Accepted Manuscript

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PII: S0223-5234(19)30480-5

DOI: https://doi.org/10.1016/j.ejmech.2019.05.063

Reference: EJMECH 11373

To appear in: *European Journal of Medicinal Chemistry*

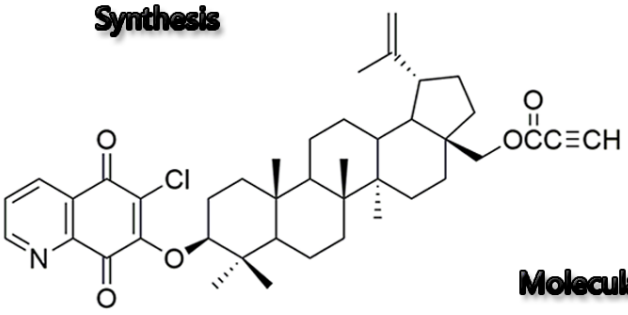
Received Date: 16 April 2019

Revised Date: 18 May 2019

Accepted Date: 23 May 2019

Please cite this article as: M. Kadela-Tomanek, E. Bębenek, E. Chrobak, K. Marciniec, Mał. Latocha, D. Kuśmierz, M. Jastrzębska, Stanisł. Boryczka, Betulin-1,4-quinone hybrids: Synthesis, anticancer activity and molecular docking study with NQO1 enzyme, *European Journal of Medicinal Chemistry* (2019), doi: https://doi.org/10.1016/j.ejmech.2019.05.063.

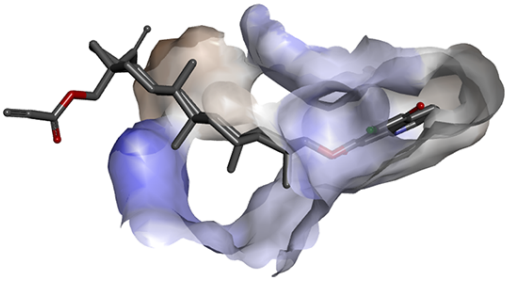
Synthesis



Anticancer activity

SNB-19, Colo-829,
C-32, MCF-7, T47D
MDA-MB-231, A549

Molecular docking study



Apoptosis assay

Transcriptional activity of:
H3, TP53, CDKN1A,
BAX, BCL-2

Betulin-1,4-quinone hybrids: synthesis, anticancer activity and molecular docking study with NQO1 enzyme

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Abstract

Betulin-1,4-quinone hybrids were obtain by connecting two active structures with a linker. This strategy allows for obtaining compounds showing a high biological activity and better bioavailability. In this research, synthesis, anticancer activity and molecular docking study of betulin-1,4-quinone hybrids are presented. Newly synthesized compounds were characterized by ^{1}H, ^{13}C NMR, IR and HR-MS. Hybrids were tested *in vitro* against a panel of human cell lines including glioblastoma, melanoma, breast and lung cancer. They showed a high cytotoxic activity depending on the type of 1,4-quinone moiety and the applied tumor cell lines. It was found that cytotoxic activities of the studied hybrids were increasing against the cell line with higher NQO1 protein level, like melanoma (C-32), breast (MCF-7) and lung (A-549) cancer. Selected hybrids were tested on the transcriptional activity of the gene encoding a proliferation marker (H3 histone), a cell cycle regulators (p53 and p21) and an apoptosis pathway (BCL-2 and BAX). The obtained results suggested that the tested compounds caused a mitochondrial apoptosis pathway in A549 and MCF-7 cell lines. The molecular docking was used to examine the probable interaction between the hybrids and human NAD[P]H-quinone oxidoreductase (NQO1) protein. The computational studies showed that the type of the 1,4-quinone moiety affected the location of the compound in the active site of the enzyme. Moreover, it was shown that an interaction of 1,4-quinone fragment with the hydrophobic matrix of the active site near Tyr128, Phe178, Trp105 and FAD cofactor could explain the observed increase of TP53 gene expression.

Keywords: 5,8-quinolinedione, betulin, cytotoxic, apoptosis assay, molecular docking, NQO1

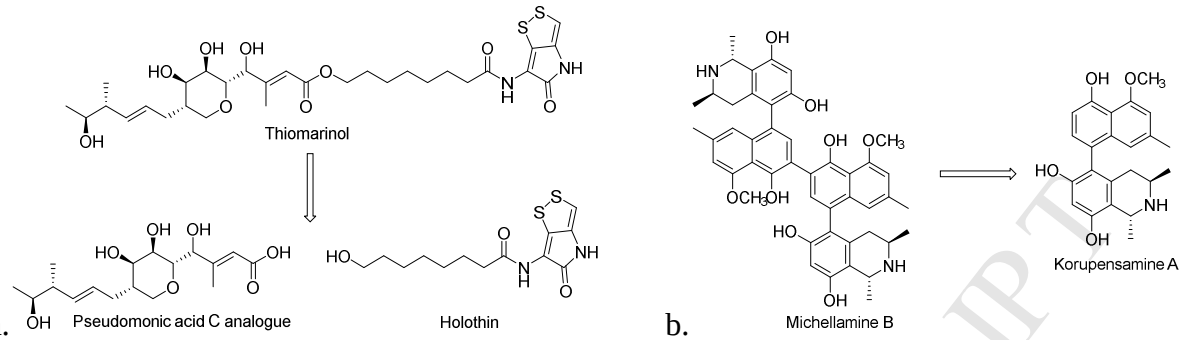
1. INTRODUCTION

Traditional medicine has been used dried materials of plant and animal origin as a main source of substances for the treatment of diseases for thousands of years. A precursor of acetylsalicylic acid, salicin, which was obtained from the bark of the willow tree *Salix alba*, had been known for more than 2400 years as a pain relieving agent [1-3]. A decoction of the birch bark was used as medicament on hypertension, high-level cholesterol, obesity, gout, kidney stones, nephritis, cystitis, digestive disorders and respiratory syndrome [4]. The main substance found in bark of birch *Betula pendula* is betulin isolated for the first time in 18th century [5]. The pharmacological activity of this pentacyclic triterpene included anticancer, antimicrobial, antiviral, and anti-inflammatory activity [6, 7]. Modification of betulin structure at C-3, C-17, C-19, C-28 and C-30 positions resulted in obtaining semisynthetic compounds with a high biological activity [8-12].

Fungi have been used as food or in alcohol production, up to 1897 year when Ernest Duchesne observed for first time that some molds could kill bacteria. More than 30 years later, Fleming observed the same effect, but he isolated the active compound called penicillin [13, 14]. One of the groups of antibiotics consists 7-amino-5,8-quinolinedione compounds, like streptomycin or lavendamycin, which were isolated from *Streptomyces*. These antibiotics exhibit a high biological activity, like anticancer, antimicrobial and antiviral activities. The mechanism of their action is based on the interaction of the 5,8-quinolinedione moiety with NAD[P]H-quinone oxidoreductase (NQO1) protein. The structure-activity relationship shows that 5,8-quinolinedione moiety is the most important structural fragment of the compounds [15-17]. Synthetic compounds containing amine or alkoxy substituent at C-6 or C-7 position of the 5,8-quinolinedione moiety exhibit a high effect against Gram-positive and Gram-negative bacteria and an antitumor activity against a broad spectrum of human and murine cancer cell lines [18-27].

Natural substances are often used as precursor structure to obtain chemical products which are characterized by a higher biological activity. It is estimated that more than 40% of the drugs used currently are products of natural origin or their derivatives [28]. Modification of natural substances allows to obtain hybrid compounds containing a partial structure of the natural product. Some hybrids were synthesized by classical organic methods, others were obtained by condensation of natural structure. However, hybrid drug was isolated for many types of organisms. For example, the marine bacterium *Alteromonas rava* is a source of antimicrobial antibiotic thiomarinol containing pseudomonic acid C analogue and holothin (Fig. 1a) [29, 30]. Michellamina B, which exhibits a strong anti-HIV activity, is a dimer of

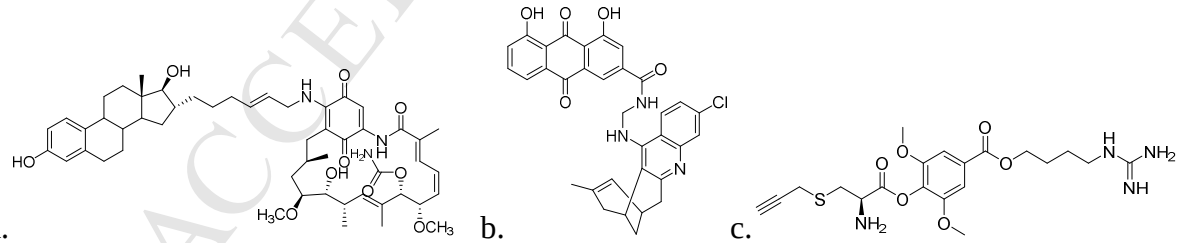
korupensamine A (Fig. 1b). Both compounds are isolated from plant material, *i.e.* *Triphyophyllum peltatum* and *Ancistrocladus korupensis*, respectively [30, 31].



a. b.

Fig. 1. Structure of natural hybrids.

Synthetic hybrids can be divided into three groups due to the type of their composition: natural-natural, synthetic-natural and synthetic-synthetic products [32]. An example of the natural-natural hybrid can be geldanamycin, isolated from *Streptomyces hygroscopicus*, combined with estradiol by linker (Fig. 2a). The obtained compound causes degradation of HER2 protein and estrogen receptor (ER) in MCF-7 breast cancer cell line [30]. Rhein, anthraquinone glycoside received from rhubarb plant, combined with huprine exhibits a strong activity against targets of Alzheimer's disease, like: BACE-1, AChE, BuChE (Fig. 2b) [32, 33]. The most important group of hybrids are compounds containing partial structure of a natural product. Pseudoalkaloid leonurine is one of active substances isolated from *Herba leonuri* exhibiting cardioprotective, anti-oxidative and anticancer activity [32, 34]. Leonurine and S-propargyl-L-cysteine conjugate (Fig. 2c) is characterized by *in vivo* cardioprotective activity and reductive oxidative stress at low concentration [32, 35]



a. b. c.

Fig. 2. Structure of synthetic hybrids.

In the literature, several conjugates of betulin with natural or synthetic substances are described. Wang et al. combined betulin with coumarin acid to obtain 28-mono and 3,28-di coumarin-3-carboxylic esters of betulin. Biological activity of these compounds is not available, but Authors suggested a potential anti-HIV activity [36]. Azidothymidine (AZT) is

a synthetic drug used in anti-HIV therapy. This compound was combined with betulin by linker creating triazole or esters derivatives (Fig. 3) [37, 38].

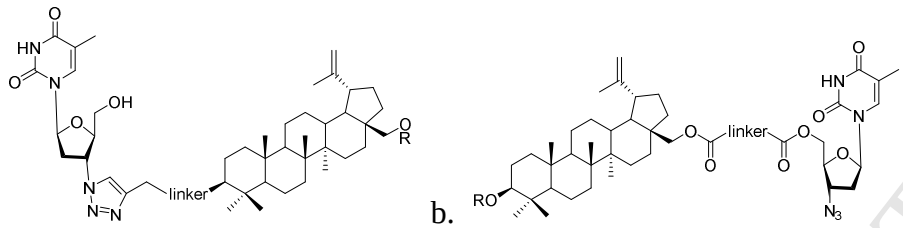


Fig. 3. Structure of betulin and AZT conjugates by triazole (a.) or ester linker (b.).

