Linum narbonense: A new valuable tool for biotechnological production of a potent anticancer lignan Justicidine B

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ABSTRACT

Background: Arylnaphthalene lignan Justicidin B is a lead compound in the management of bone cancer and osteoclastogenesis. The compound is the main cytotoxic principle of rare medicinal plant *Linum narbonense* L. (Linaceae). However, there have been no reports on the bioreactor production of justicidin B. **Objective:** to develop cost-effective biotechnology for production of this anticancer metabolite. **Materials and Methods:** The genetic transformation in hairy roots induced by *Agrobacterium rhizogenes* strain ATCC 15834, was proven by PCR analysis. The control of bioreactor was synthesized by gradient method. The optimal values of the controlling parameters were estimated with presence of technological limitation. The general structure of control system was based on "Hardware in the Loop" (HIL). **Results:** Hairy roots produced five-fold higher yields of justicidin B (7.78mg/g DW) compared to callus. A rapidly growing root line was selected for cultivation in 2-L stirred tank bioreactor. After optimization, maximum biomass of 22.5 $g.l^{-1}$ dry wt was harvested from the bioreactor culture vessel (recording about 8 times increase over initial inoculum), with 1.42 % ± 0.12 Justicidine B, greater than contents from flasks were obtained. The extracts were tested in a panel of human tumor cell lines, using the MTT-dye reduction assay, exert inhibitory effects against malignant cells. **Conclusion:** Our findings are the first work on large cultivation of *L. narbonense* hairy roots and bioreactor production of plant anticancer agent Justicidin B. To extend the research to human clinical studies, we have found a reliable biotechnological supply of plant material, produced this target compound.

Keywords: Cytotoxic lignans, justicidin B, *Linum narbonense*, stirred tank bioreactor, hairy roots

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INTRODUCTION

Lignans are a large group of natural products, which show diverse biological effects. Lignans may serve as lead compounds for the development of new therapeutic agents with cytotoxic, antiangiogenic, antiviral, antileishmanial, antifungal, hypolipidemic, antiasthmatic and antiviral activity.[1]

Justicidin B is an arylnaphthalene lignan from *Linum* spp.[2] and previously known from *Justicia* spp. (Acanthaceae) and *Haplophyllum* spp. (Rutaceae). Since the structure of justicidin B is closely related to podophyllotoxin, it was hypothesized that justicidin B may have similar cytotoxic effects as those reported for podophyllotoxin. Justicidin B was established to be the cytotoxic principle of *Justicia pectoralis*[3] and demonstrated to have strong antiviral activity.[4] The fungicidal and antiprotozoal effects of *Phyllanthus piscatorum* were attributed to justicidin B.[5] It was also reported that justicidin B significantly inhibits platelet aggregation.[6] The potent bone resorption inhibitor justicidin B was used as lead compound for new antirheumatic drugs.[7] Several tumor types including sarcomas and breast, prostate, and lung carcinomas grow in or preferentially metastasize to the skeleton where they proliferate, and induce significant bone remodeling, bone destruction, and cancer pain.[8] 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. Since there is a growing interest in justicidin B due to its various pharmacological effects, the sustainable biotechnological supply of this valuable lignan would be a

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feasible alternative. The 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 anticancer agents in plant cell cultures.

Our preliminary experiments exhibited that justicidin B is the main cytotoxic principle in the methanolic extract of *Linum narbonense* L. (Linaceae).[9] "Hairy root," an infectious plant disease caused by the soil bacteria Agrobacterium rhizogenes - a natural genetic engineer, is at the core of a promising new technique that could one day lead to "biofactories" that produce medicines derived from rare plants in huge quantities at a low cost.[10] Therefore, we decided to establish hairy roots from this species in hope of produce justicidin B in high yields.

The objective of this study is to develop cost-effective laboratory bio-technology for production of this anticancert metabolite. So we are paying attention not only to fundamental scientific tasks - to determine the lignan content in the different cell cultures from *Linum narbonense* L. (Linaceae) and to examine the cytotoxic activity of the extracts, but also to those related to some of the technological problems associated with bioreactor production of justicidin B and their optimization.

MATERIALS AND METHODS

Plant material

The seeds of *L. narbonense* were provided by the botanical garden of Zurich (Switzerland).

