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PRACA ORYGINALNA
ORIGINAL PAPER

The influence of betulin and its derivatives on the expression of TNF and its receptors in RPTEC cells

Wpływ betuliny i jej pochodnych na ekspresję TNF i jego receptorów w komórkach RPTEC

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ABSTRACT

INTRODUCTION: Despite their promising anticancer properties, betulin derivatives may have serious side effects, including nephrotoxicity. Tumor necrosis factor (TNF) and its receptors may play crucial roles in renal cells' reaction to these compounds. The aim of this study was to examine the effect of the derivatives EB5 and ECH147 on renal cell expression of TNF and its receptors.

MATERIAL AND METHODS: Human renal proximal tubule epithelial cells (RPTECs) were treated with betulin, EB5, and ECH147, as well as cisplatin and 5-fluorouracil. The transcript levels of the genes *TNF*, *TNFR1*, and *TNFR2* were assessed using real-time RT-qPCR. Protein concentrations in the culture media were determined using ELISA.

RESULTS: The transcriptional activity of the gene *TNF* was induced in cells treated with 0.5 µg/mL betulin or ECH147. Similar changes in transcriptional activity were observed for *TNFR1*. Betulin and its derivatives strongly inhibited the expression of *TNFR2*. No TNF or sTNFR2 proteins were detected in the culture media. EB5 downregulated sTNFR1 release in comparison with the other compounds.

CONCLUSIONS: EB5 at low concentrations may be less harmful to renal cells. The lower toxicity of EB5 may be a result of the altered expression of TNF and its receptors.

KEYWORDS

betulin, betulin derivatives, TNF, TNFR1, TNFR2, RPTEC

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STRESZCZENIE

WSTĘP: Pomimo obiecujących właściwości przeciwnowotworowych pochodne betuliny mogą powodować poważne skutki uboczne, w tym nefrotoksyczność. Czynnik martwicy nowotworu (*tumor necrosis factor* – TNF) i jego receptory mogą odgrywać kluczową rolę w reakcji komórek nerkowych na te związki. Celem badania było określenie wpływu pochodnych EB5 i ECH147 na ekspresję TNF i jego receptorów w komórkach nerkowych.
MATERIAŁ I METODY: Ludzkie komórki nabłonkowe kanalika proksymalnego nerki (*renal proximal tubule epithelial cells* – RPTECs) poddano działaniu betuliny, EB5 i ECH147, a także cisplatyny i 5-fluorouracylu. Poziomy transkryptów genów *TNF*, *TNFR1* i *TNFR2* oceniono z użyciem RT-qPCR w czasie rzeczywistym. Stężenia rozpuszczalnych form białek w podłożu hodowlanym określono za pomocą testu ELISA.
WYNIKI: W komórkach poddanych działaniu 0,5 µg/ml betuliny lub ECH147 stwierdzono nasilenie aktywności transkrypcyjnej genu *TNF*. Podobne zmiany w aktywności transkrypcyjnej zaobserwowano dla genu *TNFR1*. Betulina i jej pochodne silnie hamowały ekspresję *TNFR2*. W pożywkach hodowlanych nie wykryto rozpuszczalnej formy białka TNF oraz sTNFR2. EB5 zmniejszyło uwalnianie sTNFR1 w porównaniu z innymi związkami.
WNIOSKI: EB5 w niskich stężeniach może być mniej szkodliwy dla komórek nerkowych. Niższa toksyczność EB5 może wynikać ze zmienionej ekspresji TNF i jego receptorów.

