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Original article

Jatrophane diterpenes and cancer multidrug resistance – ABCB1 efflux modulation and selective cell death induction

Mariana Alves Reis[a], Omar Bauomy Ahmed[b], Gabriella Spengler[c], Joseph Molnár[c], Hermann Lage[b,d], Maria-José U Ferreira[a,*]

[a] Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Avenue Prof. Gama Pinto, 1649-003 Lisbon, Portugal
[b] Institute of Pathology, University Hospital Charité, 10117 Berlin, Germany
[c] Department of Medical Microbiology and Immunobiology, Faculty of Medicine, University of Szeged, Dóm tér 10, H-6720 Szeged, Hungary
[d] Department of Pathology, Vivantes Clinics, Berlin, Germany

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ABSTRACT

Background: Modulation of P-glycoprotein (ABCB1) and evaluation of the collateral sensitivity effect are among the most promising approaches to overcome multidrug resistance (MDR) in cancer. In a previous study, two rare 12,17-cyclojatrophanes (**1–2**) and other novel jatrophanes (**3–4**), isolated from *Euphorbia welwitschii,* were screened for collateral sensitivity effect. Herein, the isolation of another jatrophane (**5**) is presented, being the broader goal of this work to investigate the role of euphowelwitschines A (**1**) and B (**2**), welwitschene (**3**), epoxywelwitschene (**4**) and esulatin M (**5**) as ABCB1 modulators and/or collateral sensitivity agents.

Methods: Compounds **1–5** were evaluated for ABCB1 modulation ability through combination of transport and chemosensitivity assays, using a mouse T-lymphoma *MDR1*-transfected cell model. Moreover, the nature of interaction of compound **4** with ABCB1 was studied, using an ATPase assay. The MDR-selective antiproliferative activity of compound **5** was evaluated against gastric (EPG85-257) and pancreatic (EPP85-181) human cancer cells and their drug-selected counterparts (EPG85-257RDB, EPG85-257RNOV, EPP85-181RDB, EPP85-181RNOV). The drug induced cell death was investigated for compounds **4** and **5**, using the annexin V/PI staining and the active caspase-3 assay.

Results: The jatrophanes **1–5** were able to modulate the efflux activity of ABCB1, and at 2 μM, **3–5** maintained the strong modulator profile. Structure activity results indicated that high conformational flexibility of the twelve-membered ring of compounds **3–5** favored ABCB1 modulation, in contrast to the tetracyclic scaffold of compounds **1** and **2**. The effects of epoxywelwitschene (**4**) on the ATPase activity of ABCB1 showed it to interact with the transporter and to be able to reduce the transport of a second substrate. Drug combination experiments also corroborated the anti-MDR potential of these diterpenes due to their synergistic interaction with doxorubicin (combination index < 0.7). Esulatin M (**5**) showed a strong MDR-selective antiproliferative activity against EPG85-257RDB and EPP85-181RDB cells, with IC_{50} of 1.8 and 4.8 μM, respectively. Compounds **4** and **5** induced apoptosis via caspase-3 activation. A significant discrimination was observed between the resistant cell lines and parental cells.

Conclusions: This study strengthens the role of jatrophane diterpenes as lead candidates for the development of MDR reversal agents, higlighting the action of compounds **4** and **5**.

Introduction

The emergence of cancer multidrug resistance (MDR) has been pointed as one of the major hurdles for a successful chemotherapy. In such scenario, cancer cells develop resistance to drugs that are structurally unrelated and with distinct mechanisms of action.

Abbreviations: ABC, ATP binding cassette; ABCB1, ATP-binding cassette, subfamily B; EPG85-257P, parental gastric cancer cells; EPG85-257RDB, gastric cancer cells selected against daunorubicin; EPG85- 257RNOV, gastric cancer cells selected against mitoxantrone; EPP-181 RDB, pancreatic cancer cells selected against daunorubicin; EPP-181P, parental pancreatic cancer cells; EPP-181RNOV, pancreatic cancer cells selected against mitoxantrone; FAR, fluorescence activity ratio; FL-1, mean fluorescence intensity; MDR, multidrug resistance; MDR1, multidrug resistance gene 1; L5178Y-MDR, *MDR1* transfected L5178Y mouse T-lymphoma cells; L5178Y-PAR, parental L5178Y mouse T-lymphoma cells; Pi, inorganic phosphate; PI, propidium iodide; RR, relative resistance.

* Corresponding author. fax: +351 217946470.

E-mail addresses: malvesreis@ff.ulisboa.pt (M.A. Reis), omar.ahmed@charite.de (O.B. Ahmed), spengler.gabriella@med.u-szeged.hu (G. Spengler), molnar.jozsef@med.u-szeged.hu (J. Molnár), hermann.lage@vivantes.de (H. Lage), mjuferreira@ff.ulisboa.pt (M.-J.U. Ferreira).

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The adaptive responses attributed to the cancer MDR phenotype are far from being completely understood, and the general consensus is that they are likely to occur simultaneously or in a cascade of events during the establishment of the MDR phenotype (Lage, 2008; Szakács et al., 2006). Some of the most common cellular mechanisms include: alterations in the cell cycle checkpoints; failure of the apoptotic mechanisms; repair of damaged cellular targets; alterations in drug targets; drug activation and inactivation; decreased drug uptake; and reduced drug accumulation through drug efflux or vesicular sequestration by ATP binding cassette (ABC) transporters (Holohan et al., 2013; Kartal-Yandim et al., 2015). The most known and studied mode of resistance has been associated with P-glycoprotein (ABCB1/Pgp), the first human ABC transporter to be described. The overexpression of ABCB1 results in reduced intracellular concentration of anticancer drugs to levels that lead to treatment failure, causing cross-resistance to several cytotoxic drugs (Gottesman et al., 2002). To enhance the efficacy of chemotherapy, several approaches have been proposed to circumvent MDR. The development of molecules that are able to impair the drug efflux mediated by ABCB1 and development of collateral sensitivity agents lay among the most promising strategies (Callaghan et al., 2014; Szakács et al., 2014). The collateral sensitivity effect is an old concept that nowadays is gaining more strength as powerful tool to study MDR. This concept was primarily observed in bacteria, in the early 1950s by Szybalski and Bryson, which concluded that resistant *Escherichia coli* was simultaneously hypersensitive to other unrelated drugs (Szybalski and Bryson, 1952). Therefore, cancer cells that developed *in vitro* resistance to one agent can be more sensitive to alternate agents than the original parental cell line (Callaghan et al., 2014; Szakács et al., 2014). Thus, this approach can lead to the selection of compounds that are highly effective against drug-resistant phenotypes.

Natural products present a key role in drug discovery programs for cancer MDR. Consequently, a large number of compounds have been investigated (Eid et al., 2015; Palmeira et al., 2012; Wu et al., 2011). Among those, the polyoxygenated jatrophane and lathyrane-type macrocyclic diterpenes from *Euphorbia* species have shown potential anti-MDR activities, by ABCB1 modulation and/or by selective targeting of MDR cancer cells (Corea et al., 2009; Ferreira et al., 2014; Vasas and Hohmann, 2014). These encouraging results led to the development of *in silico* studies, as well as structure activity studies towards lead optimization for a promising MDR reversal agent (Ferreira et al., 2011; Matos et al., 2015; Reis et al., 2013, 2012; Sousa et al., 2012; Vieira et al., 2014). Despite all these advances, there is still a great need to explore their anti-MDR mode of action.

We have recently reported four newly 12,17-cyclojatrophane (**1–2**) and jatrophane (**3–4**) constituents of *Euphorbia welwitschii* (Reis et al., 2015). Our preliminary results on MDR-selective antiproliferative activity of these compounds pointed epoxywelwitschene (**4**) as a potential MDR reverser (Reis et al., 2015). In order to enrich the knowledge of the constituents of *E. welwitschii,* its phytochemical study was extended, giving rise to esulatin M (**5**), a known jatrophane that was reported as modulator of ABCB1 (Vasas et al., 2011). Thus, it was of interest to look further into its anti-MDR activity. Therefore, the aim of this work was to assess compounds **1–5** (Fig. 1) for their potential MDR reversal activity, using an integrative approach: through modulation of ABCB1 and the collateral sensitivity effect. In this way, euphowelwitschines A (**1**) and B (**2**), welwitschene (**3**), epoxywelwitschene (**4**) and esulatin M (**5**) were tested for modulation of ABCB1 efflux using a *MDR1*-transfected mouse T-lymphoma L5178Y cell model. In addition, the MDR-selective antiproliferative activity mode of action of epoxywelwitschene (**4**) and esulatin M (**5**) was analyzed in regard to apoptosis and caspase-3 activation events using the human tumor gastric (EPG85-257) and pancreatic (EPP85-181) cell models (drug sensitive and drug resistant sublines), well characterized for MDR (Hilgeroth et al., 2013; Lage et al., 2010; Reis et al., 2014).

Material and methods

Plant material

Aerial parts of *Euphorbia welwitschii* Boiss. & Reut. (synonym *Euphorbia paniculata* subsp. *welwitschii* (Boiss. & Reut.) Vicens, Molero & C. Blancheé) were collected in the garden of Palácio da Pena, Sintra, Portugal (June 2010). The plant was identified by Dr. Teresa Vasconcelos (plant taxonomist) of Instituto Superior de Agronomia, University of Lisbon, Portugal. A voucher specimen (no. 282/2010) has been deposited at the herbarium of Instituto Superior de Agronomia.

