Online Monitoring of Cellular Metabolism in the MCF-7 Carcinoma Cell Line Treated with Phytoestrogen Extracts

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Abstract. *Background: Phytoestrogens are naturally occurring, plant-derived, nonsteroidal phytochemicals with anticarcinogenic potential. The aim of this study was to isolate phytoestrogens from the flax root of Linum usitatissimum and to test their effect on cellular metabolism in the human mammalian carcinoma cell line MCF-7 using the Bionas® 2500 analysis system. Materials and Methods: Metabolically relevant parameters such as acidification, oxygen consumption and cell adhesion were registered continuously over 8 and 24 hours on six sensor chips in parallel at different concentrations of flax root extracts. Results: The extracts from flax roots of L. usitatissimum reduced extracellular acidification, respiration and adhesion in a concentration-dependent manner. Conclusion: The Bionas® 2500 analysis system allows multiparametric online monitoring of cellular processes and can be used to detect the mode of action of anticarcinogenic compounds in cellular metabolism.*

Phytoestrogens are a diverse group of nonsteroidal compounds synthesized by plants. The major structural classes of phytoestrogens are the isoflavones and lignans, found in high levels in various plants such as soybeans, clover or flax. Numerous *in vitro* cell culture studies and *in vivo* animal experiments have demonstrated that phytoestrogens can inhibit tumour growth (1-4). Some studies describe the influence of flax seed on tumour growth of breast cancer cells (5). A flax seed diet significantly reduced the MCF-7 tumour size in mice (5). Because of these anticancer effects, other parts of the flax plant such as leaves, stems and roots have been investigated. In a recent publication, it was demonstrated that the cell vitality of the chorion carcinoma cell line Jeg3 is inhibited more strongly after treatment with flax root extracts than with leaf and stem extracts from *Linum usitatissimum* (6). The aim of this study was to isolate phytoestrogens from the root of *L. usitatissimum* and to test their effect on acidification (glucose metabolism), respiration (oxidative phosphorylation) and adhesion (cell impedance) in the human mammalian carcinoma cell line MCF-7 using the Bionas® 2500 system (Bionas GmbH, Germany). This system allows continuous monitoring of cellular processes at a high temporal resolution and is therefore advantageous compared to conventional end-point assays.

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Materials and Methods

Extract preparation from flax roots of L. usitatissimum. The seeds from *L. usitatissimum* L., cultivar Barbara, were obtained from the Agricultural Research Institution (LUFA), Rostock, Mecklenburg-Western Pomerania, Germany. The seeds were sown in soil and grown under field conditions. When the plants reached a height of about 1 m, they flowered and the roots were harvested, frozen in liquid nitrogen and stored at –80˚C until extraction.

The extracts were prepared according to Luyengi *et al.* (7) and modified according to Matscheski *et al.* (8). Plant root (20 g) was ground with liquid nitrogen in a mortar and extracted with 180 ml methanol in a water bath for 15 min at 70˚C using a reflux condenser. The solution was cooled, filtered and evaporated to complete dryness. The residue remaining after drying of the original methanol extract was resuspended in 8 ml of distilled water and partitioned with ethyl acetate (five times) to give an ethyl acetate and a water fraction. The water fraction was withdrawn, the ethyl acetate soluble fraction was evaporated to complete dryness and dissolved in 100% ethanol to provide a stock solution of 100 mg/ml. This stock solution of the ethyl acetate fraction is hereafter referred to as 'phytoestrogen flax root extract'. Aliquots of the phytoestrogen flax root extract were added to the culture medium to give final concentrations of 0.01, 1, 20, 50, 100, 150, 200 and 1000 μg/ml (final concentration of ethanol: 1%).

Cell lines and cell culture. The human breast cancer cell line MCF-7 was obtained from Ruprecht Karls University, Heidelberg, Germany. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Switzerland) with 10% inactivated foetal calf serum (FCS) and 1% antibiotics (penicillin/streptomycin) in a humidified atmosphere (37˚C and 5% CO_2).

