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In vitro anticancer studies of a small library of cyclic lipopeptides against the human cervix adenocarcinoma HeLa cells

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Abstract: Various cyclic lipopeptides (CLPs, 23 compounds) were tested for their antitumor potential against human cervix adenocarcinoma HeLa cells. From the fast screening (tested concentrations: 0.01 and 10 μM) compound **10** ((12*S*,6*S*,10*S*,13*S*)-6-((*R*)-sec-butyl)-7-(2-(dodecylamino)-2-oxoethyl)-13-isopropyl-82-nitro-2,5,12,15-tetraoxo-4,7,11,14-tetraaza-1(1,2)-pyrrolidina-8(1,4)-benzenacyclopentadecaphane-10-carboxamide) was identified as active against HeLa cell line. The MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and CV (crystal violet) assays revealed at least five times higher cytotoxic potential of **10** (IC_{50} = 12.3±1.8 μM, MTT; 9.4±1.5 μM; CV) in comparison to control drug natural occurring CLP surfactin (IC_{50} = 64.9±0.8 μM, MTT; 76.2±1.6 μM; CV). The cell cycle analysis performed by DAPI (4',6-diamidino-2-phenylindole) assay indicated the involvement of apoptosis in HeLa cell death upon treatment with **10**, which was confirmed by apoptosis assay (annexin V/PI). Furthermore, during this process caspase activation could be detected (ApoStat assay, immunocytochemistry caspase-3 analysis). The flow cytometry analysis did not display induction of autophagy as a possible death mechanism in HeLa cells upon **10** treatment. The current findings could be used

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to design more effective CLPs based on **10** structure as potential anticancer agents.

Keywords: cancer; surfactin; proliferation; apoptosis; cell cycle; autophagy.

INTRODUCTION

Cancer is the second-most common cause of death worldwide, exceeded only by heart disease, and accounts for nearly 1 of every 4 deaths.[1–3] Due to the toxicity, and the high price of currently used therapeutics for the treatment of various types of tumours, new alternatives are extremely desired.[4–6] The development of synthetic organic chemistry over the last years had a great impact on the anticancer drug's development, in addition to antimicrobial and other medicinal drugs.[7–9] One important strategy in developing effective anticancer agents is to synthesize compounds similar to bioactive natural occurring products like lipopeptides.[10,11]

A number of bacterial and fungal species produce cyclic lipopeptides (CLPs), most of which have important biological functions.[12–14] The plentiful structural diversity of CLPs suggests that these metabolites have different natural roles, some of which may be unique to the biology of the producing organism.[15] CLPs are exhibiting a wide diversity of biological applications, ranging from antibacterial, antifungal and anticancer activities to their employment as biosurfactants, ionophores and sequestering agents.[16,17]

Surfactin (Fig. 1), a bacterial CLP, is produced by various strains of *Bacillus*.[18,19] The surfactant properties and biological activities of surfactin analogues appear very interesting in the perspective of their utilization both in cosmetic and in pharmaceutical fields. How this molecule can be effective in various biological events is still mostly unknown; however, it is theorized that the structural and lipophilic properties of surfactin may affect the stability of biological membrane.[20] On the other hand, growth inhibition in a tumor cell by surfactin could be related to induction of apoptosis and cell cycle arrest *via* the suppression of cell survival-regulating signals such as ERK and PI3K/Akt.[21,22]

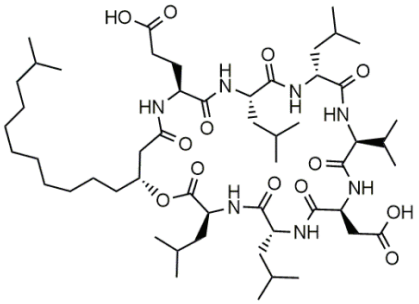

Fig. 1. Surfactin; a heptapeptide bearing a *β*-hydroxy fatty acid.

Recently, a multicomponent macrocyclization strategy was employed to synthesize natural product-like cyclic lipopeptides.[10] This specific multicomponent reaction might be used for rapid production of natural product analogues, having amide instead of ester bond(s), for biological screening. Substituting the ester bond found in natural depsipeptide CLPs with an amide bond could potentially enhance stability, alongside the benefits of macrocyclization and *N*-alkylation. In the current study, the evaluation of the anticancer activity of a selected lipopeptide library on the HeLa cancer cells were conducted. For the most active compound mechanism of action was determined.

