Lipoxygenase inhibitors suppress proliferation of G5:113 fibrosarcoma cells *in vitro* but they have no anticancer activity *in vivo*[*]

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Nordihydroguaiaretic acid (NDGA) and esculetin, both nonspecific inhibitors of lipoxygenases (LOX), were found to suppress expressivelly the *in vitro* proliferation of fibrosarcoma cells G5:113 in concentrations ranging from 10 to 50 μM. Subsequent flow-cytometric analysis of the cell cycle showed that both these drugs significantly decreased the percentage proportion of cells in the G_0/G_1-phase and simultaneously increased significantly this proportion in the S-phase. No apoptosis was detected in the whole range of concentrations studied, from 2.5 to 50 mM.

On the contrary, in experiments *in vivo*, neither NDGA nor esculetin had any curative effect if they were repeatedly injected intraperitoneally (i.p.) into mice bearing tumors growing from subcutaneously (s.c.) transplanted G5:113 cells. Pretreatment of the fibrosarcoma cells with NDGA or esculetin *in vitro* preceding their s.c. transplantation into mice did not result in suppression of the tumor growth, either. Finally, if G5:113 cells were injected intravenously and the mice were subsequently treated repeatedly with i.p. injections of NDGA, decreased survival and increased number of surface lung metastases were observed in the NDGA-treated group. Thus the suppressive action of inhibitors of LOX on the growth of fibrosarcoma cells *in vitro* was not reflected in their anti-tumor effects *in vivo*.

Key words: fibrosarcoma, lipoxygenase inhibitors.

The role of arachidonic acid (AA) metabolism in modulation of cancer development has been examined by numerous investigators using various inhibitors of AA metabolism. The attention has been focused on inhibition of metabolites produced by cyclooxygenases (COX) obtained by administration of various nonsteroidal anti-inflammatory drugs (NSAIDs). In animal experiments and clinical trials, suppression of the development and progression of solid tumors *in vivo* both in animals and in humans were found [see e.g. 7, 14, 30].

Once released, AA is converted by catalytic action of 5-, 12- or 15-lipoxygenases (LOX) into the corresponding hydroperoxyeicosatetraenoic acids (HPETEs) which are further metabolized either to hydroxyeicosatetraenoic acids (HETEs) and leukotrienes or lipoxins through additional sequential reactions. Particularly the products of 5- and 12-LOX may play important roles in tumor promotion, progression and metastatic disease [33]. It was shown that 5-LOX was universally expressed in many cancer cell lines of colon, lung, breast and prostate [2, 11]. Inhibition of 5-LOX reduced the proliferation while the addition of 5-HETE stimulated the growth of cultured lung cancer cells [2]. The ability of tumor cells to generate 12-HETE is positively correlated with their metastatic potential. Extensive studies by Honn's group have demonstrated the involvement of 12-LOX products in multiple steps of the metastatic cascade encompassing tumor cell-vasculature interactions, tumor cell motility and proliferation, proteolysis (collagenase IV release from tumor cells), intravasation/extravasation and angiogenesis [12]. A novel approach for cancer chemoprevention could also involve LOX modulators, i.e., agents that can induce the anticarcinogenic 15-LOX [32] and/or inhibit the procarcinogenic 5- and 12-LOX, thereby shifting the

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balance of LOX activities from procarcinogenic to anticarcinogenic metabolism of AA.

Nonspecific inhibitors of LOX, both nordihydroguaiaretic acid (NDGA), a plant lignan derived from *Larrea divaricata*, and esculetin, a coumarin derivative contained in many plants, such as *Artemisia capillaris*, used in our studies, are reported as inhibitors of carcinogen metabolism and may also operate as scavengers of carcinogen free radicals [34]. These properties of NDGA and esculetin are considered to be responsible for their antimutagenic and anti-tumorigenic action [1, 3, 6, 10, 13, 18, 25, 40].