Both types of hybrids were tested as anti-HIV agents. Triazole derivatives showed a low activity, while esters showed a higher or equipotent effect as bevirimat [37, 38]. Karagöz et al. synthesized hybrids of betulin with ferrocene acid and also with artesunic acid, which were tested against *P. falciparum*. It was found that a betulin-ferrocene conjugate was characterized by a higher activity than ferrocene acid alone. In the tested group of compounds, the best activity was demonstrated by the betulin-artesunic conjugate, but it was 31-times lower than that of reference chloroquine phosphate [39].

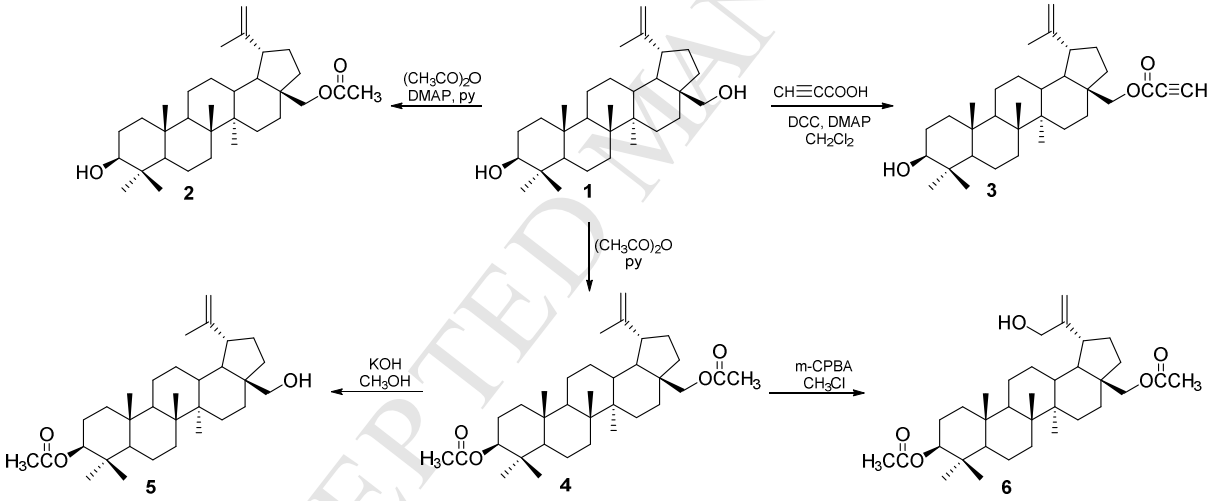
In this study, betulin and its derivatives have been combined with various compounds containing the 1,4-quinone moiety. Combination of these two active substances can lead to compounds with a higher biological activity and a better bioavailability. These hybrids were tested *in vitro* for anticancer activity against a panel of human cancer cell lines. The expression of gene encoding histone (H3), p53 (TP53) and p21 (CDKN1A) protein and apoptosis protein BCL-2 and BAX were also detected. The molecular docking study was used to examine the interaction of NAD[P]H-quinone oxidoreductase (NQO1) protein with the obtained hybrids. Moreover, it was shown that type of the 1,4-quinone system affects the interaction with the active site of the protein. The obtained results were compared with the anticancer activity against the A549, MCF-7 and C-32 cancer cell lines, which contains a higher level of the NQO1 protein.

2. RESULTS AND DISCUSSION

2.1. The synthesis of betulin-1,4-quinone hybrids

In order to synthesize betulin-1,4-quinone hybrids, betulin **1** and its four derivatives, like: 28-acetylbetulin **2**, 28-propynoylbetulin **3**, 3-acetylbetulin **5**, and 30-hydroxy-3,28-diactylbetulin **6**, were chosen.

Betulin **1** was extracted from the birch bark of *Betula pendula* collected in Poland, according to the literature method [8, 40]. As it is seen in Scheme 1, betulin **1** was converted into 28-acetylbetulin **2** in a reaction with acetic anhydride in the presence of 4-dimethylaminopyridine (DMAP) and pyridine, the yield was 71% [41]. Treatment of triterpen **1** with propiolic acid in the presence of N,N'-dicyclohexylcarbodiimide (DCC) and DMAP in dichloromethane gave 28-propynoylbetulin **3** with 60% yield [8]. 3-Acetylbetulin **5** and 30-hydroxy-3,28-diactylbetulin **6** were obtained from 3,28-diacetylbetulin **4**. The base hydrolysis of compound **4** carried out in potassium hydroxide and methanol allowed for obtaining a monoacetylated product **5** as the major product [9]. The allylic oxidation of the isopropenyl group of 3,28-diacetylbetulin **4** in the presence of m-chloroperbenzoic acid (m-CPBA) in chloroform resulted in formation of 30-hydroxy-3,28-diactylbetulin **6** [42]. All compounds **1-6** were purified using the column chromatography method and their chemical structures were confirmed by ^{1}H, ^{13}C NMR and IR spectra. The spectral data were consistent with those published in the literature [8, 9, 42].

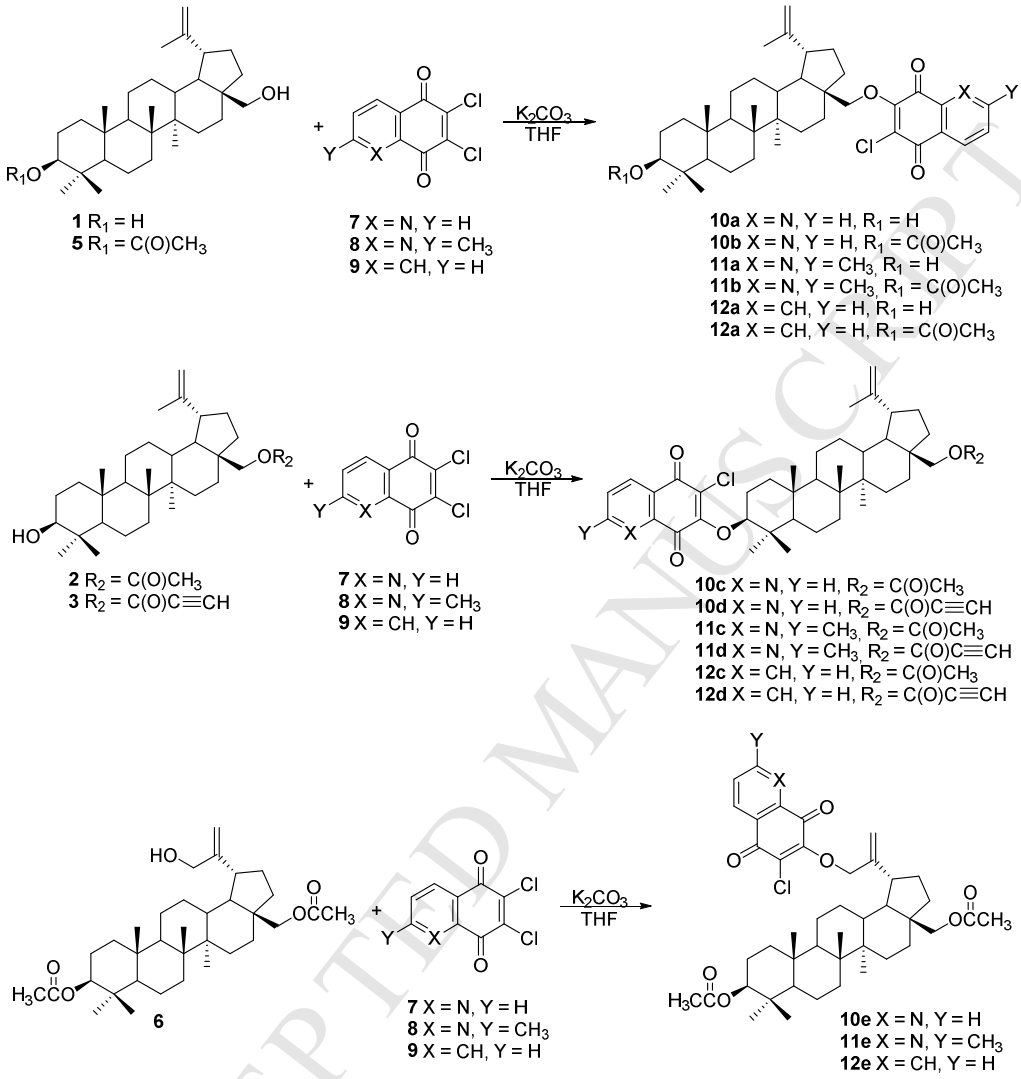


Scheme 1. Synthesis of betulin derivatives **2-6**.

Our previous research showed, that the type of the 1,4-quinone moiety may affect the biological activity of the obtained derivatives [25]. Betulin **1** and its derivatives **2-3**, **5-6** were combined with 5,8-quinolinedione, 2-methyl-5,8-quinolinedione and 1,4-naphthoquinone moieties.

6,7-Dichloro-5,8-quinolinedione **7** and 6,7-dichloro-2-methyl-5,8-quinolinedione **8** and 2,3-dichloronaphthoquinone **9** were used as starting substrates. Compounds **7** and **8** were obtained by oxidation of 8-hydroxyquinoline and 8-hydroxy-2-methylquinoline, respectively [15, 23].

Betulin-1,4-quinone hybrids **10-12** were prepared by substitution of chloride atom in the 1,4-quinone subunit with a hydroxy group in betulin compounds **1**-**3** and **5**-**6**. The reaction was carried out in the presence of potassium carbonate, in tetrahydrofuran (Scheme 2).



Scheme 2: Synthesis of betulin-1,4-quinone hybrids **10-12**.

The products **10-12** were obtained with 35-71% yields (Table 1). Structures of all new derivatives **10-12** were confirmed by ^{1}H, ^{13}C NMR, IR and HR-MS spectra.

Table 1: Yields and physical data for compounds **10-12**.

Compound	Yields [%]	M.p. [°C]	Compound	Yields [%]	M.p. [°C]	Compound	Yields [%]	M.p. [°C]
10a	58	153-154	**11a**	47	139-140	**12a**	68	122-123
10b	62	170-171	**11b**	49	169-170	**12b**	66	155-156
10c	59	165-166	**11c**	35	137-138	**12c**	34	138-139
10d	53	136-137	**11d**	41	166-167	**12d**	39	126-127
10e	59	135-136	**11e**	52	133-134	**12e**	71	132-133

2.2. Anticancer activity

The anticancer activities of the title hybrids **10**-**12** and compounds **1**, **7**-**9** were tested against seven human cancer cell lines: glioblastoma (SNB-19), melanoma (C-32 and Colo-829), breast cancer (MCF-7, T47D, MDA-MB-231) and lung cancer (A549) using WST-1 test. Cisplatin was used as a positive control. The results were expressed as the half-maximal inhibitory concentration (IC_{50}) and they are presented in Table 2.

The starting 1,4-quinone compounds **7**-**10** and betulin **1** were characterized by a low cytotoxic activity against the tested cell lines. In many cases, the insertion of betulinyloxy group into the 1,4-quinone moiety caused an increase in the anticancer activity against the tested human cancer lines (Table 2).

