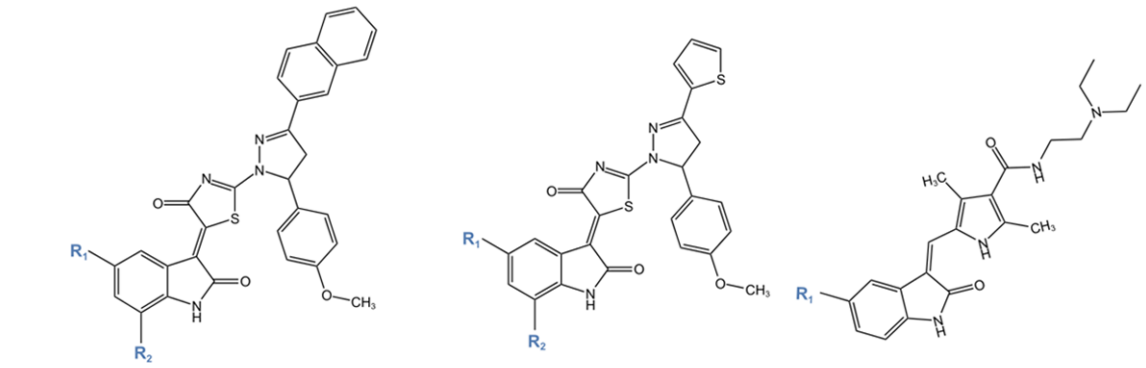
As GBM still remains incurable, it is necessary to search for new compounds that could overcome the present challenges of the treatment and could be used individually or in combination with conventional therapy. Because of high GBM vascularization level, most of targeted therapies that are being tested for GBM patients in clinical trials include antiangiogenic agents inhibiting VEGFR and PDGFR as main targets [26-29]. One of them is sunitinib, an indolin-2-one derivative inhibiting more than eight kinases with highest activity against VEGFR-1,-2,-3, PDGFR-α and PDGFR-β [30]. Since it was determined that indolin-2-one is essential for antiangiogenic properties, new compounds possessing this fragment have been developed to enhance anticancer activity. The chemical structure of sunitinib consists of 5-fluoroindolin-2-one ring, pyrrole group and amino side chain (**Figure 1**). Substitutions in indolin-2-one core and modifications of pyrrole have shown to have a significant effect on anticancer activity, VEGFR-2 inhibition and toxicity [31, 32]. Therefore, isatin/dihydropyrazole hybrids (EMAC) have been synthesized, which are suggested as more active sunitinib analogs [33].

EMAC derivatives can be categorized into two major groups: compounds possessing 2-naphthyl or 2-thiophenyl residues at C-3 position of dihydropyrazole. Additionally, new analogs possess indolin-2-one, a fragment of sunitinib, also referred as isatin (indoline-2,3-dione), which has different substitutes at C-5 and C-7 positions. We hypothesize that new sunitinib derivatives might possess stronger tyrosine kinase inhibitory properties than sunitinib and be beneficial in GBM treatment. Hence, the aim our study is to evaluate the effect of new analogs on human brain cancer cell viability, migration, and spheroid growth.

Materials and methods

Materials

EMAC derivatives were synthesized by Maccioni E. group (University of Cagliari, Italy) and pub-



	EMAC4000	EMAC4001	EMAC4002	EMAC4003	EMAC4005	EMAC4006	EMAC4007	EMAC4008	EMAC4011	EMAC4012	EMAC4014	EMAC4015	EMAC4017	EMAC4018	EMAC4019	EMAC4020	Sunitinib
R_1	-	Cl	-	CH_3	-	I	CH_3O	CH_3	-	Cl	CH_3	F	I	CH_3O	CH_3	CF_3	F
R_2	Br	-	Cl	CH_3	F	-	-	-	Br	-	CH_3	-	-	-	-	-	-

Figure 1. Tyrosine kinase inhibitor sunitinib and EMAC derivatives.

lished earlier [33]. Sunitinib malate was purchased from Sigma Aldrich (St. Louis, MO, USA).

Cell culturing

The human brain cancer U-87MG and A-172 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, USA). Glioma cells were cultures in DMEM Glutamax medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco) and 10,000 U/mL penicillin, and 10 mg/mL streptomycin (Gibco) at 37°C with 5% CO_2 in a humified atmosphere. Cell cultures were grown to 70% confluency, trypsinized with 0.125% TrypLE Express solution (Gibco) before passage and used until passage 20.

