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Alkaloids of fascaplysin are effective conventional chemotherapeutic drugs, inhibiting the proliferation of C6 glioma cells and causing their death *in vitro*

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Abstract. Glioblastoma multiforme is an invasive malignant glial brain tumor with a poor prognosis for patients. The primary reasons that lead to the development of treatment resistance are associated with tumor cells infiltrating the brain parenchyma and the specific properties of tumor stem cells. A crucial research area in medical science is the search for effective agents that are able to act on these targets. Fascaplysin alkaloids possess potent antitumor activity. Modern methods for the targeted delivery of drugs reveal extensive possibilities in terms of the clinical use of these compounds. The aim of the present study was to establish effective concentrations of fascaplysin that inhibit the growth and kill the cells of glial tumors, as well as to perform a comparative analysis of fascaplysin's effectiveness in relation to other chemotherapy drugs. C6 glioma cells were utilized as an optimal model of glioblastoma. It was established that fascaplysin at 0.5 μM has a strong cytotoxic effect, which is subsequently replaced by tumor cell death via apoptosis as the length of drug exposure time is increased. Fascaplysin kills glioma cells at a dose higher than 0.5 μM. The efficiency of fascaplysin was observed to significantly exceed that of temozolomide. Therefore, a significant feature of fascaplysin is its ability to inhibit the growth of and kill multipotent tumor cells.

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Introduction

According to the WHO classification, glioblastoma multiforme is the most common highly invasive primary glial tumor of the human brain, which is more common in the second half of life and occurs predominantly in men (1). Glioblastoma multiforme accounts for >50% of all primary brain tumors and ~20% of all intracranial tumors (2). Current treatment includes radical removal of the tumor; surgical treatment may be complemented by irradiation at a dose of up to 70 Gy and chemotherapy, which may increase the duration of relapse-free survival (3). The preferred chemotherapeutic drug is temozolomide (4). The prognosis for patients is poor and the median survival time is 12-14 months. Despite the best efforts of oncologists, only 10% of patients survive more than 18 months from the initial point of diagnosis, and the overall 5-year survival rate is close to zero (5).

A significant cause of recurrent glioblastoma is due to infiltration of brain parenchyma by the tumor cells (6). During the process of invasive growth, the neoplastic cells migrate far beyond the neoplastic node, meaning that traditional surgical methods are ineffective (7). Attempts to solve this issue by increasing the dose of radiation leads to the development of brain damage (8). Classical cytostatics are not effective at targeting cells outside the phases of mitosis (9). The application of modern targeted therapies has had no apparent success. Furthermore, the choice of drugs is limited by the selective permeability of the blood-brain barrier and the use of systemic chemotherapy is limited by the general condition of the patient (10).

The effectivity of chemotherapy may be significantly extended through the introduction of cellular and biopolymer

technologies (11). Aboody *et al* (12) described the phenomenon of 'molecular adhesion'. During this process, stem cells migrate following tumor cells that begin to metastasize to the brain parenchyma. Once the target is reached, the stem cells attach to the neoplastic cells (12). Such stem cells may be used for targeted delivery of therapeutic agents to the brain parenchyma. Stem cells may be conjugated with immunoliposomes or nanocapsules that contain drug substance (13). The implantation of such cells in a biopolymer gel that fills the defect of brain tissue following removal of the tumor provides a direct treatment for any remaining neoplastic cells, leading to prevention of relapse. The drug substance may be in biodegradable containers, and incorporated into a biopolymer gel. The specific wall thickness of the containers will provide a systematic release of the drug substance, thereby avoiding the toxic effects of chemotherapy (14).

Application of biomedical technologies extends the capabilities of chemotherapy and has led to the search for novel drugs that possess the ability to inhibit proliferation and induce cell death in glial tumors. The present study focused on a group of alkaloids, which are based on a pentacyclic system of pyrido[1,2-a:3,4-b']diindola known as fascaplysin alkaloids (15). The most well-known member of this group is the red pigment fascaplysin, which was first isolated from a marine sponge *Fascaplysinopsis* sp. in 1988 (15). This compound has a broad spectrum of biological activity, combining antimicrobial, antifungal, antiviral and antitumor activity (16). The action of fascaplysin is mediated by the activation of BH3 interacting-domain death agonist protein, which alters the conformation of proapoptotic protein bcl-2-like protein 4 leading to the release of cytochrome *c*, therefore activating caspases 3, 8, 9 and triggering apoptosis (17). Activation of the receptor family ligands of tumor necrosis factor and suppression of the anti-apoptotic B-cell lymphoma 2 (Bcl-2) protein family causes the death of vascular endothelial cells, which inhibits the formation of neoplastic vascular networks (17). These properties mean that fascaplysin may be a promising novel substance for the treatment of glioblastoma. Antitumor effects of fascaplysin alkaloids have been observed in a number of cell lines, including HeLa, BEL-7402, THP-1, SNU-C4, SK-MEL-28, DLD-1 and MDA-MB-231, as well as a number of solid tumors; however, it has not been considered as a potential agent for the treatment of glioblastoma (18,19). Modern cellular and polymer technologies allow the creation of local concentrations of substances that are capable of rendering a strong directional impact on neoplastic cells (20).