Fibrosarcoma is a tumor type that is often resistant to current therapeutical procedures. In our studies we use fibrosarcoma cells G5:113, a chemically induced cancer cell line [4], which can be both cultivated *in vitro* and grow as solid tumors forming lung metastases *in vivo*. Growth suppression of subcutaneously (s.c.) transplanted fibrosarcoma cells G5:113 after administration of NSAIDs, as well as reaction of these cells *in vitro* to the inhibition of COX pathway of AA metabolism was described in our previous study [9]. In the present study we try to bring more detailed picture of the cellular mechanisms of suppression of proliferation of G5:113 cells from the point of view of the inhibition of LOX pathway. Using NDGA and esculetin, we first performed *in vitro* studies and, subsequently, a series of *in vivo* experiments with the aim to evaluate their effects on proliferation of G5:113 cells.

Material and methods

Cell line and culture conditions. The N-methyl-N'-nitro-N-nitrosoguanidine-induced fibrosarcoma cell line G5:113 was kindly provided by Dr. Margaret L. Kripke (University of Texas, M.D. Anderson Cancer Center, Houston, TX, USA) [4].

The cells were cultivated in RPMI-1640 (Sebak, Aidenbach, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS) (PAN Systems, Nürnberg, Germany), 1 mM sodium pyruvate (ICN Biomedicals, Inc., Costa Mesa, CA), 100 μg/ml streptomycin (Gibco BRL, Paisley, Great Britain), 100 IU/ml penicillin (Gibco BRL), 0.1 mg/ml gentamicin (PAN Systems), 8 mM L-glutamine (Gibco BRL), 1% nonessential amino acids 100x (ICN Biomedicals), 5 mM HEPES (Serva, New York, NY, USA), and 50 μM 2-mercaptoethanol (Fluka, Seelz, Germany).

Chemicals. Esculetin (6,7-dihydroxycoumarin), the inhibitor of 5- and 12-LOX, (Sigma, St. Louis, MO, USA) and nordihydroguaiaretic acid (NDGA) (4,4(2,3-dimethyl-1,4butanediyl)bis(1,2-benzenediol), a general inhibitor of LOX, (Fluka) were dissolved in ethanol immediately before using.

In vitro treatment of cells. Cells were harvested by trypsinization (0.25% trypsin/0.02% EDTA, Sigma) and resuspended in cultivation medium at a density of $1x10^5$ cells per 2 ml dish. Immediately after cell seeding, drugs were added at concentrations ranging from 2.5 μM to 50 μM. All examinations were performed 48 h later when control untreated cells were in the exponential growth phase.

Proliferation and cell cycle studies. Cell number was determined using a Coulter Counter (model ZM, Coulter Electronics Ltd., UK) and expressed as percentage of control growth.

Cell viability was evaluated microscopically by 0.15% eosin exclusion assay as the percentage of viable (unstained) cells per 100 cells counted.

For cell cycle analyses the cells were fixed in 70% ethanol at 4 °C overnight. Before measurement, the cells were stained with propidium iodide in Vindelov solution [38]. Fluorescence (indicating DNA content) was measured using flow-cytometry (FACSCalibur system, Becton Dickinson, USA; equipped with an argon ion laser, excitation at 488 nm). To estimate the percentage of cell population in each phase of the cell cycle (G_0/G_1, S and G_2/M) histogram analyses of the relative DNA content were carried out with ModFit LT 2.0 software (VERITY Software House, USA).

In vivo experiments. In each *in vivo* experiment fibrosarcoma cells were washed twice with serum-free cultivation medium and adjusted to the concentration of $8x10^5$ cells/ml. Animals were anesthetized intraperitoneally (i.p.) with Narcamon/Rometar solution (5% Narcamon/2% Rometar in the ratio 2.63:1, Spofa, Prague, Czech Republic) and injected with 10^5 viable tumor cells/mouse. Three different experimental designs were used:

A) Untreated fibrosarcoma cells were injected s.c. into the flank and on day 5 after tumor injection drug administration was started. NDGA was given i.p. in the dose of 0.1 mg/mouse/day during 10 days with two-day pause after day 5 (total dose of 1 mg). Esculetin was administered in the same regimen in the dose of 0.06 mg/mouse/day (total dose of 0.6 mg).

