https://doi.org/10.15255/KUI.2021.030
KUI-10/2022
Original scientific paper
Received April 29, 2021
Accepted October 9, 2021

Antimicrobial and Antiproliferative Activities and Enzyme Binding Affinity of Xanthen-3-one Derivatives

E. Veljović,[a*] *A. Harej*,[b] *M. Klobučar*,[b] *S. Kraljević Pavelić*,[b] *S. Špirtović-Halilović*,[a] *A. Osmanović*,[a] *S. Muratović*,[a] *M. Salihović*,[a] and *D. Završnik*[a]

[a] University of Sarajevo, Faculty of Pharmacy, Zmaja od Bosne 8, 71 000 Sarajevo, Bosnia and Herzegovina

[b] University of Rijeka, Department of Biotechnology, Centre for High-Throughput Technologies, Radmile Matejčić 2, 51 000 Rijeka, Croatia

Abstract

Ten biologically active derivatives of 2,6,7-trihydroxyxanthen-3-one, previously synthesized and characterised, were investigated for their *in vitro* antimicrobial and antiproliferative activity. Compounds were tested on three bacteria, *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli*, and two fungi strains, *Candida albicans*, and *Saccharomyces cerevisiae*. The best activity against *E. coli* showed non-substituted compound **1**. The most potent against fungi strains was compound **7** with *ortho* methoxy substituent. Compound **4** exerted the most potent antiproliferative activity in the micromolar range (0.1–10 μM) on tested tumour cell lines except on SW620. Additional Western blot analyses showed increased cyclin B1 levels in HeLa cells treated with compound **4**, which is a major mitotic catastrophe's marker and decreased levels of Wee1 and Erk ½ kinases involved in regulation of the mitotic process. The most potent compounds after *in vitro* tests were subjected to molecular docking simulations to evaluate enzyme binding affinity, and provide further evidence for experimentally observed biological effects *in vitro*.

Keywords

Xanthenes, antibacterial and antifungal activity, induction of cell death, enzyme binding modes, docking study, bioavailability prediction

1 Introduction

Xanthene derivatives have many practical uses in laboratory and industry as a result of their interesting heterocyclic, oxygen-including structure.[1,2] More importantly, they constitute a number of important biologically active compounds, with many outstanding pharmacological activities.[3–8]

Nowadays, we witness growing problems in medical therapy, like absence of conclusive cure for some diseases, especially chronic ones, or cancer, and ever-growing resistance to antibiotics. In this constant race in searching for new potent drugs, many enzymes and receptors important for microbial growth and/or tumour cell development may be considered as potential targets for those new drugs. Moreover, it is largely recognised that chronic inflammation, as a consequence of microbial activity (due to release of genotoxic agents from microbes), may induce mutations which adds to tumour development.[9] For example, possible targets in microbes include the phosphoenolpyruvate phosphotransferase system (PTS) and lanosterol-14-demethylase (cytochrome P450 isoenzyme 51). PTS is omnipresent in bacteria but does not occur in eukaryotes. It catalyses the uptake and phosphorylation of carbohydrates. In the PTS phosphorylation cascade, enzyme I (EI) is the first and central component. Lack of EI in bacteria leads to severe growth defects, hence making EI a potential drug target for new antimicrobial treatment.[10] On the other hand, cytochrome P450 isoenzyme 51 is more common (from mycobacteria, fungi, plants to animals and humans). It would be ideal if antifungal drug would be selective, inhibiting only fungal CYP51 while leaving human CYP51 unaffected.[11] Further, in cancer cells, as interesting target for anticancer therapeutics (*e.g.*, doxorubicin) topoisomerase II has been widely used because of its essential role in cells.[12,13]

When these valuable molecular targets are recognised as targets for therapeutics in the current drug discovery and development process, then different *in silico* methods may be used to shed light on interactions between novel small molecules and defined target macromolecules (receptor) on cellular level. Among these *in silico* methods, molecular docking is often used to study binding of interest, and particularly the usefulness of AutoDock program has been well demonstrated so far in a different studies.[14,15]

We previously prepared and characterised ten derivatives of 9-aryl substituted 2,6,7-trihydroxyxanthen-3-one, and performed antioxidant potency evaluation.[16] Antimicrobial and antiproliferative studies on similar xanthene molecules have been previously reported indicating good biological activity.[7,8,17,18] This study was conducted in order to further evaluate biological activity, more precisely, antimicrobial and antiproliferative effects of synthesized 9-aryl substituted xanthen-3-ones. The most potent compounds were subjected to molecular docking simulations. Furthermore, to determine compounds' physicochemical properties and

* Corresponding author: Assoc. Prof. Elma Veljović
Email: elma.veljovic@ffsa.unsa.ba

their bioavailability after oral intake in humans, we applied and discussed Lipinski's rule of 5 and polar surface area for these compounds.[19,20]

2 Experimental

Structures of ten xanthen-3-one derivatives evaluated for their biological activity are shown in Fig. 1.

Derivatives were tested on several bacteria, fungi, human tumour lines of cells, as well as control normal cell line, (diploid) human fibroblasts. The latter were used to identify new molecules for pre-clinical investigation.

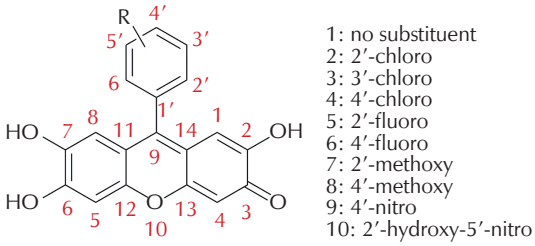

Fig. 1 – Structures of investigated compounds
Slika 1 – Strukture ispitivanih spojeva

2.1 Antimicrobial activity

To determine antimicrobial activity, diffusion and dilution methods were used against three bacteria *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538P, and *Escherichia coli* ATCC 9027, as well as against two fungi, *Candida albicans* ATCC 1023 and *Saccharomyces cerevisiae* ATCC 9763. Compounds were dissolved in 99.5 % dimethyl sulfoxide (DMSO) to prepare a stock solution (starting concentration of 12.8 mM), which were then applied to nutritious bases (Müller-Hinton and Sabouraud). At the end of an incubation period of 18 h at 37 °C for bacteria, and after 48 h at 25 °C for fungal strains, the inhibition zones were measured in millimetres. Compounds that showed good activity by diffusion method were further tested by dilution method. For this, casein soya bean digest broth (Triptic soya bujon) was used. As a reference for antimicrobial activity, tetracycline, streptomycin, and amphotericin were used in concentrations of 1.12, 0.86, and 0.54 mM, respectively. Stock solutions were diluted in a series of 12 dilutions with liquid nutritious base. After incubation for 24 h, the last tube with no growth of microorganisms was taken to represent minimum inhibitory concentration (MIC) expressed in μM. Prepared solutions concentrations ranged from 1561 to 0.6294 μM.

