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Euphorbia Species-derived Diterpenes and Coumarins as Multidrug Resistance Modulators in Human Colon Carcinoma Cells

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Abstract. *Background: Recently, many new potent multidrug resistance (MDR) reversal agents have been discovered, among them lathyrane and jatrophane diterpenes isolated from various Euphorbia species. In the present study, the cytotoxicity, P-glycoprotein inhibition activity, and MDR reversal potency of six diterpenes and two coumarins from two Euphorbia species were studied in human colon carcinoma LoVo cells, and doxorubicin-resistant, LoVo/Dx cells. Materials and Methods: Cytotoxicity of the studied compounds (alone and in combination with doxorubicin) was investigated. Inhibition of P-glycoprotein transport activity was monitored by flow cytometry. Changes in intracellular doxorubicin accumulation were observed by means of fluorescence microscopy. Results: Latilagascene B was demonstrated to be an effective P-glycoprotein inhibitor, able to increase doxorubicin accumulation in resistant cells, however not able to restore doxorubicin cytotoxicity in LoVo/Dx cells. Conclusion: The structure of latilagascene B seems to be an interesting candidate for further synthesis of new derivatives of reduced cytotoxicity and high anti-MDR potency.*

The genus *Euphorbia* (*Euphorbia*ceae), a commonly named spurge, is a large and diverse group of flowering plants, with over 2,000 species, ranging from annual herbs to trees. The spurges are distributed worldwide and present in all climates, with the majority of species occupying different habitats of the tropical and sub-tropical zone. Wounded plants secret a poisonous, white latex that contains many biologically-active compounds. Due to their richness in secondary metabolites *Euphorbia* species have a long history of traditional use as poisons and drugs (1). They have been reported as folk remedies against different skin conditions (*e.g.*, warts, excrescences and tumors), arthritis, asthma, neuralgia, gonorrhea, diarrhea, and others. Extracts from various *Euphorbia* plants are also registered modern drugs against viral infections of the upper respiratory tract and against diarrheal diseases (1). Recently, *Euphorbia* species have attracted scientific interest due to production of a wide range of polycyclic and macrocyclic diterpenes (2), many of which have been demonstrated to possess antiviral, antimicrobial, anti-inflammatory and antitumor activity as well as multidrug resistance-reversing activity (3).

Multidrug resistance (MDR), defined as intrinsic or chemotherapy-acquired simultaneous resistance of cancer cells to structurally and functionally unrelated anticancer drugs, constitutes nowadays one of the main obstacles to successful chemotherapy. Among the diverse molecular mechanisms of MDR, the changes in transmembrane drug transport caused by the overexpression of multidrug transporters, such as P-glycoprotein (P-gp, ABCB1), multidrug-resistance associated protein 1 (MRP1, ABCC1) and breast cancer resistance protein (BCRP, ABCG2), seem to predominate (4). Multidrug transporters from the ABC family are multi-specific transporters that use energy of ATP hydrolysis to pump anticancer drugs out of cells, thereby

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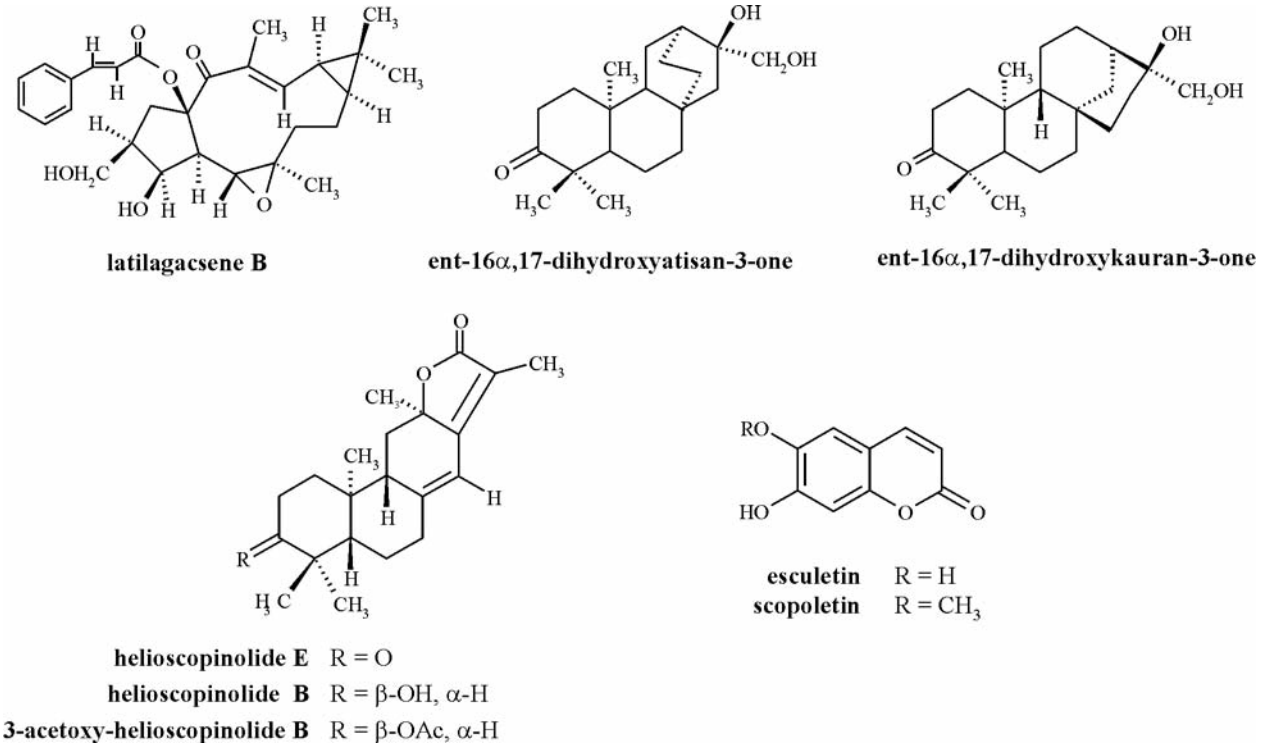

