Journal of Steroid Biochemistry & Molecular Biology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

# Steroid dimers—*In vitro* cytotoxic and antimicrobial activities

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ARTICLE INFO

*Article history:*
Received 3 December 2013
Received in revised form 23 May 2014
Accepted 6 June 2014
Available online xxx

*Keywords:*
Steroid dimers
Cytotoxic activity
Cell death
Cell cycle analysis
Caspase
Antimicrobial activity

ABSTRACT

The *in vitro* cytotoxic activity of previously synthesized steroid dimers with different spacer group (sulfide, trithiolane ring or phosphorotrithioate) and the substituent at C-17 position was tested for their possible effects against following human tumor cell lines: cervical adenocarcinoma (HeLa), chronic myelogenous leukemia (K562) and two human breast cancer cell lines (MDA-MB-361 and MDA-MB-453). These compounds, applied at micromolar concentrations, exhibited cytotoxic activity of different intensity (compared with cisplatin as a control), modality and selectivity in these malignant cell lines. The best activity against all four cell cancer lines was exhibited by dimer-sulfides. All screened compounds exerted concentration-dependent cytotoxic activity against leukemia K562 cells. The compounds which exerted the most pronounced cytotoxic action exhibited notably higher cytotoxic activities against K562, HeLa and MDA-MB-453 cells in comparison to resting and PHA-stimulated PBMC, pointing to a significant selectivity in their antitumor actions. Examination of the mechanisms of cytotoxicity on leukemia K562 cells revealed pro-apoptotic action of each of the investigated compounds applied at concentrations $2IC_{50}$. The most prominent pro-apoptotic action was exhibited by dimer-sulfide of cholest-4-en-3-one. Furthermore, almost all of the tested compounds at $IC_{50}$ concentrations induced G1 phase cell cycle arrest in K562 cells. Antimicrobial activity against Gram-positive, Gram-negative bacteria and fungal cells, and toxicity to brine shrimp *Artemia salina*, were evaluated. There was no antibacterial activity. The best antifungal activity was exhibited against *Saccharomyces cerevisiae* by dimers linked with trithiolane ring, indicating a selective activity of investigated compounds.



## 1. Introduction

One of the most fascinating challenges in modern organic chemistry is the design of structurally diverse and complex molecules which are useful for the study of important biological processes [1]. In many of them, symmetry plays a crucial role [2]. For instance, numerous proteins responsible for cell proliferation and differentiation exist as homodimers or become activated through dimerization as a key step in their respective signaling cascade [3]. For this reason, the synthesis of dimeric molecules (or bivalent ligands) capable, not only to interact with specific biologic receptors, but also to induce greater biological responses than the corresponding monomeric species have been developed.

Steroids are an important group of natural compounds widespread in almost all living organisms expressing various types of biological activity. Among them, steroid dimers form a significant group of pharmacologically active compounds that are predominantly biosynthesized by various marine organisms, and also synthesized in laboratories [4]. Dimerization of steroid skeleton renders some unique characteristics that are applicable to different areas. Dimeric steroids have micellar [5,6], detergent, and liquid–crystal properties [7], and have been used as catalysts for different types of organic reactions in which they play a key role in the rate enhancements from hydrophobic binding [8,9]. A number of dimeric steroids, *e.g.*, cephalostatins (homodimers) and ritterazines (heterodimers), are among the most potent natural cytotoxic agents [10–15]. These compounds exhibit an extraordinarily strong cytotoxic activity, with their most potent member cephalostatin 1 being 400-fold more active in *in vitro* testing than taxol, and therefore are one of the most powerful cytostatics ever to be tested by the National Cancer Institute [16]. Steroid dimers can also be used to create “molecular umbrella” for drug delivery

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http://dx.doi.org/10.1016/j.jsbmb.2014.06.005
0960-0760/

[17–19]. To study the significance of steroid carrier on the antimalarial and antiproliferative activity *in vitro*, a number of cholic acid-based tetraoxane dimers were synthesized [20].

The classification of steroid dimers has been best described in the recently published book, *Steroid Dimers: Chemistry and Applications in Drug Design and Delivery* (2012) by Nahar and Sarker [4], in which the steroid dimers have been classified according to several criteria. First, they can be broadly classified into acyclic dimers (also known as linear dimers) and cyclic dimers. Acyclic dimers involving connections between A, B, C or D rings, or via C-19, direct or through spacers, form the major group of steroid dimers. In the cyclic steroid dimers, dimerization of steroids, direct or through spacers, leads to formation of new ring systems or macrocyclic structures, *e.g.*, cyclocholates or cholaphanes. Steroid dimers can also be classified as symmetrical and unsymmetrical dimers; when a dimer is composed of two identical steroid monomeric units, it is called a symmetrical dimer, and when two different monomeric steroid units are involved or two identical monomeric steroid units are joined in a way that there is no symmetry in the resulting dimer, the dimer is known as an unsymmetrical dimer. One other way of classifying steroid dimers is to divide them into natural and synthetic dimers. However, there are two more reviews, one by Li and Dias (1997) [21] and the other one by Nahar, Sarker and Turner (2007) [22] on steroid dimers covering their chemistry and applications.

The synthetic approaches reported so far, have led to the preparation of cyclic and acyclic steroidal dimers, by connection between two cyclopentanoperhydrophenanthrene skeletons (through A–A, B–B, C–C, D–D or A–D rings) [4,21–23]. The steroidal moieties could be directly linked [24,25], linked through spacer groups [26–30] and by connection through the steroidal side chains [31–33].

In continuation of our work on modified steroid compounds we have recently reported reactions of α,β-unsaturated steroidal ketones (several cholestane, androstane and pregnane carbonyl derivatives were chosen) with Lawesson's reagent (LR: 2,4-bis(*p*-methoxyphenyl)-1,3,2,4-dithiadiphosphetane-2,4-disulfide) in which several sulfur and sulfur and phosphorus containing acyclic (linear) steroidal dimers were synthesized [34]. In all dimers obtained, two identical steroid molecules were joined via ring A–ring A connection through spacer groups. The structures of these dimeric steroids were unambiguously established from their analytical and spectral data (NMR spectroscopy). The conversion of the 4-en-3-one steroid A–ring system in starting molecules **1a–e** to the 3,5-diene system in dimers gave symmetrical 3,3′-sulfides **2a–e** and 3,3′-phosphorotrithioates **4b–e**. Dimers **3a–e** were obtained, by conversion of 4-en-3-one system to the 4-ene system, as mixtures of three possible isomers in approx. 8:1:1 ratio (deduced by comparing the peak areas of the H-4 in the corresponding $^1$H NMR spectra) differing in the configuration at C-3 and C-3′, *i.e.*, by the position of trithiolane ring which linked two steroidal molecules. After several consecutive column chromatographies, diastereomerically pure major isomer was obtained. Unfortunately, all our efforts to get the other two isomers in pure form have failed (Fig. 1).