Cell plant cultures

Seeds of *L. narbonense* were germinated under sterile conditions on hormone free MS-medium in the dark.[11] Sterile grown seedlings were used for initiation of callus cultures. Cell suspension cultures were derived from the callus cultures by transferring 5g callus cells to 50 ml medium in a 300 ml Erlenmeyer flask. Standard medium for callus and suspension cultures was MS-medium containing kinetin 2 mg/l, 2,4-dichlorophenoxyacetic acid 0.1 mg/l and indole-acetic acid 0.2 mg/l. Callus cultures were transferred every three weeks into 100 ml fresh medium. Calli were incubated under permanent light. The suspension cultures were sub cultivated every 12 days by transferring 5 g wet cells with a perforated spoon into 50 ml fresh medium. Suspension cultures were grown in dark at 120 rev./min.

Hairy root induction and cultivation in bioreactor

Hairy roots were induced by direct incubation of segments from sterile grown plants with *Agrobacterium rhizogenes* strain ATCC 15834 cultured in YMB medium[12] 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.

A rapidly growing root line of *L. narbonense,* was selected for cultivation in stirred tank bioreactor "BIOSTAT B Plus" for 40 days. After four weeks of cultivation in Erlenmeyer flasks, the hairy roots were scaled up to a 2-L bioreactor. Upon this subculture, the harvested roots were inoculated in bioreactor after being cut to of about 1.0 cm length.

Bioreactor equipment

Fermentations were carried out in 2 l jacketed glass vessel applying cultivation. Equipment of the vessel includes: sensors for temperature, pH, dissolved oxygen concentration, foam, and speed of stirrer drive system; four integrated peristaltic pumps and one external pump for flow control and feeding. Bioreactor conditions: temperature - 26°C, batch mode of cultivation, dissolved oxygen saturation (DO at 60%), marine-type impeller design with low-shear stress, speed 140 rpm. This impeller provides mixing and creates a higher oxygen mass transfer rate (Kla).

Control system

Control system has the following functions: display of all process values via schematic P&ID algorithms, digital calibration of sensors and pump dosing counters indication of sensor parameters, recalibration function of pH-probe, control loops for temperature, stirrer speed, pH, pO2, foam, level, substrate, pO2 with two stage cascade control, set point profile for substrate pumps.

Data acquisition

Data acquisition system includes the following functions: data collection, data base maintenance, visualization of the process variables by several plotting functions. This software allows starting or finished process batches, exporting database in appropriate data formants and sample data configuration.

Extraction and isolation of lignans

Lignans were extracted from powdered lyophilised cells (200mg) with MeOH (2ml). The mixture was further homogenized in an ultrasonic bath (2 × 30s) with intermediate cooling on ice. Distilled water (6ml) was added and the pH was adjusted to 5.0 by adding a few drops of 5% phosphoric acid. After adding b-glucosidase (1mg), the sample was incubated at 35°C for 1 h in a water bath. MeOH (12ml) was added and the mixture was incubated for another 10 min at 70°C in an ultrasonic bath. After

centrifugation for 7 min at 4500 rev./min the volume of supernatant was determined. One ml of the supernatant was taken and centrifuged at 13000 rev./min for 5 min at 25°C. This final solution was used for HPLC analysis.

Quantitative analysis

The HPLC determination was performed on a Thermo Quest (Egelsbach, Germany) equipped with a Spectra SYSTEM UV6000LP detector. The separation column was a GROM-SIL 120 ODS-5 ST (250 × 4mm, particle size 5μm) supplied with a precolumn (20 × 4mm, particle size 5μm); the gradient system was water with 0.01% phosphoric acid (85%) (A) and acetonitrile (B) as follows: 0 to 17 min from 40% to 67% B, from 17 to 18 min back to 40% B. The flow rate was 0.8 ml/min between 0 and 17 min, 1 ml/min between 17 and 24 min and again 0.8 ml/min after 24 min. The retention time of justicidin B is about 16.0 min, detector wavelength 290nm.

DNA analysis

DNA isolation was conducted from the dry plant material of intake plant, calli, and hairy roots according to a protocol for rapid isolation from dry and fresh samples. The isolation of DNA of *A. rhizogenes* ATCC 15834 was performed following the instructions of Qiaprep spin miniprep kit. The integration of rol A and rol C genes from *A. rhizogenes* into the plant genome, which is the genetic evidence for hairy root transformation, was proven by PCR, as described earlier.[12]

Cytotoxicity study

Cell lines and culture conditions

The antiproliferative action of the extracts was tested against panel malignant cell lines (the chronic myeloid leukemia – derived cell lines K-562 and LAMA-84, the Hodgkin lymphoma-derived HD-MY-Z and the human urinary bladder carcinoma-derived EJ cells) with etoposide as a positive control. The leukemic cells were supplied from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany), whereas the human urinary bladder carcinoma-derived cell line EJ was obtained from the American Type Culture collection (Rockville, MD, USA). The cells were maintained as suspension type culture (leukemias), semiadherent culture (HD-MY-Z) or monolayer culture (EJ) in a controlled environment: RPMI-1640 medium, supplemented with 10% fetal calf serum and 2.5 mg/ml L-glutamine in an incubator with 5% CO_2 humidified atmosphere at 37°C. The cells were kept in log-phase by trypsinization and consequent supplementation with fresh medium, 2-3 times per week.

