Journal of BUON **13:** 241-244, 2008
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ORIGINAL ARTICLE

Antiproliferative effect of synthetic lignin against human breast cancer and normal fetal lung cell lines. Potency of low molecular weight fractions

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Summary

Purpose: *Due to a lack of chemotherapeutics to efficiently control neoplastic processes, there is a need for discovering new, more efficient anticancer drugs that would distinguish malignant from normal cells.*

Materials and methods: *We studied the effect of short (4 h) and long (72 h) treatment with different concentrations of the enzymatically synthesized lignin model compound (DHP) on the proliferation of two human cell lines grown in tissue culture: breast adenocarcinoma (MCF7) and normal fetal lung fibroblast (MRC5) cell lines.*

Results: *The growth of both MRC5 and MCF7 cell lines was inhibited by DHP after 4 h-treatment, while the carcinoma cell line was also sensitive to the long-term treatment with lower dose of DHP in comparison with the fetal cells. The low molecular weight DHP fractions inhibited growth of the MRC5 cells at lower concentrations compared to the treatment with all DHP fractions.*

Conclusion: *The higher sensitivity to DHP of the human malignant cells compared to the normal transformed ones gives the possibility to further study DHP as a therapeutic agent.*

Key words: breast adenocarcinoma cell line, cytotoxicity index, DHP fractions, fetal lung cell line, lignin model compound

Introduction

Lignin, one of the main structural polymers in the cell walls of higher plants, is the second abundant organic compound on the Earth's surface. Lignin is involved in the protection of plant cell from different kinds of stress and disease resistance [1]. In the cell walls lignin is produced by oxidative coupling of the 3 phenolic alcohols (coniferyl, *p*-coumaryl, and synapyl alcohol), catalyzed by the enzyme peroxidase. The enzymatically synthesized lignin model compound, dehydrogenate polymer (DHP) from coniferyl alcohol, is the best lignin substitute, used in various experiments [2]. Although lignin exhibits a high degree of structural variability, its final structure is generally attained by aggregation of subunits obtained by polymerization of monomers [1]. Lignin structure consists of low and high molecular fraction [3,4].

Lignin was found to exert free-radical scavenging capacity [5,6]. Natural and synthetic lignin-like materials have been shown to exhibit antiviral, antitumor and immunopotentiating activities [7,8]. The copolymerization of toxic xenobiotics with lignins may be an effective mechanism of inactivation and/or immobilization of these dangerous metabolites [9].

In this work we studied the effect of different concentrations of synthetic lignin, known as DHP, on the growth of two human cell lines: breast carcinoma and normal fetal lung cell lines. The effect of short (4 h) and long (72 h) treatment duration were compared. The aim of the study was to see whether there is any inhibitory effect of DHP on the proliferation of malignant cells. The human transformed cell line served as control, to see if there is any difference in response to DHP in comparison with malignant cells. Besides, we aimed to compare the effect of higher and lower molecular weight fraction of DHP on the cell proliferation.

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Received 16-10-2007; Accepted 30-11-2007

Materials and methods

All chemicals used in the experiments were of analytical grade and obtained from Sigma Chemical Co, St Louis, USA.

DHP was synthesized from coniferyl alcohol in the presence of commercial horseradish peroxidase and hydrogen peroxide in phosphate buffer [4]. The reaction mixture contained $5 \cdot 10^{-3}$ M coniferyl alcohol, $5 \cdot 10^{-3}$ M H_2O_2 and $2.5 \cdot 10^{-8}$ M horseradish peroxidase (all Fluka Chemical Corp., New York, USA) in 50 mM phosphate buffer. The reaction mixture was prepared by simultaneous addition of H_2O_2 and coniferyl alcohol solutions to peroxidase solution. After mixing, the solution was shaken constantly for 48 h. Polymerization occurred in solution phase at 25° C. The suspension of DHP was centrifuged at 5000 rpm. The precipitate was resuspended in bidistilled water and centrifuged twice. After centrifugation the pellet (DHP) was dried in a vacuum at room temperature.

