Original Article
Published: 2025-09-29
https://doi.org/10.64253/022025jept003

Betulinic Acid Fatty Esters and Their Liposomal Formulations: Targeting Cytotoxicity Against Ovarian, Colorectal, and Lung Cancer Cells

Andreea Milan [1,2], Marius Mioc [1,2], Alexandra Prodea [1,2,*] and Codruța Șoica [1,2]

[1]Faculty of Pharmacy, Victor Babes University of Medicine and Pharmacy, 300041 Timișoara, Romania
[2]Research Center for Experimental Pharmacology and Drug Design (X-Pharm Design), "Victor Babes" University of Medicine and Pharmacy, 300041 Timișoara, Romani
*email: alexandra.ulici@umft.ro

Abstract

Despite its notable anticancer potential, betulinic acid (BA) exhibits limited bioavailability that hampers its therapeutic efficacy. To address this issue, betulinic acid fatty esters were previously synthesized by employing stearic (St-BA), palmitic (Pal-BA), and butyric acids (But-BA), and further incorporated into surface-modified liposomal formulations (St-BA-Lip, Pal-BA-Lip, But-BA-Lip). The BA derivatives, as well as their liposomal formulations, were assessed against three human cancer cell lines: colorectal adenocarcinoma Caco-2 cells, ovarian teratocarcinoma PA-1, and alveolar epithelial adenocarcinoma A549 cells. All compounds exhibited cytotoxic effects in a time- and dose-dependent manner, more potently than the positive control, 5-fluorouracil (5-FU). But-BA-Lip exhibited the best anticancer effects against all tested cancer cell lines, recording lower IC50 values than the parent compound (BA) and 5-FU (15.55 μM against Caco-2 cells, 48.16 μM against PA-1 cells, and 25.3 μM against A549 cells). *In silico* molecular docking studies revealed that Pal-BA showed superior binding affinity to AKT/PKB than the parent compound, BA, while But-BA showed enhanced interaction with EGFR1. Both derivatives achieved 93% of the native ligand's docking score, highlighting their therapeutic potential through structural modifications.

Keywords: *anticancer; liposomes; betulinic acid; triterpenes; fatty esters; cytotoxicity assay; molecular docking*

Citation: Milan A, Mioc M, Prodea A, Șoica C. Betulinic Acid Fatty Esters and Their Liposomal Formulations: Targeting Cytotoxicity Against Ovarian, Colorectal, and Lung Cancer Cells. *Journal of Experimental Pharmacology and Toxicology* 2025;2. https://doi.org/10.64253/022025jept003

1. Introduction

Cancer is considered the leading cause of death worldwide and remains a global health problem due to the increase in life expectancy and growing population [1]. Currently, cancer management has become more challenging due to multidrug resistance and the numerous adverse effects caused by conventional chemotherapeutic drugs that hamper patient compliance [2]. As a consequence, researchers have turned their attention to nature, in search of a solution [3]. Natural compounds have always been used as remedies against a wide range of pathologies, from treating infectious diseases to impeding cancer development [4].

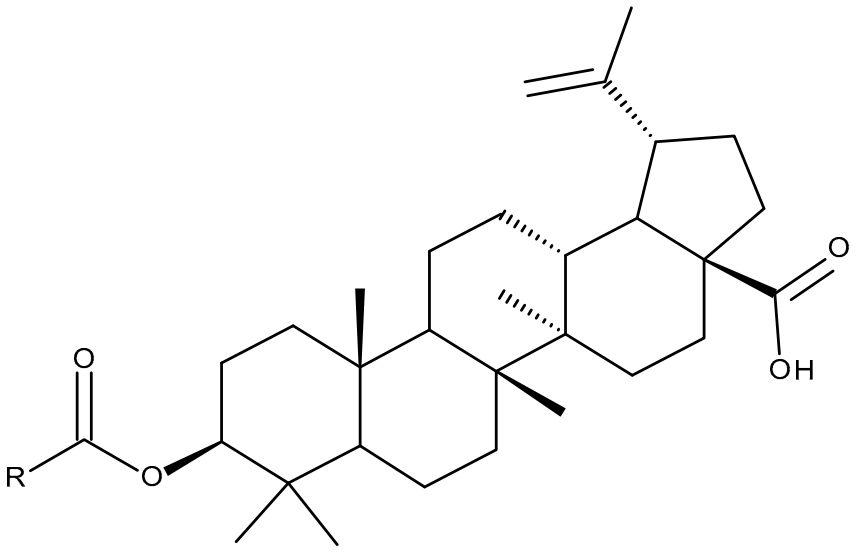
Betulinic acid (BA) is a secondary metabolite isolated from the species of the *Betulaceae* family [5] or obtained through chemical modification [6] or biotransformation [7] of betulin, a similar compound isolated in higher concentrations from the same species (up to 30% mass weight percentage) [8]. The current literature shows that BA has tremendous therapeutic potential, especially in cancer, where it showed selective cytotoxicity in numerous types of cancer [9]. However, its applicability in therapy is limited due to its low bioavailability demonstrated in both in vitro and in vivo studies [10]. Researchers have employed several strategies to resolve this limitation, including (i) structural modification [11], (ii) inclusion in liposomes [12], (iii) encapsulation in cyclodextrins [13] and (iv) formulation of metal nanoparticles [14]. Although the progress made using only one of these strategies is usually limited, an interesting strategy is to combine at least two of these strategies to obtain more potent formulations [15].

Semisynthetic derivatives of BA are mainly obtained through the modification of three key positions of BA, namely C3, C20 and C28 [10]. The derivatives synthesized until now vary greatly in terms of structural complexity, liposolubility

and cytotoxicity [16], and although the majority of the studies tried to increase the hydrophilicity of BA, promising results were also obtained in the case of more lipophilic derivatives, such as BA-fatty acid esters [17]. Some promising derivatives have also been incorporated into liposomes, highly biocompatible and biodegradable nanocarriers able to increase the solubility of a drug, enhancing their cytotoxicity even more [18]. Using surface-modified liposomes such as PEGylated liposomes can increase the lifespan of liposomes in the bloodstream and even promote drug accumulation near the tumor site [19]. Our team has synthesized and evaluated the anticancer effects of BA fatty esters using palmitic, stearic and butyric acids and their respective liposomal formulations against A375 melanoma cells, MCF-7 human breast adenocarcinoma cells, HT-29 colorectal adenocarcinoma cells and NCI-H460 non-small-cell lung adenocarcinoma cell lines, revealing strong cytotoxic results, with some of them surpassing the anticancer effects of 5-fluorouracil (5-FU) [20, 21]. These promising results led our team to expand the in vitro evaluation of these derivatives against other types of ovarian, colorectal, and lung cancer cell lines.

Furthermore, computational techniques have become indispensable tools for accelerating the process of understanding the mechanisms of action of potentially active compounds [22]. Among these, molecular docking stands out as a versatile approach. It utilizes the 3D structure of a biological target, determined through experimental methods (X-ray crystallography, NMR) to dock candidate molecules. These molecules are then ranked based on their binding affinity, assessed using a scoring function, or their complementarity within the conformational space of the target protein's active site [23]. We previously used a molecular docking-based approach to evaluate the theoretical inhibitory capacity of the three synthesized BA esters against protein targets involved in the intrinsic apoptotic pathway, like Bcl-2 and Bcl-XL. Our results showed that But-BA outperformed BA and the other two esters in terms of binding affinity.