2.2 Cell culturing

The cell lines HeLa (cervical carcinoma), SW620 (colorectal adenocarcinoma, metastatic), MiaPaCa-2 (pancreatic carcinoma), and BJ (normal diploid human fibroblasts) were cultured as monolayers and maintained in Dulbecco's modified Eagle medium (DMEM, Lonza, Austria). This medium was supplemented with 10 % foetal bovine serum (FBS), 2 mM L-glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin in a humidified atmosphere with 5 % CO_2 at 37 °C. The cells were maintained in culture until 80 % of confluence, and at that point washed with PBS (Phosphate Buffered Saline, GIBCO, USA), and detached from the surface using of 0.25 % trypsin solution (LONZA, Basel, Switzerland). Cell viability during regular passaging on an automatic cell counter (Countess, Invitrogen, USA) was determined using trypan blue solution. Light microscope (Axio Vision-Zeiss, Germany) was used to determine the morphology.

2.3 Proliferation assays

On day 0, 5000 tumour cells per well (of each cell line) were inoculated onto a series of 96-well microtiter plates. Tested compounds were then added in five, 10-fold dilutions (concentrations ranging from 0.01 to 100 μM) and incubated for 72 h. The solvent (DMSO) was also tested for inhibitory activity as a negative control (concentration of the solvent never exceeded 0.1 %). After 72 h of incubation, the growth rate of the cells was evaluated by conducting the MTT assay. Three individual experiments were performed for each test point (done in quadruplicate). Experimentally determined absorbance values (measured at 570 nm) were transformed into a cell percentage growth (PG) by formulas described previously.[21] Because this method compares the growth of treated and untreated cells in control wells on the same plate, it directly relies on control cells at the day of assay, giving the results as a percentile difference from the calculated expected value. The IC_{50} and LC_{50} values for each tested substance were calculated from dose-response curves using linear regression analysis. In case that all of the tested concentrations produce PGs exceeding the reference level of effect for a given cell line, the highest tested concentration is assigned as the default value (preceded by a ">" sign).

2.4 Cell cycle analysis

A total of 20000 cells per well were seeded in 6-well plates (10 cm in diameter, Sarstedt, Germany). After 24 h, HeLa and BJ cells were treated with compound **4** at previously titrated concentrations that cause cell cycle perturbations without immediate induction of apoptosis (0.3 and 1.5 μM or 0.5 and 2.5 μM, respectively), for 24 and 48 h. After 24 h and 48 h of incubation, the attached cells were trypsinised, washed with PBS and collected. All cells were fixed with 70 % ethanol. Immediately before the analysis, the cells were washed again with PBS and stained with 10 μg ml^{-1} of propidium iodide (PI) with the addition of 0.2 μg ml^{-1} of RNAse A. The stained cells were then analysed with Becton Dickinson FACScalibur flow cytometer (10000 counts were measured). Each test point was performed in duplicate. The percentage of the cells in each cell cycle phase was based on the obtained DNA histograms and determined using the FCS EXPRESS 4 FLOW

RESEARCH EDITION (De Novo software, USA). The concentration giving 50 % of inhibition (IC_{50}) was calculated by nonlinear regression using GraphPad-Prism7 software (GraphPad Inc., San Diego, CA). All results were expressed as means ± SD (standard deviation), taken over ≥ three independent experiments, with ≥ three replicated experiments, unless otherwise stated. Differences among means were tested for statistical significance using one-way analysis of variance (ANOVA) test. P value ≤ 0.05 was considered statistically significant.

2.5. Detection of apoptosis

For analysis of HeLa and BJ cell death, a commercial kit – Annexin-V-FLUOS Staining Kit (eBioscience, USA) was used.[22] The cells were analysed under fluorescence microscope (BX51, Olympus, USA). It was possible to detect three distinct populations of cells: cells which do not bind or weakly bind annexin V and PI (alive cells), cells that bind strongly annexin V and weakly bind PI (apoptotic cells), and cells that are in late apoptosis that highly bind annexin V and/or PI (necrotic cells are stained only with PI). For purpose of the experiment, on the 8-well cell culture plates (Falcon, USA), seeded were 50000 HeLa and BJ cells per well. After 24 h of incubation at 37 °C and 5 % CO_2, cells were treated with compound **4** at micromolar concentrations (HeLa 0.3 μM and BJ 0.5 μM) and incubated for 24 h and 48 h hours. Before analysis, cells were treated with PI and annexin according to manufacturer recommendations, and incubated 10 min in the dark. At least one hundred cells were counted for each condition.[23]

2.6. Western blot analysis

HeLa and BJ cells were seeded in a 6-well plate, at 30000 cells per well, and treated with compound **4** at micromolar concentrations (HeLa 0.3 μM and BJ 0.5 μM) for 24 h and 48 h. Protein lysates were prepared using a lysis buffer (50 mM Tris HCl (pH 8), 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS; protease and phosphatase inhibitor cocktail Roche, Switzerland). A total of 50 μg of proteins were resolved on 12 % SDS polyacrylamide gels using the Mini-protean cell (Bio-Rad, USA). Membranes were incubated with primary antibodies raised against Cyclin B1 (1 : 1000, mouse mAb, Abcam, UK), phospho (Ser 642) – Wee 1 (1 : 1000, rabbit mAb,Cell Signalling Technology, USA) dually phosphorylated pT202/pY204 + pT185/pY187 Erk ½ (1 : 1000, mouse mAb, Abcam, UK) and GAPDH (1:1000, rabbit mAb, GeneTex, USA) at 4 °C overnight. Secondary antibody linked to anti-mouse (1 : 1000, Dako, USA) and anti-rabbit (1 : 1000, Dako) were used. The signal was visualised by Western Lightening Chemiluminescence Reagent Plus Kit (Perkin Elmer, USA) on the ImageQuant LAS500 (GE Healthcare, USA). Signal intensities of particular bands were normalised with the intensity of the loading control and compared in Quantity One software (Bio-Rad, USA). The values are expressed as average ± standard deviation. Statistical analysis was performed in Microsoft Excel using the ANOVA ($p < 0.05$).[24]

2.7 Docking study and physicochemical properties calculations

The docking study was performed using AutoDock 4.2. The crystal structures of enzyme I (PDB ID: 1ZYM), CYP 51 (PDB ID: 2WX2) and topoisomerase II (PDB ID: 4FM9) from Protein Data Bank were used as the target molecule. The protein structures were optimised in AMBER03 force field, and prepared by removing water molecules and adding polar hydrogen atoms. The geometries of the xanthene molecules were optimised by the density functional theory (DFT) (B3LYP/6-31G* basis set) using Spartan 14. Lamarckian Genetic Algorithm of the AutoDock program was used to perform the flexible-ligand docking studies.[14] A blind docking approach was used, searching the whole proteins for potential binding sites. In actual calculations, following parameters were used for each docking: grid point spacing of 0.375, 10 docking runs with a maximum number of 2,500,000 energy evaluations and maximum of 27,000 generations, with step sizes of 0.2 Å for translations and 5 Å for orientations and torsions.