Drug solutions, treatment and cytotoxicity determination

Stock solutions of the extracts were freshly prepared in ethanol water and were consequently diluted with RPMI-1640 medium to yield the final concentrations. Etoposide (as a commercially sterile available dosage form) was dissolved in water for injections and accordingly diluted in RPMI-1640. Cells were seeded into 96-well plates (100 μl/well at a density of 1×10^5 cells/ml) and exposed to the tested extracts or etoposide for 72h. Cell survival was determined with the MTT dye-reduction assay as described by Mosmann[13] with some modifications.[14] Briefly, after the incubation with the test-compound, MTT-solution (10mg/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 100 μl/well of 5% formic acid in 2-propanol. Absorption was measured on an ELISA spectrophotometer (Uniscan® Titertek, Helsinki, Finland) at 540nm. For each concentration at least eight wells were used. As a blank solution 100 μl RPMI 1640 medium with 10 μl MTT stock and 100 μl 5% formic acid in 2-propanol was used. Each MTT test was run in quadruplicate.

Statistical analysis

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 $P \leq 0.05$ set as significance level.

RESULTS AND DISCUSSION

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[15] 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*.

Justicidin B [Figure 1], has been shown to be a potent cytotoxic and proapoptotic agent against the breast cancer derived cell lines MDA-MB-231 and MCF-7. This is a potential lead compound for the development of lignan-based antineoplastic agents.[16] The EI-MS of the justicidin B[12] showed an ion at m/z 364 and mass fragmentation, which is consistent with the data for an arylnaphthalene lignin.[15] 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

1H NMR spectrum showed that the proton signals at δ 7.12ppm and δ 7.05ppm 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 compound can be unambiguously identified as justicidin B.

Seeds of *L. narbonense* were germinated and shoot cultures were developed from them. From single seedlings callus and subsequently suspension cultures were initiated. Calli cultures were developed as previously described.[9] A rapidly growing root line of *L. narbonense* was selected for cultivation in stirred tank bioreactor, because they have been considered to be sensitive in mechanically agitation. The genetically modified cultures demonstrated typical hairy roots phenotype: intensive branching, hormone autotrophy and lack of geotropism. The most vigorous growth was observed when the bacterial growth stopped and there was no further necessity of antibiotic. We performed analysis of the transferred DNA (T-DNA) from *Agrobacterium rhizogenes* to *L. narbonense* transformed cells in order to confirm the hairy roots transformation using the cell line genomic DNA as template in the polymerase chain reaction (PCR) assays. The T-DNA has left (T_L) and right (T_R) borders. The T_L region in plasmid T-DNA of agropine-type strain *A. rhizogenes* 15834 contains 18 open reading frames including several loci called *rol* (root loci).[11] The products encoded by *rol A* and *rol C* genes were found to have a synergistic effect on root induction and induce increased sensitivity to auxin. PCR analysis showed that hairy roots from *L. narbonense* contain *rol C* and *rol A* genes corresponding to the positive controls obtained by DNA from *A. rhizogenes* ATCC 15834. Untransformed callus served as a negative control. The second gene system necessary for T-DNA transfer consists of virulence (*vir*) genes encoded by a region outside the T-DNA. The protein coded by *vir* D_2 provides an endonuclease that initiates the transfer process by nicking T-DNA at a specific site. *Vir* D_2 was not detected in the hairy roots, thus showing that T-DNA is incorporated in the plant genome and it is not a residual bacterial contamination.

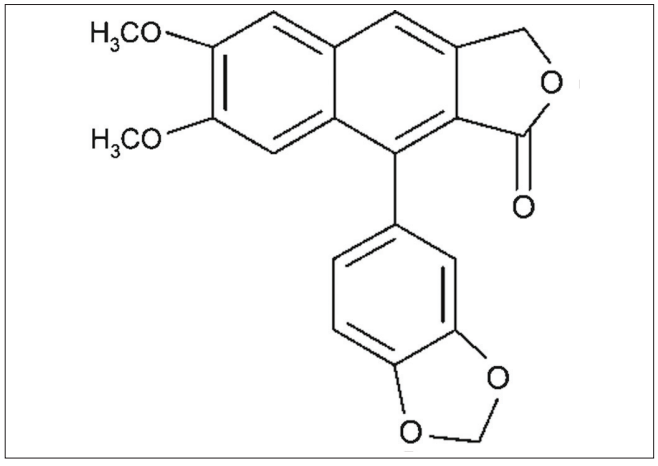

Figure 1: Structure of Justicidine B, isolated from hairy roots of L. narbonense.