The aims of the present study were to establish effective concentrations of fascaplysin by evaluating its anti-proliferative and cytotoxic activity against C6 glioma cells *in vitro*, to study the characteristics and mechanisms of the antitumor action of fascaplysin and to evaluate fascaplysin's effectiveness in comparison with other chemotherapy drugs.

Materials and methods

Materials. The present study used the red pigment fascaplysin (12,13-dihydro-13-oxopyrido [1,2-a:3,4-b']diindol-5-ium chloride), the C6 malignant glioma cell line and human fibroblasts.

Fascaplysin. All experiments described in the present study were performed with synthesized fascaplysin. Fascaplysin was synthesized according to the two-step synthesis protocol as described previously (21). The main spectral characteristics (infra-red, mass spectrometry, ^{1}H and ^{13}C nuclear magnetic resonance) of the synthesized fascaplysin were identical to those published for the natural product (22).

C6 glioma cells. Glioma cells are the most commonly used model of human glioblastoma. For the present experiment, C6 glioma cells (American Type Culture Collection, Manassas, VA, USA; CCL-107), previously frozen at -80˚C, were thawed at 37˚C for 10 min and washed to remove dimethyl sulfoxide (Ameresco, Inc., Framingham, MA, USA; Am-O231-0.5), and were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.; 41965-039) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.; 16000-036) and 100X antibiotic-antimycotic solution (Gibco; Thermo Fisher Scientific, Inc.; 15240-062). Prior to experimental use, cells were pelleted by centrifugation (120 x *g* at 20˚C for 3 min), fresh medium was added, and cells were resuspended and seeded into culture flasks. The culture was continued until a monolayer formed. Cells were removed using enzymatic dissociation by TrypLE™ Express (Gibco; Thermo Fisher Scientific, Inc.; 12604-021) at 37˚C for 10 min, followed by centrifugation (120 x *g* for 3 min at 20˚C). Subsequently, fresh medium was added and cells were resuspended. Immediately prior to the commencement of experiments, the cells underwent immunocytochemical characterization.

Immunocytochemical study of C6 glioma cells. Prior to performing the immunocytochemical analysis, cells were evaluated for viability by staining with 0.4% trypan blue (Gibco; Thermo Fisher Scientific, Inc.; 15250-061) and the cells were counted using a hemocytometer. Cells were seeded in a 24-well plate (Greiner Bio-One Ltd., Stonehouse, UK; 662892) at $25x10^3$ cells per well and cultured in DMEM for 2 days at 37˚C in an atmosphere of 5% CO_2. Cells were fixed according to the following methodology: 4% paraformaldehyde for 20 min, at 4˚C, followed by washing with a stock solution composed of phosphate-buffered saline (PBS; pH 7.4; Gibco; Thermo Fisher Scientific, Inc., 10010-023), 0.2% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA; P9416), 0.2% Triton-X-100 (Sigma-Aldrich; T8787) and 0.3% bovine serum albumin (Sigma-Aldrich; A2058) 3 times, each for 10 min.

Immunocytochemical staining was performed according to the following protocol: The primary antibodies against oligodendrocyte 4 (mouse monoclonal; dilution, 1:50; MAB1326; R&D Systems, Inc., Minneapolis, MN, USA), glial fibrillary acidic protein (GFAP; mouse polyclonal; dilution, 1:50; ab7260; Abcam, Cambridge, MA, USA), p53 (mouse monoclonal; dilution, 1:100; AHO0152; Thermo Fisher Scientific, Inc.), anti-tubulin-β-III (mouse monoclonal; dilution, 1:100; ab7751 Abcam), nestin (polyclonal rabbit; dilution, 1:100; N5413; Sigma-Aldrich), cluster of differentiation (CD)133 (rabbit polyclonal; dilution, 1:100; PA5-38014; Invitrogen; Thermo Fisher Scientific, Inc.) S100 (rabbit polyclonal; dilution, 1:100; ab868; Abcam) were incubated with cells for 18-20 h at 4˚C. Following incubation, the cells were washed by stock solution 3 times, each for 10 min. Secondary antibodies,

Alexa Fluor® 488-conjugated rabbit anti-mouse polyclonal immunoglobulin (Ig) G (dilution, 1:500; A11059; Thermo Fisher Scientific, Inc.) or Alexa Fluor® 633-conjugated goat anti-rabbit polyclonal IgG (dilution, 1:500; A21071; Thermo Fisher Scientific, Inc.), were incubated with the cells for 2 h at 37˚C. Following incubation, the samples were washed with the stock solution 3 times, each for 10 min, and cell nuclei were stained with 4',6-diamidino-2-phenylindole (Molecular Probes; Thermo Fisher Scientific, Inc.; D1306) for 7 min at 22˚C. Subsequently, an additional wash with stock solution was performed 2 times, each for 10 min, and cells were enclosed in Mowiol® 4-88 (Sigma-Aldrich; 324590). Controls were performed where cells where incubated with secondary antibodies without prior use of the primary antibodies. Primary and secondary antibodies were used according to the manufacturer's protocol.