B) The cells were first pretreated in culture either with NDGA or esculetin (10 μM) for 48 h and then injected s.c. to mice. The animals were without therapy to the end of experiment.

C) Untreated cells were injected intravenously (i.v.) into tail vein of the mice and from day 5 NDGA was administered i.p. in the regimen described in A/.

Parameters studied in vivo. In experiments A and B, incidence and approximate tumor size were recorded weekly throughout the experimental period. Tumor location was determined by palpation. Tumor size was determined weekly by measuring three dimensions of each tumor using a calliper. Approximate tumor volume (cm^3) was calculated using a formula for elliptical mass ($\pi/6$ x L x W x H; L, W and H designate tumor size for length, width and height, respectively). Survival was monitored daily up to week 15. In experiment B, number of lung metastases was assessed in the

lungs taken post mortem always on the day of death. Lungs were fixed in Bouin's solution and surface lung metastases were counted under a dissecting microscope [21]. Duration of the experiments was 15 weeks.

Statistical analysis. The significance of differences in cell number, cell viability, as well as percentage proportion of cells in individual phases of the cell cycle were assessed by Student's t-test preceded by F-test.

Differences in survival between treated and control mice were assessed by means of chi-square test. The significance of differences in tumor size was analyzed by nonparametric Mann-Whitney test.

Results

Effects of NDGA and esculetin on growth of G5:113 fibrosarcoma cells in vitro. Both nonspecific inhibitors of LOX, NDGA and esculetin, caused an expressive suppression of cell proliferation *in vitro* (Fig. 1).

Viability of G5:113 cells was preserved above 78% after 48-hour presence of NDGA in a wide range of concentrations from 2.5 to 50 μM (Fig. 1A). On the other hand, a clear concentration-dependent decline in cell number was observed 48 h after treatment of the cells with NDGA; at the concentrations of 30 and 50 μM the cell number fell below

