


SPECIAL ISSUE ARTICLE

Effective MDR reversers through phytochemical study of *Euphorbia boetica*

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Abstract

Introduction: Macrocyclic diterpenes from *Euphorbia* species were found to be promising modulators of multidrug resistance (MDR), a complex phenomenon that hampers the effectiveness of cancer therapy.

Objective: To find new effective MDR reversers through the phytochemical study of *E. boetica*, including isolation and molecular derivatisation.

Material and methods: The phytochemical study of *E. boetica* was performed through chromatographic techniques. Preliminary analysis of crude chromatographic fractions from the methanol extract was carried out by ¹H-NMR in order to prioritise the study of those having macrocyclic diterpenes. Polyamide resin was used to remove chlorophylls. Molecular derivatisation of isolated compounds comprised hydrolysis, reduction and acylation reactions. The structural identification of compounds was performed through analysis of spectroscopic data, mainly one-dimensional- and two-dimensional-NMR. The MDR reversing activity was assessed using a combination of transport and chemosensitivity assays, in mouse lymphoma (L5178Y-MDR) and Colo320 cell models.

Results: The ¹H-NMR study of crude fractions and application of a straightforward method to remove chlorophylls, allowed the effortless isolation of two lathyrane-type diterpenes in large amounts, including the new polyester, euphoboetirane B (**1**). Taking advantage of the chemical functions of **1**, 13 new derivatives were prepared. Several compounds showed to be promising modulators of P-glycoprotein (P-gp), in resistant cancer cells. Most of the compounds tested revealed to interact synergistically with doxorubicin.

Conclusion: These results corroborate the importance of macrocyclic lathyrane diterpenes as effective lead compounds for the reversal of MDR.

KEYWORDS

Euphorbia, lathyrane, macrocyclic diterpenes, multidrug resistance, P-glycoprotein

1 | INTRODUCTION

Euphorbia species (Euphorbiaceae) are reported to be used in traditional medicine to treat skin cancer and warts, digestive system disorders and infections.¹ Importantly, topical administration of *E. peplus* sap in Australian folk medicine prompted the development of Picato® (ingenol mebutate) gel, approved in 2012 by the US Food and Drug Administration (FDA) for the treatment of actinic keratosis.² In the last decades, *Euphorbia* species have been the subject of intensive phytochemical studies that have resulted in the isolation of a high diversity of compounds, some of them exhibiting very important biological activities.^{3,4} In particular, these species have afforded a large number of structurally unique macrocyclic diterpenes with the lathyrane and jatrophane skeletons.^{4,5} Apart from their importance as biogenetic and chemotaxonomic markers, since they are exclusively isolated from Euphorbiaceae plants,⁶ several studies have revealed that they are promising modulators of multidrug resistance (MDR) in tumour cells.^{4,5,7-14}

Currently, resistance towards many clinically used drugs is a major limitation to effective cancer therapies.¹⁵⁻¹⁷ Cancer resistance can be broadly divided as intrinsic, when the tumour fails to respond to the initial chemotherapy, and acquired during treatment by various therapy-induced adaptive responses.^{16,18} Resistance could also take more complex outcomes when treatments fail to respond to multiple drugs with different mechanisms of action, a phenomenon referred as multidrug resistance (MDR).¹⁹ There are several mechanisms responsible for MDR, which have been exhaustively reviewed.^{15,17,18,20-22} By far, the most studied mechanisms are related with the overexpression of several efflux membrane proteins. Among them, the ATP-Binding-Cassette (ABC) superfamily largely contributes to MDR, resulting in the increased translocation of the cytotoxic drugs out of the cell, consequently reducing their intracellular concentrations and their biological effect.^{15,19,21}

In mammalian cells, three main ABC transporter proteins are involved in MDR: P-glycoprotein (P-gp/MDR1/ABCB1), the multidrug resistant associated protein (MRP1/ABCC1) and the breast cancer resistant protein (BCRP/ABCG2).^{19,21} P-gp has been exhaustively studied, and until date, some of the most significant strategies to overcome MDR target this efflux transporter. One pharmacological approach involves the co-administration of a non-toxic P-gp modulator and the cytotoxic agent, in order to inhibit or modulate its efflux, increasing the concentration of drugs within the cells.¹⁹⁻²¹ Many synthetic and natural P-gp modulators have been reported, and some of them have reached the stage of clinical trials. However, only limited success was achieved and more studies to find new non-toxic and effective P-gp modulators are still of great significance.^{23,24} In addition, in recent years considerable progresses have been made on knowledge about mechanistic and functional aspects of ABC transporters, not only to define their substrates and inhibitors,²¹ but also to characterise P-gp structure at near-atomic resolution providing molecular basis for additional conformations and drug binding modes and sites.²³

Continuing our research for novel MDR modulators from natural sources, herein, we report the isolation of two lathyrane diterpene polyesters (**1** and **2**), from *Euphorbia boetica* aerial parts, one of them

(**1**) isolated for the first time. The straightforward and timeless isolation of compounds was only possible thanks to the use preliminary ¹H-NMR analysis of crude fractions, and removal of chlorophylls through flash chromatography over polyamide. Aiming at obtaining a set of homologous bioactive compounds, diterpene **1**, isolated in high quantity, was submitted to several chemical transformations, including hydrolysis, reduction and acylation reactions. Overall, 13 derivatives (**3-15**) were obtained, and characterised using spectroscopic methods. Their effect on modulation of P-gp efflux was evaluated by flow cytometry, measuring the rhodamine-123 accumulation in MDR mouse T-lymphoma cells and MDR human colon adenocarcinoma (Colo 320) cells. Furthermore, some of these modulators were assayed, *in vitro*, for their effects in combination with doxorubicin.

2 | EXPERIMENTAL SECTION

2.1 | General procedures

All solvents were dried according to published methods and distilled prior to use. All the other reagents were obtained from commercial suppliers and were used without further purification. Flash column chromatography (CC) was performed on polyamide CC 6 (0.05–0.16 mm, Macherey-Nagel) and silica gel (Merck 9385), or by using CombiFlash® Rf200 (Teledyne Isco). Merck silica gel 60 F254 plates were used in analytical thin-layer chromatography (TLC), with visualisation under ultraviolet (UV) light ($\lambda = 254$ and 366 nm) and by spraying with sulphuric acid/methanol (H₂SO₄/MeOH) (1:1), followed by heating. For preparative TLC, 20 cm × 20 cm silica plates were used (Merck 1.05774). Melting points were determined on a Köpffler apparatus. Specific optical rotations $[\alpha]_D^{25}$ were obtained on a Perkin-Elmer 241-MC polarimeter using quartz cells of 1 dm path length, and all samples were solubilised in chloroform (CHCl₃). Infrared (IR) spectra were determined on a Shimadzu IRAffinity-1 FTIR spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Brüker ARX-400 NMR spectrometer (¹H 400 MHz; ¹³C 100.61 MHz), using CDCl₃, CD₃OD, C₅D₅N or DMSO-*d*₆ as solvents. Chemical shifts are expressed in δ (ppm) referenced to the solvent used, and the proton coupling constants *J* in hertz (Hz). Spectra were assigned using appropriate COSY, DEPT, HMQC and HMBC sequences. High-resolution mass spectra were recorded on a FTICR-MS Apex Ultra (Brüker Daltonics) 7 T instrument. Electrospray ionisation mass spectrometry (ESI-MS) analysis were performed on a triple quadrupole (QT) Micromass Quattro Micro AP1 mass spectrometer, with an ESI ion source set in a positive ionisation mode. All tested compounds were purified to $\geq 95\%$ purity as determined by high-performance liquid chromatography (HPLC).

2.2 | Phytochemical study

Euphorbia boetica Boiss. (Euphorbiaceae) aerial parts were extracted with MeOH as previously reported.⁸ Briefly, the air-dried aerial parts were exhaustively extracted with MeOH at room temperature. The pooled extracts were evaporated under vacuum to give a residue that

was suspended in a MeOH/H₂O solution (1:1) and extracted with ethyl acetate (EtOAc). Chromatographic fractionation of the EtOAc soluble part of the MeOH extract afforded 10 crude fractions (A–J). To perform preliminary ¹H-NMR experiments, 15 mg of each fraction were solubilised in 650 μL of CDCl₃, filtered, and further analysed aiming at prioritising the study of those fractions having macrocyclic diterpenes. Fraction E (58.2 g) obtained with *n*-hexane/EtOAc (7:3) was chromatographed on a polyamide column (300 g) with mixtures of MeOH/H₂O (1:1, 3:2, 7:3, 4:1 and 1:0) as eluents. The Fraction obtained with MeOH/H₂O (3:2 and 7:3, 24.8 g) was subjected to silica gel flash chromatography [silicaon dioxide (SiO₂), 300 g], using a gradient of *n*-hexane/EtOAc (1:0 to 0:1) and EtOAc/MeOH (1:0 to 3:1). As indicated by TLC, and according to differences in composition, 12 fractions were obtained (E_A–E_L). Fractions E_B, E_C, E_D, E_E, E_G and E_H were recrystallised from EtOAc/*n*-hexane to give: euphoboetirane B (**1**, 1.88 g) and **2** (Euphorbia Factor L15, herein named euphoboetirane A, 1.72 g). The residue of fraction E_F (2.63 g) and the mother liquors of fraction E_E (1.69 g) were combined and chromatographed over SiO₂ (200 g) using mixtures of *n*-hexane/EtOAc (1:0 to 0:1) to obtain six fractions (E_{EF1a} to E_{EF1f}). The residue of fraction E_{EF1b} (1.52 g) was recrystallised with EtOAc/*n*-hexane to obtain 740 mg of the already isolated compound **2**.