The current study presents the cytotoxicity assessment of three previously synthesized BA-fatty acid esters (St-BA, Pal-BA, But-BA represented in **Figure 1**) and their liposomal formulations (St-BA-Lip, Pal-BA-Lip, But-BA-Lip) in three cell lines: PA-1 (ovarian teratocarcinoma), Caco-2 (colorectal adenocarcinoma) and A549 (lung adenocarcinoma). Moreover, herein, we propose to build upon our previous docking work by expanding the scope to include additional protein targets that play critical roles in cancer cell differentiation, migration, and survival, such as phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform (PI3Kα), protein kinase B (AKT/PKB), mammalian target of rapamycin (mTOR), phosphoinositide-dependent kinase-1 (PDK1), epidermal growth factor receptor 1 (EGFR1), vascular endothelial growth factor receptor 2 (VEGFR2) and dual specificity mitogen-activated protein kinase 1 (MEK1).



St-BA: R= CH_3-(CH_2)-$_{16}$

Pal-BA: R=CH_3-(CH_2)-$_{14}$

But-BA: R=CH_3-(CH_2)-$_2$

Figure 1. The chemical structure of St-BA, Pal-BA and But-BA.

2. Materials and Methods

2.1. Chemicals and Reagents

BA and 5-FU are commercially available products (Merck KGaA, Darmstadt, Germany) and were used without additional purification. The preparation and physicochemical characterization of BA fatty esters (St-BA, Pal-BA, But-BA) and their liposomal formulations (St-BA-Lip, Pal-BA-Lip, But-BA-Lip) were previously described [21]. Briefly, BA fatty esters were obtained through a one-way reaction between BA and the acyl chlorides of palmitic, stearic and butyric acids in the presence of 4-dimethylaminopyridine, followed by their inclusion in surfaced-modified liposomes obtained using the thin-layer hydration method.

2.2. Cell Culture

Ovarian teratocarcinoma PA-1 cells, colorectal adenocarcinoma Caco-2 cells and adenocarcinoma human alveolar basal epithelial A549 cells (purchased from American Type Culture Collection ATTC, Lomianki, Poland) were selected for our study. The cells were acquired as frozen items and stored in liquid nitrogen until further usage. A549 cells were cultured in Kaighn's Modification of Ham's F-12 Medium (F-12K) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (100 U/mL). Eagle's Minimum Essential Medium (EMEM) was added with 10% FBS and 1% antibiotic mixture to propagate both PA-1 and Caco-2 cell lines. All cells were maintained in a humidified incubator with 5% CO_2 at 37 °C. An automated cell counter (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in the presence of trypan blue was used to determine cell number.

2.3. The Evaluation of Cell Viability

The viability of PA-1, Caco-2 and A549 cells was determined using the Alamar blue colorimetric assay. All cells were seeded onto 96-well plates at an initial density of 1×10^4 and incubated until reaching 80–85% confluence. Afterward, the old medium was removed using an aspiration station. The cells were stimulated for 24 h and 48 h, respectively, using increasing concentrations (10, 25, 50, 75, 100 μM) of BA fatty esters (Pal-BA, St-BA, But-BA), their respective liposomes (Pal-BA-Lip, St-BA-Lip, But-BA-Lip), the parent compound (BA) and its liposomes (BA-Lip). After 24 h and 48 h, respectively, 20 μL of Alamar blue reagent was added to each well and the cells were again incubated for 3 h. The absorbance measurements were carried out at two wavelengths (570 and 600 nm) using a BioTek Synergy HTX Multimode Reader (Agilent, Santa Clara, CA, USA).

2.4. Statistical Analysis

The statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test (GraphPad Prism version 6.0.0, GraphPad Software, San Diego, CA, USA). The differences between the groups were considered statistically significant if $p < 0.05$, as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

2.5. Molecular Docking

Molecular docking was employed using a previously published method [24]. All protein structures, retrieved from the RCSB Protein Data Bank [25], were optimized as suitable docking targets, with Autodock Tools v1.5.6 (The Scripps Research Institute, La Jolla, CA, USA) (**Table 1**). The 2D structure files for BA and its three esters were sketched using Accelrys Biovia Draw (Dassault Systems BIOVIA, San Diego, CA, USA) and were converted into 3D structures with PyRx's Open Babel [26]. The native ligands were extracted from each protein's PDB file and saved as separate structures, after which, together with the other four ligands, all structures were converted into the docking required format. Molecular docking was achieved with PyRx v0.8 [26] virtual screening software (The Scripps Research Institute, La Jolla, CA, USA) using Vina's scoring function [27]. The docking protocol was validated by re-docking the native ligands into their original protein binding sites. The root means square deviation (RMSD) between the predicted and experimental docking pose of the native ligand was calculated. Molecular docking was performed only for cases with RMSD values below a 2 Å threshold. The grid box which delimitates the docking search space was selected to encompass the surrounding space where the native ligand was bound in each protein. Grid box coordinates are available in **Table 1**. Docking scores were recorded as binding energy values (kcal/mol). Protein–ligand binding interactions were quantified using Accelrys Discovery Studio Visualizer 4.1 (Dassault Systems BIOVIA, San Diego, CA, USA).

Table 1. Protein targets and parameters used for the docking-based screening of BA and its fatty acid esters.

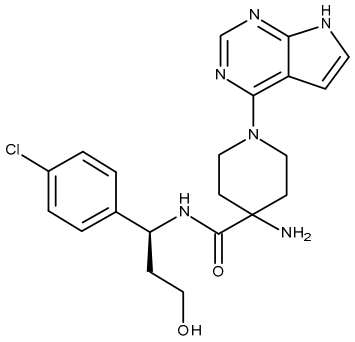
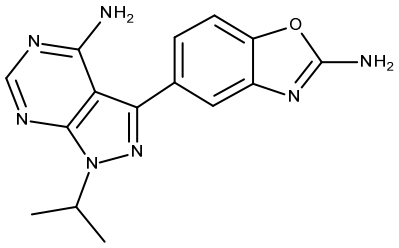
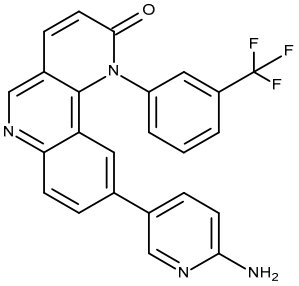
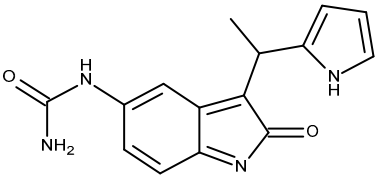
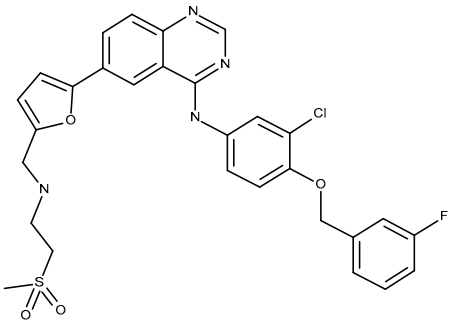
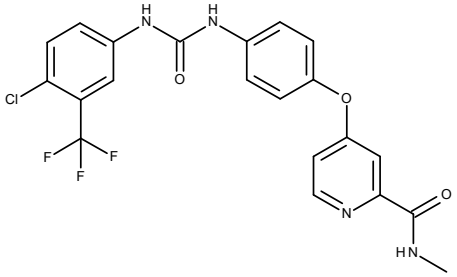
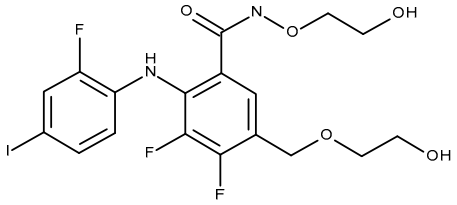
Protein (PDB ID)	Grid box center coordinates	Grid box size	Native ligand	References
AKT/PKB (4GV1)	x = −20.4343 y = 4.6659 z = 10.7795	x = 15.2922 y = 15.9257 z = 18.7420	 Capivasertib	[28]
PI3Kα (6GVF)	x = −15.9445 y = 146.113 z = 26.8339	x = 17.4660 y = 16.4602 z = 16.9395	 Sapanisertib	[29]
mTOR (4JSX)	x = 50.7082 y = −1.4947 z = −48.0854	x = 17.6426 y = 17.6426 z = 17.6426	 Torin 2	[30]
PDK1 (2PE1)	x = −6.8741 y = 43.7199 z = 44.1092	x = 17.5633 y = 17.5633 z = 17.5633	 [2-oxo-3-[1-(1H-pyrrol-2-yl)ethyl]indol-5-yl]urea	
EGFR1 (1XKK)	x = 17.0780 y = 33.6056 z = 38.1419	x = 20.8624 y = 25.0 z = 20.4376	 Lapatinib	[31]

