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Effect of justicidin B – a potent cytotoxic and pro-apoptotic arylnaphtalene lignan on human breast cancer-derived cell lines

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Justicidin B produced by genetically transformed cultures of *Linum leonii* was tested for cytotoxic activity and induction of apoptosis in MDA-MB-231 and MCF-7 breast cancer derived cell lines. The tested lignan evoked strong, concentration dependent cytotoxicity in both cell lines, whereby MCF-7 proved to be far more sensitive as compared to MDA-MB-231. The 24 h treatment of both cell lines 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 with 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.

Key words: Justicidin B, cytotoxicity, apoptosis, NFkB, MCF-7, MDA-MB-231

A number of plant-derived compounds with diverse chemical structures have played significant roles in the development of several potent anticancer drugs. The main deficiency of these compounds is their cytotoxicity for normal cells and hence side effects derived from their lack of selectivity against tumoral cells. In this regard it is necessary to investigate and prepare new more potent and less toxic analogs, with better therapeutic indices. Lignans, defined as 3,3‘-dimers of phenylpropane (C6C3) units, comprise a large group of secondary metabolites in plants [1]. This important class of natural products has drawn much attention since the discovery of the aryltetralin lignan podophyllotoxin, the establishment of its potent cytotoxic effects and the eventual successful commercialization of its semisynthetic derivatives, namely etoposide, teniposide and the pro-drug etopophos [2-4]. The latter drugs comprise an important class of antineoplastic agents used for the treatment of diverse malignancies, such as Hodgkin's disease, small cell lung cancer, testicular cancer *etc* [4-8].

The industrial semisynthetic production of the epipodophyllotoxin anticancer drugs relies solely on podophyllotoxin as starting material. For this purpose the compound is obtained from the rhizomes of *Podophyllum peltatum* and *Podophyllum hexandrum* (Berberidaceae), which, however are becoming in limited amounts only because of extensive exploitation and lack of adequate cultivation. Thus the continued supply of podophyllotoxin is not compatible with the conservation of the wild *Podophyllum* plants. On this basis, much effort has been focused upon the search for alternative sources of this rare natural lignan, including identification of alternative plant sources, as well as elaboration of biotechnological approaches for biosynthesis of podophyllotoxin [8-10]. Screening for rapid growth and high lignan yield showed that *Linum* species belonging to the Syllinum section are promising for exploitation in vitro [11-15].

The limited yeild plant-specific secondary products were long considered as a major limitation for an extensive use of plant-made pharmaceuticals in human therapy. To extend the research to human clinical studies, we needed to find a reliable supply of plant material, produced target compounds. In our laboratory, we focus on the production of some important pharmaceuticals in plant cell cultures and have successfully established cell cultures for production of anticancer agents [13,16-18]

While the most intensive search for biotechnological production of lignans is focused on podophyllotoxin and related aryltetralin lignans, suitable for the semisynthetic production of etoposide and teniposide, the intensive investigation of other lignan compounds has revealed some attractive lead compounds with potent cytotoxic and other biological activities. Important examples are the arylnaphtalene lignans justicidin A-E,

originally identified in *Justicia procumbens*. The justicidins are endowed with cytotoxic [19-21], anti-bone-resorptive, antiinflammatory and antirheumatic activities as well as antimicrobial and antiparasitic effects [1,22,23]. Our previous study has revealed that genetically transformed cultures form *Linum leonii* represent a feasible source for the biotechnological production of justicidin B (Figure 1) with high yield. This lignan has shown potent cytotoxicity against human leukemic cell lines and has been proven to induce apoptotic cell death [15].

This study is aimed at investigating the cytotoxic effects and the underlying mechanistic aspects in two human breast cancer derived cell lines – MCF-7 characterized by estrogen receptor (ER) expression and the ER-negative MDA-MB-231.

Materials and methods

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 20 μM acetosyringone for 2 days in the dark, which increased susceptibility toward infection. The fast growing hairy roots were further maintained under permanent dark on a rotary shaker as described earlier [15]. Air-dried plant material from hairy roots (20 g) was extracted with 80% methanol (200 mL for 1 h sonification at 25°C). The extract 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): 10 x 20 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. 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, due to the shielding effect of the piperonyl group from the pendant ring. Thus the isolated compound was unambiguously identified as justicidin B. The ^{1}H NMR data is in full agreement with a previous report of justicidin B [15].