Table 2. Anticancer activity of hybrids **10-12**, substrates **7-9**, betulin **1** and cisplatin.

Hybrids	**Cell lines/ IC_{50} ± SD [µM]**						
	SNB-19	**Colo-829**	**C-32**	**MCF-7**	**T47D**	**MDA-MB-231**	**A549**
10a	Neg	95.46 ± 3.94	Neg	94.83 ± 3.72	13.23 ± 1.40	80.40 ± 3.64	8.58 ± 1.70
10b	Neg	Neg	Neg	96.80 ± 4.70	Neg	Neg	14.34 ± 0.31
10c	Neg	Neg	Neg	Neg	86.82 ± 3.25	Neg	13.46 ± 1.45
10d	41.99 ± 0.11	6.67 ± 1.30	1.27 ± 0.06	14.19 ± 2.30	99.39 ± 6.98	2.43 ± 0.68	0.45 ± 0.20
10e	62.54 ± 5.86	0.13 ± 0.01	22.92 ± 0.61	11.62 ± 0.93	12.76 ± 1.35	60.43 ± 1.77	3.30 ± 0.48
11a	Neg	1.13 ± 0.03	12.65 ± 0.34	Neg	90.44 ± 2.55	10.98 ± 0.89	1.62 ± 0.91
11b	Neg	93.79 ± 1.68	1.33 ± 0.12	89.33 ± 6.61	Neg	5.88 ± 0.25	0.64 ± 0.04
11c	Neg	4.68 ± 0.33	6.52 ± 1.20	1.58 ± 0.17	11.34 ± 0.99	0.90 ± 0.01	0.59 ± 0.13
11d	10.85 ± 0.46	Neg	Neg	17.98 ± 3.67	Neg	36.85 ± 1.78	18.41 ± 1.91
11e	Neg	0.12 ± 0.03	1.14 ± 0.12	0.94 ± 0.03	10.28 ± 1.23	0.11 ± 0.01	0.84 ± 0.01
12a	9.49 ± 0.66	Neg	Neg	Neg	1.47 ± 0.32	Neg	10.63 ± 1.45
12b	3.38 ± 0.15	1.08 ± 0.06	13.83 ± 1.44	71.42 ± 3.95	12.56 ± 1.42	Neg	1.15 ± 0.10
12c	7.54 ± 0.38	0.13 ± 0.03	1.72 ± 0.22	8.72 ± 0,47	12.31 ± 0.77	Neg	0.77 ± 0.12
12d	Neg	Neg	1.04 ± 0.10	36.61 ± 1.72	96.07 ± 5.60	6.77 ± 0.85	1.28 ± 0.06
12e	Neg	Neg	24.67 ± 2.73	Neg	Neg	61.25 ± 1.81	53.90 ± 8.02
7	Neg	Neg	Neg	Neg	Neg	Neg	Neg
8	Neg	26.45 ± 1.69	Neg	Neg	Neg	Neg	Neg
9	Neg	37.93 ± 0.88	Neg	Neg	Neg	Neg	Neg
Betulin **1**	Neg	Neg	Neg	43.88 ± 3.39	20.72 ± 0.74	96.19 ± 1.47	59.73 ± 3.02
Cisplatin	23.00 ± 0.57	19.37 ± 3.10	2.73 ± 0.37	19.60 ± 1.73	21.93 ± 0.37	2.53 ± 0.27	3.50 ± 0.13

Neg – negative in the concentration used

The compounds containing a 5,8-quinolinedione moiety **10-11** were characterized by a low activity against glioblastoma (SNB-19) cells. A better activity against this cell line was shown by derivatives of 1,4-naphthoquinone **12a-e**. Comparing biological activities in this group of hybrids, one may observe that the introduction of acetyl group at C-3 position of betulin, compound **12b**, leads to a significant increase in the activity.

A comparison of the activities against melanotic (Colo-829) and amelanotic (C-32) melanoma cell lines showed that the majority of the hybrids **10-12** was characterized by a higher activity against C-32 than against Colo-829 cells. The structure-activity relationship indicates that the order of the anticancer properties against the amelanotic melanoma (C-32) cell line observed in the compounds **10-12** is as follows: 1,4-naphthoquinone > 2-methyl-5,8-quinolinedione > 5,8-quinolinedione. It means that this activity is affected by the type of the 1,4-quinone moiety.

In biological research, three lines of breast cancer used have different immunoprofiles. The MCF-7 and T47D cells belong to Luminal A class, while the MDA-MB-231 cell line is a triple negative breast cancer (Claudin-low class) [43]. In the group of 5,8-quinolinedione derivatives **10a-e**, only the compound **10d** exhibits a higher activity against MDA-MB-231 than MCF-7 and T47D. The same relationship was observed for 1,4-naphthoquinone hybrids **12a-e**. The derivative **12d** shows the highest activity against the triple negative breast cancer (MDA-MB-231). Introduction of a methyl group at C-2 position of the 5,8-quinolinedione moiety leads to a change in the activity against various breast cancer cells lines. The compounds **11a-e**, except **11d**, are more active against the MDA-MB-231 line than against other breast cancer cell lines. Comparing the compounds which contain 28-propynoyl-3-betulinyloxy fragment (**10d**, **11d** and **12d**) with 28-propynoylbetulin [44], it was observed that an insertion of the 1,4-quinone moiety at the C-3 position of the triterpene system caused an increase in the activity against the MCF-7 and T47D line.

One of the possible mechanisms of action for 1,4-benzoquinone compounds consists in an interaction with NQO1 enzyme [16, 45-49]. In our previous research, we showed that 7-mono and 6,7-dialkinyloxy derivatives of 5,8-quinolinedione exhibited a higher cytotoxic activity against a cell line with a high level of NQO1 enzymes, *e.g.*, melanoma (C-32) and breast cancer (MCF-7) cell lines. The molecular modeling study showed that alkinyloxy compounds could interact with the active site of NQO1 protein by hydrogen bond and hydrophobic interaction [24].

Continuing our research, we tested the obtained hybrids **10-12** against the cancer cell lines with different levels of NQO1 protein, like: A549, MCF-7, C-32 [50-53]. Fig. 4 shows the IC_{50} values for hybrids **10-12** tested against A549, MCF-7, C-32 cell lines. One can notice that the majority of compounds **10-12** exhibited the highest activity against the lung cancer (A549) cell line.

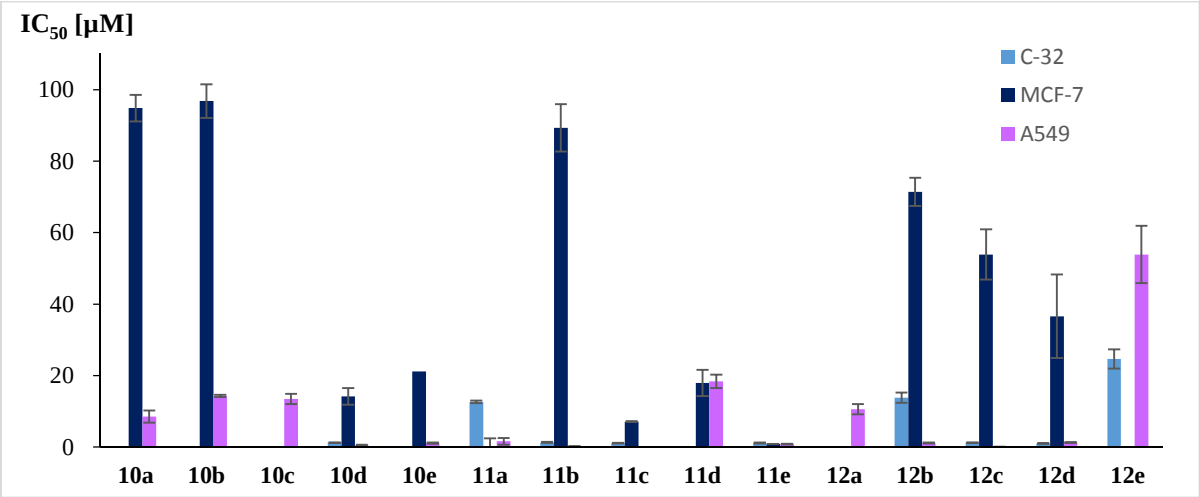


Fig. 4. Summary graph showing IC_{50} values for hybrids **10-12**. Results from the WST-1 assay performed for the C-32, MCF-7 and A549 cell lines after 72 h incubation with the tested compounds. Results are presented as the mean ± SD calculated from at least three independent experiments.

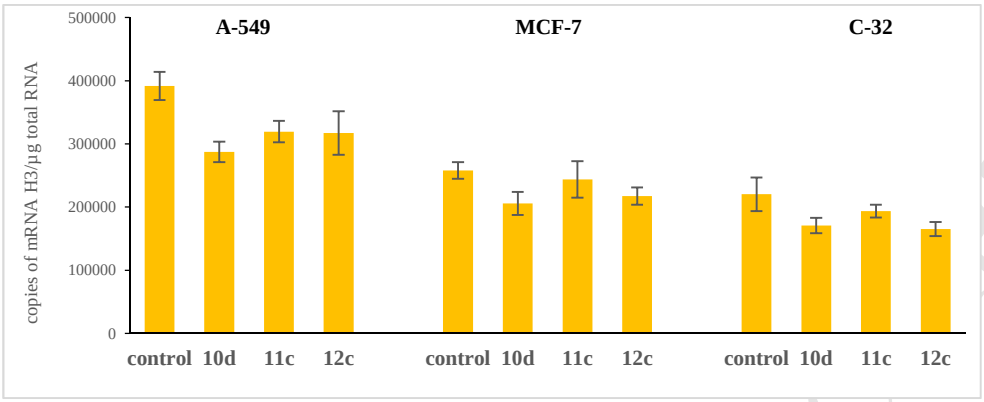
In the series of 5,8-quinolinedione compounds **10a-e**, the order of activity against lung (A549) and breast (MCF-7) cancer cell lines is as follows: 28-propynoyl-3-betulinyloxy > 3,28-diacetyl-30-betulinyloxy > 28-betulinyloxy > 28-acetyl-3-betulinyloxy > 3-acetyl-28-betulinyloxy, *i.e.* it is affected by the type of the substituent. Introduction of a methyl group at C-2 position in the 5,8-quinolinedione moiety (derivatives **11a-e**) leads to an increase in the activity against the A549 cell line (Fig. 4, Table 2). The exception is 6-chloro-2-methyl-7-(28-propynoyl-3-betulinyloxy)-5,8-quinolinedione **11d**, which exhibits a 41-time lower activity than the compound **10d**. In the group of 1,4-naphthoquinone hybrids **12a-e**, 3-chloro-2-(28-acetyl-3-betulinyloxy)-1,4-naphthoquinone **12c** is the most active compound.