*Isolation of compound **5***

Dried aerial parts of *E. welwitschii* were exhaustively extracted with methanol, as previously described (Reis et al., 2015). Briefly, the MeOH extract (595 g) was suspended in MeOH/H_2O solution and extracted with EtOAc. The ethyl acetate soluble fraction (225 g) was chromatographed (2 kg SiO_2) using mixtures of *n*-hexane-EtOAc (1:0 to 0:1) and EtOAc-MeOH (9:1 to 3:7) in increasing gradients of 5%, of 2 L each eluent. According to differences in composition as indicated by TLC, nine crude fractions were obtained (fractions A-I). Fraction G (21.3 g), eluted with *n*-hexane/EtOAc (7:3 to 13:7), was subjected to subsequent fractionation giving rise to fractions G_1–G_5, from where compounds **1–4** were isolated (Reis et al., 2015). Fraction G_3 (3.2 g) was chromatographed using reverse-phase C-18 column (100 g) chromatography (MeOH/H_2O, 1:1 to 1:0, used in increasing gradients of 5%, 300 ml each eluent), originating six fractions (G_{3A}-G_{3F}). Flash chromatography ($CHCl_3$ as eluent) of subfraction G_{3B} (549 mg) yielded 19 mg of compound **5**.

*Esulatin M (**5**)*

To characterize compound **5** the following spectroscopic data were collected: optical rotation (PerkinElmer 241 polarimeter); infrared spectra (Affinity-1, Shimadzu); NMR spectra (Bruker 400 Ultra Shield). The low resolution mass spectrometry was taken on a Triple Quadrupole mass spectrometer (Micromass Quattro Micro API, Waters).

White powder; $[\alpha]_D^{24}$ – 15.4 (c 0.1, $CHCl_3$); IR (CH_2Cl_2) ν_{max} 3524, 2949, 1765, 1375, 1215 cm^{-1}; ^1H NMR (400 MHz, C_6D_6) δ 9.59 (1H, s, 9-ONic-H-2′), 8.44 (1H, d, $J = 4.9$ Hz, 9-ONic-H-6′), 8.11 (1H, d, $J = 7.8$ Hz, 9-ONic-H-4′), 6.65 (1H, dd, $J = 7.8$, 4.9 Hz, 9-ONic-H-5′), 6.14 (1H, d, $J = 16.0$ Hz, H-11), 6.12 (1H, bs, H-5), 5.84 (1H, dd, $J = 16.0$, 9.1 Hz, H-12), 5.76 (1H, bs, H-3), 5.42 (1H, bs, H-17a), 5.29 (1H, bs, H-17b), 5.23 (1H, dd, $J = 7.3$, 2.9 Hz, H-9), 5.19 (1H, dd, $J = 6.5$, 2.9 Hz, H-7), 3.73 (1H, m, H-13), 3.33 (1H, dd, $J = 13.9$, 7.7 Hz, H-1a), 3.16 (1H, d, $J = 2.7$ Hz, H-4), 2.40 (1H, m, H-2), 2.32 (1H, m, H-8a), 2.29 (1H, m, H-8b), 1.99 (1H, m, 7-OCOC<u>H</u>$(CH_3)_2$), 1.75 (3H, s, 5-OCOC<u>H</u>$_3$), 1.74 (1H, m, H-1b), 1.71 (3H, s, 3-OCOC<u>H</u>$_3$), 1.66 (3H, s, 15-OCOC<u>H</u>$_3$), 1.43 (3H, d, $J = 6.7$ Hz, H-20), 0.95 (3H, s, H-19), 0.91 (3H, s, H-18), 0.81 (3H, d, $J = 7.0$ Hz, 7-OCOCH(C<u>H</u>$_3)_2$), 0.67 (3H, d, $J = 6.9$ Hz, H-16), 0.64 (3H, d, $J = 7.0$ Hz, 7-OCOCH(C<u>H</u>$_3)_2$) ppm; ^{13}C NMR (101 MHz, C_6D_6) δ 212.7 (C-14), 176.1 (7-O<u>C</u>OCH$(CH_3)_2$), 170.1 (15-O<u>C</u>OCH$_3$), 169.3 (3-O<u>C</u>OCH$_3$), 169.0 (5-O<u>C</u>OCH$_3$), 164.2 (9-O<u>C</u>Nic), 153.9 (9-ONic-C-6′), 151.6 (9-ONic-C-2′), 148.7 (C-6), 138.2 (C-11), 136.6 (9-ONic-C-4′), 131.5 (C-12), 126.1 (9-ONic-C-3′), 123.5(9-ONic-C-5′), 110.0 (C-17), 93.4 (C-15), 76.6 (C-3), 75.9 (C-9), 69.0 (C-7), 69.0 (C-5), 53.6 (C-4), 49.7 (C-10), 46.7 (C-1), 43.9 (C-13), 38.7 (C-2), 34.9 (C-8), 33.9 (7-OCO<u>C</u>H$(CH_3)_2$), 26.6 (C-18), 23.6 (C-19), 21.0 (5-OCO<u>C</u>H$_3$), 20.8 (3-OCO<u>C</u>H$_3$), 20.7 (15-OCO<u>C</u>H$_3$), 19.0 (7-OCOCH(<u>C</u>H$_3)_2$), 18.0

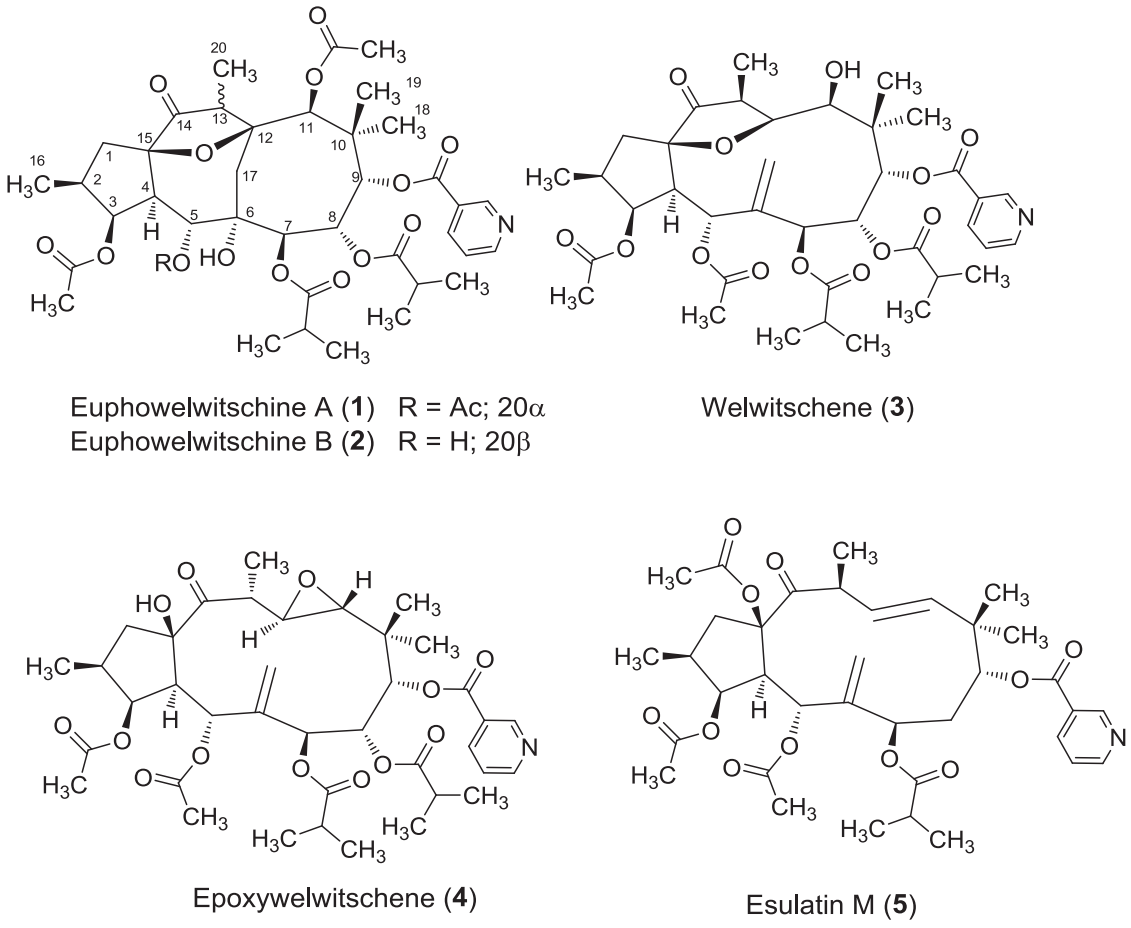

Fig. 1. Compounds evaluated for their potential MDR reversal activity.

(7-OCOCH($\underline{C}H_3$)$_2$), 13.3 (C-16) ppm; ESIMS (positive mode) *m/z* (rel. int.) 670 $[M+H]^+$ (100).

Compounds tested

Compounds **1–5** (Fig. 1) were tested for their potential as MDR modulators, against different cancer cells. The isolation and identification of compounds **1–4** have been previously reported (Reis et al., 2015). All compounds were dissolved in DMSO.