MTT test. The cell vitality of the human mammary carcinoma cell line MCF-7 treated with different concentrations of phytoestrogen extracts from *L. usitatissimum* was analysed using the MTT test, as recommended by the manufacturer (Roche, Germany). The assay is based on the cleavage of the yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) to purple formazan crystals by metabolic active cells. These salt crystals are insoluble in aqueous solution, but may be solubilised by adding the 10% sodium dodecyl sulfate in 0.01 M HC) (pH<5.0) and incubating the plates overnight in a humidified atmosphere (37˚C, 5% CO_2). The test conditions were optimised in preliminary experiments and the optimal cell number was found to be 5×10^5 cells/ml. This cell number was used in all tests. For the MTT test, the MCF-7 cells were grown in 96-well tissue plates for 24 h in the absence (controls) and presence of different concentrations of phytoestrogen flax extract at 37˚C and 5% CO_2. After incubation with MTT for 4 h at 37˚C and 5% CO_2, solubilisation solution was added and the plates were incubated overnight. The spectrophotometric absorbance of the purple formazan crystals was measured at 570 nm using a microplate ELISA reader (BioRad, Hercules, CA, USA). The reference wavelength was 670 nm.

Online monitoring of cellular metabolism with the Bionas® 2500 system. Detection system: The Bionas® 2500 system (Figure 1) allows continuous recording of physiological parameters of cells. By using the Bionas® metabolic chip SC1000 (Figure 1), acidification rate, oxygen consumption rate and adhesion (impedance) of cells can be determined from each chip in parallel. Each system can accept 6 chips (biomodules). The cells were directly seeded onto the chip surface. Modified, slightly buffered medium with 1 mM Hepes, 0.1% FCS, 10.000 U penicillin and 10 mg streptomycin/ml without bicarbonate buffer (running medium, RM) was fed over the cells at a pump rate of 56 μl/min. The breakdown products (CO_2 and lactate) released into the medium by the cells and oxygen consumption of the cells resulted in an altered pH and oxygen content of the medium. The medium flow was stopped periodically, and during this time pH and oxygen were measured (9). During the subsequent pump phase, fresh medium of a predefined pH and oxygen content was applied. This pump cycle was performed during the whole experiment. Each stop and pump phase lasted 4 min. Acidification and respiration rates were calculated in the stop phase and were defined as 100% just before the addition of phytoestrogen extracts.

In the Bionas® 2500 system, impedance measurement was used to detect the presence of electrically insulating (cell) membranes near the measurement electrodes (10). The adhesion measurement was performed continuously without pump cycles.

Preparation of sensor chip and system: Before each experiment could be started, the tube system of the Bionas® 2500 system was disinfected with 70% ethanol and then rinsed with PBS and conditioned with culture medium. The sensor chips were also disinfected with 70% ethanol, washed with PBS and conditioned with culture medium. The cell suspension was transferred directly onto the chip surfaces and the cells were cultured for at least one day in cell culture medium at 37˚C and 5% CO_2.

Implementation of measurement: The measurements in the Bionas® 2500 system were performed with running medium (see above). The pH of the running medium was adjusted to 7.4 and the osmolarity to 330 mOsmol/kg. Control experiments, namely running medium with 1% ethanol and with the highest concentration of phytoestrogen extract (blank), were always performed. In preliminary experiments, the optimal cell number was determined to be 2.0 and 1.2×10^5 cells/chip, respectively.

The experiment was started by taking the chips from the incubator and adjusting them into the biomodules of the Bionas® 2500 system. As a first step, the baselines for acidification, respiration and cell adhesion (impedance) were determined. After a stabilisation phase of 2.5-3 h, different concentrations of the flax root extract were applied and their effects on the cell line MCF-7 were measured over an incubation time of 8 h and 24 h. Thereafter, the extract was removed and a regeneration phase with running medium was initiated. At the end of the experiment, the MCF-7 cells were removed by the addition of 0.2% Triton X-100 to the running medium, which resulted in the generation of a basic signal without living cells on the sensor surfaces (negative control).

The standardized 100% value (medium without extract immediately before extract application) and the 0% value (Triton X-100 treated cells) were calculated based on 5 data points.

Statistical analysis. Statistical analysis for the MTT test was performed using Student's *t*-test for comparison of the means with the control. With the aid of one-way ANOVA, using the Tukey test, statistical comparison of the means with the neighbouring concentrations was performed. Data are presented as the mean±standard deviation. *P*-values of <0.05 were considered as being statistically significant and are denoted by asterisks for Student's *t*-test and the Tukey test.

Results

Studying the mitochondrial activity in MCF-7 cells using the MTT test, it was shown that the root extracts from *L. usitatissimum* at concentrations from 10-200 μg/ml induced a significant increase of the cell vitality (Figure 2). However, at higher concentrations of the root extract (500 and 1000 μg/ml) a significant inhibition of the cell activity was observed. The strongest inhibition of cell vitality (60%) was obtained after the addition of 1000 μg/ml root extract (Figure 2).

