„VICTOR BABEȘ” UNIVERSITY OF MEDICINE AND PHARMACY FROM TIMIȘOARA
FACULTY OF PHARMACY
DEPARTMENT II

MIOC MARIUS-NICOLAE

ABSTRACT

DESIGN, SYNTHESIS, AND BIOLOGICAL ACTIVITY ASSESSMENT OF POTENTIAL ANTITUMOR HETEROCYCLIC TRITERPENE DERIVATIVES

Scientific coordinator

PROF. UNIV. DR. ȘOICA CODRUȚA

T i m i ș o a r a
2 0 2 3

TABLE OF CONTENTS

ABSTRACT

Numerous plant species contain secondary metabolites known as pentacyclic triterpenes, which typically contain lupane, oleanane, or ursane as their primary scaffolds. The anti-inflammatory, anticancer, and immunomodulatory properties of betulinic, oleanolic, and ursolic acids have been intensively studied. To remove their disadvantages, such as poor water solubility and low oral bioavailability, a large number of semisynthetic derivatives were developed in order to eliminate these drawbacks. Consequently, pentacyclic triterpenes can be utilized as platforms for the synthesis of semisynthetic derivatives via a variety of chemical processes.

As for pentacyclic triterpenes, betulinic (BA), ursolic (UA), and oleanolic (OA) acids have been identified as effective and selective anticancer agents with well-studied mechanisms of action. Triterpenic acids exhibit very low water solubility, which was correlated to their poor oral bioavailability; in addition to various technical approaches to address this issue, such as cyclodextrin complexation or nanoformulations, various chemical modifications were performed on the molecules of the compounds, leading to the synthesis and evaluation of numerous semisynthetic derivatives.

Throughout the present doctoral research, we propose the synthesis and biological evaluation of BA, OA, and UA derivatives containing triazole rings, against melanoma. The secondary objective is to obtain a GNP-based nanoformulation of the synthesized triazole-triterpenic acid derivatives and assess their antimelanoma potential to determine if these formulations enhance the biological activity of the three triterpene derivatives. In addition, this study attempts to highlight a possible mechanism of action of the newly synthesized compounds and their GNP formulations in relation to their antiproliferative effect. Computational studies will also be caried out

to evaluate if the chemichal modiffications have a positive impact in the binding afinity of these triterpenic acids towards their targeted proteins.

In the first study of this doctoral research, we propose the synthesis and physical-chemical evaluation of 1-hydroxybenzotriazole esters BA, OA, and UA. Furthermore, given that these esters, like their parent molecules, are highly lipophilic structures, which can impede the antiproliferative potential of the molecule, and given that gold nanoparticles lack cytotoxic effects, are easily accessible, and can bind different ligands such as triazole rings (found in the structure of our esters), another goal of this research was to formulate the obtained benzotriazole-triterpenic acid ethers as GNP conjugates.

The three compounds (BA-HOBt, OA-HOBt, and UA-HOBt) were synthesized via the DCC-aided HOBt esterification of BA, OA, and UA. The synthesis pathway is shown in Figure 1.

