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Synthesis of the Vitamin E Amino Acid Esters with an Enhanced Anticancer Activity and *In Silico* Screening for New Antineoplastic Drugs

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Abstract

Tocopherols and tocotrienols belong to the family of vitamin E (VE) with the well-known antioxidant properties. For certain α-tocopherol and γ-tocotrienol derivatives used as the lead compounds in this study, antitumor activities against various cancer cell types have been

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reported. In the course of the last decade, structural analogs of VE (esters, ethers and amides) with an enhanced antiproliferative and proapoptotic activity against various cancer cells were synthesized. Within the framework of this study, seven amino acid esters of α-tocopherol (**4a-d**) and γ-tocotrienol (**6a-c**) were prepared using the EDC/DMAP reaction conditions and their ability to inhibit proliferation of the MCF-7 and MDA-MB-231 breast cancer cells and the A549 lung cancer cells was evaluated. Compound **6a** showed an activity against all three cell lines (IC_{50}: 20.6 μM, 28.6 μM and 19 μM for the MCF-7, MDA-MB-231 and A549 cells, respectively), while compound **4a** inhibited proliferation of the MCF-7 (IC_{50}=8.6 μM) and A549 cells (IC_{50}=8.6 μM). Ester **4d** exerted strong antiproliferative activity against the estrogen-unresponsive, multi-drug resistant MDA-MB-231 breast cancer cell line, with IC_{50} value of 9.2 μM. Compared with the strong activity of compounds **4a**, **4d** and **6a**, commercial α-tocopheryl succinate and γ-tocotrienol showed only a limited activity against all three cell lines, with IC_{50} values greater than 50 μM. Investigation of the cell cycle phase distribution and the cell death induction confirmed an apoptosis of the MDA-MB-231 cells treated with **4d**, as well as a synergistic effect of **4d** with the known anticancer drug doxorubicin. This result suggests a possibility of a combined therapy of breast cancer in order to improve the therapeutic response and to lower the toxicity associated with a high dose of doxorubicin. The stability study of **4d** in human plasma showed that ca. 83% initial concentration of this compound remains in plasma in the course of six hours incubation. The ligand based virtual screening of the ChEMBL database identified new compounds with a potential antiproliferative activity on MCF-7 and on multi-drug resistant MDA-MB 231 breast cancer cells.

Keywords: vitamin E, esters, anticancer, synthesis, screening

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1. Introduction

Vitamin E (VE) is a generic term that represents two families of compounds, i.e., tocopherols (T) and tocotrienols (T_3). Both families share similar chemical structure characterized by a saturated (T) or unsaturated (T_3) phytyl chain attached to the chromane ring. Moreover, each family has four isomers (α, β, γ, δ) that differ in the number and positions of methyl groups in the chromanol ring (Eitenmiller and Lee, 2004). Although the VE family members are the best known for their antioxidant activity (Jiang, 2014), recent studies have shown that they exhibit many antioxidation-independent effects such, as inhibition of protein kinase C, 5-lipoxygenase and phospholipase A2, activation of protein phosphatase 2A and diacylglycerol kinase, inhibition of cell proliferation, platelet aggregation and monocyte adhesion (Zingg and Azzi, 2014). Nowadays, special attention is paid to the antiproliferative effect of VE. Tocopherols and tocotrienols exert antitumor activity on various types of cancer cells, at the same time being selective to normal cells (Moya-Camarena and Jiang, 2012; Ling et al., 2012). Direct comparison between the two VE subclasses showed that tocotrienols are generally more potent than tocopherols in reducing proliferation of tumor cells, with δ-tocotrienol being the most potent and α-tocopherol the least potent isomer. (McIntyre et al., 2000; Guan et al., 2012; Campbell et al., 2006).

Although an exact mechanism by which the VE family members inhibit carcinogenesis remains unknown, several models are proposed, including apoptosis via the cleavages of poly (ADP-ribose) polymerase (PARP) (Park et al., 2010; Patacsil et al., 2012), or inhibition of the nuclear factor kappa-light-chain-enhancer of the activated B cells (NF-κB) signaling pathway (Sun et al., 2014). Modulation of angiogenesis is now recognized as a strategy for preventing various angiogenesis-mediated disorders, including solid tumors (Zhao and Adjei, 2015). Recent studies

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confirmed that the palm tocotrienols exhibit anti-angiogenic properties through a decreased expression of vascular endothelial growth factor in human umbilical vein endothelial cells (Selvaduray et al., 2012), human hepatocellular carcinoma cells (Siveen et al., 2014) and murine mammary cancer cells (Selvaduray et al., 2010). Synthetic analogs of tocopherols showed more potent effects than the natural forms in inducing dephosphorylation of the protein kinase B (Akt) and thereby in inhibiting the Akt-mediated signals that promote cell metabolism, proliferation, and motility (Huang et al., 2013). The experimental findings showed that the combined treatment with γ-T3 and the antihyperlipidemic drugs such, as erlotinib and gefitinib, resulted in a synergistic inhibition *in vitro* of highly malignant +SA mouse mammary epithelial cells, due to a down-regulation of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase activity, which is elevated in cancer cells (Goldstein et al., 2006; Sylvester, 2012). Furthermore, a combined treatment with a low dose of γ-T_3 and an anti-inflammatory drug celecoxib can greatly improve a therapeutic response in the treatment of breast cancer through the inhibition of the cyclooxygenase-2 (COX-2) activity (Shirode and Sylvester, 2011).

In the course of the last decade, the VE derivatives with a modified hydroxyl group have been synthesized and tested for their anticancer activity. These redox-silent analogs that lost their antioxidative potency showed an improved antiproliferative activity and stability relative to the parent compounds (Behery et al., 2010; Birringer et al., 2003; Djuric et al., 1997; Elnagar et al., 2010). The most prominent VE analog, α-tocopheryl succinate (α-TOS), was shown to exhibit anticancer properties in several cancer models, while retaining low toxicity to healthy cells (Huang et al., 2009; Dong et al., 2008). Selectivity of the VE analogs to cancer cells can be explained by higher esterase activity in normal cells that cleave α-TOS and similar agents, to produce the nonapoptogenic α-T (Neuzil et al., 2004). Due to their lesser susceptibility to

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oxidation, the succinate and acetate ester of α-tocopherol became commercially available for use in the supplements and cosmetics (Zinng, 2007). In order to enhance stability of the VE derivatives *in vivo*, the ester bond was replaced by an amide bond or by an even more resistant ether bond. The amide and ether VE derivatives showed an additional enhancement of the proapoptotic activity on the MDA-MB-453 breast cancer, Jurkat T lymphoma, the U937 leukemic and the Meso-2 malignant mesothelioma cells (Tomic-Vatic et al., 2005; Nishikawa et al., 2003).

Derivatization of the phenolic hydroxyl of α-T as an amino acid ester conjugate (Arya et al., 1998) introduces the NH_2 group that is protonated in an acidic medium of the cancer tissue (Montcourrier et al., 1994). The protonated amino group together with the long alkyl chain of α-T acts as a cationic detergent and interacts with the lysosomal and mitochondrial membranes in cancer cells, causing an increased membrane permeability and destabilizing lysosomes (Neuzil et al., 2001). Contrary to α-T and α-TOS, the α-tocopheryl-lysine ester exerts an antiproliferative activity against the human MCF-7 breast cancer cell line (Arya et al., 1998).

Within the framework of this study, we synthesized four amino acid esters of α-T (**1**) with lysine (**4a**), proline (**4b**), glutamine (**4c**), and asparagine (**4d**), and three amino acid esters of γ-T_3 (**2**) with lysine (**6a**), proline (**6b**) and glutamine (**6c**). Based on the results from our previous QSAR studies (Nikolic and Agbaba, 2009; Gagic et al., 2016), esterification of the phenol group at position C6 of the chromane ring with at least one hydrogen bond donor and/or hydrogen bond acceptor present in the ester chain, and an increased polarity of the molecule, exert positive effect on the antiproliferative activity of the synthesized compounds. This activity was tested on the human MCF-7 and MDA-MB-231 breast cancer cell lines and the A549 lung cancer cell lines, and compared with the commercially available α-TOS and γ-T_3. Since an exact anticancer

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mechanism and drug targets of these compounds are not known, we could only perform a ligand based virtual screening (LBVS), employing the ChEMBL database to identify the new compounds with a potential antiproliferative activity on human MCF-7 and MDA-MB-231 breast cancer cell lines.

2. Materials and methods

2.1. Chemistry

For the chemical synthesis, commercial α-tocopherol, Nα,Nε-di-Cbz-L-lysine, N-Cbz-D-proline, Nα-Cbz-L-asparagine, Nα-Cbz-L-glutamine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), 4-dimethylaminopyridine (DMAP) and 10% Pd on the activated carbon were used. γ-Tocotrienol was obtained from ExcelVite Sdn Bhd. (Malaysia) company (www.excelvite.com). The purity of the compounds was checked using the HPLC and TLC methods. TLC was carried out using the commercially precoated silica gel plates (Merck, Germany). The chromatographic spots were visualized in UV light at 254 nm, or with potassium permanganate/sulfuric acid as a visualizing reagent. The HPLC analysis was performed on the Zorbax Eclipse XDS-C18 column with methanol/water (97:3, v/v) as mobile phase, and the UV detection at 210 nm. Column chromatography and preparative thin-layer chromatography were performed for the purification of the compounds using *n*-hexane/AcOEt, 3:1.2, v/v (1), n-hexane/AcOEt, 3:0.8, v/v (2), AcOEt/n-hexane: 7:3, v/v (3), n-hexane/AcOEt, 3:0.6, v/v (4), CH_2Cl_2/MeOH, 100:0 → 99:1, v/v (5), AcOEt (6) as the eluent systems. The IR spectra were recorded on the Thermo Nicolet 6700 FT-IR spectrophotometer (Thermo Fisher Scientific, USA), using the ATR technique. The NMR spectra were recorded on the Varian Gemini 200 or Bruker Ascend 400 spectrometer. Chemical shifts are expressed in the parts per million (δ ppm). The splitting patterns are

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described as singlet (s), doublet (d), triplet (t) and multiplet (m). The mass spectra of the compound (MS/MS) were recorded with use of the TSQ Quantum Access MAX triple quadripole spectrometer (Thermo Fisher Scientific, USA) equipped with the electrospray ionization (ESI) source operating in the positive ionization mode. The high-resolution mass spectra (ESI-TOF analyses) of the compounds were recorded with use of the Agilent 6210 time-of-flight LC/MS system (G1969A, Agilent Technologies, USA) and the LTQ Orbitrap XL FT Mass Spectrometer (Thermo Fisher Scientific, Germany). The purity of the compounds was established as > 95%.

