New phosphate derivatives of betulin as anticancer agents: Synthesis, crystal structure, and molecular docking study

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ABSTRACT

Betulin derivatives exhibit an antiproliferative activity and have been tested for many cancer cell lines. This paper describes a new series of 3-phosphate derivatives of betulin bearing different substituents at C28 position. The synthesized compounds were tested in vitro for their antiproliferative effect against human leukemia (MV-4-11 and CCRF/CEM), lung carcinoma (A549), prostate cancer (DU 145), melanoma (Hs 294T) cell lines, and murine leukemia P388. To explore the possible mechanism of anticancer activity for the most in vitro active compounds (4, 5, 7 and 8) and betulin, molecular docking was performed to the binding sites of potential anticancer targets, described for the various triterpene derivatives, including topoisomerase I and II, epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGFR), transcription factor NF-κB, anti-apoptotic protein Bcl-2 and peroxisome proliferator-activated receptor (PPAR). According to the results of the docking, the best fit to the binding pocket of PPAR γ was shown by compound 4.

1. Introduction

Cancer is a disease which today belongs to the leading causes of death of people worldwide. The disease is characterized by loss of cellular growth control. Cancer treatment involves the use of surgery, chemotherapy and/or radiotherapy, and still is a significant challenge for modern medicine and pharmacy [1]. For many years, natural products were the main source of anticancer drugs (e.g., adriamycin or taxanes), but in the 1990s, their importance has decreased in favor of targeted therapies based on humanized monoclonal antibodies or small synthetic molecules [2]. These drugs affect all rapidly dividing cells in the body, especially the cancer cells, but also cells of normal tissues such as bone marrow or gastrointestinal tract. For this reason, the majority of the cytotoxic drugs exhibits characteristic side effects [1]. New therapies have been successful in treating certain cancers (leukemia, gastrointestinal, prostate or breast cancers), but in the case of many solid tumors, they are not suitable [2,3]. The problems arising from the use of synthetic drugs resulted in the renewed interest in natural substances, which have a high therapeutic efficacy with low cytotoxicity. More than 60% of the currently used anticancer drugs are of natural origin. They are derived from natural sources such as plants, microbes, marine organisms and animals [4,5]. Nature is not only a source of potential drugs but also the leading structures that can provide a basis and inspiration for creating new semisynthetic biologically active substances [5].

Triterpenes isolated from birch bark have various confirmed biological activities, which may be enhanced by a chemical modification of their structures. For many years, betulin and betulinic acid (Fig. 1) are a subject of interest for many researchers in the field of chemistry and medicinal chemistry.
These compounds exhibit a broad spectrum of biological activities such as anticancer, antiviral, antibacterial, anti-inflammatory and antimalarial [6]. Their antiproliferative activity was examined in relation to many cancer cell lines, including lung, breast, colorectal, pancreatic, cervical cancers, leukemia and various types of melanoma. In the case of betulin, promising results were obtained with respect to human lung carcinoma cell line A549 [7], human cervical carcinoma HeLa against skin epidermoid carcinoma A431 and breast adenocarcinoma MCF7 cells [8]. Betulinic acid is selectively cytotoxic against melanoma cell lines, cancer activity was subsequently also reported against other types of human cancers including neuroblastoma, glioblastoma, medulloblastoma, Ewing tumor, leukemia, as well as several carcinomas, i.e. head and neck, colon, breast, hepatocellular, lung, prostate, renal cell, ovarian or cervix carcinoma [9]. Due to its promising properties, betulinic acid has been extensively studied to understand the mechanism of its anticancer activity [10].

Terpenoids are capable of inhibiting the proliferation of cancer cells and inducing their death by interacting with various molecular targets. They induce apoptosis both on the extrinsic (receptor) and intrinsic (mitochondrial) pathways. The receptor pathway can be independent of the mitochondrial one or can be combined at various levels. It was described that terpene derivatives inhibit the effect of topoisomerase I and II, reduce epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGFR) expression, inhibit the transcription factor NF-κB and can suppress anti-apoptotic protein Bcl-2 [11–15].

The poor solubility of betulin and betulinic acid can be a certain limitation in the use of these compounds as therapeutic substances. Introduction of new functional groups to the molecule, creating complexes with cyclodextrins, liposomes, carbon nanotubes or gold nanoparticles, are attempts to overcome these limitations [16–18]. In order to improve various properties like bioavailability of biologically active compounds, their permeation, solubility, or to change the metabolic profile, active substances can be applied in the form of prodrugs [19]. Phosphate prodrugs are used as drugs both for parenteral and oral administration. An example of a phosphate prodrug, fosamprenavir is designed to improve the pharmacokinetics of oral antiviral drug – amprenavir [19]. Improved water solubility, compared to the basic compounds, molecular docking to various proteins playing an important role in the course of cancer processes was performed. The proposed molecular targets include topoisomerases I and IIα, EGFR, VEGFR-2, catalytic kinase IKKβ, and II, reduce epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGFR) expression, inhibit the transcription factor NF-κB and can suppress anti-apoptotic protein Bcl-2 [11–15].

Taking into account the above information, it seemed interesting to obtain novel phosphate derivatives of betulin and betulinic acid and examine their antiproliferative activity. In the present paper, synthesis of phosphate derivatives of betulin and results of an in vitro cytotoxic activity study were described. The biological activity was tested in relation to human biphenotypical B myelomonocytic leukemia (MV-4-11), lung carcinoma (A549), prostate cancer (DU 145), melanoma (Hs 294T), human acute lymphoblastic leukemia (CCRF/CEM) and human normal breast epithelial cell lines (MCF-10A), as well murine leukemia (P388) and mouse fibroblasts (Balb3T3).