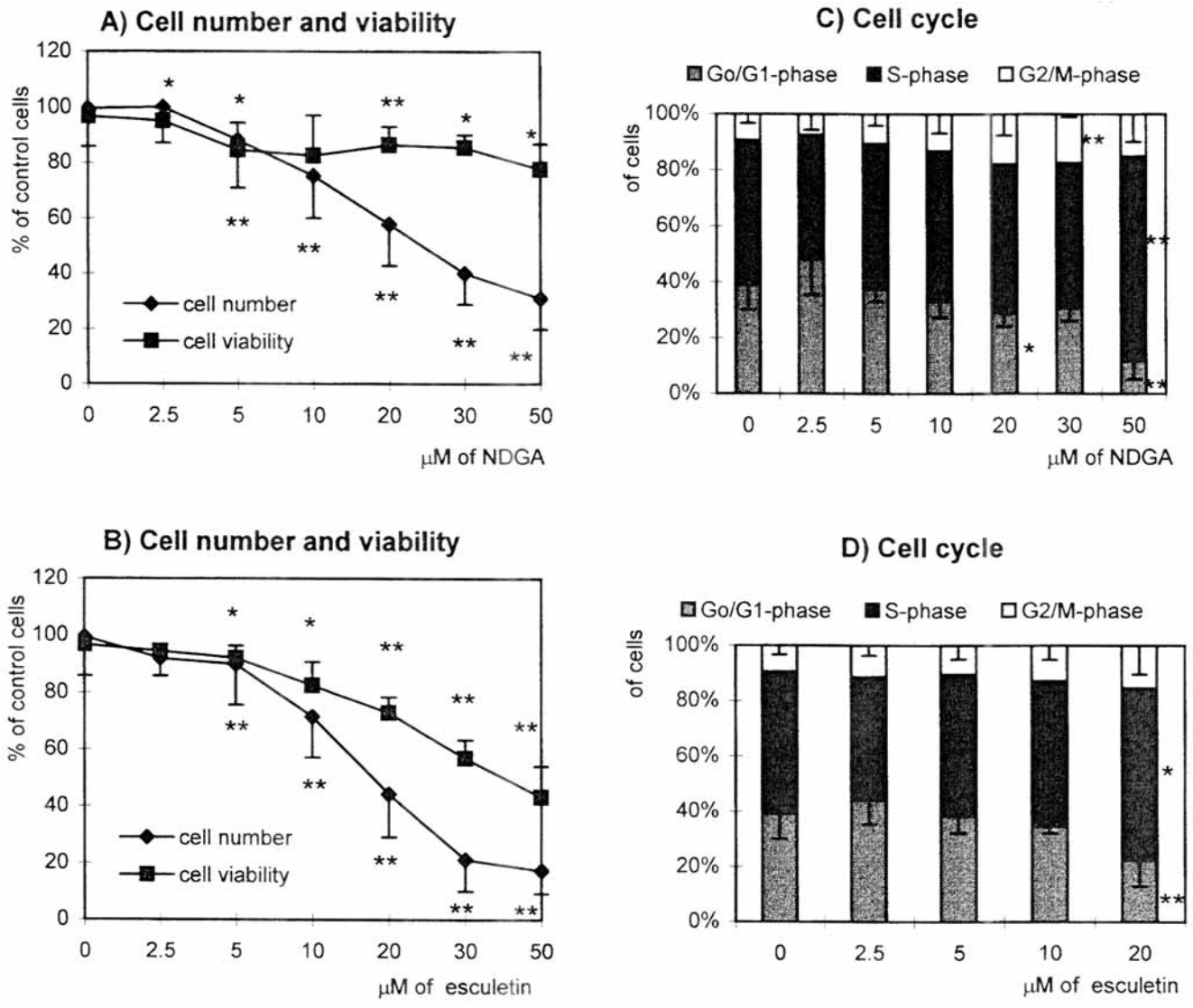

Figure 1. Effects of NDGA and esculetin on *in vitro* proliferation of G5:113 cells after 48-h treatment. Cell numbers and cell viability after treatment with different concentrations of NDGA (A) or esculetin (B). Results are expressed as percentage of control values. Cell cycle parameters after treatment with NDGA (C) or esculetin (D). Results are presented as means ± S.D. of three independent experiments. *, ** -- p<0.05, p<0.01, respectively, in comparison with untreated controls (0 μM).

