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Article

Antiproliferative effects of 13α/β-steroids on triple-negative MDA-MB-231 breast cancer cells: unraveling intracellular signaling without ERα

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This study aimed to investigate the activities of novel 20(*R*)-3,20-dihydroxy-19-norpregn-1,3,5(10)-trienes (**kuz7** and **kuz8b)** of natural 13β- and epimeric 13α-series against triple-negative MDA-MB-231 breast cancer cells. High antiproliferative activity of synthesized compounds **kuz8b** and **kuz7** against MDA-MB-231 triple-negative cancer cells was revealed. The steroid **kuz7** of natural 13β-configuration was more active against MDA-MB-231 cells than the 13α-steroid **kuz8b**. Cell cycle analysis revealed common patterns for the action of both tested compounds. The number of cells in the subG1 phase increased in a dose-dependent manner, indicating induction of apoptosis, which was also verified by PARP cleavage. In contrast, the number of cells in the G0/G1 phase decreases with increasing compound concentration. Steroid **kuz7** at micromolar concentrations reduced the expression of GLUT1, a glucose transporter. High efficacy of the combination of **kuz7** with biguanide metformin was shown, and synergistic effects on MDA-MB-231 cell growth and expression of the anti-apoptotic protein Bcl-2 were revealed. According to the obtained results, including the high activity of **kuz7** against triple-negative cancer cells, the detected induction of apoptosis, and the decrease in GLUT1 expression, 13β-steroid **kuz7** is of interest for further preclinical studies both alone and in combination with the metabolic drug metformin.

Keywords: Breast cancer. Steroids. GLUT1. Metformin. Apoptosis.

INTRODUCTION

Cancer is a leading cause of death worldwide (de Martel *et al.*, 2020). More than 19 million new cases of cancer were reported in 2020, and it has been estimated that this number will continue to rise. Breast cancer is the most common cancer among women and one of the leading causes of death among them. Breast cancer is a multifactorial disease (Momenimovahed, Salehiniya, 2019; Tao *et al.*, 2015), and the incidence and progression of this type of cancer are due to a variety of factors including genetic, environmental, lifestyle and other factors (Feng *et al.*, 2018). The development of new screening programs can detect breast cancer at an early stage, but despite this, there has not been sufficient success in the treatment of this disease.

Approximately 70% of breast tumors are hormone-dependent, which means that estrogens determine the rate of proliferation of tumor cells and the progression of the disease (Tao *et al.*, 2015). The estrogen receptor α (ERα) (Fuentes, Silveyra, 2019) is a cytoplasmic protein that, after binding to estrogens, is translocated into the nucleus and regulates the transcription of many genes. Targeted inhibition of ERα is one of the most successful therapeutic approaches. Another important target in breast cancer cells is HER2/neu, a receptor tyrosine kinase; such tumors belong to the HER2-positive group. Finally, there is a group of tumors that are characterized by the absence

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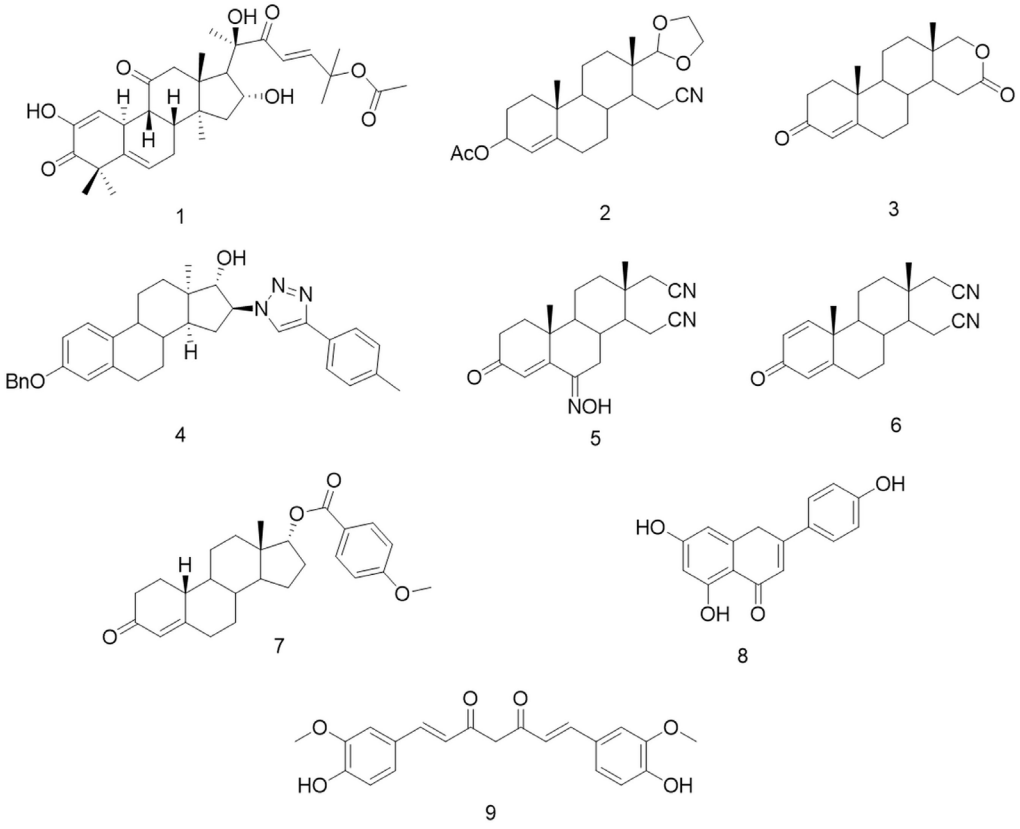
of expression of ERα , progesterone receptor and HER2/neu; these cancers are called triple-negative breast cancers (TNBC) (Eliyatkın *et al.*, 2015; Yersal, Barutca, 2014).