In the context mentioned above and as a continuation of our investigation of modified steroids as biologically active molecules [34–37], the goal of this study was to perform extensive investigation of *in vitro* cytotoxic activity of the previously synthesized steroid dimers **2a–e**, **3a–e** and **4b–e**. These compounds were tested against four human malignant cell lines: cervical adenocarcinoma (HeLa), chronic myelogenous leukemia (K562) and two human breast cancer cell lines (MDA-MB-361 and MDA-MB-453). In addition, to assess the sensitivity of normal immunocompetent cells included in the antitumor immune response, the cytotoxicity of the most potent compounds **2a–c**, **2e** and **4b** were also tested against human peripheral blood mononuclear cells (PBMC)–both unstimulated and stimulated to proliferate by the mitogen phytohemagglutinin (PHA). The specific aim of this study was to get an insight into modalities of cytotoxic

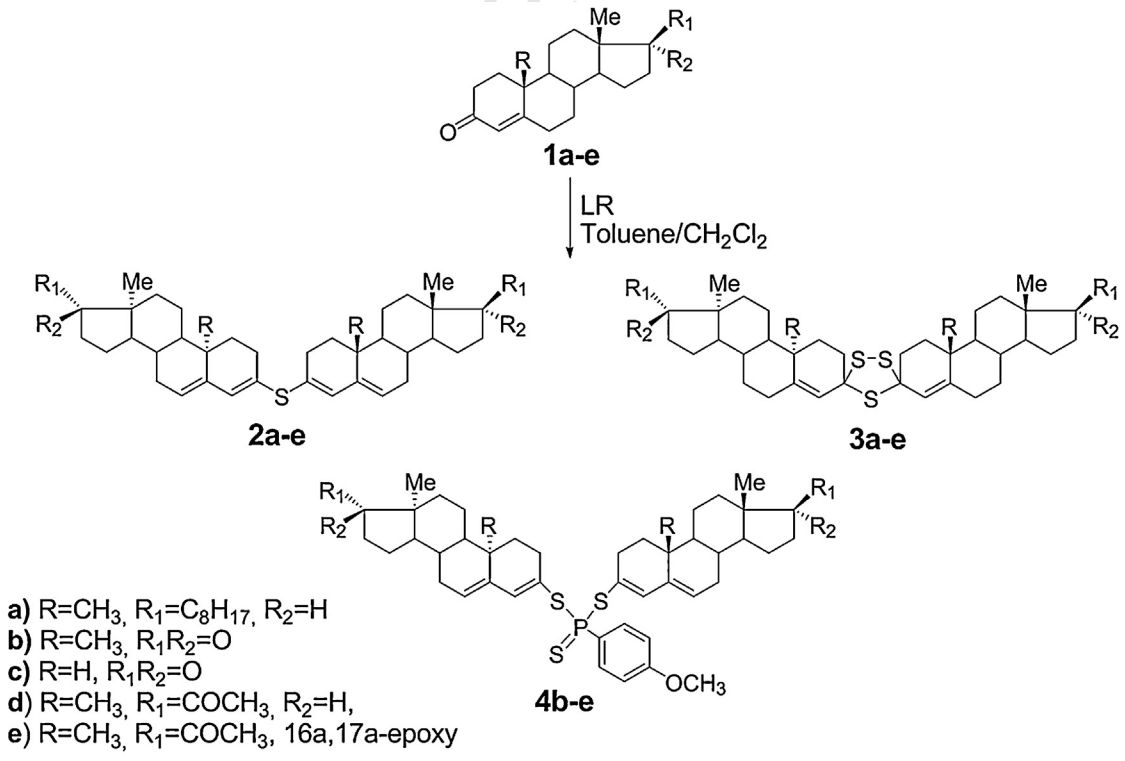


**Fig. 1.** Synthesis of steroid dimers **2a–e**, **3a–e** and **4b–e**.

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effect in targeted, highly sensitive K562 cell line. To elucidate the mechanisms of the cytotoxic effects of the tested compounds (exception of compound **3a**), the distribution of target, highly sensitive K562 cells at specific phases of the cell cycle after the actions of these agents was analyzed. To examine the mode of cell death induced by the investigated steroid compounds, the morphological analysis by fluorescence microscopy of acridine orange/ethidium bromide-stained K562 cells exposed to these compounds was performed. Elucidation of the signaling pathways implicated in the induction of apoptosis by the tested dimers was conducted by identification of target caspases. Also, antimicrobial activity against Gram-positive, Gram-negative bacteria and fungal cells, and toxicity to brine shrimp *Artemia salina* were evaluated.

## 2. Material and methods

Compounds **2a–e**, **3a–e** and **4b–e** were prepared as described earlier [34].

### 2.1. Cytotoxic activity

#### 2.1.1. Preparation of solutions of tested compounds

Stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and later diluted with a nutrient medium (RPMI 1640), supplemented with L-glutamine (3 mM), streptomycin (100 μg/mL) and penicillin (100 IU/mL), 10% heat inactivated (56 °C) fetal bovine serum (FBS) and 25 mM Hepes, adjusted to pH 7.2 by bicarbonate solution and applied to target cells to various final concentrations. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was dissolved (5 mg/mL) in phosphate buffer saline. RPMI 1640 cell culture medium, FBS, Hepes and MTT were Sigma Chemicals' products. The concentrations of tested compounds dissolved in DMSO and further diluted in nutrient medium were carefully chosen in order to eliminate any contribution of the toxic side effect of DMSO.

#### 2.1.2. Cell culture

Human cervix adenocarcinoma HeLa, human breast carcinoma MDA-MB-453 and human breast adenocarcinoma MDA-MB-361 cells were cultured as monolayers, while human chronic myelogenous leukemia K562 cells were grown in a suspension in the nutrient medium. The cells were grown at 37 °C in an atmosphere of 5% $CO_2$ and humidified air. Tested malignant cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA).

#### 2.1.3. Treatment of cancer cell lines

HeLa (2000 cells per well), MDA-MB-453 (3000 cells per well), MDA-MB-361 (10,000 cells per well) were seeded into 96-well microtiter plates and 20 h later, after the cell adherence, five different concentrations of tested compounds were added to the cells. Only nutrient medium was added to the cells in the control wells. K562 cells (5000 cells per well) were seeded 2 h before addition of compounds. Stock solutions of compounds were diluted with complete nutrient medium and applied to target cells to various final concentrations ranging from 12.50 μM to 200 μM. It should be noted that working solutions of compounds, applied to target cells, were filtered through Millipore filters, 0.45 μm, before use. All experiments were done in triplicate. Cisplatin was used as a positive control.

#### 2.1.4. Determination of target cell survival

Survival of target cells was determined by MTT test, according to the method of Mosmann, which was modified by Ohno and Abe [38,39]. Briefly, after 72 h of continuous agents' action, 10 μL of MTT solution was added to each well. The samples were incubated for further 4 h and then 100 μL of 10% SDS was added to the wells. Absorbance at 570 nm was measured the next day.

To get cell survival (*S*%), the absorbance (*A*) of a sample with cells grown in the presence of various concentrations of the investigated compounds was divided by the *A* of the control cells grown only in the nutrient medium, and multiplied by 100. It was implied that the absorbance of the blank was always subtracted from the absorbance of the corresponding sample with target cells. $IC_{50}$ concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared to a control. The $IC_{50}$ values were calculated using template file created in Microsoft EXCEL.

#### 2.1.5. Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were separated from whole heparinized blood of two healthy volunteers by Histopaque®-1077 (Sigma–Aldrich) density gradient centrifugation. Interface cells were washed three times with Haemaccel (aqueous solution supplemented with 145 mM $Na^+$, 5.1 mM $K^+$, 6.2 mM $Ca^{2+}$, 145 mM $Cl^-$ and 35 g/L gelatin polymers, pH 7.4), counted and resuspended in nutrient medium.

#### 2.1.6. Treatment of PBMC

PBMC (150,000 cells per well) were seeded into nutrient medium or in nutrient medium enriched with PHA (5 μg/mL) in 96-well microtiter plates. After 2 h, five different concentrations of the investigated compounds were added to the wells, in triplicate, except to the control wells where a nutrient medium only was added to the cells. The final concentrations of the tested compounds ranged from 12.5 μM to 200 μM. The complete nutrient medium was RPMI 1640 supplemented with 3 mM L-glutamine, 100 μg/mL streptomycin, 100 IU/mL penicillin, 10% heat-inactivated (56 °C) fetal bovine serum and 25 mM Hepes adjusted to pH 7.2 with a bicarbonate solution. PHA was obtained from INEP (Belgrade, Serbia). Cisplatin was used as a positive control.