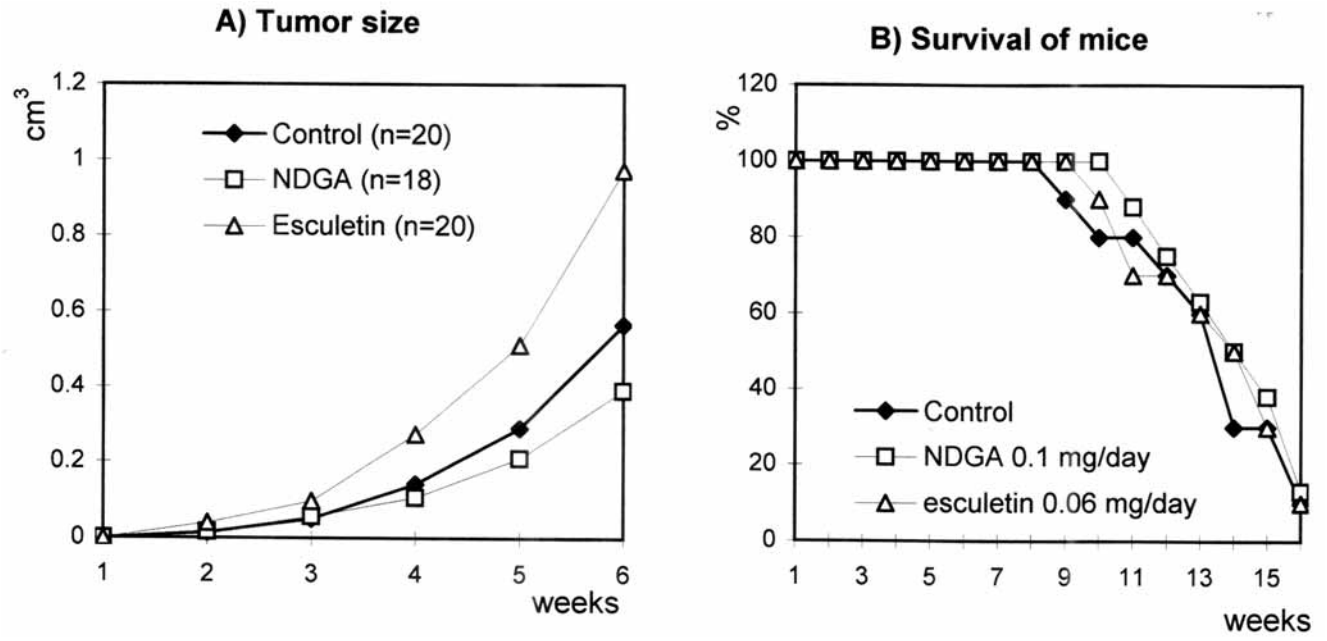

Figure 2. Growth of G5:113 cells in syngeneic mice and survival of tumor-bearing mice treated with NDGA or esculetin. Mice were injected with 10^5 tumor cells s.c. into the flank. Five days later, animals were treated i.p. with NDGA in a dose of 0.1 mg/mouse/day for 10 days (total dose of 1 mg) or with esculetin in a dose of 0.06 mg/mouse/day for 10 days (total dose of 0.6 mg). Tumor size (A) was monitored weekly by measuring the three largest diameters. Survival (B) was monitored daily. Results from two experiments were pooled. Standard deviations were omitted with regard to the clarity of the figure. n = number of experimental animals. No statistically significant differences among the groups studied were found.

40% of control untreated cells. The value of ID_{50} was 26.5 μM.

A different result was found after treatment of G5:113 cells with esculetin (Fig. 1B). Viability of the cells above 80% was recorded only for concentrations of 2.5, 5 and 10 μM. However, the treatment of the cells with 30 and 50 μM esculetin was found to be toxic because the cell viability fell down to 60% and 36%, respectively. The value of ID_{50} was 18.5 μM.

In the studies of drug effects on the cell cycle all concentrations of NDGA were used, while concentrations of esculetin of 30 and 50 μM had to be omitted because of formation of a lot of debris. Concentrations of 50 μM NDGA and 20 μM esculetin caused a significant decrease in the proportion of cells in the G_0/G_1-phase, whilst a significant increase in the S-phase was found (cell viability was comparable in the two experimental groups) (Fig. 1C, D). Moreover, in the presence of lower concentrations of NDGA, a concentration-dependent decrease in the proportion of cells in the G_0/G_1-phase and a concentration-dependent increase in the G_2/M-phase reaching statistical significance at 20 μM and 30 μM, respectively, were found.

No occurrence of apoptosis as detected either as the sub G_0/G_1 population of the cell cycle, by visualizing chromatin condensation and formation of multiple apoptotic bodies or by the specific cleavage of poly(ADP-ribose) polymerase into the 89-kDa fragment was observed (data not shown).