2.3. Apoptosis assay

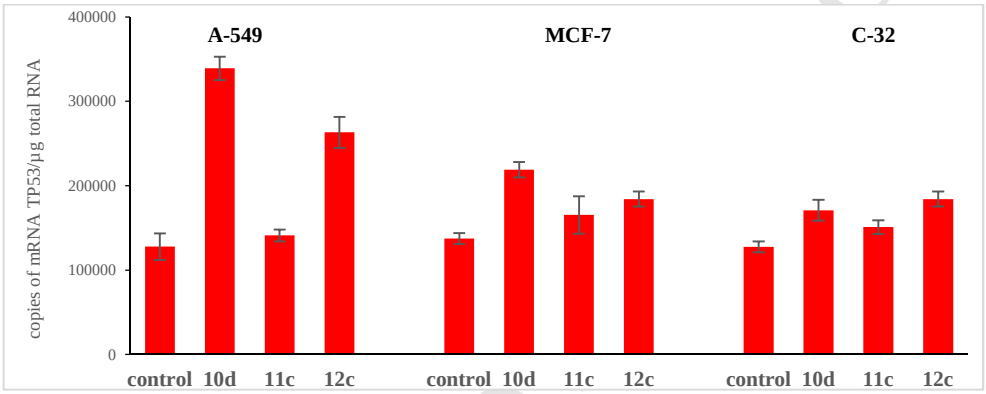
From each group of hybrids **10-12**, one compound with the highest activity against A549, MCF-7 and C-32 cell lines was selected giving a series of the following compounds: **10d**, **11c** and **12c**. The selected compounds were then tested for their transcriptional activity of the gene encoding a proliferation marker (H3 histone), cell cycle regulators (p53 and p21)

and the apoptosis pathway (BCL-2 and BAX). Analysis of H3, TP53, CDKN1A, BCL-2, and BAX genes in A549, MCF-7 and C-32 cells lines was carried out after 24h of exposure of the cultured cells to the tested compounds (half of IC_{50}).

A



B



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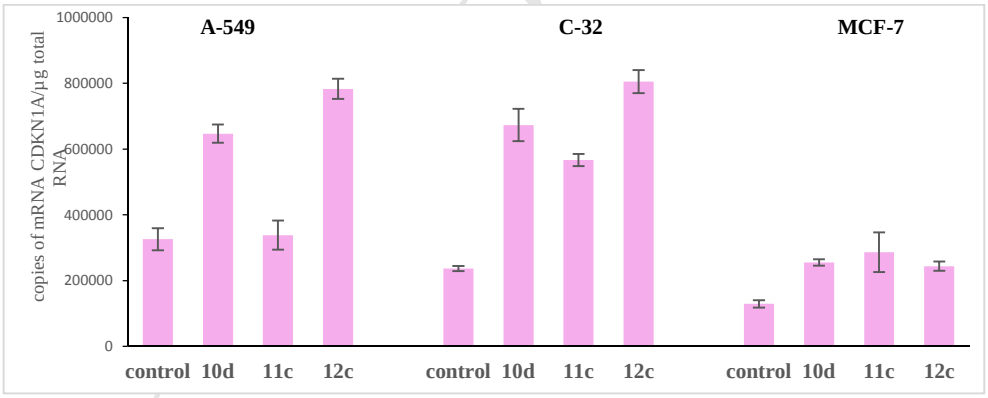


D

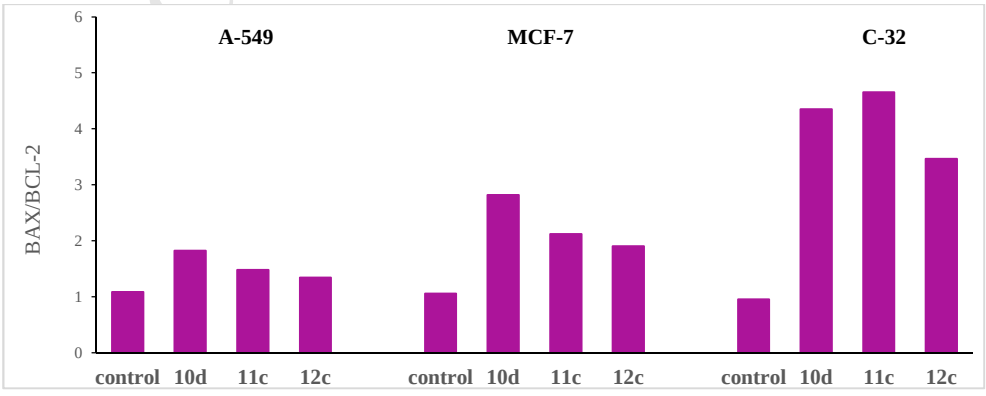


Fig. 5. The effect of hybrids **10d**, **11c** and **12c** on transcriptional activity of: H3 (A); TP53 (B); CDKN1A (C); BAX and BCL/2 (D) for A549, MCF-7, and C-32 cells.

All tested compounds caused a decrease in transcription of the gene encoding the histone H3 in A549, MCF-7 and C-32 cells. Compound **10d** reduced the proliferative activity of the used cell lines most of all (Fig. 5A).

The proposed mechanism of action for the tested hybrids assumes their interaction with NQO1 protein. Inhibition of this enzyme could stimulate formation of a superoxide, oxidative stress and might induce apoptosis [54-56]. On the other hand, inhibition of the NOQ1 enzyme could lead to a degradation of p53 protein [56, 57]. Buranrat et al. proved that dicoumarol at low concentration inhibited NQO1 activity and enhanced p53 protein level [58]. For this reason, the influence of the tested compounds on TP53 and CDKN1A genes was examined. It was found that the tested hybrids enhanced TP53 and CDKN1A genes encoding p53 and p21 proteins, respectively. The tested compounds generated a change in expression of the TP53 gene (Fig. 5B). Compounds **10d** and **12c** caused a significant increase in this gene expression for A549 and MCF-7 cells comparing to the control. Hybrid **11c** showed a slight alteration in lung cancer (A549) and breast cancer (MCF-7) cells. The tested compounds showed a slight increase in TP53 gene copies in C-32.

The p53 protein is a transcription factor of many other genes involved in the mitochondrial apoptosis pathway [59, 60]. This protein controls expression of the CDKN1A gene encoding cyclin-dependent kinase inhibitor (p21), which mediates the cell cycle G1 phase arrest in the cell [61]. Compounds **10d**, **11c** and **12c** generated significant changes in expression of the gene encoding p21 protein. Derivatives **12c** caused a high stepped-up of CDKN1A copies in A549 and MCF-7 cells (Fig. 5C). With the tested compounds containing various 1,4-quinone moieties, the observed order of the change in expression of TP53 and CDKN1A in the A-549 and MCF-7 cell lines is as follows: 5,8-quinolinedione > 1,4-naphthoquinone > 2-methyl-5,8-quinolinedione, *i.e.* it is affected by the type of the 1,4-quinone moiety.

The p53 protein could affect expression of proapoptotic (BAX) and antiapoptotic (BCL-2) gene [62-64]. A comparison of the tested compounds with the control shows that the tested compounds cause significant expression of the proapoptotic protein (BAX) gene (Fig. S1) and does not influence expression of the antiapoptotic (BCL-2) gene (Fig. S2). The exception is hybrid **11c**, which caused a decrease in expression of the BCL-2 gene for the C-32 and A-549 cell lines.

Fig. 5D shows the ratio of BAX and BCL-2 of mRNA copies for the tested compounds and the control. It was found that there is an increase in the BAX/BCL-2 ratio of mRNA copies for all cell lines. It may suggest that the tested hybrids cause the mitochondrial apoptosis pathway in the A549 and MCF-7 cell line. One can also notice that in melanoma (C-32) cell line, there is a slight change in expression of the TP53 and CDKN1A genes, while the BAX/BCL-2 ratio increases 4-5 times. Probably, the compounds **10d**, **11c** and **12c** affect the extrinsic apoptosis pathway in this cell line.

2.4. Molecular docking study

A molecular docking study was carried out to explain the differences between biological activity and molecular structure of synthesized compounds. The apoptosis study suggested that the tested hybrids caused mitochondrial apoptosis pathway. For this reason, NQO1 (1h69.pdb) enzyme was chosen as a possible molecular docking target. The obtained CHEMPLP Score value is presented in Table 3.

Table 3. Docking results showing the binding affinity of hybrids **10-12** to NOQ1 (1h69.pdb).

Ligand	CHEMPLP Score (arb. unit)	
	NQO1	
	Binding site 1 AC1	**Binding site 2 AC2**
10a	62.08	79.42
10b	79.93	83.85
10c	66.78	84.71
10d	**90.61**	**85.18**
10e	92.29	75.44
11a	66.38	62.31
11b	39.87	50.58
11c	**68.51**	**65.44**
11d	88.14	82.99
11e	65.53	72.99
12a	66.54	66.43
12b	86.92	79.08
12c	**92.44**	**79.43**
12d	95.66	88.58
12e	94.73	103.27

The nicotinamide quinone oxidoreductase 1 (NQO1) protein consists of two dimers complexed with two FAD cofactors, which are labelled as AC1 and AC2. In both dimers, the ligands were localized in the hydrophobic matrix of the active site near the side chains in positions Tyr128, Tyr126, Trp105, Po68, Phe232, and FAD cofactor (Fig. 6).

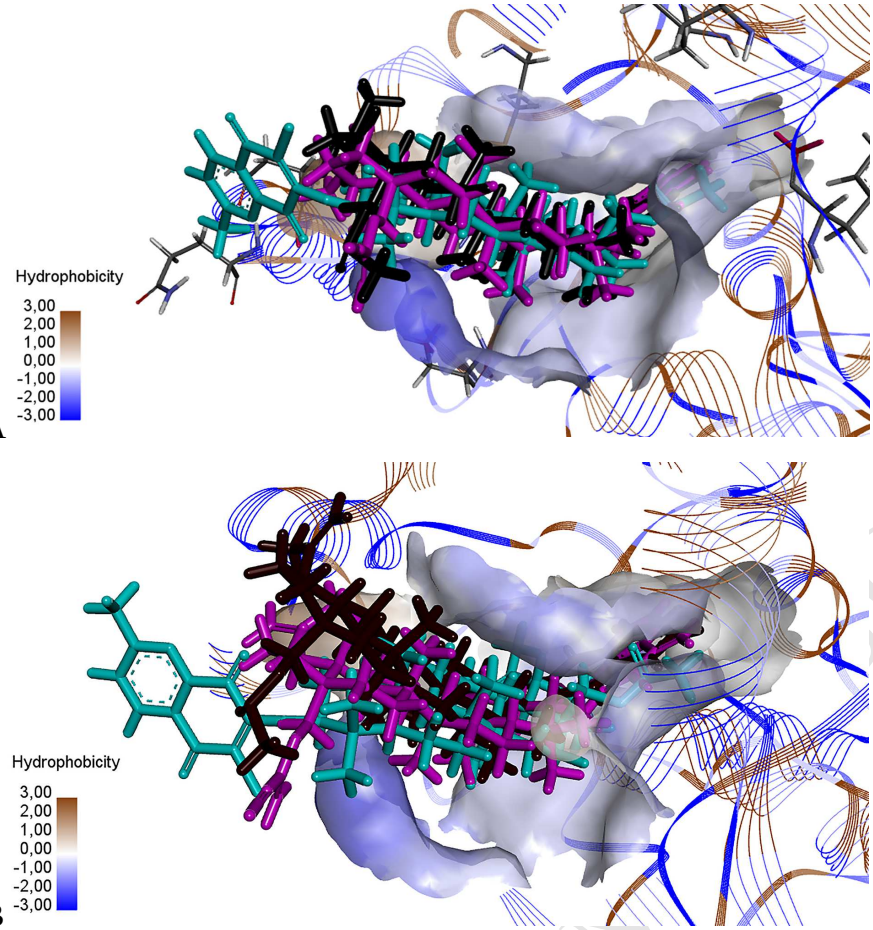


Fig. 6. The superposition of docked compounds: **10d** (violet), **11c** (blue), **12c** (black) and in the binding site of NQO1 enzyme dimers AC1 (A) and AC2 (B), respectively. Protein is colored according to its hydrophobicity scale.