Table 1. *Cont.*

Protein (PDB ID)	Grid box center coordinates	Grid box size	Native ligand	References
VEGFR2 (4ASD)	x = −24.5826 y = −0.3015 z = −11.5944	x = 17.8157 y = 14.2582 z = 20.4376	 Sorafenib	[32]
MEK1 (3DV3)	x = 40.9127 y = −14.2485 z = −3.2645	x = 15.2922 y = 15.9257 z = 18.7420	 3,4-difluoro-2-[(2-fluoro-4-iodophenyl)amino]-N-(2-hydroxyethoxy)-5-[(2-hydroxyethoxy)methyl]benzamide	[33]

3. Results

3.1. The Evaluation of the Cytotoxic Effect of Betulinic Acid Fatty Esters and Their Respective Liposomes

The viability of ovarian teratocarcinoma PA-1 cells, colorectal adenocarcinoma Caco-2 cells and adenocarcinoma human alveolar basal epithelial A549 cells were determined 24 h and 48 h after stimulation with BA fatty esters and their liposomal formulations (10, 25, 50, 75 and 100 μM) by employing the Alamar blue assay.

The incubation of A549 cells for 24 h and 48 h, respectively, has revealed that both the fatty esters and their liposomes decreased cell viability in a time- and dose-dependent manner (**Figure 2**). Pal-BA, Pal-BA-Lip, St-BA-Lip, But-BA and But-BA-Lip were able to inhibit cell viability more aggressively than the parent compound, BA, and even surpass the anticancer effects of 5-FU, expressing lower IC_{50} values 48 h post-stimulation (**Table 2**). The results showed that the liposomal formulations of BA fatty esters inhibited cell proliferation more potently than the free fatty esters, especially when the highest concentration of each compound was applied, as follows: 26.21% ± 3.43 (Pal-BA-Lip 100 μM- 48 h) vs. 28.43% ± (Pal-BA 100 μM- 48 h); 31.91% ± 3.61 (St-BA-Lip 100 μM- 48 h) vs. 48.6% ± 5.6 (St-BA 100 μM- 48 h); 21% ± 1.91 (But-BA-Lip 100 μM- 48 h) vs. 25.91% ± 5.39 (But-BA 100 μM- 48 h).

Table 2. The calculated IC_{50} values (μM) of 5-FU, BA, BA-Lip, Pal-BA, Pal-BA-Lip, St-BA, St-BA-Lip, But-BA and But-BA-Lip on A549, Caco-2 and PA-1 cell lines 24h and 48 h post-stimulation.

Compounds	A549		Caco-2		PA-1	
	24 h	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**
5-FU	64.71	58.63	72.66	67.89	87.44	75.93
BA	>100	44.19	96.7	74.84	>100	94.64
BA-Lip	74.12	40.12	49.01	46.34	91.1	70.76
Pal-BA	72.16	35.47	56.35	29.05	96.88	62.71
Pal-BA-Lip	66.39	29.73	49.66	23.11	94.7	52.62
St-BA	88.16	71.19	57.54	47.24	>100	89.7
St-BA-Lip	62.44	26.09	54.59	26.97	84.72	58.24
But-BA	70.58	38.5	27.27	27.24	87.35	58.59
But-BA-Lip	55.03	25.3	21.4	15.55	63.65	48.16

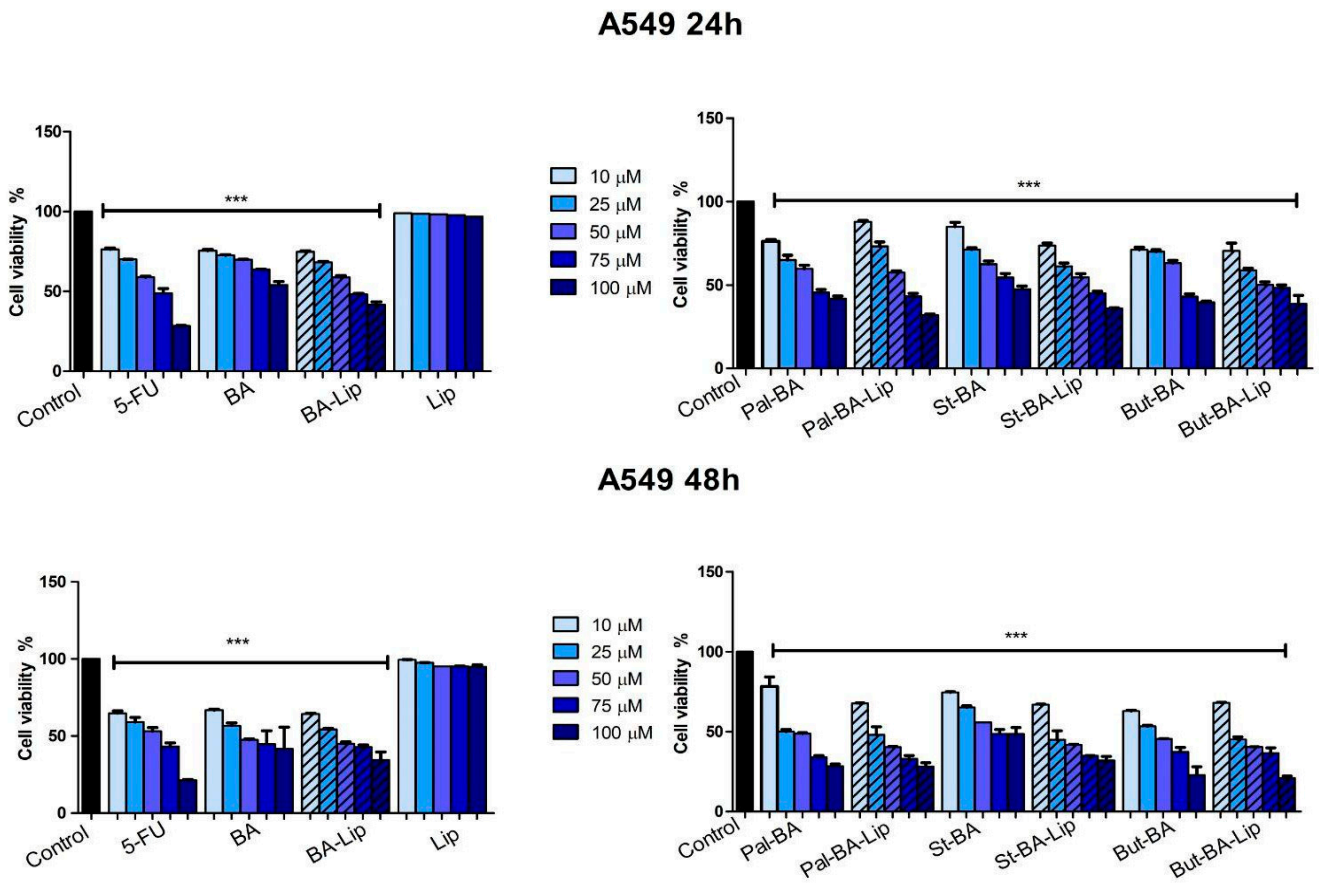


Figure 2. A549 cell viability 24 h and 48 h post-treatment with BA, BA-Lip, 5-FU, St-BA, St-BA-Lip, Pal-BA, Pal-BA-Lip, But-BA, But-BA-Lip and free Lip (10, 25, 50, 75 and 100 μM). The results are expressed as viability percentages compared to the control group (100%) (*** $p < 0.001$ vs. control cells). The data represent the mean values ± SD of three independent experiments effectuated in triplicates.