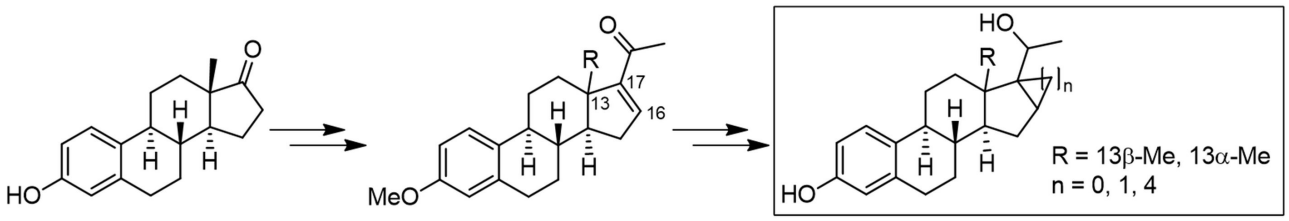
Treatment approaches for triple-negative breast cancers are very limited. It is impossible to use hormone therapy or anti-HER2 therapy for TNBC patients (Yao *et al.*, 2017). Triple-negative breast cancer is typically treated with a combination of surgery, radiation therapy, and chemotherapy. Chemotherapy is recommended for the vast majority of triple-negative and HER2-positive breast cancers because these cancers are often very aggressive. The most commonly used treatment regimens involve anthracyclines and taxanes, although cyclophosphamide, methotrexate, and 5-fluorouracil may also be used in selected patients (Ntellas *et al.*, 2019). Several studies have shown the efficacy of PARP inhibitors and immunotherapy for patients with triple-negative cancers (Barchiesi *et al.*, 2021; Vikas *et al.*, 2020). Although there are many drugs for triple-negative cancers, the effectiveness of treatment is not always high. Therefore, the development of new drugs and drug combinations for the treatment of triple-negative cancer is very relevant (Koushki *et al.*, 2021; Roshanazadeh, Babaahmadi Rezaei, Rashidi, 2021).

Steroids are widely used in the treatment of hormone-dependent breast cancer. The estrogen receptor inhibitor fulvestrant and several aromatase inhibitors are highly effective in the treatment of ERα-positive cancer. In preclinical studies, steroids and their analogs have been shown to exhibit significant antiproliferative potency against triple-negative cancer cells. Several compounds are shown in Scheme 1.



SCHEME 1 - Steroids and steroid-like compounds that inhibit triple-negative breast cancer cells, see discussion; **1** – cucurbitacin E, **2** – 3β-acetoxy-13-(1,3-dioxolan-2-yl)-16,17-seco-17-norandrost-4-ene-16-nitrile, **3** – 17-oxa-D-homoandrost-4-ene-3,16-dione, **4** – 3-benzyloxy-16β-[4-(4-tolyl)-1H-1,2,3-triazol-1-yl]-13α-estra-1,3,5(10)-trien-17α-ol, **5** – (6E)-hydroxyimino-3-oxo-16,17-secoandrost-4-ene-16,17a-dinitrile, **6** – 3-oxo-16,17-secoandrosta-1,4-diene-16,17a-dinitrile, **7** – 17α-p-methoxybenzoyloxyestra-4-en-3-one, **8** - apigenin, **9** – curcumin.

Recently, we developed a promising class of steroid compounds, which are derivatives of 3,20-dihydroxy-19-norpregn-1,3,5(10)-trienes of the natural 13β- and epimeric 13α-series (Scheme 2), and they exhibit high cytotoxicity against the hormone-dependent MCF7 breast cancer cell line and low toxicity against normal MCF-10A breast epithelial cells (Kuznetsov *et al.*, 2018a; Kuznetsov *et al.*, 2018b).



SCHEME 2 - Approach for the synthesis of 16,17-carbocycle fused and unsubstituted 3,20-dihydroxy-19-norpregn-1,3,5(10)-trienes of natural 13β- and epimeric 13α-series.

Changes in the configuration of the 13-center of the steroid core and the size of the additional carbocycle at the 16,17-positions may significantly change the antiestrogenic potential of these compounds. Moreover, several obtained steroids were active against drug-resistant aggressive cancer cells, which shows the importance of further development of the series. This study aimed to analyze the antiproliferative potency of the two lead steroids from the natural 13β- and epimeric 13α-series (Figure 1) against triple-negative MDA-MB-231 breast cancer cells and uncover the mechanisms of their action.

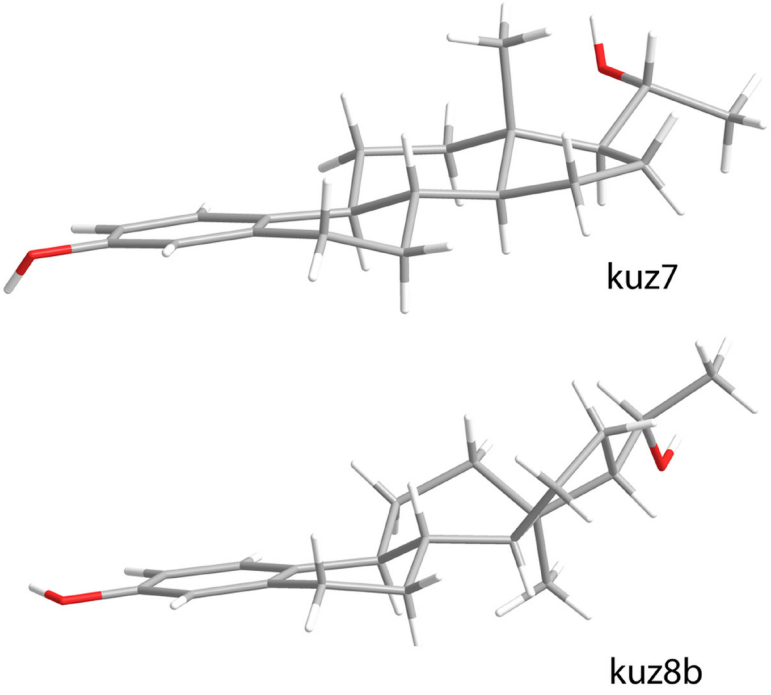


FIGURE 1 - Spatial structures of lead steroid 20(*R*)-3,20-dihydroxy-19-norpregn-1,3,5(10)-trienes of the natural 13β- (**kuz7**) and epimeric 13α-series (**kuz8b**).