Euphoboetirane B; *5α,15β*-diacetoxy-3β-propanoyloxy-lathyr-6(17),12E-en-14-one (**1**): white crystals (EtOAc/*n*-hexane); m.p. 168–171°C; [α]_D²⁵ + 213.0 (c 0.100, CHCl₃); IR (KBr): ν_{max} 2969, 1736, 1674, 1624, 1373, 1227, 907 cm⁻¹; ¹H-NMR (400 MHz, CDCl₃): δ 0.87 (3H, d, *J* = 6.7 Hz, CH₃-16), 1.12 (3H, s, CH₃-19), 1.12 (3H, t, *J* = 7.4 Hz, 3-OCOCH₂CH₃), 1.14 (3H, s, CH₃-18), 1.12 (1H, m, H-9), 1.36 (1H, dd, *J* = 8.2, 11.4 Hz, H-11), 1.56 (1H, dd, *J* = 11.6, 14.4 Hz, H-1β), 1.66 (3H, s, CH₃-20), 1.74 (1H, m, H-8a), 1.94 (3H, s, 5-OCOC-H₃), 2.00 (1H, m, H-7b), 2.04 (1H, m, H-8b), 2.06 (3H, s, 15-OCOCH₃), 2.20 (1H, m, H-7a), 2.27 (1H, m, H-2), 2.32 (2H, m, 3-OCOCH₂CH₃), 2.75 (1H, dd, *J* = 3.4, 10.2 Hz, H-4), 3.42 (1H, dd, *J* = 8.4, 14.4 Hz, H-1α), 4.70 (1H, s, H-17a), 4.96 (1H, s, H-17b), 5.57 (1H, t, *J* = 3.2 Hz, H-3), 6.05 (1H, d, *J* = 10.3 Hz, H-5), 6.46 (1H, d, *J* = 11.4 Hz, H-12); ¹³C-NMR (100.61 MHz, CDCl₃): δ 9.1 (3-OCOCH₂CH₃), 12.5 (C-20), 14.2 (C-16), 16.9 (C-19), 21.3 (5-OCOCH₃), 21.7 (C-8), 22.1 (15-OCOCH₃), 25.4 (C-10), 27.9 (3-OCOCH₂CH₃), 28.5 (C-11), 29.1 (C-18), 35.1 (C-7), 35.5 (C-9), 37.4 (C-2), 48.5 (C-1), 52.4 (C-4), 65.9 (C-5), 80.1 (C-3), 92.5 (C-15), 115.6 (C-17), 134.3 (C-13), δ 144.5 (C-6), 146.8 (C-12), 169.9 (15-OCOCH₃), 170.6 (5-OCOCH₃), 173.9 (3-OCOCH₂CH₃), 197.0 (C-14); ESI-MS *m/z* 475 [M + H]⁺. HR-ESI-MS: *m/z* 475.26163 [M + H]⁺ (calcd for C₂₇H₃₉O₇: 475.26175).

Euphoboetirane A (Euphorbia Factor L15, 2): white crystals (EtOAc/*n*-hexane); m.p. 138–140°C; [α]_D²⁵ + 230.0 (c 0.117, CHCl₃); IR (KBr): ν_{max} 2934, 1736, 1644, 1613, 1375, 905 cm⁻¹; ESI-MS *m/z* 461 [M + H]⁺.²⁵

2.3 | Preparation of 14β-hydroxylathyrane (3)

Euphoboetirane B (**1**, 0.33 mmol) was dissolved in dry tetrahydrofuran (THF, 5 mL) and the solution was cooled at 0°C. Lithium aluminium

hydride (LiAlH₄) (0.1 mol) was added and the mixture was stirred for 1 h at 0°C. The reaction was stopped with aqueous sodium hydroxide (NaOH) (10%, 1.5 mL) and stirred for 10 min to neutralise the excess of LiAlH₄. The obtained precipitate was filtrated through celite with EtOAc/H₂O 20% (*m/v*, 100 mL) and the aqueous layer was extracted with EtOAc (8 × 20 mL). The organic layers were dried with anhydrous sodium sulphate (Na₂SO₄), and evaporated to give an oil that was subjected to column chromatography (SiO₂, 12 g, *n*-hexane/EtOAc 1:0 to 0:1, CombiFlash system) to give 56 mg of a white powder (0.17 mmol, 52% yield).

14β-Hydroxylathyrane, 3β,5α,14β,15β-tetrahydroxy-lathyr-6(17),12E-ene (3): [α]_D²⁵ -134 (c 0.100, CHCl₃); IR (KBr) ν_{max} 3387, 2930, 1627, 1240, 922 cm⁻¹; ¹H-NMR (400 MHz, CDCl₃): δ 0.87 (1H, m, H-9), 0.94 (1H, m, H-8b), 1.07 (3H, d, *J* = 4.4 Hz, H-16), 1.13 (3H, s, H-18), 1.07 (3H, s, H-19), 1.32 (1H, dd, *J* = 8.4, 10.0 Hz, H-11), 1.59 (1H, m, H-7b), 1.66 (1H, m, H-8a), 1.70 (3H, s, H-20), 1.74 (1H, m, H-4), 1.78 (1H, m, H-1β), 1.91 (1H, m, H-2), 2.32 (1H, m, H-1α), 2.39 (1H, m, H-7a), 4.21 (1H, t, *J* = 3.2 Hz, H-3), 4.24 (1H, s, H-14), 4.91 (1H, s, H-17a), 4.92 (1H, d, *J* = 6.0 Hz, H-5), 5.06 (1H, s, H-17b), 5.80 (1H, d, *J* = 10.8 Hz, H-12); ¹³C-NMR (100.61 MHz, CDCl₃): δ 14.4 (C-16), 15.5 (C-19), 16.2 (C-20), 22.2 (C-10), 23.9 (C-8), 24.6 (C-11), 29.1 (C-18), 33.0 (C-9), 34.5 (C-7), 36.8 (C-2), 48.2 (C-1), 48.8 (C-4), 71.5 (C-5), 77.8 (C-14), 78.2 (C-3), 84.6 (C-15), 109.1 (C-17), 122.8 (C-12), 132.8 (C-13), 150.6 (C-6); ESI-MS *m/z* 359 [M + Na]⁺, 375 [M + K]⁺.

2.4 | Preparation of lathyrol (4)

Compound **1** (1.52 mol) in MeOH/KOH (potassium hydroxide) (5%) was stirred for 3 h at room temperature. The reaction was worked up by dilution with water (20 mL) and extraction with EtOAc (8 × 20 mL). The organic layers were dried with Na₂SO₄, evaporated and purified by column chromatography (12 g, *n*-hexane/EtOAc 1:0 to 0:1, CombiFlash system), and further recrystallisation with EtOAc/*n*-hexane to give 390 mg of **4** (1.18 mol, yield 78%). *Lathyrol, 3β,5α,15β-trihydroxy-lathyr-6(17),12E-en-14-one (4)*: m.p. 174–176°C (EtOAc/*n*-hexane); [α]_D²⁵ + 116.0 (c 0.100, CHCl₃); IR (KBr): ν_{max} 3414, 1640, 1622, 1411, 1269, 909 cm⁻¹; ESI-MS *m/z* 335 [M + H]⁺. This compound was identified by comparison of the obtained NMR spectroscopic data with literature values.²⁶

2.5 | General preparation of lathyrol derivatives

A solution of lathyrol (**4**) in dry triethylamine and dichloromethane (CH₂Cl₂) (1:1) was stirred for 5 min at room temperature before addition of the appropriate acyl chloride or anhydride and a catalytic amount of 4-dimethylaminopyridine (DMAP). The mixture was stirred for 2–18 h, at room temperature or under reflux (60°C, nitrogen atmosphere). The reaction mixture was concentrated under vacuum at 40°C and the obtained residue was purified by flash column chromatography, preparative TLC or HPLC.

2.5.1 | Preparation of euphoboetirane C (5)

Obtained from reaction of **4** (21 mg, 0.060 mmol) with 70 μ L (0.71 mmol) of 2-furoyl chloride. The mixture was purified by CC (SiO₂, 4 g, *n*-hexane/EtOAc (1:0 to 0:1), Combiflash system) and preparative TLC (*n*-hexane/EtOAc, 7:3) to afford 25 mg (0.046 mmol, 77% yield) of an amorphous white powder. $[\alpha]_D^{25} + 128$ (c 0.100, CHCl₃); ¹H-NMR (400 MHz, CDCl₃): δ 1.00 (3H, d, *J* = 6.7 Hz, CH₃-16), 1.16 (3H, s, CH₃-19), 1.20 (1H, m, H-9), 1.22 (3H, s, CH₃-18), 1.44 (1H, dd, *J* = 8.7, 11.5 Hz, H-11), 1.68 (1H, m, H-1 β), 1.84 (3H, s, CH₃-20), 1.92 (1H, m, H-7b), 2.26 (1H, m, H-7a), 2.40 (1H, m, H-2), 2.86 (1H, dd, *J* = 3.2, 10.0 Hz, H-4), 3.14 (1H, m, H-1 α), 4.95 (1H, s, H-17a), 4.97 (1H, s, H-17b), 5.79 (1H, t, *J* = 3.2 Hz, H-3), 6.14 (1H, d, *J* = 9.9 Hz, H-5), 6.43 (1H, dd, *J* = 1.6, 3.3 Hz, H-4'), 6.48 (1H, dd, *J* = 1.6, 3.3 Hz, H-4''), 7.01 (1H, d, *J* = 3.2 Hz, H-3''), 7.12 (1H, d, *J* = 3.0 Hz, H-3'), 7.48 (1H, s, H-5''), 7.55 (1H, s, H-5'); ¹³C-NMR (100.61 MHz, CDCl₃): δ 13.2 (C-20), 16.3 (C-16, C-19), 25.9 (C-10), 28.6 (C-11), 29.0 (C-18), 36.3 (C-9), 37.6 (C-2), 49.6 (C-1), 52.6 (C-4), 69.2 (C-5), 81.2 (C-3), 115.0 (C-17), 111.9 (C-4''), 112.1 (C-4'), 118.3 (C-3''), 118.5 (C-3'), 134.8 (C-13), 144.2 (C-2'), 144.0 (C-2''), 144.4 (C-6), 145.6 (C-5'), 146.5 (C-5''), 157.5 (C-1'), 157.9 (C-1'); ESI-MS *m/z* 545 [M + Na]⁺, 561 [M + K]⁺.