When tested against Caco-2 cells (**Figure 3**), both the fatty esters and their respective liposomal formulations exerted stronger cytotoxic effects compared to the parent compound BA, and even compared to the positive anticancer control, 5-FU, expressing significantly lower IC_{50} values (**Table 2**). Furthermore, it was shown that the incorporation of BA fatty esters in PEGylated liposomes led to far improved anticancer effects especially when the highest tested concentrations were applied, as follows: 6.71% ± 0.23 (Pal-BA-Lip 100 μM- 48 h) vs. 8.93% ± 0.12 (Pal-BA 100 μM- 48 h); 16.91% ± 0.46 (St-BA-Lip 100 μM- 48 h) vs. 23.6% ± 1.52 (St-BA 100 μM- 48 h); 6.01% ± 2.16 (But-BA-Lip 100 μM- 48 h) vs. 19.41% ± 0.45 (But-BA 100 μM- 48 h), compared to 41.84% ± 3.29 (BA 100 μM- 48 h) and 31.31% ± 0.69 (5-FU 100 μM- 48 h).

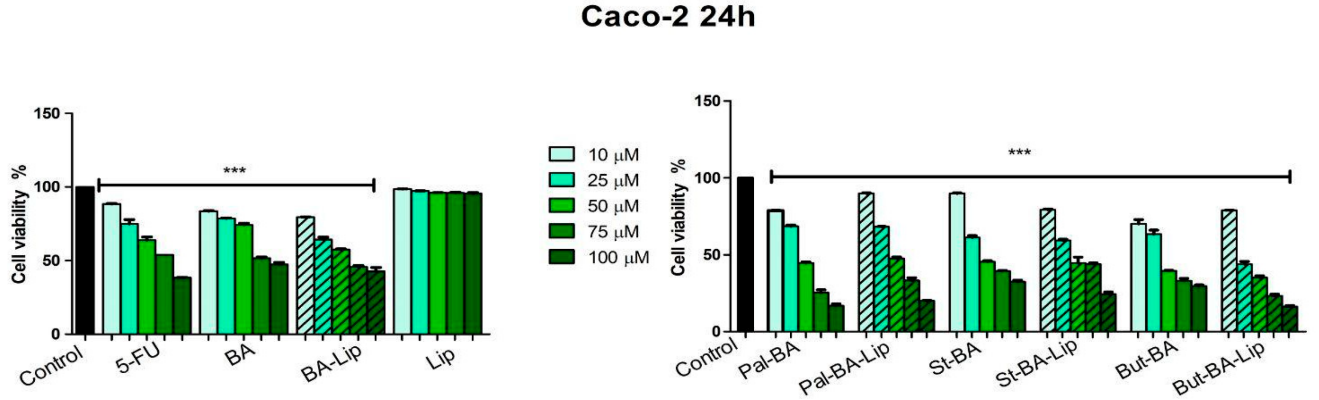


Figure 3. *Cont.*

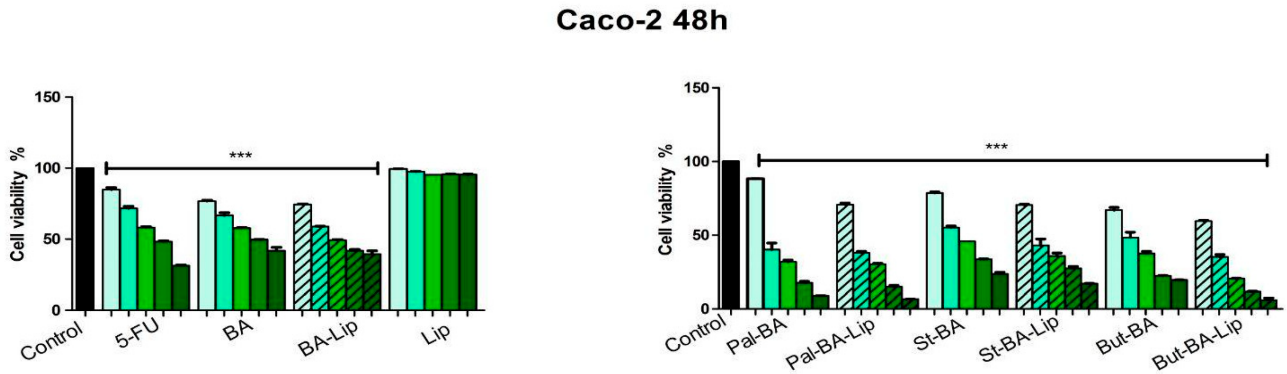


Figure 3. Caco-2 cell viability 24 h and 48 h post-treatment with BA, BA-Lip, 5-FU, St-BA, St-BA-Lip, Pal-BA, Pal-BA-Lip, But-BA, But-BA-Lip and free Lip (10, 25, 50, 75 and 100 μM). The results are expressed as viability percentages compared to the control group (100%) (*** $p < 0.001$ vs. control cells). The data represent the mean values ± SD of three independent experiments effectuated in triplicates.

In terms of anticancer activity against ovarian cancer, it was proven that except for St-BA, both the fatty esters and their respective liposomes exerted stronger cytotoxic effects compared to the positive control 5-FU and the parent compound BA (**Table 2**). The liposomes induced decreased PA-1 cell viability (**Figure 4**), superior to their respective fatty esters, especially at the highest tested concentrations: 38.21% ± 4.72 (Pal-BA-Lip 100 μM- 48 h) vs. 41.43% ± 3.79 (Pal-BA 100 μM- 48 h); 41.41% ± 3.2 (St-BA-Lip 100 μM- 48 h) vs. 48.6% ± 0.92 (St-BA 100 μM- 48 h); 31.5% ± 2.32 (But-BA-Lip 100 μM- 48 h) vs. 39.91% ± 1.67 (But-BA 100 μM- 48 h).