MATERIAL AND METHODS

Chemistry

The compounds under study were synthesized, among others in the series, from the commercially available natural hormone estrone via a multistage chemical synthesis, including etherification of estrone, epimerization of the 13-center of the steroid core (in 13α-series), building and modification of the reactive fragment at the 16- and 17-positions of the steroid, and final reduction of the ketone group and cleavage of the steroid methyl ether. Diastereoisomeric products, if they were obtained, were separated by column chromatography on silica gel. The structures and purity of the obtained compounds were confirmed by physicochemical methods (Kuznetsov *et al.*, 2018a; Kuznetsov *et al.*, 2018b).

Cell line, chemicals, and MTT assay

The MDA-MB-231 (ATCC HTB-26, Manassas, Virginia, USA) human breast cancer cells were obtained from the ATCC collection. The MDA-MB-231 cells were cultured in standard 4.5 g/L glucose DMEM medium (Gibco, Ottawa, Ontario, USA) supplemented with 10% FCS (HyClone, Marlborough, USA), 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (PanEco, Moscow, Russia) at 37 °C, 5% CO_2, and 80–85% humidity in a NuAire (Plymouth, MN, USA) incubator. Cells in the logarithmic phase of growth were used in the experiments. The growth inhibitory activities of the compounds were assessed by MTT assay based on the metabolism of the MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Applichem, Germany) in living cells, with modifications as described previously (Piven *et al.*, 2021).

Briefly, the cells were seeded in 24-well plates (Corning, New York, USA) in 900 μL of standard medium. Compounds at different concentrations in 100 μL of the appropriate medium were added, and the cells were grown for 72 h. After incubation with the compounds, the medium was removed, the MTT reagent that was dissolved in the medium was added to the final concentration of 0.2 mg/mL to each well, and the incubation was performed for 2 h. Then, the cell supernatants were removed, and purple formazan crystals were dissolved in DMSO (350 μL per well). The plates were gently shaken, and the absorbance was measured at 571 nm with a reference wavelength of 630 nm on a MultiScan reader (ThermoFisher, Waltham, MA USA). The viability of the cells was expressed as a percentage of the control. The combination index (CI) was calculated as described previously (Miladiyah *et al.*, 2020; Zhao, Au, Wientjes, 2010).

Cell cycle analysis

MDA-MB-231 breast cancer cells were seeded in 6-well plates and treated with the compounds for 48 h. Cell sediments were treated with a PI buffer containing 50 μg/mL propidium iodide (PI), 100 μg/mL RNAse A (Sigma-Aldrich, Burlington, MA, USA), 0.1% sodium citrate, and 0.3% NP-40 (Helicon, Moscow, Russia) for 30 min in the dark. Cell cycle data were acquired by measuring the DNA content using a Cytoflex flow cytometer 26 (Beckman Coulter Brea, California, USA) in the PerCP-A channel. Data analysis was performed with CytExpert (Beckman Coulter).

Immunoblotting

MDA-MB-231 cells were washed twice in phosphate buffered saline and incubated for 10 min on ice in total lysis buffer containing 50 mM Tris-HCl at pH 7.4, 1% SDS, 1% Igepal CA-630, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/mL each of aprotinin, leupeptin, pepstatin, 1 mM Na-orthovanadate, and 1 mM NaF (Sigma-Aldrich, Burlington, MA, USA). The samples were sonicated five times for 5 s each at 30% output and centrifuged for 5 min at 15 000 × g, and supernatants were then used as total cell extracts. Total protein content was determined by the Bradford method.

MDA-MB-231 cell lysates were separated in 10% SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane (GE HealthCare, Chicago, Illinois, USA), and processed according to a standard

protocol. GLUT1, CDK 4/6, Cyclin D1, full and cleaved PARP, BAD and Bcl-2 antibodies were obtained from Cell Signaling Technology; the antibodies against α-tubulin (Cell Signaling Technology) were added to standardize loading. Goat anti-rabbit IgGs (Jackson ImmunoResearch, Ely, Cambridgeshire, United Kingdom) conjugated to horseradish peroxidase were used as secondary antibodies. Signals were detected using the ECL reagent, as described in Mruk and Cheng's protocol (Mruk, Cheng, 2011), and an ImageQuant LAS4000 system (GE HealthCare, Chicago, Illinois, USA).

RESULTS

Antiproliferative activity of the 13α-/β-steroids

To assess the antiproliferative activities of the synthesized compounds, a cell line of triple-negative breast cancer MDA-MB-231 was selected. The MDA-MB-231 cells were seeded on a plate and after 24 h were treated with compounds in various doses. As shown in Figure 2, compound **kuz8b** at micromolar concentrations did not considerably affect the growth of MDA-MB-231 cells, whereas its 13β-analog **kuz7** significantly suppressed cell growth. However, 17.6 μM **kuz8b** causes 50% inhibition of the growth of MDA-MB-231 cells (Table I). The IC_{50} value of **kuz7** was slightly less than 5 μM. Cisplatin and 5-fluorouracil were used as reference drugs. As indicated in Table I, drugs had various effects on MDA-MB-231 cells. Cisplatin inhibited the growth of MDA-MB-231 cells with an IC_{50} of 12.7 μM, whereas 5-fluorouracil exhibited weak activity with an IC_{50} greater than 25 μM. Thus, compound **kuz7** was more active than cisplatin and fluorouracil; compound **kuz8b** was inferior in activity only to cisplatin.