Bindings between docked potent agents and related macromolecule were analysed using AutoDockTools program (Version 1.5.4) and PyMol-1.1 software that was used for graphical visualisation, analysing interactions of ligands and receptors, and producing images.[25]

Partition coefficient of octanol/water (log *P*), which is often used to estimate solubility and permeability, was calculated using the virtual laboratory developed by Molinspiration, and represents the sum of fragment-based contributions and correction factors (miLogP). Another important parameter, polar surface area (TPSA), was calculated from the surface areas that were occupied by either oxygen or nitrogen atoms, as well as by hydrogen atoms attached to them. Together with these, additional parameters like molecular mass (MW), hydrogen bond acceptors (*n*ON) and donors (*n*OHNH) were also calculated.

3. Results and discussion

3.1. Antimicrobial evaluation

Xanthen-3-one derivatives exhibit antibacterial and antifungal activities as tested on different bacteria and fungi strains (*E. coli* and *S. aureus*; *C. albicans*, and *S. cerevisiae)*. Results are presented in Table 1.

Compounds showed better antifungal than antibacterial activity. The most active compound against fungal strains was 9-(2-methoxyphenyl)-2,6,7-trihydroxyxanthen-3-one (**7**) with methoxy group in *ortho* position of phenyl ring (MIC 22.3 μM). Compound **8**, isomer of **7** with methoxy substituent in *para* position, showed less activity indicating the importance of substituent's position in biological events. Other derivatives (nonsubstituted, halogens, nitro) ranged in MIC 46.1–184.7 μM, being evidently less potent. The best activity against *S. aureus* was again observed for *ortho*-methoxy compound (**7**) (MIC 44.5 μM). The best activity against *E. coli* was observed for unsubstituted xanthen-3-one derivative (**1**) (MIC 24.3 μM). No antimicrobial activity was observed for tested compounds against *B. subtilis*.

Table 1 – MIC / μM and inhibition zone (mm) of the investigated compounds
Tablica 1 – MIC / μM i zona inhibicije (mm) ispitivanih spojeva

MIC / μM and inhibition zones / mm					
Compd.	*B. subtilis*	*S. aureus*	*E. coli*	*S. cerevisiae*	*C. albicans*
1	0	97.4 (15)	24.3 (16.5)	97.4 (12)	0
2	0	87.9 (19)	NT[a] (13)	43.9 (12.5)	43.9 (13)
3	0	176.2 (15)	NT[a] (12)	87.9 (11)	97.9 (12)
4	0	NT[a] (13)	0	NT[a] (10.25)	NT[a] (11)
5	0	0	46.1 (19)	184.7 (8.5)	46.1 (12)
6	0	92.2 (17)	184.7 (13.5)	92.2 (10)	NT[a] (10.5)
7	0	44.5 (19)	89.1 (14)	22.3 (16)	22.3 (18)
8	0	0	NT[a] (12)	44.5 (15)	NT[a] (11)
9	0	0	0	171.1 (9)	0
10	0	81.8 (15)	81.8 (15)	NT[a] (8)	40.9 (12)
DMSO	0	3200 (0)	6400 (0)	1600 (0)	3200 (0)
Tetracycline	NT[a]	(21)	(18)	NT[a]	NT[a]
Streptomycin	(24.4)	NT[a]	NT[a]	NT[a]	NT[a]
Amphotericin	NT[a]	NT[a]	NT[a]	(13)	(15)

[a] NT – not tested

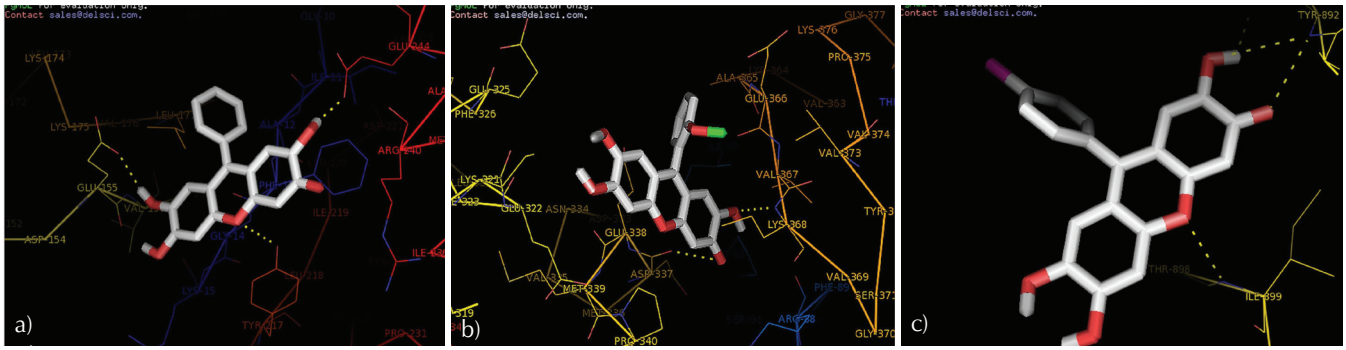

Fig. 2 – Binding modes of compounds **1**, **7,** and **4** at the active sites of: a) enzyme I, b) CYP 51, and c) topoisomerase II, respectively, as assessed by molecular docking study
Slika 2 – Načini vezanja spojeva **1, 7** i **4** na aktivnim mjestima: a) enzima I, b) CYP 51 i c) topoizomeraze II, utvrđeno *docking* studijom

Methoxy group is an electron-donating group, and reduces the acidity of the compound, thereby stabilising its structural conformation. It is probably the reason why methoxy substituent in xanthen-3-one derivatives increases the *in vitro* activity against gram-positive cocci and fungal strains. Results of antimicrobial activity showed that position of substituent is very important for antimicrobial activity of synthesized xanthen-3-on derivatives. Compounds with substituent at *para* position showed weak activity against tested microorganisms (compounds **4**, **6**, **8**, **9**), while substituents in *ortho* position increased antimicrobial activity (compounds **5** and **7**).

The antimicrobial activity of synthetic derivatives is most often related with the cell membrane interaction. The membrane-based mechanism of action refers to the ability to damage the cell membrane structures or to affect the permeability.