#### 2.1.7. Morphological evaluation of K562 cell death

To examine the mode of human chronic myelogenous leukemia K562 cell death induced by the investigated compounds, morphological analysis by microscopic examination of acridine orange/ethidium bromide-stained target cells was performed. K562 cells were seeded in 6-well plates (200,000 cells per well) in 2 mL of complete nutrient medium. After 2 h, cells were treated with investigated compounds for 24 h at concentrations corresponding to double $IC_{50}$ values that were obtained after treatments that lasted 72 h. After this period, the target cells were stained with 20 μL of a mixture of the DNA dyes acridine orange and ethidium bromide (3 μg/mL AO and 10 μg/mL EB in PBS), and visualized under a fluorescence microscope – Carl Zeiss PALM MicroBeam with Axio Observer.Z1 using AxioCam MRm (filters Alexa Fluor 489 and Alexa Fluor 546). Typical morphological features of the apoptotic cells were highly condensed and/or fragmented nuclei stained with DNA dyes. In early apoptosis only acridine orange entered the cell but ethidium bromide was excluded and the nucleus stained green. In late apoptosis along with the loss of membrane integrity, both dyes entered the cell and the nucleus became orange-red.

#### 2.1.8. Cell cycle analysis

K562 cells were exposed to two different concentrations of the examined compounds (corresponding to the $IC_{50}$ and $2IC_{50}$ values determined after 72 h treatment) for 24 h and 48 h. After these incubation times, the target cells were collected, washed with PBS and fixed in 70% ethanol on ice. The cell samples were stored at



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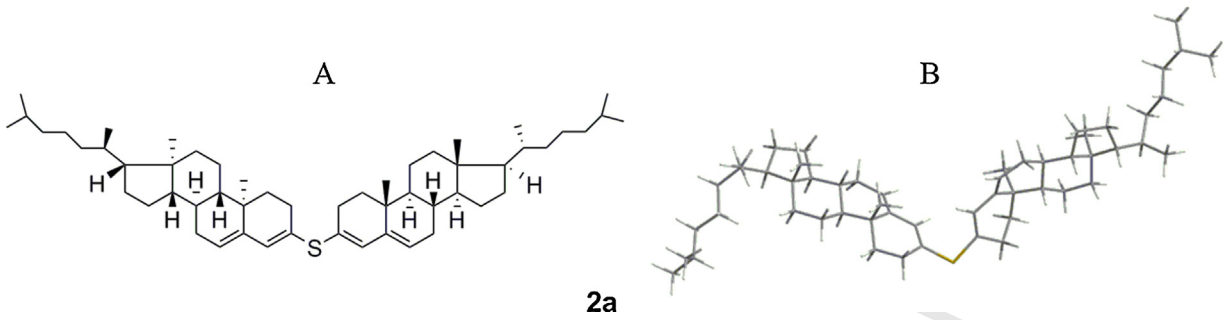


**Fig. 2.** Chemical structure (A) and 3D representation (B) of bis(cholesta-3,5-dien-3-yl) sulfide (**2a**).

−20 °C for at least one week before staining. K562 cells were collected by centrifugation, washed, resuspended in PBS containing RNase A at a final concentration of 200 μg/mL and incubated for 30 min at 37 °C. Subsequently the propidium iodide staining solution was added to the cells at a final concentration of 40 μg/mL.

Cell cycle phase distribution was determined using a FACSCalibur Flow Cytometer (BD Biosciences Franklin Lakes, NJ, USA). The data (10,000 events collected for each sample) were analyzed using CELLQuest software (BD Biosciences). The cell cycle distribution data represent the mean ± S.D of four independent experiments. The statistical significance of differences between the control and treated cell samples was evaluated using one-way ANOVA with Dunnett's post test. *p*-values below 0.05 were considered statistically significant.

### 2.1.9. Determination of target caspases

To identify the caspases involved in the apoptotic signaling pathways induced by the investigated steroid dimers, the percentages of K562 leukemia cells in the subG1 phase pretreated with caspase inhibitors were determined. K562 cells were preincubated for 2 h with specific caspase inhibitors (at a final concentration of 30 μM in 2.5 ml of complete nutrient medium). These were: Z-DEVD-FMK, a caspase-3 inhibitor, Z-IETD-FMK, a caspase-8 inhibitor and Z-LEHD-FMK, a caspase-9 inhibitor. The caspase inhibitors were purchased from R&D Systems (Minneapolis, USA). The tested compounds were applied to K562 cells at concentrations that corresponded to the double $IC_{50}$ values obtained after 72 h treatment. For each compound, one sample of K562 cells was not pretreated with an inhibitor and served as a reference sample. After 24 h of incubation, target cells were harvested and fixed in 70% ethanol on ice. The samples were stored at −20 °C for one week before PI staining. The changes in the percentages of cells in the subG1 phase were determined using a FACSCalibur Flow Cytometer and analyzed using CELLQuest software.

**Table 1**
The *in vitro* cytotoxic activity of compounds **2a–e**, **3a–e** and **4b–e**.

| Comp. | $IC_{50} \pm SD$ (μM) | | | |
|---|---|---|---|---|
| | HeLa | MDA-MB-453 | MDA-MB-361 | K562 |
| **2a** | 21.2 ± 1.6 | 19.9 ± 2.1 | 27.1 ± 3.8 | 14.9 ± 3.2 |
| **2b** | 68.2 ± 20.5 | 48.2 ± 0.6 | 173.6 ± 22.7 | 14.0 ± 5.6 |
| **2c** | 84.4 ± 2.0 | 129.6 ± 16.4 | >200 | 24.0 ± 6.7 |
| **2d** | 74.5 ± 6.9 | 173.0 ± 1.7 | >200 | 45.4 ± 2.4 |
| **2e** | 37.2 ± 0.6 | 30.1 ± 10.3 | 158.0 ± 4.4 | 12.0 ± 2.4 |
| **3a** | >200 | >200 | >200 | 186.7 ± 18.7 |
| **3b** | 89.3 ± 25.1 | 104.1 ± 28.5 | ≈200 | 43.0 ± 6.4 |
| **3c** | 110.7 ± 16.2 | 132.6 ± 39.3 | >200 | 23.7 ± 7.0 |
| **3d** | 85.9 ± 0.5 | 74.9 ± 0.1 | >200 | 20.8 ± 1.6 |
| **3e** | >200 | >200 | >200 | 26.6 ± 4.5 |
| **4b** | 79.8 ± 10.5 | 71.8 ± 8.6 | 183.9 ± 8.4 | 13.7 ± 3.5 |
| **4c** | 133.0 ± 41.7 | 114.6 ± 31.5 | >200 | 30.5 ± 7.8 |
| **4d** | 121.6 ± 32.4 | 152.8 ± 13.8 | >200 | 38.7 ± 6.0 |
| **4e** | 116.1 ± 9.17 | 134.2 ± 28.0 | >200 | 53.0 ± 0.9 |
| Cisplatin | 2.1 ± 0.2 | 3.6 ± 0.5 | 17.1 ± 1.2 | 6.2 ± 0.6 |

### 2.2. The brine shrimp test

The brine shrimp test was performed against freshly hatched nauplii of *A. salina* [40]. A teaspoon of lyophilized eggs of the brine shrimp *A. salina* was added to 0.5 L of the artificial sea water containing several drops of yeast suspension (3 mg of dry yeast in 5 mL distilled water) and air was passed through the suspension under illumination for 48 h. The hatched nauplii were used in experiments. The compounds were dissolved in $CH_2Cl_2$, then in various amounts (0.5–0.0125 mg) applied to filter paper discs (8 mm in diameter), and the solvent was evaporated. Paper discs were placed on the bottom of the glass vial into which 1 mL of artificial sea water, and about 15–20 hatched nauplii were added. The vials were left at room temperature under illumination for 24 h, and afterward live and dead nauplii were counted. All samples were done in triplicate. $LC_{50}$ was defined as a concentration of compounds that causes death of 50% nauplii.