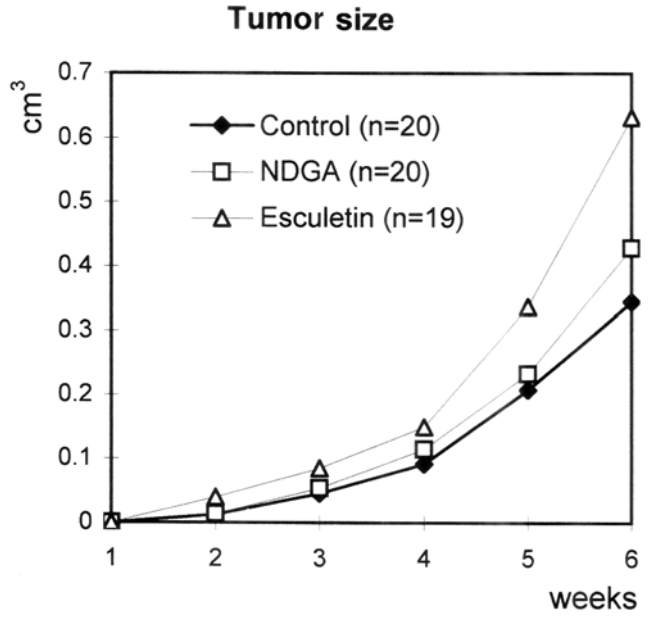

Figure 3. Effect of *in vitro* pretreatment of G5:113 cells with NDGA and esculetin on their ability to form solid tumors in mice after s.c. transplantation. Cells were pretreated with NDGA or esculetin (10 μM) in culture for 48 h before s.c. transplantation. Mice were injected with 10^5 tumor cells s.c. into the flank and they were mantained without therapy to the end of experiments. Tumor size was monitored weekly by measuring the three largest diameters. Standard deviations were omitted with regard to the clarity of the figure. n = number of experimental animals. No statistically significant differences among the groups studied were found.

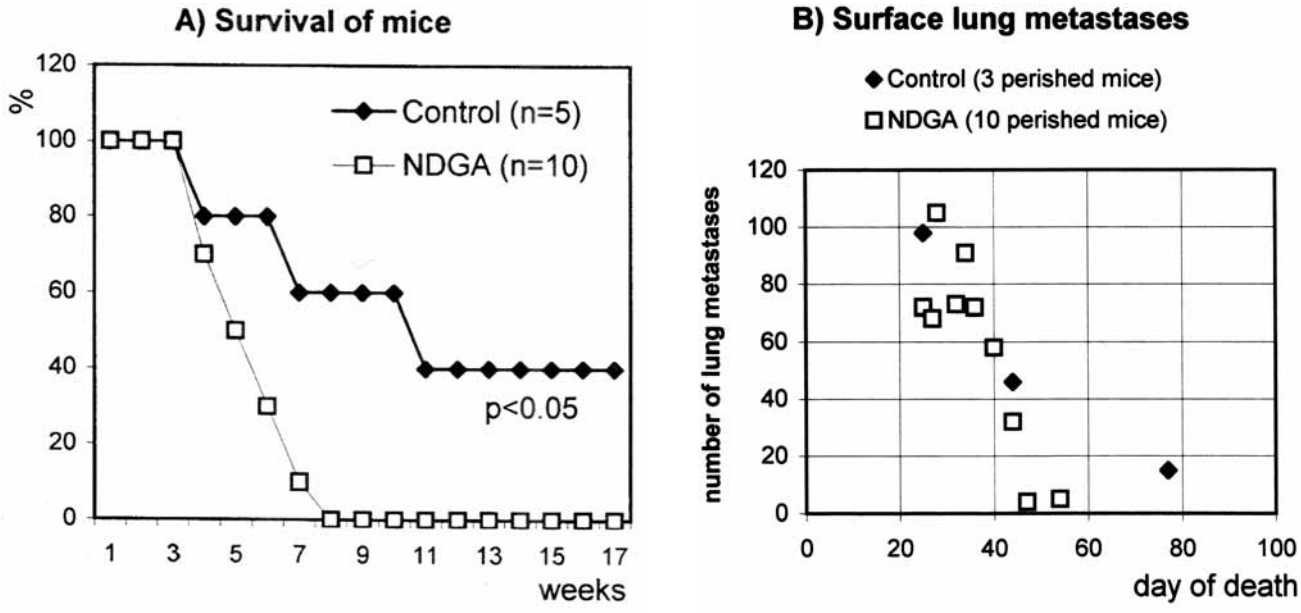

Figure 4. Survival of mice treated with NDGA after i.v. injection of G5:113 cells and the number of surface lung metastases in mice. Mice were injected with 10^5 tumor cells i.v. into the tail vein and NDGA was administered from day 5 i.p. in a dose of 0.1 mg/mouse/day for 10 days (total dose of 1 mg). Survival of mice (A) was monitored daily. n = number of experimental animals. The number of surface lung metastases (B) was assessed in the lungs taken *post mortem* on day of death.

