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Inhibition of P-glycoprotein Transport Activity in a Resistant Mouse Lymphoma Cell Line by Diterpenic Lactones

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Abstract. *Multidrug resistance (MDR) is believed to be a major reason for the failure of cancer treatment. It is in most cases caused by the activity of the various ABC transporters, multidrug resistance (MDR) gene-encoded p-glycoproteins that pump anticancer drugs out of the cells. P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP1) are the most important and widely studied members of the ABC superfamily of transporters. The ability of four diterpenic lactones isolated from Euphorbia species to modulate the transport activity of P-gp in mouse lymphoma cells was evaluated by flow cytometry. The reversion of MDR was investigated by using a standard functional assay with rhodamine 123 as a fluorescent substrate analogue of doxorubicin. Verapamil was applied as a positive control. All the compounds were able to reverse the MDR of the tested human MDR1 gene-transfected mouse lymphoma cells, in a concentration-dependent manner from 4 to 40 μg/mL, in a short-term experiment below the cytotoxic doses.*

Multidrug resistance (MDR) is believed to be a major reason for the failure of treatment of infectious diseases and cancer. In resistant cancer cells, it is often associated with the overexpression of P-glycoprotein (P-gp or MDR1) and MRP1. P-glycoprotein is a 170-kDa trans-membrane ATP-dependent glycoprotein, that pumps a variety of hydrophobic antitumour compounds from target cancer cells and thereby prevents their cytotoxic effects. In addition to its role in multidrug resistance, P-gp seems to have several physiological functions, because it is also expressed in many non-tumoral tissues such as epithelial cells of the gastrointestinal tract, adrenal gland, biliary canaliculi and brain capillaries. Thus, it is possible that tumours in these tissues present higher levels of P-gp expression and, thereby, a stronger resistance to chemotherapeutic agents (1).

To overcome this form of drug resistance, effective inhibitors of these efflux pumps are being searched for. Despite the large number of known reversal agents, there are currently none available clinically. The first generation of P-gp modulators being tested clinically include the calcium-channel blocker, verapamil and the immuno-suppressant, cyclosporin A. They are able to restore the intracellular drug accumulation by competing with cytostatic drugs for P-gp binding sites. Despite their activity, they show dose-limiting side-effects that restrict their clinical utility. Thus, the search for new compounds able to modify MDR is of great importance since the simultaneous administration of a P-gp inhibitor and an anticancer drug may improve the effectivity of anticancer therapy (2, 3). Recently, besides the large number of newly synthesised derivatives, some natural MDR reversal compounds were identified from various plants such as the *Euphorbia* species.

The *Euphorbia* species (Euphorbiaceae) have a long history of use in the treatment of cancer (4). They have provided a wide range of structurally unique polyoxygenated macrocyclic diterpenes, such as jatrophanes and lathyranes and their polycyclic derivatives. Recent investigations revealed that diterpenes of the jathophane type are promising modulators of MDR in tumour cells, (5-9) as well as microtubule-interacting agents (10). Among the diterpenes, *ent*-abietane lactones have also been reported as constituents of these species (11-13). In our previous studies, the diterpenic lactones **1** and **2** were evaluated for their capacity to inhibit the *in vitro* growth of the human tumour cell lines, MCF-7, NCI-H460 and SF-268 (11).

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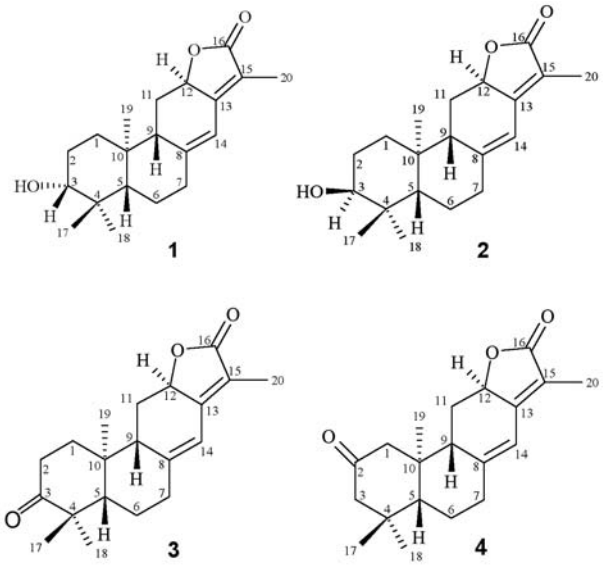

Figure 1. *Chemical structures of ent-abietane lactones* ***1-4****.*

As a continuation of our studies in the search for new anti-MDR agents from natural origins, the effects of four *ent*-abietane lactones, helioscopinolides A (**1**) B (**2**), E (**3**) and F (**4**), isolated from *Euphorbia* species, were evaluated on their cytotoxicity and inhibition of the MRD1 efflux pump overexpressed in a resistant mouse lymphoma cell line.