MDR mediated by ABCB1: rhodamine 123 accumulation assay

L5178Y mouse T-lymphoma cells (ECACC catalog no. 87,111,908, U.S. FDA, Silver Spring, MD, USA) and the L5178Y mouse T-lymphoma *MDR1*-transfected cells were established and cultured as described (Pastan et al., 1988; Reis et al., 2013). The cells were adjusted to a density of 2×10^6/ml, resuspended in serum-free McCoy's 5A medium and distributed in 500 μl aliquots. The test compounds were added at 2 and 20 μM, verapamil (positive control, EGIS Pharmaceuticals PLC, Budapest, Hungary) at 20 μM and DMSO at 2% (v/v) as solvent control. The samples were incubated for 10 min at room temperature, after which 10 μl (5.2 μM final concentration) of rhodamine-123 was added to the samples. After 20 min incubation at 37 °C, the samples were washed twice, resuspended in 500 μl phosphate-buffered saline (PBS) and analyzed by flow cytometry (Partec CyFlow® Space instrument, Partec GmbH, Münster, Germany). The resulting histograms were evaluated regarding mean fluorescence intensity (FL-1), standard deviation and peak channel of 20,000 individual cells belonging to the total and gated populations. The fluorescence activity ratio (FAR) was calculated as $FAR = (\text{L5178Y-MDR}_{\text{FL-1treated}} / \text{L5178Y-MDR}_{\text{FL-1control}}) / (\text{PAR}_{\text{FL-1treated}} / \text{PAR}_{\text{FL-1control}})$.

MDR mediated by ABCB1: ATPase assay

ABCB1 ATPase activity was determined using the SB-MDR1 PREADEASY ATPase Kit (SOLVO Biotechnology, Szeged, Hungary) according to the manufacturer's instructions. Briefly, the purified Sf9 insect membrane vesicles (4 μg protein/well), expressing high levels of human *MDR1*, were incubated in 50 ATPase assay buffer, compound **4** and 2 mM MgATP, for 10 min at 37 °C. Final concentration of DMSO in experiment was 2% (v/v). ATPase reaction was stopped and the inorganic phosphate (Pi) produced was measured colorimetrically (optical density was read at 630 nm). The amount of Pi liberated by the transporter is proportional to its activity. Hence, ATPase activities were determined as the difference of the measured Pi liberation with and without the presence of 1.2 mM sodium orthovanadate (vanadate-sensitive ATPase activity). This ATPase kit includes two different tests: the activation and inhibition assays. The activation assay detects compounds that are transported by ABCB1 and thus stimulate baseline vanadate-sensitive ATPase activity, such as, verapamil (40 μM) that was used as positive control. In the inhibition assay, the compounds were incubated in the presence of verapamil (40 μM), and thus, inhibitors may reduce the verapamil-stimulated vanadate-sensitive ATPase activity. In some cases inhibitors may inhibit the baseline transporter ATPase activity as well, such as cyclosporine A (40 μM), also used as positive control.

MDR mediated by ABCB1: antiproliferative and drug combination studies

The antiproliferative assay was based on MTT (thiazolyl blue tetrazolium bromide) staining and was performed according to what was described (Reis et al., 2013). Briefly, L5178Y mouse T-lymphoma cells (PAR and *MDR1*-transfected) were seeded at 1×10^5/ml and incubated with a concentration gradient of jatrophanes (final volume of 200 µl/well) for 72 h (5% CO_2 at 37 °C). Final concentration of DMSO in experiment was 1% (v/v). Cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). The percentage of inhibition of cell growth was determined as: $100-[(OD_{sample}-OD_{mediumcontrol})/(OD_{cellcontrol}-OD_{mediumcontrol})]\times100$. Mean IC_{50} values were obtained by best fitting the dose-dependent inhibition curves in GraphPadPrism5 program, from three independent experiments for each cell line.

Drug combinations of doxorubicin (Teva) and the compounds were designed and evaluated according to Chou using the software CalcuSyn Version 2 (Chou, 2010, 2006). Because the optimal concentration range for the combination is not known, the most appropriated experimental design is by using several concentrations of each agent, with data points above and below IC_{50} to make the assay more accurate. Therefore, the experimental design followed the checkerboard microplate method, where dilutions of doxorubicin were made in a horizontal direction and the dilutions of resistance modifiers vertically in a microtiter plate to a final volume of 200 µl of medium per well. The L5178Y-MDR cells were distributed at 2×10^5 cells/ml per well and were incubated for 48 h under the standard conditions. The cell growth rate was determined after MTT staining, as previously described (Reis et al., 2013). Each drug combination produces an effect and constant ratios ([compound]/[doxorubicin]) can be taken from the diagonals of the checkerboard. The effect of each constant ratio across a concentration gradient was computed in CalcuSyn software. Each dose–response curve (individual agents as well as combinations) was fit to a linear model using the median-effect equation (Chou, 2010, 2006): $F_a/F_u=(D/D_m)^m$ or $\log(F_a/F_u)=m\ \log(D)-m\ \log(D_m)$ (1)

In this equation, D is the dose of drug, D_m is the median– effect dose (IC_{50}), F_a is the fraction affected by dose D (% of growth inhibition), F_u is the unaffected fraction ($F_u=1-F_a$), and m is the slope of the dose–effect curve (Chou, 2010, 2006). The goodness-of-fit of the data was assessed by the linear correlation coefficient r, and only data from analyses with $r>0.90$ are presented.

As can be deduced, the dose and the effect are interchangeable since the dose (D) for any given degree of effect (F_a) can be determined if the values for D_m and m are known. Therefore, Eq. (1) can be rearranged as $D=D_m[F_a/1-F_a)]^{1/m}$ and $F_a=1/[1+(D_m/D)^m]$. These equations allow the calculation of the dose–effect relationships at IC_{50} (Chou, 2010, 2006).

The extent of interaction between drugs was expressed using the combination index (CI) for mutually exclusive drugs. The CI value is extracted through the following equation: $CI=(D)_1/(D_x)_1+(D)_2/(D_x)_2$ or $CI=(D)_1/(D_m)_1[F_a/(1-F_a)]^{1/m1}+(D)_2/(D_m)_2[F_a/(1-F_a)]^{1/m2}$. In this equation, $(D_x)_1$ and $(D_x)_2$ represent the doses of doxorubicin andcompounds alone, required to produce an effect at a determined level, and $(D)_1$ and $(D)_2$ are the doses of doxorubicin and compounds that in combination produce the same effect. The CI was computed by CalcuSyn software for each constant ration at the IC_{50} level and was classified as: $CI<0.1$: very strong synergism; $0.1<CI<0.3$: strong synergism; $0.3<CI<0.7$: synergism; $0.7<CI<0.9$: moderate to slight synergism; $0.9<CI<1.1$: nearly additive; $1.10<CI<1.45$: moderate antagonism; $1.45<CI<3.30$: antagonism (Chou, 2010, 2006).

Collateral sensitivity assays: antiproliferative assay

Cell culture procedures of the human carcinoma cell lines (EPG85-257P and EPP85-181P) and their drug-resistant sublines (EPG85-257RNOV, EPG85-257RDB, EPP85-181RNOV, EPP85-181RDB) have been described previously (Reis et al., 2014). Briefly, 5×10^3/ml (EPG85-257P and EPP85-181P) and 7.5×10^3/ml (EPG85-257RNOV, EPG85-257RDB, EPP85-181RNOV, EPP85-181RDB) were seeded. After 48 h attachment, a particular compound was added in a dilution series for 5 days incubation (5% CO_2 at 37 °C). Final concentration of DMSO in experiment was 0.3% (v/v). Cell growth was based on sulforhodamine B (SRB) staining and was performed according to what was described (Reis et al., 2014). Cell growth was measured at 562 nm against the reference wavelength of 690 nm. Mean IC_{50} values were obtained by best fitting the dose-dependent inhibition curves in GraphPadPrism5 program, from three to four independent experiments in triplicate for each cell line. Relative resistance (RR) values were determined as: $IC_{50(resistant\ cells)}/IC_{50(parental\ cells)}$.

Annexin V/PI staining and active caspase-3 assay

For detection of cytotoxic drug-induced apoptosis, a FITC Annexin V apoptosis detection kit (BD Pharmingen, BD Biosciences) was used. Detection of intracellular presence of active caspase-3 was also performed using FITC active Caspase-3 Apoptosis Kit (BD Pharmingen, BD Biosciences). Both assays followed the same experimental design. Briefly, 6×10^4 cell/ml of parental cell lines (EPG85-256P and EPP85-181P) and 1×10^5cell/ml of resistant cell lines (EPG85-256RNOV, EPG85-256RDB, EPP85-181RNOV and EPP85-181RDB) were seeded in six-well plates in complete medium and allowed to attach for 24 h. On the next day, the medium was discarded and new medium, with 30 µM of the tested compounds, was added to the gastric and pancreatic cancer cells. Final concentration of DMSO in experiment was 0.3% (v/v). Camptothecin (Cayman Chemicals, USA) was used as positive control (1 µM). Cells were further incubated for 72 h, in 5% CO_2 at 37 °C. The concentration of compounds and positive control and incubation time were optimized in order to assure a good sampling for flow cytometry measurement of the apoptotic process (data not shown). After this incubation period, cells were trypsinized, washed in PBS and stained according to each kit manufacturer's instructions. Stained cells were analyzed using BD Accuri C6 flow cytometer (BD Pharmingen, BD Biosciences) and data were processed with BD Accuri C6 software. Each sample was assessed using a collection of 10 000 events. The mean values and standard deviations were calculated from three independent experiments.

Statistical analysis

Statistical evaluation of the apoptosis assays data was performed with the two-tailed unpaired Student's t test using GraphPadPrism5 software. Probability value $p<0.05$ was considered statistically significant (further t-test details at supplementary material).