On the basis of the computer simulations it was found that the type of the 1,4-quinone fragment affects the binding affinity of the ligand to the NQO1 in both dimers. Comparing the CHEMPLP score value for compounds containing 5,8-quinolinedione group (**10a-e**) with those containing 2-methyl-5,8-quinolinedione group (**11a-e**), it is seen that the methyl group at

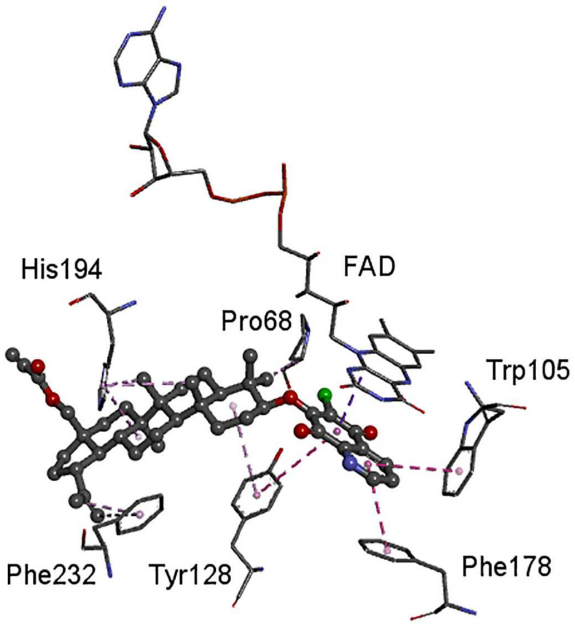
C-2 position leads to a decrease in the affinity of the derivatives to the active site of the NQO1 enzyme. Ligands **12a-e** were bonded to the active site of the protein more strongly than **10a-e** and **11a-e**.

For the selected compounds **10d**, **11c** and **12c**, complete models of the interaction in 3D views are shown in Figs. 7-9. Detailed data about the type and length of the binding interactions between these ligands and the enzyme residues are summarized in Table 4.

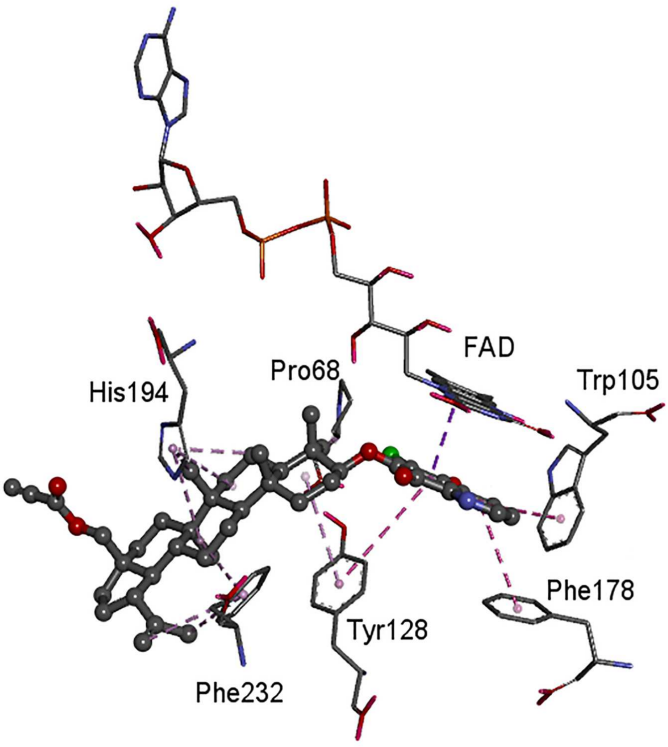
Table 4. Detailed data about binding of molecules **10d**, **11c** and **12c** to NQO1 enzyme (1H69.pdb).

Dimer	Compound	π-interaction residues and lenght (Å)	H-bonding residues and lenght (Å)
AC1	**10d**	FAD (3.56) Phe178 (4.23) Trp105 (5.50) Tyr128 (5.27, 4.63) Pro68 (4.51) His194 (4.67, 4.48, 4.12) Phe232 (5.16, 4.07)	
	11c	His161(4.96) His194 (4.88, 5.07, 4.77, 4.46) Tyr128 (3.91, 5.13, 4.83, 4.57, 4.52) Phe232 (5.30)	
	12c	FAD (3.74) Phe178 (4.53) Trp105 (4.69) Tyr128 (5.30, 4.26) Pro68 (4.36) His194 (5.10, 3.77) Phe232 (5.13, 3.75, 5.40)	
AC2	**10d**	FAD (3.52) Phe178 (4.40) Tyr128 (5.58, 4.52) Trp105 (5.49) Pro68 (4.42) Phe232 (5.37, 4.91, 4.78) His194 (5.45, 4.89, 4.12, 3.78)	
	11c	Tyr128 (4.04, 5.35, 5.04, 4.67, 4.60) Phe232 (5.37) His161 (5.12) His194 (4.62, 5.00, 4.88, 4.40)	FAD (2.78)
	12c	FAD (3.69) Tyr128 (4.85, 3.94) Trp105 (5.49) Phe232 (4.53) His194 (3.76, 4.53)	

Compound **10d** was strongly bound to both dimers of the NQO1 enzyme by a hydrophobic interaction (Fig. 7). For both dimers, the 5,8-quinolinedione moiety interacts with Trp105, Phe178, Tyr128 and FAD cofactor, while the 28-propynoilo-3-betulinyloxy moiety interacts hydrophobically with Tyr128, Phe232, His194 and Pro68.



A



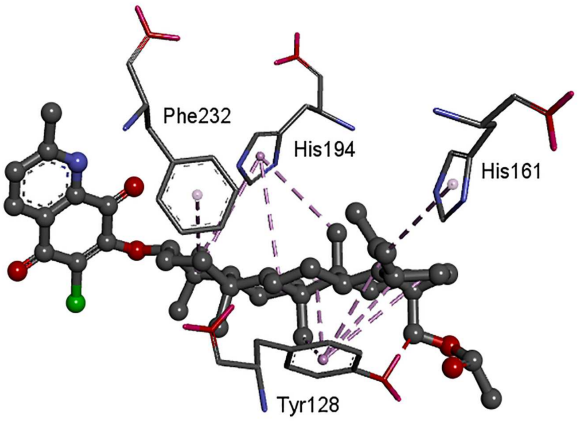
B

Fig. 7. The visualization of hydrophobic interactions (violet) between hybrid **10d** and dimers AC1 (A) and AC2 (B) of NQO1 enzyme.

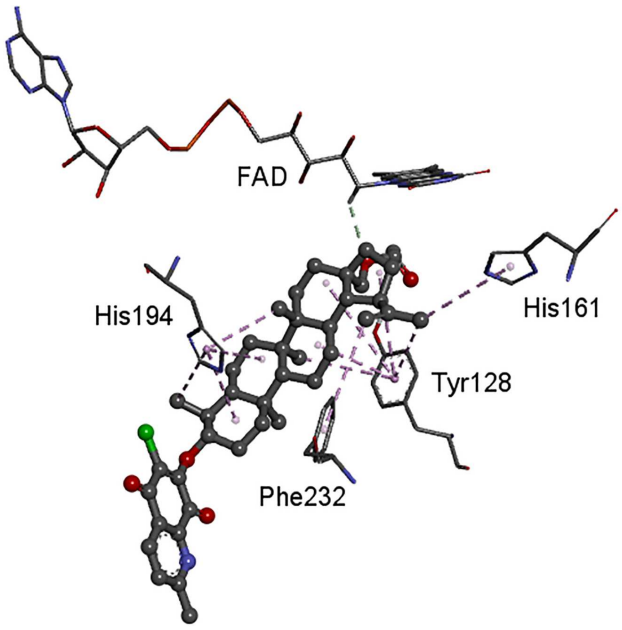
Hybrids **11c** and **12c** have different arrangement in the AC1 and AC2 dimers of the NQO1 enzyme (Figs. 8-9). In active site of AC1 dimer (Fig. 8A), 28-acetyl-3-betulinyloxy group of compound **11c** creates a hydrophobic interaction with His161, His194, Tyr128 and Phe232, while 5,8-quinolinedione system does not interact with the active site of the enzyme.

The 1,4-naphthoquinone moiety of the compound **12c** changes the orientation of 28-acetyl-3-betulinyloxy group *via* additional hydrophobic interactions (Pro68) and in consequence, this ligand creates a hydrophobic interaction between Phe178, Trp105, Tyr128, FAD cofactor and the 5,8-quinolinedione moiety (Fig. 9A).

The 6-(28-acetyl-3-betulinyloxy)-7-chloro-2-methyl-5,8-quinolinedione **11c** interacts with the AC2 dimer by a π-interaction and a hydrogen bond. The 28-acetyl-3-betulinoxy group of the compound **11c** interacts by a hydrophobic interaction with the hydrophobic matrix of enzymes (Tyr128, Phe232, His161 and His194) and by a hydrogen bond with the FAD cofactor (Fig. 8B). In the case of the ligand **12c**, 1,4-naphthoquinone moiety creates a π-interaction with Tyr128, Trp105 and FAD cofactor (Fig. 9B).

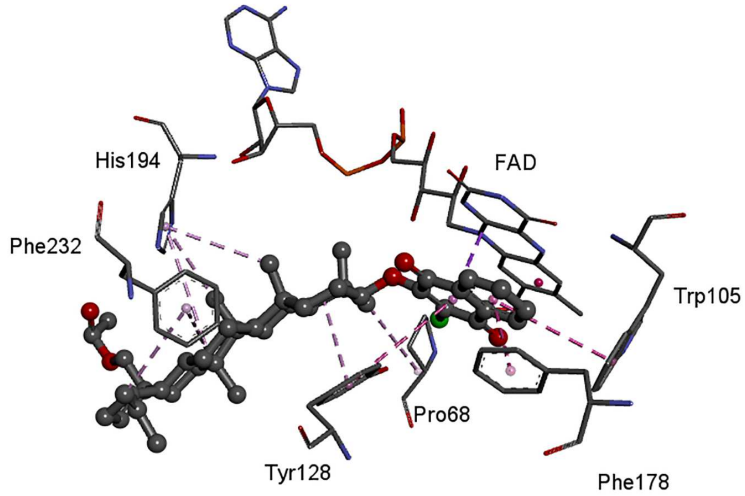


A

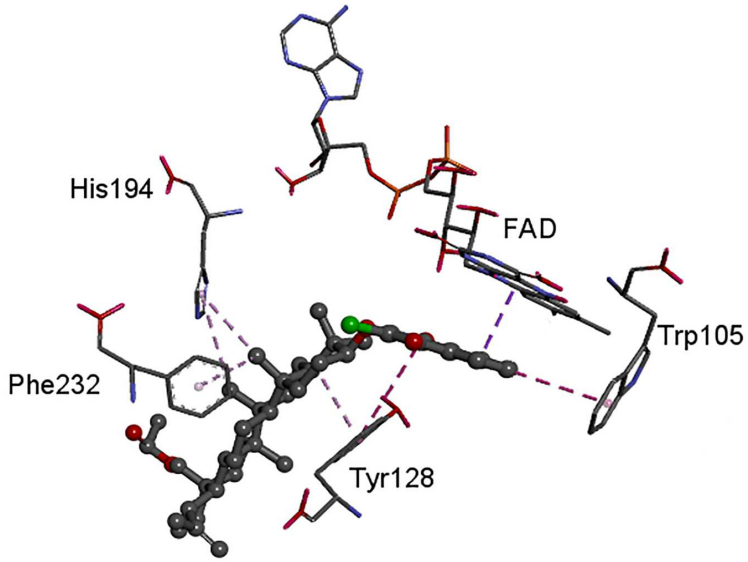


B

Fig. 8. The visualization of hydrogen bond (green) and hydrophobic interactions (violet) between hybrid **11c** and dimers AC1 (A) and AC2 (B) of NQO1 enzyme.