Figure 1. *Chemical structure of the studied compounds*.

reducing their intracellular concentration below the levels toxic to cancer cells (5). The search for compounds able to reduce multidrug resistance (MDR modulators) has been continued for many years. However, despite of many effective MDR modulators identified *in vitro*, none of them is available for the clinical practice (6).

Recently, many new potent MDR reversal agents have been discovered, among them lathyrane (7-11) and jatrophane diterpenes (12-18) isolated from various *Euphorbia* species. In the present work, the cytotoxicity, P-gp inhibition activity, and MDR reversal potency of six diterpenes and two coumarins isolated from two *Euphorbia* species was studied in human colon carcinoma LoVo, cells, and doxorubicin-resistant, LoVo/Dx cells.

Materials and Methods

Tested compounds. A set of six diterpenes and two coumarins isolated from two *Euphorbia* species was tested. The chemical structure of all compounds is presented in Figure 1. Latilagascene B, *ent*-16α,17-dihydroxyatisan-3-one, and *ent*-16α,17-dihydroxykauran-3-one were isolated from the methanol extract of *Euphorbia lagascae* aerial parts (7); esculetin and scopoletin were isolated from the methanol extract of *Euphorbia lagascae* seeds (19); helioscopinolide E and helioscopinolide B were isolated from the methanol extract of *Euphorbia tuckeyana* (15), and 3-acetoxy-helioscopinolide B was prepared, as described previously (20). All compounds were dissolved in dimethyl sulfoxide (DMSO).

Materials. Sulforhodamine B (SRB), rhodamine 123, and doxorubicin were from Sigma (Poznan, Poland). All other reagents were of analytical grade. Both rhodamine 123 and doxorubicin were dissolved in water. Other compounds were dissolved in DMSO.

Cell culture. Human colorectal adenocarcinoma cell line (LoVo), and its resistant subline (LoVo/Dx), obtained by prolonged exposure to doxorubicin (21), were cultivated in Ham's F12 medium (Cytogen, Lodz, Poland), with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), L-glutamine and antibiotics at 37˚C and 5% CO_2. Doxorubicin (100 ng/ml) was added to the culture medium of the LoVo/Dx cells. The drug was withdrawn one week before experiments. Treatment with Non-enzymatic Cell Dissociation Solution (Sigma-Aldrich, Carlsbad, CA, USA) was used to detach cells from the culture flasks.