*2.2. General procedure for synthesis of (RS)-α-tocopheryl α-(N-benzyloxycarbonylamino) carboxylates **3a-d***

To a stirred solution of α-tocopherol (2.2 mmol) in 20 mL dry CH_2Cl_2 (**3a-b**) or in a mixture of 10 mL dry CH_2Cl_2 and 10 mL THF (**3c-d**), DMAP (0.5 mmol), EDC (1.3 mmol) and the Cbz-protected amino acid (1 mmol) were added. The reaction mixture was protected from the daylight and it was left for an overnight at room temperature under the nitrogen. Then it was washed three times with water, dried over the anhydrous Na_2SO_4 and the solvent was evaporated under the vacuum. The residue was purified by the preparative TLC using the eluent systems (1), (2), (3) and (4) to obtain compounds **3a**, **3b**, **3c** and **3d**, respectively.

*2.2.1. 2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2H 1-benzopyran-6-yl 2,6-di{[(benzyloxy)carbonyl]amino}hexanoate (**3a**).* Yield:86%; ^{1}H NMR ($CDCl_3$) δ: 0.84-0.88 (m, 12H), 1.08-2.17 (m, 29H), 1.23 (s, 3H), 1.94 (s, 3H), 1.98 (s, 3H), 2.08 (s, 3H), 2.56-2.58 (m, 2H), 3.23 (m, 2H), 4.65 (m, 1H), 5.08-5.12 (m, 4H), 7.30-7.34 (m, 10H); ^{13}C NMR ($CDCl_3$) δ: 11.81, 12.11, 12.96, 19.68, 19.75, 20.59, 21.02, 22.62, 22.71, 24.44, 24.79, 27.98, 29.40, 29.69,

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31.03, 31.92, 32.18, 32.69, 32.79, 37.28, 37.38, 37.46, 37.56, 39.38, 40.53, 53.83, 66.70, 67.10, 75.13, 117.52, 123.20, 124.78, 126.51, 128.10, 128.19, 128.50, 128.53, 136.19, 136.55, 140.14, 149.63, 156.14, 156.53, 171.12; MS m/z: 827.3 $[M+H]^+$.

*2.2.2. 1-benzyl-2-(2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2H-1-benzopyran-6-yl)-pyrrolidine-1,2-dicarboxylate (**3b**).* Yield:69%; ^{1}H NMR ($CDCl_3$) δ: 0.83-0.87 (m, 12H), 1.08-1.61 (m, 21H), 1.23 (s, 3H), 1.73-1.92 (m, 4H), 2.05 (s, 3H), 2.09 (s, 3H), 2.17 (s, 3H), 2.29-2.41 (m, 2H), 2.54-2.60 (m, 2H), 3.56-3.70 (m, 2H), 4.67-4.74 (m, 1H), 5.02-5.30 (m, 2H), 7.29-7.38 (m, 5H); ^{13}C NMR ($CDCl_3$) δ: 11.79, 11.83, 12.69, 19.60, 19.70, 20.59, 21.05, 22.64, 22.73, 23.51, 24.46, 24.83, 27.99, 29.29, 30.16, 31.14, 32.72, 32.79, 37.31, 37.41, 37.43, 37.47, 39.40, 47.01, 59.30, 75.10, 117.42, 123.09, 124.85, 126.57, 127.81, 127.99, 128.10, 128.46, 136.76, 140.37, 149.52, 154.89, 171.29; MS m/z: 662.4 $[M+H]^+$.

*2.2.3. 2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2H-1-benzopyran-6-yl 2-((benzyloxycarbonyl)amino)-4-carbamoylbutanoate (**3c**).* Yield:38%; ^{1}H NMR ($CDCl_3$) δ: 0.84-0.87 (m, 12H), 1.07-1.71 (m, 21H), 1.23 (s, 3H), 1.72-1.82 (m, 2H), 1.95 (s, 3H), 1.99 (s, 3H), 2.08 (s, 3H), 2.16-2.19 (m, 1H), 2.41-2.49 (m, 3H), 2.56-2.59 (m, 2H), 4.67 (m, 1H), 5.09-5.17 (m, 2H), 7.32-7.36 (m, 5H); ^{13}C NMR ($CDCl_3$) δ: 11.83, 12.15, 12.99, 19.63, 19.77, 20.60, 21.05, 22.64, 22.73, 24.45, 24.83, 27.99, 28.44, 29.70, 31.20, 31.93, 32.72, 32.79, 37.31, 37.37, 37.40, 37.49, 37.58, 39.39, 53.82, 67.20, 75.18, 117.56, 123.24, 124.77, 126.44, 128.13, 128.26, 128.35, 128.57, 136.18, 140.14, 149.70, 156.51, 170.71, 174.26; MS m/z: 693.4 $[M+H]^+$.

*2.2.4. 2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2H-1-benzopyran-6-yl 2-((benzyloxycarbonyl)amino)-3-cyanopropanoate (**3d**).* Yield:31%; ^{1}H NMR ($CDCl_3$) δ: 0.83-

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0.87 (m, 12H), 1.05-2.01 (m, 21H), 1.24 (s, 3H), 1.73-1.83 (m, 2H), 2.04 (s, 3H), 2.09 (s, 3H), 2.16 (s, 3H), 2.57-2.63 (m, 2H), 3.20-3.24 (m, 2H), 4.85-4.89 (m, 1H), 5.17 (m, 2H), 7.33-7.38 (m, 5H); ^{13}C NMR ($CDCl_3$) δ: 11.83, 12.20, 13.04, 19.69, 19.76, 20.61, 21.03, 21.81, 22.62, 22.71, 24.45, 24.80, 27.98, 30.88, 32.70, 32.80, 37.31, 37.36, 37.40, 37.43, 37.47, 37.57, 39.39, 50.8, 67.68, 75.31, 115.94, 117.74, 123.49, 126.25, 128.21, 128.46, 128.65, 135.65, 140.05, 150.05, 155.55, 167.39; MS m/z: 661.1 $[M+H]^+$.

*2.3. General procedure for synthesis of (RS)-α-tocopheryl α-aminocarboxylates **4a-d***

To a solution of compound (1 mmol) in anhydrous ethanol (**3a-b, 3d)** or in anhydrous methanol (**3c),** 10% Pd/C catalyst (20% w/w with respect to **3a-d**) was added. The reaction mixture was stirred at ambient temperature in the atmosphere of hydrogen (2 atm H_2). Once the reaction was completed, (4 h for **3a-c** and 6 h for **3d**), the catalyst was filtered off using the membrane filter and the solvent was evaporated. Solution of HCl in dry diethyl ether was added and the white precipitate of the amine salt was filtered off and dried in a vacuum oven for 24 h at room temperature, to result in compound **4a**. Compounds **4b-4d** were isolated as free amines by purification on a chromatographic column, using the eluent systems (5), (6) and (3), to give compounds **4b**, **4c** and **4d**, respectively.

*2.3.1. 2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2H-1-benzopyran-6-yl 2,6-diaminohexanoate hydrochloride (**4a**).* Yield:82%; IR (ATR) cm^{-1}: 1764.4, 3385.3; ^{1}H NMR ($CDCl_3$) δ: 0.83-0.88 (m, 12H), 1.08-2.16 (m, 29H), 1.18 (s, 3H), 1.90 (s, 3H), 1.93 (s, 3H), 2.03 (s, 3H), 2.52-2.60 (m, 2H), 3.02-3.15 (m, 2H), 3.87 (m, 1H); ^{13}C NMR ($CDCl_3$) δ: 11.74, 12.11, 12.95, 19.63, 20.52, 20.70, 21.01, 22.60, 22.67, 23.74, 24.42, 24.76, 27.58, 27.93, 29.64, 30.93, 32.74, 33.17, 37.25, 37.40, 39.33, 39.77, 53.68, 75.03, 117.44, 123.03, 124.79, 126.49, 140.21,

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149.46, 173.77; MS m/z: 559.5 $[M+H]^+$; HRMS calcd. for $C_{35}H_{62}N_2O_3$ $[M+H]^+$ 559.48332, found 559.48060.

*2.3.2. 2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2H-1-benzopyran-6-yl pyrrolidine-2-carboxylate (**4b**).* Yield:78%; IR (ATR) cm^{-1}:1763.5, 3360.2; ^1H NMR (MeOD) δ: 0.87-0.90 (m, 12H), 1.12-2.13 (m, 23H), 1.24 (s, 3H), 2.00 (s, 3H), 2.04 (s, 3H), 2.10 (s, 3H), 2.16-2.25 (m, 2H), 2.37-2.42 (m, 1H), 2.60-2.63 (m, 2H), 2.65-2.70 (m, 1H), 3.47-3.52 (m, 2H), 4.84-4.88 (m, 1H); ^{13}C NMR (MeOD) δ: 10.70, 10.91, 11.78, 18.84, 18.90, 20.15, 20.68, 21.69, 21.78, 23.40, 24.06, 24.52, 27.76, 28.53, 32.39, 32.44, 32.53, 36.89, 36.97, 37.01, 37.09, 39.17, 45.68, 59.44, 75.00, 117.72, 122.84, 126.14, 140.04, 149.65, 168.22; MS m/z: 528.1 $[M+H]^+$; HRMS calcd. for $C_{34}H_{57}NO_3$ $[M+H]^+$ 528.44112, found 528.44130.

