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Original article

Antiproliferative and antimigratory effects of 3-(4-substituted benzyl)-5- isopropyl-5-phenylhydantoin derivatives in human breast cancer cells

Ana Obradović [a], Miloš Matić [a], Branka Ognjanović [a], Predrag Đurđević [b], Emilija Marinković [c], Gordana Uščumlić [d], Bojan Božić [e,1], Biljana Božić Nedeljković [e,*,1]

[a] Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Kragujevac, Serbia
[b] Department of Internal Medicine, Clinic for Hematology Clinical Center Kragujevac, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
[c] Department of Research and Development, Institute of Virology, Vaccines and Sera – TORLAK, Belgrade, Serbia
[d] Department of Organic Chemistry, Faculty of Technology and Metallurgy, University of Belgrade, Belgrade, Serbia
[e] Institute of Physiology and Biochemistry "Ivan Đaja", Faculty of Biology, University of Belgrade, Belgrade, Serbia

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ABSTRACT

In this study, a series of synthesized 3-(4-substituted benzyl)-5-isopropyl-5-phenylhydantoin derivatives as a potential antiproliferative and antimigratory agents were investigated. The possible antitumor mechanisms of investigated hydantoin derivatives were examined on human breast cancer cell line MDA-MB-231. The cells were treated with different concentrations of compounds (from 0.01 μM to 100 μM) during 24 h and 72 h. The proliferation index, nitric oxide production, apoptosis rate, and migration capacity were measured. The cell invasion potential was examined by measuring the level of MMP-9 and COX-2 gene expression. All tested compounds expressed antiproliferative activity and induced dose- and time-dependent increase in the level of nitrites. The investigated molecules significantly decreased cell survival rate, migration capacity and the expression levels of genes included in the process of tumor invasion. Obtained data suggest that the tested hydantoin derivatives express considerable antitumor activity by reducing cell division rate, elevating apoptosis level, and inhibiting the motility and invasiveness of breast cancer cells. The results obtained in this study indicate that investigated compounds express potential as a novel chemotherapeutic agents against breast cancer growth and progression.

1. Introduction

Breast cancer is the most common cancer type and the second leading cause of cancer-related deaths in women across the world resulting in more than half a million deaths each year (Murad et al., 2016). Despite the extensive use of multimodal chemotherapies, the level of mortality remains high, emphasizing the need for novel therapeutic approaches with higher efficiency against malignant cells and advanced selectivity towards healthy tissues. Some studies showed that different heterocyclic compounds containing nitrogen atom exhibit significant antiproliferative activity against human breast carcinoma (Gomha et al., 2016, Tao et al., 2018, Gomha et al., 2018).

Derivatives of hydantoin are commonly used for clinical treatment of epilepsy and cardiac arrhythmias (Herrera et al., 2015, Kumkamthornkul et al., 2018), but various additional properties of hydantoins such as anti-inflammatory and antitumor activities have also been noticed (Trišović et al., 2011, Obradović et al., 2013, Marinova et al., 2016, Obradović et al., 2019). One of the most prominent antitumor roles of hydantoin compounds is their significant antiproliferative potential exerted against different types of cancer cells (Cavazzoni et al., 2008, Zuliani et al., 2009, Sekulić Djaković et al., 2015). Certain, hydantoin derivatives have been shown to display strong cytotoxicity in breast cancer cells and induce growth inhibition and apoptosis (Rajic et al., 2006). Moreover, hydantoin core ring variations can be found as a moiety in many drugs used in chemotherapy. Thus selenohydantoins, derivatives of hydantoins in which one of the oxygen atoms is replaced by selenium, express the strong antitumor activity and can be used as effective antioxidants (Ivanenkov et al., 2016).

Beside uncontrolled proliferation, cancer cells display significant migratory and invasive potential which inducing metastasis.

* Corresponding author.
E-mail address: biljana@bio.bg.ac.rs (B. Božić Nedeljković).
[1] These authors contributed equally to this study.
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Various factors are involved in cancer cell division, survival, invasion, and dissemination. Tumor-associated inflammation initially promotes tumor growth and stimulates cancer cell survival, but it also enhances metastatic potential of the tumor. Cyclooxygenase 2 (COX-2) is often overexpressed in breast cancer cells (Chow et al., 2008). Although the use of non-steroidal anti-inflammatory drugs (NSAIDs) are associated with reduced risk of cancer overall and breast cancer in particular (Bowers et al., 2014), their detrimental side effects have been recorded due to low selectivity (Bundred and Barnes, 2005). The usage of new selective COX-2 inhibitors could provide satisfying anti-inflammatory effects and devoid of undesirable effects associated with classical, non-selective NSAIDs. Some recent studies are suggesting a prominent place of selective COX-2 inhibitors in the prevention of different tumors including breast cancer (Mazhar et al., 2005, Takkouche et al. 2008, Regulski et al., 2016).

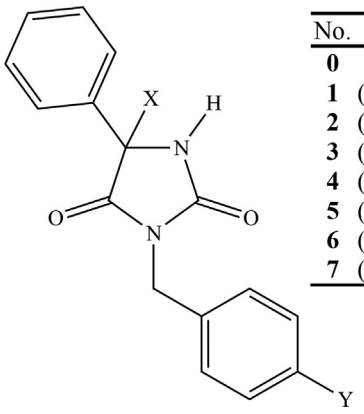
Extracellular proteinases are essential for metastasis since these enzymes degrade the components of the extracellular matrix and facilitate the disruption of intercellular adhesions, separating individual cells from solid tumor tissue (Herszényi et al., 2014). Among the matrix metalloproteinase family (MMPs), MMP-9 is known to be significantly upregulated in almost all tumor types, including breast cancer (Taguchi et al., 2014). Furthermore, MMP-9 expression positively correlates with cancer stage, grade and prognosis, making it a convenient marker, and a potential therapeutic target for preventing the invasion and metastasis of breast cancer (Roy et al., 2009, Alaseem et al., 2019).