In order to investigate a plausible mechanism of action of the most active compounds against *E. coli*, *S. cerevisiae* and *C. albicans,* docking study of compounds **1** and **7** into active sites of enzymes was performed. Docking study was carried out by taking amino terminal domain of enzyme I as a target for antibacterial activity versus *E. coli*,[10] and CYP 51 for antifungal activity.[11] The molecular modelling studies indicated that the synthesized compounds inhibited the enzyme 1 activity by binding effectively with the active sites of the enzyme 1 *via* hydrogen bonding, hydrophobic interaction, and electrostatic interactions, and confirmed their potent antimicrobial activity. Binding mode in docking study of compound **1** at the amino terminal active site of enzyme I from *E. coli* is shown in Fig. 2a.

Compound **1** binds at the active site of enzyme with binding energy of −4.68 kcal mol^{-1} by forming hydrogen bonds between OH group on C-7 and Glu 155, O-10 with Tyr

217 and OH group on C-2 with Glu 244. Binding energy of ciprofloxacin on the same receptor is −2.74 kcal mol^{-1}, by forming two hydrogen bonds with Glu 88 and Glu 84.

Binding mode of the best antifungal compound **7** at the active site of CYP 51 is shown in Fig. 2b. Molecular docking study showed that the compound **7** bound at the active site of CYP 51 with binding energy −5.69 kcal mol^{-1}. This ligand's space geometry provided relevant hydrogen bond interaction between C-3 carbonyl's oxygen and Asp 337 of the target active site and C-2 hydroxy group with Lys 368. Binding energy of fluconazole on the same receptor was −2.63 kcal mol^{-1} with no formed hydrogen bonds.

3.2 Antiproliferative evaluation

Majority of tested compounds exerted antiproliferative effects at higher tested concentrations (10–100 μM). Compounds concentrations required to inhibit tumour cell proliferation by 50 % (IC_{50} values) are presented in Table 2.

Table 2 – IC_{50} values for tested compounds
Tablica 2 – Vrijednosti IC_{50} za ispitivane spojeve

	IC_{50} / μM			
	Cell lines			
Compd.	MiaPaCa-2	SW620	HeLa	BJ
1	46.7	80.2	5.9	6.6
2	40.8	68.6	17.3	9.1
3	7.7	72.0	7.3	28.4
4	5.8	76.6	2.8	6.4
6	30.6	>100	6.8	21.9
7	73.9	43.4	36.7	7.0
9	88.3	>100	41.5	8.8
10	>100	>100	91.5	79.0

The least active compound was compound **10**, most likely because of the hydroxyl group in its structure that affects the conformation of the target molecules. Compound **4** exerted the most potent antiproliferative activity in the micromolar range (0.1–10 μM) (except on the metastatic cell line SW620), and was thus suitable for further biological evaluation on the HeLa cells. We can assume that the reason for the strongest effect of compound **4** is its structural conformation. Compound **4** has chlorine in the *para* position, and we believe that the very position of chlorine is important, because it can increase the lipophilicity of the compound, so the compound can permeate more easily through the cell membrane and localise near the pharmacological target.[26] Chlorine could also create additional interactions with the target molecule (*e.g.*, dipole-dipole interaction), which could partly explain the stronger activity of compound **4**.

Compound **6** was also cytotoxic especially to HeLa cells, probably due to fluorine in its structure which enhances chemical bonds with biomolecules. Although natural compounds rarely contain fluorine, it is still included in the structures of many drugs where it increases their stability and lipophilicity.[27] Fluorine is an electronegative element and thus increases the formation of hydrogen bonds. Therefore, the introduction of fluorine strengthens the bonds with biomolecules including the target molecules, and the action of the compound, thereby enhancing the effect of the compound.

None of the compounds showed antiproliferative activity on SW620 except at the highest tested concentration (100 μM). Interestingly, compounds **1**, **7**, and **10** increased proliferation of MiaPaCa-2 and SW620 cell lines at micromolar concentrations (0.1–10 μM).

The LC_{50} values (50 % lethal concentration, or compound concentration required to kill half the members of a tested tumour cell population) were all above 100 μM (therefore, these results are not shown), except for compound **6**, which exhibited strong cytotoxic effect against HeLa cells (LC_{50} = 9.3 μM).

Although antiproliferative, the effect of the compounds of the 2,6,7-trihydroxy-9-phenyl-3H-xanthen-3-one series was significant, all test compounds also had antiproliferative effects on BJ fibroblast cells.

The effects on the HeLa cell cycle and apoptosis induction showed that HeLa cells were arrested in G2/M phase cell death, which might be indicative for mitotic catastrophe at the lower tested concentration. A concomitant rise in cell percentage in subG1 at higher tested concentrations was also observed, which is indicative for cell death (Table 3).

Additional confirmation of mitotic catastrophe was performed by analysis of dually phosphorylated Erk ½ and activated form of Wee kinase 1 expression. Mitotic catastrophe was confirmed by Western blot as increased levels of cyclin B1 were visible in HeLa cells (Fig. 3).

As apoptosis may be induced along with mitotic catastrophe,[28] Annexin-V test was performed (Fig. 4). Results confirmed activation of apoptosis as well. Interestingly, mitotic catastrophe was not observed in normal fibroblasts BJ treated by compound **4** (data not shown).

3.3 Induction of cell death

For compound **4**, docking study was performed on the target topoisomerase II.[12,13] Binding mode of compound **4** at the active site of topoisomerase II is shown in Fig. 2c.

It is interesting to compare activities of compounds **2**, **3,** and **4**; these compounds are isomers, differing only in the position of the chlorine substituent. Although, isomer of **4**, compound **3** showed somewhat lower antiproliferative activity, indicating that chlorine substituent in *para* position was probably responsible for more potent *in vitro* interaction. Compared to **3** and **4**, their isomer **2** substituted chlorine in *ortho* position showed significantly lower activity.

Compound **4** binds at the enzyme with binding energy of −5.03 kcal mol^{-1} and provides hydrogen bonds of both

Table 3 – Cell cycle analysis of HeLa and BJ cells treated with compound **4** at concentrations 0.3 and 1.5 μM or 0.5 and 2.5 μM, respectively, for 24 and 48 h. Results are presented as percentages of cells in a certain cell cycle phase (%) ± standard deviation.

Tablica 3 – Analiza staničnog ciklusa HeLa i BJ stanica tretiranih spojem **4** u koncentracijama 0,3 i 1,5 μM odnosno 0,5 i 2,5 μM, tijekom 24 i 48 h. Rezultati su izraženi kao postotak stanica u određenoj fazi staničnog ciklusa (%) ± standardno odstupanje.