For betulin and for the most active in vitro compounds, molecular docking to various proteins playing an important role in the course of cancer processes was performed. The proposed molecular targets include topoisomerases I and IIα, EGFR, VEGFR-2, catalytic kinase IKKβ, and PPARγ as follows: Estramustine phosphate (anticancer activity) is in the third phase of clinical trials as a prodrug with an anticancer effect [21]. In recent years, such prodrugs as tedizolid phosphate (Fig. 2) (Sivextro - bacterial infections of the skin and subcutaneous tissue) and fostamatinib disodium (Tavalisse - therapy of rheumatic diseases) have been approved by USA Food and Drug Administration - FDA in 2014 and 2018, respectively [22,23]. On the other hand, the introduction of a phosphate group to improve the solubility of the drug may adversely affect the cellular uptake of the drug substance. This is due to the ionic nature of the functional group, which causes a difficulty in penetration of the drug through cell membranes. Hence, a research on derivatives containing esterified phosphate groups (noc pancanthamide) is carried out. The phosphate group, in addition to improving the bioavailability, may also have a positive impact on the pharmacological activity of compounds [24]. Moreover, introduction of a dialkylphosphate group into the drug molecule can broaden the spectrum of biological activity of the obtained derivatives compared to the starting material. Phosphate derivatives of non-steroidal anti-inflammatory drugs like sulindac, aspirin (Fig. 2), ibuprofen or flurbiprofen have been tested as anticancer agents [25]. So far, only a few betulin derivatives containing the phosphate moiety was described in the chemical literature. In order to overcome the problems associated with the administration and low bioavailability of triterpenoids, they may also be used as phosphate prodrugs, which has been shown in a patent for betulinic acid [26].

Various semisynthetic structural analogs of betulin were evaluated for their antiviral activities towards Epstein-Barr Virus (EBV), and among them, betulin 3,28-diphosphate exhibited significant activity [27]. Russian researchers reported the synthesis of betulin 3,28-diphosphate, which was tested for inhibition of the complement system, however, this compound did not work in this direction [28]. Recently, new scientific papers describing different possibilities of using betulin 3,28-diphosphate have been published. Melnikova et al. carried out a study on the physicochemical properties of this compound and its biological activity [29]. One can also find patents concerning the examination of antibacterial and antifungal activity of betulin 3,28-diphosphate [30,31]. So far, no betulin derivatives containing a dialkyl phosphate group have been described.
2. Results and discussion

2.1. Chemistry

Hitherto, several procedures for the synthesis of phosphate derivatives have been described. For this purpose, the most common reagents were used, such as phosphorus oxychloride, chlorides of phosphoric acid diesters or trialkyl phosphites with iodine. Herein, we present a convenient and general method using diethyl chlorophosphate [32].

In the first step of the synthesis, we used 28-acetylbetulin 1 as a substrate to obtain the phosphate 2 (69%), which was then subjected to hydrolysis under conventional conditions (NaOH, MeOH/THF). The reaction was carried out at room temperature for 6 h allowing for obtaining the product 3 with a good yield (83%). There was no occurrence of the hydrolysis reaction of the phosphate group leading to the formation of betulin (Scheme 1).

In the next stages of our research we have transformed the 3-diethoxyphosphorylbetulin 3 into acetylenic derivatives. A carbon–carbon triple bond is present in a large number of biologically active compounds, including natural products and some pharmaceuticals, such as, for instance, antifungal (Terbinafine) [33], contraceptive and menopausal hormone therapy (ethinyl estradiol, Norethisterone) [34], antiretroviral (Efavirenz) [35], antidepressant and Parkinson’s disease therapy (selegline) [36], or antiproliferative therapy (erlotinib) [37].

Syntheses of different betulin and betulinic acid derivatives bearing an alkyne moiety were examined for their anticancer [38], antiviral [39], hepatoprotective and anti-inflammatory activity [40].

Likewise, our previous studies have shown that in a reaction of propiolic acid with betulin and its derivatives, compounds having a promising antiproliferative activity are formed [41,42]. In this work, we carried out the synthesis of an acetylenic derivative of betulin phosphate 3 (Scheme 1). The reaction was performed according to the previously described method [41]. The 3-diethoxyphosphoryl-28-propynylbetulin 4 was obtained with a 50% yield.

The active substance should be, on one hand, lipophilic enough to overcome the membrane or the metabolic barrier, and on the other hand, it should be sufficiently hydrophilic to have a good solubility and bioavailability [43]. In order to compare the activity of neutral molecule and its charged form, deprotection of the phosphate ester 4 using bromotrimethylsilane was carried out subsequently, to give the monophosphate ester 5 with a good yield (90%).

Compounds which contain an alkyne moiety are used for a variety of further transformations leading to useful organic compounds through the construction of new bonds. Derivatives with a terminal acetylenic group participate in cycloaddition reactions (click chemistry strategy) with substituted azides to form new triazole heterocyclic systems. 1,2,3-Triazole ring is a pharmacophore group, its presence determines a broad spectrum of biological activity, such as anticancer, anti-tubercular, anti-inflammatory, antileishmanial, antitrypanosomal, antimicrobial, antiviral and antimalarial [44].

New triazole derivatives of betulin phosphate 6a-e were synthesized via the ring closure of 3-diethoxyphosphoryl-28-propynylbetulin 4 with various azides (Scheme 2). The derivatives 6a-e were obtained with yields in the range of 64–73%.