Culture of human fibroblasts. Primary cultures of human fibroblasts were used (Thermo Fisher Scientific, Inc.; S-004-5S). Cells were previously frozen at -80˚C. The cells were thawed according and cultured in complete DMEM/Ham's F12 (Gibco; Thermo Fisher Scientific, Inc., 11330-032) containing 10% FBS, 2 mM L-glutamine (Gibco; Thermo Fisher Scientific, Inc.; 25030-081), 0.8% glucose (Gibco; Thermo Fisher Scientific, Inc.; A2494001), 0.2 units/ml insulin (Gibco; Thermo Fisher Scientific, Inc.; 12585-014) and 100X antibiotic-antimycotic solution.

Investigation into cytotoxicity and cell death caused by fascaplysin. Plates (24-well) were seeded with C6 glioma cells ($7x10^4$ per well) and incubated for 48 h at 37˚C in an atmosphere of 5% CO_2 to establish concentrations of fascaplysin that exerted a cytotoxic effect. The first row of the plate wells was used as a control, and a solution of fascaplysin was added to the remaining wells at concentrations of 2, 1.5 and 1 μM. Counting of cells was performed in an automated manner using a Cell-IQ® MLF system (CM Technologies GmbH, Elmshorn, Germany).

C6 glioma cells and human fibroblasts ($5x10^3$) were cultured in three 96-well plates (Greiner Bio-One; 662120), filled with DMEM containing 10% FBS, 10 ng/ml fibroblast growth factor (Gibco; Thermo Fisher Scientific, Inc.; PHG0023), 10 ng/ml epidermal growth factor (Gibco; Thermo Fisher Scientific, Inc.; PHG0311 L) and 100X antibiotic-antimycotic solution at 37˚C in an atmosphere of 5% CO_2 to establish effective concentrations of fascaplysin that exerted an anti-proliferative effect. The first row of the plate wells was used as a control, and a solution of fascaplysin was added to the remaining wells at concentrations of 0.5, 0.05 and 0.005 μM.

The first 96-well plate was incubated for 96 h at 37˚C in an atmosphere of 5% CO_2. Counting of live and dead cells was performed in an automated manner using a Cell-IQ MLF system. The second 96-well plate was used for staining in order to observe the apoptosis of C6 glioma cells following 6 h of exposure to fascaplysin with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method, using the Click-iT TUNEL Alexa Fluor® 488 Imaging Assay (Molecular Probes; Thermo Fisher Scientific, Inc.; C10245). The third 96-well plate with C6 glioma cells was used to study the mechanisms of cell death by flow cytometry following 6 h of exposure to fascaplysin.

Flow cytometry. The present study used two methods to assess the level of apoptosis in C6 glioma cells. The first method was based on the simultaneous use of two fluorescent dyes, which were YO-PRO-1 iodide (Molecular Probes; Thermo Fisher Scientific, Inc.; Y3603) and propidium iodide (PI; Sigma-Aldrich; P4864). The dye ligand is nucleic acid, which is localized in the cytoplasm of cells (23). Penetration of YO-PRO-1 into the cell occurs via P2X purinoceptor 7 ligand-dependent ion channels that are not active in intact cells (24). Activation occurs when apoptosis commences and coincides with the violation of the asymmetry of the lipid composition of the membrane surface, which means that accumulation of YO-PRO-1 is an event that is indicative for the early stages of apoptosis (24).

To detect the later stages of apoptosis, besides using the dye YO-PRO-1, the cells were additionally stained with PI. PI does not have any specific carriers integrated into the membrane and is able to penetrate into the cytoplasm and nucleus only through damaged membranes; this typically is able to take place in the final stages of apoptosis during the formation of apoptotic bodies or cell necrosis (25). Thus, living cells in the sample were not stained by YO-PRO-1 or PI. Stained cells that have entered into apoptosis, will be positive only to YO-PRO-1, whereas cells that are in the later stages of apoptosis will be effectively stained by both dyes.

Cell staining was performed in 12x75 mm cytometric tubes (Globe Scientific, Inc., Paramus, NJ, USA; 110410) for cytometric study. A total of 5 μl YO-PRO-1 solution was added to 100 μl of the cell suspension ($1x10^6$ cells/ml) to give a final dye concentration of 250 nM. Subsequently, 10 μl of propidium iodide solution was added to the samples to give a final concentration of PI that was equal to 1 μg/ml. Staining was performed at room temperature for 10 min in the dark. Upon completion of staining, 100 μl of PBS was added to the samples and samples were analyzed by a flow cytometer (BD Accuri™ C6; BD Biosciences, Franklin Lakes, NJ, USA).

At least 10,000 single cells were analyzed for each sample. Prior to the experiment, the cells were treated using enzymatic dissociation by TrypLE™ (Gibco; Thermo Fisher Scientific, Inc.; 12604-021) at 37˚C for 5 min, followed by centrifugation (120 x *g* for 3 min, at 20˚C). Cell aggregates were discriminated from the analysis. Analysis of the results was performed using Kaluza® software (version 1.3; Beckman Coulter, Inc., Brea, CA, USA).

