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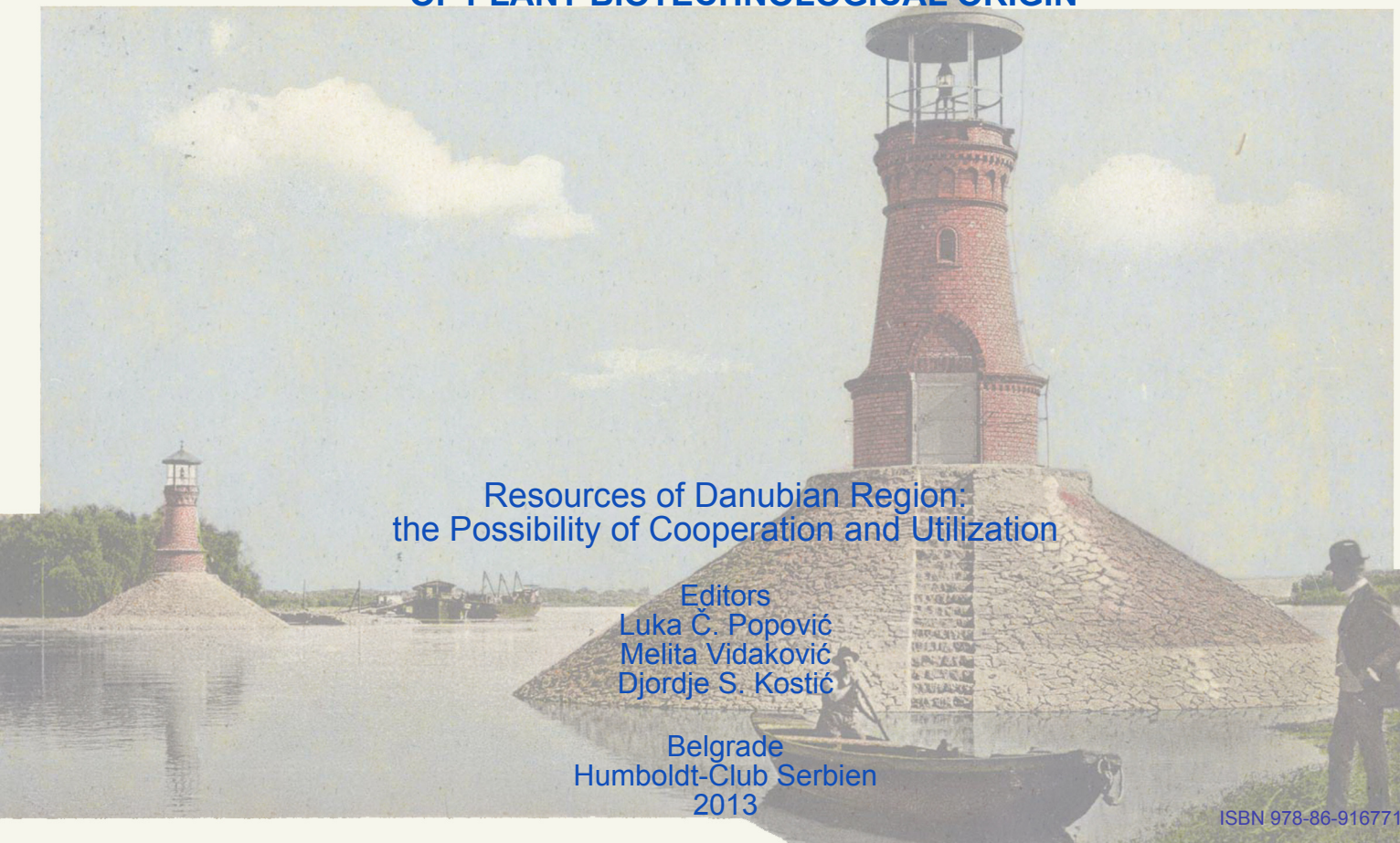
JUSTICIDIN B – A POTENTIAL ANTINEOPLASTIC DRUG OF PLANT BIOTECHNOLOGICAL ORIGIN