Taking into account much information about a high cytotoxic activity of oxidation products at C28 position of betulin, reactions of compound 3 with PCC and Jones reagent were carried out (Scheme 3). Expected 3-diethoxyphosphorylbetulinic aldehyde 7 and 3-diethoxyphosphorylbetulinic acid 8 were obtained with yields 77% and 36%, respectively.

Structure of newly synthesized derivatives were confirmed by $^1$H,


13C, 31P NMR, IR and HR MS analysis. Additionally, betulin phosphate 3, its acetylenic derivative 4 and triazole 6d, were analyzed by the X-ray diffraction.

2.2. Crystal structures

Molecular structures of 3-diethoxyphosphorylbetulin 3 and 3-diethoxyphosphoryl-28-propynoylbetulin 4 were confirmed by the X-ray diffraction method. The crystals suitable for analysis were grown by slow evaporation of an appropriate solvent, at room temperature. The molecular structures of the compounds 3 and 4 with atom numbering are shown in Figs. 3A and 4A, respectively.

The structure analysis confirms that, in the obtained product, the diethyl phosphate group is connected with the betulin molecule via an ester bond and adopts an equatorial orientation. The six-membered rings of the betulin backbone exhibit a chair conformation and are combined in a trans system. The five-membered ring assumes the form of the twisted envelope, where the C17 carbon atom is located outside the plane formed by the C18-C19-C21-C22 atoms. Compound 3 crystallizes in an orthorhombic P212121 space group. In the crystal of compound 3, molecules form along the b axis chains through hydrogen bonds between the hydroxy group at the C28 position and the oxygen atom of the phosphate group of the second molecule O2⋯H⋯O3, and thus in the “head to tail” system (Fig. 3B).

The phosphate derivative 4 crystallizes in tetragonal P42 space group. There are two molecules per asymmetric unit, one of them showing rotational disorder of the ethyl groups in phosphate moiety (Fig. 4A).

The molecular structure of 4 is stabilized by weak intermolecular C−H⋯O bonds (Fig. 4B). The molecules are connected with each other by means of weak (mostly electrostatic) bonds formed between the terminal alkyne group and the oxygen atom of the phosphate group in the next molecule (≡C133⋯H⋯O24≡P and ≡C323⋯H⋯O14≡P) (Table S3). The relatively high acidity of the Cαp−H results in ethynyl group forming some of the shortest C−H⋯O intermolecular contacts, especially when it is coupled with strong acceptors like O⋯P [45]. The molecules in the crystal of the compound 4 form springs along the c axis (Fig. 4C). The springs are connected to each other by weak hydrogen bonds (≡C241⋯H241⋯O26 and ≡C241⋯H241⋯O26).

2.3. Antiproliferative activity

All newly synthesized compounds were evaluated for their in vitro antiproliferative activity towards normal human mammary gland (MCF-10A) and cancer human cell lines of diverse etiology. Betulin and cisplatin were used as the reference compounds. In the first step of the experiments, the screening of the tested substances on cells of human biphentypic B myelomonocytic leukemia MV-4-11 was carried out. Betulin concentration causing 50% inhibition of MV-4-11 cells proliferation (IC50) was approx. 8.19 µM. Therefore, only those substances whose IC50 level was similar to or greater than betulin were selected for the second stage. The IC50 values of the examined compounds towards MV-4-11 cells are shown in Table 1.

Analyzing the obtained results, one may notice that in the case of the compound 3, the introduction of the diethyl phosphate group into the betulin molecule results in a reduction of activity against leukemia MV-4-11 cells. The values of IC50 for betulin and 3 are 8.19 and 25.60 µM, respectively. Among the tested triazole derivatives 6a-e, the lowest activity is shown by 6a and 6c which contain hydrophobic substituents at N3 position. Derivatives with polar substituents in this moiety 6c and 6d, are more strongly active. The higher activity of the compound 6b compared to a similar structure 6a results from the presence of a sulfur atom (Table 1).

In the case of derivatives, for which the concentration causing 50% inhibition of proliferation was higher than 10 µM, the studies have been terminated at the first stage of testing against the MV-4-11 cells. From the group of phosphate derivatives of betulin, eight were selected for the second stage of research. The compounds 2, 4, 5, 7, 8, 6b, 6c and 6d were evaluated for their in vitro antiproliferative activity against three cancer human cell lines A549 (non-small cell lung carcinoma), DU 145 (prostate cancer), Hs 294T (melanoma) and normal human breast epithelial cell lines – MCF-10A. The IC50 values for the tested compounds are shown in Table 2. The compounds most active against A549 cells form the following order: 8 > 4 > 7 > betulin > 6c > 2 > 6b > 5. The obtained result is consistent with the studies described in the literature, which confirmed a higher activity of betulinic acid in relation to the A549 line compared to betulin [46]. Other researchers have also reported a high activity of various tri-terpene derivatives containing a carboxylic group at the C28 position [11]. The phosphate derivative of betulinic acid 8 also showed a good activity in relation to other cell lines with values of IC50 6.10 and 7.42 µM for DU 145 and Hs 294T, respectively. The SI (Selectivity Index) was calculated for the compound 8 using the formula: SI = IC50 for normal cell line (MCF-10A)/IC50 for respective cancerous cell line. The obtained values equal to 9.34 (A549), 7.00 (DU 145) and 5.76 (Hs 294T) indicate the efficacy of 8 against cancer cells higher than their toxicity against normal cells (SI > 1.0).