The second staining approach was based on the simultaneous use of fluorescein diacetate (FDA; Sigma-Aldrich; F7378) and PI (26). It is thought that an effect of triggering apoptosis in cells is reduced activity of intracellular enzymes, primarily intracellular esterases (hydrolase class of enzymes) that are responsible for the hydrolytic cleavage of esters to alcohols and acids, with the participation of water molecules (25). FDA dye is activated by esterase and also exhibits lipophilic properties, allowing spontaneous penetration into cells from the culture medium. Following reaction with the enzymes a loss of hydroxyl groups occurs, which is accompanied by the accumulation of the dye in the cell and induction of fluorescence in response to laser light (27).

Thus, living cells possess bright fluorescence, whereas fluorescence of the FDA in cells undergoing death is considerably reduced. Additional PI staining of these cells allows discrimination between the early and late stages of apoptosis as PI is able to penetrate into the cytoplasm only in cases of violation of the integrity of the surface membrane.

Cell staining was performed in 12x75 mm cytometric tubes. A total of 1 μl of FDA was added to 100 μl of the cell suspension ($1x10^6$ cells/ml) to give a final dye concentration of 100 ng/ml. Staining was performed at room temperature for 10 min in the dark, and following staining cells were washed once by PBS containing 2% FBS (Gibco; Thermo Fisher Scientific, Inc.; 16000-036). Cells were centrifuged at 350 x *g* for 7 min at 20˚C. The cell pellet was resuspended in 200 μl PBS, after which 10 μl of propidium iodide was added to the samples, to give a final concentration of 1 μg/ml. Following completion of incubation (10 min at 20˚C), samples were analyzed on a flow cytometer (BD Accuri™ C6). At least 10,000 single cells were analyzed for each sample.

Comparison of fascaplysin with other chemotherapeutic drugs. An assessment of the antitumor effects of fascaplysin compared with other chemotherapeutic drugs was performed. A total of $3x10^4$ C6 glioma cells were seeded into 24-well plates (Greiner Bio-One Ltd.; 662160). Subsequently, 0.5 μg/ml dexamethasone, 0.5 μg/ml temozolomide and 0.5 μg/ml fascaplysin were added to the wells. A row of untreated wells with glioma C6 cells was used as a control. A cell count was performed automatically using the Cell-IQ® MLF system.

Dexamethasone. The present study used 4 mg/ml dexamethasone (Galenika a.d., Zemun, Serbia). Dexamethasone is a mandatory component of the regimen used for the treatment of glial tumors (28).

Temozolomide. Temozolomide is the standard chemotherapeutic treatment for invasive gliomas (29). The effectiveness of this drug is due to alkylation of guanine at position O6 and N7, which is disturbs the structure and synthesis of DNA (30). The drug Temodal® (Schering-Plough Labo nv; Merck Sharpe & Dohme, Hoddesdon, UK) was used in the present study.

Sample visualization. The FluoView FV 1200 MPE confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan) and LSM 710 confocal system (Zeiss GmbH, Jena, Germany) were used to visualize samples. Image processing was executed using ImageJ software version 4.1 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Statistical analysis was performed using analysis of variance, followed by Tukey's post-hoc test. Data are expressed as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference. All statistical tests were performed using GraphPad Prism version 4 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Immunocytochemistry of glioma C6. C6 glioma cells were stained with antibodies to nestin (96.7±2.8%) and p53 (88.4±3.8%; Fig. 1A-C), and the number of GFAP-positive cells was very low (4.5±1.4%). Tumor cells rapidly adhered to the bottom of the plate, and flattened and proliferated (Table I). Following 1 day of culture the number of nestin-positive cells significantly decreased (52.5±6.5%; P=0.01), and the cells became round and polygonal in shape. Cells stained positively for the calcium binding protein S100 (68.8±4.8%), oligodendrocyte 4 (46.2±3.7%) and tubulin-β-III (55.2±4.8%), as well as GFAP (38.2±3.5%); the number of cells stained positively for mutant p53 protein remained relatively unchanged.

Investigation of cytotoxic and cytostatic effects of fascaplysin in vitro. Fascaplysin at a concentration of 2-1 μM strongly inhibited proliferation and induced death of glioma cells. The cytotoxic effect became more marked with an increasing concentration of fascaplysin in the culture medium. Following 48 h of observation, the number of live cells in the sample treated with 1, 1.5 and 2 μM of fascaplysin was minimal (Fig. 2).

Fascaplysin at a concentration of 0.005 μM had no significant effect on the rate of tumor cell proliferation and showed no significant difference compared with the control (P=0.22). Tumor cells were attached and flattened on the bottom of the plate, where they proliferated and formed various morphological forms, including spindle-shaped and polygonal cells, which formed a monolayer. When the concentration of fascaplysin was increased to 0.5 μM, a marked anti-proliferative effect was observed, and with increasing concentrations of the substance a cytotoxic effect was also observed (Figs. 2 and 3). Exposure of cells for 72 h in a medium containing 0.5 μM of fascaplysin slowed the rate of fibroblast proliferation, but did not lead to a significant increase in the number of dead cells compared with glioma C6 cells treated with fascaplysin (P=0.094), indicating the preferential cytostatic and cytotoxic effects. Following 120 h in culture containing 0.5 μM of fascaplysin, morphological features of apoptosis were detected in ~50% of C6 glioma cells (Fig. 4). At the same time, following 120 h in culture containing 0.5 μM of fascaplysin, the dynamic of fibroblast growth was higher (~70%) compared with glioma C6 cells treated with fascaplysin at 120 h, indicating preferential cyctostatic and cytotoxic effects on tumor cells.