2.5.2 | Preparation of euphoboetirane D (6)

Obtained from reaction of **4** (25 mg, 0.075 mmol) with 70 μ L (0.65 mmol) of 2-thiophenecarbonyl chloride. The residue was purified by CC [SiO₂, 4 g, *n*-hexane/EtOAc (1:0 to 0:1), Combiflash system] and preparative TLC (*n*-hexane/EtOAc 7:3) to give 26 mg (0.046 mmol, 61% yield) of an amorphous white powder. $[\alpha]_D^{25} + 189$ (c 0.090, CHCl₃); ¹H-NMR (400 MHz, CDCl₃): δ 1.02 (3H, d, *J* = 6.7 Hz, CH₃-16), 1.22 (3H, s, CH₃-18), 1.16 (3H, s, CH₃-19), 1.23 (1H, m, H-9), 1.45 (1H, dd, *J* = 9.0, 11.1 Hz, H-11), 1.70 (1H, m, H-1 β), 1.87 (3H, s, CH₃-20), 2.42 (1H, m, H-2), 2.86 (1H, dd, *J* = 3.2, 10.1 Hz, H-4), 3.12 (1H, m, H-1 α), 4.95 (1H, s, H-17a), 5.00 (1H, s, H-17b), 5.79 (1H, t, *J* = 3.2 Hz, H-3), 6.16 (1H, d, *J* = 9.8 Hz, H-5), 7.01 (1H, t, *J* = 4.3 Hz, H-4''), 7.07 (1H, t, *J* = 4.2 Hz, H-4'), 7.49 (1H, d, *J* = 4.9 Hz, H-3''), 7.53 (1H, d, *J* = 4.9 Hz, H-3'), 7.58 (1H, d, *J* = 3.1 Hz, H-5''), 7.67 (1H, d, *J* = 4.8 Hz, H-5'); ¹³C-NMR (100.61 MHz, CDCl₃): δ 13.2 (C-20), 14.7 (C-16), 16.3 (C-19), 25.9 (C-10), 28.5 (C-11), 29.0 (C-18), 36.4 (C-9), 37.8 (C-2), 49.8 (C-1), 52.6 (C-4), 80.4 (C-3), 86.9 (C-15), 114.7 (C-17), 123.2 (C-2'), 123.5 (C-2''), 127.7 (C-4''), 127.9 (C-4'), 132.5 (C-3''), 132.7 (C-3'), 133.6 (C-5''), 133.7 (C-5'), 134.7 (C-13), 144.1 (C-6), 161.1 (C-1''), 161.4 (C-1'). ESI-MS *m/z* 577 [M + Na]⁺, 593 [M + K]⁺.

2.5.3 | Preparation of euphoboetirane E (7) and 12-hydroxyboetirane A (12)

Obtained from reaction of **4** (24 mg, 0.073 mmol) with 83 mg (0.44 mmol) of 2-naphtoyl chloride. The residue was purified by CC

[SiO₂, 4 g, *n*-hexane/EtOAc (1:0 to 0:1), Combiflash system] and preparative TLC (*n*-hexane/EtOAc 4:1, 2 \times) to give 19 mg of **7** (0.039 mmol, 54% yield) and 6 mg of **12** (0.012 mmol, 16% yield) as amorphous white solids. **Euphoboetirane E (7)**: $[\alpha]_D^{25} + 102$ (c 0.100, CHCl₃); ¹H-NMR (400 MHz, CDCl₃): δ 1.11 (3H, d, *J* = 6.8 Hz, CH₃-16), 1.17 (3H, s, CH₃-18), 1.22 (1H, m, H-9), 1.23 (3H, s, CH₃-19), 1.46 (1H, dd, *J* = 8.7, 11.4 Hz, H-11), 1.63 (1H, m, H-8a), 1.68 (1H, m, H-1 β), 1.83 (3H, s, CH₃-20), 1.96 (1H, m, H-8b), 1.97 (1H, m, H-7b), 2.20 (1H, m, H-2), 2.30 (1H, m, H-7a), 2.64 (1H, dd, *J* = 1.7, 10.1 Hz, H-4), 3.11 (1H, m, H-1 α), 3.15 (1H, s, 3-OH), 4.21 (1H, brs, H-3), 4.41 (1H, s, 5-OH), 5.00 (1H, s, H-17a), 5.01 (1H, s, H-17b), 6.15 (1H, d, *J* = 10.2 Hz, H-5), 8.60 (1H, s, H-3'), 7.88 (2H, d, *J* = 8.5 Hz, H-5', H-8'), 7.54 (1H, t, *J* = 7.3 Hz, H-6'), 7.60 (1H, t, *J* = 7.0 Hz, H-7'), 7.96 (1H, d, *J* = 8.0 Hz, H-10'), 8.05 (1H, d, *J* = 8.6 Hz, H-11'). ¹³C-NMR (100.61 MHz, CDCl₃): δ 13.3 (C-20), 14.5 (C-16), 16.3 (C-19), 21.3 (C-8), 25.9 (C-10), 28.4 (C-11), 29.0 (C-18), 36.3 (C-9), 37.6 (C-2), 49.3 (C-1), 53.9 (C-4), 71.4 (C-5), 79.0 (C-3), 114.7 (C-17), 125.4 (C-11'), 126.9 (C-6'), 127.0 (C-4'), 127.9 (C-8', C-5'), 128.7 (C-7'), 129.5 (C-10'), 131.6 (C-3'), 132.6 (C-13), 135.3 (C-2'), 135.8 (C-9'), 144.2 (C-6), 166.0 (C-1'). ESI-MS *m/z* 511 [M + Na]⁺, 527 [M + K]⁺.

12-Hydroxyboetirane A (12): $[\alpha]_D^{25} + 56$ (c 0.100, CHCl₃); ¹H-NMR (400 MHz, CDCl₃): δ 0.55 (1H, d, *J* = 9.1 Hz, H-11), 0.65 (1H, m, H-9), 1.04 (3H, d, *J* = 6.6 Hz, CH₃-16), 1.07 (3H, s, CH₃-18), 1.15 (3H, s, CH₃-19), 1.22 (3H, d, *J* = 7.7 Hz, CH₃-20), 1.82 (1H, m, H-1 α), 1.84 (1H, m, H-7b), 1.91 (1H, m, H-8a), 2.06 (1H, m, H-8b), 2.12 (1H, m, H-1 β), 2.16 (1H, m, H-2), 2.53 (1H, m, H-7a), 2.74 (1H, m, H-13), 3.11 (1H, dd, *J* = 3.2, 11.1 Hz, H-4), 3.51 (1H, s, 3-OH), 3.91 (1H, brs, H-3), 4.75 (1H, d, *J* = 9.4 Hz, H-12), 4.92 (1H, s, H-17a), 5.09 (1H, s, H-17b), 5.76 (1H, d, *J* = 11.1 Hz, H-5), 7.54 (1H, t, *J* = 7.0 Hz, H-6'), 7.60 (1H, t, *J* = 7.1 Hz, H-7'), 7.87 (2H, d, *J* = 8.5 Hz, H-8', H-10'), 7.95 (1H, d, *J* = 7.8 Hz, H-5'), 8.02 (1H, d, *J* = 8.6 Hz, H-11'), 8.58 (1H, s, H-3'); ¹³C-NMR (100.61 MHz, CDCl₃): δ 13.1 (C-20), 13.5 (C-16), 15.5 (C-10, C-19), 23.1 (C-8), 26.6 (C-11), 29.2 (C-18), 30.3 (C-9), 37.7 (C-2), 38.3 (C-7), 41.5 (C-13), 44.2 (C-1), 55.2 (C-4), 70.8 (C-5), 74.6 (C-12), 75.0 (C-3), 87.1 (C-15), 116.0 (C-17), 125.5 (C-11'), 126.9 (C-6'), 127.1 (C-4'), 127.9 (C-8'), 128.3 (C-10'), 128.6 (C-7'), 129.6 (C-5'), 131.7 (C-3'), 132.5 (C-9'), 135.8 (C-2'), 147.4 (C-6), 168.1 (C-1'), 220.1 (C-14); ESI-MS *m/z* 489 [M - H₂O + H]⁺.

2.5.4 | Preparation of euphoboetirane F (8)

Obtained from reaction of **4** (24 mg, 0.072 mmol) with 70 μ L (0.55 mmol) of propionic anhydride. The residue was purified by CC (SiO₂, 4 g, *n*-hexane/EtOAc (1:0 to 0:1), Combiflash system) and preparative TLC (*n*-hexane/EtOAc 4:1, 2 \times) to give 16 mg (0.036 mmol, 50% yield) of a colourless oil. $[\alpha]_D^{25} + 117$ (c 0.100, CHCl₃); ¹H-NMR (400 MHz, DMSO): δ 0.80 (3H, d, *J* = 10.4 Hz, CH₃-16), 0.92 (3H, t, *J* = 7.4 Hz, H-3''), 1.01 (3H, t, *J* = 7.5 Hz, H-3'), 1.10 (3H, s, CH₃-19), 1.11 (1H, m, H-8b), 1.16 (3H, s, CH₃-18), 1.16 (1H, m, H-7b), 1.18 (1H, m, H-9), 1.43 (1H, dd, *J* = 8.5, 11.6 Hz, H-11), 1.50 (1H, t, *J* = 12.4 Hz, H-1 β), 1.57 (3H, s, CH₃-20), 1.85 (1H, m, H-8a), 2.08

(1H, m, H-7a), 2.10 (2H, m, H-2''), 2.14 (1H, m, H-2), 2.30 (2H, m, H-2'), 2.55 (1H, dd, $J = 2.9, 10.2$ Hz, H-4), 2.87 (1H, dd, $J = 9.1, 11.9$ Hz, H-1 α), 4.69 (1H, s, H-17a), 4.90 (1H, s, H-17b), 5.34 (1H, s, H-3), 5.93 (1H, d, $J = 10.4$ Hz, H-5), 7.76 (1H, s, H-12); $^{13}\text{C-NMR}$ (100.61 MHz, DMSO): δ 8.7 (C-3''), 9.0 (C-3'), 12.4 (C-20), 14.2 (C-16), 16.3 (C-19), 21.4 (C-8), 25.1 (C-10), 26.9 (C-2''), 27.0 (C-2'), 28.1 (C-11), 28.6 (C-18), 34.8 (C-9), 35.5 (C-7), 37.0 (C-2), 49.1 (C-1), 52.5 (C-4), 66.7 (C-5), 79.7 (C-3), 87.4 (C-15), 114.8 (C-17), 133.2 (C-13), 145.0 (C-6), 152.5 (C-12), 173.0 (C-1''), 173.6 (C-1'), 203.9 (C-14); ESI-MS m/z 469 [M + Na] $^+$, 485 [M + K] $^+$.