Results

*Esulatin M (**5**)*

Compound **5**, $[\alpha]_D^{24}$ – 15.8, was isolated as an amorphous white powder. Its ESIMS presented a pseudomolecular ion, at m/z 670 $[M+H]^+$, consistent with the molecular formula, $C_{36}H_{47}NO_{11}$. The

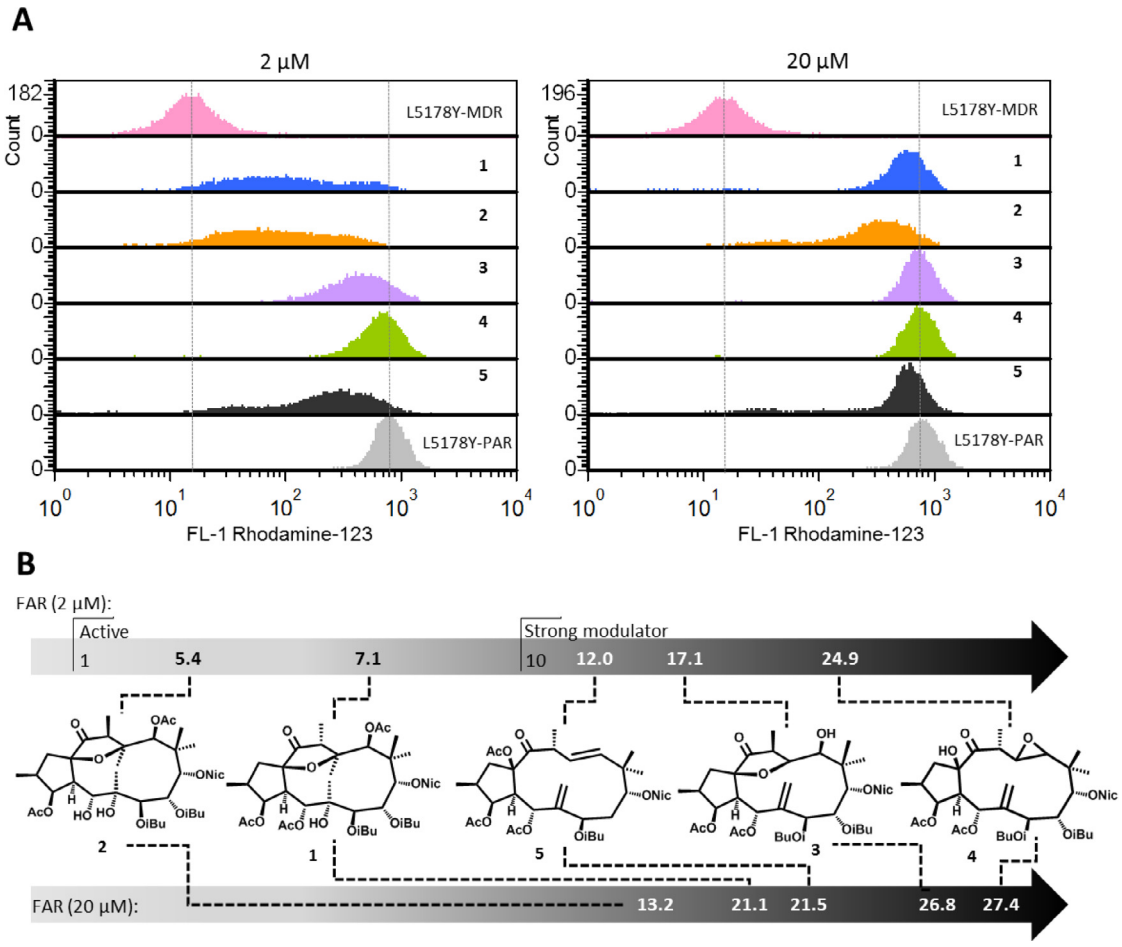

Fig. 2. Effects of compounds **1–5** on the ABCB1 mediated rhodamine-123 efflux. (A) Flow cytometry histograms of rhodamine-123 accumulation showing MDR reversion in L5178Y-MDR cells, at 2 and 20 μM. (B) Structure-activity comparisons between compounds **1–5** and their FAR values, at 2 and 20 μM. FAR = (L5178Y-$MDR_{FL\text{-}1treated}$/L5178Y-$MDR_{FL\text{-}1control}$)/($PAR_{FL\text{-}1treated}$/$PAR_{FL\text{-}1control}$). FL-1: mean fluorescence intensity of the cells. Verapamil (20 μM): FAR = 12.5. DMSO (2% v/v): FAR = 0.87.

^{1}H and ^{13}C NMR data suggested compound **5** to be a polyester Δ6(17),Δ11-jatrophane-type diterpene. From the NMR spectra, five ester residues could be identified as: three acetoxyls, one isobutyryloxyl group and one nicotinoyloxyl moiety. The conjugation of COSY, HMQC, and HMBC spectra allowed the full establishment of the diterpenic core. Therefore, the typical low field olefinic signals corresponding to the 11,12-endocyclic double bond appeared at δ_H 6.14, 5.84 and δ_C 138.2, 131.5. Moreover, the presence of two broad singlets at δ 5.42 and 5.29, with HMQC correlations with a carbon at δ 110.0, corroborated the presence of the exomethylene group. The relative stereochemistry of compound **5** was investigated through a NOESY experiment. According to its full spectroscopic data and comparison with literature data, this compound was identified as esulatin M (**5**), firstly isolated from the methanol extract of *Euphorbia esula* (Vasas et al., 2011).

Modulation of ABCB1 efflux

Aiming to evaluate the potential ABCB1 modulation effect of compounds **1**–**5**, a cell line overexpressing this transporter (L5178Y mouse T-lymphoma cell line transfected with *MDR1*) and its parental counterpart (L5178Y-PAR) were employed. The L5178Y-MDR cell line exclusively expresses ABCB1, and thus constitutes a good model for the design and evaluation of MDR reversal experiments. Therefore, compounds **1–5** were tested at 2 and 20 μM in the rhodamine-123 efflux assay. This experiment gives a direct quantitative assessment whether a compound modulates the efflux, given the fluorescence activity ratio (FAR), which is the cytoplasmic accumulation ratio of rhodamine-123 between L5178Y-MDR and L5178Y-PAR cells. ABCB1 modulation takes place when FAR value is higher than 1, hence, when this ratio is higher than 10, compounds can be classified as strong modulators (Voigt et al., 2007). Verapamil, a well-known modulator, was used as positive control in this assay. The FAR values are presented in Fig. 2.

The diterpenes **1–5** showed to be able to revert the MDR phenotype, at 20 μM (Fig. 2), being two-fold (**1** and **5**) and three-fold (**3** and **4**) more effective than the positive control verapamil (FAR = 12.5 at 20 μM). At 2 μM, only compounds **3–5** maintained the strong modulator feature (FAR > 10).

Effects on ABCB1 ATPase activity

The ABCB1 mediated efflux is coupled to ATP hydrolysis, being often stimulated by the transported substrates (Chufan et al., 2015). Measurement of this catalytic activity is an approach to investigate whether a candidate modulator acts as substrate or inhibitor (Chang et al., 2006). Therefore, epoxywelwitschene (**4**), the strongest modulator of this set of molecules, was examined for its effect on the ATPase activity of ABCB1. This activity was measured using purified insect membrane vesicles (Sf9) expressing high levels of human ABCB1. The inorganic phosphate resultant directly from ABCB1 ATP hydrolysis was assessed as vanadate sensitive ATPase activity. Vanadate is a phosphate analog that inhibits ABCB1 ATPase activity. Therefore, two complementary

Table 1
Antiproliferative activity of compounds **1–5** on L5178Y mouse T-lymphoma cells.

Compound	L5178Y-PAR IC_{50} (μM ± SD)	L5178Y-MDR IC_{50} (μM ± SD)	Relative resistance (RR)
Euphowelwitschine A (**1**)	> 50	> 50	–
Euphowelwitschine B (**2**)	> 50	> 50	–
Welwitschene (**3**)	27.86 ± 2.30	7.24 ± 1.70	0.26
Epoxywelwitschene (**4**)	48.50 ± 2.28	32.49 ± 2.25	0.67
Esulatin M (**5**)	16.64 ± 5.91	14.13 ± 0.81	0.85
Doxorubicin	–	2.98 ± 1.01	–

Values of IC_{50} are the mean ± standard deviation of three independent experiments.
Relative resistance (RR) = IC_{50} MDR cells/IC_{50} PAR cells.

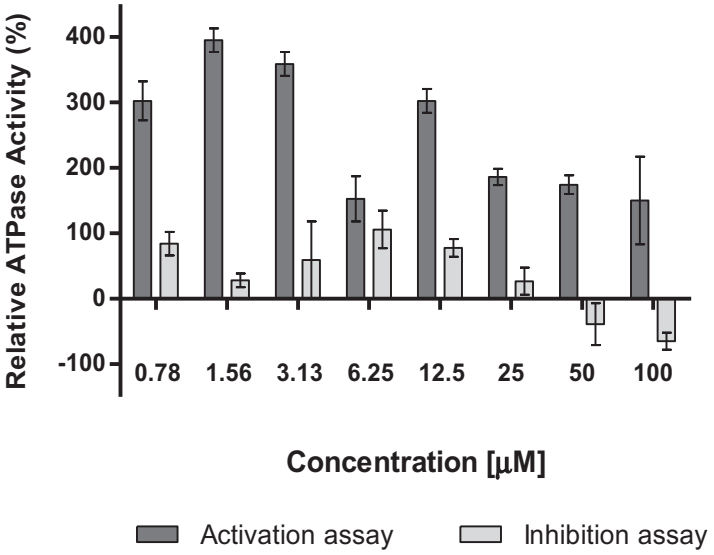

Fig. 3. Effect of epoxywelwitschene (**4**) on ABCB1 ATPase activity. Activation assay: to test the effect on the basal ATPase activity. Inhibition assay: to test the effect on drug-stimulated ATPase activity, measured in the presence of verapamil (40 μM). Results are expressed as the mean ± SD. The effects of compounds were presented as the relative ATPase activity, in which, the verapamil-stimulated vanadate-sensitive ATPase activity is taken as 100% and the baseline vanadate-sensitive ATPase activity as 0%.

assays compose the ATPase experiment: activation and inhibition assays. In the activation assay, compounds can be ranked as stimulators or inhibitors of the baseline vanadate sensitive ATPase activity. The inhibition assay, which is performed in the presence of a known ABCB1 activator, is primarily used to characterize efflux inhibitors (or slowly transported substrates) through the reduction of verapamil-stimulated vanadate-sensitive ATPase activity by the tested compound. Verapamil (40 μM) and cyclosporin A (40 μM) were used as positive controls in the activation and inhibition assays, respectively.