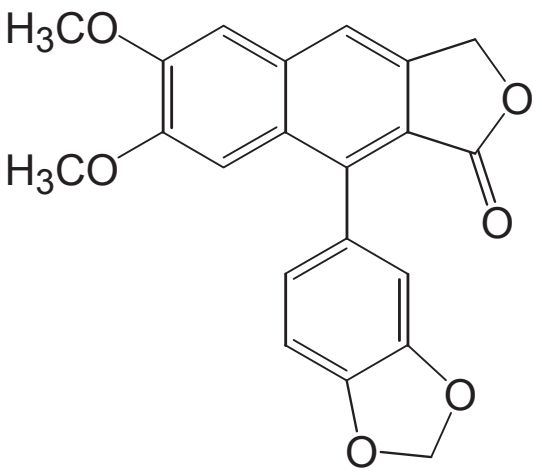

Figure 1. Chemical structure of justicidin B.

Cell lines and culture conditions. The cell lines MDA-MB-231 (ER-negative breast carcinoma) and MCF-7 (ER-positive breast adenocarcinoma) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany). The cells were grown in controlled environment – cell culture flasks at 37°C in an incubator 'BB 16-Function Line' Heraeus (Kendro, Hanau, Germany) with humidified atmosphere and 5% CO_2. Cells were kept in *log* phase by supplementation with fresh medium, two or three times a week. MCF-7 cells were grown as monolayer adherent cultures in 90% RPMI-1640 supplemented with 10% FBS, non-essential amino acids, 1 mM sodium pyruvate and 10 μg/ml human insulin. MDA-MB-231 were maintained in 90% RPMI-1640 + 10% FBS. Both cell lines were grown as monolayer cultures and were reset by trypsinization two times per week.

Cytotoxicity assessment (MTT-dye reduction assay). The cell viability was assessed using the standard MTT-dye reduction assay as described by Mosmann [24] with minor modifications [25]. The method is based on the biotransformation of the yellow tetrazolium dye MTT to a violet formazan product *via* the mitochondrial succinate dehydrogenase in viable cells. In brief, exponentially growing cells were seeded in 96-well flat-bottomed microplates (100 μl/well) at a density of 1×10^5 cells per ml and after 24h incubation at 37°C they were exposed to various concentrations of the tested compounds for 72h. For each concentration a set of at least 8 wells were used. After the exposure period 10μl MTT solution (10mg/ml in PBS) aliquots were added to each well. Thereafter the microplates were incubated for 4h at 37°C and the MTT-formazan crystals formed were dissolved through addition of 100 μl/well 5% formic acid solution in 2-propanol. The absorption was measured using a LabeximLMR-1 microplate reader at 580 nm. Cell survival fractions were calculated as percentage of the untreated control. In addition, IC_{50} values were derived from the concentration-response curves.

DNA fragmentation analysis. The characteristic for apoptosis oligonucleosomal DNA fragmentation was exam-

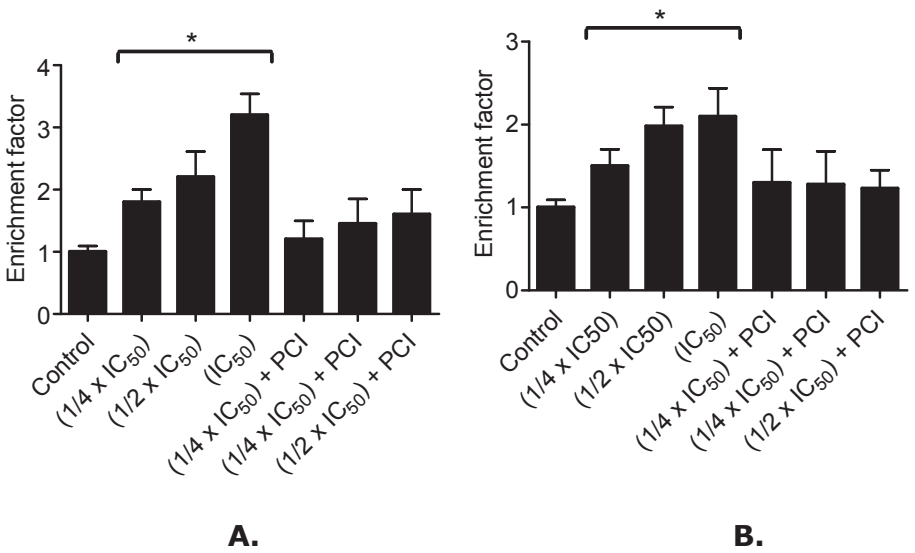

Figure 2. Proapoptotic activity of Justicidin B after 24 h treatment of MCF-7 (A.) and MDA-MB-231 (B.) cells at equitoxic concentrations, applied alone or in combination with 100 μmol/l pancaspase inhibitor (PCI). The enrichment factor is proportional to the cellular levels of histone associated mono- and oligonucleosomal DNA fragments. Each columns is representative for the arithmetic mean (± sd) of four independent experiments.

ined using a commercially available 'Cell-death detection' ELISA kit (Roche Applied Science). This method allows semi-quantitative determination of the characteristic for the apoptotic process histone-associated mono- and oligonucleosomal DNA-fragments using 'sandwitch' ELISA. Exponentially growing cells were exposed to varying concentrations of the tested compounds and thereafter cytosolic fractions of 1×10^4 cells per group (treated or untreated) served as antigen source in a sandwich ELISA, utilizing primary anti-histone antibody-coated microplate and a secondary peroxidase-conjugated anti-DNA antibody. The photometric immunoassay for histone-associated DNA fragments was executed according to the manufacturers' instructions at 405 nm, using ELISA reader (Labexim LMR-1). The results are expressed as the oligonucleosome enrichment factor (representing a ratio between the absorption in the treated vs. the untreated control samples).