2.5.5 | Preparation of euphoboetirane G (9)

Obtained from reaction of **4** (25 mg, 0.075 mmol) with 70 μL (0.55 mmol) of diethylcarbonyl chloride. The residue was purified by CC (SiO₂, 4 g, *n*-hexane/EtOAc 1:0 to 0:1, CombiFlash system) to yield 24 mg (0.055 mmol, 73% yield) of an amorphous white solid.

$[\alpha]_D^{25} + 64$ (c 0.090, CHCl₃); $^1\text{H-NMR}$ (400 MHz, CD₃OD): δ 1.07 (3H, d, $J = 6.7$ Hz, CH₃-16), 1.11 (6H, m, H-3', H-3''), 1.17 (3H, s, CH₃-18), 1.18 (3H, s, CH₃-19), 1.25 (1H, m, H-9), 1.47 (1H, dd, $J = 8.5, 11.7$ Hz, H-11), 1.56 (1H, dd, $J = 10.5, 13.7$ Hz, H-1 β), 1.64 (3H, s, CH₃-20), 1.80 (1H, m, H-8a), 1.91 (1H, m, H-8b), 2.04 (1H, m, H-7b), 2.07 (1H, m, H-2), 2.34 (1H, m, H-7a), 2.46 (1H, dd, $J = 2.7, 10.6$ Hz, H-4), 3.07 (1H, t, $J = 11.4$ Hz, H-1 α), 3.17 (1H, m, H-2''), 3.29 (1H, m, H-2'), 4.10 (1H, s, H-3), 4.67 (1H, s, H-17a), 4.87 (1H, s, H-17b), 5.93 (1H, d, $J = 10.5$ Hz, H-5), 7.60 (1H, brs, H-12). $^{13}\text{C-NMR}$ (100.61 MHz, CD₃OD): δ 12.8 (C-20), 13.5 (C-3''), 13.8 (C-3'), 14.2 (C-16), 16.7 (C-19), 22.9 (C-8), 27.1 (C-10), 29.2 (C-18), 30.1 (C-11), 35.9 (C-7), 37.9 (C-9), 39.1 (C-2), 42.2 (C-2'), 43.2 (C-2''), 50.5 (C-1), 55.8 (C-4), 69.9 (C-5), 80.6 (C-3), 90.5 (C-15), 114.1 (C-17), 135.7 (C-13), 147.3 (C-6), 154.3 (C-12), 158.1 (C-1'), 203.3 (C-14); ESI-MS m/z 456 [M + Na] $^+$, 472 [M + K] $^+$.

2.5.6 | Preparation of euphoboetirane H (10)

Obtained from reaction of **4** (17 mg, 0.052 mmol) with 70 μL (0.74 mmol) of ethylchloroformate. The residue was purified by CC (SiO₂, 4 g, *n*-hexane/EtOAc 1:0 to 0:1, CombiFlash System) to obtain 5 mg (0.012 mmol, yield 23%) of a white amorphous solid. $[\alpha]_D^{25} + 27$ (c 0.090, CHCl₃); IR (KBr) ν_{max} cm⁻¹; $^1\text{H-NMR}$ (400 MHz, CDCl₃): δ 1.12 (3H, s, CH₃-19), 1.16 (1H, m, H-8b), 1.18 (3H, s, CH₃-18), 1.20 (3H, d, $J = 6.8$ Hz, CH₃-16), 1.24 (3H, t, $J = 7.2$ Hz, CH₃-3'), 1.25 (1H, m, H-9), 1.49 (1H, dd, $J = 8.3, 11.0$ Hz, H-11), 1.75 (1H, t, $J = 13.2$ Hz, H-1 β), 1.83 (3H, s, CH₃-20), 1.99 (1H, m, H-8a), 1.97 (1H, m, H-7b), 2.18 (1H, m, H-2), 2.46 (1H, t, $J = 3.9$ Hz, H-4), 2.56 (1H, m, H-7a), 3.38 (1H, dd, $J = 6.7, 13.1$ Hz, H-1 α), 4.12 (2H, m, CH₂-2'), 4.49 (1H, t, $J = 3.6$ Hz, H-3), 5.46 (1H, d, $J = 3.1$ Hz, H-5), 5.08 (1H, s, H-17a), 5.12 (1H, s, H-17b), 6.39 (1H, d, $J = 11.2$ Hz, H-12); $^{13}\text{C-NMR}$ (100.61 MHz, CDCl₃): δ 12.5 (C-16), 12.6 (C-20), 14.3 (C-3'), 16.0 (C-19), 22.6 (C-8), 26.9 (C-10), 28.7 (C-11), 29.2 (C-18), 33.8 (C-7), 34.5 (C-9), 37.8 (C-2), 43.3 (C-1), 48.3 (C-4), 64.9 (C-2'),

74.1 (C-5), 83.1 (C-3), 90.4 (C-15), 114.4 (C-17), 132.8 (C-13), 144.9 (C-6), 145.6 (C-12), 153.2 (C-1'), 194.4 (C-14); ESI-MS m/z 407 [M + H] $^+$.

2.5.7 | Preparation of euphoboetirane I (11) and 12-hydroxyboetirane D (15)

Obtained from reaction of **4** (25 mg, 0.076 mmol) with 73 mg (0.37 mmol) of 1-adamantanecarbonyl chloride. The residue was purified by CC [SiO₂, 15 g, *n*-hexane/EtOAc (1:0 to 0:1)] and preparative TLC (*n*-hexane/EtOAc 4:1, 2 \times) to give 22 mg of **11** (0.044 mmol, 58% yield) and 5 mg of **15** (0.01 mmol, 13% yield) as amorphous white solids. *Euphoboetirane I (11)*: $[\alpha]_D^{25} + 22$ (c 0.100, CHCl₃); $^1\text{H-NMR}$ (400 MHz, CDCl₃): δ 1.13 (3H, d, $J = 4.4$ Hz, CH₃-16), 1.13 (3H, s, CH₃-18), 1.18 (3H, s, CH₃-19), 1.40 (1H, dd, $J = 8.9, 11.1$ Hz, H-11), 1.65–1.99 (15H, brs, H-3' to H-11'), 2.00 (3H, s, H-20), 2.13 (1H, m, H-2), 2.42 (1H, d, $J = 8.2$ Hz, H-4), 3.09 (2H, m, H-1), 4.06 (1H, brs, H-3), 4.35 (1H, s, H-17a), 4.87 (1H, s, H-17b), 5.72 (1H, d, $J = 9.4$ Hz, H-5); $^1\text{H-NMR}$ (400 MHz, DMSO): δ 0.94 (3H, d, $J = 6.8$ Hz, CH₃-16), 1.08 (3H, s, CH₃-18), 1.12 (3H, s, CH₃-19), 1.13 (1H, m, H-9), 1.39 (1H, dd, $J = 8.6, 11.8$ Hz, H-11), 1.44 (1H, dd, $J = 11.2, 13.2$ Hz, H-1 β), 1.54 (3H, s, CH₃-20), 1.62 (1H, m, H-8a), 1.62 and 1.75 (12H, two brs, H-3', H-5', H-7', H-8', H-10', H-11'), 1.79 (1H, m, H-8b), 1.88 (1H, m, H-7a), 1.89 (1H, m, H-2), 1.91 (3H, brs, H-4', H-6', H-9'), 2.04 (1H, m, H-7b), 2.28 (1H, dd, $J = 1.8, 10.6$ Hz, H-4), 2.92 (1H, t, $J = 10.8$ Hz, H-1 α), 3.84 (1H, d, $J = 7.2$ Hz, 3-OH), 3.95 (1H, m, H-3), 4.55 (1H, s, H-17a), 4.81 (1H, s, H-17b), 5.48 (1H, s, 15-OH), 5.99 (1H, d, $J = 9.6$ Hz, H-5), 7.53 (1H, brs, H-12); $^{13}\text{C-NMR}$ (100.61 MHz, CDCl₃): δ 13.3 (C-20), 14.6 (C-16), 16.2 (C-19), 19.0 (C-8), 25.6 (C-10), 28.0 (C-4', C-6', C-9'), 29.9 (C-18), 34.3 (C-7), 36.1 (C-9), 36.5 and 38.9 (C-3', C-5', C-7', C-8', C-10', C-11'), 37.1 (C-2), 48.7 (C-1), 55.6 (C-4), 69.9 (C-5), 77.8 (C-3), 113.9 (C-17), 135.1 (C-13), 144.5 (C-6), 178.1 (C-1'); ESI-MS m/z 519 [M + Na] $^+$, 535 [M + K] $^+$.

12-Hydroxyboetirane D (15): $[\alpha]_D^{25} + 18$ (c 0.100, CHCl₃); $^1\text{H-NMR}$ (400 MHz, CDCl₃): δ 0.50 (1H, d, $J = 9.2$ Hz, H-9), 0.59 (1H, m, H-11), 1.04 (3H, d, $J = 4.4$ Hz, H-16), 1.04 (3H, s, H-18), 1.10 (3H, s, H-19), 1.17 (3H, d, $J = 8.0$ Hz, H-20), 1.68 and 1.85 (12H, two brs, H-3', H-5', H-7', H-8', H-10', H-11'), 1.80 (1H, m, H-1 α), 1.81 (1H, m, H-8b), 1.90 (1H, m, H-8a), 1.84 (2H, m, H-7), 2.00 (3H, brs, H-4', H-6', H-9'), 2.10 (1H, t, $J = 12.6$ Hz, H-1 β), 2.35 (1H, m, H-2), 2.68 (1H, m, H-13), 2.86 (1H, dd, $J = 2.8, 11.2$ Hz, H-4), 3.46 (1H, s, 3-OH), 3.75 (1H, s, H-3), 4.70 (1H, d, $J = 9.2$ Hz, H-12), 4.70 (1H, s, H-17a), 4.96 (1H, s, H-17b), 5.38 (1H, d, $J = 11.2$ Hz, H-5); $^{13}\text{C-NMR}$ (100.61 MHz, CDCl₃): δ 13.1 (C-20), 13.5 (C-16), 15.4 (C-19), 15.5 (C-10), 23.0 (C-8), 26.6 (C-9), 28.0 (C-4', C-6', C-9'), 29.2 (C-18), 30.3 (C-11), 36.6 and 38.9 (C-3', C-5', C-7', C-8', C-10', C-11'), 37.7 (C-2), 38.2 (C-7), 41.5 (C-13), 44.2 (C-1, C-2'), 55.2 (C-4), 69.5 (C-5), 74.5 (C-12), 74.9 (C-3), 87.0 (C-15), 115.4 (C-17), 147.3 (C-6), 179.6 (C-1'), 220.1 (C-14); ESI-MS m/z 519 [M - H₂O + Na] $^+$, 535 [M - H₂O + K] $^+$.