Nitric oxide (NO) has also been considered a tumor-associated molecule. The roles of NO in tumor formation and progression remain controversial. This molecule has an important role in various stages of tumorigenesis such as DNA damage, oncogene activation, inhibition of DNA repair enzymes and tumor suppressor genes, and the modulation of apoptosis and metastasis (Fionda et al., 2016). The different roles of NO in apoptosis, cell cycle, tumor progression, angiogenesis, and metastasis are currently viewed at host tissue and tumor interface since NO was found to be strikingly associated with tumor parenchyma, as well as with the tumor microenvironment (Vannini et al., 2015). The tumor microenvironment includes the cells of the immune system and vascular tissue, and NO appears to be one of the key components of their activity (Artacho-Cordón et al., 2012). Antitumor effects of NO were also recorded in different human and animal tumors (Choudhari et al., 2013, Vahora et al., 2016). iNOS overexpression induces simulative or inhibitory effects on tumor growth, depending on the microenvironment and tumor type (Mocellin et al., 2007). Overall, the effect of NO depends on the expression level of iNOS, duration and timing of NO delivery, contents of the microenvironment, genetic background, and type of cell, but undoubtedly is involved in tumor progression (Vannini et al., 2015). The increasing data which indicate hydantoins antitumor activity suggest a beneficial role of these compounds in developing of novel chemotherapeutics. Accordingly, the aim of this research was to evaluate the effects of seven 3-(4-substitutedbenzyl)-5-isopropyl-5-phenylhydantoin derivatives (**1**–**7,** Fig. 1.) on survival ratio, type of cell death, proliferation level, NO production rate, COX-2 and MMP-9 expression profile, and migration/invasion capacity of human breast cancer cell line, MDA-MB-231.

2. Materials and methods

2.1. Cell cultivation and treatment

The human breast cancer cell line MDA-MB-231 line was obtained from American Tissue Culture Collection. These cells were propagated and maintained in DMEM (Dulbecco's Modified Eagle Medium, SIGMA-ALDRICH, USA) and supplemented with 10% Fetal Bovine Serum (FBS, SIGMA-ALDRICH, USA) and antibiotics (penicillin (100 IU/mL) and streptomycin (100 μg/mL). After several passages the cells were trypsinized, seeded in 96-well plate (10^4 cells per well) and cultured in a humidified atmosphere with 5% CO_2 at 37 °C.



No.	X	Y
0	Ph	H
1	$(CH_3)_2CH$	CH_3O
2	$(CH_3)_2CH$	CH_3
3	$(CH_3)_2CH$	H
4	$(CH_3)_2CH$	Cl
5	$(CH_3)_2CH$	Br
6	$(CH_3)_2CH$	CN
7	$(CH_3)_2CH$	NO_2

Fig. 1. Structures of investigated compounds.

After 24 h of incubation, 100 μL of working solution (concentration range: 0.01 μM – 100 μM) was added. Stock solutions of the compounds were made in dimethyl sulfoxide (DMSO) at the concentration of 1 M and diluted with DMEM to various working concentrations. The concentration of DMSO in the most concentrated working solutions was 0.01% (v/v).

2.2. Determination of cell viability

The cells were treated with various doses (0.01 μM, 0.1 μM, 1 μM, 10 μM, 50 μM, and 100 μM) of the investigated compounds for 24 h and 72 h. Non-treated cells served as control. After treatment, cell proliferation was determined by the MTT assay (Mosmann, 1983). At the end of the treatment period, 25 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, SIGMA-ALDRICH, USA) solution (5 mg/mL final concentration in PBS (GIBCO, Invitrogen, USA)) was added to each well and incubated in a humidified atmosphere with 5% CO_2 at 37 °C for three hours. The produced formazan was dissolved by overnight incubation in SDS-HCl (10% SDS in 0.01 M HCl) and the percentage of viable cells was calculated as the ratio between the absorbance at each dose of the compounds and the absorbance of the untreated control multiplied by 100 to get a percentage. The absorbance was measured at 550 nm on ELISA (2100C) 96-well plate reader (Rayto, China). We also calculated the half-maximal inhibitory concentration (IC_{50}), defined as the concentration of tested hydantoin derivatives that inhibited cell growth by 50% compared to control. All experiments were performed in triplicate. The IC_{50} values were calculated from the dose curves by the software CalcuSyn.

2.3. Determination of type of cell death

Apoptosis and necrosis were analyzed by double staining with annexin V-FITC and 7-AAD. Annexin V binds to the cells with exposed phosphatidylserine, whereas 7-AAD labels the cells with membrane damage. Apoptotic cells were detected using the Annexin V-FITC/7-AAD Kit (Apoptosis Detection Kit, Beckman Coulter, USA). Staining was performed according to the manufacturer's instructions and Shounan protocol (Shounan et al., 1998). After the treatment with compounds **0**, **3**, and **4** (Fig. 1.) at a concentration of 1 μM and 10 μM, the cells were collected, washed

in PBS and resuspended in ice cold binding buffer. Ten thousand events were analyzed on FC500 Beckman Coulter Flow Cytometer (Nyon, Switzerland). The percent of viable (Annexin $V^-7\text{-}AAD^-$) cells, early apoptotic (Annexin $V^+7\text{-}AAD^-$) cells, late apoptotic (Annexin $V^+7\text{-}AAD^+$) cells, and necrotic cells (Annexin $V^-7\text{-}AAD^+$) cells were evaluated by Flowing Software (http://www.flowing-software.com/).

2.4. Caspase activation

The activity of caspases, the enzymes involved in the execution of apoptosis, was measured by flow cytometry after labeling the cells with a cell-permeable FITC-conjugated pan-caspase inhibitor (ApoStat; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The increase in green fluorescence (FL1), as a measure of caspase activity, was determined using FACSCalibur flow cytometer (BD Biosciences, Germany), and the results were expressed as the percentage (%) of cells with active caspases.