Cell viability assay

Cell viability was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich Co., ≥ 97%) reduction assay. U-87MG and A-172 were seeded into 96-well plates (Corning) in a volume of 100 µL (5×10^3 U-87MG cells/well or 4×10^3 A-172 cells/well). After 24 hours incubation, cells were treated with 100 µL of different concentrations of sunitinib analogs and sunitinib itself. Medium without cells was used as a positive control and medium with 0.5% DMSO (Sigma-Aldrich Co., ≥ 99%) was considered as a negative control. After 72 hours, cells were incubated with 0, 5 mg/mL of MTT solution for 3 hours. The formed formazan crystals were dissolved in DMSO. The absorbance was measured spectrophotometrically at wavelengths of 570 and 630 nm. Half maximal effective concentration (EC_{50}) values were calculated using the Hill equation.

Cell migration assay

Cell migration was tested using the single-cell tracking method. U-87MG and A-172 glioma cells were seeded into collagen (Gibco) coated imaging dishes (IBIDI, Martinsried, Germany) with numbered grids in a volume of 2 mL (5×10^3 cells/mL). After 24 hours incubation, cells were treated with sunitinib analogs and sunitinib at the concentrations equal to EC_{50} values, determined as described previously. After treatment with tested compounds, photos of gridded area were taken every hour by inverted microscope (Olympus), then processed using *ImageJ* software (National Institutes of Health, USA). Coordinates of cells were estimated in the X and Y axes, then distance travelled per hour was calculated using the Pythagoras theorem equation and presented as average cell velocity.

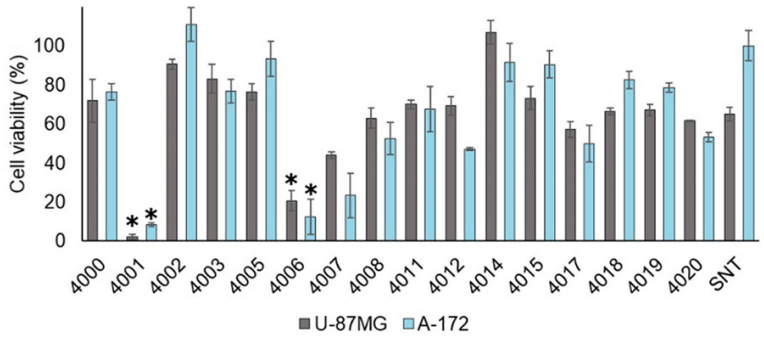


Figure 2. The effect of sunitinib analogs on human brain cancer cell viability. U-87MG and A-172 cells were treated with each compound at 1 μM concentration for 72 h and cell viability was determined by MTT assay. The asterisks (*) indicate $P < 0.05$, compared to sunitinib, n = 3. Abbreviations: SNT, sunitinib.

Spheroid growth

Spheroids were formed from U-87MG and A-172 glioma cells by the Hanging drop method. The cell suspension (5×10^5 cells/mL) was diluted with a medium containing 0.2% methylcellulose (average M_w ~90.000, Sigma-Aldrich Co.) and drops of 20 μL (1×10^3 U-87MG cells/drop or 2×10^3 A-172 cells/drop) were placed on the lid of the Petri dish (Corning). The number of cells was selected based on preliminary experiments performed at the beginning of the study. After 24 hours incubation, formed spheroids were placed into 96-well plate (Corning) coated with 2% agarose (Sigma-Aldrich Co.) and treated with 100 nM and 500 nM solutions of tested compounds. After 4 days, the medium containing solutions of tested compounds was changed by the fresh one. Images of spheroids were taken by inverted microscope (Olympus) every 24 hours for 9 days and processed with the *ImageJ* software (National Institutes of Health).

Statistical analysis

Experiments were repeated three times, calculating the mean and standard deviation. The normality of the distribution was checked using the Shapiro-Wilk test. To determine the significant differences between means of normally distributed data, the Student's t-test was performed. The analysis of abnormally distributed data was conducted using Kruskal-Wallis test. The data of migration and spheroid growth analysis is displayed in box plots in which the vertical line indicates the median and the cross indicates the mean. The level of statistical significance was set at $P < 0.05$. The data was processed using *Microsoft Office Excel 2020 (Version 2211)* and *IBM SPSS Statistics (Version 20)*.