2.5.8 | Preparation of 12-hydroxyboetirane B (13) and 12-hydroxyboetirane C (14)

Obtained from reaction of **4** (25 mg, 0.076 mmol) with 70 μL (0.47 mmol) of 4-(trifluoromethyl)benzoyl chloride. The residue was purified by CC (SiO_2 , 10 g, *n*-hexane/EtOAc (1:0 to 0:1) and preparative TLC (*n*-hexane/EtOAc 4:1, 2 \times) to give 10 mg of **13** (0.019 mmol, 25% yield) and 11 mg of **14** (0.021 mmol, 28% yield) as white amorphous solids. **12-Hydroxyboetirane B (13)**: $[\alpha]_D^{25} + 35$ (c 0.100, CHCl_3); $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 0.54 (1H, d, $J = 9.2$ Hz, H-11), 0.63 (1H, m, H-9), 1.01 (3H, d, $J = 6.4$ Hz, H-16), 1.07 (3H, s, H-18), 1.15 (3H, s, H-19), 1.20 (3H, d, $J = 8.0$ Hz, H-20), 1.68 (1H, m, H-8b), 1.77 (1H, m, H-1 α), 1.81 (1H, m, H-8a), 2.08 (1H, m, H-1 β), 2.12 (1H, m, H-7a), 2.26 (1H, m, H-2), 2.50 (1H, m, H-7b), 2.74 (1H, m, H-13), 3.11 (1H, dd, $J = 3.6, 10.5$ Hz, H-4), 3.98 (1H, d, $J = 11.2$ Hz, H-5), 4.65 (1H, s, H-17a), 4.79 (1H, d, $J = 9.2$ Hz, H-12), 4.89 (1H, s, H-17b), 5.72 (1H, t, $J = 3.2$ Hz, H-3), 7.72 (2H, d, $J = 8.2$ Hz, H-4'), 8.20 (2H, d, $J = 8.1$ Hz, H-3'); $^{13}\text{C-NMR}$ (100.61 MHz, CDCl_3): δ 13.2 (C-20), 13.9 (C-16), 15.0 (C-19), 15.4 (C-10), 23.0 (C-8), 26.9 (C-11), 29.1 (C-18), 35.1 (C-9), 37.6 (C-2), 37.8 (C-7), 41.7 (C-13), 45.4 (C-1), 56.6 (C-4), 66.3 (C-5), 74.7 (C-12), 80.8 (C-3), 86.7 (C-15), 113.3 (C-17), 121.5 (C-6'), 125.7 (C-4'), 130.3 (C-3'), 133.2 (C-5'), 134.8 (C-2'), 151.1 (C-6), 165.7 (C-1'), 220.8 (C-14); ESI-MS m/z 507 $[\text{M} - \text{H}_2\text{O} + \text{H}]^+$.

12-Hydroxyboetirane C (14): $[\alpha]_D^{25} + 38$ (c 0.100, CHCl_3); $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ 0.53 (1H, d, $J = 9.2$ Hz, H-11), 0.62 (1H, m, H-9), 1.02 (3H, d, $J = 6.6$ Hz, H-16), 1.12 (3H, s, H-18), 1.04 (3H, s, H-19), 1.20 (3H, d, $J = 7.7$ Hz, H-20), 1.79 (1H, m, H-1 α), 1.84 (1H, m, H-1 β), 1.90 (1H, m, H-8a), 1.99 (1H, m, H-2), 2.01 (1H, m, H-8b), 2.15 (1H, m, H-7a), 2.50 (1H, m, H-7b), 2.73 (1H, m, H-13), 3.07 (1H, dd, $J = 3.3, 11.2$ Hz, H-4), 3.88 (1H, t, $J = 3.2$ Hz, H-3), 4.74 (1H, d, $J = 9.2$ Hz, H-12), 4.85 (1H, s, H-17a), 5.06 (1H, s, H-17b), 5.73 (1H, d, $J = 11.2$ Hz, H-5), 7.69 (2H, d, $J = 8.2$ Hz, H-4'), 8.12 (2H, d, $J = 8.1$ Hz, H-3'); $^{13}\text{C-NMR}$ (100.61 MHz, CDCl_3): δ 13.1 (C-20), 13.5 (C-16), 15.5 (C-10, C-19), 23.1 (C-8), 26.6 (C-11), 29.2 (C-18), 30.3 (C-9), 37.7 (C-2), 38.3 (C-7), 41.5 (C-13), 44.1 (C-1), 54.9 (C-4), 71.3 (C-5), 74.6 (C-12), 75.1 (C-3), 87.0 (C-15), 116.2 (C-17), 122.5 (C-6'), 125.6 (C-4'), 130.4 (C-3'), 133.7 (C-5'), 134.9 (C-2'), 147.2 (C-6), 166.5 (C-1'), 220.5 (C-14). ESI-MS m/z 507 $[\text{M} - \text{H}_2\text{O} + \text{H}]^+$.

2.6 | Biological assays

2.6.1 | Cell lines and cultures

L5178Y mouse T-lymphoma cells (ECACC catalog no. 87111908, US FDA, Silver Spring, MD, USA) were transfected with the pHA MDR1/A retrovirus. The MDR1-expressing cell line was selected by culturing the infected cells with 60 ng/mL of colchicine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), to maintain the MDR phenotype expression. L5178Y (parental, PAR) mouse T-cell lymphoma cells and the human MDR1-transfected subline were cultured in McCoy's 5A supplemented with 10% heat-inactivated horse serum, 100 U/L L-

glutamine, and 100 mg/L penicillin/streptomycin mixture, all obtained from Sigma Aldrich. The human colon adenocarcinoma cell lines (Colo205 parental, and Colo 320/MDR-LRP expressing MDR1), were purchased from LGC Promochem, Teddington, UK. The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate and 100 mM HEPES. The semi-adherent human colon cancer cells were detached with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) for 5 min at 37°C.

2.6.2 | Antiproliferative assays

The antiproliferative effects of all compounds were tested in a range of decreasing concentrations using both mouse lymphoma and human colon adenocarcinoma cell lines as experimental models. First, the compounds were diluted in 100 μL of medium. The maximum tested concentration of each compound was 100 μM . Then cells were distributed into 96-well flat-bottomed microtiter plates at concentrations of 6×10^3 and 100 μL of medium were added to each well, with the exception of medium and cell control wells. The microtiter plates were initially incubated at 37°C for 72 h and, at the end of the incubation period, 20 μL of MTT (thiazolyl blue tetrazolium bromide, Sigma-Aldrich Chemie GmbH) solution [5 mg/ml in phosphate-buffered saline (PBS)] was added to each well and incubated for another 4 h. Then, 100 μL of 10% SDS (sodium dodecyl sulfate, Sigma) solution [10% in 0.01 M hydrochloric acid (HCl)] was added into each well, and the plates were further incubated overnight at 37°C. 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 according to equation (1). All experiments were performed in triplicate. The results were expressed as the mean \pm standard deviation (SD), and the half maximal inhibition concentration (IC_{50}) values were obtained by best fitting the dose-dependent inhibition curves in GraphPad Prism 5 software. Only data from analysis with $R^2 > 0.90$ were presented.

$$100 - \left[\frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{medium control}}}{\text{OD}_{\text{cell control}} - \text{OD}_{\text{medium control}}} \right] \times 100 \quad (1)$$

2.6.3 | Rhodamine-123 accumulation assay

Mouse T-lymphoma cells or human colon adenocarcinoma cells were adjusted to a density of 2×10^6 cells/mL, re-suspended in serum-free McCoy's 5A medium or RPMI 1640, respectively, and distributed in 500 μL aliquots into Eppendorf centrifuge tubes. Then, 10 μL of test compounds were added at two concentrations (2 or 20 μM) and verapamil (positive control, EGIS Pharmaceuticals PLC, Budapest, Hungary) was added at 20 μM . Dimethyl sulphoxide (DMSO) at 4% was also added as solvent control. The samples were incubated for 10 min at room temperature, after which 10 μL of rhodamine-123 (5.2 μM final concentration) were added. After 20 min of incubation at 37°C the samples were washed twice, resuspended in 500 μL of

PBS and analysed by flow cytometry (Partec CyFlow Space Instrument, Partec GmbH, Münster, Germany). The resulting histograms were evaluated regarding mean fluorescence intensity (FL-1), SD, both FSC and SSC parameters, and the peak channel of 20000 individual cells belonging to the total and the gated populations. The fluorescence activity ratio (FAR) was calculated on the basis of the quotient between FL-1 of treated/untreated resistant cell line (MDR1-transfected mouse lymphoma or Colo320 human colon adenocarcinoma cells) over the respective treated/untreated sensitive cell line (PAR mouse lymphoma or Colo205 human colon adenocarcinoma cells), according to equation (2).

$$\text{FAR} = \frac{\text{FL1 MDRtreated} / \text{FL1 MDRuntreated}}{\text{FL1 PARtreated} / \text{FL1 PARuntreated}} \quad (2)$$

2.6.4 | Drug combination assay

Doxorubicin (2 mg/mL, Teva Pharmaceuticals, Budapest, Hungary) was serially diluted in the horizontal direction as previously described, starting with 8 µg/mL. The resistance modifier was subsequently diluted in the vertical direction, starting with 20 µg/mL. The dilutions of doxorubicin were made in a horizontal direction in 100 µL, and the dilutions of the resistance modifiers vertically in the microtiter plate in 50 µL volume. The cells were re-suspended in culture medium and distributed into each well in 50 µL containing 1×10^4 cells, with the exception of the medium control wells, to a final volume of 200 µL per well. The plates were incubated for 48 h at 37°C in a CO₂ incubator and at the end of the incubation period, the cell growth was determined by the MTT staining method, as described earlier. Drug interactions were evaluated according to Chou using the CalcuSyn v2.2 software.²⁷ Each dose-response curve (for individual agents as well as combinations) was fit to a linear model using the median effect equation, in order to obtain the median effect value (corresponding to the IC₅₀) and slope (*m*). Goodness-of-fit was assessed using the linear correlation coefficient, *r*, and only data from analysis with *r* > 0.90 were presented. The extent of interaction between drugs was expressed using the combination index (CI), where CI = 1 represents additive effect and CI > 1 antagonism. CI < 1 represents synergism where 0.1 < CI < 0.3 = strong synergism; 0.3 < CI < 0.7 = synergism; 0.85 < CI < 0.9 = slight synergism.