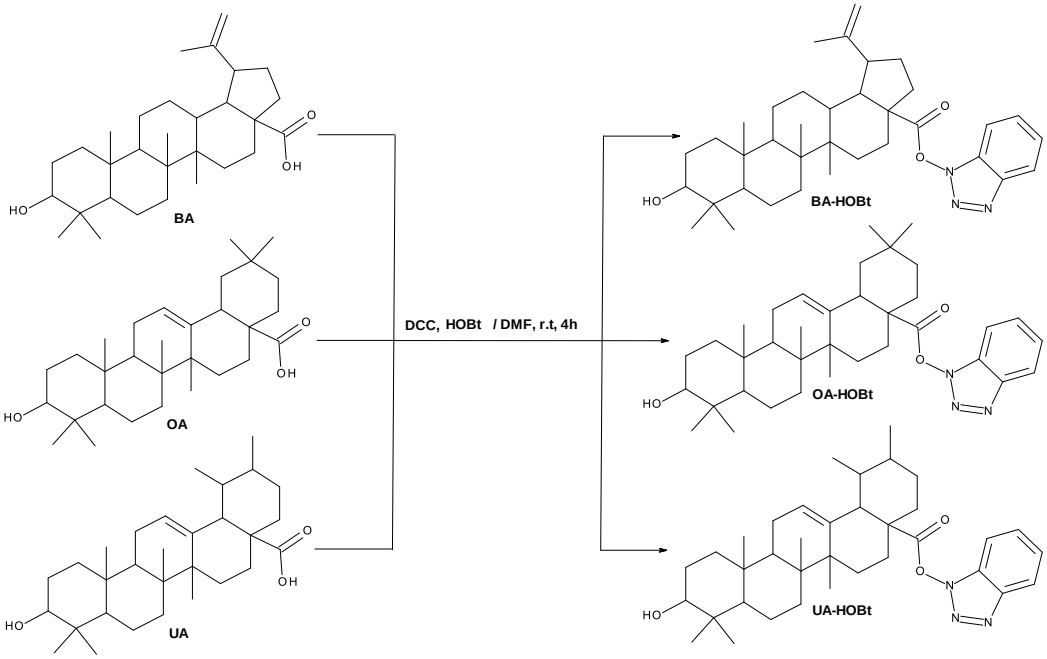

Figure 1. Synthesis pathway for the triterpenic acid-benzotriazole esters (BA-HOBt, OA-HOBt, and UA-HOBt).

The identity of the three formed compounds was validated through FTIR, LC-MS, and NMR analysis. The FTIR spectra of the benzotriazolyl esters show both characteristic signals of triterpenic acids (O-H stretch around 3440 cm^{-1}, C-H two peek stretch around 2930 and 2870 cm^{-1} and C=O stretch around 1800 cm^{-1}) and of benzotriazole as well (=C-N stretch around 1240 cm^{-1}). LC-MS analysis confirmed the masses of all three synthesized compounds.

Nevertheless, the most accurate analysis performed for the identity validation of all compounds was the NMR spectroscopy. ^{1}H NMR spectra o all synthesized compounds show the absence of the carboxyl group proton while revealing at the same time the chemical shifts of the phenyl carbocyclic protons in the 8.5-7.5 ppm region. At the same time in the ^{13}C NMR spectra, apart from the chemical shifts corresponding to the triterpenic core carbons, chemical shifts of the six phenyl carbons are observable in the 145-120 ppm region as well.

For the synthesis of citrate-capped GNP, trisodium citrate was used to reduce chloroauric acid. The functionalization of citrate-capped GNP with the triterpenic acid esters BA-HOBt, OA-HOBt, and UA-HOBt was easily accomplished through ultrasonic-assisted dispersion of the triterpenic acid derivative into the GNP nanosuspension, which allowed the 1,2,3-triazole moiety to attach to the GNP surface. Figure 2 depicts the synthesis technique utilized to create citrate-capped and triterpene-loaded GNP.