Hence, epoxywelwitschene (**4**) was tested in a dose-dependent manner and the effects were presented as the relative ATPase activity, in which the stimulated vanadate-sensitive ATPase activity is taken as 100% and the base line vanadate-sensitive ATPase activity as 0%. In this way, jatrophane **4** stimulated the ABCB1 ATPase activity, at concentrations ranging from 0.78 to 100 μM, 1.5 to 4 fold higher than verapamil, being the effect more pronounced at lower concentrations (Fig. 3, activation assay). Moreover, the inhibition assay indicated that epoxywelwitschene (**4**) inhibited the verapamil-stimulated ATPase activity, more pronouncedly at higher concentrations, being a complete inhibition attained at 50 and 100 μM (Fig. 3, inhibition assay).

Chemosensitization: reversion of drug-induced resistance

Since the studied diterpenes **1–5** showed activity as ABCB1 efflux modulators, the following step was to address what would be the MDR reversal effects after long exposure and what would be the outcomes of a combination with an antineoplastic drug, such as doxorubicin. Therefore, the antiproliferative activity of compounds **1–5** was evaluated in the L5178Y mouse T-lymphoma cell model by the MTT assay (Table 1). After 72 h incubation, the 12,17-cyclojatrophanes **1** and **2** did not have antiproliferative effect. Conversely, jatrophanes **3–5** showed an interesting activity since they were more active in ABCB1 overexpressing cells than in the parental cell line, being welwitschene (**3**) the most potent presenting an IC_{50} = 7.24 ± 1.70 μM (RR = 0.26).

Drug interaction studies were planned according to the Chou–Talalay method (Chou, 2010) and were assessed by the combination index (CI) as synergistic, additive or antagonistic (Fig. 4). Welwitschene (**3**) showed an antagonistic nature at 400:1 ratio (CI = 1.6) and nearly additive at 200:1 ratio (CI = 0.93); except for these two, all the other tested ratios synergistically enhanced the cytotoxicity of doxorubicin (CI = 0.5 – 0.4). Furthermore, euphowelwitschines A (**1**) and B (**2**), epoxywelwitschene (**4**) and esulatin M (**5**) synergistically enhanced the cytotoxicity of the drug (CI < 0.7) at all tested ratios.

Collateral sensitivity effect

In our preliminary data, compounds **1–4** were evaluated for their potential selective antiproliferative activity against parental gastric (EPG85- 257) and pancreatic (EPP-181) human cancer cells and their drug-resistant counterparts, selected against mitoxantrone (RNOV) or daunorubicin (RDB), respectively, using a proliferation assay (Reis et al., 2015). Epoxywelwitschene (**4**) was pointed as a potential MDR reverser due to its MDR-selective antiproliferative activity against EPG85-257RDB, EPP85-

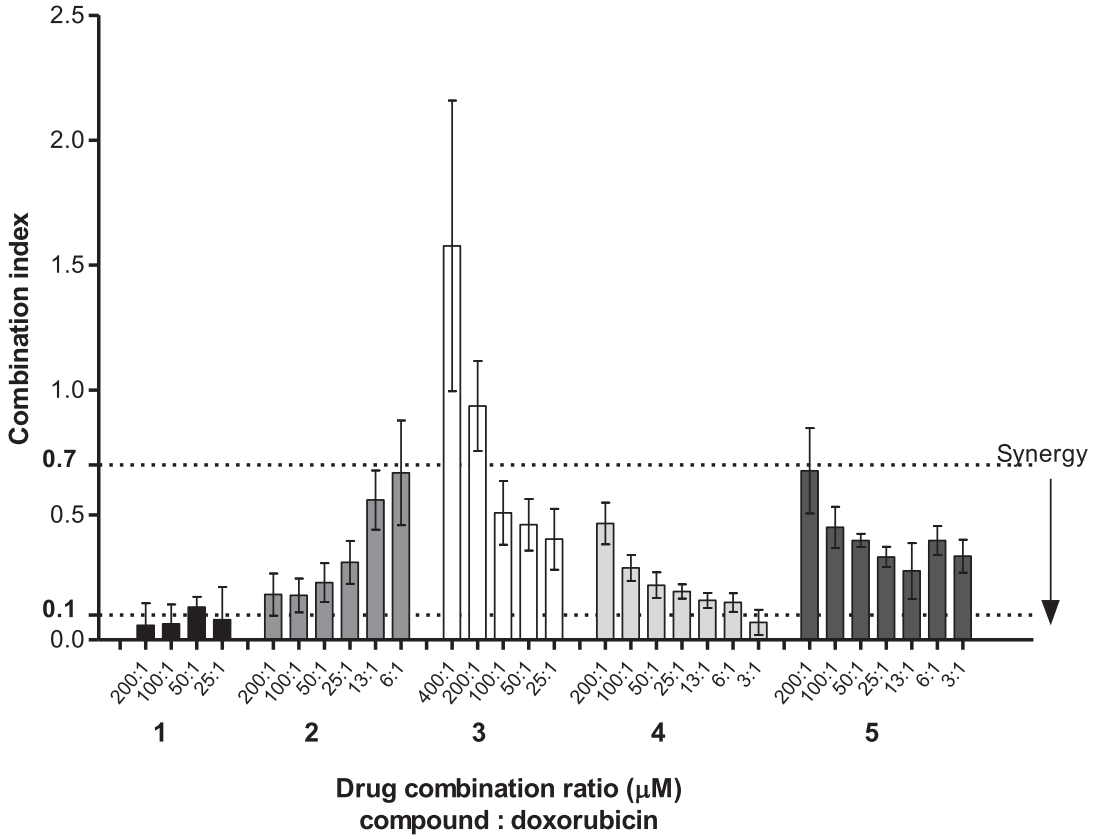

Fig. 4. Effect of compounds **1**–**5** in combination with doxorubicin in L5178Y-MDR cells. Drug combination ratio [compound]/[doxorubicin]. Combination index (CI) values are mean ± standard deviation for an inhibitory concentration of 50% (IC_{50}). CI < 0.1: very strong synergism; 0.1 < CI < 0.3: strong synergism; 0.3 < CI < 0.7: synergism; 0.7 < CI < 0.9: moderate to slight synergism; 0.9 < CI < 1.1: nearly additive; 1.10 < CI < 1.45: moderate antagonism; 1.45 < CI < 3.30: antagonism (Chou, 2010, 2006).

Table 2
Antiproliferative activity of esulatin M (**5**) against pancreatic carcinoma cells: EPP85-181P (parental), EPP85-181RNOV (MDR phenotype) and EPP85-181RDB (MDR phenotype) and on gastric carcinoma cells: EPG85-257P (parental), EPG85-257RNOV (MDR phenotype) and EPG85-257RDB (MDR phenotype).

	Esulatin M (**5**)		Cisplatin		Etoposide	
	IC_{50} (μM ± SD)	RR	IC_{50} (μM ± SD)	RR	IC_{50} (μM ± SD)	RR
EPP85-181P	12.7 ± 2.2	–	0.1 ± 0.01	–	0.6 ± 0.03	–
EPP85-181RNOV	11.2 ± 3.9	0.9	2.6 ± 0.2	34	4.5 ± 0.7	7.8
EPP85-181RDB	4.8 ± 0.7	0.4	0.1 ± 0.01	1.2	62.0 ± 4.2	106.9
EPG85-257P	10.7 ± 0.6	–	4.4 ± 0.4	–	0.1 ± 0.01	–
EPG85-257RNOV	9.7 ± 0.1	0.9	2.6 ± 0.2	0.6	1.6 ± 0.1	14.8
EPG85-257RDB	1.8 ± 0.1	0.2	4.0 ± 0.3	1	6.2 ± 0.3	59

IC_{50} value indicates the mean ± SD of $n = 3$–4 independent experiments (each concentration was performed in triplicate per experiment). Relative resistance (RR) = IC_{50} (MDR cells)/IC_{50} (parental cells).

181RNOV and EPP85-181RDB cells. Pursuing our research on this topic, esulatin M (**5**) was investigated for its potential collateral sensitivity effect on the same cancer entities. The MDR-selective activity was assessed by the relative resistance ratio ($RR = IC_{50(resistant)}/IC_{50(parental)}$). When RR < 1 indicates the compound kills MDR cells more effectively than parental cells, but if RR < 0.5, then a collateral sensitivity effect is taking place (Hall et al., 2009). The cytotoxic agents etoposide and cisplatin were used as positive controls. The antiproliferative activity and collateral sensitivity effects of esulatin M (**5**) are presented in Table 2. For the pancreatic cell lines, a MDR-selective antiproliferative effect was observed on EPG85-181RDB cells with $IC_{50} = 4.77 \pm 0.72$ μM, and RR = 0.37. A strong collateral sensitivity effect was observed towards the resistant gastric cell line EPG85-257RDB with $IC_{50} = 1.8 \pm 0.1$ μM and RR = 0.2. On this cell line, esulatin M (**5**) presented an antiproliferative activity higher than the positive controls cisplatin and etoposide (Table 2).