A total of 3 h subsequent to the beginning of the experiment, in the culture of glioma containing 0.5 μM fascaplysin, there was a marked decrease in the rate of proliferation, as well as changes in cell shape to predominantly circular or oval and a sharp deterioration in adhesiveness. After 6 h, the initial signs of nuclear fragmentation were observed in neoplastic cells. Staining by the TUNEL method revealed a large number of fluorescent apoptotic cells, which indicated oligonucleosome DNA degradation (Fig. 5).

According to the results of flow cytometry performed using two independent methods to assess the level of apoptosis, fascaplysin at a final concentration of 0.5 μM significantly increased the relative content of glioma cells at various stages of apoptosis ($P<0.001$). The number of apoptotic glioma cells stained with YO-PRO-1 (early apoptosis) and the number of dead cells stained with PI (late apoptosis) at a given concentration of fascaplysin was maximal. Furthermore, the number of live cells that were intensely stained by FDA at a concentration of 0.5 μM fascaplysin was minimal (Fig. 6; Table II).

Table I. Immunocytochemical staining of C6 glioma cells prior to and following the adhesion of cells.

Marker	Stained cells prior to adhesion, %	Stained adherent cells, %
Nestin	96.7±6.8	52.5± 6.5
Oligodendrocyte 4	-	46.2±3.7
S100	-	68.8±4.8
Tubulin-β-III	-	55.2±4.8
GFAP	4.5±1.4	38.2±3.5
p53	88.4±3.8	85.9±2.6

Data are presented as the mean ± standard deviation. GFAP, glial fibrillary acidic protein.

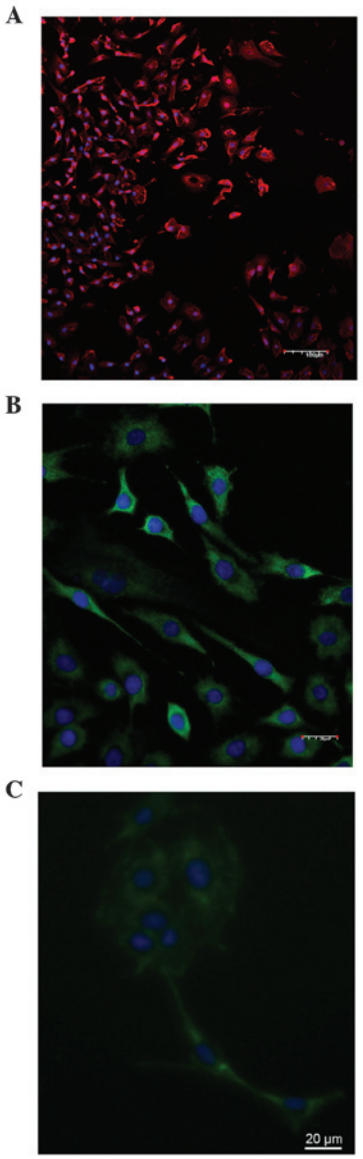

Figure 1. Immunocytochemistry of C6 glioma cells. (A) Cells were stained with nestin antibody. Nuclei were counterstained with DAPI. Magnification, x10. (B) Cells were stained with p53 antibody. Nuclei were counterstained with DAPI. Magnification, x40. (C) Cells were stained with glial fibrillary acidic protein antibody. Nuclei were counterstained with DAPI. Magnification, x40.

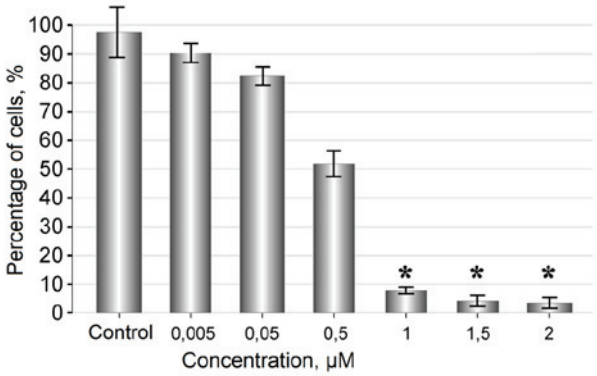

Figure 2. Growth dynamics of the total number of C6 glioma cells following 48 h of fascaplysin exposure at concentrations of 2, 1.5, 1, 0.5, 0.05 and 0.005 μM. Fascaplysin at 2, 1.5 and 1 μM strongly inhibited proliferation of glioma cells. Automated counting was performed in the Cell-IQ system (observation period, 48 h). *P<0.05 compared with the control.