Human breast adenocarcinoma cell line MCF7 and human normal fetal lung fibroblast MRC5 cell line were used in the experiments. The cells were grown in Dulbecco's Modified Eagles Medium (DMEM, GIBCO, Paisly, UK) supplemented with 5-10% fetal calf serum and antibiotics (100 IU/ml penicilin, 100 µg/ml streptomycin; ICN Galenika, Belgrade, Serbia). Cells were cultured in flasks (Costar, Vineland, USA) at 37° C in atmosphere of 100% humidity and 5% CO_2 (Heraeus, Langenselbold, Germany).

In the experiments we used DMSO as a solvent for DHP and the effect of DHP was compared between MRC5 and MCF7 lines. The effect of lower molecular weight fractions of DHP on cell proliferation was studied in methanol and acetone on the MRC5 line. DHP was soluble in DMSO, but in methanol and acetone a suspension was obtained. Therefore, the suspension was filtered through Nalgene 0.45 µm filters and the clear filtrate containing DHP lower molecular weight fractions was used for the cell treatments. Stock DHP concentrations were in the range from 1 $\mu g \cdot ml^{-1}$ to 10 $mg \cdot ml^{-1}$.

Ten microliters of stock DHP solution (in DMSO or of the filtered fraction) were added into the wells with 90 µl medium with the cells (10,000 per well), to obtain final DHP concentrations in the range 0.1 µg - 1 mg per ml.

Two experimental sets were performed. In the first set cells were exposed to DHP solutions 72 h *in continuo*; in the second, after 4 h of treatment, cells were left to recover in fresh medium for 72 h and afterwards the number of viable cells was measured.

A colorimetric method involving reduction by living cells of tetrazolium salt MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) to blue formazan product was used to estimate the number of viable cells [10]. Dark blue crystals (formazan product) were dissolved by acid-isopropanol (100 µl of 0.04 N HCl in isopropanol). After a few minutes, optical density was measured on a microplate reader (Multiscan MCC340, Labsystems, Helsinki, Finland) at 540/690 nm. The wells without cells, containing complete medium only, acted as blank. Cytotoxicity was calculated according to the formula: $"(1\text{-}A_{TEST} / A_{CONTROL}) \times 100"$, and expressed as a percent of cytotoxicity (IC %). Real (final) inhibition was calculated by subtracting the effect of the solvent itself from the overall effect of the DHP in solution.

In order to examine the molecular weight of lignin fractions present in the filtered methanol and acetone suspension, we performed SDS PAGE electrophoresis. We used 1 mm of 15% polyacrylamide gel in standard Tris-glycine buffer pH 8.3, according to the method of Laemmli [11]. Samples were mixed with 50% glycerol (1:1 v:v) and 15 µl were loaded into each well and then electrophoresed at 80 V through stacking gel for 15 min and 120 V through separating gel for 120 min. After electrophoresis silver staining was performed by the method of Merril et al. [12]. For determination of the molecular weight of the lignin fractions we used SDS marker proteins (Fermentas International Inc., Burlington, Canada).

Statistical analysis

Each treatment and the corresponding control were performed in 4 replications. Student's t-test was used to statistically evaluate differences between treated (test) and untreated (control) cells, and probability values < 0.05 were considered statistically significant.

Results

We found that the number of 10,000 cells per well was most appropriate because reproducibility was best in that case (coefficient of variation (CV) was under 10% - 9.87). Besides, 10,000 cells was a sufficient amount to enable experiment in the stretch of 72 h.

Comparing the effect of DHP on the two cell lines in the case of the short treatment duration (Figure 1A), the most profound growth inhibition was achieved by 1 $mg \cdot ml^{-1}$ DHP in both MCF5 line (IC value 47.46%) and MRC7 line (IC value 46.45%). A different response of the two cell lines was observed in the case of the long treatment duration. DHP did not induce growth inhibition of MRC5 cell line. On the contrary, the growth

of MCF7 line was inhibited, with the effective dose 0.1 mg · ml^{-1} DHP, where an IC value 58.82% was obtained (Figure 1B). The IC values for lower lignin concentrations are not presented, since the difference between values obtained for lignin and for pure solvent was not statistically significant.