Results

Sunitinib analogs effect on brain cancer cells viability

7 out of 16 new sunitinib analogs (compounds EMAC4001, 4002, 4005, 4015, 4018, 4019) reduced U87-MG cell viability stronger than A172 cell line. Out of all 16 sunitinib analogs, EMAC4001, 4006 and 4007 were the most active ones in both tested glioblastoma cell lines and reduced cell viability up to 44% (**Figure 2**). Based on their potent cytotoxic effect against brain cancer cells, EMAC4001, 4006 and 4007 were selected for further research.

After screening for potential novel sunitinib analogs, EC_{50} values of EMAC4001, EMAC4006 and EMAC4007 were calculated. Selected compounds showed from 18 to 69 times stronger effect on U87-MG cells viability and from 25 to 95 times stronger cytotoxic effect in A172 cell line, compared to sunitinib. The most active compound in both cell lines after 72 hours was EMAC4001, containing 5-chloroisatin fragment (EC_{50} values in U87-MG and A172 cell lines were 81.7 ± 6.0 nM and 72.3 ± 6.0 nM, respectively) (**Figure 3**).

Effect of sunitinib analogs on cell migration

For single-cell migration experiment, the compounds were tested at the concentrations equal to their estimated EC_{50} values. To evaluate biological response of glioma cells to new compounds, the average cells velocity was determined and compared among control and experimental groups.

EMAC4001 and EMAC4006 statistically significantly inhibited the migration of single U-87MG cells. The most active compound was EMAC4001, which reduced cell velocity 2 times, compared to the control, and was 28%

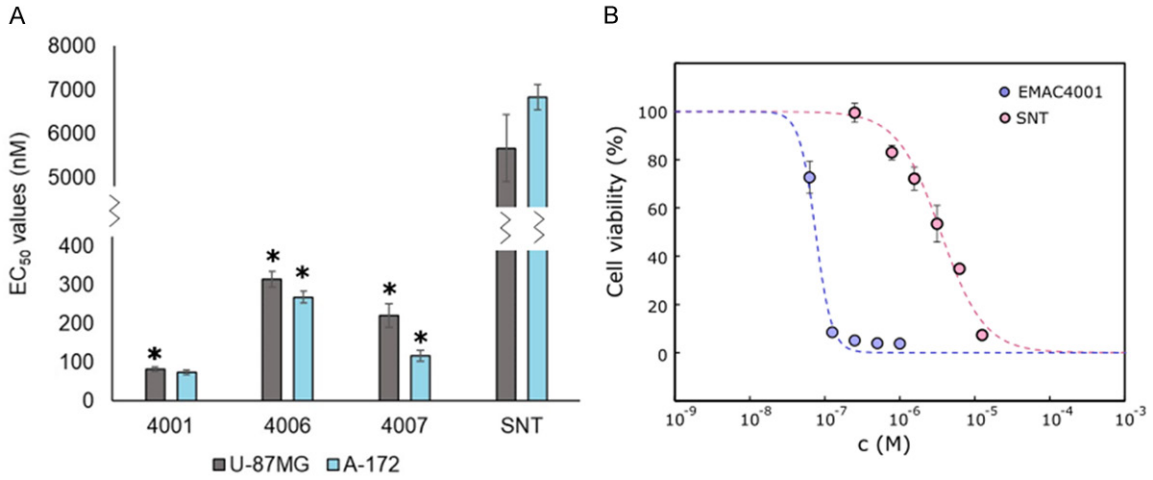


Figure 3. A. EC_{50} values of sunitinib analogs. Medium without cells was used as a positive control and cells with 0.5% DMSO served as a negative control. The asterisks (*) indicate $P < 0.05$, compared to sunitinib. B. Sunitinib and EMAC4001 effect on U-87MG cell viability after 72 h of incubation by MTT assay. Data points are experimental values (averages of three repeats) while the line is a fit of the standard inhibition model with the Hill coefficient of 6.5 (EMAC4001) and 1.5 (sunitinib). Abbreviations: SNT, sunitinib.