A



B

Fig. 9. The visualization of hydrophobic interactions (violet) between hybrid **12c** and dimer AC1 (A) and AC2 (B) of NQO1 enzyme.

Comparing the effect of **11c**, **12c** and **10d** hybrids on TP53 gene expression, it is evident, that the order of the tested compounds against lung (A549) and breast (MCF-7) cancer cells is as follows: **10d** > **12c** > **11c**. One can notice that the 5,8-quinolinedione moiety present in the molecule **10d** interacts with the active site of both dimers of the NQO1 protein, while the similar fragment of **11c** is exposed to the protein hydrophilic surface. These results suggested that the interaction of the 1,4-quinone system with Tyr128, Phe178, Trp105 and FAD cofactor could explain the increase in expression of the TP53 gene caused by the compound **12d**.

3. CONCLUSION

In this study, new betulin-1,4-quinone hybrids were synthesized, characterized and examined for their anticancer activity. It was found, that these compounds exhibited a higher activity against cancer cell lines with an increased level of the NQO1 protein, *i.e.* A549, MCF-7 and C-32. The structure-activity relationship showed, that biological activity was affected by the type of the 1,4-quinone moiety. Selected hybrids were tested on transcriptional activity of the gene encoding proliferation marker, cell cycle regulators and apoptosis pathway. The research showed that the selected hybrids initiate the apoptosis pathway. Molecular docking simulations have contributed to the characterization of the mechanism of binding of betulin-1,4-quinone hybrids to the NQO1 enzyme. The docking results showed that the interaction of the 1,4-quinone moiety with the enzyme active site led to an increase in biological activity.

4. EXPERIMENTAL

The NMR spectra were recorded on the Bruker Avance 600 spectrometer in $CDCl_3$ solvents; chemical shifts (δ) are reported in ppm and *J* values in Hz. Multiplicity is designated as singlet (s), doublet (d), doublet of doublets (dd), triplet (t) and multiplet (m). High-resolution mass spectral analysis was carried out using the Bruker Impact II instrument. Infrared spectra (IR) were determined using the IRAffinity-1 Shimadzu spectrophotometer. Melting points were designated by the Electrothermal IA 9300 melting point apparatus.

The betulin **1** and its derivatives **2-6** were obtained according the literature methods [8, 9, 42]. Compounds: 6,7-dichloro-5,8-quinolinedione **7** and 6,7-dichloro-2-methyl-5,8-quinolinedione **8** were prepared according to the method described in literature [15, 23]. All commercial substances were purchased from Sigma-Aldrich (Sigma-Aldrich, Saint Louis, MO, USA).

4.1. Synthesis of betulin-1,4-quinone hybrids. General procedure

Betulin **1** or its derivative **2-3**, **5-6** (0.439 mmol) and 1,4-benzoquinone compounds **7-9** (0.439 mmol) were dissolved in THF (5 ml). The potassium carbonate (0.121 g; 0.878 mmol) was added and the reaction mixture was stirred at the room temperature for 24 hrs. The solvent was evaporated under vacuum. The crude product was purified by silica-gel flash column chromatography (dichloromethane/ethanol, 30:1, v/v) to give pure compounds **10-12**.

*7-(28-betulinyloxy)-6-chloro-5,8-quinolinedione **10a*** Yield: 58%, m.p. 153-154 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.78 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 1.02 (s, 3H,

CH_3), 1.72 (s, 3H, CH_3), 0.87-2.31 (m, 25H, CH, CH_2), 2.43 (m, 1H, H-19), 3.21 (m, 1H, H-3), 4.24 (d, *J*=10.8 Hz, 1H, H-28), 4.62 (s, 1H, H-29), 4.78 (s, 1H, H-29), 4.88 (d, *J*=10.8 Hz, 1H, H-28), 7.73 (dd, J_{23} = 4.2 Hz, J_{34} = 7.8 Hz, 1H, H-3'), 8.51 (dd, J_{24} = 1.2 Hz, J_{34} = 7.8 Hz, 1H, H-4'), 9.07 (dd, J_{24} = 1.2 Hz, J_{23} = 42 Hz, 1H, H-2'). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 14.2, 14.9, 15.4, 16.1, 18.3, 20.8, 21.1, 25.2, 27.4, 28.0, 28.8, 29.4, 29.5, 29.6, 34.2, 34.3, 37.2, 38.7, 40.9, 42.8, 47.8, 48.0, 49.0, 50.1, 50.3, 55.3, 60.4, 74.0, 79.0, 109.9, 128.0, 128.2, 134.9, 146.6, 150.1, 154.7, 158.2, 177.8, 178.1. IR (KBr, cm^{-1}) ν_{max}: 3071-2870, 1692, 1674, 1638, 1593-1566, 1456, 1375, 1247, 1096. HR-MS (APCI) *m/z*: $C_{39}H_{52}NO_4Cl$ [$(M)^-$], Calcd. 633.3585; Found. 633.3589.

7-(3-acetyl-28-betulinyloxy)-6-chloro-5,8-quinolinedione ***10b*** Yield: 62%, m.p. 170-171 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.85 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 1.04 (s, 3H, CH_3), 1.11 (s, 3H, CH_3), 1.72 (s, 3H, CH_3), 0.87-2.25 (m, 25H, CH, CH_2), 2.07 (s, 3H, $COCH_3$), 2.43 (m, 1H, H-19), 4.26 (d, *J*=10.8 Hz, 1H, H-28), 4.49 (m, 1H, H-3), 4.62 (s, 1H, H-29), 4.71 (s, 1H, H-29), 4.88 (d, *J*=10.8 Hz, 1H, H-28), 7.73 (dd, J_{23} = 4.2 Hz, J_{34} = 7.8 Hz, 1H, H-3'), 8.51 (dd, J_{24} = 1.2 Hz, J_{34} = 7.8 Hz, 1H, H-4'), 9.07 (dd, J_{24} = 1.2 Hz, J_{23} = 4.2 Hz, 1H, H-2'). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 14.2, 15.9, 16.2, 18.5, 19.2, 20.8, 21.4, 23.7, 25.6, 28.0, 29.6, 31.0, 33.2, 34.1, 37.1, 38.7, 40.8, 42.8, 47.6, 48.0, 49.0, 50.3, 53.5, 55.4, 59.4, 68.0, 74.0, 80.9, 110.0, 128.0, 128.2, 128.8, 134.9, 146.7, 150.0, 154.7, 158.2, 171.1, 177.8, 178.1. IR (KBr, cm^{-1}) ν_{max}: 2945-2870, 1740, 1693, 1670, 1589-1557, 1462, 1367, 1246, 1094. HR-MS (APCI) *m/z*: $C_{41}H_{54}NO_5Cl$ [$(M)^-$], Calcd. 675.3690; Found. 675.3678.

7-(28-acetyl-3-betulinyloxy)-6-chloro-5,8-quinolinedione ***10c*** Yield: 59%, m.p. 165-166 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.91 (s, 3H, CH_3), 0.98 (s, 3H, CH_3), 1.05 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 1.72 (s, 3H, CH_3), 0.87-2.25 (m, 25H, CH, CH_2), 2.10 (s, 3H, $COCH_3$), 2.46 (m, 1H, H-19), 3.88 (d, *J*=10.8 Hz, 1H, H-28), 4.27 (d, *J*=10.8 Hz, 1H, H-28), 4.61 (s, 1H, H-29), 4.71 (s, 1H, H-29), 4.81 (m, 1H, H-3), 7.71 (dd, J_{23} = 4.2 Hz, J_{34} = 7.8 Hz, 1H, H-3'), 8.50 (dd, J_{24} = 1.2 Hz, J_{34} = 7.8 Hz, 1H, H-4'), 9.04 (dd, J_{24} = 1.2 Hz, J_{23} = 4.2 Hz, 1H, H-2'). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 14.8, 15.9, 16.0, 16.5, 18.2, 19.1, 20.8, 21.1, 25.1, 26.6, 27.0, 28.0, 29.7, 31.0, 33.5, 34.2, 36.7, 37.8, 38.5, 39.5, 40.9, 42.7, 47.7, 48.7, 49.7, 50.3, 53.5, 55.5, 62.8, 92.8, 109.9, 127.9, 128.9, 134.9, 146.7, 150.1, 154.6, 158.1, 171.6, 177.8, 178.1. IR (KBr, cm^{-1}) ν_{max}: 3070-2870, 1728, 1691, 1670, 1593-1562, 1464, 1373, 1240, 1093. HR-MS (APCI) *m/z*: $C_{41}H_{53}NO_5Cl$ [$(M)^-$], Calcd. 675.3690; Found. 675.3665.

6-chloro-7-(28-propynoyl-3-betulinyloxy)-5,8-quinolinedione ***10d*** Yield: 53%, m.p. 136-137 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.91 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 1.05 (s, 3H, CH_3),

1.07 (s, 3H, CH_3), 1.72 (s, 3H, CH_3), 0.87-2.25 (m, 27H, CH, CH_2), 1.92 (s, 1H, C≡CH), 2.45 (m, 1H, H-19), 4.04 (d, *J*=10.8 Hz, 1H, H-28), 4.43 (d, *J*=10.8 Hz, 1H, H-28), 4.62 (s, 1H, H-29), 4.72 (s, 1H, H-29), 4.82 (m, 1H, H-3), 7.72 (dd, J_{23} = 4.2 Hz, J_{34} = 7.8 Hz, 1H, H-3'), 8.50 (dd, J_{24} = 1.2 Hz, J_{34} = 7.8 Hz, 1H, H-4'), 9.03 (dd, J_{24} = 1.2 Hz, J_{23} = 4.2 Hz, 1H, H-2'). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 14.7, 15.7, 16.3, 18.2, 19.1, 20.8, 21.1, 24.6, 26.6, 27.0, 29.5, 30.6, 33.5, 34.5, 36.7, 37.7, 38.5, 39.8, 40.8, 42.7, 46.5, 47.7, 48.7, 48.8, 50.2, 53.5, 55.5, 64.9, 74.8, 92.7, 110.1, 127.9, 128.1, 134.9, 149.9, 153.3, 154.6, 158.1, 177.8, 178.1. IR (KBr, cm^{-1}) ν_{max}: 2943-2870, 2118, 1713, 1670, 1639, 1591-1560, 1457, 1252, 1227, 1065. HR-MS (APCI) *m/z*: $C_{42}H_{52}NO_5Cl$ [$(M)^-$], Calcd. 685.3534; Found. 685.3451.