### 2.3. Antimicrobial activity

The antimicrobial activity of all steroidal dimers was evaluated against Gram-positive bacteria *Micrococcus luteus* (ATCC 4698), Gram-negative bacteria *Escherichia coli* (ATCC 25922), yeast species *Candida albicans* (ATCC 10231) and *Saccharomyces cerevisiae* (ATCC 9763) and against *Penicillium* sp. (isolated from *Calendulae* flos) using disk diffusion method, according to the NCCLS [41].

All the tested compounds were dissolved in $CH_2Cl_2$ (2 mg/mL), and then 100 μL of solution was applied to filter paper discs (8 mm in diameter), and the solvent was evaporated so that the compounds were tested at the dose of 200 μg per disc.

Antibacterial assays were performed in Mueller-Hinton agar (Biolab). In each sterile Petri dish (90 mm diameter) 22 mL of Mueller-Hinton agar and 100 μL of bacterial suspension ($10^6$ cells per mL) were added. Tetracycline (Institute of Virology, Vaccines and Sera, Torlak, Belgrade) 30 μg per filter paper disc (8 mm in diameter) was used as a positive control, while the discs of the same diameter impregnated with 100 μL of $CH_2Cl_2$ were used as a negative control. Filter paper discs with the investigated compounds were placed on the agar. Petri dishes were incubated for 24 h at 37 °C. Measured susceptibility zones were the clear zones around the disk inhibiting the microbial growth. Zone of inhibition was measured in millimeters, including the disc.



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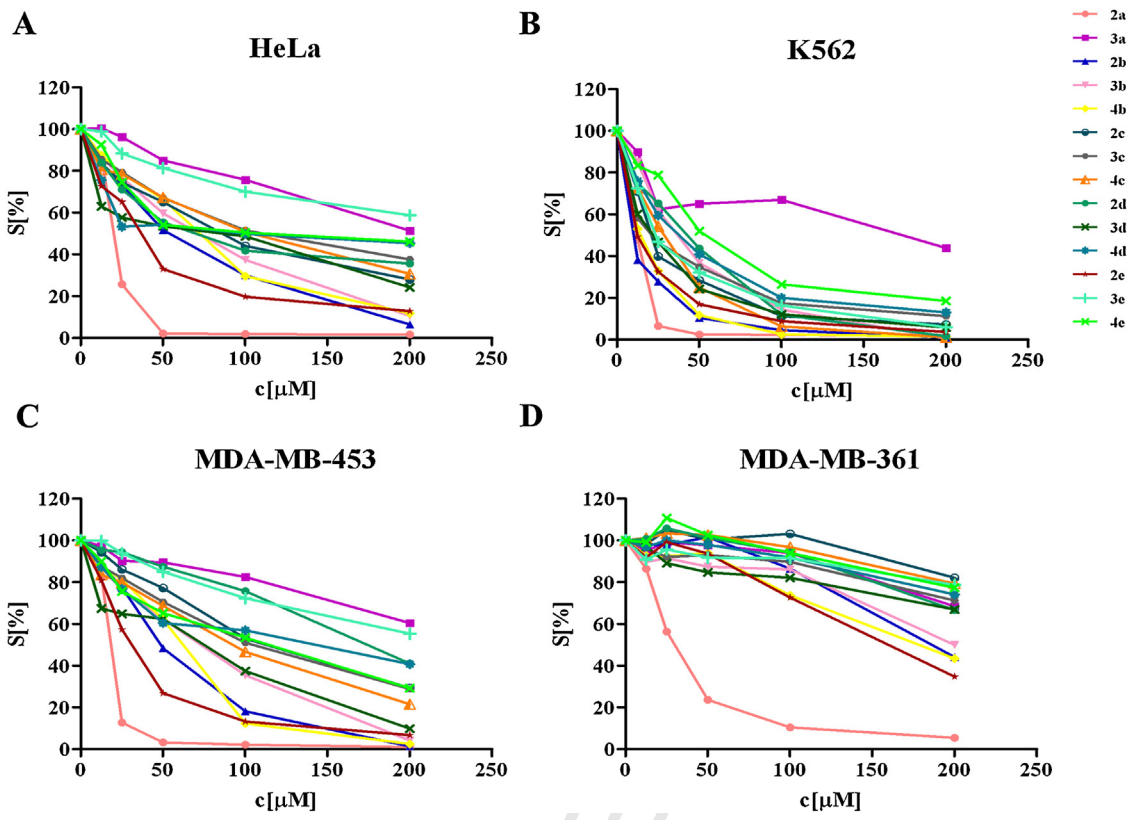


**Fig. 3.** The concentration–response curves of compounds **2a**–**e**, **3a**–**e** and **4b**–**e** on tumor cells.
Survival of HeLa (A), K562 (B), MDA-MB-453 (C) and MDA-MB-361 cells (D) grown for 72 h in the presence of increasing concentrations of investigated compounds, determined by MTT test as described in Section 2. Representative graphs are shown. Three independent experiments were conducted in triplicate. S(%): survival.

Antifungal assays were performed in Sabouraud dextrose agar (Institute of Virology, Vaccines and Sera, Torlak, Belgrade). In each sterile Petri dish (90 mm diameter) 22 mL of the previously prepared agar suspension (according to the manufacturer's instructions) was poured, and 100 μL of fungi suspension ($10^5$ spores per mL) was added. Paper discs of investigated compounds were placed on the agar with the fungi. Nystatin (Hemofarm-STADA, Vršac), 30 μg per filter paper disc (diameter of 8 mm) was used as a positive control, while disc with 100 μL of $CH_2Cl_2$ was used as a negative control. Petri dishes were incubated for 48 h at 28 °C. Zone of inhibition was measured in millimeters, including the disc.

## 3. Results and discussion

### 3.1. Cytotoxic activity

The *in vitro* cytotoxic activity of the previously synthesized steroid dimers **2a**–**e**, **3a**–**e** and **4b**–**e** was tested against four malignant cell lines: human cervix adenocarcinoma HeLa, two human breast cancer cell lines (originally estrogen receptor-positive and progesterone receptor-positive MDA-MB-361 cells and estrogen receptor-negative and progesterone receptor-negative MDA-MB-453 cells) and chronic myelogenous leukemia K562 cells. The selected compounds were also screened for cytotoxic activity against normal huma peripheral blood mononuclear cells (PBMC) non-stimulated and stimulated with phytohaemaglutinin (PHA).

In general, dimer-sulfides **2a**–**e** (as well as cisplatin, which served as a positive control) exhibited the highest cytotoxic actions against tested malignant cell lines. Among them, dimer-sulfide of cholest-4-en-3-one (**2a**) (Fig. 2) proved to be the most potent against all cell lines with $IC_{50}$ from 14.9 μM to 27.1 μM (Table 1).

Toward K562 cells, dimer-sulfides **2a**, **2b** and **2e** exerted a strong, concentration-dependent cytotoxic activity with $IC_{50}$ values of 14.9, 14.0 and 12.0 μM, respectively. Dimers **2c** and **2d** were less, but still very active with $IC_{50}$ values of 24.0 and 45.4 μM, respectively. A very good activity against HeLa cells was exhibited by **2a** and **2e** with $IC_{50}$ 21.2 μM and 37.2 μM while dimer-sulfides **2b**–**d** showed a moderate activity against this cell line with $IC_{50}$ 68.2, 84.4 and 74.5 μM, respectively. Against ER- and PR-negative MDA-MB-453 cells dimers **2a** and **2e** showed a very good activity with $IC_{50}$ 19.9 μM and 30.1 μM, respectively, compound **2b** exhibited a good activity with $IC_{50}$ 48.2 μM while compounds **2c** and **2d** showed a very poor activity with $IC_{50}$ 129.6 and 173.0 μM, respectively. Compound **2a** exerted a very good cytotoxic activity against ER- and PR-positive MDA-MB-361 cells with $IC_{50}$ 27.1 μM, dimer-sulfides **2b** and **2e** showed a very poor activity against this cell line with $IC_{50}$ 173.6 and 158.0 μM, respectively, while **2c** and **2d** were practically inactive.