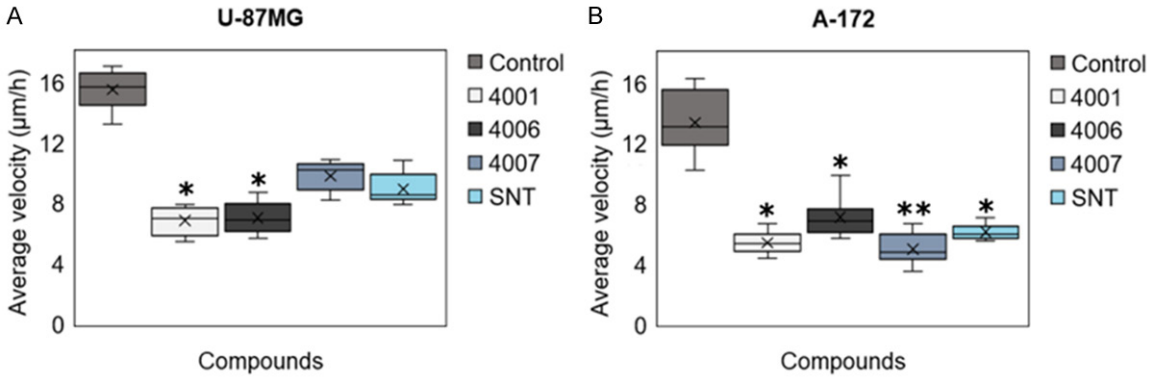


Figure 4. Effect of sunitinib analogs on (A) U-87MG cell and (B) A-172 cell migration. Cells were treated with compounds at their EC_{50} values for 8 hours. The asterisks (*) indicate $P < 0.05$ and the double asterisk (**) indicates $P < 0.01$, compared to the control. Abbreviations: SNT, sunitinib.

more active than sunitinib. Regarding the migration of A172 cells, it was found that all tested compounds showed statistically significant inhibition of cell movement from 2 to 2.5 times, compared to the control. The most active compound in A172 cell line was compound EMAC4007 (**Figure 4**). Over 6 hours, the average U-87MG cell velocity increased steadily in the control group (**Figure 5A**) and in sunitinib group cell movement started decreasing after 3 hours of treatment (**Figure 5B**). After treatment with EMAC4001 and EMAC4006, U-87MG cell movement maintained reduced for the 6 hours (**Figure 5C** and **5D**). No movement trends were observed in cell migration after treatment of EMAC4007 (**Figure 5E**). A-172 cells had similar migration routine over six hours (Data not shown).

Effect of sunitinib analogs on spheroid growth

In 3 days both cell lines were able to form compact spheroids with a diameter ranging from 260 μm to 290 μm. U-87MG spheroids were more irregularly shaped and slightly larger in the start of the experiment (day 0), however, after the treatment A172 spheroids entered the log phase and surpassed U-87MG spheroids in size in 3 days (**Figures 6** and **7**).

After treatment with 100 nM solutions of tested compounds, U-87MG spheroids continued to grow after day 5 of the experiment, with the