3 | RESULTS AND DISCUSSION

3.1 | Phytochemical study

Euphorbia boetica aerial parts were studied with the aim of finding novel effective compounds for overcoming MDR in cancer cells. Briefly, the powdered plant was exhaustively extracted with MeOH. This crude residue was suspended in MeOH/H₂O (1:1) and extracted with EtOAc. The EtOAc soluble fraction was subjected to silica gel flash chromatography to afford 10 crude fractions.⁸ Due to the high complexity of these fractions, preliminary ¹H-NMR analysis was

performed in order to prioritise those that were of the most interest.²⁸ Although as complex as the spectra appeared, preliminary ¹H-NMR spectra showed that fraction E, obtained with *n*-hexane/EtOAc (7:3) was very rich in macrocyclic diterpenes (Supporting Information, Figure S1). Characteristic signals could be observed at δ 6.5–4.5, which were assignable to olefinic protons and protons geminal to acyl functions, found in macrocyclic diterpenes that generally appear as polyesters. Moreover, it could also be recognised the signals for vinylic and acetyl methyls displayed as singlets at δ 2.1–1.6, and other methyl group signals at δ 1.2–0.7. Nevertheless, the isolation of macrocyclic diterpenes is a difficult and laborious task since these compounds often appear as a complex mixture of structurally related polyesters, chlorophylls, triterpenes and steroids. In particular, the removal of chlorophylls is amongst the most troublesome and time consuming process in natural products isolation and purification. Several methodologies have been proposed to remove chlorophylls from crude extracts or fractions, which include liquid-liquid partition, repeated column chromatography, solid phase extraction using different adsorbents, or the use of activated charcoal that carries the risk of loss of important compounds. Polyamide resins have been used to adsorb apolar compounds, in batches or packed in chromatographic columns.²⁸ Therefore, in order to remove chlorophylls, fraction E was subjected to a polyamide-6 column chromatography eluted with mixtures of MeOH/H₂O of decreasing polarity (1:1, 3:2, 7:3, 4:1 and 1:0). Fractions obtained with MeOH/H₂O (3:2 and 7:3) contained the bulk of diterpenes, as showed by a TLC analysis. As a result of the removal of chlorophylls, it was possible to observe on the TLC plates, the typical black or dark brown spots after spraying with H₂SO₄/MeOH (1:1) followed by heating. Fractions eluted with MeOH/H₂O (4:1 and 1:0) were rich in chlorophylls and were studied no further. This procedure allowed the straightforward isolation, in larger amounts, of the new diterpene polyester euphoboetirane **1** (*Euphorbia* factor L15 (herein named euphoboetirane A, **2**) that was previously isolated from *Euphorbia lathyris*.²⁵

Compounds **1** and **2** (Figure 1) were obtained as white crystals and displayed very similar spectroscopic data. The ¹³C-NMR and DEPT spectra of euphoboetirane **1** exhibited 27 signals corresponding to: seven methyl groups, five methylenes, seven methines (two oxygenated at δ_C 65.9 and 80.1 and one olefinic at δ_C 146.8) and eight quaternary carbons (two olefinic at δ_C 134.3 and 144.5, one oxygenated at δ_C 92.5, one carbonyl at δ_C 197.0 and three ester carbonyl groups at δ_C 169.9, 170.6 and 173.9). The ¹H-NMR spectrum showed signals for four methyl groups: one secondary at δ_H 0.87 (d, *J* = 6.7 Hz), two tertiary (δ_H 1.12 and 1.14) and one vinylic methyl group displayed as singlet at δ_H 1.66. Two oxymethine protons (δ_H 5.57 t, *J* = 3.2 Hz; 6.05, d, *J* = 10.3 Hz) and three olefinic protons (δ_H 4.70 s, 4.96 s; 6.46 d, *J* = 11.4 Hz) could also be observed. The major difference between NMR data of compounds **1** and **2** was the presence of signals corresponding to a propanoyl group in compound **1** (δ_H 2.32 m; 1.12 t, *J* = 7.4 Hz and δ_C 173.9, 27.9, 9.1) instead of an acetyl group located at C-3 in compound **2**. The structure of **1** was confirmed by ¹H-¹H COSY, HMQC and HMBC experiments that allowed the unequivocal assignment of all ¹H and ¹³C signals. The

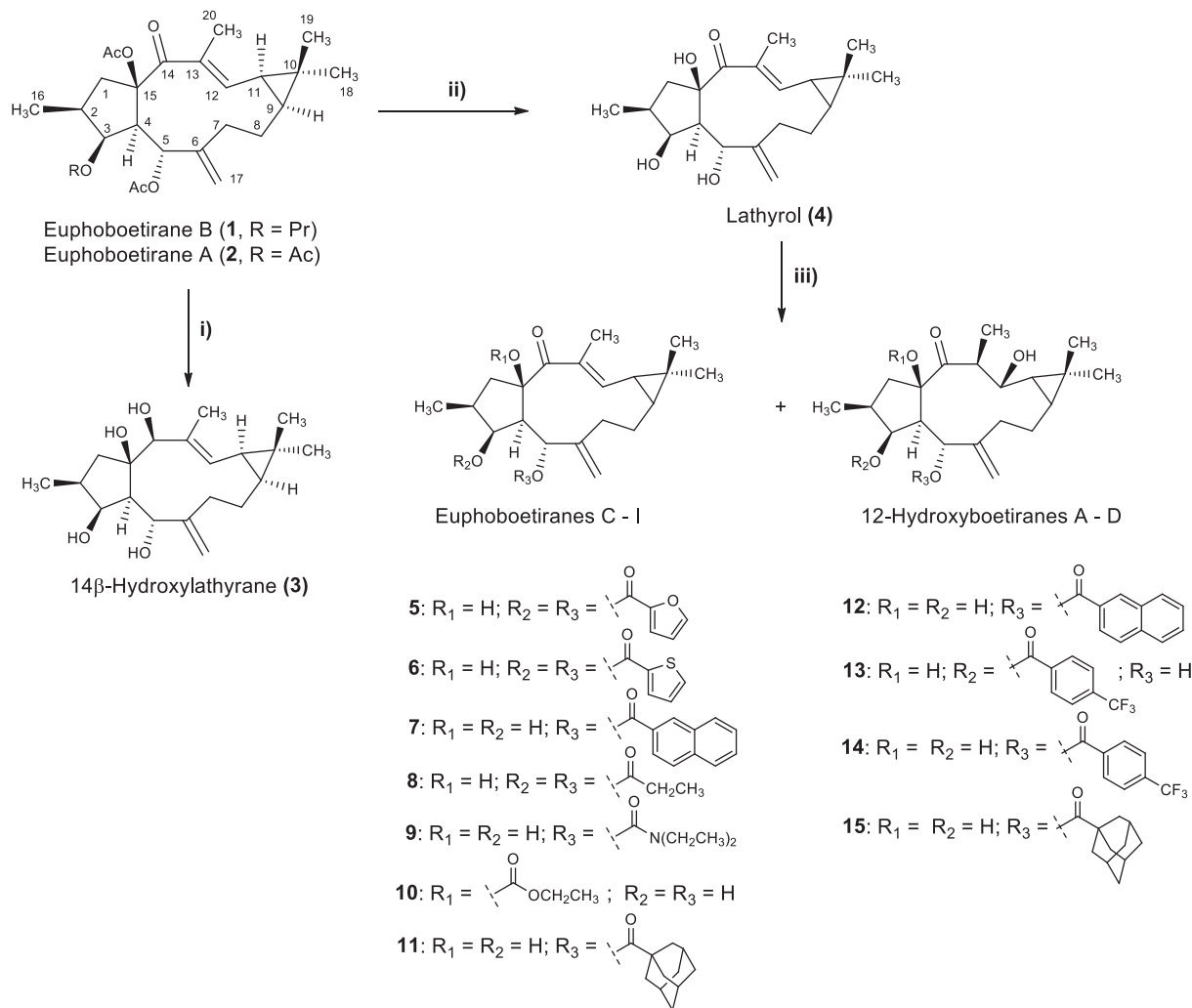


FIGURE 1 Chemical structures of lathyrane diterpenes (**1** and **2**) isolated from *Euphorbia boetica* aerial parts and preparation of 14β-hydroxylathyrane (**3**), lathyrane (**4**) and derivatives (**5–15**). Reagents and conditions: (i) LiAlH₄, THF, 0°C, 1 h; (ii) KOH/MeOH (5%), room temperature, 3 h; (iii) acylating reagent, DMAP (cat.), TEA/CH₂Cl₂ (1:1), room temperature or under reflux (60°C, nitrogen atmosphere), 2–18 h

relative stereochemistry of all tetrahedral centres was found to be identical to those of euphoboetirane A (**2**)²⁵ through a NOESY spectrum.

3.2 | Derivatives of lathyrane

Molecular derivatisation of macrocyclic diterpenes is regarded as an important way to obtain a high number of homologous compounds towards an increasing knowledge on structure–activity relationships in P-gp-modulating activity. Therefore, in order to obtain a small set of lathyrane derivatives and taking advantage of the chemical functions of this compound, euphoboetirane B (**1**) was reduced, using LiAlH₄, and hydrolysed in a MeOH solution of KOH, to afford 14β-hydroxylathyrane (**3**), a new lathyrane-type polyalcohol, and lathyrane (**4**), respectively (Figure 1). Using lathyrane (**4**) as a starting material and different acylation reagents, seven new derivatives were obtained: three diacylated, named euphoboetiranes C, D and F (**5**, **6** and **8**) and four monoacylated, named euphoboetiranes E, G and H

(**7**, **9–11**). While performing the referred reactions, some acylated derivatives were further modified undergoing conjugate addition of water to the α,β-unsaturated system, giving rise to four new compounds, named 12-hydroxyboetiranes A–D (**12–15**), (Figure 1).