EXPERIMENTAL

Chemicals

CLPs were collected from the compounds library of the Leibniz Institute of Plant Biochemistry Halle, Germany. The structures of the selected CLPs were confirmed by ^{1}H- and ^{13}C-NMR spectroscopy as well as with mass spectrometry and are in accordance with the compounds previously reported by Wessjohann and coworkers.[23] Digitonin (Riedel De Haen Seelze, Germany), surfactin, crystal violet (CV – histochemical stain, bind to duplex DNA), paraformaldehyde, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT – reduced to formazan by metabolically active cells), acridine orange (AO – monitor and quantify autophagy by assessing changes in the acidity of cellular compartments), 4',6-diamidino--2-phenylindole (DAPI – nuclear staining in cells, Sigma–Aldrich, Germany), RPMI (Roswell Park Memorial Institute) medium 1640, tryban blue stain 0.4 %, caspase-3 rabbit Ab, secondary anti-rabbit Alexa Fluor 488 antibody (Life Technologies, Germany), Dulbecco's phosphate-buffered saline (DPBS), trypsin EDTA (PAN Biotech, Germany), 4-amino-5-methylamino-2,7'-diflurofluorescin diacetate (DAF-FM – detection and quantification of low concentrations of nitric oxide, Cayman chemical company, Germany), fetal calf serum, penicillin/strepetomycin, HEPES (PAA laboratories, Germany), DMSO (Duchefa Biochemie, Germany), acetic acid 33 % (Roth company, Germany), dihydrorhodamine (DHR – detection of ROS and RNS), carboxyfluorescein succinimidyl ester (CFSE – utilized to track different generations of actively dividing cells through the process of dye dilution), ApoStat (identify and measure caspase activity in apoptotic cells), Annexin V (protein, labeled with a fluorochrome FITC, that has a high affinity for phosphatidylserine, a phospholipid that is exposed on the outer surface of the cell membrane during early stages of apoptosis) and PI (a DNA-intercalating dye, impermeable to live cells) were obtained commercially (DB Biosciences, USA). Stock solutions of CLPs, including surfactin, were prepared in DMSO at concentration of 20 mM. Human cervix adenocarcinoma (HeLa) and NIH3T3 mouse fibroblast cells were obtained from Ontochem (Halle, Germany). HeLa and NIH3T3 cells were grown in RPMI 1640 nutrition medium supplemented with 10 % fetal calf serum (FCS) and 1 % penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5 % CO_2. For MTT and CV assays, HeLa and NIH3T3 cells were seeded at density 2.000 and 10.000 cells/well, respectively, in 96 well plates, while for flow cytometry experiments at 1×10^5 HeLa cells/well in the 6 well plates. Working concentrations were prepared from stock solutions in nutrition medium.

Fast screening and determination of IC_{50} *concentrations (MTT and CV assays)*

After the HeLa cells were seeded and incubated for 24 h, 100 μL of various concentrations of CLPs, in quadruplicate, were added into the wells. For the fast screening, two con-

centrations (0.01 and 10 μM) of CLPs were used, while in experiments for IC_{50} values determination using different concentrations (0.625, 1.25, 2.5, 5, 10, 20, 40 and 80 μM). NIH3T3 cells were seeded and incubated for 24 h, afterwards treated with the IC_{50} concentrations of **10** and surfactin. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and CV (crystal violet) assays, upon 72 h of treatment, were performed as described in the literature.[24] All experiments were performed in biological triplicates. The absorbance was measured with an automated microplate reader (Spectramax from Molecular Devices) at 540 nm (reference 670 nm). IC_{50} values, defined as the concentrations of the compound at which 50 % of cell inhibition occur were calculated using four-parameter logistic function and presented as mean from three independent experiments.