Western blot. Non-treated controls of both cell lines parallel with samples treated with different concentrations of justicidin B were frozen at the 6th and 24th hour after treatment. The cell pellets were then lysed and the total protein concentration in each sample was measured by BCA Protein Assay (Pierce), according to the manufacturer's protocol. Volumes of each sample, containing 50 μg protein were loaded on a gradient 4-12% polyacrylamide gel Lonza and the electrophoresis was carried out at 120 V for 2 hours. The proteins were then transferred to a PVDF membrane which was thereafter blocked for 1 hour in PBS (phosphate buffered saline) with 5% skimmed milk. The membrane was next incubated for 2,5 hours with a primary antibody against NFkB (sc-372 – rabbit polyclonal) purchased from Santa Cruz Biotechnology in PBS with 0,5% skimmed milk and 0,1% Triton X. The primary antibodies were diluted 400 times. After the first incubation the membrane was washed 4 times for 5 minutes with 0,1 % Triton X in PBS. The membranes were then incubated with secondary anti-rabbit (Santa Cruz Biotechnology- goat anti-rabbit antibody – sc-2004) horseradish peroxidase-conjugated IgG secondary antibody for 45 minutes. The membrane was washed again 4 times for 5 minutes and then visualized using Western Blotting Luminol Reagent (sc-2048, Santa Cruz Biotechnology).

Data processing and statistics. The cytotoxicity assays were carried out in eight separate experiments, whereas the apoptosis induction evaluation was conducted in quadruplicate. The MTT data were normalized as percentage of the untreated control (set as 100%) and fitted to sigmoidal concentration–response curves and the corresponding IC_{50} values were calculated using non-linear regression analysis (GraphPad Prizm software package). Statistical processing exploited Student's t-test with $p\leq0.05$ set as significance level.

Results

Justicidin B was tested for cytotoxic activity in both breast cancer derived cell lines after 72 h continuous exposure, by means of the MTT-dye reduction assay for cell viability. The data were fitted to sigmoidal dose-response curves (not shown) and the corresponding IC_{50} values were calculated using non linear regression (curve fit). The tested lignan evoked strong, concentration dependent cytotoxicity in both cell lines, whereby MCF-7 (IC_{50} 38.7 μM) proved to be far more sensitive as compared to MDA-MB-231 (IC_{50} 106.9 μM). The IC_{50} values of the reference cytotoxic agent etoposide were as follows 57.2 μM in MCF-7 and 63.1 μM in MDA-MB-231.

The results from the proapoptotic activity determination are depicted in Fig 2. The 24 h exposure of both cell lines is consist-

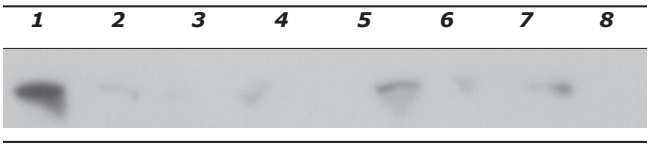

Fig 3. Effects of Justicidin B and etoposide on NFkB expression in MDA-MB-231 cells. (1) Untreated control; (2) Justicidin B – ½IC_{50} (6 h); (3) Justicidin B –IC_{50} (6 h); (4) Etoposide – IC_{50} (6 h); (5) Justicidin B - ½IC_{50} (24 h); (6) Justicidin B IC_{50} (24 h); (7) Etoposide – ½ IC_{50} (24 h); (8) Etoposide – IC_{50} (24 h)

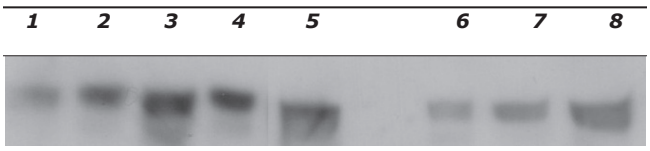

Figure 4. Effects of justicidin B and etoposide on NFkB expression in MCF-7 cells. (1) Untreated control; (2) Justicidin B - ¼IC_{50} (6 h); (3) Justicidin B - ½ IC_{50} (6 h); (4) Justicidin B - IC_{50} (6 h); (5) Etoposide IC_{50} (6 h); (6) Justicidin B - ¼IC_{50} (24 h); (7) Justicidin B - ½ IC_{50} (24 h); (8) Justicidin B - IC_{50} (24 h).

ent with statistically significant increase in the level of DNA fragmentation (expressed as enrichment factor, in arbitrary units). These findings unambiguously indicate that the induction of cell death through apoptosis plays pivotal role for the cytotoxic mode of action of the tested lignan. The ER-positive cell line MCF-7 proved to be more responsive in this respect, which well correlates to the established higher chemosensitivity of this cell line in comparison to MDA-MB-231.