Resources of Danubian Region:
the Possibility of Cooperation and Utilization

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Belgrade
Humboldt-Club Serbien
2013

ISBN 978-86-916771-1-4

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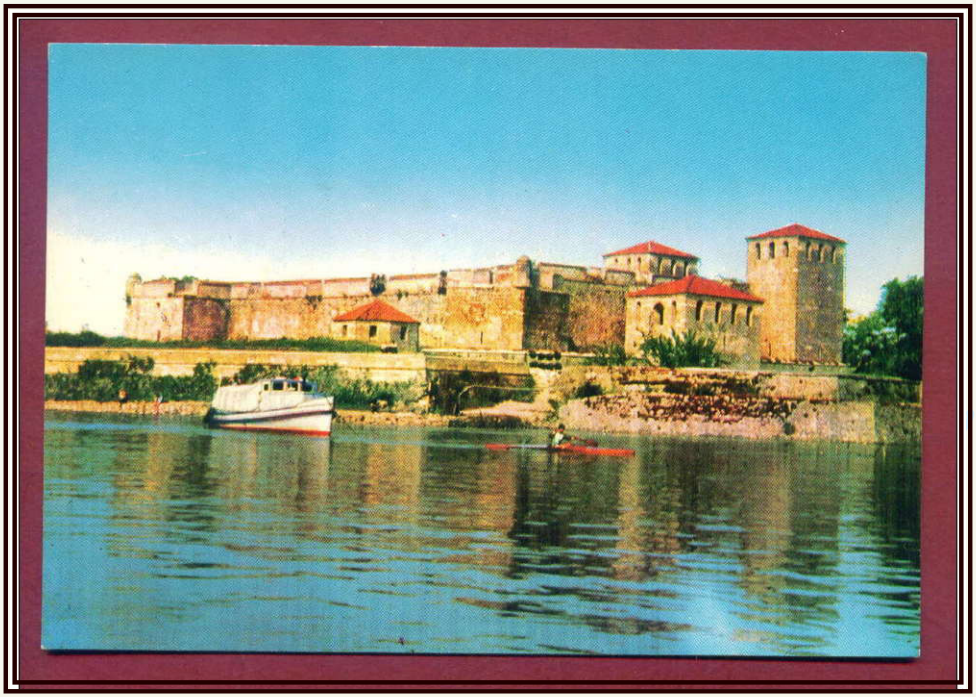

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Abstract. Justicidin B produced by genetically transformed cultures of *Linum leonii* was tested for cytotoxic activity in EOL-1, HL-60 and HL-60/Dox AML derived cell lines and for overcoming of drug resistance in HL-60/Dox.
The tested lignan evoked strong, concentration dependent cytotoxicity in all cell lines, whereby HL-60/Dox proved to be far more sensitive as compared to HL-60. Exposure of the all three cell lines with Etoposide was used as a reference for the cytotoxicity of the investigated arylnaphthalene lignan and leads to concentration dependent decrease in the cell viability although no MDR overcoming in the resistant cell line was observed.

Introduction

Justicidin A and justicidin B are closely related lignan derivatives. *Justicia procumbens* is a traditional herbal remedy which was produced in the south-western and southern provinces of China and Taiwan province used to treat fever, pain, and cancer. Justicidin A is an arylnaphthalide lignan isolated from *Justicia procumbens*.