Treatment	subG1	G1	S	G2/M
HeLa control 24 h	15.97 ± 3.20	50.71 ± 5.58	25.38 ± 1.89	23.91 ± 3.68
HeLa 0.3 μM 24 h	18.33 ± 0.07	50.46 ± 0.21	27.25 ± 2.71	22.30 ± 2.50
HeLa 1.5 μM 24 h	27.38 ± 10.66	38.28 ± 11.03	30.04 ± 2.91*	31.67 ± 13.94
HeLa control 48 h	13.90 ± 2.01	63.33 ± 5.95	18.85 ± 3.00	17.83 ± 2.95
HeLa 0.3 μM 48 h	16.16 ± 2.49*	51.30 ± 4.27	16.48 ± 9.43	32.22 ± 5.17*
HeLa 1.5 μM 48 h	19.23 ± 1.71*	63.38 ± 7.79	26.53 ± 11.79	9.37 ± 3.99
BJ control 24 h	27.93 ± 1.33	39.97 ± 0.26	34.19 ± 0.42	25.85 ± 0.68
BJ 0.5 μM 24 h	22.39 ± 2.09	39.00 ± 1.35	30.33 ± 1.61	28.37 ± 2.96
BJ 2.5 μM 24 h	35.97 ± 13.95	39.84 ± 1.58	29.56 ± 3.78	30.60 ± 2.21
BJ control 48 h	40.54 ± 1.10	50.90 ± 0.87	29.97 ± 0.61	19.14 ± 0.26
BJ 0.5 μM 48 h	25.09 ± 1.29*	54.38 ± 1.12*	28.21 ± 0.06	17.42 ± 1.05
BJ 2.5 μM 48 h	46.11 ± 0.40*	59.57 ± 0.40*	23.23 ± 0.16*	17.20 ± 0.14*

* Statistically significant at $p < 0.05$

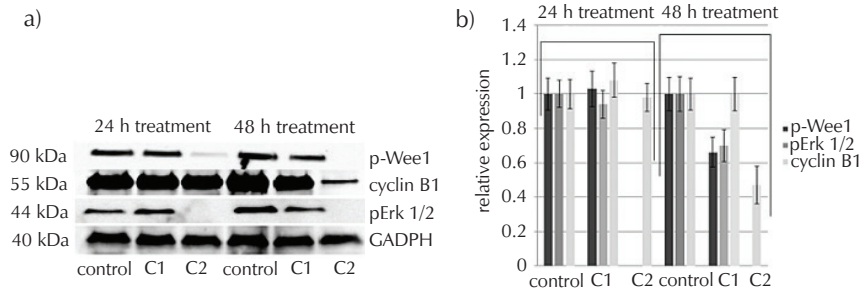

Fig. 3 – a) Western blot analysis of p-Wee 1, pErk ½, and cyclin B1 relative expression in control HeLa cells, HeLa cells treated with compound **4** at two micromolar concentrations C1 and C2 (0.3 and 1.5 μM) for 24 h and 48 h. b) Relative expression (expressed as the average ± standard deviation) of p-Wee 1, pErk 1/2 and cyclin B1 in control HeLa cells, and HeLa cells treated with compound **4** at two micromolar concentrations C1 and C2 (0.3 μM and 1.5 μM) for 24 h and 48 h.

Slika 3 – Relativna ekspresija p-Wee 1, pErk ½ i ciklina B1 u kontrolnim HeLa stanicama, te HeLa stanicama tretiranim spojem **4** pri mikromolarnim koncentracijama C1 i C2 (0,3 i 1,5 μM) tijekom 24 i 48 h: a) *Western blot* analiza i b) prosječna vrijednost relativne ekspresije ± standardna devijacija

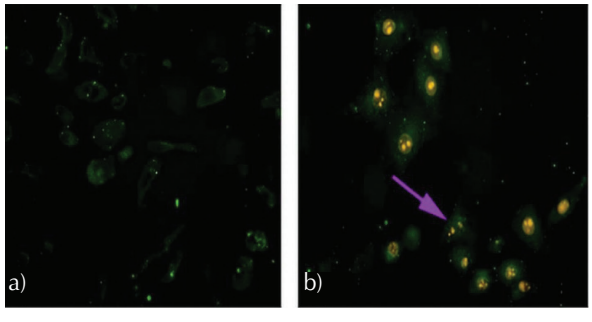

Fig. 4 – Annexin-V assay results: HeLa cells treated with compound **4** at a concentration of 0.3 μM for (a) 24 h and (b) 48 h

Slika 4 – Rezultati Aneksin-V testa: HeLa stanice tretirane spojem **4** koncentracije 0,3 μM tijekom 24 h (a) i 48 h (b)

C-2 hydroxy and C-3 carbonyl's oxygen with Tyr 892, and O-10 with Ile 899. Doxorubicin as referent compound binds on receptor with binding energy −5.09 kcal mol^{-1}, while forming one hydrogen bond with Lys 798. As assessed by docking study, essential ligand-receptor interactions seem to come only from xanthene core. Importance of oxygen groups (C-2 and C-7 hydroxy, C-3 carbonyl, and O-10 ether) of xanthene core for interaction with the receptor is evident. However, different biological potency of isomers indicates importance of substituent's position on the 9-aryl part of the molecule as well.

Further *in vitro* results were in line with docking study. Indeed, inhibition of topoisomerase enzyme II may cause either apoptosis and/or mitotic catastrophe in tumour cells.[29] Western blot analyses showed increased cyclin B1

levels in HeLa cells treated with compound **4**, which is a major mitotic catastrophe's marker,[30] and decreased levels of Wee1 and Erk ½ kinases involved in regulation of the mitotic process. Indeed, in normal circumstances, a large amount of cyclin B1 in the form of inactive cyclin B1/Cdk1 complex is present in the cell just prior to mitosis. The cyclin B1/ Cdk 1 complex is inactivated by phosphorylation of Cdk 1 by Wee 1 kinase on Tyr 15 residue, which inhibits complex activity during the G2 phase of cell cycle and allows proper transition from G2 to M phase of cell cycle.[31,32] Premature entry of active cyclin B1/ Cdk1 complex into nucleus can cause premature chromatin condensation and mitotic catastrophe,[28,33] which is in line with observed results. In addition, previous studies have demonstrated that Erk inhibition increases activity of Myt 1 protein, which is a negative regulator of Cdk1-cyclin B complexes, leading to G2/M phase arrest.[34] Our Western blot results have clearly shown lack of activated Wee-1 and pErk 1/2 in compound **4**-treated HeLa cells both after 24 and 48 h. The observed cell death mechanisms of compound **4** on HeLa cells included both mitotic catastrophe and apoptosis, which is in line with literature data that showed antiproliferative effects of xanthone derivatives through induction of cell cycle arrest and apoptosis.[35]

3.4. Physicochemical properties and bioavailability

Bioavailability of tested compounds was assessed using Lipinski's rule of 5. According to this rule, most molecules that are used in therapy have lipophilicity, logP ≤ 5, molecular weight, *MW* ≤ 500, number of hydrogen bond acceptors, *n*ON ≤ 10, and number of hydrogen bond donors, *n*OHNH ≤ 5.[19,20] If molecules violate more than one of these rules there may be a problem with bioavailability. Along with lipophilicity, another important property for the prediction of oral bioavailability of drug molecules is polar surface area (*PSA*). Values of *PSA* appeared to be a very good descriptor characterising drug absorption, including intestinal absorption, bioavailability, Caco-2 permeability, and blood brain barrier penetration. It is closely related to the hydrogen bonding potential of a compound.[36] Therefore, we have calculated physicochemical properties for compounds, and compared them with the values obtained for standard drugs ciprofloxacin, fluconazole, and doxorubicin (Table 4).