2.5. Determination of NO production

The spectrophotometric determination of nitrites - NO_2^- (an indicator of the nitric oxide level) was performed by using the Griess method (Griess, 1879). Griess reaction is based on the coupling of NO-generated diazonium ion with *N*-(1-napthyl) ethylenediamine where a chromophoric azo product is formed with an absorption maximum at 550 nm and measured by ELISA microplate reader. The concentrations of nitrites were calculated from the appropriate standard curves for nitrites.

2.6. Cell migration

The cell migration capacity was determined by measuring the ability of cells to pass the pores of polycarbonate membranes (pore size 8 μm; Greiner Bio-One, Switzerland) at the bottom of transwell chambers. The migration test was performed according to the protocol described by Chen (Chen, 2005). The cells were exposed to 1 μM and 10 μM concentration of compounds **0**, **3**, and **4** for 24 h and 72 h, respectively. The control cells were cultured only in DMEM. After the treatments, all groups of treated cells were trypsinized and placed in the upper chambers at a density of 1×10^5 cells/well in 500 μL of DMEM with 10% FBS. The lower chambers contained 750 μL of DMEM supplemented with 10% FBS. After 6 h of incubation at 37 °C, the cells from the upper surface of the membrane were completely removed with gentle swabbing. The remaining migrated cells were fixed for 20 min at room temperature in 4% paraformaldehyde and stained with 0.1% crystal violet in 200 mM 2-(N-Morpholino) ethanesulfonic acid (pH 6.0) for 10 min. Absorbance was measured by a microplate reader at 595 nm. The migration index was calculated as the ratio of absorbance of the treated cells divided by the absorbance of the control cells and multiplied by 100 to give the percentage.

2.7. Quantitative PCR (qPCR)

Total cell RNA was isolated from cells using TRIzol reagent according to Chomczynski and Sacchi protocol (Chomczynski and Sacchi, 1987). The purity and concentrations of obtained RNA were evaluated spectrophotometrically by measuring solution absorbance at 260 and 280 nm. The A_{260}/A_{280} ratio of the samples was $\geq$ 1.8. Extracted RNA (2 μg) from each sample were reversely transcribed using Superscript II RT enzyme (Invitrogen) in a total reaction volume of 20 μL. The obtained complementary DNA (cDNA) was PCR-amplified and the final 20 μL reaction volume contained 0.5 μM primers, 1 × SYBR Green PCR Master Mix (Applied Biosystems) and 2 μL of the cDNA. cDNA amplification was performed by qRT-PCR method (quantitative real-time PCR) in 7500 Real Time System (Thermo Fisher Scientific) using the primer pairs (Obradović et al., 2019).

The qPCR reaction program was set in accordance with the manufacturer's instructions (Applied Biosystems): activation of the polymerase for 3 min at 95 °C, 40 cycles of 15 sec at 95 °C and 60 sec at 55 °C. Relative expression value was calculated according to formula ΔCT - ΔΔCT where ΔCT refers to the difference between the cycle threshold values (CT) obtained for the target gene in treatment sample and non-treated control, respectively, and ΔΔCT represents the difference of β-actin gene expression levels (endogenous control) (Livak and Schmittgen, 2001). The negative control samples (without cDNA) were also amplified in qPCR reaction to confirm that the contamination with genomic DNA was in the acceptable range (Cheeseman et al., 2012). The results were expressed as the percentage of the change compared to control values.

2.8. Statistical analysis

All values are expressed as mean ± SE. Statistical evaluation was calculated by Paired Sample T-Test, SPSS. The magnitude of the correlation between variables was done using an SPSS (Chicago, IL) statistical software package (SPSS for Windows, ver. 17). For all comparisons $p < 0.05$ control *vs*. treatment was considered significant. The data are the result of three independent (individual) experiments, performed in triplicate for each dose.

3. Results

3.1. Antiproliferative potential of isopropyl-phenyl hydantoins

At our previous research (Trišović et al., 2011, Obradović et al., 2013) compound 3-benzyl-5,5-diphenylhydantoins, marked as compound **0**, showed the strongest activity in the previously tested hydantoin derivatives series. Accordingly, this compound was used for comparison of the effects with the new series of isopropyl-phenyl hydantoins. In Tables 1 and 2 antiproliferative effects of these compounds at various concentrations on the human breast cancer cell line, MDA-MB-231, after 24 and 72 h of incubation are presented. All compounds showed significant antiproliferative activities on MDA-MB-231 cells compared to non-treated cells. All compounds showed considerable dose- and time-dependent inhibition of MDA-MB-231 cell proliferation. Antiproliferative activity of compound **3** was the highest after 24 h treatment and compound **4** showed the strongest effects after 72 h.

Since the compounds used in this study show significant antiproliferative effects on MDA-MB-231 cells, these molecules should be further investigated for the mechanisms of their antiproliferative action. The results of the antiproliferative activity of all investigated compounds on MDA-MB-231 cells were expressed as IC_{50} values and presented in Table 3. After 72 h treatment, all examined compounds showed stronger antiproliferative activity compared to short-term (24 h) treatment. After long-term treatment, all compounds showed higher antiproliferative potential compared to compound **0**, from the previous study, with IC_{50} values<10 μM, except for the compound **1**.

3.2. Determination of cell apoptosis by Annexin V-FITC/7-AAD staining

The type of cell death induced by treatment with investigated hydantoin derivatives was determined by flow cytometric analysis of treated cells stained with Annexin V FITC and 7-AAD. The strongest antiproliferative effect has been exerted by compound **3** and **4,** and the effect was significantly stronger compared to compound **0**,

Table 1
Effect of investigated compounds on proliferation index of MDA-MB-231 cells expressed as percentage of proliferation level after 24 h compared to non-treated control cells (100%).