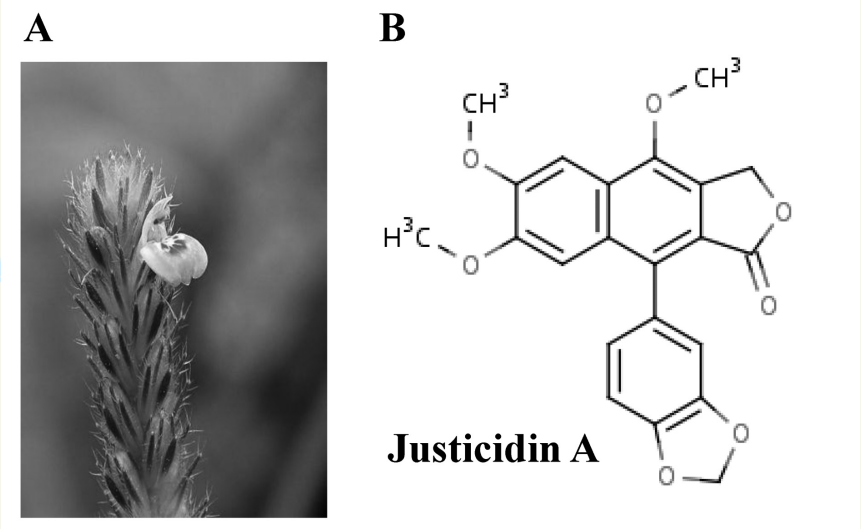

Figure 1.
Chemical structure of Justicidin A and the plant Justicia procumbens (endemic to India).

A new compound 6'-hydroxy justicidin A (JR6) from *Justicia procumbens*, which showed obvious anti-cancer effects, was identified (He, Zhang et al. 2012). A sensitive and accurate analytical method was developed and validated of for the determination of 6'-hydroxy justicidin A (HJA), a potential antitumor active component isolated from *Justicia procumbens* in rat plasma using a simple liquid-liquid extraction (LLE) method for sample preparation (Qiu, Zhou et al. 2012).

Justicidin A has been reported to suppress in vitro growth of several tumor cell lines as well as hepatoma cells. Moreover, justicidin A caused activation of caspase-8, tBid increase, disrupted mitochondrial membrane potential (Delta psi(m)), and release of cytochrome c and Smac/DIABLO in Hep 3B and Hep G2 cells. Justicidin A also reduced Bcl-x(L) and increased Bax and Bak in mitochondria. Caspase-8 inhibitor (Z-IETD) attenuated the justicidin A-induced disruption of Delta psi(m). Growth of Hep 3B implanted in NOD-SCID mice was suppressed significantly by oral justicidin A (20 mg/kg/day). These results indicate that justicidin A-induced apoptosis in these cells proceeds via caspase-8 and is followed by mitochondrial disruption (Su, Huang et al. 2006).

Justicidin A is toxic to many different types of cells, including human colorectal cancer (HT-29 and HCT 116), cervix carcinoma (SiHa), breast adenocarcinoma (MCF7), bladder carcinoma (T24), human embryonic kidney epithelial cells (HEK293) and PBMCs. Interestingly, all cancer cells tested were particularly more sensitive to justicidin A than non-tumorigenic normal cells. For example, the IC_{50} for T24 bladder carcinoma cells was 0.004 mM which is 6250-fold lower than that for PBMCs (25 mM). This result suggests that justicidin A can be used for chemotherapy for cancers. IC_{50} values for colorectal cells (HT-29 and HCT 116) were determined to be in the range between 0.11 and 0.4 mM, respectively. Justicidin A is believed to kill cancer cells by inducing apoptosis (caspase 3 and 9 activation) (Lee, Lee et al. 2005). Justicidin A was able to suppress the expression of prohibitin 1 in transitional cell bladder cancer in vitro (Wu, Wu et al. 2007).

Arylnaphthalene lignans from a Vietnamese acanthaceae, *Justicia patentiflora* were found to exert cytotoxic activity against human tumor cell lines such as KB, HCT116, and MCF-7 (Susplugas, Hung et al. 2005).