SŁOWA KLUCZOWE

betulina, pochodne betuliny, TNF, TNFR1, TNFR2, RPTEC

INTRODUCTION

Betulin, a lupane-type triterpenoid (lup-20(29)--en-3*β*,28-diol), is a compound obtained from birch bark and known for its anti-inflammatory, antioxidant and anticancer properties [1,2]. However, this compound is characterized by poor bioavailability, which prompts the search for derivatives with better pharmacokinetic properties and greater activity. A large pool of candidates is betulin derivatives, with anticancer activity against various cell lines of breast cancer, lung cancer, prostate cancer, colon cancer, and human and murine leukemia cells [3,4,5,6,7]. Structurally, these derivatives indicate that the preferred modification of the betulin structure is to introduce substituents containing a carbon–carbon triple bond [8,9,10]. Numerous studies have confirmed that introducing this type of moiety into the C-28 position of betulin produces compounds with desirable pharmacological parameters. Examples of alkynyl betulin derivatives with promising anticancer activity are the compounds EB5 (a 28-propynoyl derivative) and ECH147 (a 29-diethylphosphonate analog) [11,12,13,14,15]. However, betulin derivatives, like most anticancer drugs, may have side effects due to negative impacts on normal tissues that can lead to disorders such as nephrotoxicity [16].
Depending on the drug, the particular mechanism of nephrotoxicity may be crystal precipitation and drug accumulation in renal tubules, increased oxidative stress, induction of tubular injury, or proximal tubule dysfunction [16]. A recent study showed that the derivatives EB5 and ECH147 influence the viability of human renal proximal tubule epithelial cells (RPTECs) and change their antioxidant status through different mechanisms than those that drive betulin or cisplatin responses [17]. This difference is promising and provides hope for the development of safe anticancer drugs. However, the toxicity of these derivatives may result from other molecular changes, including altered expression of cytokines such as tumor necrosis factor (TNF).
TNF is a pleiotropic cytokine that is involved in the activation of many intracellular pathways through two receptors: TNFR1 and TNFR2 [18]. It may induce cell proliferation or cell death or may activate genes involved in processes such as inflammatory response. The influence of TNF on a particular cell type depends on receptor expression and signal transduction in that cell; these features may differ between cell types. TNF and its receptors are expressed by many cells, though the expression and activation of TNFR2 are restricted to specific cell types [18]. Previous research has shown that genes encoding TNF and its receptors are active in human RPTECs [19] and that an amphotericin B–copper II ion complex (AmB–Cu^{2+}) influences the expression of TNF and its receptors in a different way than amphotericin alone. In RPTECs, the complex was less toxic than amphotericin and promoted different expressions of genes involved in intracellular signaling. In the current study, we examined whether betulin derivatives have different effects than betulin itself regarding the expression of TNF and its receptors. To date, the effects of these compounds on the genes encoding TNF, TNFR1, and TNFR2 in kidney cells have not been investigated.

MATERIAL AND METHODS

Synthesis of EB5 and ECH147

The betulin derivatives EB5 (a 28-propynoyl derivative) and ECH147 (a 29-diethylphosphonate

analog) were synthesized at the Department of Organic Chemistry of the Faculty of Pharmaceutical Sciences in Sosnowiec (SUM), according to previously described procedures. The compound ECH147 was obtained through a several-stage modification of the betulin molecule: it was transformed into a 3,28-diacetyl derivative, then a bromine atom was introduced in the C-30 (allyl) position, and then replaced with a diethylphosphonate group in the Michaelis–Arbuzov reaction. Deacetylation (C-3 and C-28 positions) combined with isomerization to the vinyl system (C-29) resulted in 29-diethoxyphosphorylbetulin [14]. The ECH147 and EB5 used in the research were created in a reaction with propiolic acid (29-diethoxyphosphorylbetulin and betulin, respectively) [11,15]. The synthesis was carried out using the Steglich method, which is suitable for esterifying substrates which are sensitive to strongly acidic environments [20].

The target compounds were purified by column chromatography. Their identity and purity were assessed by determining their melting points and analyzing their ^{1}H and ^{13}C NMR spectra; for the ECH147, its ^{31}P NMR spectrum was also analyzed. The results corresponded to literature data [11,15].

Conditions of cell culturing

Normal human RPTECs (CC-2553) were cultured with the use of a REGM Bullet Kit CC-3190 (renal epithelial basal medium [REBM]), supplements, and growth factors (SingleQuots) at 37°C in a 5% CO_2 incubator (Direct Heat CO_2; Thermo Fisher Scientific). The RPTECs were seeded 5×10^5 per well on 6-well plates (Greiner Bio-One GmbH) and left overnight. Following previous research, the cells were treated for 24 h with two concentrations of each tested compound: 0.1 and 0.5 μg/mL [17]. Each experiment variant was performed in triplicate.

Total RNA extraction

Total cellular RNA was extracted using TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. The RNA extracts were purified and subjected to qualitative and quantitative analysis.

Real-time RT-qPCR

The transcripts' levels were assessed with the following oligonucleotide Taq-Man® Assays (Thermo Fisher Scientific): TNFA (Assay ID: Hs00174128_m1), TNFRSF1A (Assay ID: Hs01042313_m1), and TNFRSF1B (Assay ID: Hs00961750_m1). The real-time RT-qPCR analysis was carried out using a GoTaq® Probe 1-Step RT-qPCR System (Promega Corporation) and a LightCycler® 480 Instrument II (Roche). The mRNA copy numbers were recalculated per 1 μg of total RNA.