No	0.01 μM	0.1 μM	1 μM	10 μM	50 μM	100 μM
0	85 ± 3.8 *	75 ± 3.6*	72 ± 1.1 *	69 ± 2.9 *	53 ± 1.8 *	42 ± 2.3 *
1	82 ± 2.8 *	78 ± 2.0 *	75 ± 1.0 *	68 ± 2.6 *	73 ± 4.8 *	57 ± 4.3 *
2	84 ± 2.3 *	70 ± 5.1 *	75 ± 4.1 *	70 ± 4.2 *	57 ± 6.0 *	53 ± 6.9 *
3	81 ± 1.2 *	68 ± 3.2 *	67 ± 2.6 *	65 ± 4.9 *	56 ± 1.2 *	40 ± 2.1 *
4	83 ± 1.8 *	75 ± 2.4 *	60 ± 4.6 *	58 ± 4.8 *	46 ± 2.4 *	41 ± 3.3 *
5	78 ± 2.2 *	68 ± 2.8 *	70 ± 3.5 *	67 ± 3.3 *	61 ± 2.1 *	56 ± 2.4 *
6	87 ± 1.5 *	77 ± 3.7 *	66 ± 2.4 *	64 ± 3.6 *	53 ± 4.8 *	45 ± 3.2 *
7	79 ± 2.0 *	70 ± 2.7 *	76 ± 2.4 *	71 ± 1.4 *	56 ± 3.0 *	44 ± 4.6 *

The results are expressed as the mean ± SE from three independent experiments performed in triplicate. (*p < 0.05 different concentrations of compound *vs.* control)

Table 2
Effect of investigated compounds on proliferation index of MDA-MB-231 cells expressed as percentage of proliferation level after 72 h compared to non-treated control cells (100%).

No	0.01 μM	0.1 μM	1 μM	10 μM	50 μM	100 μM
0	79 ± 1.2 *	65 ± 3.3 *	58 ± 3.0 *	52 ± 1.7 *	50 ± 3.9 *	39 ± 2.8 *
1	71 ± 4.2 *	70 ± 3.3 *	60 ± 4.0 *	50 ± 2.7 *	49 ± 2.8 *	55 ± 1.8 *
2	63 ± 3.6 *	60 ± 1.2 *	59 ± 3.7 *	56 ± 4.2 *	45 ± 1.3 *	40 ± 2.7 *
3	70 ± 1.9 *	65 ± 1.3 *	55 ± 4.30 *	50 ± 1.1 *	45 ± 3.1 *	32 ± 5.8 *
4	60 ± 3.7 *	57 ± 3.3 *	55 ± 2.8 *	49 ± 1.7 *	37 ± 3.1 *	31 ± 2.5 *
5	59 ± 2.0 *	50 ± 2.5 *	46 ± 1.8 *	55 ± 1.6 *	40 ± 2.1 *	38 ± 3.7 *
6	65 ± 9.9 *	55 ± 0.4 *	43 ± 4.9 *	53 ± 8.7 *	42 ± 4.7 *	35 ± 6.0 *
7	68 ± 3.2 *	63 ± 3.8 *	53 ± 4.2 *	45 ± 8.4 *	43 ± 4.3 *	40 ± 4.9 *

The results are expressed as the mean ± SE from three independent experiments performed in triplicate. (*p < 0.05 different concentrations of compound *vs.* control)

Table 3
The half-maximal inhibitory concentration IC_{50} (μM) of investigated compounds against MDA-MB-231 cells proliferation.

No.	24 h	72 h
0	87 ± 0.2	14 ± 0.03
1	> 100	78 ± 0.1
2	> 100	10 ± 0.1
3	74 ± 0.1	6 ± 0.1
4	21 ± 0.2	0.90 ± 0.1
5	> 100	1.40 ± 0.04
6	> 100	1.20 ± 0.1
7	> 100	3.70 ± 0.1

The cells treated with various concentrations of compounds during a 24 and 72 h. The results are expressed as the mean ± SE from three independent experiments performed in triplicate.

the selected molecule from the previous study (Trišović et al., 2011). Subsequently, compounds, **3** and **4**, their structure was shown in Fig. 1, were selected for further research and determining of the antitumor mechanisms, in concentrations of 1 μM and 10 μM where the antiproliferative effect is clearly evident. MDA-MB-231 cells were incubated during 24 and 72 h. At both examined concentrations, all three compounds induced a statistically significant increase of apoptosis rate in MDA-MB-231 cells, after 24 h treatment, (shown in Fig. 2), and 72 h treatment (shown on Fig. 3). The time- and dose-dependent effects of the compounds are respectively exhibited.

3.3. Determination of caspase activation

MDA-MB-231 cells were treated with 1 μM and 10 μM of selected derivatives **0**, **3**, and **4** during 24 and 72 h. At both examined concentrations, all three compounds have stimulated the activation of the apoptosis executing enzymes, caspases, as demonstrated by an increase in Apostat fluorescence signal. The data are shown in Figs. 4 and 5 represent caspases activation.

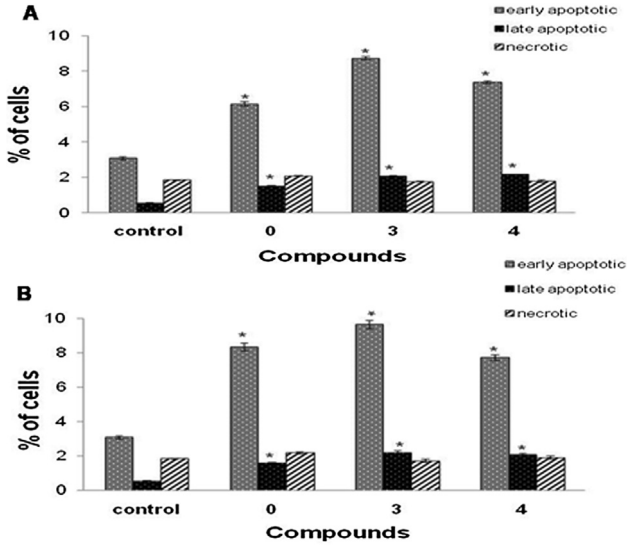


Fig. 2. Flow cytometric analysis of Annexin V-FITC/7-AAD stained MDA-MB-231 cells for 24 h with compounds **0**, **3,** and **4** at concentrations 1 μM (A) and 10 μM (B). The percentages of early apoptotic (Annexin V^+7-AAD^-, lower right quadrant), late apoptotic (Annexin V^+7-AAD^+, upper right quadrant) and necrotic cells (Annexin V^-7-AAD^+, upper left quadrant) in non-treated and treated cells are indicated on dot plots). The results are expressed as the mean ± SE from three independent experiments performed in triplicate. (*p < 0.05 different concentrations of compound *vs.* control).