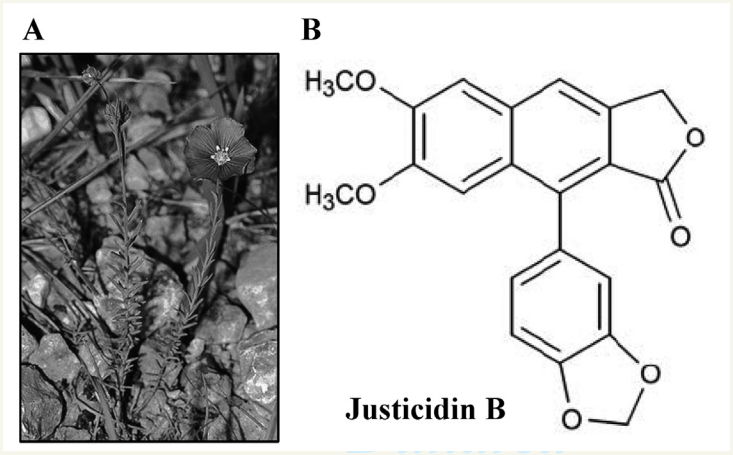

Figure 2.
Chemical structure of Justicidin B and the plant Linum leonii.

Justicidin B is another not as broadly reported pharmacologically active lignan.

Cell suspension cultures of *Linum perenne* L. Himmelszelt accumulate justicidin B as the main component together with glycosides of 7-hydroxyjusticidin B (diphyllin). A hypothetical biosynthetic pathway for these compounds was suggested. Justicidin B 7-hydroxylase (JusB7H) catalyzes the last step in the biosynthesis of diphyllin by introducing a hydroxyl group in position 7 of justicidin B. This enzyme was characterized from a microsomal fraction prepared from a *Linum perenne* suspension culture for the first time (Hemmati, Schneider et al. 2007).

Justicidin B is an arylnaphthalene lignan that exerts cytotoxic (Joseph, Gleye et al. 1988), antiviral (Asano, Chiba et al. 1996), (MacRae, Hudson et al. 1989), fungicidal, antiprotozoal (Gertsch, Tobler et al. 2003), and antiplatelet properties (Chen, Hsin et al. 1996). The potent bone resorption inhibitor justicidin B was used as a lead compound for the design of new antirheumatic drugs (Baba, Kawamura et al. 1996). Several tumor types including sarcomas and breast, prostate, and lung carcinomas grow in or preferentially metastasize the skeleton where they proliferate and induce significant bone remodeling, bone destruction, and cancer pain (Sabino, Ghilardi et al. 2002). Thus, justicidin B may have significant clinical utility as a lead compound in the management of bone cancer and osteoclastogenesis, due to its cytotoxic and bone resorption inhibitory properties. Justicidin B was first isolated from Justicia spp. (Acanthaceae) and Haplophyllum spp. (Rutaceae) (Mohagheghzadeh, Schmidt et al. 2002), (Okigawa, Maeda et al. 1970). Justicidin B has further been isolated from different *Phyllanthus* species (Euphorbiaceae) (Pettit and Schaufelberger 1988), (Bachmann 1993). It was shown that cell cultures of *Linum austriacum* produce justicidin B, which was the first report on the occurrence of arylnaphthalene lignans in a species of the Linaceae (Mohagheghzadeh, Schmidt et al. 2002).

Callus and hairy root cultures of *Linum leonii* accumulate the arylnaphthalene lignan justicidin B as a major constituent. Hairy roots produce 5-fold higher yields of justicidin B. Justicidine B was found to induce apoptosis in tumor cells (Vasilev, Elfahmi et al. 2006).

Justicidin B produced by genetically transformed cultures of Linum leonii evoked strong, concentration dependent cytotoxicity in two breast cancer cell lines, whereby the estrogen receptor positive MCF-7 proved to be far more sensitive as compared to triple negative MDA-MB-231 cell line. The 24 h treatment increased the level of apoptotic DNA fragmentation; however the proapoptotic activity is completely inhibited if the cells are co-incubated with the non-selective pan-caspase inhibitor Boc-Asp(OMe)-fluoromethyl ketone (PCI), which implies that justicidin B, activates programmed cell death via caspase –dependent mechanisms. Exposure of MDA-MB-231 cells to justicidin B leads to concentration dependent decrease in the expression of NFkB; whereas the treatment of MCF-7 is consistent with strong increase in the expression of this transcription factor (Momekov, Konstantinov et al. 2011). The pharmacological properties and the plausibility for effective, high-yield production of justicidin B from genetically transformed cultures of *L. leonii* warrant for further more detailed investigation of this agent as a potential compound for development of new antineoplastic drugs.