Concentration of proteins

The concentrations of TNF, sTNFR1, and sTNFR2 proteins in the culture medium were determined with the use of immunoenzymatic tests (R&DSystems Inc.) according to the supplied protocols: a Human TNF-alpha Quantikine ELISA Kit, a Human TNF RI/TNFRSF1A Quantikine ELISA Kit, and a Human TNF RII/TNFRSF1B Quantikine ELISA Kit. The concentrations were calculated from optical density readings at 450 nm with a BioTek Epoch Microplate Spectrophotometer (BioTek Instruments, Agilent Technologies).

Statistical analysis

The statistical analysis was performed using Statistica v. 13.3 software (TIBCO Software Inc.). The normality of the distribution was assessed by the Shapiro–Wilk test. The Kruskal–Wallis test (ANOVA) followed by the Mann–Whitney U test were used to evaluate differences between the groups of cells in the level of mRNA and the protein concentrations of TNF, TNFR1, and TNFR2. All results are expressed as median and quartile range (significance was set at $p < 0.05$).

RESULTS

TNF mRNA

Treatment with 0.5 μg/mL of ECH147 or betulin increased the expression of the gene *TNF* compared to 0.5 μg/mL of 5-FU ($p = 0.0027$ and $p = 0.0024$, respectively), cisplatin ($p = 0.0081$ and $p = 0.0033$, respectively), or EB5 ($p = 0.0171$ and $p = 0.0108$, respectively), as well as the control cells ($p = 0.0036$ and $p = 0.0033$, respectively; Figure 1). Cisplatin at 0.1 μg/mL downregulated *TNF* expression compared to the untreated control cells ($p < 0.001$) and compared to 0.1 μg/mL of betulin ($p = 0.0004$), ECH147 ($p = 0.0006$), EB5 ($p = 0.0062$), or 5-FU ($p = 0.0004$). Both betulin and cisplatin showed dose-dependent responses, in which higher concentrations induced greater *TNF* gene expression ($p = 0.0108$ and $p = 0.0272$, respectively).

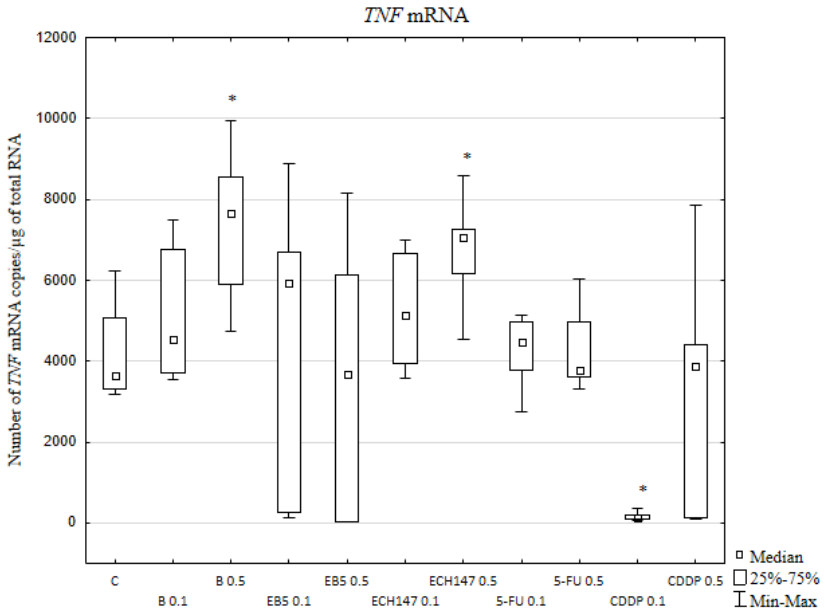

Fig. 1. Number of *TNF* mRNA copies (per 1 µg of total RNA) in the RPTECs after treatment with the tested compounds. C – control (untreated cells); B 0.1 – betulin at 0.1 µg/mL conc.; B 0.5 – betulin at 0.5 µg/mL conc.; EB5 0.1 – EB5 at 0.1 µg/mL conc.; EB5 0.5 – EB5 at 0.5 µg/mL conc.; ECH147 0.1 – ECH147 at 0.1 µg/mL conc.; ECH147 0.5 – ECH147 at 0.5 µg/mL conc.; 5FU 0.1 – 5-fluorouracil at 0.1 µg/mL conc.; 5FU 0.5 – 5-fluorouracil at 0.5 µg/mL conc.; CDDP 0.1 – cisplatin at 0.1 µg/mL conc.; CDDP 0.5 – cisplatin at 0.5 µg/mL conc.; *statistical significance ($p < 0.05$) in comparison to the controls; data represents medians and quartile ranges.