Cell cycle analysis

HeLa cells (1×10^5 cells/well) were cultivated with the IC_{50} or $2\times IC_{50}$ doses of active compounds for 72 and 48 h, respectively. Then the supernatant (which contains the floating dead cells) from each well and the attached cells belong to the same well were collected in 5 mL test tubes. Cells were washed with phosphate buffered saline (PBS) and fixed in ethanol 70 % at 4 °C overnight. Afterwards, cells were washed in PBS and the cell pellet was taken and resuspended in 1 mL of DAPI (4',6-diamidino-2-phenylindole) solution (1 vol. % Triton X-100 and 1 μg mL^{-1} DAPI in PBS) for staining. Prepared samples were stored in the dark for 10 min then the distribution of cells in different cell cycle phases was determined with FACSAria III (DB Biosciences).[25]

Annexin V/PI assay

HeLa cells were incubated with IC_{50} or $2\times IC_{50}$ doses of active compounds for 72 h. Cells were washed and trypsinated, then collected and washed with PBS. Cells had been stained with 100 μL of working Ann V/PI solution (93 μL of 1×Annexin binding buffer (ABB) + 5 μL Ann V + 2 μL PI per sample) for 15 min at 37 °C in dark. Afterwards, staining was stopped by adding 900 μL of 1×ABB per sample. Treated and untreated cells were examined by flow cytometry analysis with FACSAria III (DB Biosciences).[26]

ApoStat staining

Investigated cancer cells were incubated with IC_{50} or $2\times IC_{50}$ doses of active CLPs for 72 h. Cells were washed and trypsinated, then collected and washed with PBS. Cells were incubated with 100 μL of working ApoStat solution (99 μL of PBS 5 % FCS + 1 μL ApoStat per sample) for 30 min at 37 °C. Later, 1 mL of PBS per sample was used to stop staining and washing out unbound ApoStat. Examine cells were suspended in 1 mL PBS for flow cytometry analysis with FACSAria III (DB Biosciences).[25]

Immunocytochemistry

Cells (1×10^5 cells/well) were cultivated with the $2\times IC_{50}$ dose of active compounds for 48 h. The medium was removed and cells were fixed with paraformaldehyde 4 % for 15 min at room temperature. Permeability was improved by incubating the treated cells with 0.25 % Triton X-100 in PBS for 10 min then cells had been washed with PBS 3 times for 5 min. In order to avoid unspecific antibody binding, 10 % FCS in PBS-T (PBS with 0.1 % Triton X-100) was added for 30 min at room temperature. Afterwards, cells were incubated with Rabbit anti--caspase 3 (1:400 dilution in 1 % bovine serum albumin PBS-T) over the night at 5–10 °C. Anti-rabbit Alexa Flour 488 secondary antibody was used in dilution 1:200 in 1 % BSA PBS-T for 30 min at RT in dark place. Nucleus staining was performed with DAPI 1 μg mL^{-1} in PBS

by incubating the cells with DAPI for 1 min in dark place. Finally, the preparations were used to take the images by fluorescence microscope.

Analysis of autophagy

For this purpose, cells were incubated with IC_{50} or $2\times IC_{50}$ doses of active compounds for 72 and 48 h, respectively. Cells were washed and trypsinated, consequently collected and washed with PBS before staining in 10 μM acridine orange solution for 15 min at 37 °C in dark. Afterwards, cells were washed with PBS and the cell pellet was resuspended in 500 μL PBS for flow cytometry analysis with FACSAria III (DB Biosciences).[25]

CFSE staining

The assay was performed as described by supplier.[27] Cells were stained with 1 μM of carboxyfluorescein succinimidyl ester (CFSE) for 15 min at 37 °C and then exposed to the IC_{50} dose of active compounds for 72 h. At the end of cultivation, cells were washed with PBS and trypsinated, washed in PBS and resuspended in 500 μL of PBS for measurement. Proliferation activity of the treated cells was analysed by FACSAria III (DB Biosciences).

Investigation of ROS/RNS

For detection of reactive oxygen species/reactive nitrogen species (ROS/RNS), dihydrorhodamine (DHR) which is redox-sensitive dye was used.[28] The cells were stained with 1 μM DHR for 20 min at 37 °C. Afterward, the cells were treated with the IC_{50} dose of investigated compounds for 72 h. Subsequently, the cells were collected by trypsinization and washed with PBS before flow cytometry analysis.