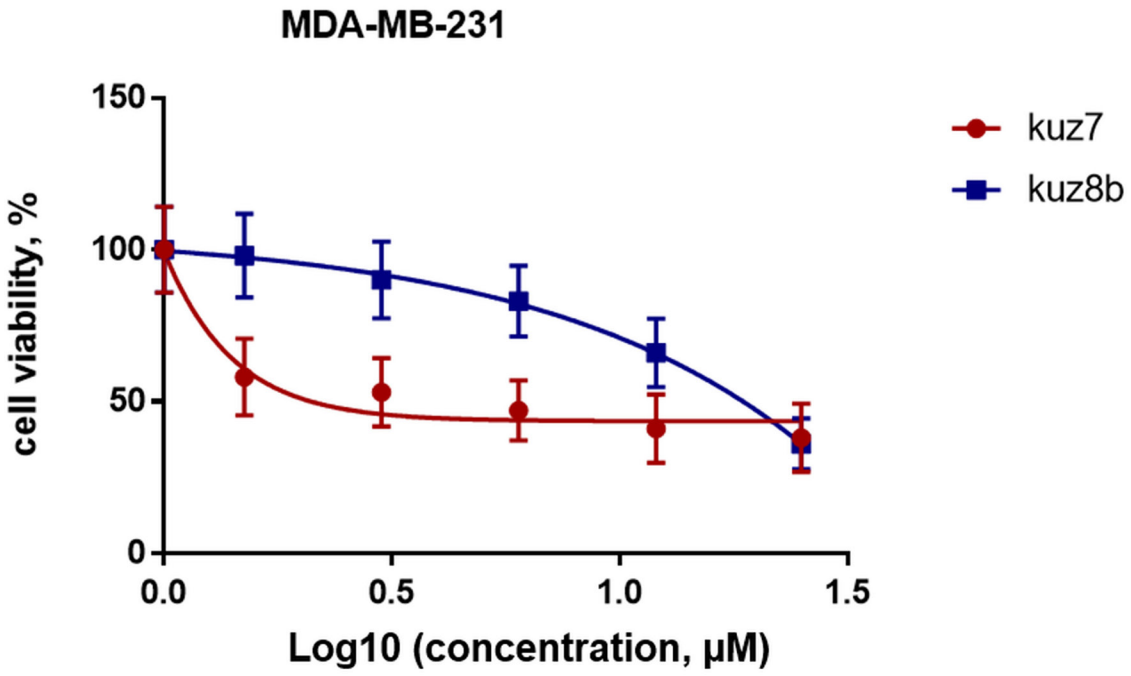


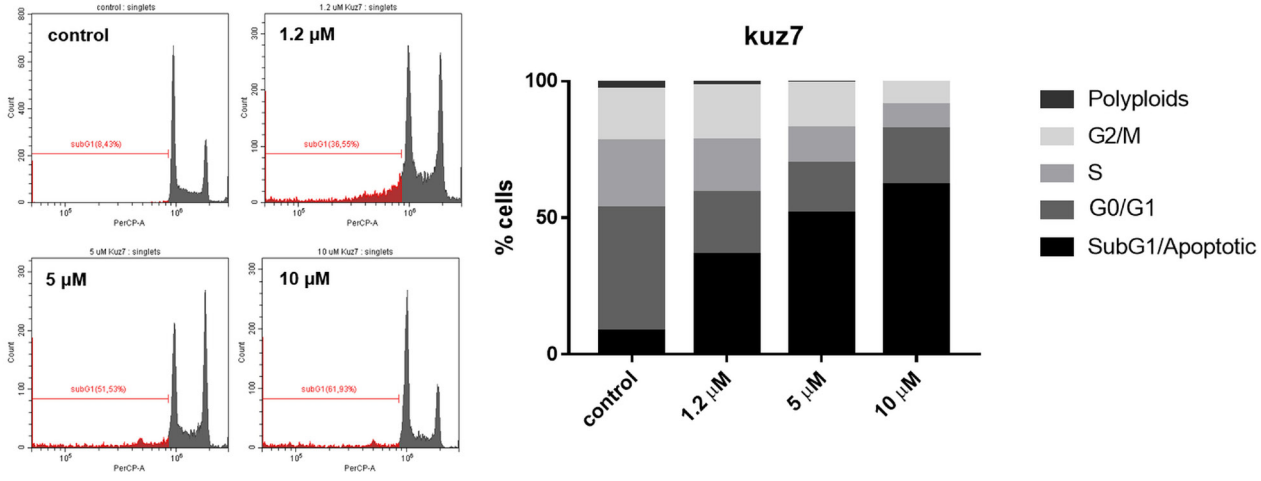
FIGURE 2 - Antiproliferative activity of compounds **kuz7** and **kuz8b** against MDA-MB-231 cells (the cell viability was assessed by MTT assays after 72 h of growth with steroids).

TABLE I - IC_{50} values of compounds **kuz7** and **kuz8b** and reference chemotherapeutics

entry	compound	IC_{50} values against MDA-MB-231 cells, μM
1	**kuz8b**	17.6 ± 1.6
2	**kuz7**	4.2 ± 0.5
3	cisplatin	12.7 ± 1.0
4	5-fluorouracil	> 25

Cell cycle analysis of MDA-MB-231 treated with 13α-/β-steroids

We used the TNBC cell line MDA-MB-231 to investigate the effects of **kuz7** and **kuz8b** on the cell cycle.



	subG1	G0/G1	S	G2/M	Polyploids
Control	8.4%	44.9%	21.8%	19.0%	3.2%
1.2 μM kuz7	36.5%	22.5%	18.1%	19.7%	2.0%
5 μM kuz7	51.5%	18.1%	12.5%	16.4%	1.0%
10 μM kuz7	61.9%	20.5%	8.5%	8.4%	0.5%

FIGURE 3 - Analysis of the cell cycle of MDA-MB-231 after incubation with compound **kuz7** for 48 h.