The amounts of justicidin B were determined as aglycone after enzymatic hydrolysis with β-glucosidase. The presence of justicidin B was verified using HPLC. The content of justicidin B in the *L. narbonense* intact roots is about 0.5mg/g DW, in callus cultures is 1.57mg/g dwt. Suspensions of *L. narbonense* contain much smaller amounts of justicidin B, 0.09mg/g dwt [Table 1]. Hairy roots produced 5-fold higher justicidin B (7.78mg/g DW) compared to callus. To our knowledge, no reports on induction of hairy roots from *L. narbonense* and bioreactor cultivation have been published until now.

Bioreactor strategy for transformed roots of *L. narbonense*

The aim of this study was to establish a bioreactor culture of *Linum narbonense* (Linaceae) as a renewable source of potent anticancer arylnaphtalene lignan justicidin B. A rapidly growing selected root line of *L. narbonense* was grown in 2-L stirred tank bioreactor for period of forty days. In order to reach effective production we have to combine two factors – both lignan content and biomass growth. Cell growth dynamics and production of justicidine B of *Linum narbonense* cells cultivated under different conditions in shake-flasks and in a 2-L bioreactor were compared. It was recognized that a transformed roots showed a growth-associated product formation and have low oxygen demand, unlike suspension cultures where change in oxygen level can significantly affect growth and productivity. Rheological characteristics of heterogeneous system should also be taken into consideration during mass scale culturing of hairy roots. Concerning the establishment of a bioreactor system it was of importance to find an appropriate aeration level for maintaining the dissolved oxygen sufficiently high without creating a shear-intensive environment. Several control schemes for

Table 1: Content of justicidin B in cell cultures from *L. narbonense*

Culture type	Justicidin B (mg/g DW)	Variation (CV %)	
		Intra-day (n = 6)	Inter-day (n = 5)
Callus	1.57	7.4	1.8
Suspension	0.09	3.6	0.5
Hairy roots in 300 ml flasks	7.78	4.9	3.4
Hairy roots in 2 L-bioreactor	7.89	3.7	2.5

bioprocess have been investigated up to this date, a good compendium of them can be found in.[17] Our investigation addresses the study of a control approach for regulation of the cultivation process in bioreactor of *L. narbonense* hairy roots. In this study the control of bioreactor was synthesized by gradient method. The optimal values of the controlling parameters were estimated with presence of technological limitation. We have investigated the controllability and observable ability of bioreactor plant system: "BIOSTAT B Plus" and *L. narbonense* hairy roots. Controlling system achieved accuracy in dynamic regimes 8% errors, in static regimes - 2.5% errors. The general structure of control system is based of "Hardware in the Loop" (HIL) and is presented in Figure 2. Input-Output interface is based on RT-DAC4/PCI - INTECO (Intelligent Technology for Control). Control algorithm included several integrators blocks. Several simulations were carried out to test this scheme using an experimentally identified process model.

After forty days of sterile run cultivation of hairy root culture in bioreactor, 22.5g.l^{-1} dry wt of root biomass was harvested from the bioreactor culture vessel, recording about 8 times increase over initial inoculum (3.0g), with 1.42% ± 0.12 Justicidine B, greater than contents from a 300ml flasks. Our findings are the first work on large cultivation of *L. narbonense* hairy roots and bioreactor production of plant anticancer agent Justicidin B, employing bioreactors for high biomass production to meet the industrial requirement, led to the successful commercialization of plant cell bioprocesses.

Cytotoxic study

The cytotoxic potential of the *L. narbonense* hairy root extracts was investigated after 72 h continuous exposure, by means of the MTT-dye reduction assay for cell viability in a panel of human cancer cell lines, representative for some common human malignancies. Up to now the *L. narbonense* hairy root extracts were not tested for the cytotoxic potential on the human tumor cell lines, used in this study. The data were fitted to sigmoidal dose-response curves and the corresponding IC_{50} values were calculated using non-linear regression (curve fit).