As the induction of apoptosis is associated with cascade activation of the caspase enzymes, which represent a family of specific proteases, we aimed at investigating the level of DNA-fragmentation following co-incubation of the cell lines with the non-selective pan-caspase inhibitor Boc-Asp(OMe)-fluoromethyl ketone (PCI). As evident from the data presented in figure 2 the concomitant treatment of cells with the lignan and the caspase inhibitor is consistent with total abrogation of the proapoptotic activity of justicidin B, which implies that the latter activates programmed cell death via caspase –dependent mechanisms.

To more precisely evaluate the cellular events following exposure to justicidin b we investigated the levels of expression of NFkB. The treatment of MDA-MB-231 cells with justicidin B leads to a concentration dependent-decrease in the expression of NFkB transcription factor; the referent drug etoposide similarly leads to down regulation of the target protein as well (Fig 3). In a dissimilar fashion the exposure of the ER-positive cell line (MCF-7), is consistent with strong increase in the NFkB expression, detected after both short and longer treatment periods (Fig 4).

Discussion

As a class of plant-derived biologically active compounds the lignans are considered as perspective lead compounds for development of antineoplastic drugs in line with their complex pharmacological properties including cytotoxicity, estrogen receptor binding and modulation, activation of cell death signaling pathways, immune and inflammatory response modulations etc.

The presented paper deals with a detailed pharmacological evaluation of the arylnaphtalene lignan justicidin B, in breast cancer-derived cell lines -MCF-7 and MDA-MB-231.

The result from the MTT-dye reduction assay following 72 h continuous exposure clearly point out that the estrogen receptor expression largely influences the responsiveness of breast cancer cells to the lignan compound. The ER-positive MCF-7 proved to be far more sensitive as compared to MDA-MB-231, which could be ascribed to an estrogen-receptor modulation by justicidin B. Lignans including those abundant in Linum species have been well established as phytoestrogens and estrogen receptor modulators [26-28], and most probably the established alternative cellular events at the signal transduction level in MCF-7 vs. MDA-MB-231 could be attributed to the estrogen receptor expression in the former cell lines. These discrepancies between the two cell lines nevertheless need further clarification.

Induction of cell death through apoptosis in sub-cytotoxic concentrations has been well established as a possible explanation of the selective antineoplastic cytotoxicity of anticancer drugs. For this reason we sought to determine whether justicidin B activates the apoptotic cell signaling pathways after 24 h at equitoxic concentrations. To meet this objective we tested the ability of justicidin B to trigger histone-associated fragmentation of genomic DNA, which is due to the action of specific DNA endonucleases, activated during the apoptotic process and is considered as a key hallmark feature of programmed cell death. The established pro-apoptotic effects are in correspondence with our previous findings for induction of apoptosis by justicidin B in leukemic cell lines [15]; the structurally related justicidin A has been also described to trigger apoptotic cell death in malignant cells [29,30].

NFkB is a transcription factor which is implicated in some of the key elements of tumor cell biology including increased proliferation, angiogenesis and resistance to apoptosis [31-33]. The established down-regulation in MDA-MB-231 cells after justicidin B exposure correlates well with our previously described findings for NFkB inhibition after treatment with a podophyllotoxin-related lignan 4'-demethyl-6-methoxypodophyllotoxin in HeLa cells [34]. Nevertheless the MCF-7 cells were found to be more susceptible to the pro-apoptotic effects of the lignan although it failed to down regulate NFkB in this particular cell line. These findings need further clarification but could be ascribed to possible modulation of ER-dependent cell signaling in MCF-7 by justicidn B.

Finally, the presented results have indicated that justicidin B, an arylnaphtalene lignan biotechnologically produced by hairy root cultures of *Linum leonii* proved a potent cytotoxic

and proapoptotic agent against the breast cancer derived cell lines MDA-MB-231 and MCF-7. The lignan has distinct effects on the expression of the transcription factor NFkB in the two cell lines. These findings well correlate to the previously described prominent antileukemic and proapoptotic activities of Justicidin B in SKW-3, K-562 and LAMA-84 cells. Taken together, the presented 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.

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