3.4. Determination of NO production by Griess assay

The data shown in Fig. 6 represent nitrite concentrations in supernatants of MDA-MB-231 cells treated with 1 μM and 10 μM of selected compounds **0**, **3**, and **4** after 24 and 72 h. All three compounds showed a significant increase in production of NO by MDA-MB-231 cells measured by nitrite concentrations compared to NO values in supernatants of non-treated cells. The strongest activity has been shown on compound **3** after 24 h of

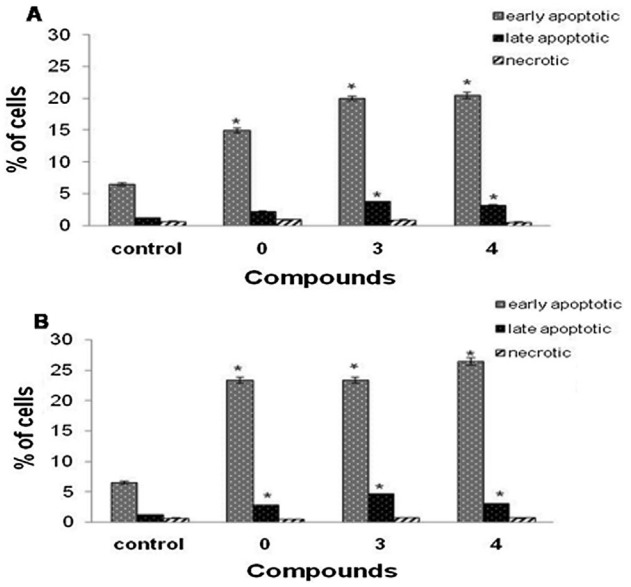


Fig. 3. Flow cytometric analysis of Annexin V-FITC/7-AAD stained MDA-MB-231 cells for 72 h exposure with compounds **0**, **3,** and **4** at concentrations 1 μM (A) and 10 μM (B). The percentages of early apoptotic (Annexin $V^+7^-AAD^-$, lower right quadrant), late apoptotic (Annexin $V^+7^-AAD^+$, upper right quadrant) and necrotic cells (Annexin $V^-7\text{-}AAD^+$, upper left quadrant) in untreated and treated cells are indicated on dot plots).The results are expressed as the mean ± SE from three independent experiments performed in triplicate. (*p < 0.05 different concentrations of compound *vs.* control).

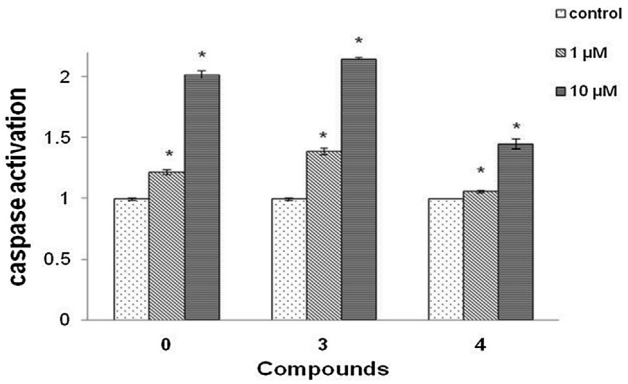


Fig. 4. The antitumor activity of investigated compounds against MDA-MB-231 cells *in vitro* after 24 h. MDA-MB-231 cell line were treated with compounds **0**, **3,** and **4** at concentrations 1 μM and 10 μM during 24 h exposure compared to non-treated control cell (100%). The cell viability was determined using caspase activation by flow cytometry. The results are expressed as the mean ± SE from three independent experiments performed in triplicate. (*p < 0.05 different concentrations of compound *vs.* control).

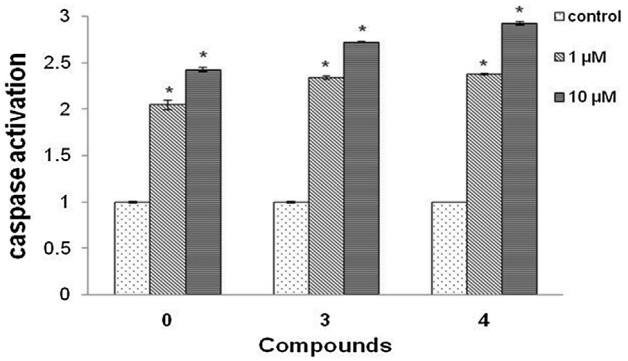


Fig. 5. The antitumor activity of investigated compounds against MDA-MB-231 cells *in vitro* after 72 h. MDA-MB-231 cell line were treated with compounds **0**, **3,** and **4** at concentrations 1 μM and 10 μM during 72 h exposure compared to non-treated control cell (100%). The cell viability was determined using caspase activation by flow cytometry. The results are expressed as the mean ± SE from three independent experiments performed in triplicate. (*p < 0.05 different concentrations of compound *vs.* control).

treatment, while after 72 h treatment compound **4** showed the strongest effect.

3.5. Expression of the inducible nitrite oxide synthase (iNOS) gene

In Fig. 7, the changes in the iNOS gene expression profile induced by synthesized hydantoin compounds **0**, **3**, and **4** are presented. The results show that long-term treatment (72 h) with all three hydantoin derivatives at concentrations of 1 μM and 10 μM caused statistically significant increase in gene expression compared to non-treated cells.

3.6. Migration capacity (Boyden chamber assay)

To examine the effects of hydantoin derivatives on the migration capacity of human breast cancer cell line MDA-MB-231, the transwell migration assay was performed. The results presented in Fig. 8 show a significant decrease in migration index of MDA-MB-231 cells exposed to compounds **0**, **3**, and **4** at a concentration of 1 μM and 10 μM for 24 and 72 h, respectively, compared to the non-treated cells. Long-term exposure to compounds **0**, **3**, and **4** induced stronger reduction of migration index compared to short-term exposure suggesting a time-dependent effect.