Calculated logP values of tested compounds were between 2.60 and 3.61; according to Lipinski's rule, for the drugs to be able to penetrate through biological membranes this value should not exceed 5. In addition, *PSA* values above 140 Å^2 indicate poor intestinal absorption. Table 4 shows that all tested xanthene derivatives, except **10** (which is also the least biologically active in this study), were within this limit. However, logP and *PSA* values are important, although not sufficient, criteria for predicting oral absorption of a drug.[37] To support this contention, it should be noted that none of the tested compounds had presented a violation of the Lipinski's rule of 5. Two or more violations of the Rule of 5 would suggest a probable problem in bioavailability.[19]

Table 4 – Calculated physicochemical properties of tested compounds

Tablica 4 – Izračunata fizikalno-kemijska svojstva ispitivanih spojeva

Compd.	*PSA* / Å^2	logP	*MW* / g mol^{-1}	*n*ON	*n*OHNH
1	90.89	2.93	320.30	5	3
2	90.89	3.56	354.75	5	3
3	90.89	3.58	354.75	5	3
4	90.89	3.61	354.75	5	3
5	90.89	3.04	338.29	5	3
6	90.89	3.09	338.29	5	3
7	100.13	2.94	350.33	6	3
8	100.13	2.98	350.33	6	3
9	136.72	2.89	365.30	8	3
10	156.95	2.60	381.30	9	4
Ciprofloxacin	74.57	−0.70	331.35	6	2
Fluconazole	81.66	−0.12	306.28	7	1
Doxorubicin	206.08	0.57	543.52	12	7

4 Conclusion

Ten 9-aryl substituted 2,6,7-trihydroxyxanthen-3-one derivatives were previously synthesized by reliable one-pot synthesis procedure. Synthesized derivatives showed antibacterial and antimycotic activities against different bacteria and fungi species. Tested compounds exerted better antifungal activity than antibacterial potential, where compound **7** was the most potent against fungi strains and *S. aureus*, indicating that *ortho* positioned methoxy group greatly influenced antimicrobial activity. Unsubstituted compound **1** showed best activity against *E. coli*. Tested compounds showed no activity against *B. subtilis*.

Majority of tested compounds exerted antiproliferative effects on tumour cell lines at higher tested concentrations (10–100 μM). *Para*-chloro substituted compound **4** exerted the most potent antiproliferative activity in the micromolar range (0.1–10 μM) on all tested cell lines except on the metastatic cell line SW620. *Para* position of chlorine substituent contributed to the best antiproliferative activity compared to *ortho* and *meta* chlorine substituted derivatives (**2** and **3**, respectively). The major mechanism of action of cell death mechanism on HeLa cells, that were the most sensible cells towards compound **4**, included mitotic catastrophe and apoptosis, which is in line with docking study.

Docking studies for the most potent compounds were carried out by taking amino terminal domain of enzyme I as a target for antibacterial activity against *E. coli*, CYP 51 for antifungal activity, and topoisomerase II for anticancer activity against HeLa tumour cells. Docking study showed sites of importance in forming hydrogen bonds with re-

ceptors. Synthesized compounds inhibited the enzyme 1 activity by binding effectively with the active sites of the enzyme 1 *via* hydrogen bonding, hydrophobic interaction, and electrostatic interactions, and confirmed their potent antimicrobial activity. Calculated physicochemical properties support good bioavailability properties for application in humans, and suggest that synthesized 9-aryl substituted xanthenes are prominent candidates for further pre-clinical research.

ACKNOWLEDGEMENTS

We greatly appreciate access to equipment in possession of University of Rijeka within the project "Research Infrastructure for Campus-based Laboratories at University of Rijeka", financed by European Regional Development Fund (ERDF) and University of Rijeka research grant 13.11.1.1.11.