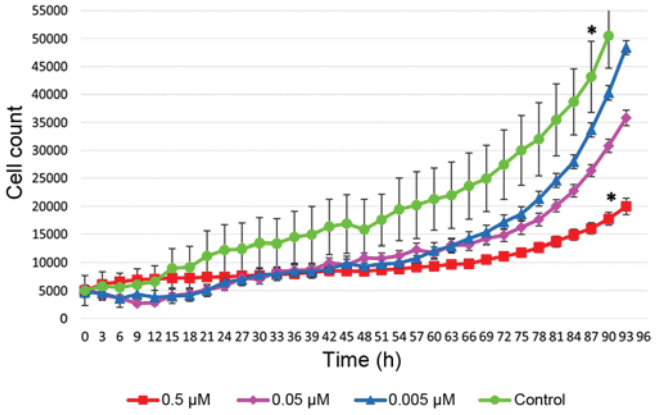

Figure 3. Proliferation of C6 glioma cells following exposure to fascaplysin at concentrations of 0.5, 0.05 and 0.005 μM. (A) The growth dynamics of C6 glioma cells, assessed by cell counting (observation period, 96 h). Fascaplysin at concentration 0.5 μM significantly inhibited cell proliferation. *P<0.05 compared with the control.

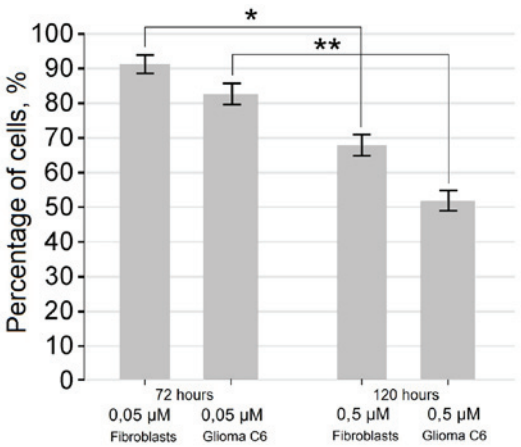

Figure 4. Growth dynamics of the total number of C6 glioma cells and fibroblasts (observation period, 72 and 120 h) following exposure to fascaplysin at 0.005 and 0.5 μM. ***P<0.05 compared different concentrations of fascaplysin between glioma C6 cells and fibroblasts at different time intervals.

Table II. Ratio of live and dead cells in C6 glioma cell culture with 0.5 μM fascaplysin according to flow cytometry data.

Time, h	Live C6 glioma cells, % FDA stained	Apoptotic C6 glioma cells, % YO-PRO-1 stained
24	65.12±9.4	24.61±5.4
48	48.13±11.2	31.7±7.3
72	24.6±14.2	53.7±8.3

Data are presented at the mean ± standard deviation. FDA, fluorescein diacetate.

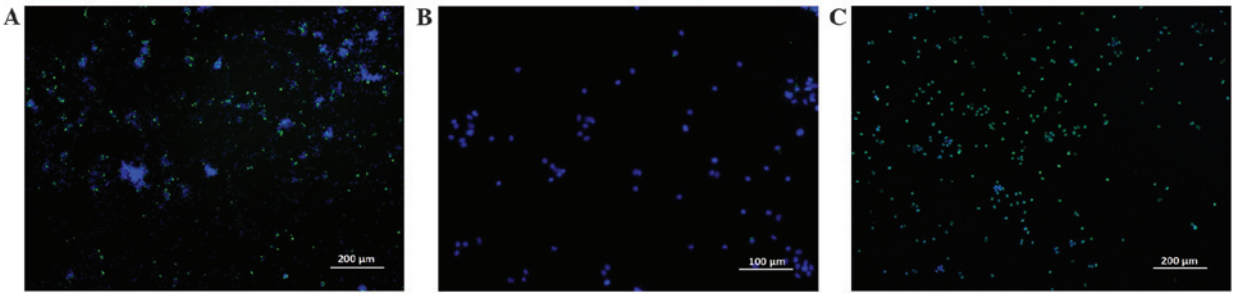

Figure 5. Staining by terminal deoxynucleotidyl transferase dUTP nick end labeling method following 6 h exposure to 0.5 μM fascaplysin in C6 cell culture. (A) Initial first signs of nuclear fragmentation of neoplastic cells, with a large number of fluorescent apoptotic cells, which indicated oligonucleosomic DNA degradation (green). (B) Control (positive) cells. Prior to staining, cells were pretreated with DNAse to induce apoptosis. (C) Control (negative) glioma C6 cells not treated by fascaplysin.