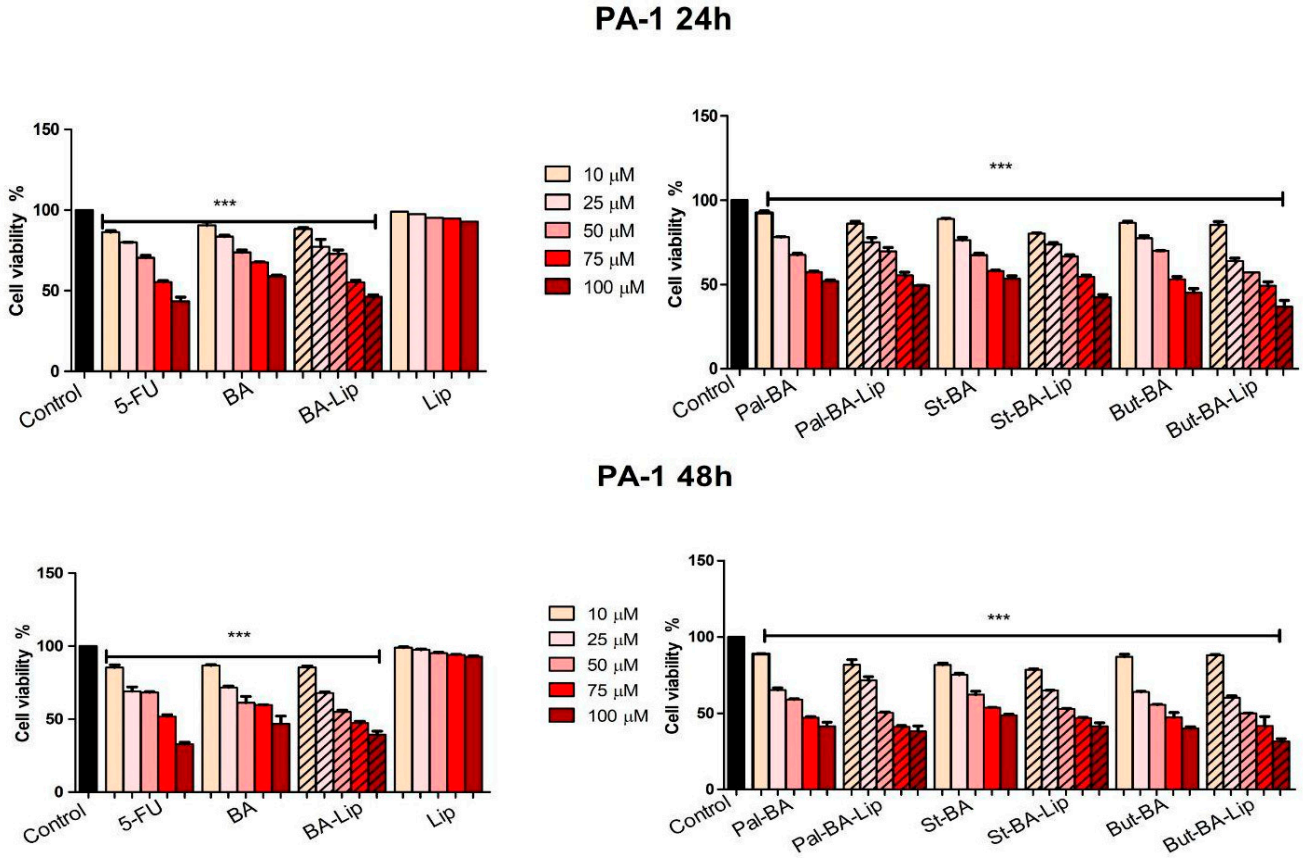


Figure 4. PA-1 cell viability 24 h and 48 h post-treatment with BA, BA-Lip, 5-FU, St-BA, St-BA-Lip, Pal-BA, Pal-BA-Lip, But-BA, But-BA-Lip and free Lip (10, 25, 50, 75 and 100 μM). The results are expressed as viability percentages compared to the control group (100%) (*** $p < 0.001$ vs. control cells). The data represent the mean values ± SD of three independent experiments effectuated in triplicates.

Free liposomes did not induce any significant cytotoxic effect against either tested cancer cell line.

3.2. Molecular Docking

The current study used a molecular docking-based approach to determine possible protein targets for the three BA esters. Subsequently, we docked the BA-derived esters, BA and the native ligands (NLs) (used as positive controls) against the seven protein targets mentioned above (**Table 1**). The obtained docking scores are presented in **Table 3**.

Table 3. Recorded docking scores for BA and its fatty acid esters (binding energy, ΔG kcal/mol).

Compound	Binding free energy ΔG (kcal/mol)						
	PI3KA	**AKT/PKB**	**MTOR**	**PDK1**	**EGFR1**	**VEGFR2**	**MEK1**
NL	−8.8	−9.4	−11.2	−8.8	−10.2	−12	−9.3
BA	−6.6	−7.5	−8.5	−6.8	−9.1	5.2	−5.6
But-BA	−5.9	−8.2	−7.7	−6.7	−9.5	4.5	−6.2
Pal-BA	−6	−8.8	−8.1	−4.8	−7.1	2.2	−5.5
St-BA	−6.3	−7.7	−7.8	−4.3	−6.9	2.2	−5.3

None of the docked compounds exhibited binding affinities lower than those of the NLs, which were used as positive controls. Except in the case of VEGFR2, where all compounds recorded positive binding energy values, and thus cannot form a ligand–protein complex, all docked structures can potentially inhibit each protein target to a certain degree. However, it is difficult to assert which inhibitory effect is stronger since values for the docked NLs vary between −8.8 and −12 kcal/mol. To better assess a more accurate ranking and certain affinities toward a specific protein target, the docking scores were normalized as percentages relative to their respective NL's docking score. These normalized values were then visualized using a radar graph, with each corner representing one of the seven protein targets used (**Figure 5**).

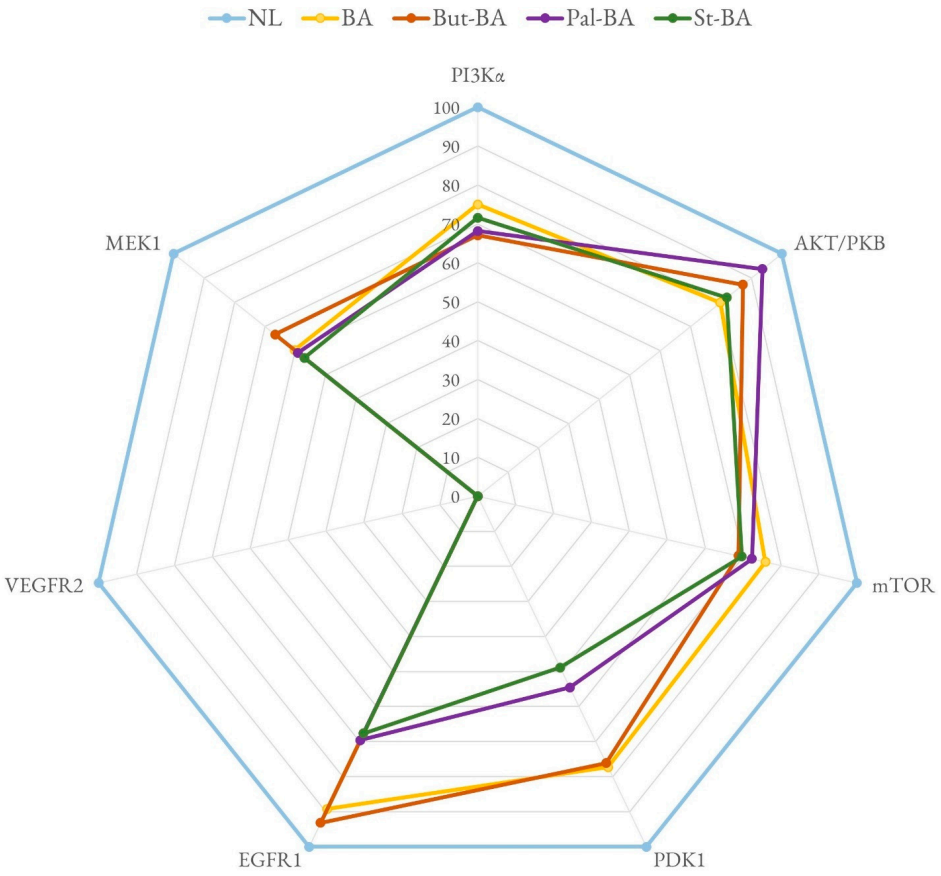


Figure 5. Radar graphs of NL-normalized docking scores (lines) for all docked compounds, against 7 protein targets (corners).

The graph shows that BA derivatives exhibit a general trend toward AKT inhibition, where Pal-BA exceeds the 90% threshold. The same threshold is exceeded by But-BA in the case of EGFR1. In both of these mentioned cases, the two esters scored close to the native ligands (93% in both cases) which are biological inhibitors of their respective proteins.

Pal-BA, due to its large hydrophobic sidechain, interacts with neighboring amino acid residues from the AKT binding site, through multiple hydrophobic interactions (**Figure 6**). On the other hand, But-BA is considerably smaller and thus reaches deep into the EGFR1 binding site. The binding pattern observed here is quite the opposite, where But-BA interacts with binding site amino acids solely through hydrogen bonds (**Figure 7**).