The highest activity in the studied group of derivatives in relation to DU 145 and Hs-294T cell lines was demonstrated by the compound 4. Its activity was significantly higher than that of betulin and higher or comparable to the values for cisplatin (Table 2). This result is consistent with previous reports of a high activity of compounds containing an acetylene group in their structure [41]. A conversion of the acetylene group to a triazole ring with various substituents at the N3 position (compounds 6b-d) resulted in a decrease in the activity. A similar situation was described by the Csuk et al., for betulin derived compounds bearing an ethynyl side chain at the C28 position [38].

The lowest activity against the tested cell lines is exhibited by the phosphate 5. The compound 5 showed a poor inhibition of the A549, DU 145 and Hs 294T cells, although its activity was satisfactory in the initial study against leukemia (MV4-11). Additionally, compounds 4 and 5 were evaluated for activity against other human (CCRF/CEM) and murine leukemia (P388) lines (Table 3). With respect to these cell lines, the compound 5 exhibited a better activity (values of IC50 6.87 and 5.46 µM, respectively), however, the derivative 4 showed an inhibition of cell proliferation at lower concentrations (IC50 0.78 and 0.73 µM, respectively).

Comparing the obtained results with the previously described values obtained for 28-O-propynoylbetulin in relation to the same cancer cell lines, it can be noticed that the presence of the C3 diethylphosphate
Fig. 3. (A) Molecular structure of the compound 3 showing the atom numbering scheme. Displacement ellipsoids are drawn at the 50% probability level, (B) packing diagram of 3. Dashed lines indicate hydrogen bonds.
Fig. 4. (A) Molecular structure of the compound 4 showing the atom numbering scheme. Displacement ellipsoids are drawn at the 50% probability level, (B) packing diagram of crystal 4 projected parallel to the ab plane, (C) Spring arranged along the c axis.
group (compound 4) causes a significant increase in the cytotoxicity relative to the tested CCRF/CEM and P388 lines. The selectivity of phosphates 4 and 5 is comparable or higher than that for 28-O-propynoylbetulin.

2.4. Molecular docking

Molecular modeling carried out in our work was an attempt to predict the possible mode of action of the synthesized compounds and to determine future directions of research in the field of optimizing the structure of the compounds. Four of the most in vitro active compounds (4, 5, 7 and 8) and betulin were docked to binding sites of various reported triterpene derivatives targets. Studies carried out so far, have confirmed the ability of terpenes to inhibit proliferation and induce apoptosis, but the mechanism responsible for causing the above effects is not always known. Numerous scientific papers on the study of the mechanism of action of terpenes describe their ability to inhibit topoiso...
for betulin, and this is due to the larger number of hydrogen bonds formed by phosphoryl (with Arg487 and deoxyadenine) and carboxyl groups (with Asp541, Gly617 and Leu616). The compound 8, like betulin, binds to Tyr805 via alkyl-π interactions which are not formed by other ligands. All binding interactions of the phosphate derivatives (4, 5, 7 and 8) and betulin with residues of Top II α and nucleotides of double cleaved DNA are presented in Table S6.

Induced by a signal from the outside, so-called “extrinsic”, a pathway leading to cell apoptosis, is associated with membrane death receptors. Receptor tyrosine kinases (RTKs), transmembrane surface receptors regulate numerous cell functions including apoptosis, metabolism and proliferation. These receptors are an important element of the signaling pathway associated with various cancers, e.g., prostate, breast, lung and ovarian cancer [13]. As potential receptor molecular targets for the docking of betulin and phosphate ligands (4, 5, 7 and 8) and betulin with residues of Top II α and nucleotides of double cleaved DNA are presented in Table S6.

Betulin formed, with the ATP-active site of EGFR, a network of hydrogen bonds formed by phosphoryl (with Arg487 and deoxyadenine) and carboxyl groups (with Asp541, Gly617 and Leu616). The compound 8, like betulin, binds to Tyr805 via alkyl-π interactions which are not formed by other ligands. All binding interactions of the phosphate derivatives (4, 5, 7 and 8) and betulin with residues of Top II α and nucleotides of double cleaved DNA are presented in Table S6.

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Peroxisome proliferator-activated receptors (PPARα, PPARβ/δ and PPARγ) belong to the family of nuclear receptors that act as transcription factors. Their main role is to control the metabolism of fatty acids and to maintain glucose homeostasis. In addition, they play an important role in cell proliferation and differentiation and are a key regulator of cellular differentiation [53]. The results of in vitro tests on various human cancer cell lines, including breast cancer, lung carcinoma, hematopoietic cancer, glioma, colon, liver cancer, pancreatic cancer, thyroid cancer and prostate cancer indicate that PPAR-γ is often overexpressed in these cancer cells. Compounds which are PPAR-γ ligands have an antiproliferative, pro-apoptotic and differentiation-promoting activities and can be useful in treatment of cancer [54].

The PPARγ receptor is composed of five regions named A-E, arranged from the N- to C-terminus of the protein. Region E contains two functional domains, LBD (ligand binding domain) and AF-2 (ligand-dependent activation function). The ligand binding domain is formed of 13 α-helices and one β-sheet. The LBD secondary structure studies have shown that the ligand-binding pocket is T-shaped or Y-shaped with three branches named I, II and III, and is larger than the binding pockets in other nuclear receptors. In addition, it consists mainly of hydrophobic amino acids which makes it able to bind large hydrophobic molecules [53,54].