**Table 2**
The *in vitro* cytotoxic activity of **2a**–**c**, **2e** and **4b** on PBMC.

| Comp. | $IC_{50} \pm SD$ (μM) | |
|---|---|---|
| | PBMC[a] | PBMC + PHA[a] |
| **2a** | 164.8 ± 42.5 | 166.2 ± 67.6 |
| **2b** | 196.2 ± 7.6 | 188.0 ± 23.9 |
| **2c** | >200 | 200 |
| **2e** | 177.9 ± 34.6 | 172.7 ± 54.5 |
| **4b** | 181.3 ± 13.9 | 165.8 ± 23.6 |
| Cisplatin | >50 | 47.3 ± 4.2 |

[a] From four independent experiments.

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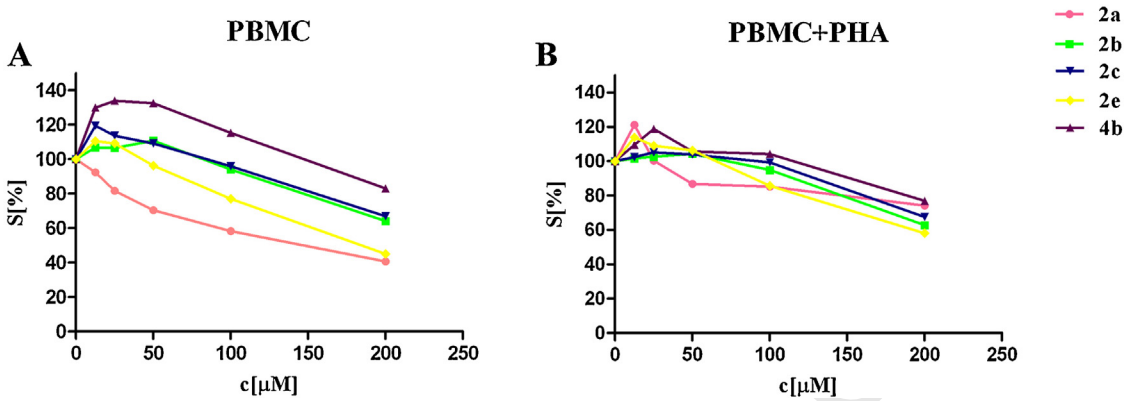


**Fig. 4.** The concentration–response curves of compounds **2a–e**, **3a–e** and **4b–e** on healthy immunocompetent PBMC.
Survival of resting PBMC (A) and PHA-stimulated PBMC (B) grown for 72 h in the presence of increasing concentrations of investigated dimers, determined by MTT test as described in Section 2. Representative graphs are shown. Four independent experiments were conducted in triplicate. S(%): survival.

Dimers **4b–e** with phosphorotrithioate ring as a spacer group displayed less pronounced cytotoxicity in comparison to dimersulfides **2a–e**; exception is K562 cell line against which these compounds showed a good cytotoxic activity with $IC_{50}$ from 13.7–53.0 μM. Dimers **3a–e** with trithiolane ring had, in general, the lowest cytotoxic action. Compound **3a** was practically inactive against all tested malignant cell lines, including K562 cells, against which the other trithiolane dimers exerted a good activity with $IC_{50}$ from 20.8 μM to 43.0 μM (Table 1 and Fig. 3).

On the other hand, if we consider the type of steroid units which participates in the dimer construction the best results in general were obtained with androst-4-en-3,17-dione dimers (**2–4**)**b** (Table 1).

Androstene dimers **2b** and **4b** exerted a strong, concentration-dependent cytotoxic activity against K562 cells with $IC_{50}$ values of 14.0 and 13.7 μM, respectively, while dimer **3b** showed a good activity with $IC_{50}$ 43.0 μM. Against HeLa cells, all androstene dimers exhibited moderate activity with $IC_{50}$ from 68.2 μM to 89.3 μM. Dimer **2b** showed good activity against ER- and PR-negative MDA-MB-453 cells with $IC_{50}$ 48.2 μM while compounds **3b** and **4b** showed a moderate activity with $IC_{50}$ 104.1 and 71.8 μM, respectively. Toward ER- and PR-positive MDA-MB-361 cell line dimers **2b** and **4b** showed very poor activity with $IC_{50}$ 173.6 and 183.9 μM, respectively while **3b** was practically inactive.

With regard to the specific sensitivities of the different cells to the cytotoxic activities of the tested dimers, it is important to note that K562 cells were the most sensitive to the cytotoxic actions of almost all investigated compounds. HeLa and MDA-MB-453 cells exhibited a lower sensitivity, while the sensitivity of breast cancer MDA-MB-361 cell line to the toxic actions of the tested compounds was the lowest (Table 1 and Fig. 3).

The compounds which exerted the most pronounced cytotoxic action against malignant cells, especially strong against myelogenous leukemia K562 cells, were selected for further evaluation of their anticancer potential through examination of cytotoxicity against healthy immunocompetent PBMC – both unstimulated and stimulated to proliferate by the mitogen phytohemagglutinin (PHA) (Table 2 and Fig. 4). Each of the tested compounds (**2a–c**, **2e** and **4b**) exhibited notably higher cytotoxic activities against K562, HeLa and MDA-MB-453 cells in comparison to resting and PHA-stimulated PBMC[1].

High selectivities in the antitumor action of all of the examined compounds were observed against leukemia K562 cells, which were the most sensitive to their cytotoxic effects. It is noteworthy that compounds **2a** and **2e** displayed a very good selectivity in the anticancer effect against human cervix adenocarcinoma HeLa and human breast carcinoma MDA-MB-453 cells. In addition, compounds **2b**, **2c** and **4b** showed considerably higher intensities of cytotoxic action against HeLa and MDA-MB-453 cells than against normal immunocompetent PBMC (Table 3).

The observed pronounced cytotoxic activities against HeLa, MDA-MB-453 and particularly against K562 cells in addition to remarkable selectivities in their cytotoxic actions against target malignant cells in comparison to healthy PBMC point out the prominent anticancer potential of these compounds, especially of compounds **2a** and **2e**.

### 3.2. Morphological analysis of K562 cell death mode

The results mentioned above, as well as a high coefficient of selectivity (Cs) in the antitumor action of most of the investigated compounds prompted us to get a deeper insight into the mode of cell death induced by the investigated agents.

In order to determine whether the investigated dimers have pro-apoptotic activities, morphological analysis by fluorescence microscopy of acridine orange/ethidium bromide-stained K562 cells exposed for 24 h to these compounds (exception of compound **3a**) were performed. The morphological examination revealed that each of the tested dimers applied at a concentration of double $IC_{50}$ triggered apoptosis in exposed cells. The alterations in the structure, size and shape of the cell nucleus were detected.