Lathyrane (**4**) was identified by comparison of its spectroscopic data with those reported in the literature.²⁶ 14β-Hydroxylathyrane (**3**) was obtained as white amorphous powder with [α]_D²⁵ –134.0. The spectroscopic data of **3** resembled those achieved for lathyrane (**4**).²⁶ As expected, in the ¹H-NMR spectrum of **3**, the most remarkable difference was the presence of a new singlet at δ_H 4.24 (H-14). In the ¹³C-NMR, the presence of a signal at δ_C 77.8 and the disappearance of the ketone resonance at δ_C 207.1, together with the upfield shifts of C-12 (Δδ = –17.1 ppm), C-13 (Δδ = –4.4 ppm) and C-15 (Δδ = –3.4 ppm) were consistent with the introduction of a new hydroxyl group at C-14. The relative stereochemistry of all tetrahedral centres was found to be identical to those of compound **1**, except at C-14, the new tetrahedral centre. In this way, assuming the α-orientation for H-4 as a reference point,²⁶ the NOESY cross-peaks between H-4/H-2, H-3/H-2 and H-2/H-14 established the α-

configuration of these protons. No correlation was found between H-4 and H-5, which corroborated the preservation of the β -configuration for H-5.

The structural elucidation of euphoboetiranes C-I (**5–11**) was achieved by comparison of their spectroscopic data with those of lathyrol (**4**) and euphoboetirane A (**1**). However, it should be noted that due to the poor spectral resolution of some compounds when dissolved in CDCl_3 , other solvents had also to be used to overcome this problem, namely CD_3OD , pyridine- d_5 and $\text{DMSO}-d_6$ (Supporting Information).

Besides the signals due to the different acyl groups, the analysis of $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra showed, as expected, very similar data regarding the diterpenic core. When comparing with lathyrol (**4**), the most remarkable differences in the $^1\text{H-NMR}$ spectrum were related to the H-3 and H-5 chemical shifts that were displayed downfield when these positions were acylated. These differences were in agreement with the effects expected for the acylation of the hydroxyl groups and were also observed in euphoboetirane B (**1**). Regarding the $^{13}\text{C-NMR}$ spectra, it was crucial to analyse the carbonyl signals in order to make conclusions on the number of acyl groups. The location of the acyl groups was definitely confirmed by the long-range correlations between the carbonyl signals and the corresponding H-3 and/or H-5 oxymethine protons. The relative stereochemistry of all tetrahedral centres was found to be identical to those of euphoboetirane B (**1**).

When comparing the NMR spectroscopic data of 12-hydroxyboetiranes A-D (**12–15**) to those of the already described ester derivatives **1**, **2** and **5–11**, several differences could be indicated. Indeed, in the $^1\text{H-NMR}$ spectra of compounds **12–15** both H-12 and the olefinic methyl (CH_3 -20) signal resonances disappeared, while new signals at δ_{H} 4.70–4.79 (t, $J \approx 9.2$ Hz) and δ_{H} 1.17–1.22 (d, $J \approx 7.9$ Hz) could be observed. Likewise, these differences were also obvious in the $^{13}\text{C-NMR}$ spectra, which showed the presence of two extra methine carbons at $\delta_{\text{C}} \approx 74.6$ and $\delta_{\text{C}} \approx 41.5$, together with the disappearance of the signals corresponding to the olefinic carbons C-12 and C-13. Moreover, a downfield chemical shift ($\Delta\delta + 23$ ppm) was also observed for the ketone signal that was in agreement with the absence of the α,β -unsaturated system. These structural features were confirmed by the analysis of $^1\text{H}-^1\text{H}$ COSY, HMQC and HMBC spectra that allowed the unambiguous assignment of all proton and carbon resonances. These spectroscopic data led to conclusion that 12-hydroxyboetiranes A-D (**12–15**) differed from the remaining lathyrol derivatives by having a hydroxyl group at C-12 instead of the C-12/C-13 endocyclic double bond. The relative configuration of 12-hydroxyboetiranes A-D (**12–15**) was deduced through the analysis of their NOESY spectra and by comparison with euphoboetirane B (**1**), assuming, as usual, the α -orientation for H-4. In this way, the strong nuclear Overhauser interactions between H-4/H-3, H4/H-2, H-2/ CH_3 -18 and at CH_3 -18/H-11 established the α configuration of these protons. The α -orientation of the new chiral centres C-12 and C-13 was supported by nuclear Overhauser interactions between CH_3 -18/H-12, H-12/H-11, H-12/H-13 and H13/H11. The β -orientation of H-5 was

suggested by the absence of a NOESY correlation between this proton and H-4 and was corroborated by $J_{4,5}$ value which were similar to that of related diterpenes.^{25,26}

3.3 | Biological activity

The diterpenes **1–15** were investigated for their antiproliferative activity in order to select non-cytotoxic concentrations to perform the P-gp modulation experiments. Antiproliferative assays were performed using the MTT test on chemosensitive (PAR) and human MDR1-gene transfected mouse lymphoma cells (MDR), and on sensitive and resistant human colon adenocarcinoma cell lines (sensitive Colo205 and Colo320 MDR cells). The results are summarised in Table 1 and expressed in IC_{50} values. The selectivity index [$\text{SI} = \text{IC}_{50}(\text{MDR cells})/\text{IC}_{50}(\text{PAR cells})$] was also calculated. As can be observed, except for euphoboetirane D (**6**) that showed an IC_{50} value of 6.9 μM against Colo205 cell line, all compounds exhibited weak antiproliferative activities (IC_{50} values higher than 10 μM). Moreover, the compounds did not show significant IC_{50} disparities between the assayed parental and MDR cell lines (SI values between 2.37 and 0.80), indicating that they were similarly active against both parental and resistant cells.

The ability of compounds to modulate the transport activity of P-gp was evaluated on both human MDR1-gene transfected L5178Y mouse lymphoma and on MDR human colon adenocarcinoma cells. Reversion of MDR was performed by flow cytometry, using a standard functional assay that measures rhodamine-123 (a fluorescent analogue of the anti-cancer drug doxorubicin) accumulation on the cells. Verapamil (20 μM) was used as positive control, since it was a well-known MDR modifier. The compounds were tested at two concentrations (2 and 20 μM). The FAR values were used to assess the P-gp modulating potential. Compounds were considered to be active when presenting FAR values higher than 1, and assigned as strong P-gp modulators when FAR values are higher than 10.^{29,30} However, it should be emphasised that FAR values obtained with L5178Y-MDR mouse lymphoma cells, where P-gp is highly expressed, could not be comparable with those obtained on human Colo320 MDR cells because P-gp expression is much lower on the latter.

The results are summarised in Table 2. As it can be observed, when tested at 20 μM euphoboetiranes A (**2**), euphoboetiranes C–G (**5–9**) and 12-hydroxyboetiranes A–C (**11–14**) were found to be strong P-gp modulators on L5178Y-MDR mouse lymphoma cells, displaying FAR values ranging from 12.0 to 83.8. At this concentration, the strongest effects were found for euphoboetiranes C (**5**, FAR = 83.8), D (**6**, FAR = 82.2) and E (**7**, FAR = 64.7), which showed a manifold activity when compared to that of verapamil (FAR = 17.7 at 20 μM). Comparing the FAR values of the acylated diterpenes **5–11** and the parental alcohol lathyrol (**4**, FAR = 2.0 at 20 μM), the majority of them showed a 6 to 42-fold increase of the activity. 14 β -Hydroxylathyrene (**3**) and euphoboetirane B (**1**) were found to be barely active, even at the highest concentration (FAR = 3.3 and 1.3, respectively).

TABLE 1 Antiproliferative activity of compounds **1–15** on mouse T-lymphoma (L5178Y-PAR and L5178Y-MDR) cells and human colon adenocarcinoma (Colo205 and Colo320) cells

Compound	L5178Y mouse T-lymphoma cells			Colon adenocarcinoma cells		
	IC ₅₀ ^a (μM)			IC ₅₀ ^a (μM)		
	PAR	MDR	SI ^b	Colo205	Colo320	SI ^b
Euphoboetirane B (1)	15.2 ± 2.4	18.2 ± 3.3	1.20	23.1 ± 4.1	25.4 ± 0.5	1.10
Euphoboetirane A (2)	18.6 ± 0.3	16.0 ± 2.0	0.86	55.0 ± 4.7	> 50	–
14β-hydroxylathyrane (3)	60.7 ± 6.8	59.7 ± 3.4	0.98	> 50	65.1 ± 7.6	–
Lathyril (4)	78.7 ± 2.7	62.8 ± 16.1	0.80	> 100	> 100	–
Euphoboetirane C (5)	27.6 ± 0.9	29.6 ± 1.2	1.07	15.3 ± 2.4	22.0 ± 0.4	1.44
Euphoboetirane D (6)	36.4 ± 1.0	45.7 ± 6.5	1.26	6.9 ± 0.4	16.4 ± 0.2	2.37
Euphoboetirane E (7)	40.9 ± 4.9	50.6 ± 0.4	1.24	11.2 ± 1.1	12.9 ± 1.2	1.15
Euphoboetirane F (8)	32.0 ± 2.4	45.4 ± 1.6	1.42	22.9 ± 3.2	24.6 ± 1.0	1.07
Euphoboetirane G (9)	> 100	> 100	–	39.1 ± 4.9	58.7 ± 4.6	1.50
Euphoboetirane H (10)	37.0 ± 6.8	> 100	–	> 100	> 100	–
Euphoboetirane I (11)	34.2 ± 1.6	49.4 ± 5.4	1.44	10.4 ± 0.6	12.7 ± 0.1	1.22
12-Hydroxyboetirane A (12)	54.4 ± 3.8	> 100	–	> 50	> 50	–
12-Hydroxyboetirane B (13)	57.2 ± 2.2	70.5 ± 16.5	1.23	46.5 ± 3.2	47.9 ± 3.8	1.03
12-Hydroxyboetirane C (14)	57.3 ± 4.0	> 100	–	55.8 ± 8.7	> 50	–
12-Hydroxyboetirane D (15)	48.0 ± 2.8	52.2 ± 3.2	1.09	48.9 ± 4.6	> 50	–
DMSO (1%)	> 100	> 100	–	> 100	> 100	–

^aValues of IC₅₀ are the mean ± standard deviation of three independent experiments.