Given the promising results shown by esulatin M (**5**) and epoxywelwitschene (**4**) (Reis et al., 2015), compounds **4** and **5** were selected for apoptosis induction studies.

Apoptosis induction and active caspase-3 assays

Once the antiproliferative effects were determined, it was of interest to assess whether the mechanism of cell death was apoptosis or necrosis. The membrane phospholipid phosphatidylserine was used as marker of apoptosis. In the earlier stages of apoptosis, phosphatidylserine is translocated from the inner to the outer leaflet of the plasma membrane. Therefore, the annexin V/propidium iodide (PI) staining allows the identification of early

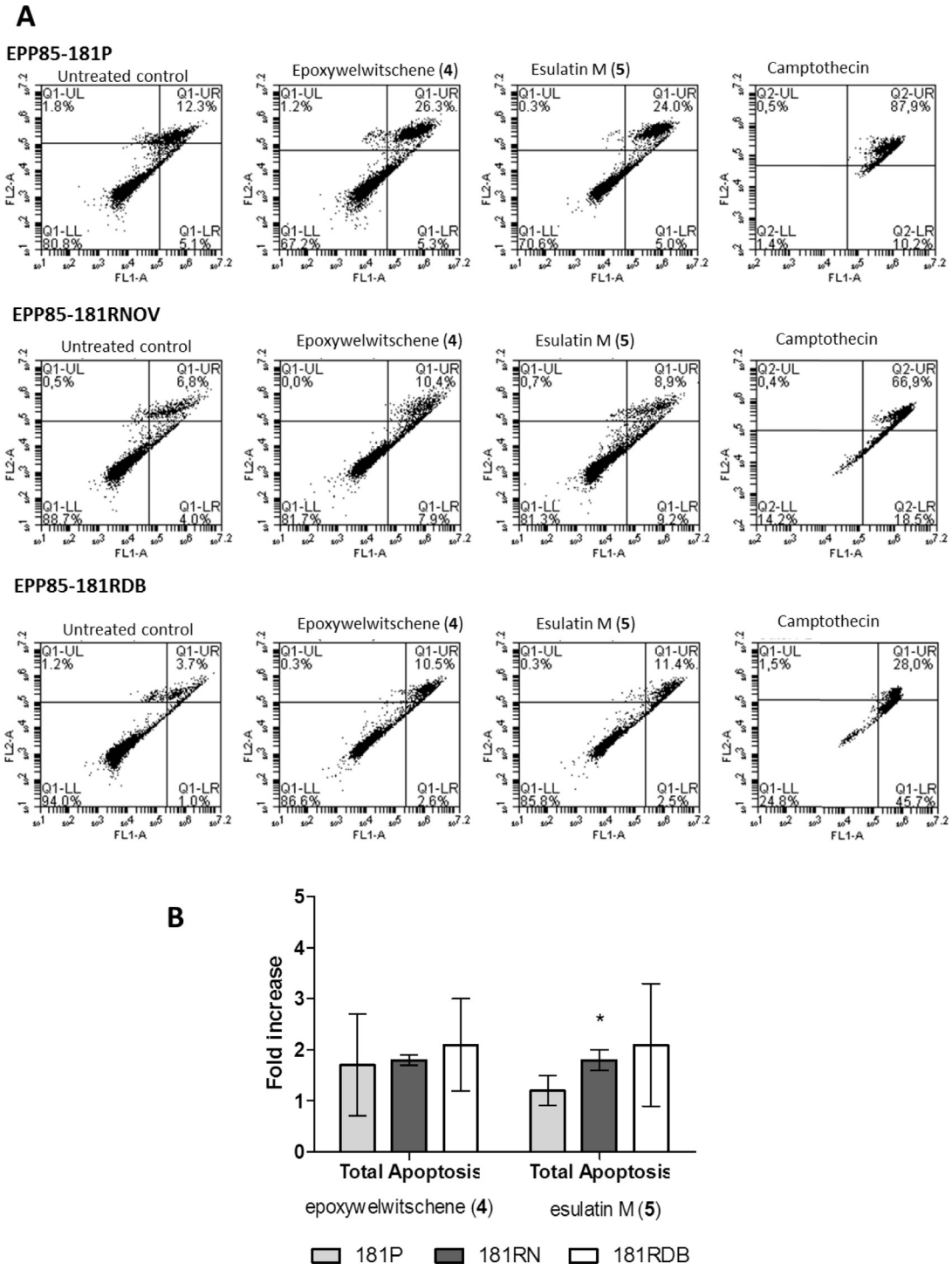

Fig. 5. Induction of apoptosis in pancreatic cancer cell lines after 72 h incubation with compounds **4** and **5** (30 μM). (A) Representative flow cytometry analysis after annexin V-FITC/PI staining. The FL1 and FL2 axis represent the fluorescence intensities of Annexin V-FITC and PI, respectively. Camptothecin (1 μM) was used as internal positive control. (B). Total apoptosis was considered the sum of early and late apoptotic events (cells annexin V-FITC positive/PI negative plus cells annexin V-FITC positive/PI positive). The results were expressed as the ratio between treated samples with untreated. Each column represents the mean ± SD of three independent experiments. Statistical significance was calculated for the difference between treated resistant cell lines and treated parental cells using a two-tailed unpaired Student's *t* test. Level of significance $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$.

apoptotic cells (PI negative, annexin V positive) and late apoptotic cells (PI positive, annexin V positive), where membrane integrity is lost (cells with intact membranes exclude PI) (Chen, 2009). The assessment of induction of apoptosis by compounds **4** and **5** (30 μM) was measured with the annexin V/PI assay, for 72 h (Figs. 5 and 6). The results were presented as total apoptosis (early and late apoptotic events) and the effects were expressed as fold increase (ratio between treated samples and untreated samples).

Regarding the pancreatic cancer cells, compounds **4** and **5** were able to induce apoptosis in about 2-fold (Fig. 5B). Statistical significant discrimination between resistant pancreatic cell lines (EPP85-181RNOV; EPP85-181RDB) and EPP85-181P cells could not

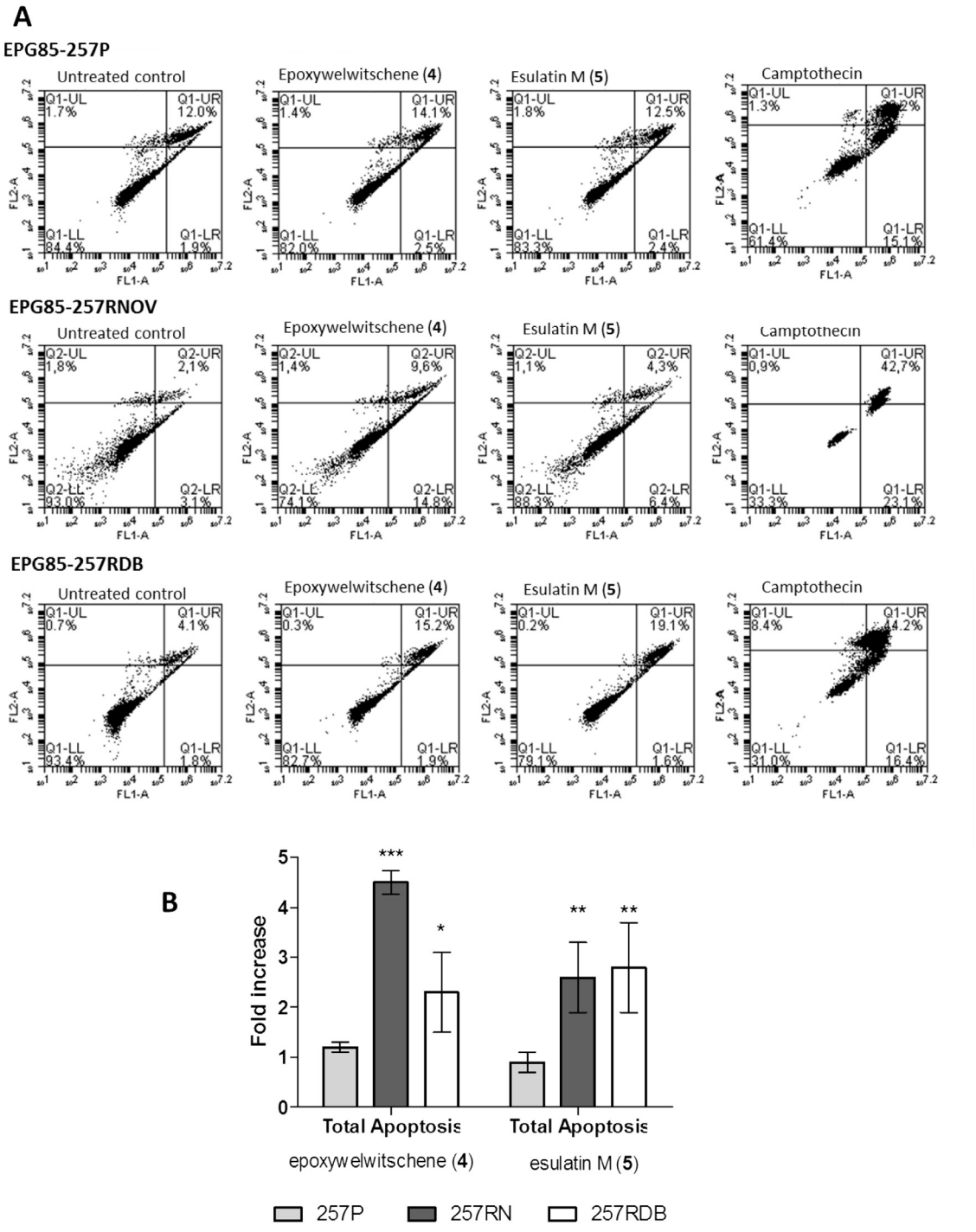

Fig. 6. Induction of apoptosis in gastric cancer cell lines after 72 h incubation with compounds **4** and **5** (30 μM). (**A**) Representative flow cytometry analysis after annexin V-FITC/PI staining. The FL1 and FL2 axis represent the fluorescence intensities of Annexin V-FITC and PI, respectively. Camptothecin (1 μM) was used as internal positive control. (B). Total apoptosis was considered the sum of early and late apoptotic events (cells annexin V-FITC positive/PI negative plus cells annexin V-FITC positive/PI positive). The results were expressed as the ratio between treated samples with untreated. Each column represents the mean ± SD of three independent experiments. Statistical significance was calculated for the difference between treated resistant cell lines and treated parental cells using a two-tailed unpaired Student's *t* test. Level of significance $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$.