Acute myeloid leukemia is one of most common leukemia by adults. It is a clonal heterogeneous disease of the hematopoietic progenitor and stem cells characterized by an irrepressible proliferation, differentiation arrest and lack of self-regeneration (Appelbaum, Rowe et al. 2001), (Appelbaum, Gundacker et al. 2006). The disease progress leads to accumulation of premature myeloid precursor

cells in the bone marrow and a cumulative replacement of the normal myelocytes, megakaryocytes and erythrocytes. This process interferes with the normal bone marrow functions and is a reason for complications as hemorrhages, anemia and infections (McKenzie 2005).

Materials and methods

Drugs and compounds. The stock solution of justicidin B was prepared in DMSO and stored at 4°C. Justicidine B was dissolved in DSMO. Etoposide was supplied at a quality for clinical applications from the respective manufacturer and stored at 4°C.

Cell line and culture conditions. The cytotoxic activity of justicidin B was tested on a panel of acute myeloid leukemia cell lines. The cell lines EOL-1 (ACC-386) and HL-60 (ACC-3) were purchased from the German Collection of Microorganisms and Cell Cultures (Leibnitz-Institut-DSMZ GmbH, Braunschweig, Germany). The MRP1 expressing resistant cell line HL-60/Dox was cultured in the presence of the anthracycline doxorubicin (200 nM). Cells were maintained in RPMI-1640 medium (Invitrogen, USA) containing 10 % fetal calf serum (Biochrom, Germany) and 2 mM L-Glutamine (Invitrogen, USA) and passaged not more than ten times during the performance of the experiments. Cells were grown as suspension cultures (37°C, humidified atmosphere, 5% CO_2), and kept in log phase before treating with the appropriate substances and drugs.

Biotechnological production of justicidin B. The seeds of *L. leonii* were a kind gift by the botanical garden Nancy (France). Hairy roots were induced by direct incubation of segments from sterile grown plants with *Agrobacterium rhizogenes* strain ATCC 15834 cultured in YMB medium in the presence of 20μM acetosyringone for 2 days in the dark, which increased susceptibility toward infection. The fast growing hairy rots were further maintained under permanent dark on a rotary shaker as described earlier (Vasilev, Elfahmi et al. 2006). Air-dried plant material from hairy roots (20g) was extracted with 80% methanol (200ml for 1 h sonification at 25° C). The extracted was separated with 3 x 200 ml dichloromethane. Dichloromethane layers were filtered (Na_2SO_4 was used as a drying agent), combined, concentrated under reduced pressure at 50° C, dried and kept at -20° C. The initial amount of hairy roots yielded 780 mg dry dichloromethane extract. The dichloromethane extract was subjected to preparative TLC separation using Silica gel 60 F_{254} (Merck):10x20 cm, 2 mm, toluene: acetone 10:1, development length: 9 cm and λ = 254 nm. The most abundant fraction (R_f = 0.45) was pooled and evaporated to dryness consequently. The residue was further purified by re-crystallization in cold methanol to yield 7.06 mg justicidin B. The main component in hairy roots of *L. leonii* was isolated by preparative TLC and consequent re-crystallization in cold methanol. This isolate was analyzed by means of GC-MS and NMR. The EI-MS of the isolated compound showed an ion at m/z 364 and mass fragmentation, which is consistent with the data for an arylnaphtalene lignan (Momekov, Konstantinov et al. 2011). Further NMR experiments were performed in order to distinguish between justicidin B and isojusticidin B as these two isomers have slightly different MS fragmentation pattern. A closer look at the ^{1}H NMR spectrum showed that the proton signals at δ 7.12 ppm and δ 7.05 ppm appeared as singlets which is indicative only for 4,5-dimethoxy substitution. Therefore the resonance signals at δ 7.12 ppm and δ 7.05 ppm were assigned to H-6 and H-3 respectively, dye to the shielding effect of the piperonyl group from

the pendant ring. Thus the isolated compound was unambiguously identified as justicidin B (Vasilev, Elfahmi et al. 2006), (Momekov, Konstantinov et al. 2011).