**Table 3**
Coefficient of selectivity (Cs) in the antitumor action of **2–c**, **2e** and **4b** as a ratio of $IC_{50}$ values for PBMC and malignant cells.

| Comp. | Cs | | | | | |
|---|---|---|---|---|---|---|
| | PBMC/ HeLa | PBMC + PHA/ HeLa | PBMC/ MDA-MB-453 | PBMC + PHA/ MDA-MB-453 | PBMC/ K562 | PBMC + PHA/ K562 |
| **2a** | 7.78 | 7.85 | 8.27 | 8.34 | 11.05 | 11.15 |
| **2b** | 2.88 | 2.76 | 4.07 | 3.90 | 14.03 | 13.44 |
| **2c** | 2.37 | 2.37 | 1.54 | 1.54 | 8.33 | 8.33 |
| **2e** | 4.78 | 4.64 | 5.90 | 5.73 | 14.81 | 14.38 |
| **4b** | 2.27 | 2.08 | 2.52 | 2.31 | 13.2 | 12.06 |

[1] It must be mentioned that one person's PBMCs have shown much more pronounced sensitivity for the cytotoxic action of all examined compounds, especially compounds **2a** and **2e**, indicating existence of the interindividual differences in the sensitivity of normal PBMC. The $IC_{50}$ values for these compounds determined for non-stimulated PBMC were 113.1 and 127.2 μM, respectively. PHA-stimulated PBMC were even less resistant to cytotoxic action of the compounds **2a** and **2e** with $IC_{50}$ values of 64.8 and 90.9 μM, respectively.

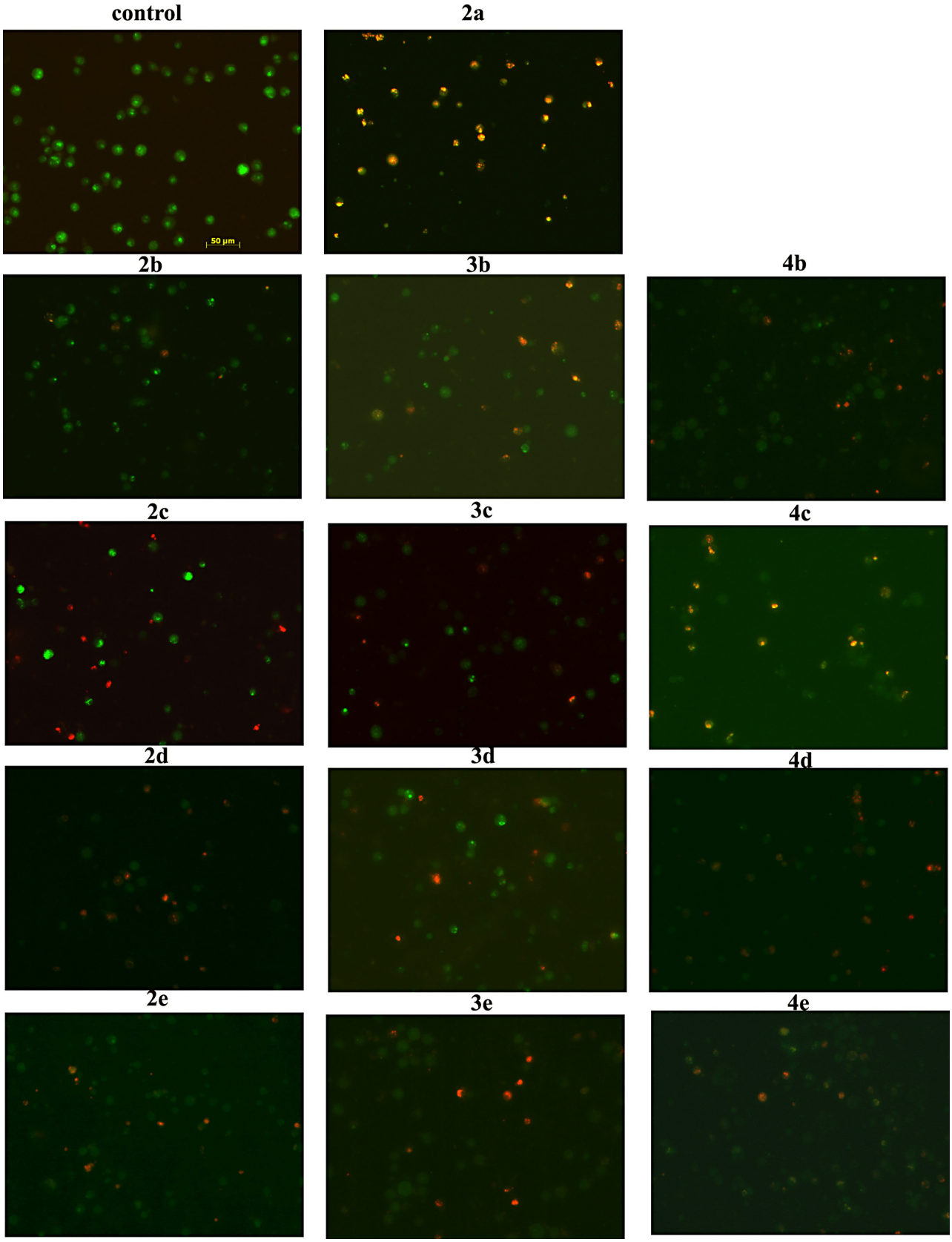


**Fig. 5.** Induction of apoptosis and/or necrosis in K562 cells by compounds **2a–e**, **3b–e** and **4b–e**.
Photomicrographs of acridine orange/ethidium bromide-stained control K562 cells and K562 cells exposed for 24 h to investigated compounds **2a–e**, **3b–e**, **4b–e** as described in the Section 2. Applied concentrations of tested compounds corresponded to $2IC_{50}$ values determined for 72 h. It can be seen that each of the examined dimers induces apoptosis in target myelogenous leukemia K562 cells. Typical hallmarks of pro-apoptotic effects of the steroid dimers are orange-red stained K562 cells in late stages of apoptosis or secondary necrosis, as well as K562 cells with highly condensed or even fragmented nuclei.

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Chromatins of condensed appearance, cell shrinkage and nuclear fragmentation as well as a formation of apoptotic bodies were thus observed. Compound **2a** exhibited the strongest pro-apoptotic activity, as it could be seen in Fig. 5. Other tested compounds also displayed the ability to induce apoptotic cell death in target K562 cells.

Typical hallmarks of pro-apoptotic actions of the examined compounds were orange-red stained K562 cells in late stages of apoptosis or secondary necrosis, as well as green stained apoptotic cells with highly condensed or even fragmented nucleus. It is noteworthy that late apoptosis followed by secondary necrosis, two phenomena which are naturally interconnected, are sometimes seen [42].

To elucidate the mechanisms of the cytotoxic actions of the examined steroid dimers, the assessment of changes in the cell cycle phase distribution of myelogenous leukemia K562 cells treated with compounds for 24 and 48 h was done. Cell cycle analysis revealed that compounds **2a**, **2d**, **3b**, **4b–e** applied at $IC_{50}$ concentrations induced significant increase in the percentages of K562 cells in the subG1 phase ($p < 0.05$) after 24 h exposure in comparison to control cell sample (Fig. 6). The compounds **2e** and **4e** at $IC_{50}$ concentration caused significant accumulation ($p < 0.05$) of target cells in the G1 cell cycle phase. It is noteworthy that treatment with steroid dimers **2a–c**, **3b–e**, **4b–d** led to notable increase in the percentages of cells in the G1 phase, although mentioned differences were not statistically significant. After 48 h of continuous action at $IC_{50}$ concentrations, all tested compounds except **3c** triggered off significant increase in the percentages of subG1 cells ($p < 0.05$) in relation to control cell sample. Furthermore, each of the examined steroid dimers applied at double $IC_{50}$ concentrations induced statistically significant increase in the percentages of subG1 cells after 24 h and 48 h exposure. It is important to mention that the most prominent pro-apoptotic action at higher tested concentrations ($2IC_{50}$) was exerted by compound **2a**.