TNFR1 mRNA

The changes observed in the transcriptional activity of *TNFR1* in the RPTECs were similar to those observed for *TNF* (Figure 2). Compared to control cells, treatment with betulin at 0.5 µg/mL or ECH147 at 0.5 µg/mL or 0.1 µg/mL induced the expression of *TNFR1* ($p = 0.0062$, $p = 0.0045$, and $p = 0.0485$, respectively). This expression was downregulated after treatment with 0.1 µg/mL of cisplatin ($p = 0.0004$) or 0.5 µg/mL of EB5 ($p = 0.0171$).

Both betulin and cisplatin showed dose-dependent responses, where higher concentrations induced greater expression of *TNFR1* ($p = 0.0062$ and $p = 0.0104$, respectively). Conversely, the higher concentration of 5-FU (0.5 µg/mL) downregulated *TNFR1* gene expression ($p = 0.0006$).

As in the case of *TNF*, treatment with 0.5 µg/mL of betulin increased the number of mRNA copies of *TNFR1* compared to cells treated with 0.5 µg/mL of EB5 ($p = 0.0004$), 5-FU ($p = 0.0004$), or cisplatin ($p = 0.0047$). Treatment with 0.1 µg/mL of betulin or 5-FU decreased *TNFR1* expression compared to cells treated with 0.1 µg/mL of cisplatin ($p = 0.0004$). ECH147 at 0.1 or 0.5 µg/mL increased the *TNFR1* mRNA level compared to cells treated with 5-FU ($p = 0.0006$ and $p = 0.0006$, respectively) or cisplatin ($p = 0.0006$ and $p = 0.0045$, respectively). Treatment with 0.1 µg/mL of ECH147 increased *TNFR1* expression compared to cells treated with EB5 ($p = 0.0006$).

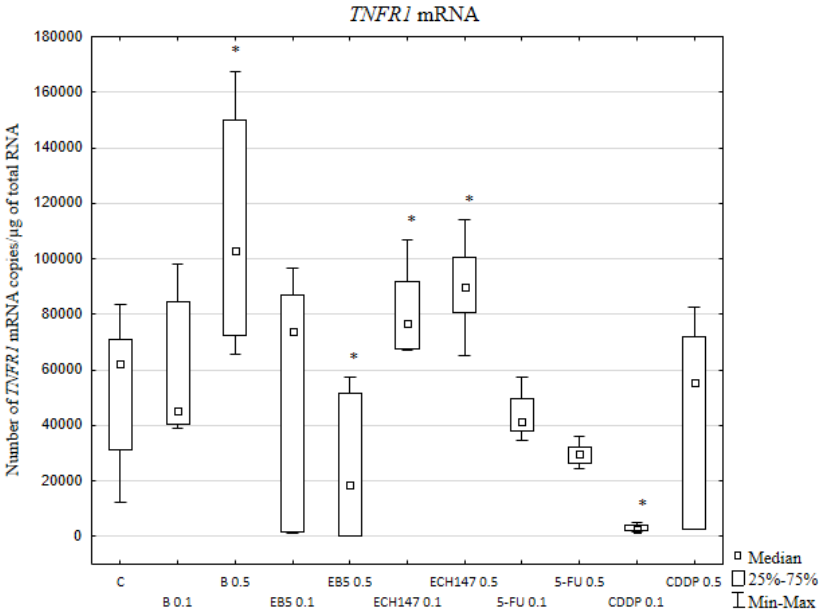

Fig. 2. Number of *TNFR1* mRNA copies (per 1 µg of total RNA) in the RPTECs after treatment with the tested compounds. C – control (untreated cells); B 0.1 – betulin at 0.1 µg/ml conc.; B 0.5 – betulin at 0.5 µg/ml conc.; EB5 0.1 – EB5 at 0.1 µg/ml conc.; EB5 0.5 – EB5 at 0.5 µg/ml conc.; ECH147 0.1 – ECH147 at 0.1 µg/ml conc.; ECH147 0.5 – ECH147 at 0.5 µg/ml conc.; 5FU 0.1 – 5-fluorouracil at 0.1 µg/ml conc.; 5FU 0.5 – 5-fluorouracil at 0.5 µg/ml conc.; CDDP 0.1 – cisplatin at 0.1 µg/ml conc.; CDDP 0.5 – cisplatin at 0.5 µg/ml conc.; *statistical significance ($p < 0.05$) in comparison to the controls; data represents medians and quartile ranges.