*2.3.3. 2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2H-1-benzopyran-6-yl 2-amino-4-carbamoylbutanoate (**4c**).* Yield:68%; IR (ATR) cm^{-1}: 1644.1, 1734.4, 3321.8; ^1H NMR (MeOD) δ: 0.87-0.90 (m, 12H), 1.11-1.56 (m, 21H), 1.26 (s, 3H), 1.80-1.83 (m, 2H), 1.99 (s, 3H), 2.02 (s, 3H), 2.10 (s, 3H), 2.17-2.20 (m, 2H), 2.49-2.53 (m, 2H), 2.62-2.65 (m, 2H), 3.86-3.90 (m, 1H); ^{13}C NMR (MeOD) δ: 10.67, 10.97, 11.85, 18.81, 18.86, 20.17, 20.71, 21.66, 21.75, 24.05, 24.51, 27.76, 28.17, 29.31, 29.70, 30.72, 31.24, 32.53, 37.00, 37.05, 37.09, 37.13, 39.16, 54.68, 74.84, 117.51, 122.55, 124.80, 126.34, 140.39, 149.25, 173.31, 176.39; MS m/z: 559.5 $[M+H]^+$; HRMS calcd. for $C_{34}H_{58}N_2O_4$ $[M+H]^+$ 559.44693, found 559.44685.

*2.3.4. 2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2H-1-benzopyran-6-yl 2-amino-3-cyanopropanoate (**4d**).* Yield:28%; IR (ATR) cm^{-1}: 1753.1, 2159.3, 3292.1; ^1H NMR (DMSO-*d*6) δ: 0.81-0.85 (m, 12H), 1.06-1.53 (m, 21H), 1.19 (s, 3H), 1.73-1.75 (m, 2H), 1.92 (s, 3H), 1.94 (s, 3H), 2.02 (s, 3H), 2.55-2.58 (m, 2H), 2.95-2.98 (m, 2H), 4.03-4.06 (m, 1H); ^{13}C

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NMR (DMSO-*d*6) δ: 12.03, 12.30, 13.15, 20.04, 20.10, 20.39, 20.84, 22.93, 23.02, 23.40, 24.16, 24.60, 27.84, 31.08, 32.39, 32.47, 36.98, 37.07, 37.14, 37.18, 37.21, 37.35, 39.23, 51.62, 75.34, 117.92, 118.90, 122.37, 125.37, 126.75, 140.47, 149.24, 171.89; MS m/z: 527.1 $[M+H]^+$; HRMS calcd. for $C_{33}H_{54}N_2O_3$ $[M+H]^+$ 527.42072, found 527.41897.

2.4. General procedure for synthesis of γ-tocotrienyl α-(N-benzyloxycarbonylamino) carboxylates ***5a-c***

To a stirred solution of **γ** -tocotrienol (2.2 mmol) in 20 mL dry CH_2Cl_2 (**5a-b**) or in a mixture of 10 mL dry CH_2Cl_2 and 10 mL THF (**5c**), DMAP (0.5 mmol), EDC (1.3 mmol) and the Cbz-protected amino acid (1 mmol) were added. The mixture was protected from the daylight and left for an overnight at room temperature under the nitrogen. The reaction mixture was washed three times with water, dried over the anhydrous Na_2SO_4 and the solvent was evaporated under the vacuum. The residue was purified by the preparative TLC using the eluent systems (1), (2) and (3), to obtain compounds **5a**, **5b** and **5c**, respectively.

*2.4.1. 2,7,8-trimethyl-2-((3E,7E)-4,8,12-trimethyltrideca-3,7,11-trien-1-yl)-3,4-dihydro-2H-1-benzopyran-6-yl-2,6-di((benzyloxycarbonyl)amino)hexanoate (**5a**).* Yield:83%; ^{1}H NMR ($CDCl_3$) δ: 1.26 (s, 3H), 1.60 (s, 3H), 1.60 (s, 3H), 1.63 (s, 3H), 1.68 (s, 3H), 1.53-2.13 (m, 20H), 1.99 (s, 3H), 2.10 (s, 3H), 2.69 (m, 2H), 3.21 (m, 2H), 4.59-4.60 (m, 1H), 5.08-5.15 (m, 7H), 6.54 (s, 3H), 7.33-7.34 (m, 10H); ^{13}C NMR ($CDCl_3$) δ: 11.95, 12.67, 15.92, 16.02, 17.69, 22.20, 22.44, 24.08, 25.70, 26.62, 26.78, 29.45, 31.03, 32.27, 39.70, 39.73, 40.05, 40.56, 53.85, 66.70, 67.12, 75.90, 118.54, 118.57, 124.19, 124.22, 124.43, 126.03, 126.87, 128.10, 128.13, 128.21, 128.52, 128.55, 131.25, 134.99, 135.25, 136.23, 136.60, 141.26, 149.74, 156.09, 156.54, 171.54; MS m/z: 807.3 $[M+H]^+$.

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*2.4.2. 1-benzyl-2-(2,7,8-trimethyl-2-((3E,7E)-4,8,12-trimethyltrideca-3,7,11-trien-1-yl)-3,4-dihydro-2H-1-benzopyran-6-yl)-pyrrolidine-1,2-dicarboxylate (**5b**).* Yield:72%; ^{1}H NMR ($CDCl_3$) δ: 1.26 (s, 3H), 1.59 (s, 3H), 1.60 (s, 3H), 1.68 (s, 3H), 1.68 (s, 3H), 1.63-2.18 (m, 16H), 2.04 (s, 3H), 2.11 (s, 3H), 2.19-2.27 (m, 1H), 2.35-2.39 (m, 1H), 2.63-2.72 (m, 2H), 3.55-3.71 (m, 2H), 4.58-4.65 (m, 1H), 5.09-5.21 (m, 5H), 6.60 (s, 1H), 7.30-7.38 (m, 5H); ^{13}C NMR ($CDCl_3$) δ: 11.91, 12.59, 15.92, 16.02, 17.69, 22.20, 23.59, 24.13, 24.47, 25.70, 26.62, 26.78, 29.29, 31.05, 39.73, 39.95, 47.04, 58.92, 67.22, 75.81, 118.51, 118.75, 124.18, 124.23, 124.42, 125.86, 126.88, 127.82, 127.99, 128.48, 131.25, 134.98, 135.24, 136.76, 141.27, 149.57, 154.91, 171.67; MS m/z: 642.4 $[M+H]^+$.

*2.4.3. 2,7,8-trimethyl-2-((3E,7E)-4,8,12-trimethyltrideca-3,7,11-trien-1-yl)-3,4-dihydro-2H-1-benzopyran-6-yl 2-((benzyloxycarbonyl)amino)-4-carbamoylbutanoate (**5c**).* Yield:29%; ^{1}H NMR ($CDCl_3$) δ: 1.27 (s, 3H), 1.59 (s, 3H), 1.60 (s, 3H), 1.68 (s, 3H), 1.68 (s, 3H), 1.61-2.18 (m, 16H), 2.00 (s, 3H), 2.10 (s, 3H), 2.39-2.46 (m, 2H), 2.70-2.71 (m, 2H), 4.63 (m, 1H), 5.09-5.17 (m, 5H), 6.56 (s, 1H), 7.31-7.37 (m, 5H); ^{13}C NMR ($CDCl_3$) δ: 11.96, 12.70, 15.92, 16.02, 17.69, 22.19, 22.21, 24.09, 25.70, 26.62, 26.78, 28.54, 31.01, 31.80, 39.70, 39.73, 40.06, 53.74, 67.22, 75.92, 118.53, 118.55, 124.18, 124.21, 124.42, 126.05, 126.84, 128.16, 128.27, 128.58, 131.26, 135.00, 135.27, 136.17, 141.22, 149.82, 171.07, 174.02; MS m/z: 673.3 $[M+H]^+$.

*2.5. General procedure for synthesis of γ-tocotrienyl α-aminocarboxylates **6a-c***

To a solution of compound (1 mmol) in anhydrous ethanol (**5a-b**) or in anhydrous methanol (**5c**), 10% Pd/C catalyst (20% w/w with respect to **5a-c**) was added. The reaction mixture was stirred

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for 2 h at ambient temperature in the atmosphere of hydrogen (2 atm H_2). The catalyst was filtered off using the membrane filter and the solvent was evaporated. Solution of HCl in dry diethyl ether was added and the white precipitate of the amine salt was filtered off to result in compound **6a**. Compounds **6b** and **6c** were isolated as free amines by purification on a chromatographic column using the eluent system (6).

2.5.1.

*2,7,8-trimethyl-2-((3E,7E)-4,8,12-trimethyltrideca-3,7,11-trien-1-yl)-3,4-dihydro-2H-1-benzopyran-6-yl 2,6-diaminohexanoate hydrochloride (**6a**).* Yield:78%; IR (ATR) cm^{-1}: 1765.9, 3396.4; ^{1}H NMR (DMSO-*d*6) δ: 1.23 (s, 3H), 1.54 (s, 3H), 1.54 (s, 3H), 1.56 (s, 3H), 1.63 (s, 3H), 1.07-2.04 (m, 20H), 1.98 (s, 3H), 2.06 (s, 3H), 2.69 (m, 2H), 2.80 (m, 2H), 4.29 (m, 1H), 5.06-5.13 (m, 3H), 6.71 (s, 1H); ^{13}C NMR (DMSO-*d*6) δ: 12.18, 12.98, 16.08, 16.27, 18.01, 21.87, 22.17, 22.97, 24.21, 25.95, 26.41, 26.66, 28.92, 29.85, 30.87, 38.64, 40.44, 40.65, 40.76, 52.20, 76.27, 119.04, 119.31, 124.35, 124.59, 124.64, 125.44, 127.12, 131.07, 134.81, 134.87, 141.20, 149.68, 172.39; MS m/z: 539.4 $[M+H]^+$; HRMS calcd. for $C_{34}H_{54}N_2O_3$ $[M+H]^+$ 539.42072, found 539.41883.

2.5.2.