DAF-FM assay

For NO production evaluation, cells were treated with IC_{50} and $2\times IC_{50}$ for 72 h. The medium was discarded from all wells which had been washed with PBS 2 times. Then, 1 mL per well of working DAF-FM (4-amino-5-methylamino-2,7'-diflurofluorescin diacetate) solution (5 μM DAF-FM in white medium with 10 % FCS) was added and incubated with cells at 37 °C for 1 h for staining. The staining solution was dropped and wells washed with PBS 2 times before incubated with the white medium for 15 min at 37 °C. Afterwards, cells had been trypsinated and collected from all wells in tubes for FACS analysis.[25]

RESULTS AND DISCUSSION

Cytotoxicity and toxicity studies

Various CLPs (23 synthetic compounds) and surfactin (natural occurring compound, herein control) were at first fast screened against HeLa cell lines, using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and CV (crystal violet) assays in order to select the most active compounds (72 h treatment, Figs. 2 and S-1of the Supplementary material to this paper). From all tested synthetic CLPs only compound **10** (Fig. 3a) exhibited antitumor activity against HeLa cell line at the higher investigated concentration (10 μM).

Furthermore, the MTT and CV assays were carried out in order to determinate cytotoxic potential of selected synthetic and naturally occurring compounds (Fig. 3b). Based on the recommendation of National Cancer Institute, active compounds have IC_{50} value < 30 μg mL^{-1}.[29] The activity of **10** is in the range of active compounds (IC_{50} = 12.3/9.4 μM, MTT/CV, corresponds to 9.8/

/7.5 μg mL^{-1}, MTT/CV). Surprisingly, surfactin expressed much lower activity than **10** against HeLa cells. The lower activity of surfactin, a cyclic ester, in comparison to **10**, instead of lactone an *N*-substituted amide moiety is present, may be due to the fact that surfactin is more susceptible to hydrolysis/proteolysis reactions in which ring-opened compounds are produced. Such products are more exposed to degradative process in the physiological medium, thus in that way bioavailability is decreased. However, amide-containing compounds are also susceptible to hydrolysis, but it occurs at an extremely slower rate than the hydrolysis of lactone.[30] The cyclic moiety of CLPs is important for their high surfactant activity and destruction of the cyclic structure in surfactin may lead to decrease its cytotoxic activity.[31]

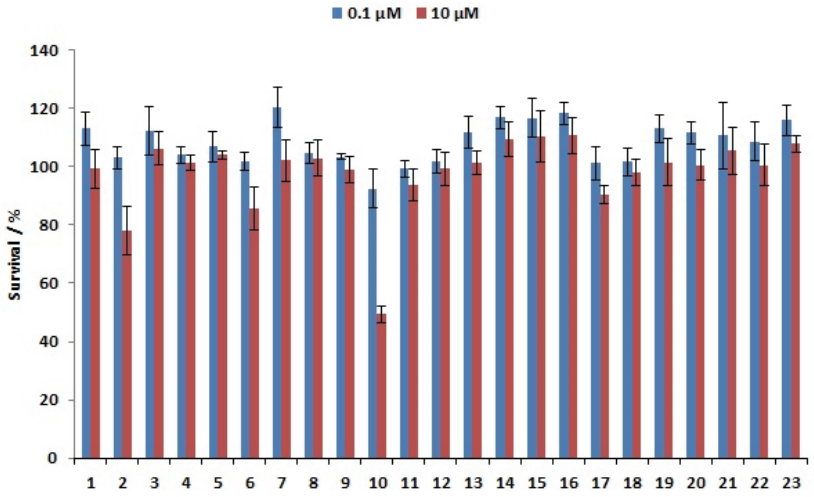

Fig. 2. The viability of HeLa cells treated with CLPs (CV assay). Cells were treated with 2 concentrations (0.1 and 10 μM) of CLPs for 72 h. Data represent absorbance measurements normalized to control-untreated cells. Values are expressed as means and standard deviations obtained from independent experiments. For MTT results please see supplementary material (Fig. S-1).

In order to explore does **10** or surfactin have an effect on normal cells, mouse embryonic NIH3T3 fibroblast cells were incubated with IC_{50} concentrations of selected compounds (Fig. 3c). Both MTT and CV assays demonstrated that surfactin was more toxic to NIH3T3 cells than compound **10**. The survival rate of the cells treated with **10** (91 %) showed that this synthetic CLP selectively acts on tumor cells (at the same concentration 50 % of HeLa cells are death). On the other hand, the IC_{50} concentration of surfactin, determined on HeLa cells, decreased the cell viability of normal cells to 15 %, thus is more active against normal than tumor cells.