Effects of NDGA and esculetin in in vivo experiments with G5:113 cells. Determination of tumor size was performed in mice which were transplanted s.c. with G5:113 fibrosarcoma cells and subsequently treated with NDGA or esculetin. Statistical evaluation was done up to week 6 after tumor cell transplantation which was the time of the first tumor-related deaths. Tumor growth in mice treated with NDGA was slightly suppressed and in mice treated with esculetin was slightly stimulated in comparison with control untreated tumor-bearing animals; however, the differences did not reach the level of statistical significance (Fig. 2A).

No differences were observed in the survival of tumor-bearing mice, either. Animals treated with NDGA or esculetin, as well as control untreated tumor-bearing mice showed very similar survival curves and approximately 10% survival on week 16 after s.c. transplantation of fibrosarcoma cells (Fig. 2B).

Since the results of *in vitro* experiments showed suppressing effects of NDGA and esculetin on the proliferation of G5:113 cells, an attempt was made to find out whether pretreatment of the fibrosarcoma cells with NDGA or esculetin *in vitro* preceding their s.c. transplantation into mice would influence the tumor growth. As shown in Figure 3, the general picture was very similar to that observed after transplantation of cells not pretreated with the drugs. No statistical differences were found up to week 6 after tumor cell transplantation.

The effect of NDGA was tested also in another experimental setting in which the G5:113 cells were injected i.v. and the animals were subsequently treated with the drug. Under these conditions the survival of mice given NDGA was significantly impaired in comparison with drug-untreated animals (Fig. 4A). Moreover, in this experiment we looked for the presence of surface lung metastases in mice on day of their death. A relatively high number of lung metastases in mice treated with NDGA was observed in weeks 4 to 6 of the experiment (Fig. 4B). However, it did not reach significance as compared to the controls.

Discussion

The antiproliferative action of NDGA and esculetin has been demonstrated in several *in vitro* models. Esculetin was found to inhibit the survival of human promyelocytic leukemia HL-60 cells [3]. Recently it has been found that NDGA induced DNA degradation of lympholeukemic P-388 line cells [15] and completely inhibited arachidonic acid-stimulated growth of prostate cancer cells [8]. Moreover, both NDGA and esculetin suppressed proliferation of human breast cancer cells [5, 6, 25], rat mammary tumor cells [18, 22] and monoblastoid U937 cells [1, 10].

We report here that also the proliferation activity of fibrosarcoma cells G5:113 *in vitro* was extensively suppressed after treatment with both NDGA and esculetin in a wide range of concentrations. However, esculetin showed a dose-dependent cytotoxic effects on G5:113 cells, which corresponds with the findings of the influence of this drug on both

human retinoblastoma cells [37] and leukemia cells HL-60 [3].

It is known that NDGA exhibits many LOX-unrelated effects such as blocking voltage-actived Ca^{2+} currents and inhibiting respiration rate of ascites tumor cells by preventing electron flow through the respiratory chain. Consequently, ATP levels, cell viability and culture growth rates are decreased. These effects suggest that NDGA may have antineoplastic activity, which would be mediated through processes not connected with production of LOX metabolites [27, 36]. However, most of the authors suggest that the NDGA-induced inhibition of cell proliferation is mediated primarily via its LOX activity. Taken into consideration that 5-LOX is also expressed in G5:113 cells (our unpublished data) we can judge that *in vitro* antitumor effects of NDGA and esculetin observed in our experiments are, at least in part, due to their LOX-inhibiting activity.