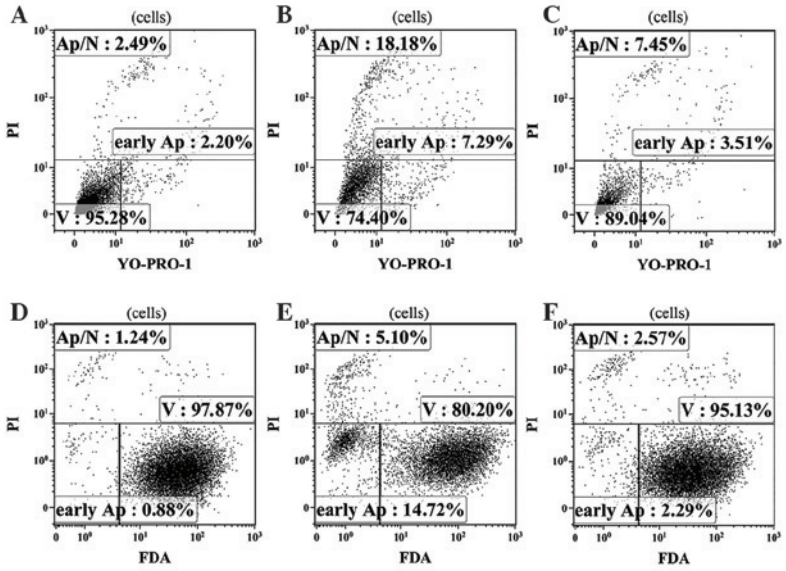

Figure 6. Histograms of the apoptosis of C6 glioma cells following 6 h of exposure to fascaplysin. (A) C6 glioma cells were incubated in culture medium (negative control). (B) C6 glioma cells following 6 h of exposure to 0.5 μM fascaplysin. (C) C6 glioma cells following 6 h exposure to fascaplysin at concentration 0.05 μM. The abscissa shows the fluorescence intensity of YO-PRO-1 iodide (λ=533±30 nm), and the ordinate shows the fluorescence intensity of propidium PI (λ>670 nm). The 'V' population of living cells was not stained by any of the dyes; 'early Ap' cells were stained only by YO-PRO-1 iodide, which corresponds to the early stages of apoptosis; 'Ap/N' dead cells were positively stained with both dyes, which corresponds to the later stages of apoptosis and necrosis. The result is expressed as a percentage of the total cells analyzed. (D) C6 glioma cells incubated in culture medium (negative control). (E) C6 glioma cells following 6 h of exposure to 0.5 μM fascaplysin. (F) C6 glioma cells following 6 h of exposure to 0.05 μM fascaplysin. The abscissa shows the fluorescence intensity of FDA (λ=533±30 nm), and the ordinate shows the fluorescence intensity of PI (λ>670 nm).'V' represents the population of living cells, which were intensely stained by FDA; 'Ap' cells were weakly by stained FDA and negative for PI, which corresponds to the early stages of apoptosis; 'Ap/N' dead cells were weakly stained by the FDA and intensely incorporated PI, which corresponds to the later stages of apoptosis and necrosis. The result is expressed as a percentage of the total cells analyzed. FDA, fluorescein diacetate; PI, propidium iodide.

It should also be noted that the decrease in FDA fluorescence in C6 glioma cells during the early stages of apoptosis, when cells have not yet lost their plasma membrane integrity, indicates a shortage of adenosine triphosphate (ATP) in the cytoplasmic compartment of the cell, as the early stages of apoptosis are considered to be ATP-dependent (31). Therefore,

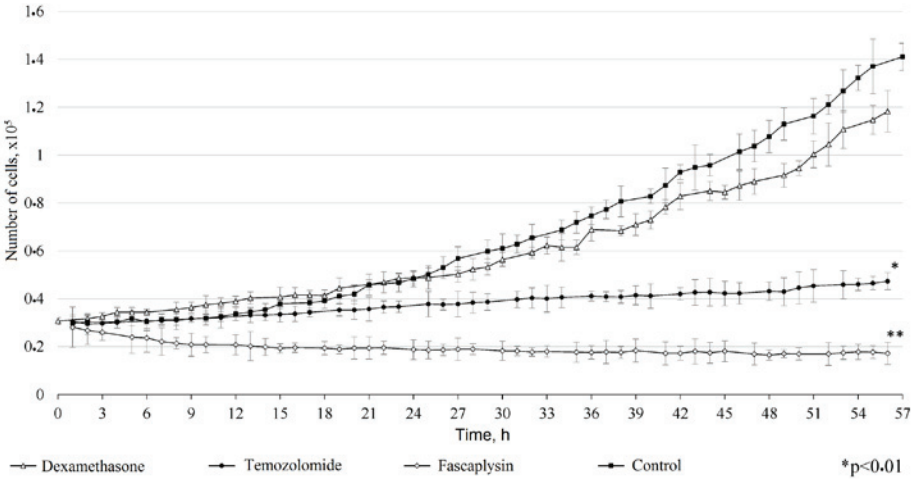

Figure 7. Comparative assessment of the antitumor effects of 0.5 μM fascaplysin, 0.5 μM dexamethasone and 0.5 μM temozolomide on C6 glioma cells. Fascaplysin at concentration 0.5 μM more effectively inhibited cell proliferation and caused cell death.

it may be assumed that a direct and/or indirect influence of fascaplysin treatment on cells is associated with impaired mitochondrial membrane potential and a decrease in the efficiency of ATP-synthetase, which is accompanied by a decrease in the overall level of ATP in the cytoplasm and induction of apoptosis.

The results of the present study confirm the data obtained by the Cell-IQ MLF system. Reducing the final concentration of fascaplysin to 0.005 μM was accompanied by a decrease in the number of cells undergoing apoptosis.