Figure 3 shows the distribution of the MDA-MB-231 cell line within the cell cycle after incubation with **kuz7** for 48 h. The number of apoptotic cells (the subG1 phase of the cell cycle) significantly increased as the concentration of the active compound increased: 8% apoptotic cells of the control cells population versus 62% apoptosis level in the sample treated with 10 μM steroid **kuz7**. Moreover, the tested compound **kuz7** caused a considerable reduction of the G0/G1 phase population of cells treated with 1.2–10 μM of **kuz7** simultaneously with a gradual decrease in the S phase of the cell cycle. Of note, the percentage of cells in the G2/M phase did not change in the range of low concentrations and was reduced due to high-concentration treatment with **kuz7**. Thus, the effect of **kuz7** on the cell cycle of MDA-MB-231 cells is characterized by a significant increase in the number of apoptotic cells with a simultaneous reduction of the G0/G1 and S phase of cells as the compound concentration increases.

	subG1	G0/G1	S	G2/M	Polyploids
control	6.8%	52.6%	19.3%	18.1%	3.1%
1.2 μM kuz8b	18.3%	42.3%	19.6%	17.5%	2.2%
2.5 μM kuz8b	24.1%	39.1%	19.2%	15.0%	2.5%
10 μM kuz8b	35.8%	21.2%	19.0%	20.6%	3.5%
20 μM kuz8b	51.1%	17.6%	16.6%	11.9%	2.8%

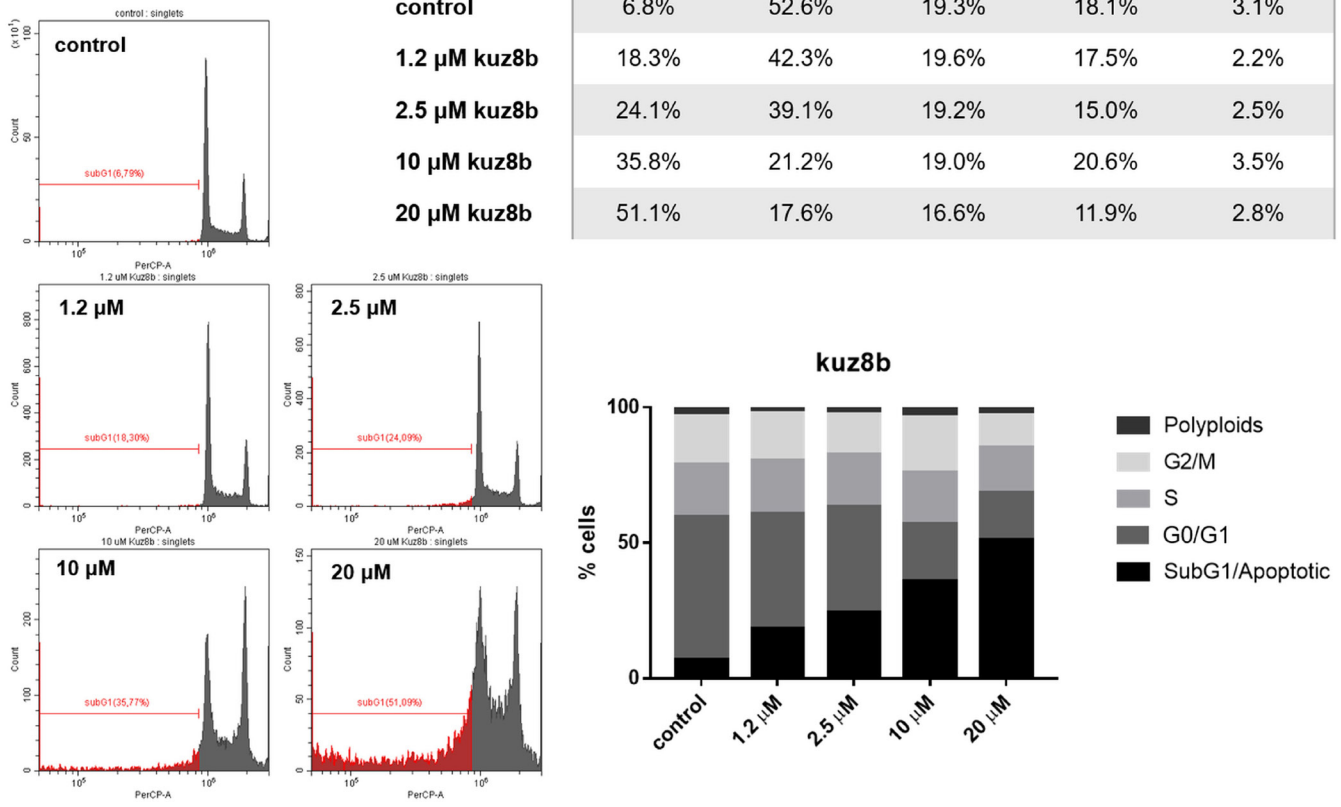


FIGURE 4 - Analysis of the cell cycle of MDA-MB-231 after incubation with compound **kuz8b** for 48 h.

The analysis of the distribution of MDA-MD-231 cells in populations, according to the DNA content after treatment with **kuz8b**, is shown in Figure 4. Here, we identified similar patterns characteristic of the steroid **kuz7** from the 13ß-series. Compound **kuz8b** was tested in the range of concentrations from 1.2 to 20 μM. Of note, we used a higher concentration of **kuz8b** because it had a slightly lower cytotoxic effect than the previously described **kuz7**. We demonstrated that an increase in the effective concentration of **kuz8b** caused the accumulation of a fraction of apoptotic MDA-MB-231 cells. The reduction of the MDA-MB-231 cell population by half was achieved after treatment with 20 μM **kuz8b** for 48 h. In addition to the increase in apoptosis, there was a decrease in the percentage of cells in the G0/G1 phase: 17.6% in 20 μM **kuz8b**-treated cells vs. 52.6% in control cells. Unlike **kuz7**, there was no significant change in cell distribution within the S and G2/M phases of the cell cycle in the range of tested concentrations for **kuz8b**. Thus, **kuz8b** can be characterized by two main trends of action in MDA-MB-231 cells involving a cytotoxic effect: an increase in the apoptotic fraction and a parallel reduction in the percentage of the G0/G1 phase of the cell cycle.