Materials and Methods

Compounds. Four diterpenic lactones (Figure 1) were tested for reversal of MDR: helioscopinolide A (**1**), helioscopinolide B (**2**), helioscopinolide E (**3**) and helioscopinolide F (**4**). Compound **1** was isolated from the methanol extract of *Euphorbia pubescens* Vahl and identified, as previously described (11). Compounds **2** and **3** and compound **4** were isolated from the acetone extracts of *Euphorbia tuckeyana* Steud and *Euphorbia portlandica* L., respectively, and identified by spectroscopic methods (IR, ^{1}H-NMR, ^{13}C-NMR and MS). Their isolation and identification will be reported elsewhere. The purity of the compounds was more than 95% by GC and/or HPLC analysis. All compounds were dissolved in DMSO.

Cells. The L5178 Y mouse T-lymphoma parental cell line was transfected with the pHa MDR1/A retrovirus, as previously described (14). The L5178 MDR cell line and the L5178 Y parental cell line, (obtained from Prof. M. Gottesmann, NCI and FDA, USA), were grown in McCoy's 5A medium with 10% heat-inactivated horse serum, *L*-glutamine and antibiotics. MDR1-expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain expression of the MDR phenotype. Cell viability was determined by trypan blue.

Rhodamine 123 (R123) uptake assay. The cells were adjusted to a density of $2x10^{6}$/mL, resuspended in serum-free McCoy's 5A medium and distributed in 0.5 mL aliquots into Eppendorf centrifuge tubes. Two to 16.0 μl of the 2.0 mM stock solutions of the compounds in DMSO were then added and the samples were incubated for 10 min at room temperature. A total of 10 μl (5.2 μM final concentration) of rhodamine 123 (R123; Sigma, St. Louis, MO, USA) was next added to the samples and the cells were incubated for a further 20 min at 37˚C, washed twice and subsequently resuspended in 0.5 mL phosphate-buffered saline for analysis. The fluorescence of the cell population was measured by flow cytometry with a Beckton Dickinson FACScan instrument. Verapamil (5 μl of a 2.0 mM solution) was used as a positive control in the rhodamine 123 exclusion experiments. The mean fluorescence intensity was calculated as a percentage of the control for the parental and MDR cell lines as compared to untreated cells. An activity ratio (R) was calculated on the basis of the measured fluorescence values (FL-1) calculated *via* the following equation (15, 16):

$$R = \frac{\text{MDR-treated / MDR-control}}{\text{parental-treated / parental control}}$$

Assay for cytotoxic effect. The cells were distributed in a 96-well microplate at $2x10^{4}$ density/ 100 μl medium / well, with the exception of the medium control wells. The plates were incubated for 24 h. The compounds were diluted and added to each well in the suitable concentration. The culture plates were further incubated at 37˚C for 24 h; at the end of the incubation period, 15 μL of MTT (thiazolyl blue tetrazolium bromide, Sigma) solution (from a 5 mg/mL stock) was added to each well. After incubation at 37˚C for 4 h, 100 μL of sodium dodecyl sulfate (SDS) (Sigma) solution (10%) was added into each well and the plates were further incubated at 37˚C overnight. The cell growth was determined by measuring the optical density (OD) at 550 nm (ref. 630 nm) with a Dynatech MRX vertical beam ELISA reader. Inhibition of cell growth (as a percentage) was determined according to the formula:

$$100 - \frac{\text{OD sample} - \text{OD medium control}}{\text{OD cell control} - \text{OD medium control}} \times 100$$

Table I. *Cytotoxicity values of helioscopinolides* ***1-4*** *on the human MDR1-transfected mouse lymphoma cell line.*

Compound	Cytotoxicity (ID_{50}, μg/mL)
Helioscopinolide A (**1**)	32.99
Helioscopinolide B (**2**)	16.62
Helioscopinolide E (**3**)	17.80
Helioscopinolide F (**4**)	33.48
DMSO	19.35