The tested extracts inhibited the proliferation of the malignant cells (the chronic myeloid leukemia-derived cell lines K-562 and LAMA-84, the Hodgkin lymphoma-derived HD-MY-Z and the human urinary bladder carcinoma-derived EJ cells) in a concentration-dependent manner, which allowed the construction of dose-response curves (not shown) and the calculation of the corresponding IC50 (mg/ml) values (concentrations causing 50% decrease of cell viability), summarized in Table 2. The extracts showed a moderate cytotoxicity to all tested cell lines with IC50 in the range from 0.029 to 0.812mg/ml. The cell lines displayed differential sensitivity towards tested extracts whereby LAMA-84 cells were found to be most susceptible, K-562 and HD-MY-Z were less responsive and the urinary-bladder carcinoma EJ proved to be the most resistant cell line among the panel investigated. The cytotoxic effects may be directly linked to the content of justicidin B.

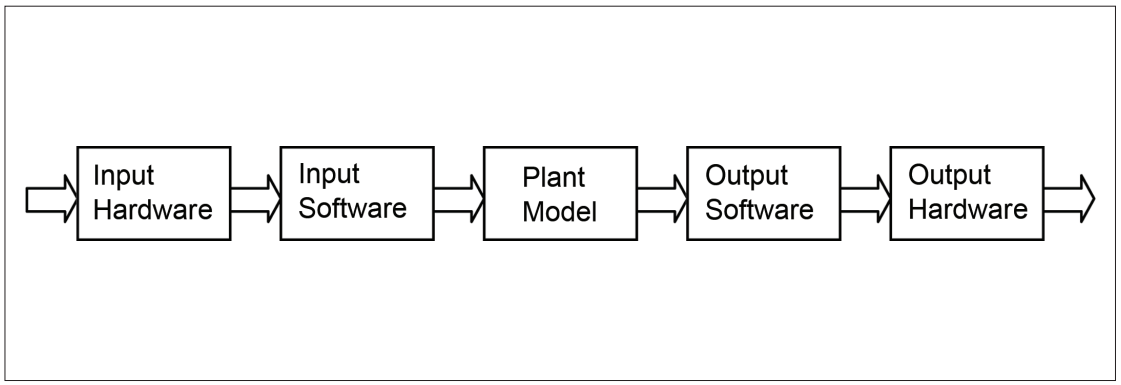

Figure 2: The general structure of control system is based of „Hardware in the Loop" (HIL)

Table 2: Cytotoxicity of the tested *L. narbonense* hairy roots extracts in a panel of human tumor cell lines after 72h exposure (MTT- assay)

Extract/Compound	IC_{50} (µg/ml)[a]			
	LAMA-84	K-562	HD-MY-Z	EJ
L. narbonense hairy roots	0.029 ± 0.05	0.597 ± 0.019	0.620 ± 0.022	0.812 ± 0.031
Etoposide[b]	0.124 ± 0.102	0.311 ± 0.092	0.247 ± 0.04	0.379 ± 0.044

[a] Data represent the mean values (± standard deviation) from four independent assays; [b]Positive control

CONCLUSION

Some of the most effective cancer treatments to date are natural products or compounds derived from plant products. Recently, the interest of international pharmaceutical industries has been directed more and more to plant based anticancer compounds. Isolation of anticancer pharmaceuticals from plants is difficult due to their extremely low concentrations. The industry currently lacks sufficient methods for producing all of the desired plant-derived pharmaceutical molecules. Some substances can only be isolated from extremely rare plants. Plant cell cultures are an attractive alternative source to whole plant for the production of high-value secondary metabolites.[18] Our results indicate that hairy root culture is a valuable alternative approach for obtaining of potential anticancer agent Justicidine B from *L. narbonense*.

Bioreactor studies represent the final step leading to commercial production of economically important phytochemicals from plant cell cultures. Although a number of economically important compounds have been produced by plant cell and tissue culture techniques, production of compounds on an industrial scale is still restricted.[18]

In conclusion, these results prove that the selected high productive hairy root clone of *L. narbonense,* inducing by *Agrobacterium rhizogenes* ATCC 15834 can be a valuable alternative approach for the production of arylnaphthalene lignan Justicidin B. This bioreactor system is a feasible alternative for the production of the cytotoxic metabolites of *L. narbonense* on a large scale. Using Input-Output interface, based on RT-DAC4/PCI - INTECO (Intelligent Technology for Control), including several integrators blocks, a relatively high lignan production can be achieved. Justicidin B has been shown to be a potent cytotoxic and proapoptotic agent against the human chronic myeloid leukemia and breast cancer derived cell lines.[19] To extend the research to human clinical studies, we have found a reliable biotechnological supply of plant material, produced this target compounds.

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