TNFR2 mRNA

Treating the RPTECs with betulin or its derivatives, regardless of the concentration, strongly inhibited the expression of the gene *TNFR2* (Figure 3). This inhibition was statistically significant compared to the expression in the control cells, for both concentrations of 5-FU and for cisplatin at 0.5 µg/mL ($p < 0.001$). *TNFR2* gene expression was higher in the cells treated with 0.1 µg/mL of 5-FU than in those treated with 0.1 µg/mL of CDDP ($p = 0.0181$).

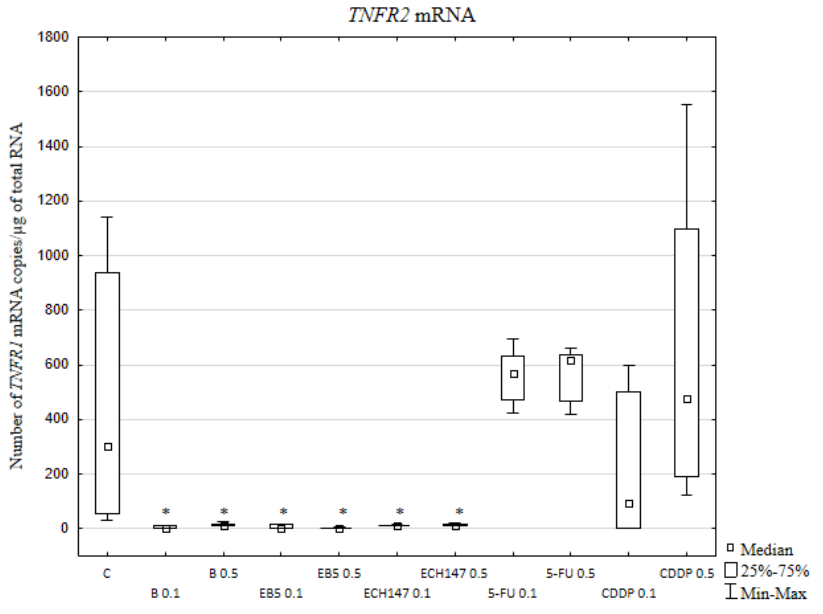

Fig. 3. Number of *TNFR2* mRNA copies (per 1 µg of total RNA) in the RPTECs after treatment with the tested compounds. C – control (untreated cells); B 0.1 – betulin at 0.1 µg/ml conc.; B 0.5 – betulin at 0.5 µg/ml conc.; EB5 0.1 – EB5 at 0.1 µg/ml conc.; EB5 0.5 – EB5 at 0.5 µg/ml conc.; ECH147 0.1 – ECH147 at 0.1 µg/ml conc.; ECH147 0.5 – ECH147 at 0.5 µg/ml conc.; 5FU 0.1 – 5-fluorouracil at 0.1 µg/ml conc.; 5FU 0.5 – 5-fluorouracil at 0.5 µg/ml conc.; CDDP 0.1 – cisplatin at 0.1 µg/ml conc.; CDDP 0.5 – cisplatin at 0.5 µg/ml conc.; *statistical significance ($p < 0.05$) in comparison to the controls; data represents medians and quartile ranges.

Protein concentrations in culture media

Neither TNF nor sTNFR2 was detected in the culture media. The concentration of sTNFR1 was higher in the medium from cells treated with cisplatin at either concentration ($p < 0.006$), those treated with ECH147 ($p < 0.006$), 0.1 µg/mL of 5-FU ($p = 0.0051$), 0.1 µg/mL of betulin ($p = 0.005$), or 0.5 µg/mL of EB5 ($p = 0.0082$), compared to the untreated control cells (Figure 4). Treatment with 0.1 µg/mL of EB5 downregulated sTNFR1 release compared to treatment with 0.1 µg/mL of ECH147, betulin, 5-FU, or cisplatin ($p < 0.006$). Treatment with 0.1 µg/mL of betulin upregulated sTNFR1 compared to treatment with ECH147 and cisplatin ($p < 0.007$). Treatment with 0.5 µg/mL of ECH147 increased sTNFR1 release compared to treatment with EB5 or 5-FU ($p < 0.006$). A dose-dependent effect was noted only for 5-FU, where the lower concentration caused a higher level of sTNFR1 in the culture medium ($p = 0.005$).