According to the results from the molecular docking studies for PPARγ, all phosphate derivatives (4, 5, 7 and 8) showed significantly better scores when compared with betulin (Table 4). The docked poses of betulin and the phosphate derivatives within the active site of PPARγ were in the inside branch III, as its experimentally obtained co-crystallized ligand, betulinic acid. Betulinic acid is a native ligand of crystal structure of this receptor registered in the PDB with ID 5LSG. In the structure described, the carboxyl group present at C17 of the betulin system forms two strong hydrogen bonds with Ser289 (carbonyl oxygen atom) and Tyr327 (oxygen atom of the hydroxyl group) [55]. The PPARγ ligand binding domain with the compounds 4, 5 and betulin molecule docked inside branch III are shown in Fig. 7.

The major difference in the binding mode of compound 4 compared to betulinic acid is to maintain only one hydrogen bond with Ser289 and carbonyl atom of the ester group present at C17 of the betulin. However, the stability of the complex is increased as a result of additional strong hydrogen bond formed between the oxygen atom of the phosphoryl group and Arg280. The major structural differences between betulin and the compounds 4 and 5 are their hydrophobicity, and the size of the substituent at the C3 and C28 positions. The best docking result was obtained for the compound 4. The compound 5, more polar due to the presence of free phosphoric acid groups, assumed a different position and did not get deep into the binding pocket (Fig. 8C). The hydroxy group formed a hydrogen bond with carbonyl oxygen of Glu291. This compound also showed hydrophobic interactions with Cys285, Arg288, Ile341 (steric) and with Phe287 (alkyl-π) (Fig. 8D).

Betulin has adapted to the ligand pocket of PPARγ through hydrophobic interactions as well as a hydrogen bond with Arg280 (Fig. 8E).
and F). The binding interactions of the phosphate derivatives and betulin with PPAR-γ residues are presented in Table S10.

3. Conclusions

In the present study, a new group of betulin 3-phosphate derivatives was synthesized and tested against five human cancer cell lines. The compounds 4 and 8 showed higher activity against four cell lines (MV-4-11, A549, DU 145 and Hs 294T) compared to betulin. In relation to the Hs 294T line, the compound 4 revealed higher activity than cisplatin used as a reference compound (the IC50 value was equal to 3.01µM for 4 and 6.33µM for cisplatin). Among the studied compounds, the derivative 5 showed the most selectivity, i.e. weakly inhibited A549, DU 145 and Hs 294T cells, simultaneously, revealed the activity towards various types of leukemias, human (MV-4-11, CCRF/CEM) and murine (P388). In the studies, carried out against CCRF/CEM cells, the compound 5 was markedly more active than betulin (IC50 values were 6.78µM and 62.73µM for 5 and betulin, respectively). Against the same leukemia cell line, the compound 4 has a higher activity referred to that previously described for 28-O-propynoylbetulin which does not have a diethoxyphosphoryl moiety. Considering the literature reports on various potential mechanisms of anticancer activity of triterpenes as well as cancer cell lines used for in vitro research, molecular modeling to the five potential molecular targets has been carried out. The best fit, expressed in arbitrary units of GOLD for all selected molecular targets, showed the compound 4. The values of the fitness score function were in the range of 57.7–71.5 and did not differ remarkable from the other phosphate ligands and betulin. However, in molecular docking to peroxisome proliferator-activated receptor PPARγ, a significant difference between betulin and the phosphate ligands was observed (fitness score: 71.5 and 69.0 for the compound 4 and 5, respectively; 46.3 for betulin). This effect suggests a potential...
molecular target which determines antiproliferative activity of the studied compounds.

4. Experimental

4.1. Biological activity

4.1.1. Cell culture and medium—is included in the supplementary materials

4.1.1.1. Antiproliferative assay. The in vitro cytotoxic effect of the examined substances was determined by means of MTT assay for leukemia cells or SRB assay for adherent cells as it was described by Wietrzyk et al. [56]. Stock solutions of the tested compounds at a concentration of 10 mg/mL were prepared for each experiment ex tempore by dissolving 1 mg of substance in 100 µL of DMSO. The solvent for further dilution test was the medium. The compounds were tested in concentration ranges from 0.1 to 100 mg/mL.

The results of experiments were presented in the form of inhibitory concentration 50 (IC50) - the dose of the tested compounds which inhibits proliferation of 50% of the cell population. Calculation of the IC50 values were performed separately for each sample, the average values and standard deviation were included in the tables. The compounds in each concentration was tested in triplicate in a single experiment, which was made at least three times.

4.2. Synthesis

4.2.1. Materials and methods—is included in the supplementary materials

28-Acetylbetulin 2 was obtained according to described method. Melting point, 1H and 13C NMR spectra data for compound 2 were consistent with the literature information [57].

4.2.2. Synthesis of 28-acetoxy-3-diethoxyphosphorylbetulin 3

Diethyl chlorophosphate (0.28 mL, 1.9 mmol) was added dropwise to a stirred solution of 1 (0.48 g, 1 mmol) and 4-dimethylaminopyridine (DMAP) in pyridine (6 mL), cooled to 0 °C in an ice-water bath. Next, mixture was allowed to warm to room temperature and stirred overnight in an argon atmosphere. After completion the reaction, pyridine was removed in vacuum and the residue was dissolved in 30 mL of dichloromethane and washed with 10% HCl, saturated NaHCO3 and water. The organic fraction was dried (anhydrous Na2SO4), filtered, and the solvent removed under reduced pressure. The crude product was purified by column chromatography on silica gel (dichloromethane/ethanol, 40:1 v/v) to give the compound 2.

Yield: 69%, m. p. 67–70 °C.

TLC (dichloromethane/ethanol, 40:1, v/v): Rf = 0.27.