With the Bionas 2500® system it was shown that the treatment of the MCF-7 cells with phytoestrogen root extracts from *L. usitatissimum* resulted in drastic alterations of cellular processes such as glycolysis (acidification), respiration (oxygen consumption) and adhesion (impedance) (Figure 3, 4; results are expressed relative to the 100% standardised value). After 8 h exposure of MCF-7 cells to low concentrations of phytoestrogen extracts (0.01, 1, 20, 50 μg/ml), no or only weak inhibition of the glycolysis, respiration and adhesion were observed, whereas high concentrations (100, 150, 200,

Figure 1. *Bionas® 2500 system showing the Bionas® metabolic chip SC1000 in the inset.*

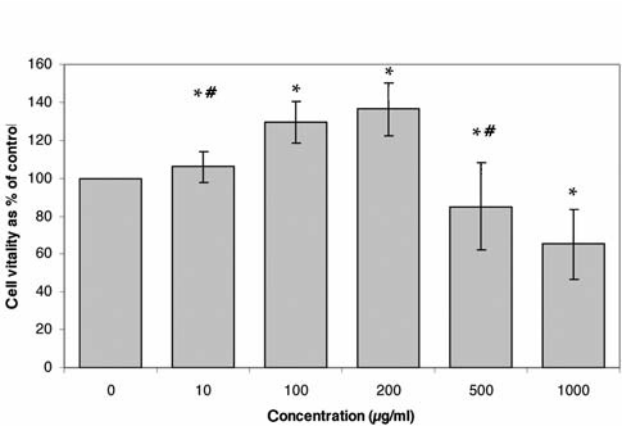

Figure 2. *Effect of different concentrations of flax root extracts on cell vitality (MTT test, 24 h exposure) of MCF-7 cells (5 x 10^5 cells/ml). Data represent the % relative formation of formazan from MTT in comparison to the control (100%) [mean±standard deviation, (n=9)]. Significant difference between *treated cells and the control and #between adjacent concentrations ($p<0.05$).*

1000 µg/ml) strongly inhibited these cellular processes (Figure 3 shows the results of acidification due to glycolytic activity in the presence of 1, 50 and 1000 µg/ml phytoestrogen extract). After addition of the phytoestrogen extracts (50 and 1000 µg/ml) two acidification peaks occurred. Thereafter a rapid decrease of acidification was visible. At 1 µg/ml extract no changes of the acidification rate were measured (Figure 3). Acidification and alkalisation occured in a concentration-dependent manner: the higher the applied concentration of flax root extract, the higher was the amplitude or the stronger was the alkalisation. After removing the flax root phytoestrogen extract from the medium (RM), no regeneration effects were observed.

By displaying acidification rate, respiration rate and cell adhesion in a single graph, dynamic effects of the flax root extract (100 and 200 µg/ml) on cellular metabolism of MCF-7 cells over 24 h were apparent (Figure 4). The overall picture arising from this experimental approach was that after 24 h, almost no acidification and no respiration could be observed, and cell adhesion was only weak. While acidification and respiration continuously decreased after extract application, the cell adhesion was initially (7.5 h) improved after the addition of 200 µg/ml phytoestrogen extract. Thereafter, however, the cellular impedance also decreased. In detail, acidification and respiration rates decreased to ~25% and 40% after 24 h exposure to 100 µg/ml and to ~10% and 15% with 200 µg/ml, respectively. The cell adhesion was reduced to about 40% and 10% by 24 h after exposure to 100 and 200 µg/ml flax root extract, respectively. Regeneration effects were not observed after removing the phytoestrogen extract from the medium.

The IC_{50} value indicates the concentration when 50% of the cells are killed by a drug or an extract after a defined time. The dynamic IC_{50} curves demonstrated the changes of the IC_{50} values in the course of 24 h influence of the flax root extract (Figure 5). The IC_{50} values for acidification, respiration and cell adhesion were about 120 µg/ml, 160 µg/ml and 225 µg/ml at 12 h exposure to the flax root extract, respectively, whereas extract concentrations of 20 µg/ml, 70 µg/ml and 75 µg/ml induced 50% inhibition when the application time lasted 24 h, respectively (Figure 5).