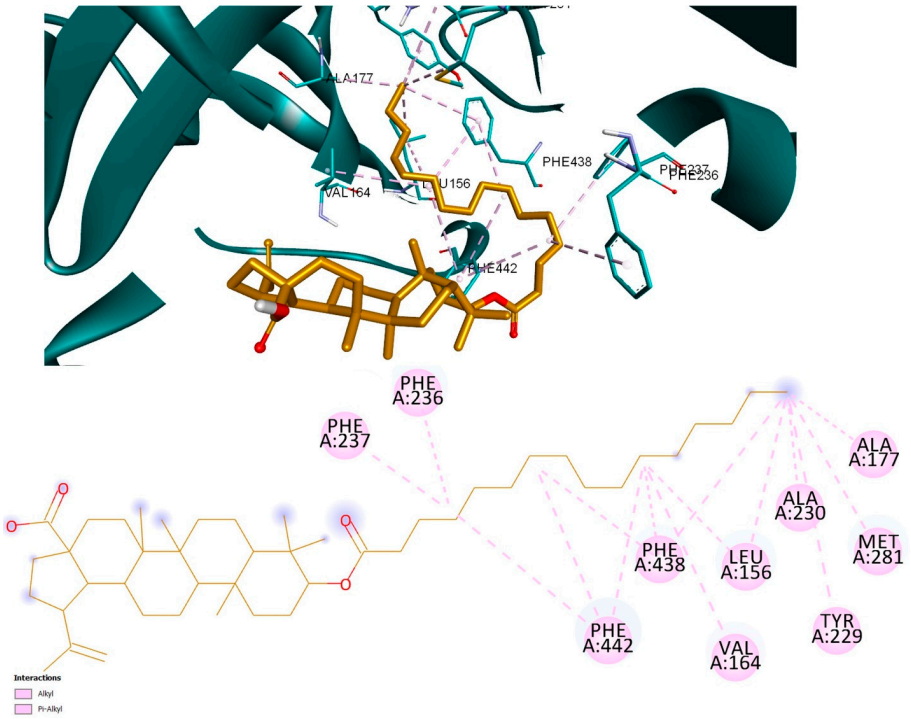


Figure 6. The 3D and 2D representations of Pal-BA docked in the binding site of AKT/PKB (PDB ID: 4GV1).

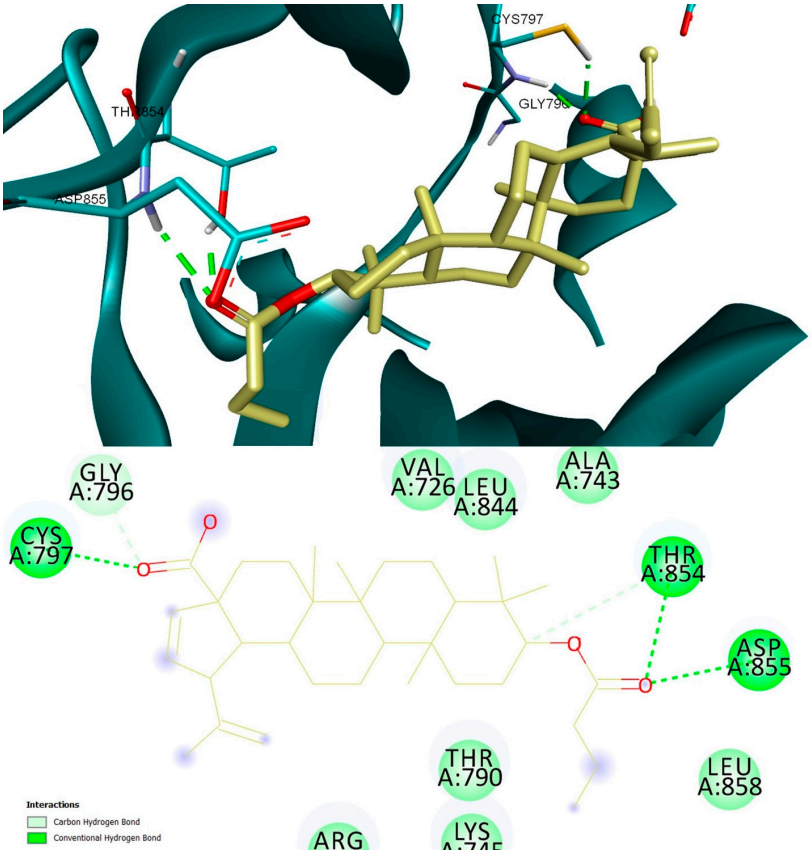


Figure 7. The 3D and 2D representations of But-BA docked in the binding site of EGFR1 (PDB ID:1XKK).

4. Discussions

Natural compounds play an important role in cancer therapy; many plant-derived anticancer agents have emerged within pharmaceutical drug design in recent decades due to their lack of severe side effects, lower cost, and chemical diversity [34]. Pentacyclic triterpenes have been extensively researched due to their valuable medical potential. BA can be considered one of the most promising representatives of the lupane-type class of pentacyclic triterpenes, frequently being reported in the literature for its tremendous pharmacological potential [35]. Despite its potential to become a suitable treatment for several types of maladies, its low bioavailability hampers its therapeutic use [16].

Chemical derivatizations using fatty acids as reaction partners have become an interesting alternative for obtaining bioconjugates with improved biological effects and biocompatibility; an important contribution could be attributed to the intrinsic antifungal, antiviral and anticancer effects of fatty acids [36–38]. Similarly, the fatty ester of lupeol, namely lupeol tricosanoate, was extracted from *S. persica* seeds by El-Desouky et al. and assessed against MCF-7, HT-29 and HepG2 cell lines, revealing potent cytotoxic activity against all tested cancer cells, displaying low IC_{50} values (9.4 μg/mL against MCF-7, 6.85 μg/mL against HT-29 and 12.74 μg/mL against HepG2 cells). To enhance their bioavailability, and hence their biological effects, the synthesized BA fatty esters were encapsulated in surface-modified liposomes with PEG fragments. PEGylated liposomes have been designed to overcome drug leakage and organ accumulation that characterize conventional liposomes [39] in addition, PEG fragments attached to the liposomes' surfaces provide increased stability due to their ability to evade the immune system and lack interaction with plasmatic proteins [40]. Jin et al. has demonstrated the anticancer effect of a cocktail containing BA, honokinol, ginsenoside Rh2 and parthenolide, which were further encapsulated in PEGylated liposomes and tested both in vitro against A549 cells and in vivo on A549 xenografted nude mice. The results revealed synergistic inhibitory effects for the cocktail compared to the phytocompounds alone; moreover, the encapsulation in liposomal formulations led to much improved anticancer and antitumor effects, superior even to those recorded in the cisplatin-treated group [41].

Our team has previously synthesized and tested the BA fatty esters (Pal-BA, St-BA, But-BA) and their respective liposomal formulations (Pal-BA-Lip, St-BA-Lip, But-BA-Lip) against human keratinocyte HaCaT cells, A375 human melanoma cells, MCF-7 human breast adenocarcinoma cells, HT-29 colorectal adenocarcinoma cells and NCI-H460 non-small-cell lung adenocarcinoma cells [20, 21]. The results revealed that both the fatty esters and their respective liposomal formulations induced strong cytotoxic effects in all tested cancer cell lines after prolonged exposure and especially after stimulation with the highest tested concentrations, in some cases even surpassing the cytotoxic effects of the positive control 5-FU. In addition to this, we have decided to further investigate their anticancer potential against other different cancer cell lines, namely PA-1 ovarian teratocarcinoma cells, Caco-2 colorectal adenocarcinoma cells and A549 adenocarcinomic human alveolar basal epithelial cells. The cytotoxic potential of BA fatty esters and their respective liposomes was assessed by employing the Alamar blue assay. It was shown that all compounds inhibited the proliferation of cancer cells in a time- and dose-dependent manner, exhibiting more aggressive cytotoxic effects than the parent compound (BA) and even the positive anticancer control, 5-FU. Furthermore, it was found that in all cases the encapsulation in PEGylated liposomes has led to stronger anticancer effects than the ones observed post-stimulation with the unencapsulated fatty esters. The results obtained from the viability assessment were consistent with the ones proven on the other tested cancer cell lines, demonstrating that the derivative But-BA-Lip exhibited the strongest anticancer effects, displaying much lower IC_{50} values than the parent compound and 5-FU.