Cell viability assay. Cells were seeded (3×10^5 cells/well) onto 96-well plates in 75 μl of medium and allowed to attach (60 min, 37˚C). Then, 75 μl of medium containing an appropriate concentration of the studied compounds was added and cells were incubated for another 72 h. The sulforhodamine B assay was performed according to the method of Skehan *et al*. (22). Absorbance at 492 nm was measured and the percentage of cell survival was calculated as: (A_{492} of treated cells/A_{492} of control cells) ×100%. The control cells were treated with medium only. The influence of DMSO (maximal concentration in the

Table I. *Influence of the studied diterpenes and coumarins on reversal of multidrug resistance in LoVo/Dx cells and on viability of LoVo and LoVo/Dx cells. Viability was defined as the percentage of cells surviving 72-h cultivation in the presence of the studied compound.*

Compound	Concentration (μM)	FIR	LoVo cells survival (%)	LoVo/Dx cells survival (%)
Diterpenes				
Latilagascene B	10	1.53±0.31	71.66±15.12	105.26±14.87
	100	6.51±1.30	10.80±1.39	7.08±9.34
ent-16α,17-dihydroxyatisan-3-one	10	1.54±0.31	98.05±2.84	106.76±1.83
	100	1.40±0.28	72.15±3.44	81.01±13.69
ent-16α,17-dihydroxykauran-3-one	10	1.87±0.37	99.20±5.39	120.97±10.86
	100	1.93±0.39	68.26±0.86	94.31±15.20
Helioscopinolide E	10	1.10±0.22	93.27±3.65	101.64±11.72
	100	1.77±0.35	62.75±11.12	50.04±12.59
Helioscopinolide B	10	1.11±0.22	93.39±4.58	113.36±10.96
	100	1.72±0.34	63.52±14.79	41.08±5.96
3-acetoxy-helioscopinolide B	10	1.19±0.24	74.36±3.27	91.44±14.93
	100	0.94±0.19	65.99±9.73	63.07±5.81
Coumarins				
Esculetin	10	0.84±0.17	96.32±4.58	101.42±8.71
	100	1.31±0.26	56.81±5.42	68.42±7.56
Scopoletin	10	1.06±0.24	95.70±7.45	103.97±13.25
	100	0.94±0.19	75.60±8.84	102.76±14.94

samples 0.3%) on the cells was also monitored. Experiments were repeated at least three times.

Accumulation of rhodamine 123. LoVo or LoVo/Dx cells (3×10^5 cells/ml in serum-free and phenol red-free medium) were incubated with the appropriate concentration of the modulators (15 min, RT). Next, rhodamine 123 (2 μM) was added and cells were incubated for further 60 min at 37˚C. The fluorescence of the cell population (washed and resuspended in PBS) was measured by flow cytometry using a Beckton Dickinson (Sunnyvale, CA, USA) FACSCalibur instrument equipped with a 488 nm argon laser. Fluorescence was recorded *via* a 530/30 nm band pass filter. A total of 5,000 events were registered and analyzed with the use of Cell Quest® software (Beckton Dickinson). The influence of DMSO (maximal concentration in samples 0.8%) on the cells was also checked. Fluorescence intensity ratio (FIR) was calculated from the following equation on the basis of measured fluorescence intensity values (FL).

$$FIR = \frac{(FL_{LoVoDx\ treated})/(FL_{LoVoDx\ control})}{(FL_{LoVo\ treated})/(FL_{LoVo\ control})}$$

Control samples were treated with medium only (without modulator).

Doxorubicin localization in cancer cells. The cells were seeded (15,000 cells/well) onto an 8-well μ-Slide microscopy chamber (Ibidi, Munich, Germany). After 48 h 50 μM of doxorubicin (plus 100 μM of the modulator in treated samples) was added in a fresh portion of medium and the incubation was continued for 60 min at 37˚C. Cells were washed with PBS, and then with serum- and phenol red-free F12 medium. Images were collected with a Nikon Eclipse TE2000-E microscope (Nikon Instruments, Amstelveen, the Netherlands), equipped with a PlanFluor 40× (0.60) objective. Fluorescence was excited in a range 528-553 nm, and collected in a range 578-633 nm.

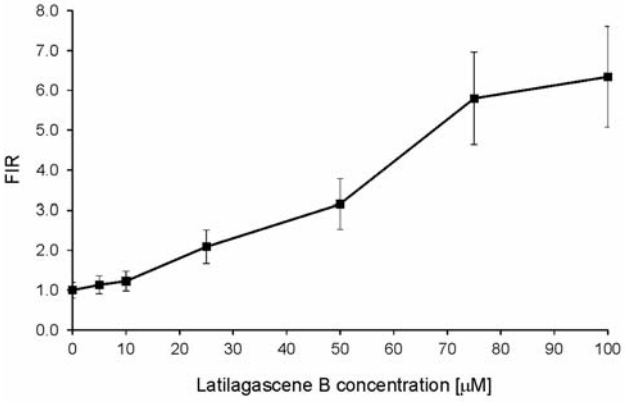

Figure 2. *Influence of latilagascene B on rhodamine 123 accumulation in cancer cells. Means±S.D. of three experiments are presented.*