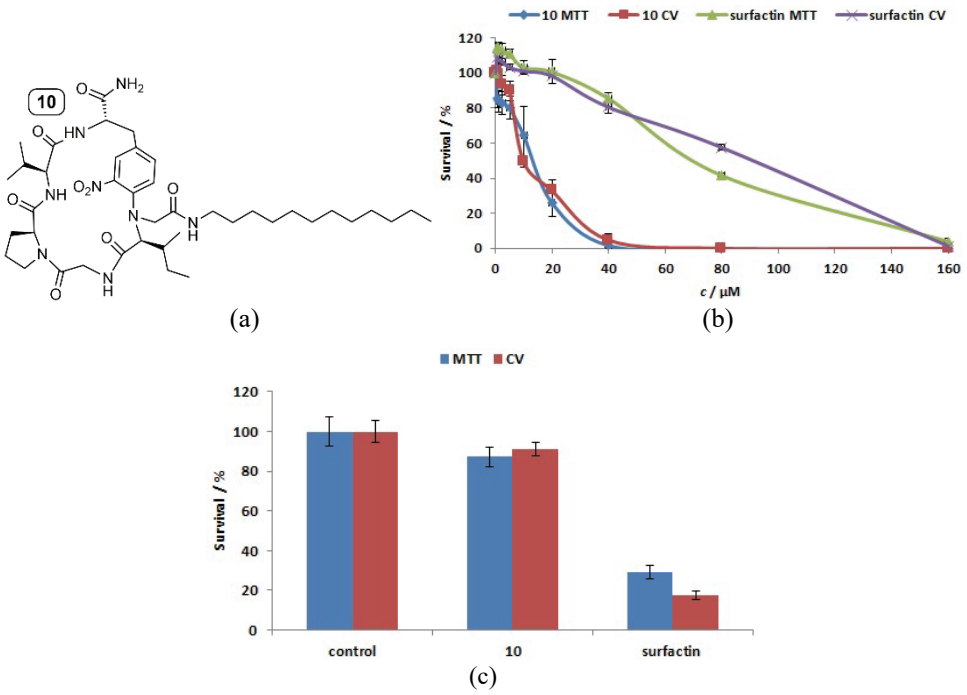

Fig. 3. a) The structure of **10**; b) dose-dependent response of the HeLa cells upon treatment with CLPs (MTT and CV assays). HeLa cells were treated with serial dilutions of **10** and surfactin for 72 h. IC_{50}: **10**, 12.3±1.8 μM (MTT) and 9.4±1.5 μM (CV); surfactin, 64.9±0.8 μM (MTT) and 76.2±1.6 μM (CV). The data represent absorbance measurements normalized to control-untreated cells. The dose-response curves and IC_{50} values show mean values ± *SD* of three independent experiments with six parallel measurements in each case; c) the effect of **10** and surfactin on the viability of normal mouse NIH3T3 fibroblasts. NIH3T3 cells were untreated or treated with the IC_{50} concentrations of **10** and surfactin for 72 h then cell viability was determined by MTT and CV assays. Values are expressed as means and standard deviations obtained from three independent experiments.

Mechanism of action

To investigate the mode of action HeLa cells were treated with both synthetic and naturally occurring compounds, **10** and surfactin, and at first distribution of the cells in the cell cycle was analysed using flow cytometry (DAPI assay, Fig. 4a).[25] Based on the obtained results, cells treated with **10** as well as surfactin, showed enhanced cell number in sub-G1 phase which might indicate an involvement of apoptosis in cell death induced by these compounds.[32] Both compounds do not express cytostatic potential, thus their action is mainly due to the cytotoxic action (Fig. 4b). The apoptotic mode of cell death was confirmed with Annexin V/PI assay (Fig. 4c). There was a recognized increase in early and late apoptotic cell populations (Ann^+/PI^- and Ann^+/PI^+, respectively) in the HeLa