Results and Discussion

Chemotherapeutics are the most effective in treatment for many types of tumours (17). However, the ability of cancer cells to become progressively resistant to structurally and functionally unrelated anticancer drugs remains a significant

Table II. *Multidrug resistance reversing activity of compound **1** on human MDR1 gene-transfected mouse lymphoma cells.*

Samples	Conc. (μg/mL)	FSC[a]	SSC[a]	FL-1[a]	Fluorescence activity ratio
PAR +R123	-	517.43	145.32	924.46	-
MDR + R123	-	583.02	190.45	8.04	-
Verapamil	10	576.49	192.00	137.30	18.68
Heliosconolide A (**1**)	4	550.47	177.50	18.17	2.47
	40	533.44	166.57	180.27	24.52
DMSO control	20 μL	532.18	172.43	5.93	0.80

[a]FSC: Forward Scatter Count; SSC: Side Scatter Count; FL-1: Fluorescence Intensity
[b]Par: A parental cell without MDR gene.
[c]MDR: A parental cell transfected with human MDR1 gene.

Table III. *Multidrug resistance reversing activity of helioscopinolides **2**, **3**, and **4** on human MDR1 gene-transfected mouse lymphoma cells.*

Samples	Conc. (μg/mL)	FSC[a]	SSC[a]	FL-1[a]	Fluorescence activity ratio
PAR +R123[b]	-	530.99	145.37	957.08	-
MDR + R123[c]	-	562.30	191.33	8.45	-
Verapamil	10	563.34	190.96	88.61	12.23
Heliosconolide B (**2**)	4	553.85	192.90	14.25	1.96
	40	534.26	181.90	370.28	51.14
Heliosconolide E (**3**)	4	539.88	194.53	50.99	7.04
	40	543.23	178.36	503.96	69.61
Heliosconolide F (**4**)	4	545.86	187.68	421.37	58.20
	40	540.64	189.42	1003.16	138.56
DMSO control	20 μL	511.42	180.90	7.32	1.01

[a]FSC: Forward Scatter Count; SSC: Side Scatter Count; FL-1: Fluorescence Intensity.
[b]Par: A parental cell without MDR gene.
[c]MDR: A parental cell transfected with human MDR1 gene.

obstacle to successful chemotherapy. The multidrug resistance phenotype is usually the result of higher levels of cellular transport proteins and thereby higher efflux of the drugs from the cell.

The ability of compounds which can inhibit the function of the MDR-efflux proteins such as MDR1 and MRP, to improve the cytotoxic activity of anticancer drugs, prompted the search for MDR reversing natural compounds. The ability of helioscopinolides A, B, E and F (**1-4**), isolated from the acetone or methanol extracts of *Euphorbia pubescens*, *Euphorbia tuckeyana* and *Euphorbia portlandica*, to modulate the transport activity of P-gp in mouse lymphoma cells was evaluated by flow cytometry. The reversion of MDR was investigated and evaluated by using a standard functional assay with rhodamine 123 as a fluorescent substrate analogue of doxorubicin. Verapamil was applied as a positive control. As can be observed in Table I, the tested compounds did not show significant toxic effect on the human MDR1-transfected mouse lymphoma cell line because the inhibitory doses 50 (ID_{50}) values of each compound were high in long-term experiments (48 h).

In short-term experiments where drug exposition and measurement of efflux inhibition took only 30-35 min, compounds **2-4** were able to reverse the MDR of the tested human MDR1 gene-transfected mouse lymphoma cells in a concentration-dependent manner (Tables II and III). Compound **4** displayed very strong activity (fluorescence activity ratio R=58.20 at 4 μg/mL; R=138.56 at 40 μg/mL) compared with the positive control verapamil (R=12.23 at 10 μg/mL).

The four compounds tested have very similar structures, differing only in the substitution pattern of ring A; compounds **1** and **2** are epimers only differing in the configuration of their stereocenter at C-3 and compounds **3** and **4** are constitutional isomers having the carbonyl function of ring A in a different position. They are hydrophobic compounds with calculated log P=3.9 (**1** and **2**) and 3.8 (**3** and **4**) and with three hydrogen bond acceptors and one hydrogen bond donor in the case of **1** and **2**. By comparing the activity of **3** with that of **4**, it seems that the higher inhibitory effect of **4** might be derived from the

carbonyl position at C-2 – most probably due to the conformational and functional changes in the P-gp induced by the particular structures of helioscopinolides.