The effect of the low molecular weight DHP fractions on the MRC5 cells is presented in Figure 2A. We found 5 DHP fractions in methanol, with molecular weights ranging from 670 to 8000 D, while in acetone there were 4 fractions ranging from 670 to 6000 D. In both solvents, 4 fractions had identical molecular weight (Figure 2B). The most effective DHP concentration in acetone was 0.1 mg · ml^{-1} (IC value 43.78%) in the case of the short-term treatment (Figure 2A). In the case of long-term treatment, the effective DHP concentrations, 0.1 mg ml^{-1} and 0.5 mg · ml^{-1}, induced 80-90% inhibition. Low molecular weight DHP fractions in methanol were ineffective in the short-term treatment, but showed a 50% effective dose in the long-term treatment, at the concentration 0.5 mg · ml^{-1} (IC value 48.33%). Consequently, this dose can be considered for further studies.

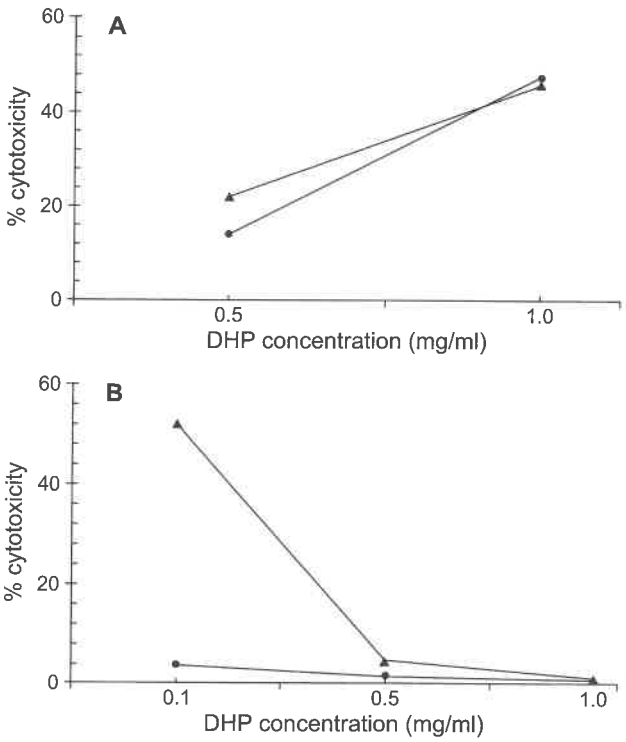


Figure 1. Effect of short-term (4 h) treatment (A) and long-term (72 h) treatment (B) with DHP solution in DMSO, on the growth of human normal fetal lung fibroblast MRC5 (•), and human breast adenocarcinoma MCF7 (▲) cell line. p = 0.036 (0.332) and p < 0.01 (< 0.01) for the difference between effect of DHP concentrations 0.5 and 1 mg/ml, in short and long-term treatment duration, for MRC5 (MCF7) cell line, respectively. p = 0.172 (< 0.01) for the difference between 0.1 and 0.5 mg/ml, in long- term treatment duration, for MRC5 (MCF7) cell line. For details see Materials and Methods.