1H NMR (600 MHz, CDCl3) δ (ppm): 4.71 (d, J = 1.8 Hz, 1H, H-29), 4.61 (d, J = 1.8 Hz, 1H, H-29), 4.26 (d, J = 10.8 Hz, 1H, H-28), 4.13 (m, 4H, 2x OCH2CH3), 3.99 (m, 1H, H-3), 3.88 (d, J = 10.8 Hz, 1H, H-28), 3.81 (m, 1H, H-19), 2.09 (s, 3H, CH3), 1.70 (s, 3H, H-30), 1.43 (m, 6H, 2x OCH2CH3), 1.05 (s, 3H, CH3), 1.02 (s, 3H, CH3), 0.98 (s, 3H, CH3), 0.86 (s, 3H, CH3), 0.83 (s, 3H, CH3), 0.75 (m, 1H, H-5). 

13C NMR (150 MHz, CDCl3) δ (ppm): 171.7; 150.2; 109.9; 86.3; 63.5; 62.8; 55.3; 48.7; 47.7; 46.3; 42.7; 40.9; 38.8; 38.7; 38.4; 37.6; 36.9; 34.5; 34.1; 29.7; 28.6; 28.0; 25.4; 25.1; 21.1; 20.8; 19.1; 18.3; 16.2; 16.2; 16.1; 15.0; 14.9; 13.7. 

31P NMR (243 MHz, CDCl3) δ (ppm): −1.31. 

IR (KBr, cm−1) ν: 1740 (C=O), 1234 (P=O), 1034 (P=OeC). 

HR-MS (APCI) m/z: C36H60O6P [(M−H)−], Calc. 619.4127; Found 619.4123.

4.2.3. Synthesis of 3-diethoxyphosphorylbetulin 3

Sodium hydroxide solution (0.25 M) in the water/tetrahydrofuran/methanol system (50 mL in the ratio of 1:2 v/v) and 0.62 g (1 mmol) of compound 2 are placed in a round-bottomed flask, the whole mixture was stirred at room temperature for 1 h. Then, dichloromethane (15 mL) was added to the mixture and washed with 10% HCl and water. The organic layer was dried with anhydrous sodium sulfate (VI), then concentrated till dry. The product was purified by column chromatography (SiO2, dichloromethane/ethanol, 40:1 v/v) yielding compound 3.

Yield: 83%, m. p. 210–211 °C.

TLC (dichloromethane/ethanol, 40:1, v/v): Rf = 0.24.

1H NMR (600 MHz, CDCl3) δ (ppm): 4.70 (d, J = 1.8 Hz, 1H, H-29), 4.60 (d, J = 1.8 Hz, 1H, H-29), 4.10 (m, 4H, 2x OCH2CH3), 3.98 (m, 1H, H-3), 3.81 (d, J = 10.8 Hz, 1H, H-28), 3.35 (d, J = 10.8 Hz, 1H, H-28), 2.39 (m, 1H, H-19), 0.90–2.00 (m, 24H, CH2, CH3), 1.70 (s, 3H, CH3), 1.35 (m, 6H, 2x OCH2CH3), 1.07 (s, 3H, CH3), 1.05 (s, 3H, CH3), 0.86 (s, 3H, CH3), 0.83 (s, 3H, CH3), 0.75 (m, 1H, H-5). 

Fig. 7. The PPARγ ligand binding domain (PDB entry 5LSG) with the compound 4 molecule docked inside branch III. In the insert 1 – betulin. In the insert 2 – the compound 5.
1.01 (s, 3H, CH₃), 0.95 (s, 3H, CH₃), 0.83 (s, 3H, CH₃), 0.75 (m, 1H, H-5). ¹³C NMR (150 MHz, CDCl₃) δ (ppm): 150.5; 109.7; 86.3; 63.5; 63.4; 60.5; 55.3; 50.3; 48.7; 47.8; 42.7; 40.9; 38.8; 38.4; 37.3; 36.9; 34.2; 34.0; 29.7; 29.2; 28.0; 27.0; 25.3; 25.1; 20.9; 19.1; 18.3; 16.2; 16.1; 16.0; 14.7. ³¹P NMR (243 MHz, CDCl₃) δ (ppm): −1.35. IR (KBr, cm⁻¹) ν: 3455 (O–H), 1262 (P=O), 1036 (P=O–C). HR-MS (APCI) m/z: C₃₄H₅₈O₅P [(M−H)−], Calc. 577.4022; Found 577.4011.

4.2.4. Synthesis of 3-diethoxyphosphoryl-28-propynoylbetulin 4

Compound 3 in the amount of 0.29 g (0.5 mmol) was dissolved in 2.5 mL of methylene chloride. The obtained solution was cooled in an ice-water bath to −10 °C, then 0.037 mL (0.59 mmol) of a propiolic acid was added, and next a solution of 0.123 g (0.59 mmol) of DCC (N,N′-dicyclohexylcarbodiimide) and 0.005 g (0.04 mmol) of DMAP (4-dimethylaminopyridine) in 0.5 mL of methylene chloride was added dropwise. The reaction was carried out under argon atmosphere for 5 h in a cooling bath, and then at room temperature. After 24 h, the reaction mixture was filtered, the filtrate was concentrated till dry on a vacuum evaporator. The crude product was purified by column chromatography (SiO₂, dichloromethane/ethanol, 40:1, v/v), yielding ester 4.

Yield: 50%, m. p. 167–168 °C.
4.2.6.2. 3-Diethoxyphosphoryl-28-(1-phenylthiomethyl-1H-[1,2,3]-triazol-4-yl)-carbonylbetulin 6b.