Results

The first step in studying the biological activity of diterpenes and coumarins isolated from *Euphorbia* species was to characterize their cytotoxicity on cancer cells. Human adenocarcinoma LoVo cells were used, as well as its resistant subline, LoVo/Dx, obtained by prolonged exposure to doxorubicin. All compounds were tested in the concentration range 10-100 μM. The results for the lowest and the highest concentrations tested are presented in Table I. At 10 μM none of the studied compounds was seriously toxic to LoVo

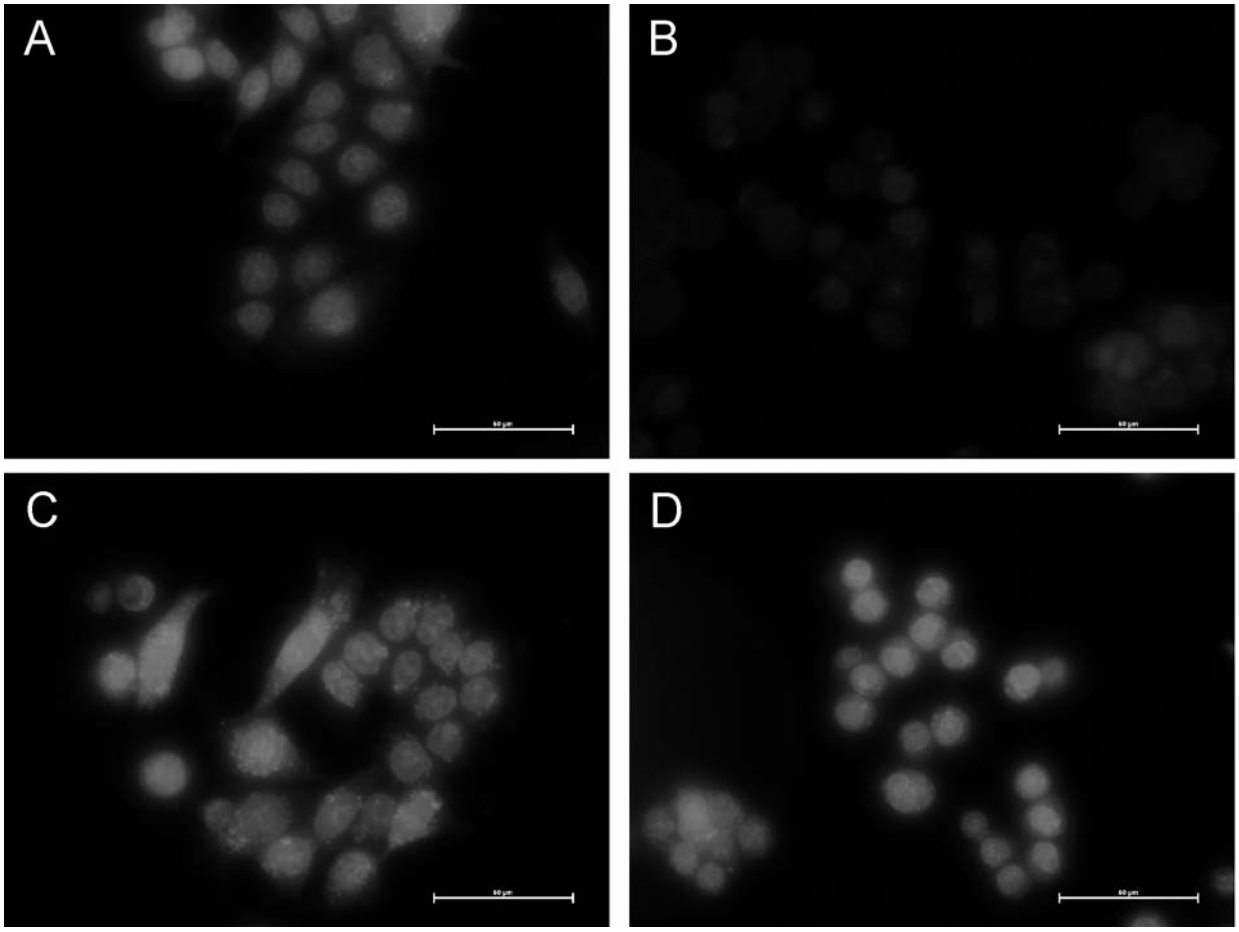

Figure 3. *Fluorescence microscopy images of LoVo (A, C) and LoVo/Dx cells (B, D) treated with 50 μM doxorubicin (A, B) and doxorubicin plus 100 μM latilagascene B (C, D). Scale bar is 50 μm. Illumination conditions were the same for all images.*

and LoVo/Dx cells. At 100 μM latilagascene B was highly cytotoxic to both LoVo and LoVo/Dx cells. The application of the other compounds at 100 μM for 72 h resulted in 50%-75% of LoVo cells surviving the experiment and 40%-100% of LoVo/Dx cells. Usually the studied compounds were more cytotoxic to sensitive LoVo cells than to doxorubicin-resistant LoVo/Dx cells.