Molecular docking serves as a valuable technique for predicting the potential molecular mechanisms of action of active biological compounds by targeting specific proteins [42]. Our observations based on computational analysis suggest that BA fatty esters, especially Pal-Ba and But-BA, have anticancer potential through the specific inhibition of key proteins involved in cancer development. But-BA showed a superior effect compared to BA in inhibiting EGFR1, a member of the EGFR transmembrane glycoprotein family, which is considered a critical target for the development of anticancer drugs [43]. BA inhibited the EGFR pathway in a wild-type EGFR non-small-cell lung cancer cell line, showing a potent affinity toward a wild type of EGFR. Furthermore, BA combined with erlotinib/gefitinib synergistically blocked EGFR and induced tumor suppression in vivo [44], suggesting that But-BA, which, in our study showed superior EGFR affinity compared to BA, could be combined with other EGFR inhibitors for superior cytotoxicity.

5. Conclusions

Current challenges in cancer management emphasize the need for better alternatives to the current drug arsenal. BA's therapeutic potential proven for many types of cancer is hampered by its low bioavailability. An interesting strategy to overcome this limitation is the encapsulation of fatty ester derivatives of BA into PEGylated liposomes. Our study described the biological assessment of BA fatty esters previously synthesized using palmitic, stearic and butyric acids and their respective PEGylated liposomes against Caco-2 colorectal adenocarcinoma cells, PA-1 ovarian teratocarcinoma

cells and A549 adenocarcinomic human alveolar basal epithelial cells. All tested compounds revealed strong cytotoxic effects after prolonged exposure with the highest tested concentrations, with most of them being able to surpass the anticancer effects of the parent compound and the positive control 5-FU by displaying much lower IC_{50} values, and with But-BA-Lip demonstrating the most impressive effects against all tested cancer cell lines. Molecular docking has revealed that 3-O esterification with butyric acid may increase BA's inhibitory potential against EGFR1 while a palmitic acid ester of BA can theoretically outperform BA in terms of AKT/PKB inhibition. Future studies are required to establish the intrinsic molecular mechanism of action responsible for the anticancer effects of both BA fatty esters and their liposomal formulations and assess their in vivo potential effects and their toxicological profile.

Funding

This research received no external funding.

Author contributions

Conceptualization, A.M.; Methodology, A.M., M.M. and A.P.; Software, A.M. and M.M.; Validation, A.M. and A.P.; Formal Analysis, A.M. and M.M.; Data Curation, A.M. and A.P.; Writing—Original Draft Preparation, A.M., M.M. and A.P.; Writing—Review and Editing, C.Ș.; Visualization, A.M. and C.Ș.; Supervision, C.Ș. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Data availability statement

The original contributions presented in the study are included within the article; further questions can be directed to the corresponding author.

Institutional review board statement

Not applicable.

Informed consent statement

Not applicable.

Sample availability

Not applicable.

Additional information

Received: 2025-07-14
Accepted: 2025-09-05
Published: 2025-09-29

Publisher's note

The Journal of Experimental Pharmacology and Toxicology remains neutral with regard to jurisdictional claims in published maps and institutional affiliations. All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright

© 2025 copyright by the authors. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

References

1. Guo J, Huang M, Hou S, Yuan J, Chang X, Gao S, et al. Therapeutic potential of terpenoids in cancer treatment: targeting mitochondrial pathways. Cancer Rep. 2024;7(9):e70006.