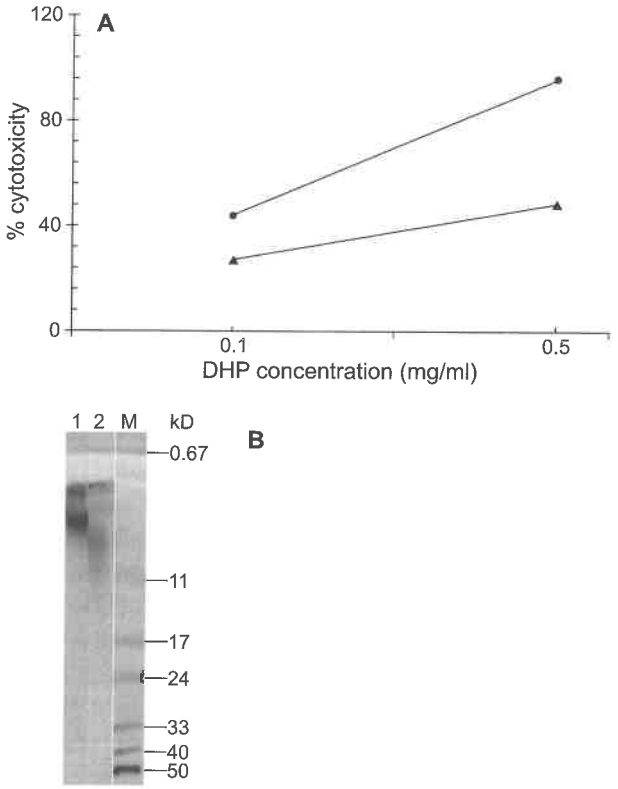


Figure 2. A: effect of short-term (•) and long-term (▲) treatment with solution of low molecular weight DHP fractions, on the growth of MRC5 cell line (p = 0.224 and 0.503, respectively); the solvent was methanol (▲) or acetone (•). B: SDS-PAGE gel of DHP in methanol (lane 2) and acetone (lane 1) filtrate, after silver staining. M: SDS molecular weight markers. The molecular weights are indicated to the right.

Discussion

The fact that in DMSO solvent there was an effective inhibition by 1 mg · ml^{-1} DHP for only 4 h for both normal MRC5 cell line and human breast adenocarcinoma cell line MCF7 shows the similarity of the reaction of the two cell lines towards DHP. However, the growth of carcinoma cell line was also found to be sensitive to the long-term treatment with the lower DHP dose (0.1 mg · ml^{-1}) in comparison with the normal fetal cells. These data are important, depicting the difference in response between the normal fetal and malignant cells and they might be a basis for further studies of synthetic lignin as an agent that selectively influences the growth of malignant cells. Artificial lignin, synthesized by polymerization of ferulic acid, was previously found to inhibit tumor growth in mouse skin [13]. The polymer was dissolved in acetone and the effective concentration was 25 mg ·

ml^{-1}, which is considerably higher than the concentrations found to be effective in the presented work. In the pine cone extract, which showed inhibitory effect on the growth of ascites and solid tumor cells transplanted in mice, and also had antiviral and antimicrobial activities, lignin-related structures complexed with sugars and polysaccharides were found to be most effective components [7]. Synthetic lignins have been found to show anti-HIV activity [14]. The lignin-like substance suberin, extracted from *Querkus suber* cork, showed antimutagenic activity in *Euglena gracilis* [15], and the authors proposed that this effect may be due to the scavenging of reactive oxygen species [15]. Some previous results indicated radical scavenging activities of lignin [5,6]. Sakagami et al. [16] showed that ^{125}I-labeled natural and synthetic lignins administered orally to mice are absorbed through the digestive tract and excreted via urine, in addition to feces.

It was previously shown that in the solution of DHP in DMSO all polymer fractions are present, both low molecular weight (2000-8000) and high molecular weight (60,000-100,000) [3,4]. In filtered solutions of DHP in both methanol and acetone, only low molecular weight fractions were present (Figure 2B), namely oligomer and low molecular weight polymer. The results show the potency of these low molecular weight fractions to inhibit cell growth (Figure 2), even at lower DHP concentrations than in the case when all fractions were present (Figure 1). Lignans (short oligomers from the lignin family) have been proposed to possess a phytoestrogenic activity in mammals, but generally more experiments are needed to better assess their potential beneficial effects [17]. On the other hand, MCF 7 cell line is estrogen receptor-positive, therefore the inhibitory effect of lignin on its proliferation might be attributed to an antiestrogenic effect. An inhibitory effect of lignans on E2-induced growth of MCF7 tumors has been recently shown [18].

The effect of purified individual low molecular weight fractions of DHP is to be studied in our further experiments. The synthesized lignin model compound, DHP, or its particular fractions, is a pure substance, and there is a possibility of controlling and changing the conditions of its synthesis. This is important for its application in further studies on its effect on cell proliferation.

Acknowledgement

This work was supported by the grants of the projects 143043 and 1346 of the Ministry of Science and Environmental protection of Serbia.

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