Discussion

Over the last 25 years, there has been a growing interest in the role of phytoestrogens in health and disease. The major classes of phytoestrogens that have been examined are the isoflavones and lignans, found in high levels in soybean, flax seed and in various other plant organs, including roots, stems, leaves, flowers, fruits and seeds (11, 12, 6). Previous HPLC-MS analysis demonstrated that the root, stem and leaf extracts from the flax species of *L. usitatissimum* contain measurable concentrations of lignans such as secoisolariciresinol, matairesinol, pinoresinol, lariciresinol, isolariciresinol and arctigenin, and isoflavones such as genistein, daidzein and biochanin A (6). It was shown that the extracts from *L. usitatissimum* contained more representatives of lignans compared to isoflavones, as has been shown for other *Linum* species (4). The lignan and isoflavone compounds were found as aglycones or as glycosides (6).

Several studies have examined the effects of phytoestrogen extracts and isolated isoflavones and lignans on breast cancer, colon cancer and other cancers (12, 13). In preceding

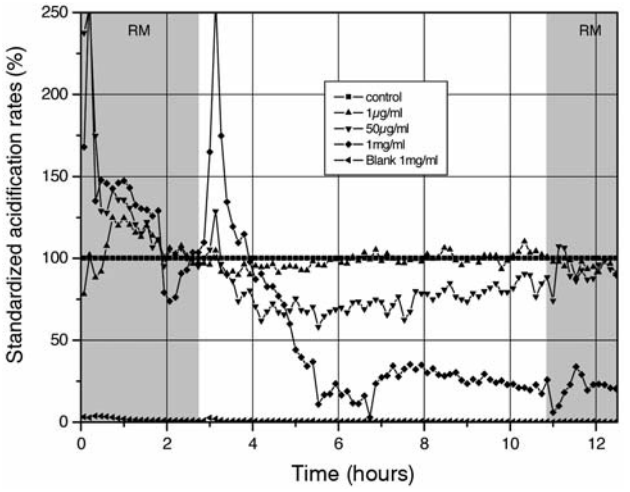

Figure 3. *Alterations of acidification rates (%) of MCF-7 cells (2.0×10^5 cells/chip) after treatment with 1, 50 and 1000 µg/ml phytoestrogen root extract from Linum usitatissimum (8 h exposure to the extract, n=2; RM, running medium).*

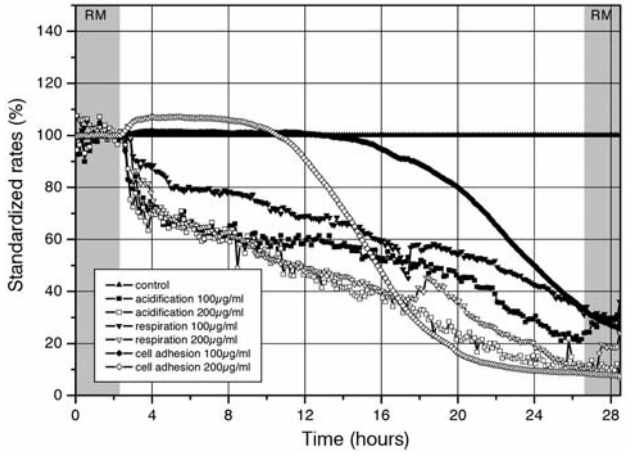

Figure 4. *Acidification, respiration rates and cell adhesion (%) of MCF-7 cells (1.2×10^5 cells/chip) during 24 h exposure to 100 and 200 µg/ml phytoestrogen root extract from Linum usitatissimum (n=2; RM, running medium).*

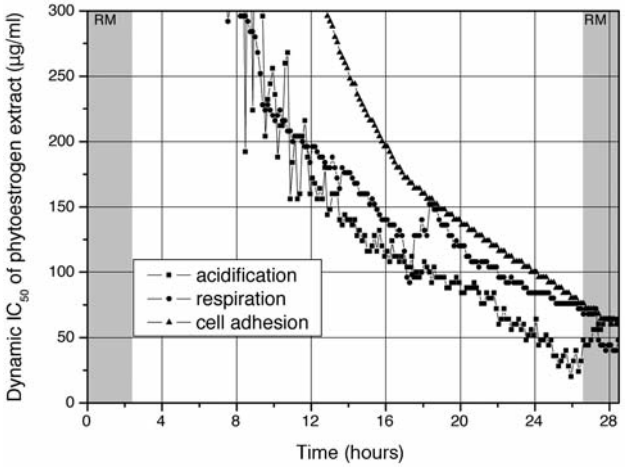

Figure 5. *The dynamic IC_{50} values of phytoestrogen root extract from Linum usitatissimum (µg/ml) for acidification, respiration and cell adhesion in MCF-7 cell lines (1.2×10^5 cells/chip, 24 h exposure to the extract (n=2; RM, running medium).*

publications, the anticarcinogenic influence of flax and elm bark phytoestrogen extracts have been demonstrated on the human chorion carcinoma cell line Jeg3, the endometrial carcinoma cell line RL95-2 and the human carcinoma breast cell lines MCF-7 and BT 20 (6, 14, 15). Respective investigations were performed using the well-known and accepted MTT, BrdU and LDH assays. With these test systems, the measurable parameter is usually determined at the end of incubation (end-point determination). To assess the dynamic changes occurring at the molecular and cellular level during the incubation period, a continuous measurement is necessary.