*2,7,8-trimethyl-2-((3E,7E)-4,8,12-trimethyltrideca-3,7,11-trien-1-yl)-3,4-dihydro-2H-1-benzopyran-6-yl pyrrolidine-2-carboxylate (**6b**).* Yield:55%; IR (ATR) cm^{-1}: 1766.1, 3369.4; ^{1}H NMR ($CDCl_3$) δ: 1.27 (s, 3H), 1.58 (s, 3H), 1.59 (s, 3H), 1.60 (s, 3H), 1.68 (s, 3H), 1.69-2.13 (m, 14H), 2.01 (s, 3H), 2.11 (s, 3H), 2.18-2.31 (m, 4H), 2.63-2.73 (m, 2H), 2.96-3.0 (m, 1H), 3.14-3.17 (m, 1H), 4.01-4.04 (m, 1H), 5.09-5.15 (m, 3H), 6.56 (s, 1H); ^{13}C NMR ($CDCl_3$) δ: 11.96, 12.63, 15.91, 16.02, 17.69, 22.20, 22.67, 24.12, 25.55, 25.70, 26.62, 26.78, 30.58, 31.06, 39.71, 39.73, 40.02, 47.03, 59.80, 75.85, 118.47, 118.65, 123.96, 124.22, 124.43, 125.94, 126.95, 131.25,

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134.98, 135.23, 141.45, 149.60, 174.55; MS m/z: 508.4 $[M+H]^+$; HRMS calcd. for $C_{33}H_{49}NO_3$ $[M+H]^+$ 508.37852, found 508.37874.

2.5.3..

*2,7,8-trimethyl-2-((3E,7E)-4,8,12-trimethyltrideca-3,7,11-trien-1-yl)-3,4-dihydro-2H-1-benzopyran-6-yl 2-amino-4-carbamoylbutanoate (**6c**).* Yield:68%; IR (ATR) cm^{-1}: 1667.2, 1739.3, 3357.5; ^1H NMR ($CDCl_3$) δ: 1.27 (s, 3H), 1.59 (s, 3H), 1.60 (s, 3H), 1.66 (s, 3H), 1.68 (s, 3H), 1.61-2.18 (m, 14H), 2.01 (s, 3H), 2.11 (s, 3H), 2.32-2.43 (m, 2H), 2.47-2.55 (m, 2H), 2.69-2.71 (m, 2H), 3.72-3.77 (m, 1H), 5.10-5.14 (m, 3H), 6.55 (s, 1H); ^{13}C NMR ($CDCl_3$) δ: 11.96, 12.72, 15.90, 17.68, 22.19, 22.23, 24.10, 25.74, 26.61, 26.77, 29.91, 31.02, 32.15, 39.69, 39.73, 39.90, 53.96, 75.90, 118.53, 118.58, 124.17, 124.37, 124.42, 126.01, 126.84, 131.24, 134.96, 135.24, 141.37, 149.68, 170.96, 174.61; MS m/z: 539.3 $[M+H]^+$; HRMS calcd. for $C_{33}H_{50}N_2O_4$ $[M+H]^+$ 539.38433, found 539.38390.

2.6. Biological assays

2.6.1. Cell culture

All cell lines used in this study were obtained from the American Type Culture Collection (Rockville, MD), unless specified otherwise. The human lung cancer (A549) cells and human normal lung tissue (MRC5) cell lines were maintained as a monolayer culture in the Roswell Park Memorial Institute (RPMI) 1640 medium, while the human breast cancer cell lines (MCF7 and MDA-MB-231) were maintained in the Dulbecco's Modified Eagle medium (DMEM). The powdered RPMI 1640 medium and DMEM were purchased from Sigma Chemicals Co, USA. The nutrient medium RPMI 1640 was prepared in sterile deionized water, supplemented with penicillin (192 U/mL), streptomycin (200 µg/mL), 4-(2-hydroxyethyl) piperazine-1-

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ethanesulfonic acid (HEPES) (25 mM), L-glutamine (3 mM) and 10% heat-inactivated fetal calf serum (FCS) (pH 7.2). The nutrient medium DMEM was prepared in sterile deionized water supplemented with penicillin (192 U/mL), streptomycin (200 µg/ mL) and 10% heat-inactivated FCS. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2 with twice-weekly subculture.

2.6.2. MTT assay

The antiproliferative activity of the tested compounds was assessed using the MTT assay (Mosmann, 1983; Ohno and Abe, 1991). All cell lines were seeded at a density of 7000 cells/well in the 96-well plates and left for 24 h to rest, followed by an addition of the tested compounds at various concentrations (0.6-50 µM). Stock solutions of the compounds (80 mM) were prepared in DMSO immediately before the experiment, whereas further dilutions were performed with the fresh medium. Each concentration was tested in triplicate during the 48 hours incubation. After the treatment in the 96-well plates, the MTT solution (3-(4, 5-dimethylthiazol-2-yl)-2, 5-dyphenyl tetrazolium bromide) (Sigma-Aldrich, St. Louis, USA) (20 µL/well) was added to each well. Samples were incubated for further 4 h, followed by an addition of 100 µL 10% sodium dodecyl sulfate (SDS). The absorbance was measured on the next day at 570 nm and the cell survival (S %) was calculated as an absorbance (A570 nm) ratio between the treated and the control cells multiplied by 100. IC_{50} was defined as the concentration of the agent that inhibited cell survival by 50%, compared with the vehicle control.

2.6.3. Flow-cytometric analysis of cell cycle phase distribution

Quantitative analysis of the cell cycle phase distribution was performed by the flow-cytometric analysis of the DNA content in the fixed MDA-MB-231 cells after staining with propidium

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iodide (PI) (Sigma-Aldrich, US) (Ormerod, 1994). The tested cell line in the exponential phase of growth, plated at a density of 2 x 10^5 cells/Petri dish (60 x 15 mm, NUNC), was continually exposed to compound **4d**, Dox and their combination at the IC_{50} concentrations. After 48 hours continuous treatment, cells were collected by trypsinization, washed twice with the ice-cold PBS and fixed for 30 min in 70% EtOH. The fixed cells were washed again with PBS and incubated with RNase A (1 mg/mL) for 30 min at 37 °C. The cells were stained with PI (400 µg/mL) immediately before the analysis. The cell cycle phase distribution was analyzed using the FACS (Becton Dickinson, USA) flow cytometer and the Cell Quest computer software.

2.6.4. Apoptotic assay

Apoptosis of the MDA-MB-231 cells treated with compound **4d**, Dox and their combination was evaluated by the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences, Pharmingen San Diego, CA, USA). Briefly, after incubation for 48 h with 1x IC_{50} of the investigated compounds, cells were washed twice with the cold PBS and resuspended in 200 µL of the binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1 x 10^6 cells/mL. A total of 100 µL of the solution (1 x 10^5 cells) was transferred to a 5 mL culture tube and 5 µL Annexin V–FITC and 5 µL PI was added. The cells were gently vortexed and incubated for 15 min at 25 °C in the dark. Finally, 400 µL of the binding buffer was added to each tube and the analysis was carried out using the FACS (Becton Dickinson, USA) flow cytometer and the Cell Quest computer software.

2.7. Stability in human plasma

Plasma was prepared by centrifugation of human blood obtained from a healthy adult volunteer. A stability study in human plasma was conducted by the procedure described elsewhere (Behery

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et al., 2010; Konsoula and Jung, 2008). The freshly prepared human plasma (1.5 mL) was preheated at 37ºC on a shaking water bath. The reaction was initiated by adding the tested compound to reach final concentration of 200 μg/mL. Samples (75 μL) were taken at 0, 0.5, 1, 2, 4, 6, and 24 h and added to 300 μL acetonitrile in order to deproteinize the plasma. The samples were vortexed for 1 min and then centrifuged at 4°C for 15 min at 14,000 rpm. Incubations were conducted in duplicate. Clear supernatants were analyzed by HPLC using the Zorbax Eclipse XDB-C18 column and acetonitrile/water (95:5, v/v) as mobile phase. The mobile phase flow rate was set at 2 mL/min and the UV detection wavelength at 215 nm. Chromatographic analyses were performed at 30ºC. This HPLC method was developed earlier for the analysis of α-tocopheryl esters (Marra et al., 2009) and then modified for the purpose of this study. Diluted concentrations (in the range of 1–500 μg/mL) of the stock solutions of the compounds in plasma were used to construct the calibration curves.

2.8. Ligand based virtual screening

Two LBVS models were developed using the FLAP software (Molecular Discovery Ltd., 2015) to identify the compounds with a potential antiproliferative activity on the MCF-7 cells (model 1) and the MDA-MB 321 cells (model 2). Compounds **3a** and **4d**, which have been shown to exert a strong antiproliferative activity on these cell lines, were used as templates for screening in model 1 and model 2, respectively. The dataset was generated using the ChEMBL database (https://www.ebi.ac.uk/chembl), which currently is the most extensive public database containing 1 463 270 differing bioactive compounds. In order to validate the model, compounds with the strongest antiproliferative activity on the MCF-7 and MDA-MB 321 cells should be taken into the account. Thus, we included in the dataset the newly synthesized compounds with an experimentally determined antiproliferative activity on the cancer cells and 53 compounds

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from our previous QSAR study (Gagic et al., 2016; Djuric et al., 1997; Elnagar et al., 2010; Arya et al., 1998; Liu et al., 2013).

FLAP calculates the GRID Molecular Interaction Fields (Cross et al., 2010) (MIFs) using H, O, N1, and the DRY probes to describe the shape, the hydrogen bond acceptor, the hydrogen bond donor, and the hydrophobic interactions. Each molecule is described as a set of quadruplets derived by the combination of all atoms, four by four, in an exhaustive way. All combinations of the quadruplets are then used to generate different superpositions of a test molecule onto a template molecule and the MIF similarity is calculated. Compounds from the database were ranked by different similarity scores, i.e., by the probe scores (representing a degree of an overlap of the MIFs for each probe individually and for the combinations thereof), by the distance score (representing an overall difference of the probe score between the ligand and the template), and the two global scores (Glob-Prod and Glob-Sum). The enrichment plot was used to determine the best similarity score based on the AUC value that holds for the measure of a successful discrimination between the known active and inactive compounds in a given template.