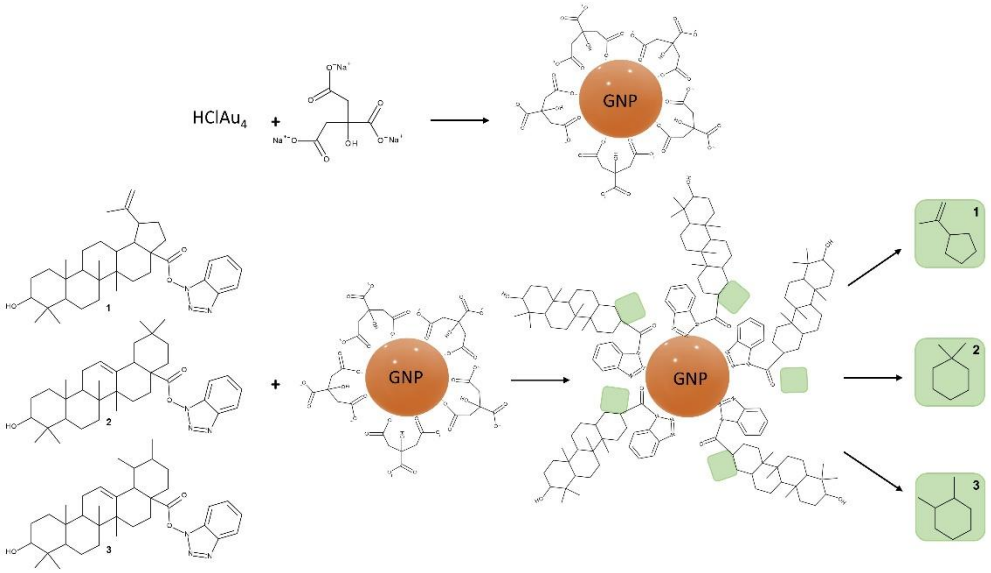

Figure 2. Synthesis of citrate-capped GNP, BA-HOBt (1) loaded GNP, OA-HOBt (2) loaded GNP, and UA-HOBt (3) loaded GNP; conditions: i. H2O, boiling; ii. Ultrasound-assisted dispersion, 1 hour.

UV-VIS spectroscopy was used to observe GNP synthesis and functionalization. The analysis validated the synthesis of GNP by the citrate reduction technique. The triterpene derivative functionalization of GNP was confirmed using FTIR spectroscopy. The FTIR spectra of GNP are comparable with recent results on citrate GNP and exhibit the two distinctive symmetric and anti-symmetric stretch vibration peaks of citrate carboxylate (1593 and 1402 cm^{-1}, respectively) in addition to the O-H stretch vibration at 3439 cm^{-1}. In each instance, the FTIR spectra demonstrate that GNP was successfully functionalized by the triterpenic acid derivative.

TEM examination uncovered stable spherical GNP nanoparticles with diameters between 13 and 25 nm; triterpene functionalization of GNP did not affect the shape or diameter of the functionalized nanoparticles (Figure 13). However, dynamic light scattering (DLS) studies showed that BA-HOBt GNP, OA-HOBt GNP, and UA-HOBt GNP increased in hydrodynamic size and had a slightly higher polydispersity index (PDI).

In the second study triterpene-benzotriazole esters and their GNP conjugates were tested against melanoma cells in this context. In this section, in addition to evaluating the antimelanoma cytotoxic activity of the obtained samples, we also explored a potential mechanism of action associated with the antiproliferative activity.

Using the Alamar Blue assay, the effects of BA-HOBt (**1**), OA-HOBt (**2**), and UA-HOBt (**3**), their corresponding acids (BA, OA, and UA), and GNP conjugates (BA-HOBt GNP, OA-HOBt GNP, and UA-HOBt GNP) on the viability of healthy human keratinocytes HaCaT and human melanoma A375 cells were determined following a 24-hour treatment period.

The triterpene derivatives and their GNP conjugates exhibited significantly greater dose-dependent cytotoxicity against the A375 melanoma cell line than the parent compounds. The samples examined had minimal to no cytotoxic effects on non-cancerous cells (HaCaT).

All of the samples affected the morphology of the cells in a manner consistent with apoptosis. This was primarily observed in the examined cancer cell line, while the normal cells subjected to this analysis showed signs of apoptosis only at the highest tested concentrations and only few cases.

PCR results revealed a decrease in the relative fold expression of the antiapoptotic Bcl-2 and an increase in the pro-apoptotic Bax genes, in cancer cells treated with the test compounds and GNP-conjugates, confirming the pro-apoptotic effect hypothesis.

Triterpene derivatives and their GNP conjugates are selective inhibitors of mitochondrial function in A735 melanoma cells, as determined by high-resolution respirometry, where normal cells treated with the same did not show any signs of mitochondrial impairment.

In the last study of the current work, considering the biological data obtained for our three triterpene derivatives, we employed molecular docking to evaluate the potential of these esters to inhibit the anti-apoptotic protein Bcl-2. We also docked the parent structures, BA, OA and UA respectively, to comparatively assess if there is an increase in the theoretical affinity towards Bcl-2 due to the employed structural changes. In comparison to their respective parent compounds BA, OA, and UA, compounds 1-3 exhibit a notable decrease in binding energy and an increase in affinity for Bcl-2, as revealed by docking results. We can also observe that the same compounds 1–3 have docking scores that are relatively close to those of the native ligand (NL). This aspect is closely associated with the structural transformation that has occurred and the interactions between these compounds and the active site of Bcl-2.