Elucidation of molecular mechanisms of the cell death triggered in K562 leukemia cells by the steroid dimers was done by identification of possible target caspases implicated in the main apoptotic signaling pathways. Flow cytometric analysis showed that pretreatment with specific caspase-3 inhibitor of K562 cells exposed to the investigated steroid dimers led to decrease in the percentage of subG1 K562 cells in comparison to percentage of subG1 phase K562 cells in treated samples which were not preincubated with mentioned inhibitor (Fig. 7). The notable reduction of the apoptotic K562 cells in the presence of caspase-3 inhibitor was observed for all of the tested compounds except for **2d**. The observed mild decrease indicates that compound **2d** might activate not only caspase-dependent apoptosis but perhaps caspase-independent apoptosis or some other types of cell death as well. Moreover, presence of caspase-8 inhibitor or caspase-9 inhibitor also reduced the levels of apoptotic subG1 K562 cells treated with each of the tested compounds. The smallest decrease was observed in the cell samples exposed to compounds **2d** and **3d**.

### 3.3. The brine shrimp test

In addition, the results of the brine shrimp test are in good correlation with cytotoxicity against K562 leukemia cells. All compounds exerted a moderate activity with $IC_{50}$ in the range of

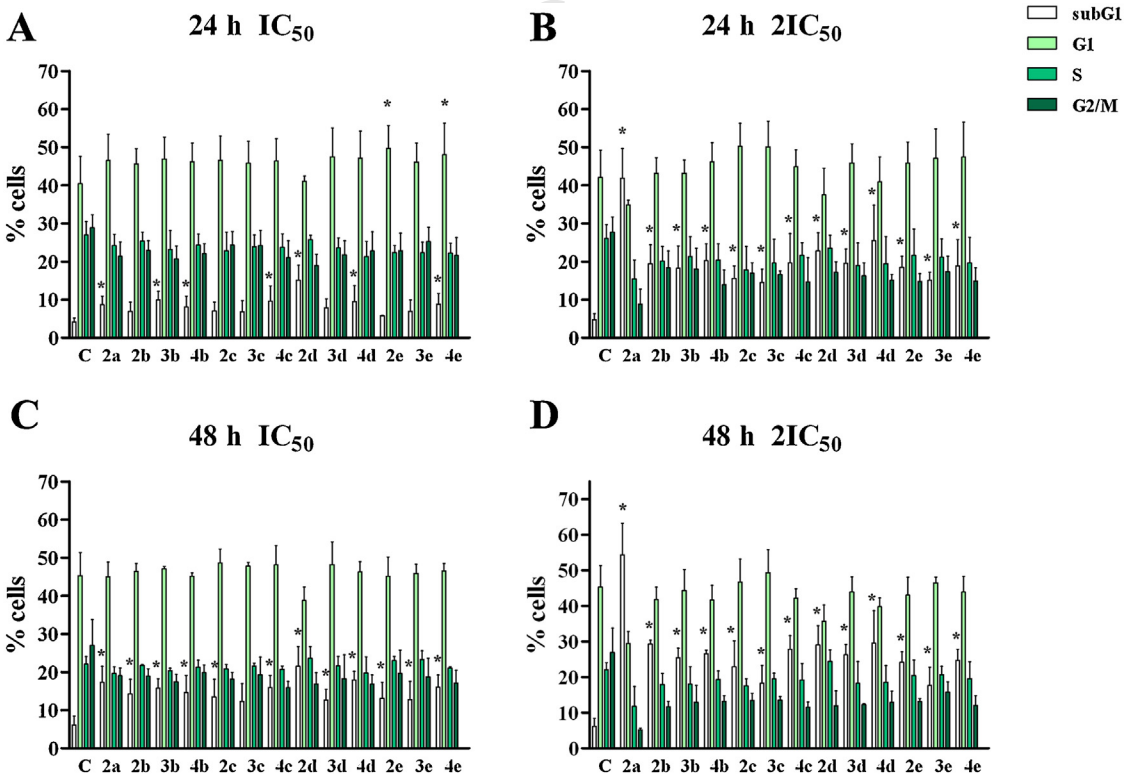


**Fig. 6.** Changes in the cell cycle phase distribution of K562 cells.
Changes in the cell cycle phase distribution of myelogenous leukemia K562 cells, induced by the investigated compounds **2a–e**, **3b–e**, **4b–e** after 24 h (A, B) and 48 h (C, D) treatment. (in the graphs C denotes control). Applied concentrations of tested compounds corresponded to $IC_{50}$ (A, C) and $2IC_{50}$ (B, D) values determined for 72 h. The data shown represent the mean ± S.D of four independent experiments. Significant differences between cell cycle distribution of control and treated cells are indicated by * ($p < 0.05$)

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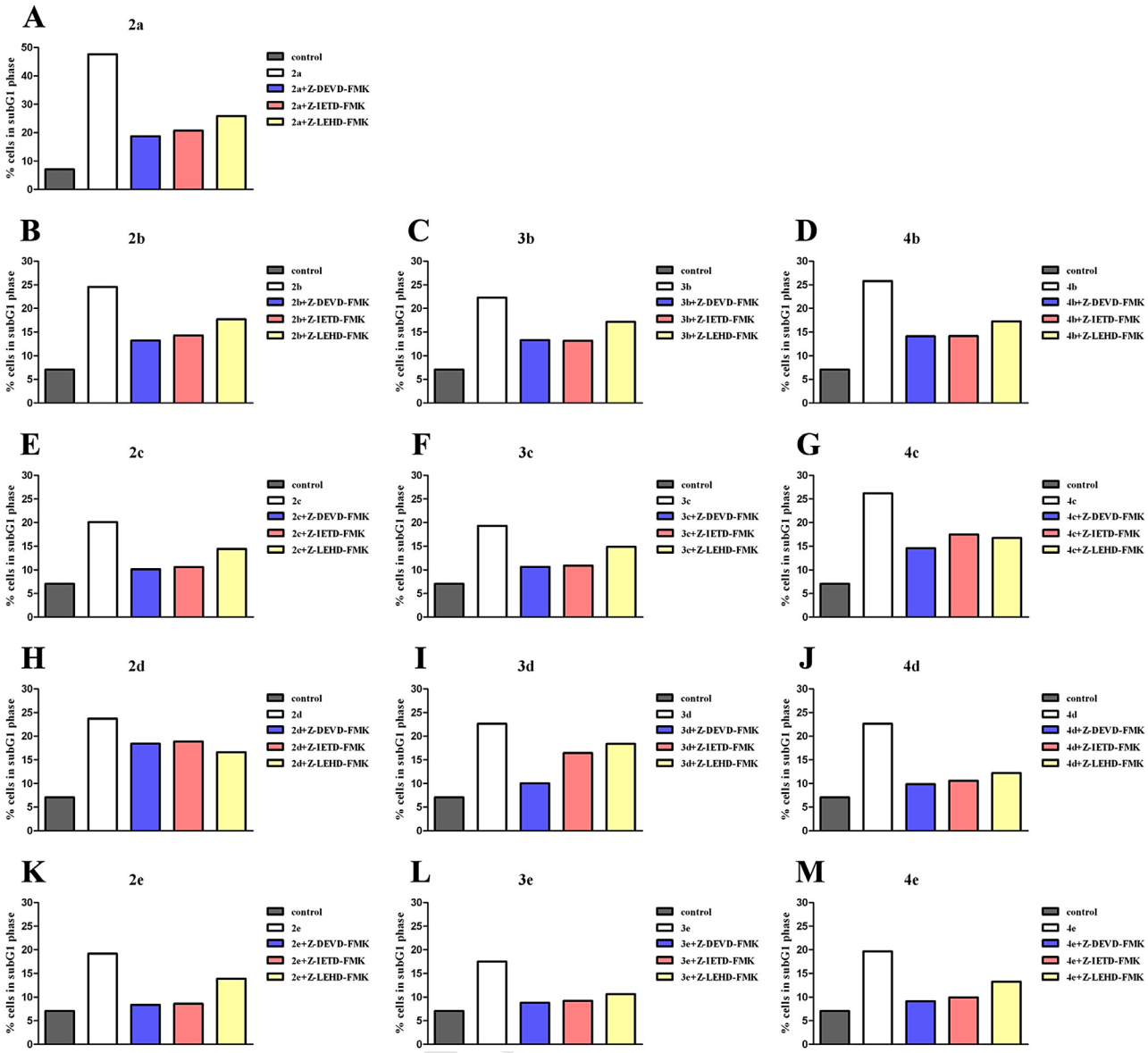


**Fig. 7.** Identification of target caspases involved in the apoptotic signaling pathways induced by the investigated steroid dimers **2a–e**, **3b–e** and **4b–e**. Effects of specific caspase inhibitors on the percentages of subG1 K562 cells treated with steroid dimers for 24 h. (Z-DEVD-FMK – caspase-3 inhibitor; Z-IETD-FMK – caspase-8 inhibitor; Z-LEHD-FMK – caspase-9 inhibitor). Applied concentrations of tested compounds corresponded to $2IC_{50}$ values determined for 72 h.