The results from this study has identified diterpenic lactones, obtained from *Euphorbia* species, as effective compounds for the reversal of MDR. However, the structure-activity relationship for these *ent*-abietanes needs further evaluation. The differences of activity observed within the set of diterpenic lactones tested highlight that stereochemistry features and small structural differences can be important in the inhibitory action of the compounds.

Compound **1** was a moderate/weak growth inhibitor of all the cell lines tested. Compound **2** was shown to be inactive against the SF-268 cell line (11).

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References

1 Grossi A and Biscardi M: Reversal of MDR by verapamil analogues. Hematology *9*: 47-56, 2004.
2 Teodori E, Dei S, Scapecchi S and Gualtieri F: The medicinal chemistry of multidrug resistance (MDR) reversing drugs. Il Farmaco *57*: 385-415, 2002.
3 Robert J and Jarry C: Multidrug resistance reversal agents. J Med Chem *46*: 4805-4817, 2003.
4 Hartwell JL: Plants used against cancer. A survey. Lloydia *62*: 153-205, 1969.
5 Valente C, Ferreira MJU, Abreu PM, Gyémant N, Ugocsai K, Hohmann J and Molnar J: Pubescenes, jatrophane diterpenes, from *Euphorbia pubescens* with multidrug resistance reversing activity on mouse lymphoma cells. Planta Med *70*: 81-84, 2004.
6 Madureira AM, Gyémant N, Ugocsai K, Ascenso JR, Abreu PM, Hohmann J, Molnar J and Ferreira MJU: Rearranged jatrophane-type diterpenes from *Euphorbia* species. Evaluation of their effect on the reversal of multidrug resistance. Planta Med *70*: 45-49, 2004.
7 Hohmann J, Molnar J, Rédei D, Evanics F, Forgo P, Kalman A, Argay Gy and Szabo P: Discovery and biological evaluation of a new family of potent modulators of multidrug resistance: reversal of MDR of mouse lymphoma cells by new natural jatrophane diterpenoids isolated from *Euphorbia* species. J Med Chem *45*: 2425-2431, 2002.
8 Corea G, Fattorusso E, Lanzotti V, Taglialatela-Scafati O, Appendino G, Ballero M, Simon PN, Dumontet C and Di Pietro A: Modified jatrophane diterpenes as modulators of multidrug resistance from *Euphorbia dendroides*. Bioorg Med Chem *11*: 5221-5227, 2003.
9 Corea G, Fattorusso E, Lanzotti V, Motti R, Simon PN, Dumontet C and Di Pietro A: Jatrophane diterpenes as modulators of multidrug resistance. Advances of structure-activity relationships and discovery of the potent lead pepluanin A. J Med Chem *47*: 988-992, 2004.
10 Miglietta A, Gabriel L, Appendino G and Bocca C: Biological properties of jatrophane polyesters, new microtubule-interacting agents. Cancer Chemother Pharmacol *51*: 67-74, 2003.
11 Valente C, Pedro M, Duarte A, Nascimento MSJ, Abreu PM and Ferreira MJU: Bioactive diterpenoids, a new jatrophane and two *ent*-abietanes, and other constituents from *Euphorbia pubescens*. J Nat Prod *67*: 902-904, 2004.
12 Marco JA, Sanz-Cervera JF, Yuste A and Jakupovic J: Isoterracinolides A and B, novel bishomoditerpene lactones from *Euphorbia terracina*. J Nat Prod *62*: 110-113, 1999.
13 Sutthivaiyakit S, Thapsut M and Prachayasittikul V: Constituents and bioactivity of the tubers of *Euphorbia sessiliflora*. Phytochemistry *53*: 947-950, 2000.
14 Cornwell MM, Pastan I and Gottesmann MM: Certain calcium channel blockers bind specifically to multidrug-resistance human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. J Biol Chem *262*: 2166-2170, 1987.
15 Weaver JL, Szabo G, Pine PS, Gottesman MM, Goldenberg S and Aszalos A: The effect of ion channel blockers, immunosuppressive agents, and other drugs on the activity of the multidrug transporter. Int J Cancer *54*: 456-461, 1993.
16 Kessel D: Explorating multidrug resistance using Rhodamine 123. Cancer Commun *1*: 145-149, 1989.
17 Frenkel GD and Caffrey PB: A prevention strategy for circumventing drug resistance in cancer chemotherapy. Curr Pharm Des *7*: 1595-1614, 2001.

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