Cell survival test. The cell survival fraction was estimated with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye reduction assay as described by Mosmann (Mosmann 1983) with some modifications. Cells were seeded at 10^3 cells/well in 96-well plates and incubated for 24, 48 h or 96 h with ten different concentrations of justicidine B (upm to 200 μM) or etoposide. Briefly after incubation with the appropriate drug or combination, MTT-solution (10 mg/ml in PBS) was added (10 μl/well). Plates were further incubated for 4 h at 37°C and the formazan crystals formed were dissolved by adding 110 μl/well of 0,04 N HCl in 2-propanol.Absorbtion was measured by an enzyme-lynked immunosorbant (ELISA) reader (Labexim, Austria) at 540 nm, reference filter 690 nm. For each concentration at least 8 wells were used. 100 μl RPMI-1640 medium with 10 μl MTT stock-solution and 110 μl 0.04 N HCl in 2-propanol was used as blank solution. Data obtained were presented as percentage of untreated control (untreated control = 100) as shown below.

$$\% \text{ of control} = \frac{E_{\text{(extinction of viable cells in treated samples)}}}{E_{0\text{ (extinction of viable cells in untreated control)}}} \; x\,100$$

Statistical analysis. Experiments were performed in triplicate and results are expressed as means with corresponding standard deviations. The significance of differences in the data was analyzed using Student's *t*-test ($^*P<0.05$).

Results

Cytotoxic activity of justicidin B in comparison with classical cytostatics. The cytotoxic activity of justicidin B was determined on three acute myeloid leukemia cell lines. IC_{50} values were calculated after 24, 48 and 74h incubation of the appropriate cell line with 10 different concentrations of the compound ranging from 0.39 μM up to 200 μM. The cytotoxic activity of justicidin B was shown to be time and concentration dependant as evidenced by the IC_{50} values, presented in Table 1. The most sensitive cell line toward justicidin B was the myeloid cell line EOL-1, where IC_{50} was 15.52 μM after 48h incubation with the compound. The cell lines HL-60 and HL-60/Dox were less sensitive toward justicidin B. IC_{50} for HL-60 cells was calculated - 101.6 μM and for HL-60/Dox – 53.59 μM after 48h incubation. After 72h incubation the lowest levels of extinction were measured and the corresponding IC_{50} values were also lower as compared to the values calculated after 48 h incubation – 8.18 μM for EOL-1, 100.3 μM for HL-60 and 25.8 μM for HL-60/Dox. Interestingly, the MRP1 expressing cell line HL-60/Dox was more sensitive to justicidin B than HL-60 cells. The classical cytostatic etoposide was used as a referent drug for the activity of justicidin B. Etoposide showed higher activity in HL-60 cells (IC_{50} = 2.45 μM after 48h and IC_{50} = 0.77 μM after 72h incubation) but was less potent in the resistant cell line HL-60/Dox as compared to justicidin B after the longest incubation period of 72h (IC_{50} = 41.97 μM).

		AML-derived cell lines		
Compounds	Incubation period	EOL-1	HL-60	HL-60/Dox
Justicidin B	24h		192.0	135.0 (118.1 – 154.3)
	48h	15.52 (14.54 – 16.57)	101.6 (76.8 – 134.3)	53.59 (47.85 – 60.02)
	72h	8.18 (7.66 – 8.74)	100.3 (76.13 – 132.1)	25.8 (24.11 – 27.62)
Etoposide	24h		9.87 (7.59 – 12.83)	104.4 (99.92 – 109.0)
	48h		2.45 (1.81 – 3.32)	44.16 (41.76 – 46.71)
	72h		0.77 (0.67 – 0.88)	41.97 (40.27 – 43.75)

Table 1. Citotoxicity and IC50 values (μM) with 95% confidence intervals, determined by MTT dye reduction assay. Three different AML-derived cell lines were used – EOL-1, HL-60 and HL-60/Dox. The citotoxic activity of Justicidin B and Etoposide was measured after 24, 48 and 72h exposure.