6-chloro-7-(3,28-diacetyl-30-betulinyloxy)-5,8-quinolinedione ***10e*** Yield: 59%, m.p. 135-136 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.85 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 1.08 (s, 3H, CH_3), 0.87-2.25 (m, 25H, CH, CH_2), 2.08 (s, 3H, $COCH_3$), 2.11 (s, 3H, $COCH_3$), 2.48 (m, 1H, H-19), 3.85 (d, *J*=10.8 Hz, 1H, H-28), 4.28 (d, *J*=10.8 Hz, 1H, H-28), 4.47 (m, 1H, H-3), 5.09 (s, 1H, H-29), 5.20 (m, 3H, H-29, 2xH-30), 7.73 (dd, J_{23} = 4.2 Hz, J_{34} = 7.8 Hz, 1H, H-3'), 8.50 (dd, J_{24} = 1.2 Hz, J_{34} = 7.8 Hz, 1H, H-4'), 9.06 (dd, J_{24} = 1.2 Hz, J_{23} = 4.2 Hz, 1H, H-2'). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 14.8, 16.0, 18.2, 20.9, 21.1, 23.7, 25.6, 27.0, 28.0, 29.7, 31.0, 34.3, 37.0, 38.4, 40.8, 42.7, 46.5, 49.8, 50.2, 53.5, 55.3, 62.4, 68.0, 80.9, 111.9, 128.0, 134.9, 146.6, 149.4, 154.8, 157.0, 171.1, 177.6, 178.1. IR (KBr, cm^{-1}) ν_{max}: 2949-2876, 1734, 1589-1558, 1365, 1246, 1074. HR-MS (APCI) *m/z*: $C_{43}H_{56}NO_7Cl$ [$(M)^-$], Calcd. 733.3745; Found. 733.3714.

7-(28-betulinyloxy)-6-chloro-2-methyl-5,8-quinolinedione ***11a*** Yield: 37%, m.p. 139-140 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.78 (s, 3H, CH_3), 0.85 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 1.03 (s, 3H, CH_3), 1.72 (s, 3H, CH_3), 0.87-2.31 (m, 25H, CH, CH_2), 2.42 (m, 1H, H-19), 2.81 (s, 3H, CH_3'), 3.21 (m, 1H, H-3), 4.26 (d, *J*=10.8 Hz, 1H, H-28), 4.61 (s, 1H, H-29), 4.70 (s, 1H, H-29), 4.82 (d, *J*=10.8 Hz, 1H, H-28), 7.57 (d, J_{34} = 7.8 Hz, 1H, H-3'), 8.38 (d, J_{34} = 7.8 Hz, 1H, H-4'). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 14.2, 14.8, 15.4, 16.1, 18.5, 19.2, 20.9, 21.1, 25.6, 27.0, 27.4, 28.0, 29.6, 34.2, 37.7, 38.9, 40.9, 42.8, 47.9, 49.0, 50.1, 50.4, 53.5, 55.3, 60.4, 68.0, 73.9, 79.0, 109.9, 126.0, 127.9, 128.7, 135.0, 146.2, 150.1, 157.9, 165.2, 171.2, 178.0, 178.5. IR (KBr, cm^{-1}) ν_{max}: 2941-2868, 1734, 1716, 1655, 1578, 1456, 1375, 1252, 1076. HR-MS (APCI) *m/z*: $C_{40}H_{54}NO_4Cl$ [$(M)^-$], Calcd. 647.3741; Found. 647.3695.

7-(3-acetyl-28-betulinyloxy)-6-chloro-2-methyl-5,8-quinolinedione ***11b*** Yield: 49%, m.p. 169-170 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.85 (s, 3H, CH_3), 0.87 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.07 (s, 3H, CH_3), 1.72 (s, 3H, CH_3), 0.87-2.25 (m, 25H, CH, CH_2), 2.07 (s, 3H,

$COCH_3$), 2.41 (m, 1H, H-19), 2.81 (s, 3H, CH_3'), 4.25 (d, *J*=10.8 Hz, 1H, H-28), 4.49 (m, 1H, H-3), 4.61 (s, 1H, H-29), 4.70 (s, 1H, H-29), 4.82 (d, *J*=10.8 Hz, 1H, H-28), 7.57 (d, J_{34} = 7.8 Hz, 1H, H-3'), 8.37 (d, J_{34} = 7.8 Hz, 1H, H-4'). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 14.2, 14.8, 16.2, 16.5, 18.5, 19.2, 20.8, 21.4, 23.7, 25.2, 27.1, 27.9, 29.6, 34.3, 37.8, 38.4, 40.9, 42.8, 47.9, 48.9, 50.3, 55.4, 60.4, 73.9, 80.9, 109.9, 126.0, 127.9, 128.7, 135.0, 146.2, 150.1, 157.9, 165.2, 171.2, 177.9, 178.5. IR (KBr, cm^{-1}) ν_{max}: 2949-2876, 1732, 1689, 1598-1546, 1470, 1375, 1246, 1074. HR-MS (APCI) *m/z*: $C_{42}H_{56}NO_5Cl$ [$(M)^-$], Calcd. 689.3847; Found. 689.3807.

7-(28-acetyl-3-betulinyloxy)-6-chloro-2-methyl-5,8-quinolinedione ***11c*** Yield: 35%, m.p. 137-138 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.89 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 1.05 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 1.71 (s, 3H, CH_3), 0.87-2.25 (m, 25H, CH, CH_2), 2.09 (s, 3H, $COCH_3$), 2.47 (m, 1H, H-19), 2.81 (s, 3H, CH_3'), 3.87 (d, *J*=10.8 Hz, 1H, H-28), 4.26 (d, *J*=10.8 Hz, 1H, H-28), 4.62 (s, 1H, H-29), 4.71 (s, 1H, H-29), 4.77 (m, 1H, H-3), 7.55 (d, J_{34} = 7.8 Hz, 1H, H-3'), 8.38 (d, J_{34} = 7.8 Hz, 1H, H-4'). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 14.7, 15.9, 16.3, 18.2, 19.1, 20.9, 21.3, 25.2, 26.6, 27.9, 28.1, 29.0, 29.7, 33.5, 34.6, 36.9, 37.6, 39.7, 40.9, 42.7, 46.3, 47.7, 48.8, 50.4, 55.6, 62.8, 68.0, 79.0, 92.7, 109.9, 126.0, 127.9, 128.6, 129.5, 135.0, 146.3, 150.2, 157.9, 165.0, 171.7, 177.9, 178.5. IR (KBr, cm^{-1}) ν_{max}: 2945-2868, 1740, 1717, 1695, 1597-1558, 1456, 1385, 1234, 1072. HR-MS (APCI) *m/z*: $C_{42}H_{56}NO_5Cl$ [$(M)^-$], Calcd. 689.3847; Found. 689.3768.

6-chloro-7-(28-propynoyl-3-betulinyloxy)-2-methyl-5,8-quinolinedione ***11d*** Yield: 41%, m.p. 166-167 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.90 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 1.04 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 1.72 (s, 3H, CH_3), 0.87-2.25 (m, 27H, CH, CH_2), 1.91 (s, 1H, C≡CH), 2.45 (m, 1H, H-19), 2.80 (s, 3H, CH_3'), 4.02 (d, *J*=10.8 Hz, 1H, H-28), 4.40 (d, *J*=10.8 Hz, 1H, H-28), 4.63 (s, 1H, H-29), 4.72 (s, 1H, H-29), 4.75 (m, 1H, H-3), 7.55 (d, J_{34} = 7.8 Hz, 1H, H-3'), 8.36 (d, J_{34} = 7.8 Hz, 1H, H-4'). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 14.7, 15.7, 16.3, 18.2, 19.1, 20.8, 21.1, 24.6, 26.6, 27.0, 29.5, 30.6, 33.5, 34.5, 36.7, 37.7, 38.5, 39.8, 40.8, 42.7, 46.5, 47.7, 48.7, 48.8, 50.2, 53.5, 55.5, 64.9, 74.8, 92.7, 110.1, 127.9, 128.1, 134.9, 149.9, 153.3, 154.6, 158.1, 171.6, 177.8, 178.1. IR (KBr, cm^{-1}) ν_{max}: 2951-2870, 2120, 1716, 1674, 1599-1574, 1456, 1375, 1224, 1074. HR-MS (APCI) *m/z*: $C_{43}H_{54}NO_5Cl$ [$(M)^-$], Calcd. 699.3690; Found. 699.3612.

6-chloro-7-(3,28-diacetyl-30-betulinyloxy)-2-methyl-5,8-quinolinedione ***11e*** Yield: 52%, m.p. 133-134 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.85 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 1.07 (s, 3H, CH_3), 0.87-2.25 (m, 25H, CH, CH_2), 2.08 (s, 3H, $COCH_3$), 2.11 (s, 3H,

$COCH_3$), 2.50 (m, 1H, H-19), 2.81 (s, 3H, CH_3'), 3.87 (d, *J*=10.8 Hz, 1H, H-28), 4.33 (d, *J*=10.8 Hz, 1H, H-28), 4.48 (m, 1H, H-3), 5.08 (s, 1H, H-29), 5.16 (m, 2H, 2xH-30), 5.22 (s,1H, H-29), 7.57 (d, J_{34} = 7.8 Hz, 1H, H-3'), 8.37 (d, J_{34} = 7.8 Hz, 1H, H-4'). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 14.2, 16.2, 18.2, 20.9, 21.3, 23.7, 25.2, 27.0, 29.8, 34.3, 37.5, 38.4, 40.9, 42.7, 46.4, 49.8, 50.2, 55.4, 60.4, 62.5, 80.9, 126.0, 28.1, 135.0, 156.8, 171.1, 177.6, 178.1. IR (KBr, cm^{-1}) ν_{max}: 2949-2872, 1734, 1692, 1670, 1589-1558, 1365, 1246, 1074. HR-MS (APCI) *m/z*: $C_{44}H_{58}NO_7Cl$ [$(M)^-$], Calcd. 747.3901; Found. 747.39815.

3-(28-betulinyloxy)-2-chloro-1,4-naphthoquinolinedione ***12a*** Yield: 68%, m.p. 122-123 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.78 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 1.03 (s, 3H, CH_3), 1.72 (s, 3H, CH_3), 0.87-2.31 (m, 25H, CH, CH_2), 2.44 (m, 1H, H-19), 3.21 (m, 1H, H-3), 4.20 (d, *J*=10.8 Hz, 1H, H-28), 4.61 (s, 1H, H-29), 4.71 (s, 1H, H-29), 4.77 (d, *J*=10.8 Hz, 1H, H-28), 7.76 (m, *2*H, H-6', H-7'), 8.12 (dd, J_{78} = 1.8 Hz, J_{56} = 4.8 Hz, 1H, H-5'), 8.18 (dd, J_{78} = 1.8 Hz, J_{56} = 4.8 Hz, 1H, H-8'). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 14.2, 14.9, 15.4, 18.3, 20.8, 21.1, 25.2, 27.1, 28.0, 28.8, 29.4, 34.3, 37.3, 37.8, 38.7, 39.7, 40.9, 42.8, 47.8, 48.0, 49.0, 50.1, 50.3, 50.4, 55.3, 60.4, 74.0, 79.0, 109.9, 126.9, 127.0, 129.4, 130.9, 133.8, 134.3, 149.7, 150.2, 157.4, 178.8, 179.9. IR (KBr, cm^{-1}) ν_{max}: 2941-2870, 1734, 1717, 1699, 1676, 1595, 1456, 1375, 1251, 1093. HR-MS (APCI) *m/z*: $C_{40}H_{53}ClO_4$ [$(M)^-$], Calcd. 632.3632; Found. 632.3603.