3.7. Expression of genes involved in invasion (COX-2 and MMP-9)

The rate of gene expression is directly connected with the levels of protein synthesis important for regulation of migration/invasion of breast cancer cells. The gene expression levels were determined by qRT PCR method using the fluorescently labeled Syber Green dye that generates the signal and quantifies the amount of expressed mRNA (mRNA). The expression of the genes was determined only after long-term treatment (72 h).

The effects of synthesized derivatives **0**, **3**, and **4** in concentrations of 1 μM and 10 μM on the change in expression of COX-2 gene in MDA-MB-231 cells after long-term exposure (72 h) were shown in Fig. 9**A**. The results show that the treatment with these three derivatives at concentrations of 1 μM and 10 μM caused statistically significant reduction in COX-2 gene expression compared to control cells.

Moreover, the results obtained from expression of MMP-9, which plays an important role in the progression of breast cancer cells (Duffy et al., 2000), indicate that tested compounds can affect expression of the corresponding gene. The changes induced by compounds **0**, **3**, and **4** in the level of MMP-9 gene expression compared to control cells are shown in Fig. 9**B**. The results show that long-term treatment with compounds **0**, **3**, and **4** at concentrations of 1 μM and 10 μM induced a statistically significant reduction in the expression of the MMP-9 gene compared to control cells.

4. Discussion

Despite various new therapeutic approaches, chemotherapy remains the most common form of breast cancer treatment, with considerable limitations regarding excessive toxicity of frequently used drugs and increasing resistance of cancer cells. Many studies have implied the correlation between hydantoin structure and

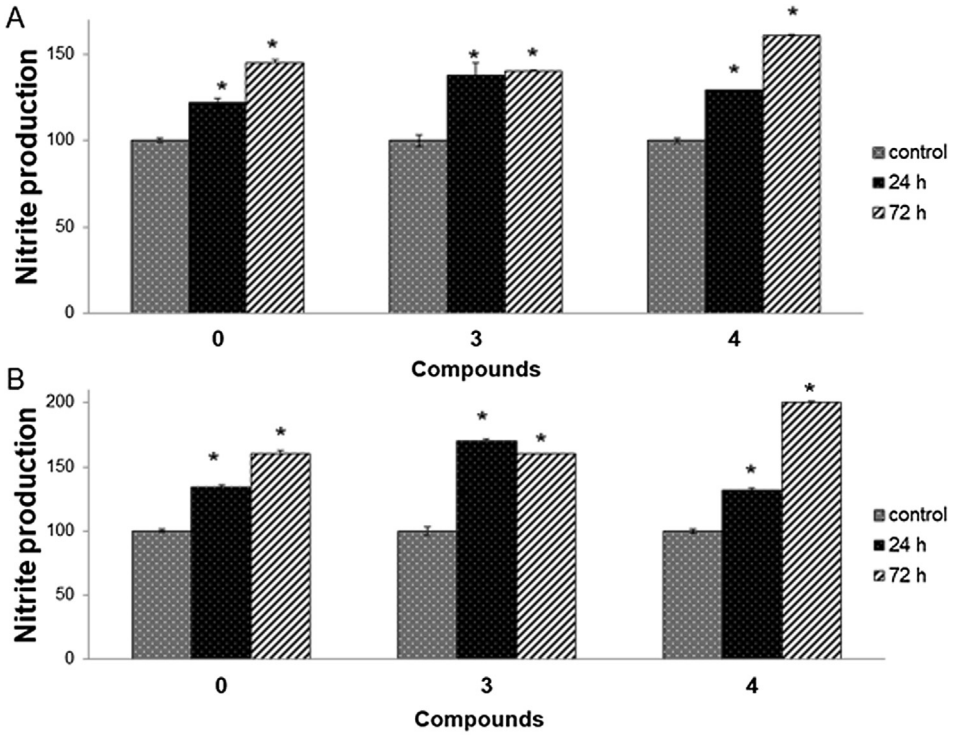


Fig. 6. Effect of investigated compounds on NO production in the MDA-MB-231 cells during 24 h and 72 h exposure compared to non-treated control cell (100%). The cells were treated with compounds **0**, **3,** and **4** at concentrations 1 μM (A) and 10 μM (B). The results are expressed as the mean ± SE from three independent experiments performed in triplicate. (*p < 0.05 different concentrations of compound *vs.* control).

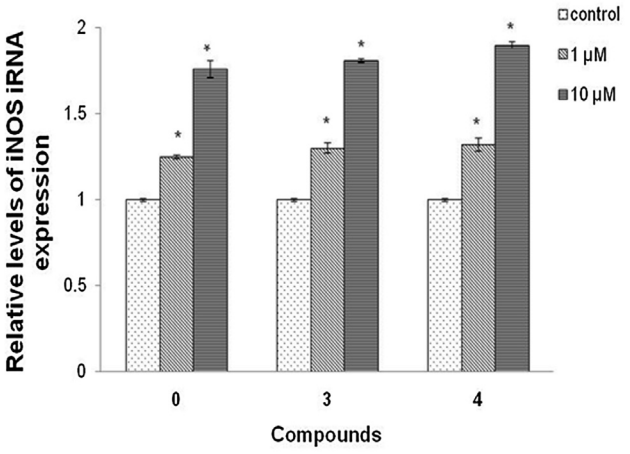


Fig. 7. Relative levels of iNOS mRNA expression following treatment with 1 μM and 10 μM of investigated compounds **0**, **3,** and **4** in MDA-MB-231 cells after 72 h. The results are expressed as the mean ± SE from three independent experiments performed in triplicate. (*p < 0.05 different concentrations of compound *vs.* control).

their various biological effects, so growing number of experiments are focused on the synthesis of novel hydantoin derivatives with the aim of reinforcing their present antitumor potential (Jansen et al., 2003, Kumar et al., 2017). Aromatic units at C5 position are important structural property for interactions with biological structures and studies have shown that 5-benzyliden-hydantoins can function as 4-anilinokinazolin bioisosters already approved for the treatment of lung cancer (Carmi et al., 2006, Cavazzoni et al. al., 2008). The significant antiproliferative effect of certain diazaspiro-bicyclo hydantoin derivatives on human leukemia cells are also indicated (Kumar et al., 2009).