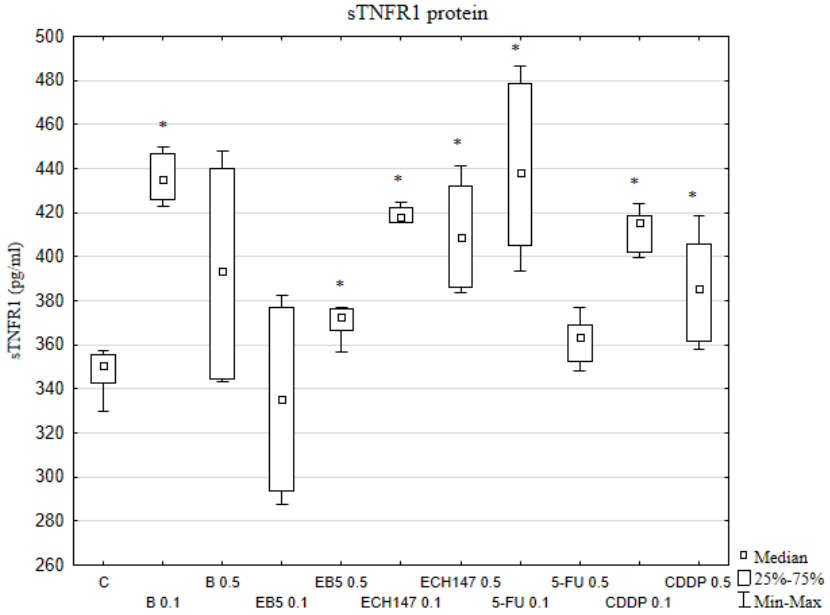

Fig. 4. Concentration of sTNFR1 in the RPTECs' culture media after treatment with the tested compounds. C – control (untreated cells); B 0.1 – betulin at 0.1 µg/ml conc.; B 0.5 – betulin at 0.5 µg/ml conc.; EB5 0.1 – EB5 at 0.1 µg/ml conc.; EB5 0.5 – EB5 at 0.5 µg/ml conc.; ECH147 0.1 – ECH147 at 0.1 µg/ml conc.; ECH147 0.5 – ECH147 at 0.5 µg/ml conc.; 5FU 0.1 – 5-fluorouracil at 0.1 µg/ml conc.; 5FU 0.5 – 5-fluorouracil at 0.5 µg/ml conc.; CDDP 0.1 – cisplatin at 0.1 µg/ml conc.; CDDP 0.5 – cisplatin at 0.5 µg/ml conc.; *statistical significance ($p < 0.05$) in comparison to the controls; data represents medians and quartile ranges.

DISCUSSION

Despite significant progress in the development of new anticancer drugs, cancer treatment remains a major challenge. The problem is not only the effectiveness of treatment, but also several adverse side effects that anticancer compounds have on normal cells. The most common issues are cardiotoxicity, hepatotoxicity, and nephrotoxicity [16]. The nephrotoxicity caused by anticancer drugs has been the subject of numerous studies and some of the mechanisms of its development are now known. For example, 5-FU increases apoptosis of mesangial cells and necrosis of tubular cells [21], while cisplatin, ifosfamide, and pemetrexed cause proximal tubulopathy [22]. Cisplatin has also been shown to induce oxidative stress in tubular cells and to increase the expression of several pro-inflammatory factors, including TNF, which plays an important role in the cisplatin-induced apoptosis of tubular cells [23]. However, the mechanisms of the nephrotoxicity induced by many drugs remain unclear. Therefore, molecular studies aimed at identifying potential mechanisms of nephrotoxicity of new compounds with anticancer activity are essential when assessing their potential use as treatments.