Discussion and conclusion

Justicidin B is an arylnaphtalene lignan, a representative of the plant-derived biologically active compounds with antineoplastic activity. It is considered as a perspective lead compound due to its cytotoxicity, activation of cell death signaling pathways, immune and inflammatory response modulation.

A detailed pharmacological investigation of its cytotoxicity in AML derived cell lines is performed and described in this paper. Furthermore a comparison to the cytotoxic activity of etoposide in these two cell lines (HL-60 and the resistant HL-60/Dox) is done. The referent substance etoposide is an already known anticancer drug. It is a synthetic topoisomerase inhibitor closely related to the plant derived podophyllotoxin.

The results from the MTT-dye reduction assay following 24, 48 and 72h exposure proved a dose and time-dependent cytotoxic activity of both compounds. EOL-1 cells appear with highest sensitivity to the citotoxicity of the arylnaphtalene lignan. HL-60 and their derivative HL-60/Dox cells show significant but lower sensitivity. The obtained results point out justicidin B as a potent antineoplastic compound, which is in line with already published experimental data (Vasilev, Elfahmi et al. 2006), (Momekov, Konstantinov et al. 2011).

MDR1 (Multidrug resistance protein) also known as P-glycoprotein 1 (permeability glycoprotein, P-gp or Pgp) or ATP-binding cassette sub-family B member 1 (ABCB1) or cluster of differentiation 243 (CD243) is a glycoprotein that in humans is encoded by the *ABCB1* gene. P-gp is a member of the superfamily of ATP-binding cassette (ABC) transporters (MDR/TAP subfamily). ABC proteins transport various molecules across extra- and intra-cellular membranes. As a member of the MDR/TAP subfamily Pgp is involved in multidrug resistance. This protein is an ATP-dependent drug efflux pump for xenobiotic compounds with broad substrate specificity. It is responsible for decreased drug accumulation in multidrug-resistant cells and often mediates the development of resistance to anticancer compounds (Santos and Paulo 2013), (O'Connor 2007). Its close congener called multidrug resistance related protein 1 (MRP-1) is also responsible for clinically relevant drug resistance.

HL-60/Dox is a MRP-1 expressing cell line. As it is already known HL-60/Dox is less sensitive to the antineoplastic therapy than the normal HL-60 one. This claim is supported by the results, obtained after the application of etoposide. But the application of justicidin B in these resistant cells showed much higher activity of the compound than the activity of etoposide (collateral sensitivity). HL-60 cells are more than 50 fold more sensitive to etoposide than HL-60/Dox, while HL-60/Dox have 1.5 fold (for 24h exposure), 2 fold (for 48h exposure) and 4 fold (for 72h exposure) lower IC_{50} values than the reference. This data leads to an assumption that justicidin B is a possible mean for overcoming multidrug resistance. It could be supposed that the lignan acts via inhibition of the MRP-1 expression. This rare and interesting find nevertheless needs further clarification.

Taken together the presented pharmacological properties of justicidin B and the results of the performed research indicate that the arylnaphtalene lignan biotechnologically produced by hairy root cultures of *Linum leonii* possesses definite and potent anticancer activity. The lignan has effects on the drug resistance, observed in HL-60/Dox cells. The possibility for overcoming multidrug resistance is related with further detailed and profound investigation of the mechanism of action of this plant derived molecule.

Financial support from Ministry of Education and Science, Sofia, Bulgaria (grant DFNI-B01/3-2012) is acknowledged.

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