List of abbreviations and symbols
Popis kratica i simbola

PTS – phosphotransferase system
– sustav fosfotransferaze

EI – enzyme I
– enzim I

DMSO – dimethyl sulfoxide
– dimetil sulfoksid

MIC – minimum inhibitory concentration
– minimalna inhibitorna koncentracija

DMEM – Dulbecco's modified Eagle medium
– Dulbeccov modificirani Eagle medij

FBS – foetal bovine serum
– fetalni goveđi serum

PBS – phosphate buffered saline
– fiziološka otopina puferirana fosfatom

PG – percentage growth
– postotni rast

TPSA – topological polar surface area
– topološka polarna površina

MW – molecular mass
– molekularna masa

*n*ON – number of hydrogen bond acceptors
– broj prihvata vodikovih veza

*n*OHNH – hydrogen bond donors
– donori vodikovih veza

References
Literatura

1. *C. G. Knight, T. Stephens,* Xanthene-dye-labelled phosphatidylethanolamines as probes of interfacial pH. Studies in phospholipid vesicles, Biochem. J. **258** (3) (1989) 683–687, doi: https://doi.org/10.1042/bj2580683.
2. *M. Ahmad, T. A. King, D. K. Ko, B. H. Cha, J. Lee*, Performance and photostability of xanthene and pyrromethene laser dyes in sol-gel phases, J. Phys. D: Appl. Phys. **35** (13) (2002) 1473–1476, doi: https://doi.org/10.1088/0022-3727/35/13/303.
3. *K. R. M. Naidu, B. S. Krishna, M. A. Kumar, P. Arulselvan, S. I. Khalivulla, O. Lasekan*, Design, synthesis and antiviral potential of 14-aryl/heteroaryl-14H-dibenzo [a, j] xanthenes using an efficient polymer-supported catalyst, Molecules **17** (6) (2012) 7543–7555, doi: https://doi.org/10.3390/molecules17067543.
4. *H. N. Hafez, M. I. Hegab, I. S. Ahmed-Farag, A. B. A. El-Gazzar*, A facile regioselective synthesis of novel spiro-thioxanthene and spiro-xanthene-9′,2-[1,3,4] thiadiazole derivatives as potential analgesic and anti-inflammatory agents, Bioorg. Med. Chem. Lett. **18** (16) (2008) 4538–4543, doi: https://doi.org/10.1016/j.bmcl.2008.07.042.
5. *D. J. Jiang, Z. Dai, Y. J. Li,* Pharmacological effects of xanthones as cardiovascular protective agents*,* Cardiovasc. Drug. Rev. **22** (2) (2004) 91–102, doi: https://doi.org/10.1111/j.1527-3466.2004.tb00133.x.
6. *A. Ilangovan, K. Anandhan, K. M. Prasad, P. Vijayakumar, R. Renganathan, D. A. Ananth, T. Sivasudha*, Synthesis, DNA-binding study, and antioxidant activity of 14-aryl-14 H-dibenzo [a, j] xanthene derivatives, Med. Chem. Res. **24** (1) (2015) 344–355, doi: https://doi.org/10.1007/s00044-014-1124-8.
7. *H. Marona, N. Szkaradek, E. Karczewska, D. Trojanowska, A. Budak, P. Bober, W. Przepiórka, M. Cegla, E. Szneler*. Antifungal and antibacterial activity of the newly synthesized 2-xanthone derivatives, Arch. Pharm. **342** (1) (2009) 9–18, doi: https://doi.org/10.1002/ardp.200800089.
8. *X. Z. Wang, J. H. Yao, G. B. Jiang, J. Wang, H. L. Huang, Y. L. Liu*, Synthesis, characterization, cytotoxicity, apoptosis and cell cycle arrest of dibenzoxanthenes derivatives, Spectrochim. Acta A **133** (2014) 559–567, doi: https://doi.org/10.1016/j.saa.2014.05.054.
9. *F. Grasso, T. Frisan,* Bacterial genotoxins: merging the DNA damage response into infection biology, Biomolecules **5** (3) (2015) 1762–1782, doi: https://doi.org/10.3390/biom5031762.
10. *K. J. Huang, S. H. Lin, M. R. Lin, H. Ku, N. Szkaradek, H. Marona, A. Hsu, D. Shiuan,* Xanthone derivatives could be potential antibiotics: virtual screening for the inhibitors of enzyme I of bacterial phosphoenolpyruvate-dependent phosphotransferase system, J. Antibiot. **66** (8) (2013) 453–458, doi: https://doi.org/10.1038/ja.2013.30.
11. *M. Iman, A. Davood*, Homology modeling of lanosterol 14α-demethylase of Candida albicans and insights into azole binding, Med. Chem. Res. **23** (6) (2014) 2890–2899, doi: https://doi.org/10.1007/s00044-013-0769-z.
12. *K. Y. Jun, E. Y. Lee, M. J. Jung, O. H. Lee, E. S. Lee, H. Y. P. Choo, Y. Na, Y. Kwon*, Synthesis, biological evaluation, and molecular docking study of 3-(3′-heteroatom substituted-2′-hydroxy-1′-propyloxy) xanthone analogues as novel topoisomerase IIα catalytic inhibitor, Eur. J. Med. Chem. **46** (6) (2011) 1964–1971, doi: https://doi.org/10.1016/j.ejmech.2011.01.011.
13. *S. Alam, F. Khan*, QSAR and docking studies on xanthone derivatives for anticancer activity targeting DNA topoisomerase Iiα, Drug Des. Devel. Ther. **8** (2014) 183, doi: https://doi.org/10.2147/dddt.s51577.
14. *S. Cosconati, S. Forli, A. L. Perryman, R. Harris, D. S. Goodsell, A. J. Olson*, Virtual screening with AutoDock: theory and practice, Expert Opin. Drug Dis. **5** (6) (2010) 597–607, doi. https://doi.org/10.1517/17460441.2010.484460.
15. *H. M. Kumalo, S. Bhakat, M. E. Soliman*, Theory and applications of covalent docking in drug discovery: merits and pitfalls, Molecules **20** (2) (2015) 1984–2000, doi: https://doi.

org/10.3390/molecules20021984.