Yield: 75%, m. p. 110–111°C.

TLC (chloroform/ethanol, 15:1, v/v): Rf = 0.60.

1H NMR (600 MHz, CDCl3) δ (ppm): 7.95 (s, 1H, CH-triazol), 7.27 (m, 5H, H5a), 5.59 (s, 2H, CH2), 4.64 (s, 1H, H-29), 4.54 (d, J = 10.8, 1H, H-28), 4.04 (m, 5H, 2x OCH2CH3, H-28), 3.91 (m, 1H, H-3), 2.43 (m, 1H, H-19), 0.83–2.00 (2m, 21H, CH2, CH3), 1.63 (s, 3H, H-30), 1.27 (m, 6H, 2x OCH2CH3, 0.98 (s, 3H, CH3), 0.92 (s, 3H, CH3), 0.91 (s, 3H, CH3), 0.78 (s, 3H, CH3), 0.74 (s, 3H, CH3), 0.66 (m, 1H, H-5). 13C NMR (150 MHz, CDCl3) δ (ppm): 163.1; 150.0; 138.1; 136.7; 127.4; 111.3; 89.8; 86.8; 85.2; 84.5; 63.5; 62.1; 61.7; 60.0; 59.7; 50.3; 48.9; 47.6; 42.7; 40.9; 38.4; 37.7; 37.4; 36.9; 28.0; 16.8; 16.1; 14.8; 12.6; 12.5. 31P NMR (243 MHz, CDCl3) δ (ppm): −1.33. IR (KBr, cm−1): ν = 2945 (CH), 1734 (C = O), 1541 (C = N), 1456 (N = N), 1263 (N = N), 1220 (P = O). HR-MS (APCI) m/z: C44H65N3O6PS [(M−H)−], Calc. 794.4332; Found 794.4314.

4.2.6.3. 3-Diethoxyphosphoryl-28-(1-(3′-deoxythymidine-5′-yl)-1H-[1,2,3]-triazol-4-yl)-carbonylbetulin 6c.

Yield: 83%; m. p. 163–165°C.

TLC (chloroform/ethanol, 15:1, v/v): Rf = 0.16.

1H NMR (600 MHz, CDCl3) δ (ppm): 8.18 (s, 2H, NH-AZT), 8.09 (s, 1H, CH-triazol), 7.36 (s, 1H, AZT), 6.00 (t, 1H, J = 6.6 Hz, AZT), 5.45 (m, 1H, AZT), 4.64 (s, 1H, H-29), 4.54 (m, 1H, H-29), 4.50 (d, J = 10.8, 1H, H-28), 4.34 (m, 1H, AZT), 4.07 (m, 6H, 2x OCH2CH3, H-28, 1H-AZT), 3.76 (m, 2H, AZT), 3.02 (m, 1H, AZT), 2.49 (m, 1H, AZT), 2.43 (m, 1H, H-19), 1.96 (m, 1H, AZT), 1.86 (s, 3H, CH2-AZT), 0.83–1.84 (m, 22H, CH2-CH3), 1.63 (s, 3H, CH3-H20), 1.26 (m, 6H, 2x OCH2CH3), 1.01 (s, 3H, CH3), 0.93 (s, 3H, CH3), 0.92 (s, 3H, CH3), 0.78 (s, 3H, CH3), 0.77 (s, 3H, CH3), 0.66 (m, 1H, H-5). 13C NMR (150 MHz, CDCl3) δ (ppm): 160.9; 150.0; 146.0; 134.2; 129.7; 126.8; 110.0; 86.3; 63.5; 63.4; 55.3; 54.5; 50.3; 48.8; 47.8; 46.7; 42.7; 40.9; 38.8; 38.4; 37.7; 37.6; 36.9; 36.8; 34.3; 30.9; 29.8; 28.0; 27.8; 26.0; 19.1; 18.3; 16.2; 16.1; 14.8; 14.8. 13C NMR (150 MHz, CDCl3) δ (ppm): −1.36. IR (KBr, cm−1): ν = 2945 (CH), 1734 (C = O), 1541 (C = N), 1456 (N = N), 1263 (N = N), 1220 (P = O), 1037 (P = O–C). HR-MS (APCI) m/z: C44H65N3O6PS [(M−H)−], Calc. 794.4332; Found 794.4314.
Compound 3 in the amount of 0.29 g (0.5 mmol) was dissolved in 6 mL of dichloromethane and 0.65 g (3 mmol) of pyridinium chloride/ethanol, 20:1 v/v, yielding compound 7.

Yield: 77%, m. p. 109–112°C.

TLC (dichloromethane/ethanol, 40:1, v/v): Rf = 0.33.

1H NMR (600 MHz, CDCl3) δ (ppm): 9.61 (d, J = 1.2 Hz, 1H, CHO), 4.69 (br, s, 1H, H-29), 4.56 (br, s, 1H, H-29), 4.03 (m, 4H, 2x OCH2CH3), 3.91 (m, 1H, H-3), 2.81 (m, 1H, H-19), 0.95–2.10 (m, 23H, CH2, CH3), 1.69 (s, 3H, H-30), 1.27 (m, 6H, 2x OCH2CH3), 0.94 (s, 3H, CH3), 0.89 (s, 3H, CH3), 0.84 (s, 3H, CH3), 0.78 (s, 3H, CH3), 0.74 (s, 3H, CH3), 0.67 (m, 1H, H-5). 13C NMR (150 MHz, CDCl3) δ (ppm): 172.8 (C), 126.5 (P), 110.2; 86.3; 63.5; 63.4; 62.8; 55.3; 51.0; 50.3; 48.9; 47.8; 42.7; 40.9; 38.8; 38.4; 37.9; 36.9; 34.7; 34.1; 29.8; 29.6; 28.0; 27.1; 25.4; 25.2; 20.8; 19.1; 18.3; 16.2; 16.1; 16.0; 14.8. 31P NMR (243 MHz, CDCl3) δ (ppm): −1.33. IR (KBr, cm−1): 2945 (CH), 1757 (C=O), 1558 (C=O), 1718 (C=N), 1632 (P=O), 1265 (P=O), 1045 (P=O−C). HR-MS (APCI) m/z: C41H56O6P [(M−H)−], Calc. 758.4509; Found 758.4500.