^bSelectivity index (SI) = IC₅₀ (MDR cells)/IC₅₀ (PAR cells) or IC₅₀ (Colo320 cells)/IC₅₀ (Colo 320 cells).

When tested at 2 μM, euphoboetirane D (**6**) exhibited a remarkable reversion activity (FAR = 64.5). Moreover, at the lowest concentration, euphoboetiranes C (**5**, FAR = 14.2), E (**7**, FAR = 13.3) and G (**9**, FAR = 13.4) showed also significant MDR reversal activities (Table 2). Concerning the activity on MDR human colon adenocarcinoma cells (Colo320), euphoboetiranes A (**2**), C–F (**5–8**) and I (**11**), and 12-hydroxyboetiranes A–C (**12–14**) were the most effective, when tested at 20 μM (FAR values between 3.1 and 4.9), although less active than verapamil (FAR = 9.0). At 2 μM, the most active compounds were euphoboetiranes D (**5**, FAR = 2.3) and E (**7**, FAR = 2.5).

Further studies were conducted in order to assess the combined effect of diterpenes **1–15** and the anticancer drug doxorubicin, and evaluate the type of interaction, using the checkerboard microplate method on L5178Y-MDR mouse lymphoma cells. The extent of interactions between the anticancer drug and compounds **1–15** was calculated as proposed by Chou,²⁷ and expressed using the CI values (Table 3). All compounds had a synergistic interaction with the anticancer drug (CI values among 0.19 and 0.86). Strong synergistic effects were found for compounds **5**, **6** and **8–13**. Interestingly, the majority of the most active compounds on the P-gp modulation assay were also those that developed higher synergism with doxorubicin. However, the polyalcohols **3** and **4** also exhibited low CI values (0.29 and 0.27, respectively) even though they displayed a weak P-gp modulation activity (FAR = 1.3 and 2.0 respectively, at 20 μM), suggesting that different mechanisms may be involved in this process. The drug combination assay was also applied on Colo320 cell line

for diterpenes **1**, **2**, **5–8** and **11–14**, which revealed the highest MDR *in vitro* modulation activities on the referred cell line. Curiously, all compounds displayed a synergistic interaction, excepting euphoboetiranes C (**5**) and E (**7**). In fact, diterpenes **5** and **7** showed additive (CI = 0.95) and antagonistic effects (CI = 1.18), respectively, despite being two of the most active modulators of P-gp activity on Colo320 cell line (FAR values 3.9 and 4.9, respectively, at 20 μM).

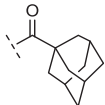
Regarding the chemical structure of the diterpenic core, the analysed diterpenes could be divided in two main sets: compounds with Δ^{6,17} and Δ¹² double bonds (**1–4**, and **5–11**, euphoboetirane series) and compounds with an exocyclic Δ^{6,17} double bond and absence of the Δ¹² unsaturation, being hydroxylated at C-12 (**13–15**, 12-hydroxyboetirane series). Among the two sets, the compounds differ in the type, number and location of the acyl groups. These structural features led to different physicochemical properties, such as lipophilicity, molecular volume and topological polar surface area, which may condition the P-gp modulatory ability of the compounds, and were generally considered to be important non-specific requirements for MDR reversal activity.^{31,32} Nevertheless, the identification of specific structural characteristics of the different diterpenic scaffolds is also of major importance to better understand structure–activity relationships in P-gp modulation activity. Accordingly, our group reported an improved pharmacophore model based on the analysis of several in-house macrocyclic diterpenes isolated from *Euphorbia* species.^{10,33,34} In these studies, *in silico* approaches identified several structural features that may correlate with experimental modulation

TABLE 2 Effect of compounds **1–15** on the P-glycoprotein (P-gp) mediated rhodamine-123 efflux, on L5178Y-MDR mouse T-lymphoma cells and on MDR human colon adenocarcinoma (Colo320)

Compound	R ₁	R ₂	R ₃	Conc (μM)	FAR	
					L5178Y	Colo320
Euphoboetirane B (1)				2 20	1.0 3.3	2.1 2.7
Euphoboetirane A (2)				2 20	4.6 23.7	1.8 3.5
14β-hydroxylathyrane (3)	—	—	—	2 20	0.9 1.3	1.3 1.5
Lathyrol (4)	H	H	H	2 20	1.1 2.0	0.7 0.7
Euphoboetirane C (5)	H			2 20	14.2 83.8	1.9 3.9
Euphoboetirane D (6)	H			2 20	64.5 82.2	2.3 3.6
Euphoboetirane E (7)	H	H		2 20	13.3 64.7	2.5 4.9
Euphoboetirane F (8)	H			2 20	3.0 57.2	1.7 4.1
Euphoboetirane G (9)	H	H		2 20	13.4 56.5	1.3 2.7
Euphoboetirane H (10)		H	H	2 20	1.2 1.9	0.8 1.6
Euphoboetirane I (11)	H	H		2 20	2.1 12.0	1.1 3.1
12-hydroxyboetirane A (12)	H	H		2 20	2.5 44.0	1.6 4.4
12-hydroxyboetirane B (13)	H		H	2 20	3.3 24.2	1.9 3.1
12-hydroxyboetirane C (14)	H	H		2 20	1.8 36.1	1.8 3.4

(Continues)

TABLE 2 (Continued)

Compound	R ₁	R ₂	R ₃	Conc (μM)	FAR	
					L5178Y	Colo320
12-hydroxyboetirane D (15)	H	H		2 20	1.9 6.9	1.0 1.4

Verapamil 20 μM (positive control): FAR (L5178Y-MDR cells) = 17.7, FAR (Colo320 cells) = 9.0; DMSO 2% (neg. control): FAR (L5178Y-MDR cells) = 0.8, FAR (Colo320 cells) = 0.6

TABLE 3 Type and strength of the interaction between compounds 1–15 and doxorubicin on L5178Y-MDR mouse T-lymphoma cells and on MDR human colon adenocarcinoma (Colo320)

Compound	L5178Y-MDR		Colo320 cells	
	CI ^a	Interaction	CI ^a	Interaction
Euphoboetirane B (1)	0.42	Synergism	0.52	Synergism
Euphoboetirane A (2)	0.32	Synergism	0.34	Synergism
14β-hydroxylathyrane (3)	0.29	Strong synergism	–	–
Lathyrol (4)	0.27	Strong synergism	–	–
Euphoboetirane C (5)	0.22	Strong synergism	0.95	Additive
Euphoboetirane D (6)	0.16	Strong synergism	0.29	Strong synergism
Euphoboetirane E (7)	0.42	Synergism	1.18	Antagonism
Euphoboetirane F (8)	0.22	Strong synergism	0.39	Synergism
Euphoboetirane G (9)	0.20	Strong synergism	–	–
Euphoboetirane H (10)	0.19	Strong synergism	–	–
Euphoboetirane I (11)	0.25	Strong synergism	0.37	Synergism
12-Hydroxyboetirane A (12)	0.29	Strong synergism	0.44	Synergism
12-Hydroxyboetirane B (13)	0.25	Strong synergism	0.55	Synergism
12-Hydroxyboetirane C (14)	0.86	Slight synergism	0.64	Synergism
12-Hydroxyboetirane D (15)	0.44	Synergism	–	–

Data are shown as the best combination ratio between the tested compounds and doxorubicin.

^aCombination index (CI) values at 50% of growth inhibition (ED₅₀) were determined by using the CalcuSyn software to plot four to five data points to each ratio. The extent of interaction between drugs was expressed using the CI. CI = 1 and CI > 1 represent additive effect and antagonism, respectively. CI < 1 represents synergism where 0.1 < CI < 0.3 = strong synergism; 0.3 < CI < 0.7 = synergism; 0.85 < CI < 0.9 = slight synergism.

of P-gp. Therefore, the presence of a hydrophobic core, hydrogen bond acceptor groups and one or two aromatic moieties was highlighted as essential features for the interaction with P-gp drug binding site.^{10,33} Moreover, the conformation of the macrocyclic scaffold, the charge distribution within the molecule and the acyl or hydroxyl substitution patterns are also key factors for the biological activity and considered to be responsible for the increased affinity that some molecules display with P-gp.³⁴

It is interesting to note that the most active diterpenes were those with aromatic moieties (5–7, 12 and 14) exhibiting FAR values ranging from 44.0 (12) to 83.8 (5) for L5178Y MDR cells and from 3.6 (6) to 4.9 (7) for Colo320 cells (at 20 μM). Euphoboetiranes C (5) and D (6) are bioisosteric compounds, diacylated at C-3 and C-5, which differ only by the presence of oxygen instead of a sulphur atom at the five membered aromatic ring of the ester moieties. This fact clearly changed the log *P* (5.3 vs. 6.5), topological polar surface area (116.2

vs. 89.9) and molecular volume (474 vs. 493) values, which seems to contribute to the different MDR modulating activity, particularly evident when tested at low concentration (FAR = 14.2 for **5** and 64.5 for **6**). When comparing the C-5 naphtoyl derivatives, euphoboetirane E (**7**) and 12-hydroxyboetirane A (**12**), it can be noticed a decrease of activity for compound **12** at both concentrations tested and on both cell lines (Table 2). Surprisingly, euphoboetirane F (**8**), with two propanoyl groups at C-3 and C-5 and euphoboetirane G (**9**) with a carbamoyl function at C-5, showed also a strong activity at 20 μ M, although not possessing any aromatic group.

According to these and previous results, macrocyclic diterpenes possessing the lathyrene and jatrophane scaffolds have great potential as P-gp modulators on MDR cancer cells. Moreover, most of them, when combined with antineoplastic drugs, such as doxorubicin, synergistically enhance their effect, providing evidence that they may be valuable as lead compounds and are worthy of further studies in order to increase their potency and selectivity.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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