In the current study, for the first time, we evaluated the influence of the betulin derivatives EB5 and ECH147 on the expression of genes encoding TNF and its receptors in human RPTECs at the mRNA and protein levels (soluble forms in culture media). The involvement of TNF in renal damage has been proven in many studies [24]. For example, this cytokine causes apoptosis of renal cells (including tubular cells) while stimulating the expression of proinflammatory factors and the production of reactive oxygen species (ROS) [24]. Our previous research assessing the oxidative status of RPTECs showed that EB5 and ECH147 may be less harmful than betulin itself, which has similar effects on RPTEC antioxidant systems to those of cisplatin [17]. However, treatment with betulin and its derivatives caused significantly higher concentrations

of malondialdehyde (MDA) compared to the untreated control cells or those treated with 5-FU or cisplatin [17]. MDA forms adducts with proteins, resulting in modified intracellular signaling. MDA-acetaldehyde--protein activates protein kinase C, leading to the activation of NFκB [25]. It also activates intercellular adhesion molecule 1 and vascular adhesion molecule factors, which consequently leads to increased TNF expression [26]. In a previous study conducted on an animal model, Liu et al. [27] showed that cisplatin treatment induced the generation of ROS and MDA in the kidneys, while also activating the NFκB pathway and consequent expression of inflammatory cytokines, such as TNF, IL-6, and IL1β. This report prompted us to assess whether betulin and its derivatives are able to influence the expression of genes encoding TNF and its receptors, as these genes play crucial roles in the activation of pro-inflammatory pathways.

Our previous research showed that EB5 and ECH147 stimulated the expression of genes encoding antioxidant enzymes, suggesting an influence of these derivatives on molecular processes within the cell [17]. Interestingly, these compounds significantly increased the expression of each of these genes compared to control cells. In the current study, the expression of TNF and its receptors was higher or lower, depending on the compound and its concentration. It should also be stated that the expression of these genes was significantly lower than for those encoding antioxidant enzymes. In another study, we showed that betulin and its derivatives downregulated *TGFB1*, *BMP2*, and *GDF15* in RPTECs at the mRNA level [28]. Also, mRNA levels were significantly lower than those observed for genes encoding antioxidant enzymes. These results confirm that betulin and its derivatives may adversely influence gene transcription or may influence mRNA stability. However, other mechanisms responsible for changes in gene expression should also be taken into account. In addition, it is possible that for TNF and its receptors, as well as TGF-beta family members, fewer protein molecules are needed to obtain a cell response than in the case of antioxidant enzymes. In the current study, betulin and ECH147 caused a concentration-dependent stimulation of the expression of *TNF* and *TNFR1* at the transcriptional level (as indicated by the statistically significant differences compared to the untreated control cells), with 0.5 μg/mL of betulin having the strongest effect. Interestingly, the lower concentration of cisplatin tested here strongly downregulated the expression of these two genes. Both betulin and cisplatin showed a dose-dependent influence on *TNF* and *TNFR1* gene expression; however, the expression profile of *TNFR2* was totally unlike that of *TNF* and *TNFR1*. Betulin and its derivatives strongly downregulated the level of *TNFR2* mRNA at both concentrations. This downregulation effect was statistically significant when compared with the expression in the control cells, but also when compared to cells treated with 5-FU (either 0.1 or 0.5 μg/mL) and 0.5 μg/mL of cisplatin. We observed a similar effect in our previous study, where there was lower gene expression, especially of *BMP2* and *GDF15*, in cells treated with betulin and its derivatives.

Little is known about the expression of *TNFR2* in tubular cells exposed to cisplatin or 5-FU. In colorectal cancer cells, the TNFR2/NF-κB pathway plays an important role in the development of resistance to 5-FU [29]. Zhang et al. [30] showed that 5-FU induces *TNFR2* expression in RKO cells, which are sensitive to this drug. In our research, 5-FU treatment of RPTECs increased *TNFR2* gene transcription, as indicated by its higher mRNA level, suggesting that this gene has a protective role in the response of renal cells to this drug. However, Ramesh and Reeves [31], in studies conducted in a mouse model (C57BL/6), revealed that a lack of the gene *TNFR2* caused only minor renal dysfunction after treatment with cisplatin when compared to *TNFR1*-deficient and wild-type mice. They observed reduced apoptosis and necrosis of renal epithelial cells, as well as a diminished inflammatory response in the kidneys. These previous findings may suggest that both cisplatin and 5-FU can induce TNFR2-mediated apoptosis or necrosis of RPTECs. In our research, betulin and its derivatives strongly inhibited the expression of *TNFR2*, indicating that their impact on renal cells is not mediated by this receptor. However, the molecular mechanism of this effect needs further investigation, including analysis of the signaling pathways involved in gene regulation and *TNFR2* mRNA stability.