4.2.8. Synthesis of 3-diethoxyphosphorylbetulinic acid

As a solution of 0.29 g (0.5 mmol) of compound 3 in 16 mL of acetone was cooled for 15 min in an ice-water bath, then 1.4 mL of Jones reagent was added dropwise gradually so as to maintain the temperature not exceeding 0°C. Stirring was continued for 1.5 h at room temperature. Then, the flask was placed in a water bath with a temperature of 10°C, 6 mL of ethanol was added gradually and mixed for another 30 min. After this time, the mixture was poured into 15 mL of water with crushed ice, obtaining a pale-green precipitate. The precipitate was filtered, washed with water and dried in air. The crude product was purified by column chromatography (SiO2, dichloromethane/ethanol, 20:1 v/v), yielding compound 8.

Yield: 36%, m. p. 244–245°C.

TLC (dichloromethane/ethanol, 40:1, v/v): Rf = 0.25.

1H NMR (600 MHz, CDCl3) δ (ppm): 4.67 (br, s, 1H, H-29), 4.54 (br, s, 1H, H-29), 4.02 (m, 4H, 2x OCH2CH3), 3.91 (m, 1H, H-3), 0.95–2.10 (m, 24H, CH2, CH3), 1.74(s, 3H, H-30), 1.36 (m, 6H, 2x OCH2CH3), 0.92 (s, 3H, CH3), 0.89 (s, 3H, CH3), 0.86 (s, 3H, CH3), 0.77 (s, 3H, CH3), 0.73 (s, 3H, CH3), 0.75 (m, 1H, H-5). 13C NMR (150 MHz, CDCl3) δ (ppm): 180.9; 150.4; 140.7; 109.7; 86.4; 63.5; 63.4; 55.3; 50.4; 49.3; 46.9; 42.4; 40.7; 38.9; 38.4; 38.7; 36.9; 34.3; 32.2; 30.6; 29.7; 28.0; 25.5; 25.4; 20.9; 19.3; 18.3; 16.2; 16.2; 16.1; 16.0; 4.7. 31P NMR (243 MHz, CDCl3) δ (ppm): −1.15. IR (KBr, cm−1): ν: 3068 (OH), 1715 (C=O), 1243 (P=O), 1019 (P=O−C). HR-MS (APCI) m/z: C41H56O6P [(M−H)−], Calc. 758.4380; Found 759.3789.

4.3. X-ray diffraction experiment

A single crystal X-ray diffraction study was conducted for compound 3 and 4. Colourless crystals were obtained from slow evaporation of a solution in acetone/DMF 10:1 and hexane/acetone 3:1, for 3 and 4 respectively.

The crystallographic data, data collection and structure refinement details as well as selected hydrogen-bond parameters are given in Tables S1–S3.

4.4. Molecular docking

All target macromolecules for molecular docking studies were obtained from the Protein Data Bank (https://www.rcsb.org/). We used 3D crystal structure of topoisomerase I (ID: 1TLS), topoisomerase IIα (ID: 5GWK), EGFRK (ID: 1M17), VEGFR2 (ID: 1Y6A), IKKβ (ID: 3RZF), Bcl-2 (ID: 2W3L) and PPARγ (ID: 5LSG).

The three-dimensional (3D) structures of all studied compounds required for virtual screening were generated in their low-energy conformation using Gaussian 16 (revision A.03) computer code [58] at the density functional theory (DFT, B3LYP [59]) and 6-31+G(d,p) basis sets. In case of compounds 3 and 4, calculations were performed using the X-ray coordinates as the input structure. Genetic Optimisation for Ligand Docking (GOLD) 5.6.3 [60] was used for the docking. The Hermes visualiser in the GOLD Suite was used to further prepare receptors for docking. The region of interest used for Gold docking was defined as all the protein residues within the 10 Å of the reference ligands that accompanied the downloaded protein complexes. Default values of all other parameters were used and the complexes were submitted to 10 genetic algorithm runs using the GOLDScore fitness function. Molecular docking details were visualized using the BIOVIA Discovery Studio virtual environment [61].

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Appendix A. Supplementary material

Supplementary files contain the following information: Experimental data such as information on cell culture and medium for biological activity tests as well as materials and methods used in the synthesis. The methodology for X-ray analysis (Table S1), monocryystals characteristics (Table S2) and selected hydrogen-bond parameters (Table S3). The detailed crystallographic data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures. The crystal structures have been allocated the deposition numbers CCDC: 1853751 for 3 and 1853752 for 4. The 1H, 13C, 31P NMR spectra of selected compounds. Interactions of betulin and phosphate derivatives with amino acids residues in binding pocket of topoisomerase I and IIα, EGFRK, VEGFR2, IKKβ, Bcl-2 and PPAR-γ (Tables S4–S10). Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.03.060.

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