3. Results and discussion

3.1. Chemistry

The synthetic route for the target compounds is outlined in Scheme 1. The four α-T amino acid esters (lysine, proline, glutamine and asparagine) and the three γ-T_3 amino acid esters (lysine, proline, and glutamine) were prepared in two steps and their structures were confirmed by the IR, ^{1}H-NMR, ^{13}C-NMR, MS and HRMS spectroscopic analyses.

The first step in the synthetic route was the coupling of α-T/γ-T_3 with the corresponding Cbz-protected amino acid by employing the Steglich esterification method (Neises and Steglich,

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1978). In this paper, an alternative reaction employing EDC instead of DCC was used, because of an enhanced solubility of EDC and more particularly, because the urea by-product formed in the course of the reaction can easily be removed from the reaction mixture by extraction with water. In an earlier study, the influence of the reactants concentration (acid, alcohol and a catalyst) on the progress of esterification and on the urea by-product formation was studied (Tsvetkova et al., 2006). We employed the acid/alcohol/catalyst molar ratio as equal to 0.01:0.022:0.0050 that was found optimal for the preparation of the esters. Asparagine and glutamine are known to undergo the nitrile formation via dehydration of the carboxamide side-chain, when using carbodiimides as the activating agents (Katsoyannis et al., 1958). With asparagine, this reaction course is almost exclusive, so we decided to prepare the α-T-asparagine ester in the form of the nitrile. With glutamine, dehydration results in a smaller yield, so we were able to isolate the glutamine esters as the amides.

The Cbz-protecting groups were removed from each coupled product (**3a-d** and **5a-c)** by catalytic hydrogenation, using 10% Pd/C as a catalyst. Hydrogenation of the γ-T_3 esters (**5a-c**) was carefully monitored because of the risk of reduction of double bonds. Samples were collected in the 30-min intervals and analyzed by means of TLC, HPLC and NMR. We found out that two hours is an optimal period for hydrogenation, which does not cause the reduction of double bonds and at the same time, it provides satisfactory reaction yields. Compounds **3a** and **6a** were precipitated as hydrochloride salts after acidification with the HCl solution in dry diethyl ether and then the precipitate was filtered off. Precipitation did not occur with the other compounds so they were isolated as free amino derivatives by purification with use of the preparative TLC or column chromatography.

3.2. In vitro biological evaluation

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The antiproliferative activity of all synthesized compounds was tested against the human cancer cell lines, i.e., against the estrogen-responsive MCF-7 breast cancer, the estrogen-unresponsive MDA-MB-231 breast cancer, and the A549 lung cancer cell line, and the results were compared with those obtained for the commercially available α-TOS and γ-T_3. The proliferation inhibitory activities were evaluated by the MTT assay after 48 h and expressed as the IC_{50} values (Table 1). Moreover, all the compounds were screened for the cytotoxic activity against the normal human fetal lung fibroblast MRC-5 cell line.

It was established that compounds **4a**, **4d** and **6a** exert a good antiproliferative activity, with the IC_{50} values lower than 30 μM (Table 1). As already mentioned before, an antiproliferative activity of the α-T-lysine ester (**4a**) against the MCF-7 cell line has already been reported elsewhere (Arya et al., 1998) and our results remain in good agreement with those already published. Moreover, compound **4a** showed very good activity against the A549 cell line. Compound **6a** was the only one active against all three cell lines, but **4a** was more potent against the MCF-7 and A549 cell lines. The proline esters of α-T (**4b**) and γ-T_3 (**6b**) showed moderate activity against the MCF-7 cell line, with the cell survival of 67.1% and 66.1% at 20 μM, respectively (Fig. 1). In contrast with the synthesized compounds **4a**, **4d** and **6a** able to effectively inhibit proliferation at the selected cancer cell lines, the parent γ-T_3 (**2**) and the commercial α-TOS showed a limited activity against all three cell lines only, with the IC_{50} values higher than 50 μM, in spite of an earlier demonstrated anticancer activity of these two compounds against various cancer cells (Ling et al., 2012; McIntyre et al., 2000; Guan et al., 2012; Patacsil et al., 2012; Sun et al., 2014).

Compound **4d** was the only one which exerted a strong selective antiproliferative activity against the estrogen-unresponsive, multi-drug resistant MDA-MB-231 breast cancer cell line. In order to

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obtain more information regarding the events related to the antiproliferative activity of **4d** and to investigate a potential synergistic effect with the anticancer drug doxorubicin (Dox), changes in the cell cycle progression were evaluated using the fluorescence-activated cell sorting (FACS) analysis. For this purpose, the MDA-MB-231 cells were treated for 48 hours with **4d**, Dox, as well as with the combination thereof (**4d**-Dox). Each of these two agents was applied at the IC_{50} concentration. It needs to be mentioned that the IC_{50} value of Dox against the MDA-MB-231 cells was 4 μM (data not presented). After 48 hours continuous treatment, **4d** and Dox induced a percentage increase of the apoptotic cells (sub-G1 peak) and a strong percentage decrease of the cells in the G1 and S phases, and in the S and G2/M phases, respectively (Fig. 2). The highest percentage (45%) of apoptotic cells was found with the MDA-MB-231 cells upon 48 hours treatment with the combination **of 4d** and Dox. This effect was accompanied by the reduction of cells in both, the S and G2/M phases of the cell cycle (Fig. 2).

Then we performed the FACS analysis of the cell death induction on the MDA-MB-231 cells using **4d**, Dox and the combination thereof. The cells were incubated with the investigated compounds for 48 hours and stained with Annexin V and propidium iodide (PI) for the identification of the early and late apoptotic cells. Treatment with **4d**, Dox, and especially with the combination of **4d** and Dox resulted in the onset of a massive apoptotic cell death (Fig 3.). Interestingly, while the percentage of the early apoptotic cell population increased after the **4d**-Dox co-treatment, the late apoptotic and necrotic cell populations were almost lost (the Annexin V positive/PI positive and the Annexin V negative/PI positive cells, respectively), probably suggesting a delay in the activation of the apoptotic execution machinery (Fig. 3). These results suggest that the combined **4d**-Dox treatment of the highly metastatic breast cancer could lead to

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an enhanced therapeutic response and lower the toxicity associated with a high dose of the Dox monotherapy.

3.3. Stability in human plasma

Due to the high levels of non-specific esterases, the VE esters could be hydrolyzed in the liver. The sensitivity of α-tocopheryl acetate and the α-tocopheryl asparagine ester to hepatic hydrolysis were previously verified using the porcine liver esterase (Marra et al., 2009). The half-life of commercial α-tocopheryl acetate was established as equal to ca. 1 h, and the α-tocopheryl asparagine ester has proved as significantly more stable against hepatic enzymes with the half-life of 6 h (Marra et al., 2009). In order to avoid an extensive liver metabolism and to achieve high concentrations of the VE esters in plasma, the parenteral formulations of the VE esters were proposed (dos Santos et al., 2012; Weber et al., 2002). Recently, the liposomal parenteral formulations of the VE derivatives were developed to increase the half-life and ensure their safe delivery to the tumor cells. An anticancer effect of these formulations has been successfully demonstrated in the pre-clinical models *in vivo* (Turanek et al., 2009; Koudelka et al., 2015; Neophytou and Constantinou, 2015).

Since it is possible that the enzymes can hydrolyze the VE esters in blood plasma, we tested the stability of our most active compound **4d** and the commercial α-TOS in the freshly prepared human plasma. The results depicted on Fig. 4 show that both compounds slowly decomposed in plasma. Concentration of the examined esters decreased to about 83% of the initial concentration within the 6 hours incubation. After 24 h, 39% of the initial **4d** concentration and 69% of the initial α-TOS concentration were determined in plasma. These results point out to an acceptable metabolic stability of **4d** and the related VE esters in human plasma. Based on the all above mentioned facts, it could be concluded that parenteral application of the examined VE esters

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should provide enough stable concentration in plasma to warrant effective distribution and sufficient therapeutic concentrations of the compounds in tumor tissue.

3.4. Ligand based virtual screening

Virtual screening is a powerful technique to identify potentially active compounds from molecular databases. To identify the novel hit compounds with potential antiproliferative activity on the MCF-7 cells (model 1) and the MDA-MB 321 cells (model 2), **3a** and **4d,** respectively, were used as the templates for LBVS of the ChEMBL database (https://www.ebi.ac.uk/chembl). Moreover, the synthesized compounds and the compounds from our previous QSAR study (Gagic et al., 2016) were added to the dataset. The GRID probes H, DRY, O, and N1 included in FLAP (Molecular Discovery Ltd., 2015) were used for modeling of the ligands and templates. The compounds were ranked based on the similarity score. According to the enrichment plot, the highest AUC value was achieved using the DRY score (AUC= 0.78) for model 1 and the N1*DRY score (AUC= 0.75) for model 2. Thus, it can be concluded that hydrophobicity and the presence of the H-bond acceptors are the important factors for discriminating the antiproliferative activity of the screened compounds. The five top ranked compounds from model 1 (CHEMBL3105824, CHEMBL477744, CHEMBL1825075, CHEMBL1190700 and CHEMBL1825079) and the five top ranked compounds from model 2 (CHEMBL81504, CHEMBL317957, CHEMBL3105824, CHEMBL3183599 and CHEMBL3091854) were selected as the hit leads with the highest potential to show an antiproliferative activity on the MCF-7 and MDA-MB 321 breast cancer cell lines, respectively (Fig. 5). The screening identified CHEMBL1825079 as the potentially active compound against the MCF-7 cell line, and its antiproliferative activity on this cell line has already been reported (Carvalho et al., 2011), in that way confirming the validity of the model. Besides, CHEMBL3105824, CHEMBL477744,

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CHEMBL1825075, CHEMBL81504 and CHEMBL317957 have also been tested and they were shown active against various different cancer cell lines. Both models identified CHEMBL3105824 as a compound potentially active against the MCF-7 and MDA-MB 321 cells.