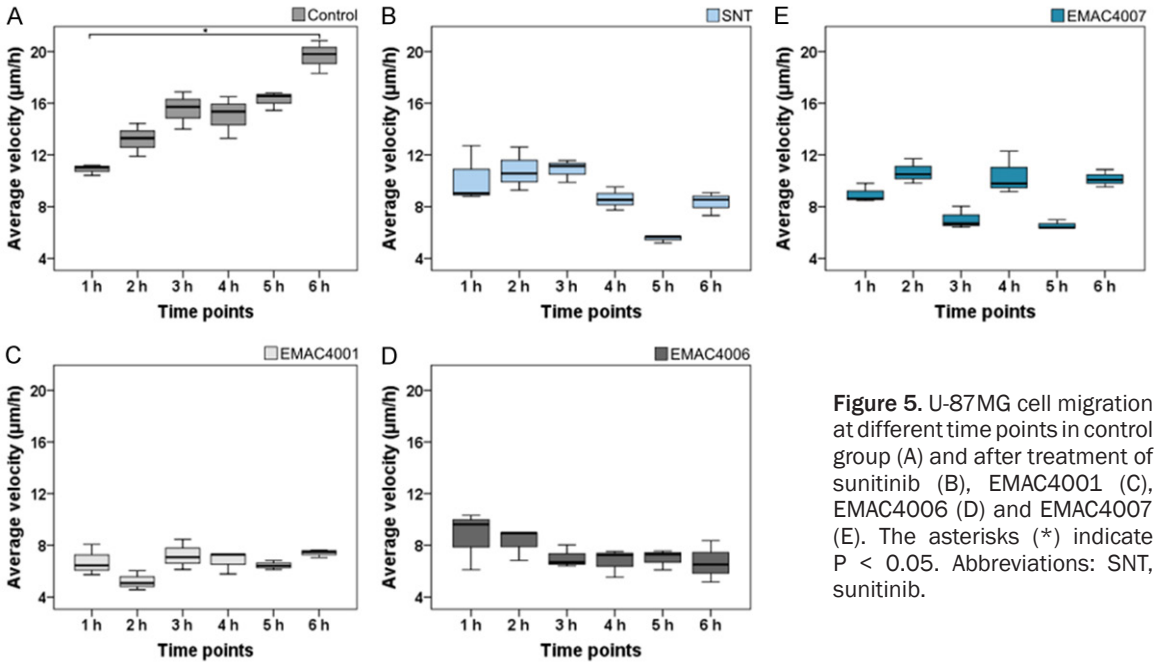


Figure 5. U-87MG cell migration at different time points in control group (A) and after treatment of sunitinib (B), EMAC4001 (C), EMAC4006 (D) and EMAC4007 (E). The asterisks (*) indicate $P < 0.05$. Abbreviations: SNT, sunitinib.

sunitinib group as an exception, as it showed steady decrease in spheroid size after day 2. A lower concentration was sufficient to reduce the size of the U-87MG spheroids from 9.6 to 34.2%, however only in sunitinib group the size change was statistically significant compared to the control. Contrary to the results in 2D models, all compounds except EMAC4001 had significant effect on U-87MG spheroid growth at higher concentration (**Figure 6**). As in migration studies, compound EMAC4007 has shown a selective effect on the A172 cell line. It was the only compound that had statistically significant impact on glioma spheroid growth at lower concentration, similarly to sunitinib (**Figure 6**).

Discussion

Our results showed that sunitinib analogs EMAC4001, EMAC4006, EMAC4007 possess stronger anticancer effect on tested GBM cell lines (U-87MG and A-172) in monolayer cells, compared to sunitinib. Chemical structure of all three compounds consists of 2-naphthyl substituent in position 3 of the dihydropyrazole ring and halogen or methoxy group in position 5 of isatin, suggesting these chemical features are beneficial for the antiproliferative activity of GBM cells. New substituents significantly increased cytotoxicity compared to the effect of sunitinib and EC_{50} values of tested analogs were up to 250 times lower in U87-MG cell line and up to 600 times lower in A172 cell line, compared to temozolomide, the main agent of GBM chemotherapy [34].

In vitro studies have shown that presence of chlorine in isatin fragment usually results in higher activity of different hybrid molecules against cancer cells [35], as halogen improves permeability to lipid membranes, increasing cellular concentration of compound. Nevertheless, optimizing the position of the substituents is essential for stronger activity. Studies performed on various compounds containing an isatin core have shown that inclusion of substituents at C-5 and C-7 positions of isatin is a promising direction for the development of hybrid molecules [36, 37]. In our case, modifications in C-5 position had clearly greater impact on the cytotoxicity against GBM cells, as EMAC4002 bearing chlorine at C-7 almost completely lost its activity in both cell lines compared to EMAC4001 containing chlorine at C-5. Additionally, inclusion of bromine at C-7 (EMAC4000) was not sufficient to achieve a strong cytotoxic effect on glioma cells, even though, bromo-substituents has shown to be beneficial for different isatin derivatives activity against myeloma and breast cancer cells *in vitro* studies [38, 39]. In our case, presence of iodine at C-5 was more favorable as EMAC4006 showed strong anticancer activity in 2D cell cultures.