be observed (Fig. 5B), despite the collateral sensitivity effect shown by esulatin M (**5**) (Table 2). A different scenario was found for the gastric cell lines; the incubation with jatrophanes **4** or **5** did not caused early or late apoptosis in EPG85-257P cells (parental) (Fig. 6B). Nevertheless, both compounds (**4** and **5**) were able to elicit statistical significant differences in the gastric MDR phenotypes (Fig. 6B). For EPG85-257RNOV cells, compound **4** caused a 4.5-fold increase of total apoptosis and compound **5** showed a 2.6-fold increase. In terms of EPG85-257RDB cells, both compounds presented a similar effect causing apoptosis in about 2.5 fold.

Once epoxywelwitschene (**4**) and esulatin M (**5**) killed the cells by apoptosis, it was interesting to follow if this involved

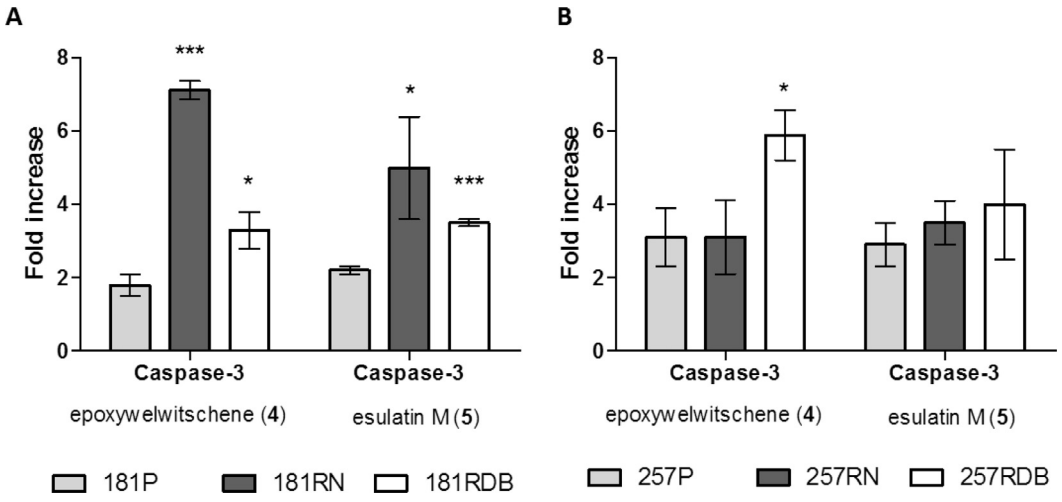

Fig. 7. Active caspase-3 in pancreatic (A) and gastric (B) cancer cell lines after 72 h incubation with compounds **4** and **5** (30 μM). The results were expressed as the ratio between treated samples with untreated. Each column represents the mean ± SD ($n = 3$). Statistical significance was calculated for the difference between treated resistant cell lines and treated parental cells using a two-tailed unpaired Student's t test. Level of significance $^{*}p < 0.05$. $^{**}p < 0.01$. ***, $p < 0.001$. Representative flow cytometric analysis of populations after active caspase-3 staining at Supplementary material.

caspase activation. Activated caspases cleave and activate other downstream caspases, as well as other targets in the cytoplasm (*e.g.* Bcl-2), and in the nucleus (*e.g.* PARP). Caspase-3 plays a central role in the execution of apoptosis, being pro-caspase-3, the penultimate enzyme for execution of the apoptotic process (Tan et al., 2009). Therefore, the quantification of active caspase-3 was assessed by flow cytometry after 72 h of exposure, at 30 μM (Fig. 7). The results were expressed as fold increase (ratio between treated samples and untreated samples).

In terms of active caspase-3 in the pancreatic cells, a statistical significant discrimination was observed between the resistant cell lines and parental cells (Fig. 7A). Compound **4** caused a 7-fold increase in EPP85-181RNOV and about 3-fold in EPP85-181RDB. As for jatrophane **5,** a 3.5-fold increase was observed for EPP85-181RNOV and 5-fold for EPP85-181RDB cells. No significant differences were found between the gastric cancer phenotypes (Fig. 7B).

Discussion

The research on potent ABCB1 reversal agents has been subject of massive endeavor, nevertheless no ABCB1 modulator is currently in clinical usage. Some of the drawbacks were attributed to the toxic effects derived from simultaneous inhibition of ABCB1 and the drug metabolizing cytochrome enzyme P450 (CYP3A4 isoform), and to the inability of modulators/inhibitors to discriminate between ABCB1 expressed in normal tissues and ABCB1 expressed in cancerous ones (Callaghan et al., 2014). Such selective action is one of the most desired traits of anti-cancer MDR therapy. In the course of drug discovery process, collateral sensitivity can offer new insights into this problem. The mechanisms of MDR-selectivity are still under investigation. For instance, some works associated it to the expression of ABC transporters; nevertheless, others found it to be independent (Hall et al., 2009; Szakács et al., 2014). Therefore, it is of relevance to study the effect of compounds in ABCB1 and collateral sensitivity.

In a previous study, we had investigated the potential collateral sensitivity effect of the two rare 12,17-cyclojatrophanes (**1–2**) and other novel jatrophanes (**3–4**) isolated from *Euphorbia welwitschii* (Reis et al., 2015). In order to continue this study, we further evaluated the MDR mechanisms of these compounds (**1**–**4**) on ABCB1 modulation, along with esulatin M (**5**). This compound was isolated during the extension of the phytochemical study of *E. welwitschii.* Despite the fact that esulatin M (**5**) has been reported as modulator of ABCB1 (Vasas et al., 2011), it was of interest to look into its anti-MDR activity in more detail and compare it with the other novel compounds. As referred, ABCB1 modulation results indicated compounds **1**–**5** as potential efflux modulators (Fig. 2A). Interesting structure-activity findings were obtained from the FAR values at 2 μM (Fig. 2B). As could be observed, the high conformational flexibility of the twelve-membered ring of jatrophanes **3–5** promotes ABCB1 modulation, in contrast to the 5/8/8 fused ring system of euphowelwitschines A (**1**) and B (**2**). Similar observations were also found for rearranged polycyclic jatrophanes, based on segetane, paraliane and pepluane skeletons. Those showed a lower ABCB1 modulatory efficiency when compared with molecules with the macrocyclic jatrophane-type scaffold (Corea et al., 2009; Ferreira et al., 2014).

Some physicochemical properties (molecular weight, molecular volume, logP, molar refractivity, topological polar surface area, accessible solvent area) of compounds **1–5** were also computed. However, due to the low variability obtained, no conclusions could be established (data not shown).

Epoxywelwitschene (**4**) was found to be the strongest efflux modulator of this set (**1**–**5**) of molecules. Therefore, in order to understand the relation between its activity and ABCB1 ATP hydrolysis, the catalytic activity of this transporter was evaluated in the presence and absence of a second transported substrate. The results of the ATPase activity indicated that epoxywelwitschene (**4**) interacted with ABCB1, and reduced verapamil transport. Hence, taking together the results, it might be concluded that compound **4** was able to impair the transport of rhodamine-123 and of verapamil due to direct interaction with ABCB1. Rhodamine-123 and verapamil have distinct binding sites within the drug binding pocket of ABCB1 (Chufan et al., 2015). Epoxywelwitschene (**4**) might affect the affinity of these molecules to ABCB1 by acting as a modulator (Ferreira et al., 2013; Zinzi et al., 2014) and/or a slowly transported substrate. These assumptions were also corroborated by the combination assay results. The synergic effect of epoxywelwitschene (**4**)/doxorubicin restored the cytotoxicity of doxorubicin in the ABCB1-mouse T-lymphoma cell line. This is a relevant finding because doxorubicin's cytostatic efficacy is known to be hampered by ABCB1 activity (Gottesman et al., 2002; Szakács et al., 2006). It is also worth to note that the other tested compounds (**1–3** and **5**), besides their effect on rhodamine-123 efflux, also presented a synergistic interaction with doxorubicin. This

work highlights the importance of these compounds as ABCB1-MDR reversers, because their activity is in accordance with the assumption that a promising ABCB1 modulator will consequently be able to increase the action of a cytotoxic drug, due to greater efflux impairment.