3-(3-acetyl-28-betulinyloxy)-2-chloro-1,4-naphthoquinolinedione ***12b*** Yield: 66%, m.p. 155-156 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.86 (s, 3H, CH_3), 0.88 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.07 (s, 3H, CH_3), 1.75 (s, 3H, CH_3), 0.87-2.25 (m, 25H, CH, CH_2), 2.07 (s, 3H, $COCH_3$), 2.23 (m, 1H, H-19), 4.20 (d, *J*=10.8 Hz, 1H, H-28), 4.49 (m, 1H, H-3), 4.62 (s, 1H, H-29), 4.71 (s, 1H, H-29), 4.74 (d, *J*=10.8 Hz, 1H, H-28), 7.78 (m, *2*H, H-6', H-7'), 8.12 (dd, J_{78} = 1.8 Hz, J_{56} = 4.8 Hz, 1H, H-5'), 8.18 (dd, J_{78} = 1.8 Hz, J_{56} = 4.8 Hz, 1H, H-8'). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 14.2, 14.9, 15.9, 18.2, 20.8, 21.1, 23.4, 27.1, 28.0, 29.4,]34.3, 37.3, 37.8, 38.3, 40.9, 42.8, 47.8, 48.0, 50.3, 50.4, 55.3, 60.4, 73.1, 73.5, 80.9, 109.9, 126.9, 127.0, 129.4, 131.0, 131.2, 133.8, 134.2, 134.7, 150.2, 157.3, 171.6, 178.7, 180.0. IR (KBr, cm^{-1}) ν_{max}: 2945-2872, 1732, 1676, 1595-1570, 1458, 1375, 1248, 1080. HR-MS (APCI) *m/z*: $C_{42}H_{55}ClO_5$ [$(M)^-$], Calcd. 674.3738; Found. 674.3713.

3-(28-acetyl-3-betulinyloxy)-2-chloro-1,4-naphthoquinolinedione ***12c*** Yield: 34%, m.p. 138-139 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.73 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 1.72 (s, 3H, CH_3), 0.87-2.25 (m, 25H, CH, CH_2), 2.01 (s, 3H, $COCH_3$), 2.38 (m, 1H, H-19), 3.78 (d, *J*=10.8 Hz, 1H, H-28), 4.17 (d, *J*=10.8 Hz, 1H, H-28),

4.52 (s, 1H, H-29), 4.62 (s, 1H, H-29), 4.65 (m, 1H, H-3), 7.67 (m, *2*H, H-6', H-7'), 7.99 (dd, J_{78} = 1.8 Hz, J_{56} = 4.8 Hz, 1H, H-5'), 8.08 (dd, J_{78} = 1.8 Hz, J_{56} = 4.8 Hz, 1H, H-8'). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 14.2, 14.7, 15.9, 16.0, 16.2, 16.3, 16.5, 18.2, 20.9, 21.1, 23.7, 25.0, 25.1, 27.0, 28.1, 29.6, 29.7, 34.2, 34.6, 37.1, 37.5, 38.5, 39.7, 40.9, 42.7, 46.3, 47.7, 48.8, 49.7, 50.3, 55.6, 60.4, 62.8, 92.0, 109.9, 126.9, 127.0, 129.7, 131.0, 131.2, 133.8, 134.2, 134.7, 150.2, 157.3, 171.6, 178.7, 180.0. IR (KBr, cm^{-1}) ν_{max}: 2951-2851, 1740, 1674, 1593-1565, 1456, 1386, 1251, 1096. HR-MS (APCI) *m/z*: $C_{42}H_{55}ClO_5$ [$(M)^-$], Calcd. 674.3738; Found. 674.3683.

2-chloro-3-(28-propynoyl-3-betulinyloxy)-1,4-naphthoquinolinedione ***12d*** Yield: 39%, m.p. 126-127 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.82 (s, 3H, CH_3), 0.98 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 1.09 (s, 3H, CH_3), 1.71 (s, 3H, CH_3), 0.87-2.25 (m, 27H, CH, CH_2), 2.01 (s, 1H, C≡CH), 2.45 (m, 1H, H-19), 4.02 (d, *J*=10.8 Hz, 1H, H-28), 4.41 (d, *J*=10.8 Hz, 1H, H-28), 4.65 (s, 1H, H-29), 4.73 (s, 1H, H-29), 4.75 (m, 1H, H-3), 7.76 (m, *2*H, H-6', H-7'), 8.08 (dd, J_{78} = 1.8 Hz, J_{56} = 4.8 Hz, 1H, H-5'), 8.17 (dd, J_{78} = 1.8 Hz, J_{56} = 4.8 Hz, 1H, H-8'). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 14.2, 14.7, 16.0, 16.3, 18.2, 20.8, 21.1, 25.0, 25.9, 26.0, 27.0, 28.0, 29.5, 34.2, 34.5, 37.1, 38.6, 39.7, 41.0, 42.7, 46.4, 47.7, 48.8, 50.3, 55.6, 60.4, 64.9, 74.8, 92.0, 110.0, 126.8, 127.0, 129.7, 131.0, 131.2, 133.8, 134.2, 149.9, 153.3, 157.2, 171.2, 178.7, 180.0. IR (KBr, cm^{-1}) ν_{max}: 2949-2868, 2118, 1716, 1699, 1591-1558, 1456, 1373, 1224, 1078.

HR-MS (APCI) *m/z*: $C_{43}H_{53}ClO_5$ [$(M)^-$], Calcd. 684.3581; Found. 684.3525.

2-chloro-3-(3,28-diacetyl-30-betulinyloxy)-1,4-naphthoquinolinedione ***12e*** Yield: 71%, m.p. 132-133 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 0.82 (s, 3H, CH_3), 0.87 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 1.08 (s, 3H, CH_3), 0.87-2.25 (m, 25H, CH, CH_2), 2.010 (s, 3H, $COCH_3$), 2.12 (s, 3H, $COCH_3$), 2.45 (m, 1H, H-19), 3.88 (d, *J*=10.8 Hz, 1H, H-28), 4.28 (d, *J*=10.8 Hz, 1H, H-28), 4.48 (m, 1H, H-3), 5.07 (s, 1H, H-29), 5.11 (s, 2H, 2xH-30), 5.20 (s, 1H, H-29), 7.77 (m, *2*H, H-6', H-7'), 8.11 (dd, J_{78} = 1.8 Hz, J_{56} = 4.8 Hz, 1H, H-5'), 8.17 (dd, J_{78} = 1.8 Hz, J_{56} = 4.8 Hz, 1H, H-8'). ^{13}C NMR (150 MHz, $CDCl_3$) δ: 14.2, 14.8, 16.0, 16.2, 16.5, 18.2, 20.9, 21.1, 23.7, 25.6, 27.0, 27.9, 29.8, 31.0, 31.5, 34.3, 37.0, 37.8, 38.4, 40.9, 42.7, 46.5, 49.8, 50.2, 55.4, 60.4, 62.5, 80.9, 110.9, 126.9, 127.0, 128.3, 133.9, 134.4, 157.0, 171.2, 178.9, 180.1. IR (KBr, cm^{-1}) ν_{max}: 2949-2876, 1734, 1734, 1716, 1595-1558, 1367, 1246, 1074. HR-MS (APCI) *m/z*: $C_{44}H_{57}ClO_7$ [$(M)^-$], Calcd. 732.3793; Found. 732.3751.

4.2. Biological study

4.2.1. Anticancer activity

The compounds **1**, **7-9** and hybrids **10-12** were tested for cytotoxic activity *in vitro* against seven cancer cell lines: SNB-19 (human glioblastoma, DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), Colo-829 (human malignant melanoma, ATCC, Rockville, MD, USA), C-32 (human amelanotic melanoma, ATCC), MCF-7 (human breast adenocarcinoma, ATCC, Rockville, MD, USA), T47D (human ductal carcinoma, ATCC, Rockville, MD, USA), MDA-MB-231 (human breast adenocarcinoma, ATCC, Rockville, MD, USA), A549 (human lung carcinoma, ATCC, Rockville, MD, USA). As a references compound was used cisplatin. The cultured cells were kept at 37 °C and 5% CO_2. The cells were seeded ($5x10^4$ cells/well/100 mL DMEM supplemented with 10% FCS and streptomycin and penicillin) using 96-well plates (Nunc Thermo Fisher Scientific, Waltham, MA, USA). The tested compounds **7-12** and betulin **1** with the concentration of 0.1-100 µg/mL DMSO were inducted with the cancer cells for 72 hrs. The WST-1-formazan (proliferation reagent WST-1 assay, Roche Diagnostics, Mannheim, Germany) was detected using a microplate reader at 450 nm. Results were expressed as a mean value of at least three independent experiments performed in triplicate.

4.2.2. Apoptosis assay

Transcriptional activity of genes (H3, TP53, CDKN1A, BAX and BCL-2) was rated by real time RT-QPCR using Opticon™ DNA Engine system (MJ Research, Watertown, NY, USA) and QuantTect® SYBR® Green RT-PCR Kit (Qiagen, Hilden, Germany). Cultured cell was incubated with tested compound by 24 h. RNA was extracted using Quick-RNA™ MiniPrep kit columns (Zymo Research, Irvine, CA, USA). The extracted RNA was assessed qualitatively and quantitatively. Amount and purity of the total RNA in extracts was determined spectrophotometrically (HP8452A apparatus, Hewlett Packard, Waldbronn, Germany).

4.3. Molecular docking study

Molecular docking study was done on the crystal structure of human NQO1 protein, which was collected from the Protein Data Bank (PDB) database with the PDB identifier 1H69 [65]. In the experiment, atomic coordinates of 1H69.pdb dimer named AC composed of two identical monomers (A and C) were used. Ligand's molecule was fitted separately into two almost identical binding sites of 1H69.pdb AC dimer (named AC1 and AC2). During docking the FAD molecules were presented into AC binding sites as cofactors.

The 3D structure of hybrids was calculated using Gaussian 16 (revision A.03) program package [66]. Geometry optimization was carried out using the B3LYP exchange–correlation functional with the 6-311+G(d.p) basis set [67]. Molecular docking study was performed with

the Genetic Optimisation for Ligand Docking (GOLD) 5.6.3 [68]. Hermes visualizer was used to prepared the protein to molecular docking study. The active site of protein was defined for the protein residues within 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. All obtained results were visualized in the BIOVIA Discovery Studio software package [69].

Acknowledgements

This work was supported by Medical University of Silesia in Katowice, Poland. Grant No KNW-2-020/N/8/K. Calculations have been carried out using resources provided by Wroclaw Centre for 818 Networking and Supercomputing (http://wcss.pl), Grant No. 382.

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Highlights

- Synthesized a series of betulin-1,4-quinone hybrids
- Evaluated anticancer *in vitro* activity against panel of the human cell line.
- Apoptosis study for selected compound
- Molecular docking showed the most possible interactions between hybrids and NQO1 protein