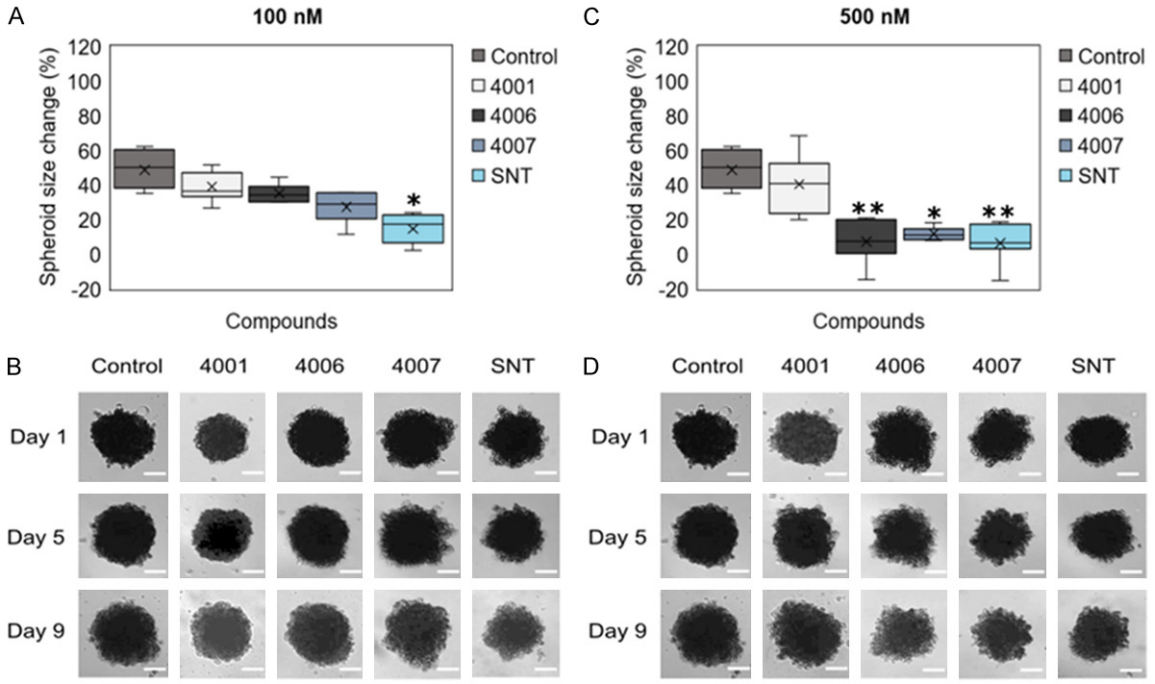


Figure 6. Effect of sunitinib analogs on U-87MG spheroid growth at (A, B) 100 nM and (C, D) 500 nM concentrations. The asterisks (*) indicate $P < 0.05$ and the double asterisk (**) indicates $P < 0.01$, compared to the control. Scale bar = 100 μm. Abbreviations: SNT, sunitinib.