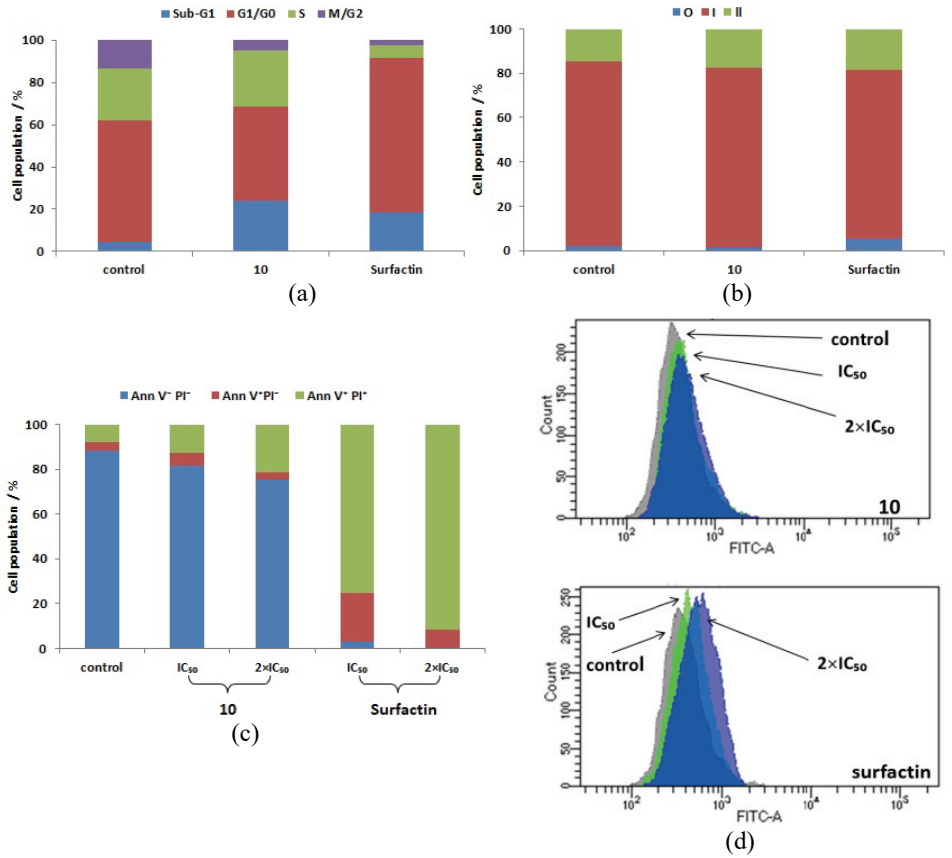

Fig. 4. a) Flow cytometry analysis of cell cycle distribution of HeLa cells. Cells were treated with DMSO as vehicle control or IC_{50} concentrations of **10** and surfactin for 72 h then stained with DAPI. The DNA content was then determined by flow cytometry. The cell population was analyzed as cell numbers at each cell cycle phase relative to the total population; b) flow cytometer analysis for cell proliferation assay of CFSE-labelled HeLa upon treatment with DMSO as vehicle control, IC_{50} concentrations of **10** or surfactin for 72 h.; c) detection of apoptosis with Annexin-V-FITC and propidium iodide staining. HeLa cells were treated with DMSO as vehicle control, the IC_{50} or $2\times IC_{50}$ concentrations of **10** and surfactin for 72 h then stained with Annexin V and PI for FACS analysis. Annexin V positive cells represent percentages of late and early apoptotic HeLa cells; d) representative images for caspase activation in HeLa cells upon treatment with CLPs (ApoStat assay). HeLa cells were treated with DMSO as vehicle control, the IC_{50} concentrations of **10** or surfactin for 72 h, stained with ApoStat and measured by flow cytometry to determine the percentage of cells expressing active caspases. Results are expressed as means obtained from three independent experiments (a, b and c).

cells treated with **10**. On the other hand, massive late apoptosis was detected for the HeLa cells treated with surfactin. Apoptosis initiation and execution are accompanied by complex cascades of intra-cellular events that may include

members of the caspase family.[33] Flow cytometry was used to evaluate activation of caspases in HeLa cells upon treatment (ApoStat assay). An obvious upregulation of caspases could be noted upon treatment with both compounds (Fig. 4d). Moreover, activation of caspase-3 executor could be confirmed using immunocytochemistry analysis (Fig. 5). Fluorescent examination of treated HeLa cells gives definite proof of involvement of caspase in the apoptotic mode of cell death using selected CLPs.