The ability of the compounds isolated from *Euphorbia* to inhibit transport activity of the multidrug transporter, P-gp, was investigated by means of functional testing in which the accumulation of fluorescent P-gp substrate, rhodamine 123, inside cancer cells was measured. Not surprisingly, flow cytometry experiments showed that more rhodamine 123 was more accumulated inside LoVo cells than in LoVo/Dx cells (mean fluorescence intensity of sensitive cells population was approximately 6-7-times higher of the resistant cells). Among the studied compounds only latilagascene B was found to increase the accumulation of rhodamine 123 in resistant LoVo/Dx cells (Table I and Figure 2). The obtained results suggested that latilagascene B inhibited P-gp in a concentration-dependent manner.

Fluorescent microscopy allowed for the investigation of intracellular localization of doxorubicin in LoVo and LoVo/Dx cells. The fluorescent signal recorded in sensitive cells was stronger than in resistant cells (Figure 3A and B). Doxorubicin accumulated mainly within nuclei of LoVo cells, while the drug staining was observed in the perinuclear region in LoVo/Dx cells. After 60 min of incubation with latilagascene B the amount of doxorubicin accumulated within LoVo/Dx cells increased compared to incubation with doxorubicin only (Figure 3B and D). The addition of latilagascene B did not influence the fluorescent signal recorded in LoVo cells (Figure 3C).

Additionally, experiments were performed in which the influence of 5 μM of latilagascene B on doxorubicin cytotoxicity in LoVo and LoVo/Dx cells was studied. The

compound affected cytotoxicity of doxorubicin neither in sensitive LoVo cells nor in doxorubicin-resistant LoVo/Dx cells (data not shown).

Discussion

The cytotoxicity of compounds studied in the present article has been previously tested in human gastrointestinal cancer cell lines: gastric (EPG85-257), pancreatic (EPP85-181) and colon (HT-29) carcinomas (15, 23, 24). In these studies cancer cells were incubated with the *Euphorbia*-derived compounds for a five-day period and it was found that he majority of studied compounds was moderately cytotoxic to cancer cells. Esculetin (23) and latilagascene B (24) were identified as the most cytotoxic compounds in the studied gastrointestinal cancer cell lines. In opposition to the results obtained in the present study the majority of *Euphorbia*-derived compounds were more cytotoxic to drug-resistant sublines of gastric, pancreatic and colon carcinomas than to their corresponding parental, drug-sensitive cell lines (23, 24). The observed differences might be explained by the use of different cancer cell lines as model systems. Resistant cancer cell lines used in this and in the previous studies were also likely to display different mechanisms of MDR.

The lathyrane diterpene, latilagascene B, was shown to increase the accumulation of rhodamine 123 as well as doxorubicin in resistant LoVo/Dx cells. Unfortunately, it did not reverse doxorubicin cytotoxicity in these cells in the studied concentration range. Previously, latilagascene B, has been identified as an effective P-gp inhibitor in drug-resistant human colon adenocarcinoma cell line (COLO 320 MDR) (25) and in mouse T lymphoma cells transfected with a human *MDR1* gene expression construct (7). Three other diterpenes were tested in the same model system. Helioscopinolide B was demonstrated to be a P-gp inhibitor (26), while, *ent*-16α,17-dihydroxyatisan-3-one and ent-16α,17-dihydroxykauran-3-one turned out to be inactive (7). Latilagascene B was also demonstrated to increase doxorubicin cytotoxicity in resistant mouse lymphoma cells (8). Additionally, it was proven to be a potent apoptosis-inducing agent in this cell line.

In conclusion, from the studied set of *Euphorbia*-derived compounds, latilagascene B was shown to be an effective P-gp inhibitor in human colon carcinoma cells. Its relatively high cytotoxicity prevented us from demonstrating its direct MDR reversal effect in this model system. The structure of this compound seems to be an interesting candidate for further synthesis of new derivatives of reduced cytotoxicity and anti-MDR potency comparable or higher to the parent compound. Additionally, encapsulation of latilagascene B and its derivatives in liposomes might be likely to reduce their intrinsic cytotoxicity and to allow for their *in vivo* application.

Acknowledgements

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