Tumor cells evade apoptosis, excessively proliferate and survive under hypoxic conditions, acquiring the resistance to therapeutic agents (Liang et al., 2018). All examined hydantoin derivatives in this study exerted antiproliferative activities in dose-dependent manner until the concentration of 10 μM where the plateau was reached. The compound **4** with chloro-substituent has shown the strongest antiproliferative effect which is in accordance with literature data (Sekulić Djaković et al., 2015). All investigated derivatives have shown satisfying biocompatibility (Obradović et al., 2019), implying their safe use as potential drugs for treatment of breast cancer. Apoptosis-inducing agents are expected to be effective antitumor drugs since apoptosis is an efficient protective mechanism against tumor development that remove genetically damaged cells before they undergo clonal expansion (Hassan et al., 2014). The selected derivatives (**0**, **3** and **4**) at both used concentrations (1 μM and 10 μM) induced statistically significant increase of apoptosis rate and caspase activation in MDA-MB-231 cells in time-dependent manner. These results imply that some of antitumor mechanisms of these derivatives is based on triggering of apoptosis and inhibiting the division of breast cancer cells.

Some previous studies have demonstrated that NO can induce cell death by affecting apoptosis-related mitochondrial proteins (Messmer et al., 1996, Snyder et al., 2009). The data acquired in our study suggest that significant proapoptotic levels in MDA-MB-231 cells could be mediated via increased NO production induced by examined derivatives which could trigger the components of intrinsic proapoptotic pathways. Besides, excessive generation of NO promotes formation of extremely aggressive peroxynitrite ($ONOO^-$), which is one of the key molecules for bimodal effects of NO in physiological and pathological conditions (Pacher et al., 2007). Our results suggest that in this study investigated hydantoin compounds are potent inducers of apoptosis of breast cancer cell line which may correspond to NO-generating potential of these molecules. Elevated levels of NO have also been shown to inhibit metastasis in different tumor type which could imply that NO generation has beneficial role in antitumor therapies (Aranda et al., 2012). Our results indicate increase levels of NO in

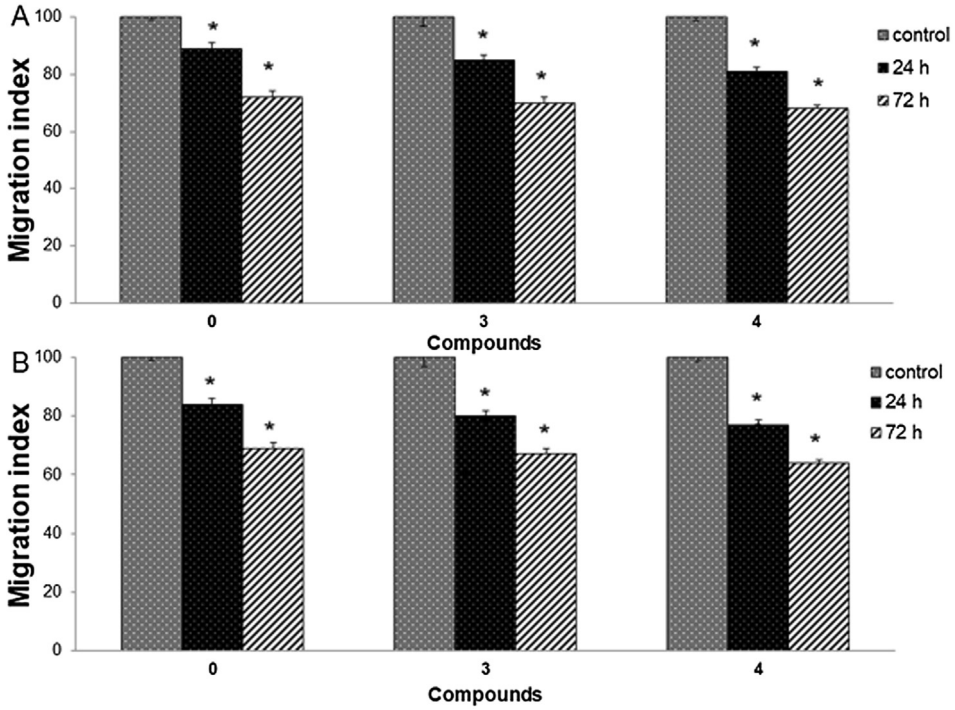


Fig. 8. Effect of exposure to investigated compounds **0**, **3,** and **4** on migration index of MDA-MB-231 cells. The cells were treated at concentrations of 1 μM (A) and 10 (B) μM during a 24 h and 72 h exposure. The results are expressed as the mean ± SE from three independent experiments performed in triplicate. (*p < 0.05 different concentrations of compound *vs.* control).

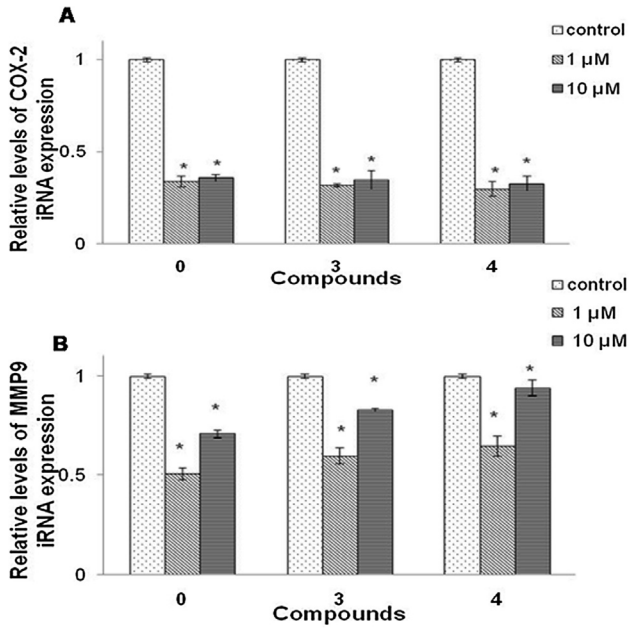


Fig. 9. Relative levels of COX-2 (A) and MMP-9 (B) iRNA expression following treatment with 1 μM and 10 μM investigated compounds **0**, **3,** and **4** in MDA-MB-231 cells after 72 h. The results are expressed as the mean ± SE from three independent experiments performed in triplicate. (*p < 0.05 different concentrations of compound *vs.* control).