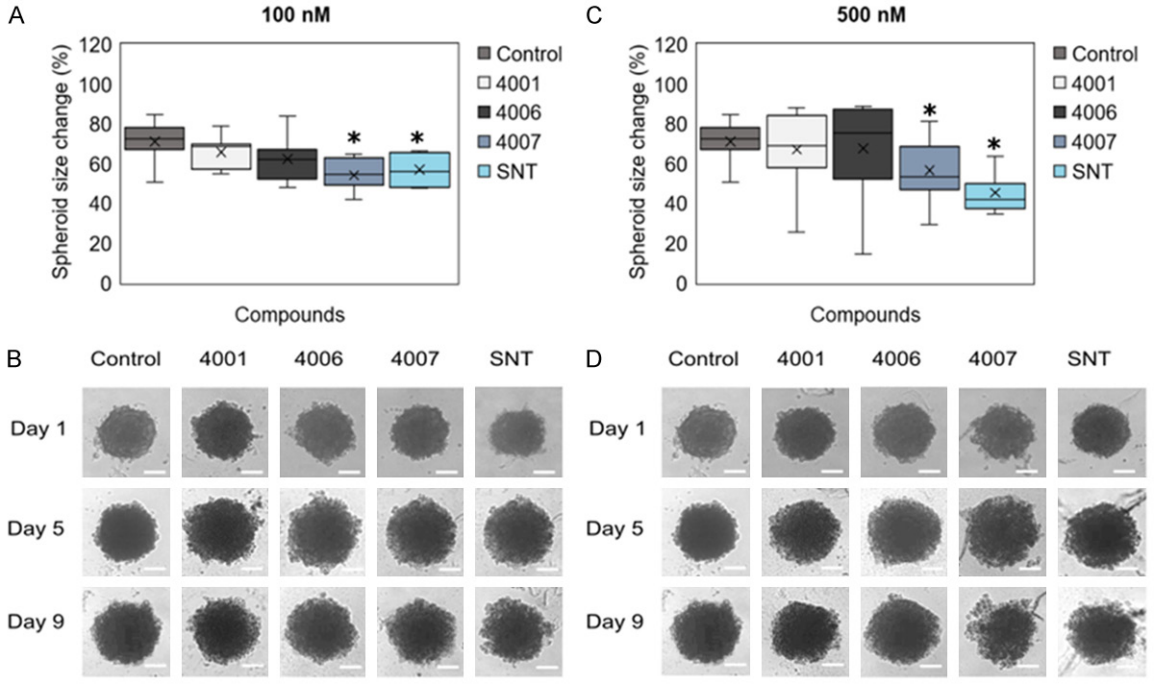


Figure 7. Effect of sunitinib analogs on A-172 spheroid growth at (A, B) 100 nM and (C, D) 500 nM concentrations. The asterisks (*) indicate $P < 0.05$, compared to the control. Scale bar = 100 μm. Abbreviations: SNT, sunitinib.

Hypervascularization of GBM depends on endothelial cells migration, which is proven to be regulated by VEGFRs, PDGFRs and their growth factors [40-42]. Like sunitinib, our tested derivatives contain flat aromatic group essential for binding to the ATP-binding pocket in kinases

[31, 33], thus we hypothesized that hybrid molecules with lipophilic substituents in isatin might have stronger kinase inhibitory effect in GBM cells, compared to sunitinib. Our results showed that addition of chlorine at C-5 position resulted in strong inhibitory effect on cell migration of EMAC4001, which surpassed sunitinib in U-87MG cell line and had similar effect to sunitinib in A-172 cell line. Iodine substituent in EMAC4006 showed advantage in reducing U-87 single cell movement, compared to sunitinib and EMAC4007. EMAC4007 containing methoxy substituent showed reduced activity in U-87MG, however it was the most active compound in A172 cell line. It should be noted that average U-87MG cell velocity remained the same for 6 hours after treatment of EMAC4001 and EMAC4006, while treatment with EMAC4007 resulted in variation in cell velocity. Interestingly, EMAC derivates had faster effect on U-87MG cell movement, as sunitinib reduced migration only after 3 hours. As it was established that analogs had different effect on single cells movement based on the cell line, it is possible that compounds have different selectivity for kinases expressed in tested glioma cells. There is evidence that U-87MG and A-172 cells express both VEGF receptors, VEGFR-1 and VEGFR-2 [21], however PDGFR-α expression is associated with U-87MG cells and PDGFR-β is upregulated in A-172 cells [43].

As expected, new derivatives showed encouraging results in human brain cancer 2D models, however, their effect on 3D structures may be described as inconsistent. EMAC4001 outperformed sunitinib in inhibiting tested GBM cells viability and migration suggesting more potent effect on RTKs essential for GBM development. Unfortunately, new sunitinib derivative did not show a significant effect in GBM spheroids meaning further investigation of permeability is needed to understand the reason for failure. Although, it is not uncommon for new molecules to lose potential activity in 3D cultures as it is proven cancer cells monolayer is more sensitive to traditional chemotherapy [44]. Hypoxia is one of the main features of tumor which is highly associated with activation of different signaling pathways leading to resistance to therapy and has been observed in 200-300 μm diameter spheroids meaning compounds that are active in 3D models are more likely to be active clinically [44, 45]. EMAC4007 was the only compound which not only surpassed sunitinib in cell viability and migration testing, but also was similarly active in 3D cultures of A-172 cells. We discovered that halogen substituents contribute to activity in 2D cultures, but methoxy group is more beneficial in 3D cultures of GBM and can be associated to selectivity for A-172 cell line. However, considering high GBM heterogeneity, the anticancer effect of EMAC4007 alone is likely to be insufficient, thus further studies of new sunitinib derivative effect in combinations with other therapeutic agents are needed.

Conclusions

Sunitinib analogs possess the anticancer activity in brain cancer U-87MG and A172 cell lines. Compounds had a stronger effect on brain cancer cell viability and migration based on the cell line. However, none of the new tested compounds were more active in 3D cell cultures than sunitinib.

Disclosure of conflict of interest

None.

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