16. *E. Veljović, S. Špirtović-Halilović, S. Muratović, L. Valek Žulj, S. Roca, S. Trifunović, A. Osmanović, D. Završnik*, 9-Aryl substituted hydroxylated xanthen-3-ones: Synthesis, structure and antioxidant potency evaluation, Croat. Chem. Acta **88** (2) (2015) 121–127, doi: https://doi.org/10.5562/cca2595.
17. *E. Veljović, S. Špirtović-Halilović, S. Muratović, M. Salihović, I. Novaković, A. Osmanović, D. Završnik*, Antimicrobial Activity and Docking Study of Synthesized Xanthen-3-on Derivatives, Res. J. Pharm. Biol. Chem. Sci. **9** (5) (2018) 777–783, url: https://rjpbcs.com/pdf/2018_9(5)/[96].pdf.
18. *E. Veljović, S. Špirtović-Halilović, S. Muratović, A. Osmanović, S. Haverić, A. Haverić, M. Hadžić, M. Salihović, M. Malenica, A. Šapčanin, D. Završnik*, Antiproliferative and genotoxic potential of xanthen-3-one derivatives, Acta Pharm. **69** (4) (2019) 683–694, doi: https://doi.org/10.2478/acph-2019-0044.
19. *C. A. Lipinski, F. Lombardo, B. W. Dominy, P. J. Feeney*, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings 1PII of original article: S0169-409X (96) 00423-1, Adv. Drug Deliv. Rev. **46** (1-3) (2001) 3–26, doi: https://doi.org/10.1016/s0169-409x(00)00129-0.
20. *C. A. Lipinski*, Lead-and drug-like compounds: the rule-of-five revolution, Drug Discovery Today: Technol. **1** (4) (2004) 337–341, doi: https://doi.org/10.1016/j.ddtec.2004.11.007.
21. *T. Gazivoda, S. Raić-Malić, S. Krištafor, D. Makuc, J. Plavec, S. Bratulić, S. Kraljević Pavelić, K. Pavelić, L. Naesens, G. Andrei, R. Snoeck, J. Balzarini, M. Mintas*, Synthesis, cytostatic and anti-HIV evaluations of the new unsaturated acyclic C-5 pyrimidine nucleoside analogues. Bioorg. Med. Chem. **16** (10) (2008) 5624–5634, doi: https://doi.org/10.1016/j.bmc.2008.03.074.
22. *M. Malenica Staver, I. Ratkaj, D. Broznić, I. Jerković, Z. Marijanović, D. Željezić, S. Kraljević Pavelić*, Bioactivity of *Satureja montana* L. honey extracts and their profile screening, RSC Adv. **4** (88) (2014) 47329–47340, doi: https://doi.org/10.1039/c4ra08368g.
23. *T. Gazivoda Kraljević, N. Ilić, V. Stepanić, L. Sappe, J. Petranović, S. Kraljević Pavelić, S. Raić-Malić*, Synthesis and in vitro antiproliferative evaluation of novel *N*-alkylated 6-isobutyl-and propyl pyrimidine derivatives, Bioorg. Med. Chem. Lett. **24** (13) (2014) 2913–2917, doi: https://doi.org/10.1016/j.bmcl.2014.04.079.
24. *A. Bistrović, L. Krstulović, A. Harej, P. Grbčić, M. Sedić, S. Koštrun, S. Kraljević Pavelić, M. Bajić, S. Raić-Malić*, Design, synthesis and biological evaluation of novel benzimidazole amidines as potent multi-target inhibitors for the treatment of non-small cell lung cancer, Eur. J. Med. Chem. **143** (2018) 1616–1634, doi: https://doi.org/10.1016/j.ejmech.2017.10.061.
25. *M. A Lill, J. Danielson*, Computer-aided drug design platform using PyMOL, Comput. Aided Mol. Des. **25** (1) (2011) 13–19, doi: https://doi.org/10.1007/s10822-010-9395-8.
26. *K. Naumann*, Influence of chlorine substituents on biological activity of chemicals, J. Prakt. Chem. **341** (5) (1999) 417–437, doi: https://doi.org/10.1002/(SICI)1521-3897(199907)341:5<417::AID-PRAC417>3.0.CO;2-A.
27. American Academy of Pediatric Dentistry, Guideline on fluoride therapy, Pediatric dentistry **35** (5) (2013) E165–168.
28. *M. Castedo, J. L. Perfettini, T. Roumier, K. Andreau, R. Medema, G. Kroemer*, Cell death by mitotic catastrophe: a molecular definition, Oncogene **23** (16) (2004) 2825–2837, doi: https://doi.org/10.1038/sj.onc.1207528.
29. *A. K. Larsen, A. Skladanowski*, Perturbations of Cellular Functions by Topoisomerase II Inhibitors, in D. A. Gewirtz, S. Holt, S. Grant (eds.): Apoptosis, Senescence, and Cancer, 2nd Ed., Humana Press Inc., Totowa, 2007, pp. 407–422, doi: https://doi.org/10.1007/978-1-59745-221-2_21.
30. *H. Vakifahmetoglu, M. Olsson, B. Zhivotovsky*, Death through a tragedy: mitotic catastrophe, Cell Death Differ. **15** (7) (2008) 1153–1162, doi: https://doi.org/10.1038/cdd.2008.47.
31. *L. D. Berry, K. L. Gould*. Regulation of Cdc2 activity by phosphorylation at T14/Y15, in: Progress in Cell Cycle Research (Eds. L. Meijer, S. Guidet and L. Vogel), Springer, Boston, 1996, pp. 99–105, doi: https://doi.org/10.1007/978-1-4615-5873-6_10.
32. *V. A. Smits, R. H. Medema*, Checking out the G2/M transition, BBA–Gene Struct. Expr. **1519** (1-2) (2001) 1–12, doi: https://doi.org/10.1016/s0167-4781(01)00204-4.
33. *L. Shen, W. Y. Au, K. Y. Wong, N. Shimizu, J. Tsuchiyama, Y. L. Kwong, R. H. Liang, G. Srivastava*, Cell death by bortezomib-induced mitotic catastrophe in natural killer lymphoma cells, Mol. Cancer Ther. **7** (12) (2008) 3807–3815, doi: https://doi.org/10.1158/1535-7163.mct-08-0641.
34. *J. C. Chambard, R. Lefloch, J. Pouysségur, P. Lenormand*, ERK implication in cell cycle regulation, BBA–Mol. Cell Res. **1773** (8) (2007) 1299–1310, doi: https://doi.org/10.1016/j.bbamcr.2006.11.010.
35. *N. Pouli, P. Marakos*. Fused xanthone derivatives as antiproliferative agents, Anti-Cancer Agent. Me. **9** (1) (2009) 77–98, doi: https://doi.org/10.2174/187152009787047699.
36. *D. E. Clark*, Rapid calculation of polar molecular surface area and its application to the prediction of transport phenomena. 1. Prediction of intestinal absorption, J. Pharm. Sci. **88** (8) (1999) 807–814, doi: https://doi.org/10.1021/js9804011.
37. *V. N. Viswanadhan, A. K. Ghose, G. R. Revankar, R. K. Robins*, Atomic physicochemical parameters for three dimensional structure directed quantitative structure-activity relationships. 4. Additional parameters for hydrophobic and dispersive interactions and their application for an automated superposition of certain naturally occurring nucleoside antibiotics, J. Chem. Inf. Comput. Sci. **29** (3) (1989) 163–172, doi: https://doi.org/10.1021/ci00063a006.

SAŽETAK

Antimikrobno i antiproliferativno djelovanje derivata ksanten-3-ona i njihov afinitet vezivanja za enzime

Elma Veljović,[a*] *Anja Harej*,[b] *Marko Klobučar*,[b] *Sandra Kraljević Pavelić*,[b] *Selma Špirtović-Halilović*,[a] *Amar Osmanović*,[a] *Samija Muratović*,[a] *Mirsada Salihović*[a] i *Davorka Završnik*[a]

Za deset prethodno sintetiziranih biološki aktivnih derivata 2,6,7-trihidroksiksanten-3-ona ispitivano je *in vitro* antimikrobno i antiproliferativno djelovanje. Mikrobiološka aktivnost spojeva ispitana je na tri bakterije: *Staphylococcus aureus*, *Bacillus subtilis* i *Escherichia coli*, te dva soja gljiva: *Candida albicans* i *Saccharomyces cerevisiae*. Najbolju antibakterijsku aktivnost protiv *E. coli* pokazao je nesupstituirani spoj **1**. Najveću antifungalnu aktivnost pokazao je spoj **7** s *orto* metoksi supstituentom. Spoj **4** pokazao je najjače antiproliferativno djelovanje u mikromolarnom rasponu (0,1 – 10 μM) na testiranim linijama tumorskih stanica, osim na SW620. Dodatne *Western blot* analize pokazale su povišenu razinu ciklina B1 u HeLa stanicama tretiranim spojem **4**, koji je glavni marker mitotske katastrofe, i smanjenu razinu Wee1 i Erk ½ kinaza uključenih u regulaciju mitotskog procesa. Najpotentniji spojevi podvrgnuti su molekularnim *docking* simulacijama da bi se procijenio afinitet vezanja za enzime i pružili daljnji dokazi o eksperimentalno opaženim biološkim učincima *in vitro*.

Ključne riječi
Ksanteni, antibakterijska i antifungalna aktivnost, indukcija stanične smrti, načini vezanja za enzime, docking studija, predviđanje bioraspoloživosti

[a] *Univerzitet u Sarajevu, Farmaceutski fakultet, Zmaja od Bosne 8, 71 000 Sarajevo, Bosna i Hercegovina*
[b] *Sveučilište u Rijeci, Odjel za biotehnologiju, Centar za visokopropusne tehnologije, Radmile Matejčić 2, 51 000 Rijeka, Hrvatska*

Izvorni znanstveni rad
Prispjelo 29. travnja 2021.
Prihvaćeno 9. listopada 2021.