We also measured the amounts of the proteins TNF, sTNFR1, and sTNFR2 released into the culture media. Interestingly, TNF and sTNFR2 were not detectable in the culture media from cells treated with any of the test compounds or the controls. This is consistent with our previous research, in which we showed that neither unstimulated nor AmB-treated RPTECs released soluble forms of these proteins [19]. TNF receptors are responsible for mediating several functions of TNF, including the activation of cell death; however, they can also activate the MAP kinase and NFκB pathways, which promote cell survival, proliferation, and inflammation [18]. Overall, the result of TNF-induced signaling depends on the balance between TNFR1 and TNFR2 surface molecules and the recruitment of the proteins involved in forming intracellular signaling complexes [18]. TNFR2 is mostly involved in the promotion of cell survival and proliferation [18]; however, it can also indirectly induce cell death mediated by TNFR1. The sequestration of TRAF2 (TNF receptor associated factor) by TNFR2 may influence the formation of signaling complexes of activated TNFR1 [18]. TRAF2 is crucial for the

recruitment of the cellular inhibitors of the apoptosis proteins cIAP 1 and cIAP2, which are key factors in the activation of cell survival signaling via the NFκB, JNK, and p38 pathways [32].

The functions of TNF receptors also depend on the TNF form. Membrane-bound TNF (mTNF) may interact with soluble forms of TNF receptors to mediate reverse signaling, including activation of the pro-survival NFκB pathway [33]. Moreover, mTNF may interact with both soluble and transmembrane forms of TNFR2, leading to opposite effects [34]. Our studies cannot rule out the possibility that RPTECs retained mTNF and TNFR2 on their surfaces and that the cleavage of TNFR1 receptor molecules was crucial for their survival when exposed to the compounds tested in the present study. Nevertheless, a high level of sTNFR1 in the serum is a predictive factor in renal diseases [35,36,37]. In our study, a higher sTNFR1 concentration was observed in the culture media from RPTECs treated with ECH147 or cisplatin. By contrast, the effect of treatment with betulin, EB5, or 5-FU depended on the concentration. EB5 at 0.1 μg/mL downregulated the shedding of sTNFR1 when compared to the effects of ECH147, betulin, 5-FU, and cisplatin. This may indicate that EB5 may be less harmful to renal cells than ECH147 or betulin. However, we evaluated only the soluble forms of proteins released into the culture media; thus, a key limitation of our study is that it did not assess the transmembrane forms of TNF, TNFR1, and TNFR2. Moreover, without analyzing the activation of particular signaling pathways, we cannot conclude that EB5 is not harmful to renal cells. Negative effects may take longer to become apparent. This aspect needs further research that considers different doses and incubation times, because changes in intracellular signaling may depend on them. A further limitation is that we conducted our studies only on renal tubular epithelial cells, whereas the nephrotoxic effect of drugs may result from damage to other types of renal cells (e.g., mesangial cells) [21]. Additionally, conducting research on in vitro models precludes the study of critical interactions and changes occurring in intact kidney tissues, including the infiltration of immune cells, which can completely change these interactions. Therefore, our research is preliminary and these results should be treated with caution. However, our study shows for the first time the influence of betulin and EB5 and ECH147, its derivatives, on the expression of TNF and its receptors in RPTECs. Due to the essential and pleiotropic role of this cytokine in the regulation of many biological processes, the results may help to direct further studies.

CONCLUSIONS

This preliminary research suggests that the betulin derivative EB5, when supplied at a low concentration, may be less harmful to human RPTECs than betulin itself, indicating the possibility of its future use in therapy. The possible mechanism that provides this lower toxicity may result, at least in part, from altered expression of TNF and its receptors. However, the safety of EB5 use requires further research.

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Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contribution

Study design – J.M. Gola, E. Bębenek, E. Chrobak
Data collection – C. Kruszniewska-Rajs, J. Adamska, J. Szota-Czyż
Data interpretation – J.M. Gola, C. Kruszniewska-Rajs, B. Strzałka-Mrozik
Statistical analysis – J.M. Gola
Manuscript preparation – J.M. Gola, E. Bębenek, E. Chrobak
Literature research – J.M. Gola, B. Strzałka-Mrozik

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