2. Rahman MM, Sarker MT, Alam Tumpa MA, Yamin M, Islam T, Park MN, et al. Exploring the recent trends in perturbing the cellular signaling pathways in cancer by natural products. Front Pharmacol. 2022;13:950109. doi: 10.3389/fphar.2022.950109
3. Pavithra R, Khan MR, Khan MS. Recent advancements in natural compounds for cancer therapy and prevention. Phytochem Rev. 2024;23:1835–59. doi: 10.1007/s11101-024-09940-0
4. Sauter ER. Cancer prevention and treatment using combination therapy with natural compounds. Expert Rev Clin Pharmacol. 2020;13(3):265–85. doi: 10.1080/17512433.2020.1738218
5. Cunha AB, Batista R, Castro MÁ, David JM. Chemical strategies towards the synthesis of betulinic acid and its more potent antiprotozoal analogues. Molecules. 2021;26(4):1081. doi: 10.3390/molecules26041081
6. Lou H, Li H, Zhang S, Lu H, Chen Q. A review on preparation of betulinic acid and its biological activities. Molecules. 2021;26(18):5583. doi: 10.3390/molecules26185583
7. Gökfiliz-Yıldız P, Çalışkan S, Yıldırım H, Uzel A. Biotransformation of betulin to betulinic acid by Fusarium lacertarum BRF59, an endophytic fungus isolated from the genus Betula. S Afr J Bot. 2024;167:62–71. doi: 10.1016/j.sajb.2024.01.070
8. Chen H, Xiao H, Pang J. Parameter optimization and potential bioactivity evaluation of a betulin extract from white birch bark. Plants. 2020;9(3):392. doi: 10.3390/plants9030392
9. Aswathy M, Vijayan A, Daimary UD, Girisa S, Radhakrishnan KV, Kunnumakkara AB. Betulinic acid: a natural promising anticancer drug, current situation, and future perspectives. J Biochem Mol Toxicol. 2022;36(12):e23206. doi: 10.1002/jbt.23206
10. Zhong Y, Liang N, Liu Y, Cheng MS. Recent progress on betulinic acid and its derivatives as antitumor agents: a mini review. Chin J Nat Med. 2021;19(9):641–7. doi: 10.1016/S1875-5364(21)60097-3
11. Tang L, Lv S, Wu Z, Qian M, Xu Y, Gao X, et al. Role of betulinic acid derivative SH-479 in triple negative breast cancer and bone microenvironment. Oncol Lett. 2021;22(2):605. doi: 10.3892/ol.2021.12866
12. Liu Y, Gao D, Zhang X, Liu Z, Dai K, Ji B, et al. Antitumor drug effect of betulinic acid mediated by polyethylene glycol modified liposomes. Mater Sci Eng C Mater Biol Appl. 2016;64:124–32. doi: 10.1016/j.msec.2016.03.080
13. Soica C, Danciu C, Savoiu-Balint G, Borcan F, Ambrus R, Zupko I, et al. Betulinic acid in complex with a gamma-cyclodextrin derivative decreases proliferation and in vivo tumor development of non-metastatic and metastatic B164A5 cells. Int J Mol Sci. 2014;15(5):8235–55. doi: 10.3390/ijms15058235
14. Oladimeji O, Akinyelu J, Daniels A, Singh M. Modified gold nanoparticles for efficient delivery of betulinic acid to cancer cell mitochondria. Int J Mol Sci. 2021;22(10):5072. doi: 10.3390/ijms22105072
15. Dutta D, Paul B, Mukherjee B, Mondal L, Sen S, Chowdhury C, et al. Nanoencapsulated betulinic acid analogue distinctively improves colorectal carcinoma in vitro and in vivo. Sci Rep. 2019;9(1):11506. doi: 10.1038/s41598-019-47743-y
16. Nistor G, Trandafirescu C, Prodea A, Milan A, Cristea A, Ghiulai R, et al. Semisynthetic derivatives of pentacyclic triterpenes bearing heterocyclic moieties with therapeutic potential. Molecules. 2022;27(19):6552. doi: 10.3390/molecules27196552
17. Pinzaru I, Trandafirescu C, Szabadai Z, Mioc M, Ledeti I, Coricovac D, et al. Synthesis and biological evaluation of some pentacyclic lupane triterpenoid esters. Rev Chim. 2014;65(7):848–51.
18. Nsairat H, Khater D, Sayed U, Odeh F, Al Bawab A, Alshaer W. Liposomes: structure, composition, types, and clinical applications. Heliyon. 2022;8(5):e09394. doi: 10.1016/j.heliyon.2022.e09394
19. Khan AA, Allemailem KS, Almatroodi SA, Almatroudi A, Rahmani AH. Recent strategies towards the surface modification of liposomes: an innovative approach for different clinical applications. 3 Biotech. 2020;10(4):163. doi: 10.1007/s13205-020-2144-3
20. Milan A, Mioc M, Mioc A, Gogulescu A, Mardale G, Avram Ş, et al. Cytotoxic potential of betulinic acid fatty esters and their liposomal formulations: targeting breast, colon, and lung cancer cell lines. Molecules. 2024;29(14):3399. doi: 10.3390/molecules29143399
21. Milan A, Mioc M, Mioc A, Marangoci N, Racoviceanu R, Mardale G, et al. Exploring the antimelanoma potential of betulinic acid esters and their liposomal nanoformulations. Processes. 2024;12(2):416. doi: 10.3390/pr12020416
22. Kazakova O, Mioc A, Smirnova I, Baikova I, Voicu A, Vlaia L, et al. Novel synthesized N-ethyl-piperazinyl-amides of C2-substituted oleanonic and ursonic acids exhibit cytotoxic effects through apoptotic cell death regulation. Int J Mol Sci. 2021;22(20):10967. doi: 10.3390/ijms222010967
23. Cheng T, Li Q, Zhou Z, Wang Y, Bryant SH. Structure-based virtual screening for drug discovery: a problem-centric review. AAPS J. 2012;14(1):133–41. doi: 10.1208/s12248-012-9322-0
24. Jianu C, Goleţ I, Stoin D, Cocan I, Bujancă G, Mişcă C, et al. Chemical profile of ruta graveolens, evaluation of the antioxidant and antibacterial potential of its essential oil, and molecular docking simulations. Appl Sci. 2021;11(24):11753. doi: 10.3390/app112411753
25. Berman HM. The protein data bank. Nucleic Acids Res. 2000;28(1):235–42. doi: 10.1093/nar/28.1.235
26. Dallakyan S, Olson AJ. Small-molecule library screening by docking with PyRx. Methods Mol Biol. 2015;1263:243–50.
27. Trott O, Olson AJ. AutoDock vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem. 2010;31(2):455–61. doi: 10.1002/jcc.21334
28. Addie M, Ballard P, Buttar D, Crafter C, Currie G, Davies BR, et al. Discovery of 4-Amino-N-[(1S)-1-(4-chlorophenyl)-3-hydroxypropyl]-1-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)piperidine-4-carboxamide (AZD5363), an orally bioavailable, potent inhibitor of Akt kinases. J Med Chem. 2013;56(5):2059–73. doi: 10.1021/jm301762v
29. Ouvry G, Clary L, Tomas L, Aurelly M, Bonnary L, Borde E, et al. Impact of minor structural modifications on properties of a series of mTOR inhibitors. ACS Med Chem Lett. 2019;10(11):1561–7. doi: 10.1021/acsmedchemlett.9b00401
30. Yang H, Rudge DG, Koos JD, Vaidialingam B, Yang HJ, Pavletich NP. mTOR kinase structure, mechanism and regulation. Nature. 2013;497(7448):217–23. doi: 10.1038/nature12122
31. Wood ER, Truesdale AT, McDonald OB, Yuan D, Hassell A, Dickerson SH, et al. A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib). Cancer Res. 2004;64(18):6652–9. doi: 10.1158/0008-5472.CAN-04-1168
32. Mctigue M, William B, Chen JH, Deng Y li, Solowiej J, Kania RS. Molecular conformations, interactions, and properties associated with drug efficiency and clinical performance among VEGFR TK inhibitors. Proc Natl Acad Sci USA. 2012;109(45):18281–9. doi: 10.1073/pnas.1207759109

33. Tecle H, Shao J, Li Y, Kothe M, Kazmirski S, Penzotti J, et al. Beyond the MEK-pocket: can current MEK kinase inhibitors be utilized to synthesize novel type III NCKIs? Does the MEK-pocket exist in kinases other than MEK? Bioorg Med Chem Lett. 2009;19(1):226–9. doi: 10.1016/j.bmcl.2008.10.108
34. Nobili S, Lippi D, Witort E, Donnini M, Bausi L, Mini E, et al. Natural compounds for cancer treatment and prevention. Pharmacol Res. 2009;59(6):365–78. doi: 10.1016/j.phrs.2009.01.017
35. Oliveira-Costa JF, Meira CS, das Neves MVG, Dos Reis BPZC, Soares MBP. Anti-inflammatory activities of betulinic acid: a review. Front Pharmacol. 2022;13:883857. doi: 10.3389/fphar.2022.883857
36. uimarães A, Venâncio A. The potential of fatty acids and their derivatives as antifungal agents: a review. Toxins. 2022;14(3):188. doi: 10.3390/toxins14030188
37. Feng Y, Yang Y, Zou S, Qiu S, Yang H, Hu Y, et al. Identification of alpha-linolenic acid as a broad-spectrum antiviral against zika, dengue, herpes simplex, influenza virus and SARS-CoV-2 infection. Antiviral Res. 2023;216:105666. doi: 10.1016/j.antiviral.2023.105666
38. Du A, Wang Z, Huang T, Xue S, Jiang C, Qiu G, et al. Fatty acids in cancer: metabolic functions and potential treatment. MedComm Oncol. 2023;2(1):e25. doi: 10.1002/mog2.25
39. Sofou S. Surface-active liposomes for targeted cancer therapy. Nanomedicine. 2007;2(5):711–24. doi: 10.2217/17435889.2.5.711
40. Mineart KP, Venkataraman S, Yang YY, Hedrick JL, Prabhu VM. Fabrication and characterization of hybrid stealth liposomes. Macromolecules. 2018;51(8):3184–92. doi: 10.1021/acs.macromol.8b00361
41. Jin X, Yang Q, Cai N, Zhang Z. A cocktail of betulinic acid, parthenolide, honokiol and ginsenoside Rh2 in liposome systems for lung cancer treatment. Nanomedicine. 2019;15(1):41–54. doi: 10.2217/nnm-2018-0479
42. Agu PC, Afiukwa CA, Orji OU, Ezeh EM, Ofoke IH, Ogbu CO, et al. Molecular docking as a tool for the discovery of molecular targets of nutraceuticals in diseases management. Sci Rep. 2023;13(1):13398. doi: 10.1038/s41598-023-40160-2
43. Singh D, Kumar Attri B, Kaur Gill R, Bariwal J. Review on EGFR inhibitors: critical updates. Mini-Rev Med Chem. 2016;16(14):1134–66. doi: 10.2174/1389557516666160321114917
44. Wang H, Du X, Liu W, Zhang C, Li Y, Hou J, et al. Combination of betulinic acid and EGFR-TKIs exerts synergistic anti-tumor effects against wild-type EGFR NSCLC by inducing autophagy-related cell death via EGFR signaling pathway. Respir Res. 2024;25(1):215. doi: 10.1186/s12931-024-02844-9