4. Conclusions

Seven amino acid esters of α-tocopherol and γ-tocotrienol were synthesized and their *in vitro* antiproliferative activity was tested on the human MCF-7 and MDA-MB-231 breast cancer cell lines, and the A549 lung cancer cell line. Compound **4a** effectively inhibited proliferation of the MCF-7 and A549 cells, while **6a** exerted an antiproliferative effect on the three investigated cell lines. Interestingly, **4d** inhibited proliferation of the multi-drug resistant MDA-MB-231 cells at the doses that had no effect on the normal human MRC-5 cells. In contrast with good activity of **4a**, **4d** and **6a**, the commercial α-TOS and γ-T3 samples showed only a limited activity against the three investigated cell lines. The cell cycle and the cell death induction studies demonstrated synergistic effect of the combined treatment of **4d** with the anticancer drug doxorubicin, indicating a possible combination therapy with the lower dose of Dox to reduce toxicity. Based on an acceptable metabolic stability of **4d** and the related VE esters in human plasma, a conclusion can be drawn that parenteral application of the VE esters should provide stable enough concentrations thereof in plasma to warrant their effective distribution and high therapeutic concentrations in tumor tissues, and therefore the VE esters seem very good candidates for the further *in vivo* testing.

The virtual screening studies identified new compounds with a potential antiproliferative activity on the human MCF-7 and MDA-MB-231 cells.

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Scheme captions

Scheme 1. The synthetic route of amino acid esters of α-tocopherol and γ-tocotrienol. Reagents and conditions: (a) CH_2Cl_2, DMAP, EDC, di-Cbz-Lys; (b) CH_2Cl_2, DMAP, EDC, Cbz-Pro; (c) CH_2Cl_2/THF, DMAP, EDC, Cbz-Glu; (d) CH_2Cl_2/THF, DMAP, EDC, Cbz-Asp; (e) H_2, 10% Pd/C.

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Figure captions

Figure 1. Antiproliferative activity of VE derivatives against human (A) MCF-7, (B) MDA-MB-231 breast cancer and (C) A549 lung cancer cell lines.

Figure 2. Cell cycle phase distribution of MDA-MB-231 cells: (A) untreated cells, (B) cells treated with compound **4d**, (C) cells treated with Dox, (D) cells treated with **4d**-Dox combination. Cells were exposed to IC_{50} concentrations of **4d** and Dox for 48 h. Increased cell populations at sub-G1 phase of treated cells confirm apoptosis. M1, M2, M3 and M4 correspond to sub-G1, G1, S and G2/M phases respectively.

Figure 3. Contour diagram of FITC-Annexin V/PI flow cytometry of MDA-MB-231 cells after 48 h treatment: (A) untreated cells, (B) cells treated with compound **4d**, (C) cells treated with Dox, (D) cells treated with **4d**-Dox combination.

Figure 4. Stability profile of **4d** and α-TOS in human plasma.

Figure 5. Lead compounds obtained by virtual screening of ChEMBL database with potential antiproliferative activity on (A) MCF-7 and (B) MDA-MB 231 cells.

Table captions

Table 1. IC_{50} values of VE derivatives against human MCF-7, MDA-MB-231 breast cancer and A549 lung cancer cell lines.

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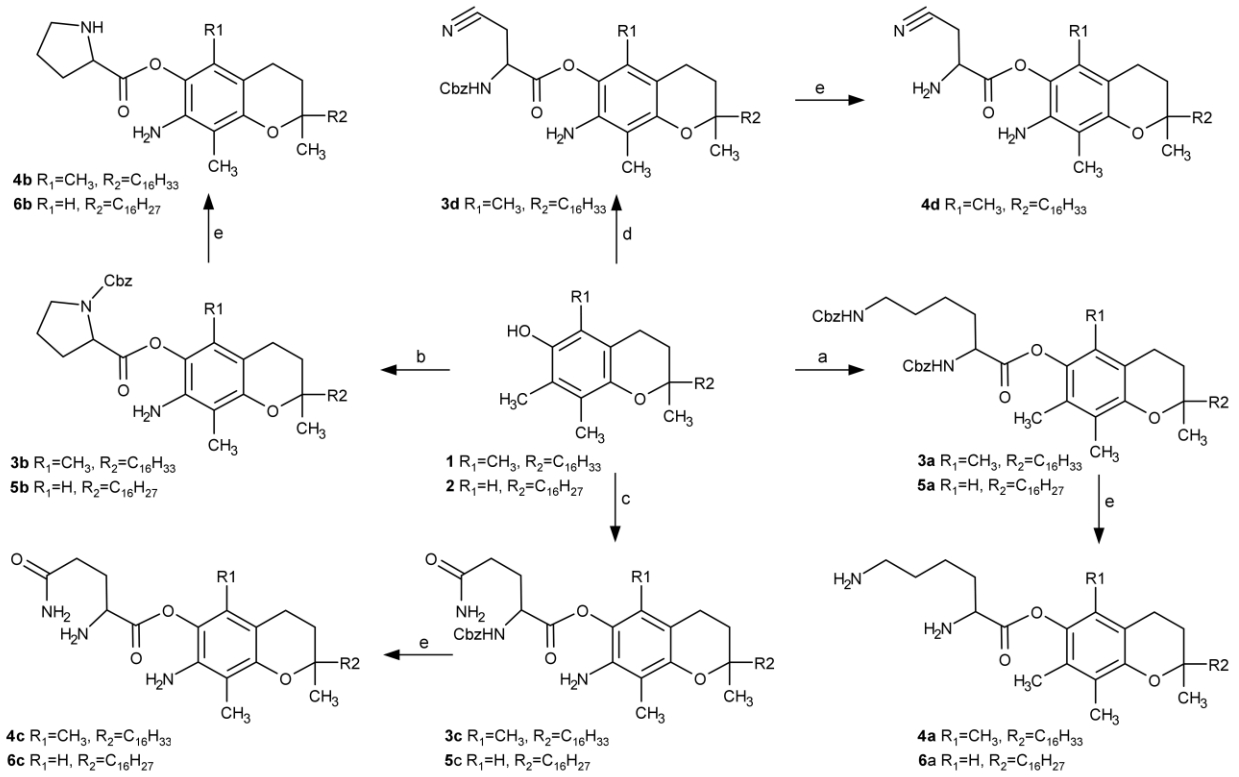

Scheme 1

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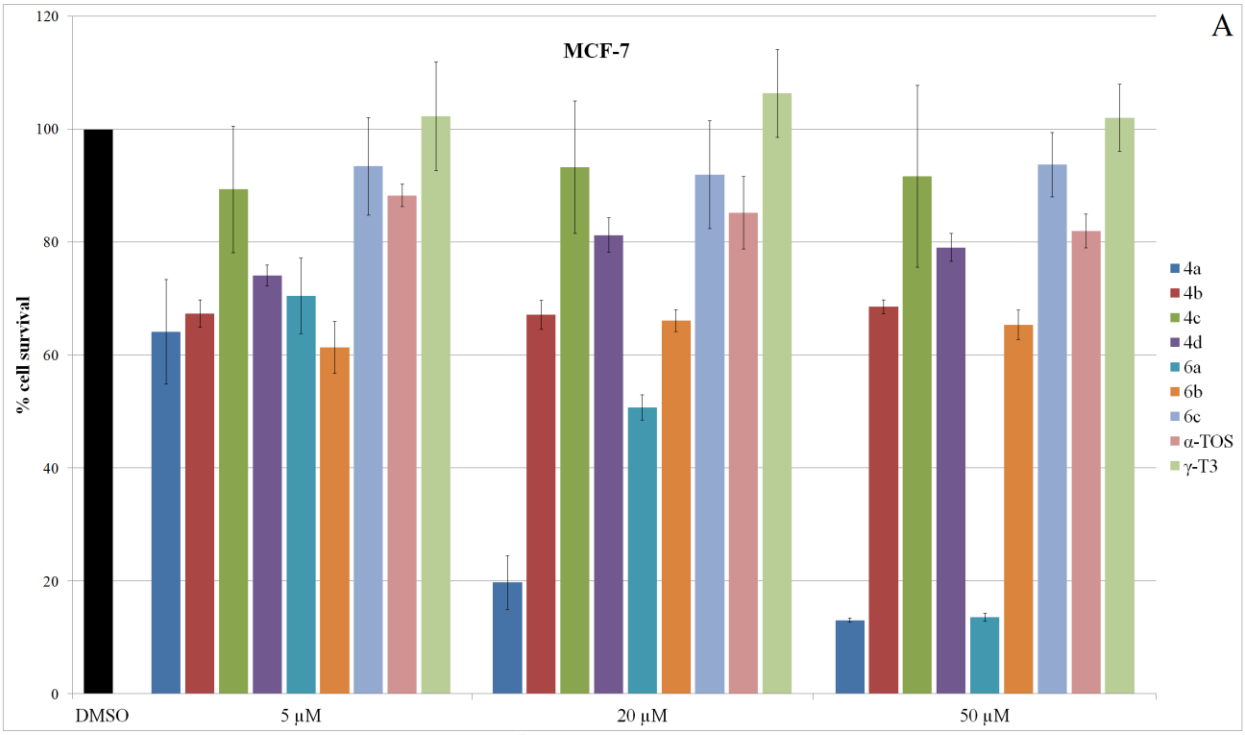