**Table 4**
The brine shrimp test results of **2a–e**, **3a–e** and **4b–e**.

| Comp. | $IC_{50}$ (mg/disc) |
|---|---|
| **2a** | 0.060 ± 0.005 |
| **2b** | 0.048 ± 0.004 |
| **2c** | 0.048 ± 0.002 |
| **2d** | 0.063 ± 0.004 |
| **2e** | 0.068 ± 0.006 |
| **3a** | 0.028 ± 0.003 |
| **3b** | 0.031 ± 0.004 |
| **3c** | 0.026 ± 0.005 |
| **3d** | 0.032 ± 0.003 |
| **3e** | 0.033 ± 0.004 |
| **4b** | 0.052 ± 0.006 |
| **4c** | 0.049 ± 0.007 |
| **4d** | 0.046 ± 0.003 |
| **4e** | 0.053 ± 0.005 |

0.026–0.068 mg per disc (Table 4). The compounds **2a**, **2b**, **2d**, **2e**, **4b**, and **4e** seem more promising candidates for potential therapeutic application as the toxicity was lower.

### 3.4. Antimicrobial activity

The antimicrobial activity of all steroidal dimers was evaluated against Gram-positive bacteria *M. luteus* (ATCC 4698), Gram-negative bacteria *E. coli* (ATCC 25922), yeast species *C. albicans* (ATCC 10231) and *S. cerevisiae* (ATCC 9763) and against *Penicillium* sp.

The data of the disc susceptibility test showed that there was no antibacterial activity.

Against *C. albicans* compounds **2a**, **3a**, **3c**, and **4e** showed a very good activity, comparable with nystatin. The inhibition zone ranged from 14 mm to 38 mm, similar to 30 mm for nystatin. Similar activities (from 14 mm to 32 mm) were exhibited against *S. cerevisiae* by compounds **2a**, **2b**, **2e**, **3a–e**, **4b**, and **4e**. There was no activity against *Penicillium* sp. (Table 5).

**Table 5**
The antifungal activity of **2a**–**e**, **3a**–**e** and **4b**–**e**.

| Comp. | Inhibition zone (mm) | | |
|---|---|---|---|
| | *C. albicans* | *S. cerevisiae* | *Penicillium* sp. |
| **2a** | 38 | 30 | 0 |
| **2b** | 0 | 30 | 0 |
| **2c** | 0 | 0 | 0 |
| **2d** | 0 | 0 | 0 |
| **2e** | 0 | 14 | 0 |
| **3a** | 30 | 30 | 0 |
| **3b** | 0 | 30 | 0 |
| **3c** | 27 | 26 | 0 |
| **3d** | 0 | 30 | 0 |
| **3e** | 0 | 30 | 0 |
| **4b** | 0 | 24 | 0 |
| **4c** | 0 | 0 | 0 |
| **4d** | 0 | 0 | 0 |
| **4e** | 14 | 32 | 0 |
| Nystatin | 30 | 54 | 38 |

## 4. Conclusion

In this study the cytotoxic and antimicrobial activity of previously synthesized steroid dimers with different linkers, **2a**–**e**, **3a**–**e**, and **4b**–**e** were evaluated. These compounds, applied at micromolar concentrations, exhibited cytotoxic activity of different intensity (compared with cisplatin as a control), modality and selectivity in malignant cell lines used in this study. Hence, the trithiolane dimer **3a** was the least active (practically inactive) toward all four examined malignant cell lines, with $IC_{50}$ over 200 μM. Other trithiolane dimers **3b**–**e** as well as the dimers with phosphorotrithioate ring as a spacer group **4b**–**e** have also shown a poor activity against HeLa, MDA-MB-453 and MDA-MB-361 cancer cell lines with $IC_{50}$ not below 70 μM. The best activity against these three cell cancer lines was exhibited by dimer-sulfides **2a**–**e.** It is noteworthy that HeLa, MDA-MB-453 and MDA-MB-361 cells were the most sensitive to the cytotoxic action of compound **2a**. Furthermore, the compound **2e** showed a high cytotoxic effect on HeLa and MDA-MB-453 cells. All screened compounds (exception is dimer **3a**) exerted strong, concentration-dependent cytotoxic activity against leukemia K562 cells with $IC_{50}$ values ranging from 12.0 μM to 53.0 μM. The compounds which exerted the most pronounced cytotoxic action were further screened for cytotoxic activity against normal human peripheral blood mononuclear cells (PBMC), unstimulated or PHA-stimulated to proliferate. Each of the tested compounds (**2a**–**c**, **2e** and **4b**) exhibited notably higher cytotoxic activities against K562, HeLa and MDA-MB-453 cells in comparison to resting and PHA-stimulated PBMC, pointing to a significant selectivity in their antitumor actions.

The examination of the mechanisms of cytotoxic action of steroid dimers in myelogenous leukemia K562 cells revealed pro-apoptotic action of each of the investigated compounds applied at double $IC_{50}$ concentrations. The identification of target caspases implicated in the activation of apoptosis indicates that the tested steroid dimers trigger apoptosis in K562 cells employing both the extrinsic and intrinsic signaling pathways.

The results of our *in vitro* study may indicate the anticancer potential of steroid compounds **2a**–**c**, **2e** and **4b** owing to their pronounced antiproliferative and pro-apoptotic activities especially high against leukemia K562 cells, in addition to good selectivity in their antitumor actions against malignant cells in comparison to normal immunocompetent PBMC.

Although we are dealing with, in essence, steroid molecules with similar structures (all dimers), it is impossible from this perspective to generalize the possible mechanisms of their effects upon K562 cells. Obviously, the spacer group (sulfide, trithiolane or phosphorotrithioate) affects their activity, as well as the substituent at C-17 position. However, further molecular based investigation is necessary to determine the possible mechanisms of action of the investigated compounds.

The data of the disc susceptibility test showed that there was no activity against Gram-negative bacteria *E. coli* or against Gram-positive bacteria *M. luteus*, indicating that these compounds are not promising antibacterial agents for further research. Also, there was no activity against *Penicillium* sp. However, against *C. albicans* compounds **2a**, **3a**, **3c**, and **4e** showed very good activity, comparable with nystatin. Similar activities were exhibited against *S. cerevisiae* by compounds **2a**, **2b**, **2e**, **3a**–**e**, **4b**, and **4e**.

Unlike cytotoxic activity the best antifungal activity was exhibited by dimers linked with trithiolane ring **3a**–**e,** indicating a selective activity of investigating compounds. Thus, further analyses of structure–activity relationships of these compounds would make it possible to design novel synthetic antifungal agents.

## Acknowledgement

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grants 172055 and 175011).

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