Signaling pathways regulated by 13α-/β-steroids

In subsequent experiments, we analyzed the signaling pathways that are regulated by the synthesized compounds. MDA-MB-231 cells were treated with compounds, and then, the expression of signaling proteins was determined by immunoblotting. The data obtained are shown in Figure 5. CDK4/6 and cyclin D1 are the main regulators of the cell cycle. In MDA-MB-231 cells treated with the compounds, we found no significant changes in the expression of these proteins. A slight decrease in

CDK4 expression was observed for cells treated with the steroid **kuz7**. Glucose supply is very important for MDA-MB-231 breast cancer cells, and the growth of such cancer depends on the availability and transport of glucose. Glucose enters cells through its transporters, and GLUT1 is one of the proteins responsible for glucose uptake. We found that the steroid **kuz7** reduces the expression of GLUT1, as shown in Figure 5.

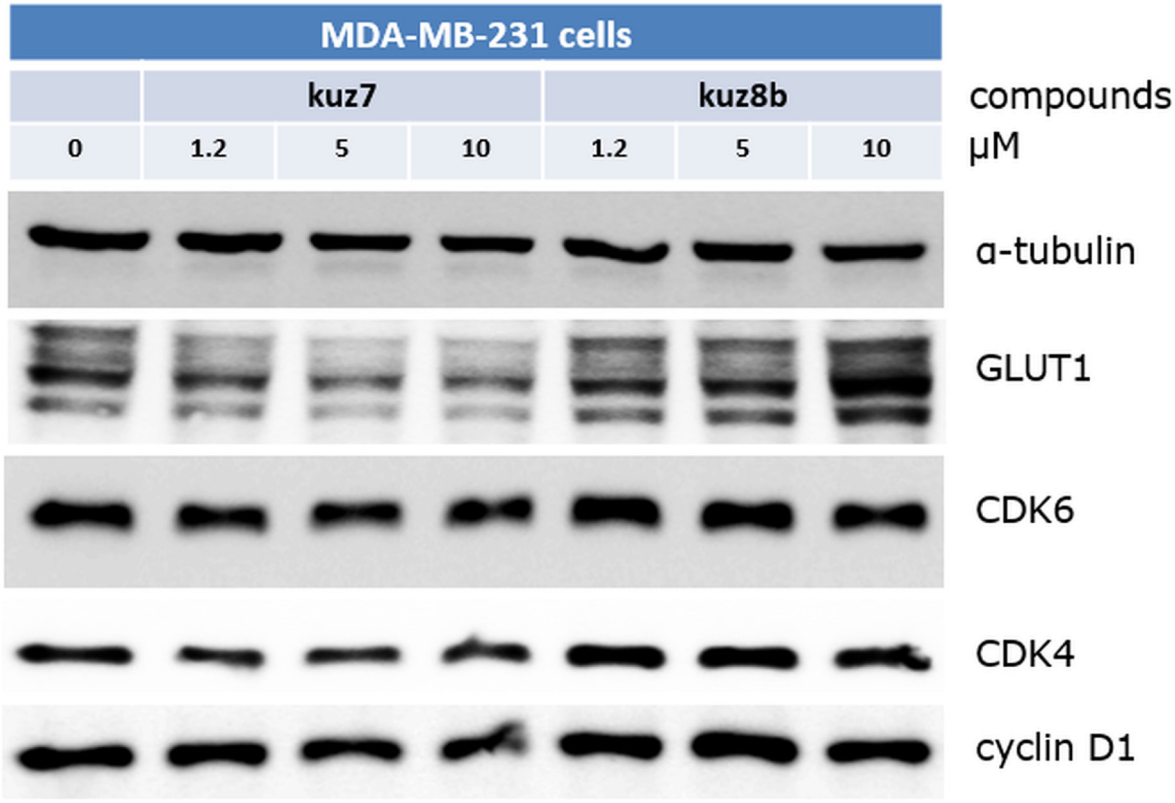


FIGURE 5 - Signaling pathways regulated by 13α-/β-steroids. MDA-MB-231 cells were treated with 0, 1.2, 5, and 10 μM of tested compounds. The GLUT1, CDK 4/6, and cyclin D1 expressions were detected by immunoblotting. α-Tubulin was used as a loading control.

Considering that **kuz7** is more active than **kuz8b**, we conducted further experiments with **kuz7** alone. It is known that inhibition of glucose transport can lead to the activation of oxidative phosphorylation (OxPhos). The application of **kuz7** in combination with a respiratory chain inhibitor may provide significant antiproliferative effects against MDA-MB-231 cells. As the OxPhos inhibitor, we chose metformin (MF), which is used to treat diabetes mellitus. The key target of metformin is complex I of the mitochondrial respiratory chain (Feng *et al.*, 2022).

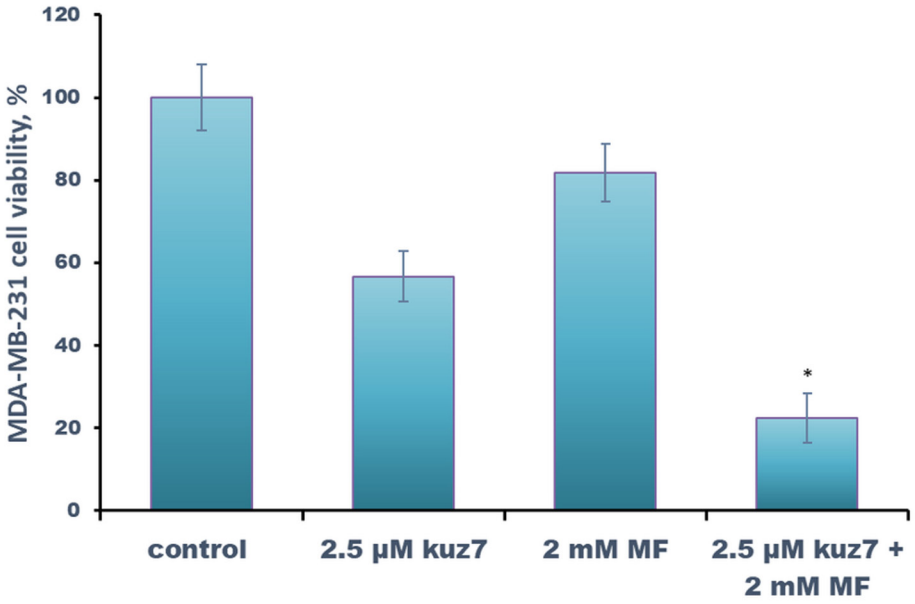


FIGURE 6 - Combinatorial synergism of **kuz7** and metformin (MF). MDA-MB-231 cells were exposed to the combination of **kuz7** and MF or each treatment alone for 72 h, and cell viability was assessed using MTT assay (the combination index was <0.8, when the concentration ratio was 800). Error bars in the figure represent mean ± SD. P<0.05: * – versus vehicle control and each drug alone.