MDA-MB-231 cells treated by investigated hydantoin derivatives. One of the key genes involved in tumorigenesis is iNOS that generates NO in significant amounts in breast cancer cell (Choudhari et al., 2013). A lot of antitumor agents have been aimed to trigger iNOS gene expression in order to inhibit growth and induce apoptosis of metastatic tumor cells (Vannini et al., 2015). The hydantoin derivatives tested in our study have increased the expression level for almost 100% compared to control levels, which explains the high concentration of NO recorded in the study. These data indicate that hydantoin derivatives are potential triggers of NO production which could contribute to partly explain their antiproliferative and proapoptotic properties. The results show that long-term treatment (72 h) of MDA-MB-231 cells with selected hydantoin derivatives (**0**, **3**, and **4)** caused statistically significant increase of iNOS gene expression compared to non-treated cells indicating that high concentration of NO may be caused by *de novo* synthesis of these signal molecule.

The tested hydantoin derivatives in our study reduced the migration capacity of MDA-MB-231 cells. Based on these data, we proposed that one of the mechanisms by which these compounds inhibit migration of human breast cancer cells is elevating the bioavailability of NO. This molecule is involved in the regulation of cell adhesion molecules, presumably the integrins, important for cell motility (Roberts et al., 2008). Previously mentioned elevation in NO production could indicate the role of NO in migration mechanisms of this cell type. Although the majority of studies have reported invasion-promoting effects of NO in various cell types, there are several studies which reported antimigratory and antiproliferative effects of NO (Aranda et al., 2012). Reduction in migration capacity could also be correlated with increased expression of the inducible isoform of cyclooxygenase, COX-*2* gene detected in the study. Majumder and colleagues have implied that elevated COX-2 expression level had to promote effect on migration capacity of breast cancer cells (Majumder et al., 2016). In our study reduced migration index could be caused by a decrease in COX-2 expression level recorded in the treatments. The compound **4** with chloro-substituent has shown the strongest reduction of the migratory potential of these cells. However, these data suggest that tested hydantoin derivatives exert significant antimigratory effects on breast cancer cells making them promising antitumor agents.

Matrix metalloproteinases (MMPs), as regulators of the tumor microenvironment, are also important contributors to cancer progression, representing the crucial enzymes in tumor cell invasion by leading to epithelial-mesenchymal transition. MMP-9 is strongly associated with aggressive and metastatic breast cancer (Mehner et al., 2014). MMP-9 and other MMPs as biomarkers of breast cancer stage are used in the monitoring of therapeutic responses on different individuals to estimate the efficiency of particular chemotherapeutic regimen (Said et al., 2014). The results of our study have shown that long-term treatment (72 h) of MDA-MB-231 cells with selected compounds (**0**, **3**, and **4**) induced statistically significant reduction of expression level of the MMP-9 gene compared to control cells, suggesting that these compounds may reduce invasion capacity of MDA-MB-231 cells by downregulation of MMP-9 expression.

Non-steroidal anti-inflammatory drugs NSAIDs are the most used medicaments against inflammation, but besides their benefits, serious side effects prompted the development for more selective COX-2 inhibitors such as Coxibs and NO-releasing NSAIDs (Arun and Goss, 2004, Tołoczko-Iwaniuk et al., 2019). Inflammatory molecules and factors involved in the wound healing process are considered the key regulatory signals in numerous models of tumorigenesis (Coussens and Werb, 2002, Philip et al., 2004). COX-2 has been shown to represent an indicative link between inflammation and progression of many types of cancer, including breast cancer, and a lot of contemporary chemotherapies are aimed at down-regulation of COX-2 activity (Koki et al., 2002, Hashemi et al., 2019). Literature data suggest that COX-inhibitors can reduce tumor invasion, representing reinforcement to standard cytotoxic therapy (Liu et al., 2015). Certain hydantoin compounds such as 5,5-diarylhydantoin derivatives have shown more selective inhibition of COX-2 enzyme (Zarghi and Arfaei, 2011). Our results show that compounds **0**, **3**, and **4** caused a statistically significant reduction in COX-2 gene expression compared to non-treated cells, which additionally may promote one of the possible modes of their antitumor action (Mehner et al., 2014). The comparative studies with NSAID selective inhibitors are yet to be done, but we can suggest that these novel hydantoin derivatives are promising agents in reducing prostaglandin production in breast cancer cells MDA-MB-231.

5. Conclusions

The investigated hydantoin derivatives have shown significant antitumor activity against MDA-MB-231 cells measured by different parameters of cell physiology. Almost all tested compounds exerted significant antiproliferative and proapoptotic effects in a dose- and time-dependent manner as one of the most important antitumor mechanisms. Also, these compounds have reduced cell migration and invasion capacity, and shown significant selectivity on proliferation index regarding non-malignant cells. Thus, our study exhibited that investigated 3-(4-substituted benzyl)-5-iso propyl-5-phenylhydantoin derivatives, owing to their pleiotropic effects, had the potential for the treatment of breast cancer and can be considered as a promising novel tumor therapy in humans, whereby compounds **3** and **4** being particularly prominent and which remains to be further explored.

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Disclosure of interest

The authors declare that there is no conflict of interest.

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