The Bionas® 2500 system allows the determination of cellular parameters every 8 minutes. With this measurement system, it is therefore possible to interpret the dynamic processes of the cells such as the effects of phytoestrogen extracts on cellular acidification, respiration and adhesion.

The multiparametric online monitoring of cellular processes with the Bionas® 2500 system after treatment of human carcinoma cells with bioactive compounds has been investigated by several research groups (16-18). To the best of the Authors' knowledge, this is the first study investigating the influence of phytoestrogen extracts from roots of *L. usitatissimum* on the cellular metabolism in human mammalian carcinoma MCF-7 cell lines using the Bionas® system.

The experiments described in this paper showed that low concentrations of phytoestrogen flax root extracts (1-50 µg/ml) induced no or only little inhibition of the glycolysis, respiration and adhesion in MCF-7 cells (Figure 3). Higher extract concentrations (100-1000 µg/ml), however, resulted in dramatic metabolic and morphological inhibition (Figure 3 and 4).

Regeneration effects were not observed and therefore the reactions were not reversible. Two observations were interesting to note. First, regarding the sequence of events, acidification preceded respiration, which preceded cell adhesion processes. Secondly, in the first 7.5 hours after extract application, an improved cell impedance/adhesion was observed. Both results indicate important differences and should be followed up by further investigations.

With the Bionas® 2500 system it was possible to calculate the dynamic IC_{50} values according to Thedinga *et al.* (18), which demonstrated the changes of the IC_{50} values in the course of 24 h influence of the flax root extract

(Figure 5). These values in MCF-7 cells demonstrated that the longer the incubation time with the flax root extract, the lower the IC_{50} value is. After 12 h treatment with the phytoestrogen flax root extract, the IC_{50} value for acidification amounted 120 μg/ml, whereas after 24 h exposure only 20 μg/ml of the extract would be necessary to kill 50% of the MCF-7 cells.

The mitochondrial activity was determined in MCF-7 cells which were exposed to phytoestrogen extracts with two different test systems: (i) end-point determination with MTT test, and (ii) online measurement of the respiration using the Bionas® 2500 system. These experiments demonstrated that after addition of root extracts of *L. usitatissimum* at high concentrations (500 and 1000 μg/ml), the cell vitality (MTT test) of the MCF-7 cell line was inhibited significantly after 24 h influence in a concentration-dependent manner, whereas low concentrations (10, 100 and 200 μg/ml) induced a significant activation of the process (Figure 2). According to the oxygen consumption rate of MCF-7 cells after application of 100 and 200 μg/ml extract obtained with the Bionas® 2500 *analyzing system*, a contradictory result was obtained (reduced respiration, Figure 4). It can be envisaged that the continuously registered oxygen consumption is more sensitive and more reliable at reflecting the impairment of mitochondrial function than is the end-point MTT test.

In a recent publication (15), it was shown that after 24 h treatment with 0.01 and 50 μg/ml flax root extract, only 4% apoptotic MCF-7 cells are generated, a value equivalent to the controls of untreated cells. However, the application of 500 μg/ml root extract resulted in a significant increase of apoptosis (25%) and induction of cell death (~30%, LDH test). These results confirm the data obtained using the Bionas® 2500 system that recorded changes of cell adhesion after addition of low (0.01, 1, 50 μg/ml) and high concentrations (100, 200, 1000 μg/ml) of root extracts of *L. usitatissimum*.

It can be assumed that single compounds of the ethanol root extract of *L. usitatissimum* in combination exert the biological activity measured in the *in vitro* cell experiments with the Bionas® 2500 system. Forthcoming research should be directed to testing individual lignan and isoflavone compounds obtained from the flax root extract with the Bionas® 2500 system, as well as testing their effects on primary cells and other hormone-dependent carcinoma cells.

Conclusion

The Bionas® 2500 system has been demonstrated to be a powerful tool for online, label-free, and non-invasive monitoring of cellular parameters of the human mammalian carcinoma MCF-7 cell line and can be used to detect the mode of action of anticarcinogenic compounds in the cellular metabolism.

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