Figure 1a

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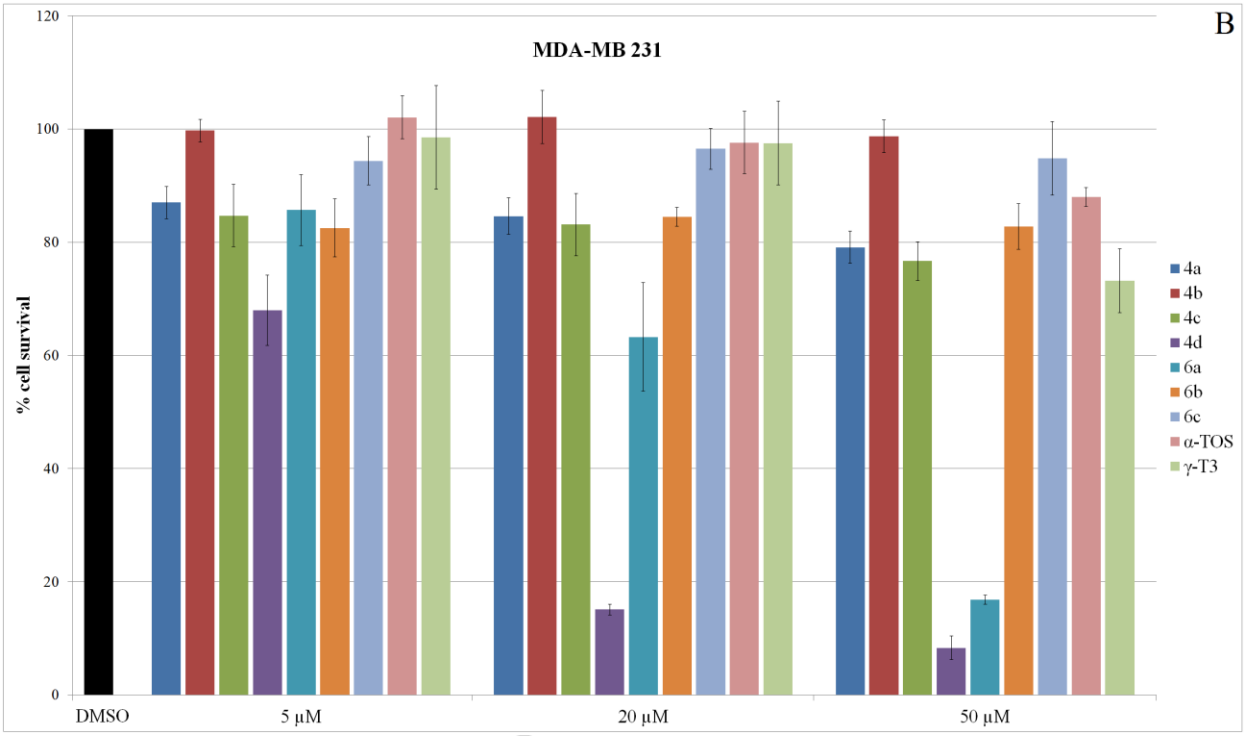

Figure 1b

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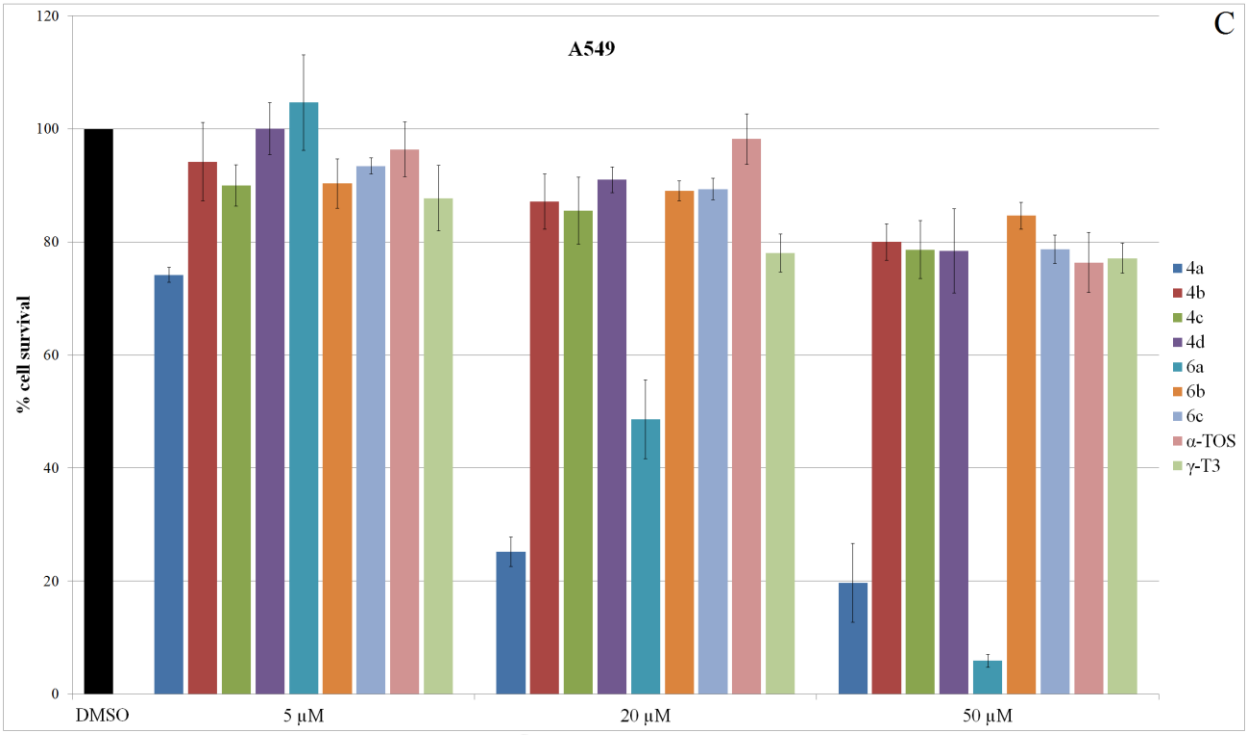

Figure 1c

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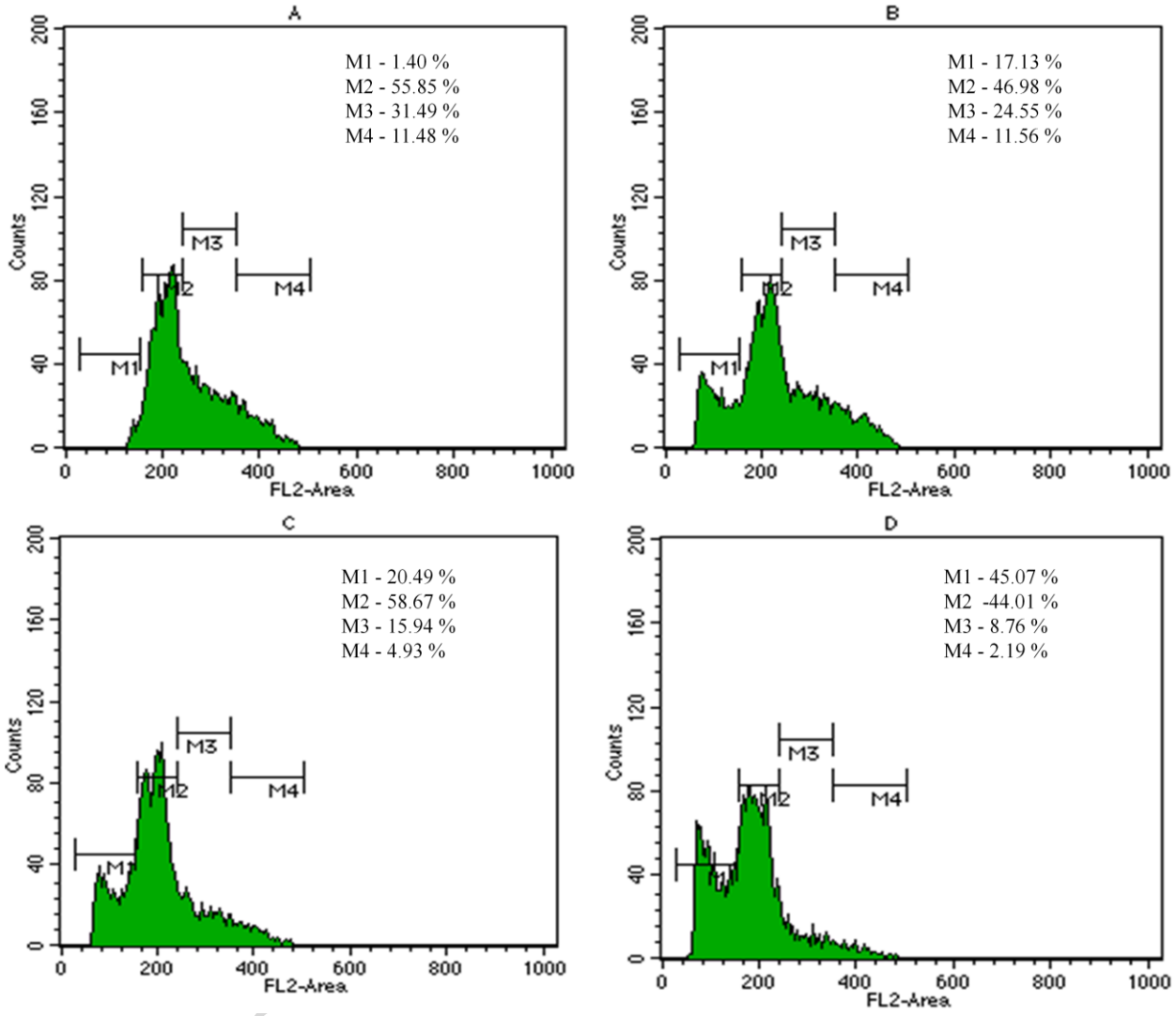