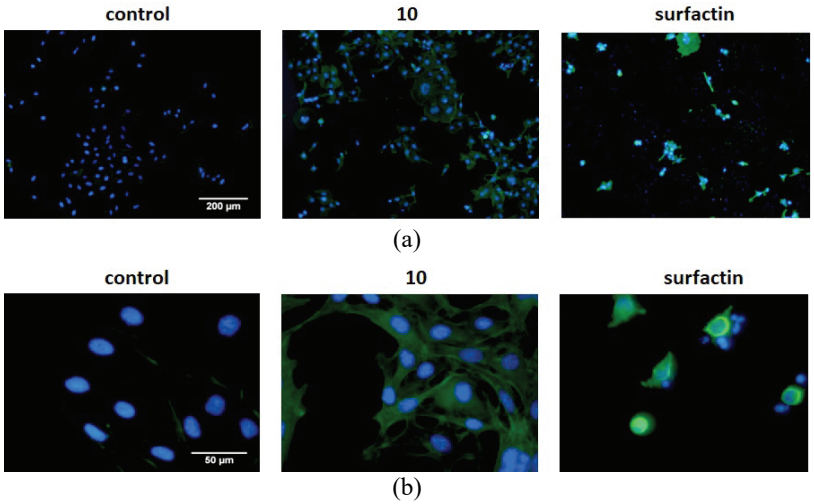

Fig. 5. Representative fluorescence images, a) 10× and b) 40×, of caspase-3 activation HeLa cells upon treatment with **10** and surfactin (2×IC_{50}, 48 h, nuclei – DAPI stained, caspase-3 – primary rabbit antibody, secondary anti-rabbit Alexa Fluor 488 antibody).

Autophagy might be involved in various cellular processes such as cell differentiation as well as in a cell death mechanism involving autophagosomal/lysosomal degradation of cellular components.[34,35] In the current study, it was sought to determine whether treatment of cells with active CLPs results in an induction of autophagy of cancer cells. For this purpose, cell previously treated with active CLPs were collected and analysed using flow cytometry (acridine orange assay, Fig. S-2 of the Supplementary material).[25] Upon treatment with **10** or surfactin, HeLa cells did not show any elevated presence or acidic vehicles, autophagosomes, meaning that autophagy is not involved in the death process.

To investigate whether ROS/RNS, as well as NO species, are generated by CLPs, DHR (dihydrorhodamine) or DAF-FM (4-amino-5-methylamino-2,7'-difluorofluorescin diacetate), respectively, prestained HeLa cells were incubated with selected CLPs (Fig. 6a and b). Flow cytometry analysis showed no increase in the ROS/RNS formation, moreover, **10** suppressed the formation of the men-

tioned species. Surfactin enhanced the production of NO, while **10** had no impact on its production.

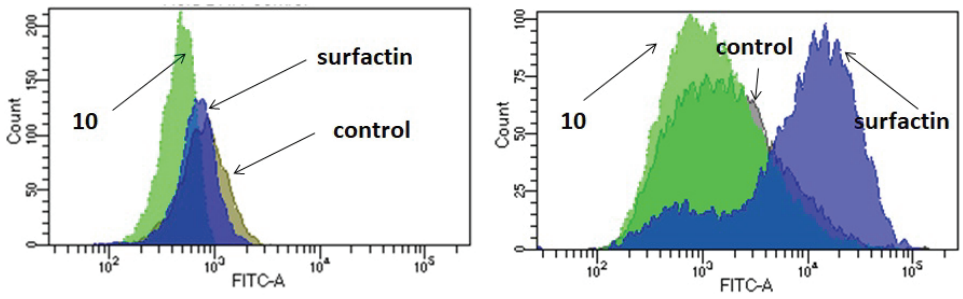

Fig. 6. a) Flow cytometry analysis for ROS/RNS production in HeLa cells upon treatments with DMSO as vehicle control, IC_{50} concentrations of **10** or surfactin for 72 h (DHR assay); b) NO generation in treated and untreated HeLa cells. HeLa cells were treated with DMSO as vehicle control, IC_{50} concentrations of **10** or surfactin for 72 h then stained with DAF-FM solution for FACS analysis. Representative images are shown.