As shown in Figure 6, **kuz7** caused approximately 40% inhibition of cell growth, and the effects of MF were weak when it was used alone. Using the agents in combination resulted in significant suppression of MDA-MB-231 cell growth.

We hypothesized that the chosen combination of compounds would provide an effect on apoptosis proteins.

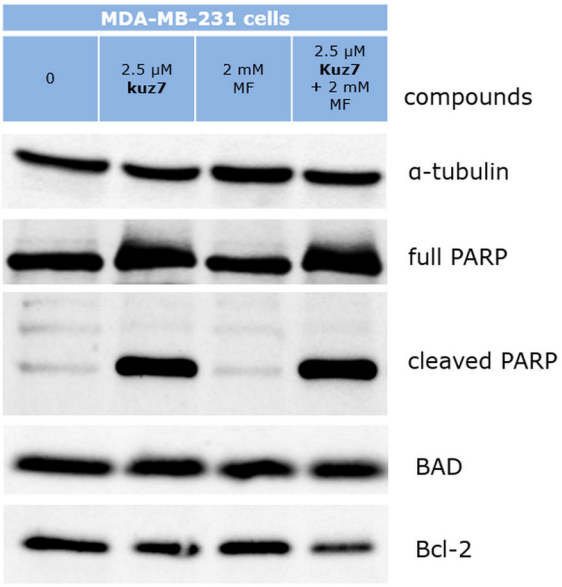


FIGURE 7 - Apoptosis proteins in MDA-MB-231 cells treated with the steroid **kuz7**, metformin (MF), or their combination.

The results of the experiment revealed that compound **kuz7** alone causes an increase in the level of cleavage of poly(ADP-ribose) polymerase (PARP) (Figure **7**), which is a biomarker of induced apoptosis (Moghtaderi *et al.*, 2018; Saraste, Pulkki, 2000). MF treatment did not result in PARP cleavage compared to that in control samples. The combination treatment induced PARP cleavage as high as that in MDA-MB-231 cells treated with **kuz7** alone. Among the factors that restrain programmed cell death are the disbalance of Bcl-2/BAD proteins, reduced caspase function, and disrupted death receptor signaling (Elmore, 2007). The expression of pro-apoptotic protein BAD was high and did not change after incubation of cells with **kuz7**, MF, or their combination. The expression of the anti-apoptotic protein Bcl-2 did not change when MDA-MB-231 cells were treated with **kuz7** or MF, whereas the drug combination significantly reduced the expression of this factor. Thus, the combination of **kuz7** with MF has a significant antiproliferative effect on MDA-MB-231 cells and induces apoptosis, including through a decrease in Bcl-2 expression.

DISCUSSION

TNBC is the most aggressive type of breast cancer and accounts for 15–25% of all breast tumors; it is associated with a poor prognosis of the course of the disease (da Silva *et al.*, 2020; Medina *et al.*, 2020). In many cases, this type of cancer is resistant to treatment; hence, the investigation of new compounds and the development of new therapeutic approaches targeted to TNBC treatment is a promising area of research (Moghtaderi *et al.*, 2018; Nikolić *et al.*, 2015; Won, Spruck, 2020). Currently, novel compounds (including those in the steroid series) are being actively designed and studied by scientists around the world (Fröhlich *et al.*, 2018; Kovačević *et al.*, 2016; Moghtaderi *et al.*, 2018; Nikolić *et al.*, 2018; Won, Spruck, 2020). Scheme 1 demonstrates some of the natural and synthesized compounds exhibiting anticancer activity.

Cucurbitacins are a class of highly oxidized tetracyclic triterpenes present in several plants used in traditional Chinese medicine treatments. Analysis of the antiproliferative properties of cucurbitacins was performed by several research groups (Alghasham, 2013; Garg, Kaul, Wadhwa, 2018; Kong *et al.*, 2014, Lan *et al.*, 2013). The first significant study of cucurbitacin E (CuE, Scheme 1, Compound **1**) on TNBC cells was described previously (Lan *et al.*, 2013). CuE inhibits the growth of MDA-MB-231 breast cancer cells in a dose and time-dependent manner. Further analysis showed that CuE induces G2/M phase arrest and apoptosis. CuE induced the cleavage of caspase 3 and upregulated p21 and p27. CuE enhanced the antiproliferative potency of cisplatin (Lan *et al.*, 2013). In another study, Yanjie Kong *et al.* confirmed that CuE significantly inhibited the growth of TNBC cells, leading to the arrest in the G2/M phase of the cell cycle (Kong *et al.*, 2014). CuE treatment resulted in a decrease in the number of EdU-positive proliferating cells in a dose-dependent manner and further apoptosis within 24 h. The described compound showed a strong growth-inhibiting effect on five TNBC cell lines with IC_{50} values from 10 to 70 nM. CuE in concentrations from 100 to 200 nM reduced the expression of proteins such as cyclin D1 (cell cycle regulator), survivin, Mcl-1, XIAP, and Bcl-2 (anti-apoptotic proteins) (Kong *et al.*, 2014). Thus, CuE has a higher antiproliferative potency than that of the obtained steroids **kuz7** and **kuz8b** and reference compounds in our study. CuE induced G2/M cell cycle arrest, whereas the tested steroids increased the subG1 cell population, decreased the G0/G1 and S phase cell population at lower doses and only decreased G2/M cell population at higher doses. CuE alone and steroid **kuz7** in combination with MF were able to reduce Bcl-2 expression in cancer cells.