Figure 2

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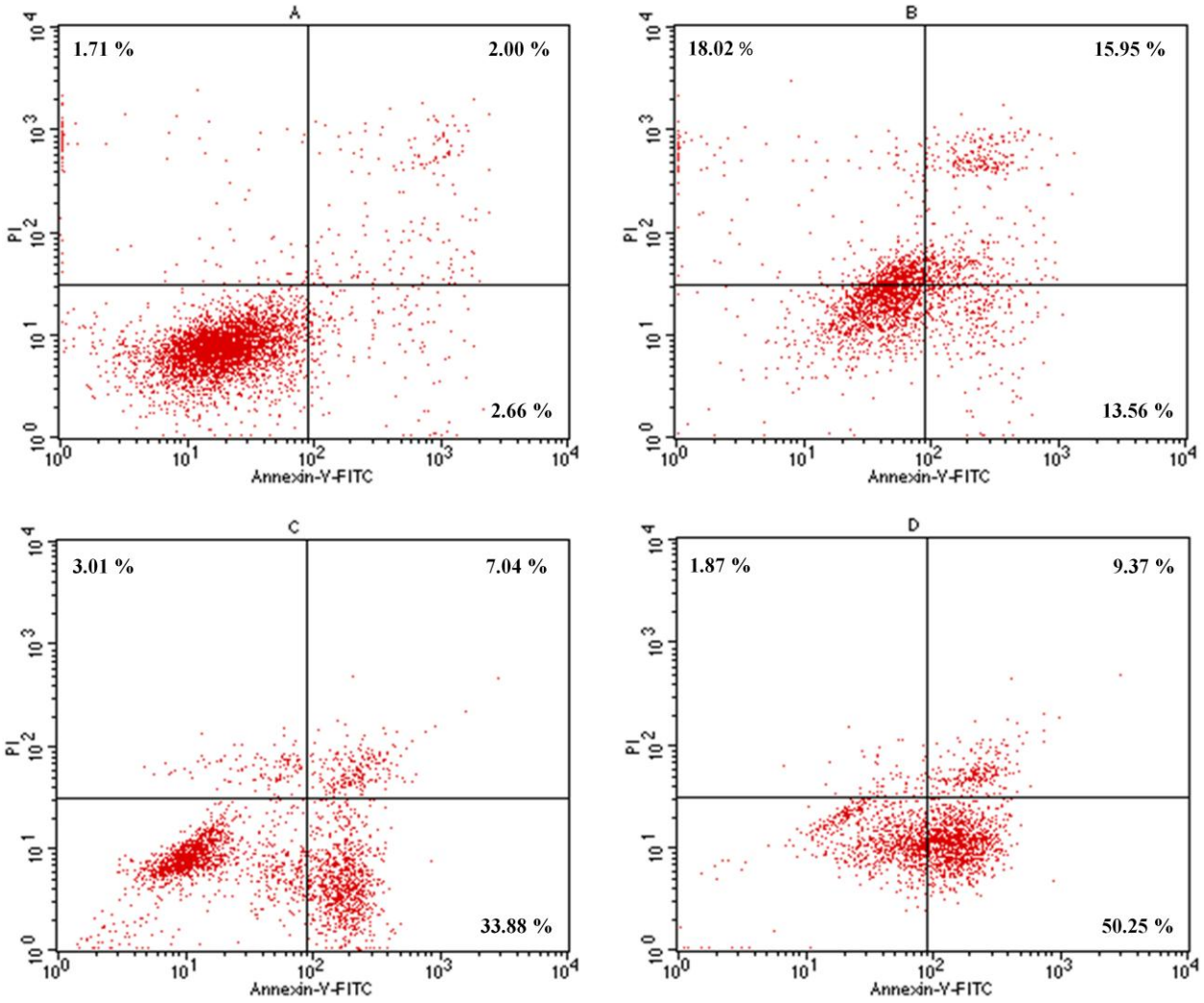

Figure 3

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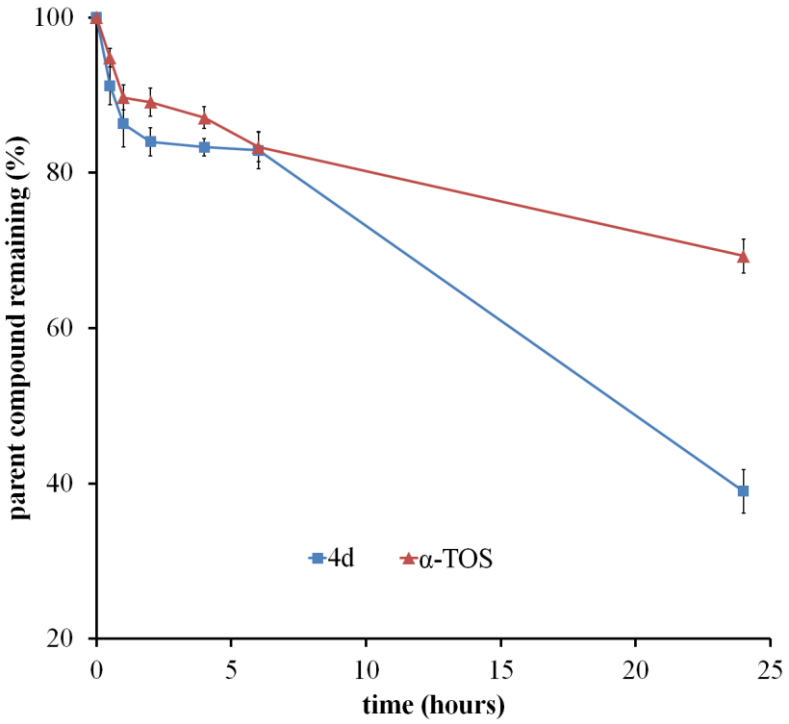

Figure 4

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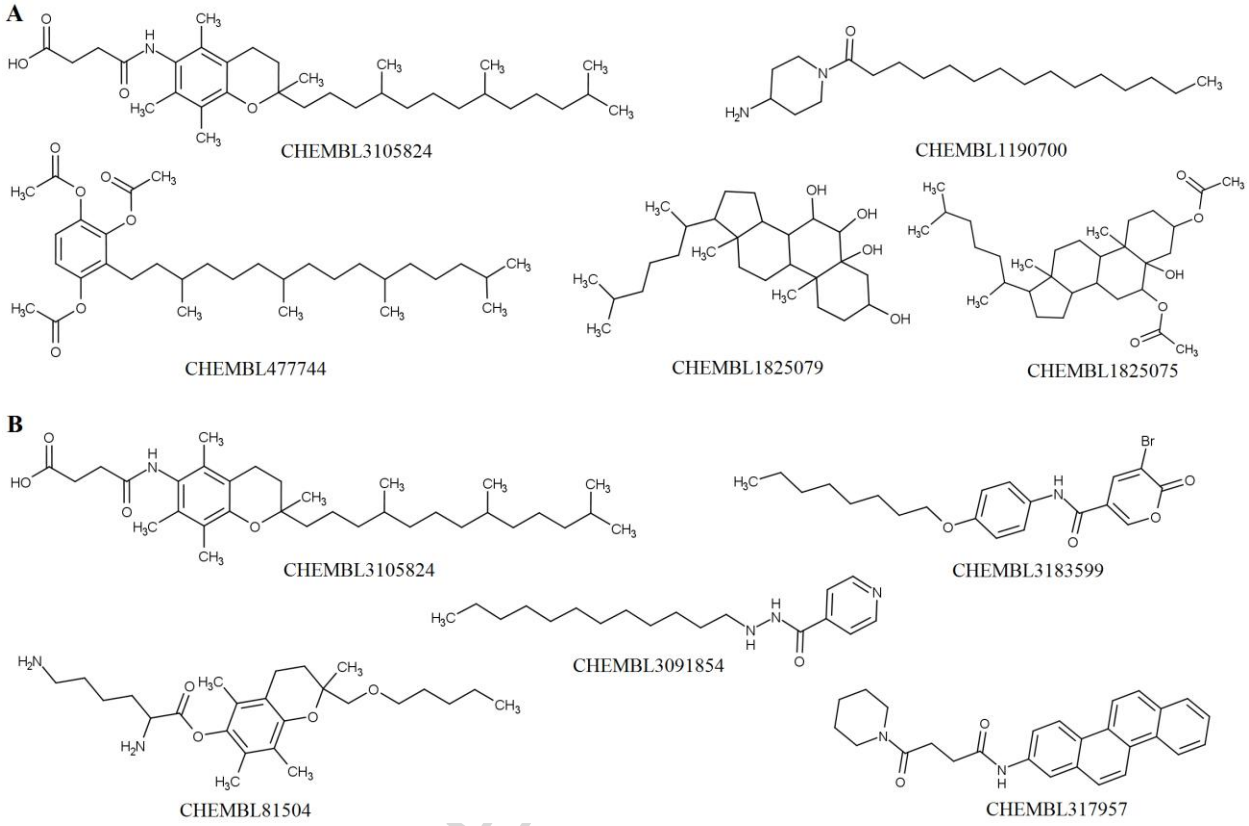

Figure 5

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Graphical abstract

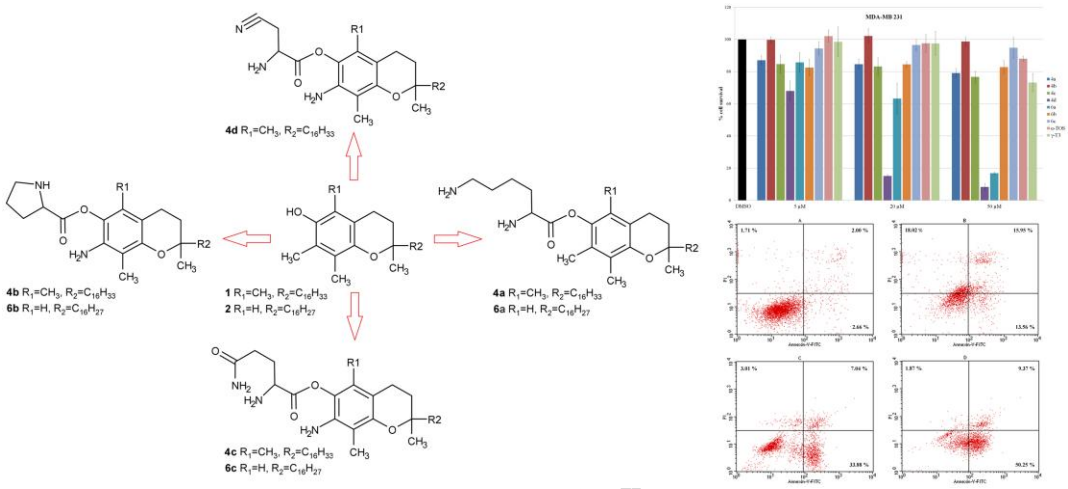

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Table 1. IC_{50} values of VE derivatives against human MCF-7, MDA-MB-231 breast cancer and A549 lung cancer cell lines.

Compound	MCF-7 IC_{50} (μM)	MDA-MB-231 IC_{50} (μM)	A549 IC_{50} (μM)
4a	8.6	>50	10.6
4b	>50	>50	>50
4c	>50	>50	>50
4d	>50	9.2	>50
6a	20.6	28.6	19
6b	>50	>50	>50
6c	>50	>50	>50
γ-T3	>50	>50	>50
α-TOS	>50	>50	>50

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