Compound **10** shows an activity surpassing that of the natural compounds surfactin or even emodin (four-fold).[25] On the other hand, **10** exhibits lower activity than cisplatin against HeLa cells,[36] however with notable advantages. In contrast to cisplatin, **10** displays selectivity by being inactive against normal fibroblast cells. While cisplatin has severe side effects, optimizing the chemical structure of compound **10** could enhance its cytotoxicity. In modern anticancer therapy, it is also important to prioritise selectivity over potency in drug development. Compound **10** may act by reducing its nitro group, producing free-radical intermediates and reactive metabolites that interact with DNA, leading to the inhibition of nucleic acid and protein synthesis.[37,38] The deadly effects on HeLa cancer cells and the cytotoxic action of **10** may be related to the covalent binding of the reduced metabolites to cellular macromolecules or interactions with cytoplasmic and membrane enzymes. Analysis of cells treated with **10** revealed an increase in late and early apoptotic HeLa cells. Among the nitro group-containing compounds investigated herein, only compound **10** demonstrated relevant activity. The incorporation of the nitro-phenyl group into a peptide cycle makes the possibility of intercalation of **10** into DNA highly improbable, as there is no conformation that permits flat aromatic intercalation.

CONCLUSION

Lipopeptides represent a class of microbial surfactants, that has achieved increasing scientific, therapeutic and biotechnological interest. The main aim of the current work was *in vitro* anticancer investigation for a library of synthetic CLPs. For this purpose, the HeLa cell line was used and cytotoxicity was assessed

with MTT and CV assays. As a result, 23 compounds were examined and many of them exhibited a high cytotoxic response during the primary screening.

The IC_{50} value was established for active CLPs in order to attain the most important structural features that may contribute to their cytotoxic activity. Synthetic CLP (**10**) revealed a better cytotoxic profile (12.3±1.8 μM (MTT) and 9.4±1.5 μM (CV)) in comparison to natural CLP surfactin (64.9±0.8 μM (MTT) and 76.2±1.6 μM (CV)) due to structural modifications, such as lactone group replacement, the introduction of nitro functional group and lipophilicity improvement. Additionally, active CLP showed a higher safety profile against NIH3T3 than surfactin.

Accumulation of treated cells in the sub-G phase might indicate apoptosis as a possible mechanism of action. This assumption was confirmed by annexin V/PI assay. In addition, active CLP had been displayed to provoke late apoptosis and cell death in HeLa cells. Results reported in this research contribute to understanding CLPs cytotoxicity, the structural features that might be involved in their activity and their possible mechanisms of action. Finally, these findings may be used to design more effective and less harmful CLPs as potential anticancer agents.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/12766, or from the corresponding author on request.

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ИЗВОД

IN VITRO АНТИКАНЦЕРОГЕНО ИСПИТИВАЊЕ МАЛЕ БИБЛИОТЕКЕ ЦИКЛИЧНИХ ЛИПОПЕПТИДА НА HELA ХУМАНИМ ЋЕЛИЈАМА АДЕНОКАРЦИНОМА ГРЛИЋА МАТЕРИЦЕ

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Антитуморски потенцијал разних цикличних липопептида (CLP, 23 једињења) је испитиван на HeLa хуманим ћелијама аденокарцинома грлића материце. Из брзог скрининга (испитане концентрације: 0,01 и 10 μM) једињење **10** ((12*S*,6*S*,10*S*,13*S*)-6-((*R*)-сек-бутил)-7-(2-(додециламино)-2-оксоетил)-13-изопропил-82-нитро-2,5,12,15-тетраоксо--4,7,11,14-тетрааза-1(1,2)-пиролидина-8(1,4)-бензенциклопентадекафан-10-карбоксамид)

је идентификовано као активно на HeLa ћелијама. MTT (3-(4,5-диметилтиазол-2-ил)--2,5-дифенилтетразолиум-бромид) и CV (кристал виолет) тестови показали су да једињење **10** има најмање 5 пута већи цитотоксични потенцијал (IC_{50} = 12,3±1,8 μM, MTT; 9,4±1,5 μM; CV) у поређењу са природним CLP контролним леком, сурфактин (IC_{50} = = 64,9±0,8 μM, MTT; 76,2±1,6 μM; CV). Анализа ћелијског циклиса, извршена помоћу DAPI (4'6-диамидино-2-фенилиндол) теста, указује на учешће апоптозе у смрт HeLa ћелија након третирања са **10**, што је потврђено тестом за апоптозу (Annexin V/PI). Поред тога, током овог процеса може се детектовати активација каспазе (ApoStat тест и имуноцитохемијска каспаза-3 анализа). Анализа проточне цитометрије није показала индукцију аутофагије као могући механизам смрти HeLa ћелија након третирања са **10**. Ови налази могу да се користе у дизајнирању побољшаних CLP, заснованих на структури једињења **10**, као потенцијалних агенаса против рака.

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