Evgenija Djurendić *et al.* evaluated the effect of D-seco and D-homo androstane derivatives on TNBC cells. Compounds **2** (an IC_{50} of 10 μM) and **3** (an IC_{50} of 9 μM) showed moderate antiproliferative activity against MDA-MB-231 cells and a low cytotoxic effect on the estrogen-positive cell line MCF7 (IC_{50} >100 μM) (Djurendić *et al.*, 2009). Strahinja Kovačević et al. used a method of quantitative structure–activity relationship (QSAR) to predict the antiproliferative activity against breast cancer cells of A- and B-modified D-homo lactone and D-seco androstane derivatives (Kovačević *et al.*, 2016). In the first group, there were compounds with D-homolactone function in the steroid nucleus, while

in the second group, there were steroids with D-seco function. Eventually, the studied compounds showed antiproliferative activity against MDA-MB-231 with IC_{50} values from 10 μM and higher (Kovačević *et al.*, 2016).

In the study by Erzsébet Mernyák *et al.*, it was demonstrated that trans-16-triazolyl-13α-methyl-17-estradiol diastereomer (Scheme 1, compound **4**) had a potent cytotoxic effect on the MDA-MB-231 cells with IC_{50} values of 6.5 μM (Mernyák *et al.*, 2015).

Andrea Nikolić *et al.* investigated the antitumor activity of novel 6-substituted 4-en-3-one D-seco-steroidal dinitriles. Among them, compound **5** showed a good cytotoxic effect on the MDA-MB-231 cell line with an IC_{50} value of 2.8 μM, and early and late apoptosis was also observed by different methods. Moreover, the described compounds did not affect the proliferation of normal MRC-5 cells (Nikolić *et al.*, 2018). The same group reported the antitumor potential of steroid 16,17-seco-16,17a-dinitriles. Compound **6**, the 3-keto-1,4-diene derivative, has been shown as a selective agent with antiproliferative activity against estrogen-dependent (MCF7) and estrogen-independent (MDA-MB-231) breast cancer cell lines at submicromolar concentrations with IC_{50} values of 0.52 and 0.11 μM, respectively (Nikolić *et al.*, 2015). Novel diastereomeric 16-hydroxymethyl-19-nortestosterone derivatives prepared by Birch reduction from the corresponding 3-methoxy-16-hydroxymethylestra-1,3,5(10)-trien-17-ol isomers with known configurations were described by Gyula Schneider (Schneider *et al.*, 2016). One of the obtained compounds, 17α-p-methoxybenzoyloxy19-nortestosterone estra-4-en-3-one (Scheme 1, compound **7**), at a concentration of 30 μM exhibited 71% growth inhibition of MDA-MB-231 cells and was as active as the chemotherapy drug cisplatin (Schneider *et al.*, 2016). Thus, the IC_{50} values of steroids and steroid-like compounds against TNBC vary considerably. Usually, these compounds inhibit cancer cell growth at doses of 1 μM and slightly higher, except for compounds with very high activity, such as CuE. The antiproliferative potency of the 13β-steroid **kuz7** is similar to that described for several lead compounds, whereas the 13α-steroid **8b** is less active.

Compounds isolated from natural products can provide hormonal and antihormonal effects, mimicking the action of hormones. Such compounds are also considered potent agents that inhibit the growth of triple-negative breast cancers. The activity of the phytoestrogen apigenin (compound **8**, Scheme 1) has been described in several recent papers (Bauer *et al.*, 2020; Lee *et al.*, 2019; Nasir *et al.*, 2020). We demonstrated the activity of apigenin against various cancer cell lines (Scherbakov, Andreeva, 2015). Cultivation of HER2-positive breast cancer SK-BR-3 (HTB-30) cells in the presence of apigenin resulted in a decrease in HER2/neu expression, accompanied by cleavage of an apoptosis substrate PARP. The search for novel combinations of steroid-like agents to inhibit cancer is actively being conducted. For instance, Hassan Moghtaderi *et al.* showed that the combination of gallic acid and curcumin (compound **9**, Scheme 1) strongly decreased MDA-MB-231 cell growth (Moghtaderi *et al.*, 2018). Moreover, the applied combination increased the ROS level and cytotoxic activity along with the glutathione depletion in MDA-MB-231 cells. Vivek Kumar Soni *et al.* described that metabolite transporters and receptors (GLUT1, MCT-1, MCT-4, and HCAR-1) were upregulated in high glucose-exposed HepG2 cells (Soni *et al.*, 2021). Curcumin **9** inhibited the elevated expression of these enzymes, transporters, and receptors in tumor cells.

Curcumin has an effect on expression and activity of GLUT1 at sufficiently high doses [*i.e.*, 20 μM (Dai *et al.*, 2021), 75 μM (Gunnink *et al.*, 2016), and 15 and 30 μM (Liao *et al.*, 2015)], whereas steroid **kuz7** proved to be a more effective GLUT1 inhibitor exhibiting activity at a concentration of 1.2 μM and higher.

CONCLUSIONS

This investigation was performed to explore the effect of 13α- and 13β-steroids on the growth of triple-negative breast cancer cells. The antiproliferative activities of the obtained compounds **kuz8b** and **kuz7** against MDA-MB-231 cells were evaluated. The 13β-steroid **kuz7** selected for study was more active against MDA-MB-231 than the 13α-steroid **kuz8b**. Analysis of the cell cycle revealed common patterns regarding the effects of both steroids. After treatment with the compounds, MDA-MB-231 cells were in the subG1 phase, which

indicates the induction of apoptosis. Compound **kuz7** at micromolar concentrations reduced the expression of GLUT1, a transmembrane protein responsible for the facilitated diffusion of glucose across a cell membrane. The combination of **kuz7** with biguanide MF was shown to be synergistic. Taking into account the results of our experiments, including the high activity of **kuz7** against triple-negative cancer cells, the detected induction of apoptosis, and decrease in GLUT1 expression, the 13β-steroid **kuz7** is of interest for further preclinical studies, alone or/and in combination with the complex I inhibitor MF.

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