Comparison of the antitumor effect of fascaplysin with other drugs. Comparison of the antitumor effect of fascaplysin with other drugs used for the treatment of tumors revealed a number of features. *In vitro*, dexamethosone and temozolomide demonstrated inferior cytotoxic efficiency compared with fascaplysin. Dexamethasone weakly inhibited the growth of glioma cells and did not show a significant difference compared with the control (P=0.15). Addition of dexamethasone to the culture medium did not alter the morphology of the tumor cells, but increased the time of adhesion to the surface of the cultural plate. Temozolomide prevented the proliferation of tumor cells, but its cytotoxic effect was dependent on the exposure time. Overall, the inhibition of tumor cell growth by fascaplysin was more marked compared with the other drugs investigated (Fig. 7; P<0.001).

Discussion

Glioblastoma multiforme is one of the most aggressive human brain tumors (32). An important reason for the development of resistance to treatment in glioblastoma is associated with tumor stem cells (33). A number of arguments have provided evidence in favor of the origin of tumor stem cells of glioblastoma from the neural stem cells of the human brain. This is indicated by the similarity in basic cell surface markers and other immunocytochemical clusters of differentiation in tumor cells compared with neural stem cells, which has been reported to be 63.5% (34).

Adhesion to the substrate is a strategically important regulatory mechanism of tumor stem cells (35). In the present study, the majority of free-floating 'gliomaspheres' were stained with antibodies against nestin, which is found in intermediate filaments and is one of the key markers of neural stem cells (36). An important distinction of C6 glioma cells from neural stem cells is the presence of the mutant protein p53 (37). The ability to transform into other cell immunophenotypes indicates multipotency, which is also a key feature of stem cells (38). Thus, the culture of C6 glioma cells used in the present study contained a high number of stem cells, allowing the assumption that the described method will be effective for treatment of cells of this type.

Invasion is one of the main features of glioblastoma progression (39). One of the most important conditions for the initiation of the invasive process is the predominance of proliferation over apoptosis, allowing the tumor to accumulate the necessary quantitative potential for penetration into the surrounding tissues and organs (39). The tumor acquires the capacity to invade surrounding tissues as a result of genetic and biochemical changes, which allow cells to undergo proliferation (40). Fascaplysin is characterized by a strong anti-proliferative activity against C6 glioma cells. This effect is in direct proportion to the exposure time and the concentration of the substance. A gradual increase of fascaplysin concentration in the culture of tumor cells from 0.005 to 0.5 μM causes a distinct reliable cytostatic effect, and death of the majority of the cell population with a further increase in time of exposure in the culture medium. Increasing the concentration of fascaplysin up to 2 μM is accompanied by increased apoptosis of neoplastic cells. It may be assumed that the local release of fascaplysin will have a profound anti-proliferative action against tumor cells infiltrating the brain, exceeding the effects of irradiation and chemotherapy. Increasing the exposure time to fascaplysin and increasing the local concentration leads to the death of neoplastic cells and the inhibition of tumor growth in contrast to the majority of modern drugs.

Creating local concentrations of fascaplysin by targeted transport of substances in tumor foci may have a pronounced cytoreduction effect, which is particularly important for inoperable tumors (41). In the present study, a feature of the cytostatic and cytoreduction effects of fascaplysin was a

predominant targeting of actively proliferating tumor cells, with a lesser impact on fibroblasts. Our present study further suggests that fascaplysin, as used in the present study but released from capsules, will not have a negative effect on fibroblasts and other cellular elements that penetrate the modern biodegradable matrix and form loose connective tissue. This may be identified in future studies by delivering fascaplysin by capsules and the use of biodegradable matrices.

The antitumor effect of fascaplysin against C6 glioma cells surpasses tested substances. In theory, the activation of glucocorticoid receptors by corticosteriods, such as dexamethasone, shifts the balance from Bcl-2 family proteins in favor of pro-apoptotic proteins, inhibiting the migration of tumor cells by influencing the Akt/mammalian target of rapamycin/Ras homolog gene family, member A signaling pathway (42). Dexamethosone typically inhibits the growth of tumor cells. However, the addition of dexamethasone to the culture medium in the present study had virtually no effect on the rate of proliferation of glioma cells. This substance is potentially applicable for local anti-relapse therapy of glioblastoma multiforme, but it is significantly inferior in efficiency to temozolomide (43). Temozolomide is the most commonly used drug in the treatment of gliomas and metastatic tumors (44). It has a strong cytotoxic effect due to alkylation of guanine at position O6 and optional alkylation in position N7 (45). In a previous study by the present authors, temozolomide increased the life expectancy of rats with experimentally-induced glioblastoma (46). The combined use of methods of targeted delivery of fascaplysin and traditional chemotherapeutic agents requires further research, as this may potentially result in strong antitumor responses.

In conclusion, the present study suggests the following: Fascaplysin in a concentration from 0.05 to 1 μM has a marked cytostatic effect against C6 glioma cells, which is replaced by tumor cell death via apoptosis with increasing exposure time. Fascaplysin causes the death of C6 glioma cells at doses exceeding 0.5 μM. The anti-proliferative effect of fascaplysin is significantly superior to temozolomide. However, the most important finding of the present study is the ability of fascaplysin to inhibit the growth of and kill poorly differentiated multipotent tumor cells. Compared with other substances investigated *in vitro*, fascaplysin showed marked inhibition of cell growth and proliferation, inducing apoptosis in tumor cells.

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