Compound 1 most closely resembles the NL in terms of interactions with amino acid residues (Asp70, Phe71, and Met74), with the benzotriazole ring acting similarly to the tetrahydroisoquinoline ring in the NL structure (Figure 3). While compound 3 also forms hydrophobic interactions with Ala108 and Arg105 and has an orientation opposite to that of compound 1 (Figure 3), compound 2 behaves somewhat differently. In this instance, the benzotriazole ring interacts with Arg105 via multiple hydrogen bonds (HB), and the structure also interacts with Asp70 at the opposite pole, firmly anchoring the molecule within the binding pocket (Figure 3). Nevertheless, the benzotriazole ring could be a useful option in the design of extremely potent triterpene-based Bcl-2 inhibitors.

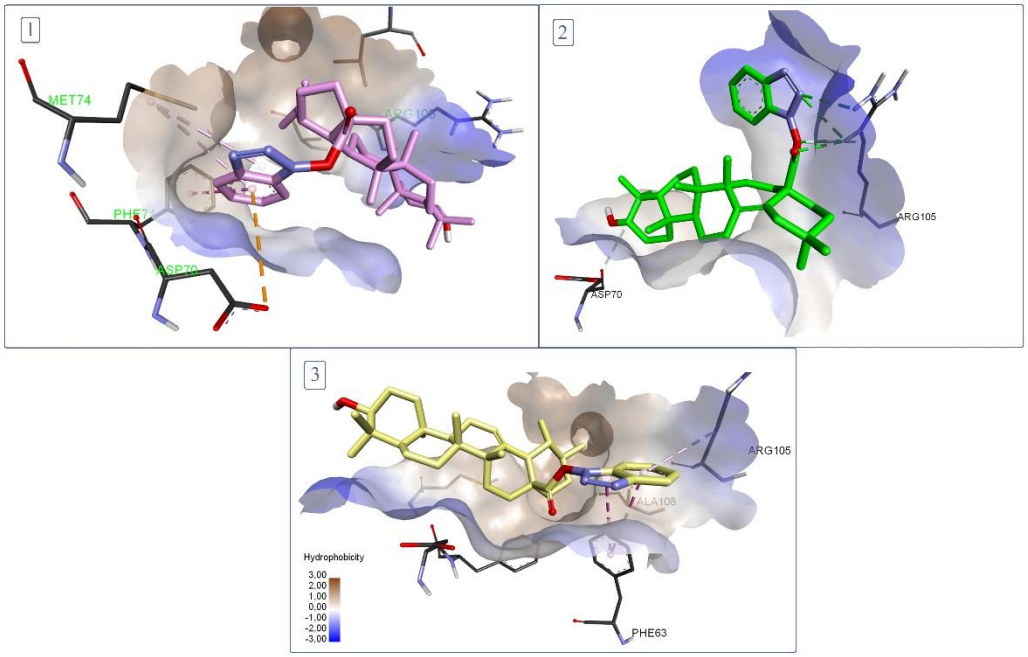

Figure 3. Bcl-2 (2W3L) in complex with compounds 1 (pink), 2 (green), and 3 (yellow); HB interactions are represented by green dotted lines, hydrophobic interactions by purple dotted lines, and electrostatic interactions by orange dotted lines; interacting amino acids are depicted as dark gray sticks

Collectively, we conclude that hydroxi-benzotriazole esterification is a practical approach that increases the apoptotic effect of triterpenic acids in melanoma and can be used as a valuable tool in the development of future semisynthetic triterpenoid compounds with enhanced anticancer potential.

The antimelanoma effect of benzotriazole-triterpenic acid esters can be further enhanced by GNP formulation. Additional research is nevertheless required to see if GNP functionalization of other triazole-bearing triterpene derivatives also increases their antimelanoma effects. It is possible that this could pave the way for a novel and efficient way for the development of GNP-aided delivery of triterpenoid compounds, and also add valuable information to the lacking literature regarding the use of GNP as versatile carrier for pentacyclic triterpenes.