In respect to the selective targeting of MDR phenotypes, esulatin M (**5**) showed collateral sensitivity effect in both pancreatic and gastric cell lines resistant to daunorubicin. This compound (**5**) and epoxywelwitschene (**4**), whose collateral sensitivity effects have been evaluated in a previous study (Reis et al., 2015), were selected for further investigation. Indeed, it was verified that, in the pancreatic MDR phenotypes, the selective activity of epoxywelwitschene (**4**) and esulatin M (**5**) occurred through an apoptotic caspase-dependent pathway. As for the gastric cancer cells, although discrimination between resistant and parental cell lines was not so evident in terms of active caspase-3, it can be considered that the selective cytotoxicity was also modulated by the same apoptotic pathway.

In summary, this study demonstrates the role of macrocyclic jatrophanes as lead candidates for the development of MDR reversal agents. The compounds epoxywelwitschene (**4**) and esulatin M (**5**) appear particularly interesting, due to their dual activity: as ABCB1 modulators and MDR-selective antiproliferative compounds.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2016.05.007.

References

Callaghan, R., Luk, F., Bebawy, M., Cuperus, F.J.C., Claudel, T., Gautherot, J., Halilbasic, E., Trauner, M., 2014. Inhibition of the multidrug resistance P-glycoprotein: time for a change of strategy. Drug Metab. Dispos. 42, 623–631.

Chang, C., Bahadduri, P.M., Polli, J.E., Swaan, P.W., Ekins, S., 2006. Rapid identification of P-glycoprotein substrates and inhibitors. Drug Metab. Dispos. 34, 1976–1984.

Chen, T., 2009. A Practical Guide to Assay Development and High-Throughput Screening in Drug Discovery. CRC Press, Boca Raton, Florida.

Chou, T.C., 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol. Rev. 58, 621–681.

Chou, T.C., 2010. Drug combination studies and their synergy quantification using the Chou–Talalay method. Cancer Res. 70, 440–446.

Chufan, E.E., Sim, H., Ambudkar, S.V, 2015. Molecular basis of the polyspecificity of P-glycoprotein (ABCB1): recent biochemical and structural studies. In: Adv Cancer Res. Elsevier Inc., pp. 71–96.

Corea, G., Di Pietro, A., C., Dumontet, E., Fattorusso, Lanzotti, V., 2009. Jatrophane diterpenes from *Euphorbia* spp. as modulators of multidrug resistance in cancer therapy. Phytochem. Rev. 8, 431–447.

Eid, S.Y., El-Readi, M.Z., Fatani, S.H., Mohamed, E.E., Wink, M., 2015. Natural products modulate the multifactorial multidrug resistance of cancer. Pharmacol. Pharm. 06, 146–176.

Ferreira, M.J.U., Duarte, N., Reis, M., Madureira, A.M., Molnár, J., 2014. *Euphorbia* and *Momordica* metabolites for overcoming multidrug resistance. Phytochem. Rev. 13, 915–935.

Ferreira, R.J., dos Santos, D.J.V., Ferreira, M.J.U., Guedes, R.C., 2011. Toward a better pharmacophore description of P-glycoprotein modulators, based on macrocyclic diterpenes from *Euphorbia* species. J. Chem. Inf. Model. 51, 1315–1324.

Ferreira, R.J., Ferreira, M.J.U., dos Santos, D.J.V., 2013. Molecular docking characterizes substrate-binding sites and efflux modulation mechanisms within P-glycoprotein. J. Chem. Inf. Model. 53, 1747–1760.

Gottesman, M.M., Fojo, T., Bates, S.E., 2002. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat. Rev. Cancer 2, 48–58.

Hall, M.D., Handley, M.D., Gottesman, M.M., 2009. Is resistance useless? Multidrug resistance and collateral sensitivity. Trends Pharmacol. Sci. 30, 546–556.

Hilgeroth, A., Baumert, C., Coburger, C., Seifert, M., Krawczyk, S., Hempel, C., Neubauer, F., Krug, M., Molnár, J., Lage, H., 2013. Novel structurally varied N-alkyl 1,4-dihydropyridines as ABCB1 inhibitors: structure-activity relationships, biological activity and first bioanalytical evaluation. Med. Chem. 9, 487–493.

Holohan, C., Van Schaeybroeck, S., Longley, D.B., Johnston, P.G., 2013. Cancer drug resistance: an evolving paradigm. Nat. Rev. Cancer 13, 714–726.

Kartal-Yandim, M., Adan-Gokbulut, A., Baran, Y., 2015. Molecular mechanisms of drug resistance and its reversal in cancer. Crit. Rev. Biotechnol. 8551, 1–11.

Lage, H., 2008. An overview of cancer multidrug resistance: a still unsolved problem. Cell. Mol. Life Sci. 65, 3145–3167.

Lage, H., Duarte, N., Coburger, C., Hilgeroth, A., Ferreira, M.J.U., 2010. Antitumor activity of terpenoids against classical and atypical multidrug resistant cancer cells. Phytomedicine 17, 441–448.

Matos, A.M., Reis, M., Duarte, N., Spengler, G., Molnár, J., Ferreira, M.J.U., 2015. Epoxylathyrol derivatives: modulation of ABCB1-mediated multidrug resistance in human colon adenocarcinoma and mouse T-lymphoma cells. J. Nat. Prod. 78, 2215–2228.

Palmeira, A., Sousa, E., Vasconcelos, M.H., Pinto, M.M., 2012. Three decades of P-gp inhibitors: skimming through several generations and scaffolds. Curr. Med. Chem. 19, 1946–2025.

Pastan, I., Gottesman, M.M., Ueda, K., Lovelace, E., Rutherford, V., Willingham, M.C., 1988. A retrovirus carrying an MDR1 cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells. Proc. Natl. Acad. Sci. USA 85, 4486–4490.

Reis, M.A., Paterna, A., Ferreira, R.J., Lage, H., Ferreira, M.J.U., 2014. Macrocyclic diterpenes resensitizing multidrug resistant phenotypes. Bioorg. Med. Chem. 22, 3696–3702.

Reis, M., Ferreira, R.J., Santos, M.M.M., dos Santos, D.J.V., Molnár, J., Ferreira, M.J.U., 2013. Enhancing macrocyclic diterpenes as multidrug-resistance reversers: structure-activity studies on jolkinol D derivatives. J. Med. Chem. 56, 748–760.

Reis, M., Ferreira, R.J., Serly, J., Duarte, N., Madureira, A.M., J.V.A. Santos, D., Molnar, J., U. Ferreira, M.J., 2012. Colon adenocarcinoma multidrug resistance reverted by *Euphorbia* Diterpenes: structure-activity relationships and pharmacophore modeling. Anticancer Agents Med. Chem. 12, 1015–1024.

Reis, M.A., André, V., Duarte, M.T., Lage, H., Ferreira, M.J.U., 2015. 12,17-cyclojatrophane and jatrophane constituents of *Euphorbia welwitschii*. J. Nat. Prod. 78, 2684–2690.

Sousa, I.J., Ferreira, M.-J.U., Molnár, J., Fernandes, M.X., 2012. QSAR studies of macrocyclic diterpenes with P-glycoprotein inhibitory activity. Eur. J. Pharm. Sci. 48, 542–553.

Szakács, G., Hall, M.D., Gottesman, M.M., Boumendjel, A., Kachadourian, R., Day, B.J., Baubichon-Cortay, H., Di Pietro, A., 2014. Targeting the achilles heel of multidrug-resistant cancer by exploiting the fitness cost of resistance. Chem. Rev. 114, 5753–5774.

Szakács, G., Paterson, J.K., Ludwig, J.A, Booth-Genthe, C., Gottesman, M.M., 2006. Targeting multidrug resistance in cancer. Nat. Rev. Drug Discov. 5, 219–234.

Szybalski, W., Bryson, V., 1952. Genetic studies on microbial cross resistance to toxic agents I, cross resistance of *Escherichia coli* to fifteen antibiotics. J. Bacteriol. 64, 489–499.

Tan, M.L., Ooi, J.P., Ismail, N., Moad, A.I.H., Muhammad, T.S.T., 2009. Programmed cell death pathways and current antitumor targets. Pharm. Res. 26, 1547–1560.

Vasas, A., Hohmann, J., 2014. *Euphorbia* diterpenes: isolation, structure, biological activity, and synthesis (2008–2012). Chem. Rev. 114, 8579–8612.

Vasas, A., Sulyok, E., Rédei, D., Forgo, P., Szabó, P., Zupkó, I., Berényi, Á., Molnár, J., Hohmann, J., 2011. Jatrophane diterpenes from *Euphorbia esula* as antiproliferative agents and potent chemosensitizers to overcome multidrug resistance. J. Nat. Prod. 74, 1453–1461.

Vieira, C., Duarte, N., Reis, M.A., Spengler, G., Madureira, A.M., Molnár, J., Ferreira, M.J.U., 2014. Improving the MDR reversal activity of 6,17-epoxylathyrane diterpenes. Bioorg. Med. Chem. 22, 6392–6400.

Voigt, B., Coburger, C., Monár, J., Hilgeroth, A., 2007. Structure-activity relationships of novel N-acyloxy-1,4-dihydropyridines as P-glycoprotein inhibitors. Bioorg. Med. Chem. 15, 5110–5113.

Wu, C., Ohnuma, S., Ambudkar, S.V., 2011. Discovering natural product modulators to overcome multidrug resistance in cancer chemotherapy. Curr. Pharm. Biotechnol. 12, 609–620.

Zinzi, L., Capparelli, E., Cantore, M., Contino, M., Leopoldo, M., Colabufo, N.A., 2014. Small and innovative molecules as new strategy to revert MDR. Front. Oncol. 4, 1–12.