We found that the percentages of cells in the S-phase of the cell cycle after treatment with higher concentrations of both NDGA and esculetin were increased. Detailed mechanisms of effects of these drugs need further study. The binding of ribonucleoproteins to nuclear matrix affecting the replication of DNA was found to be influenced by intranuclear level of phospholipids [20, 21]. Although molecular basis of this effect is not well understood, DNA replication was associated with a decreased content of phospholipids in the cell nucleus. Thus, when processing of phospholipids is slowed down due to general inhibition of the LOX pathway, the level of phospholipids may become high enough to inhibit DNA replication and manifest itself by the accumulation of cells in the S-phase described here.

Several studies showed also *in vivo* antitumor effects of NDGA and esculetin. For example, esculetin suppressed mammary carcinoma incidence as well as prolonged tumor doubling time in rats [13] and NDGA exhibited antitumor promoting activities in the skin of mice [40]. It was also reported that NDGA inhibited experimental adhesion of B16 melanoma cells to fibronectin in an *in vitro* model of experimental metastasis [26] as well as *in vivo* [28]. Moreover, 5-HPETE reversed the inhibition of invasion of B16 cells pretreated by NDGA and esculetin. Similarly, 5-HPETE prevented the reduction of colagenase IV production by an inhibitor of LOX, while 12- and 15-HPETE had no effects [28].

In our studies we noted an apparent contrast between our *in vitro* and *in vivo* findings. In contradistinction to both the extensive inhibition of proliferation of G5:113 cells *in vitro* and the *in vivo* findings observed by others, we showed that neither subcutaneously growing fibrosarcomas nor formation of lung metastases after i.v. injection of tumor cells were suppressed by NDGA and esculetin. Our *in vitro* studies were undertaken in order to better understand how metabolites of LOX might affect fibrosarcoma cell proliferation *in vitro*, where cell-mediated immunity would not be involved. However, the millieu of the whole organism encompasses many additional factors which may influence the outcome of *in vivo* therapy with the compounds tested.

Generally, two possibilities exist how to explain the absence of antitumor effects of the inhibitors of LOX administered *in vivo*. The first possibility is the toxicity of the pharmacological treatment. The dosing used in our experiments was derived from that used by SUZUKI et al [35]; these authors administered repeatedly a daily dose of 5 mg NDGA per 1 g of mouse average body weight in the experimental B16 melanoma which corresponds to 0.1 mg/mouse in 25 g mice used by us. Individual *in vivo* doses of esculetin were set at a lower level of 0.06 mg/mouse taking into account its toxicity *in vitro* at higher concentrations.

The second possibility consists in the interference of the LOX inhibition caused by some pathway(s) of AA metabolism responsible for anti-tumor immune reactions of the organism. Several literature data suggest that this line of explanation may be reasonable. There exists a regulatory balance between the negatively (prostaglandins) and positively (leukotrienes) acting eicosanoids in the control of hematopoiesis [16]. It is concievable that the inhibition of the LOX pathway caused by NDGA and esculetin is followed by shunting the AA metabolism to the COX pathway [35]. Inhibitors of LOX can thus actually stimulate the production of immunosuppressive prostaglandins [16, 24]. Results of several studies confirm this hypothesis: Interruption of the LOX pathway by esculetin or NDGA resulted in decreased colony formation by both lymphoid and myeloid stem cells *in vitro* [16, 17, 23, 39]. Additionally, NDGA and esculetin reversibly inhibited the natural killer-mediated lysis and the maintenance of natural killer activity [19, 29].

Significantly impaired survival of mice with i.v. administered fibrosarcoma cells was observed when the animals were subsequently treated with NDGA. This finding clearly suggests that systemic administration of NDGA in the state of dissemination of fibrosarcoma cells in the organism is therapeutically disadvantageous. The cause of this unambiguously undesirable effect of NDGA is unknown at present. Tumor type or the way of NDGA administration may be responsible for this finding: REICH and MARTIN [28] observed a positive action of NDGA on the suppression of formation of lung metastases by B16 melanoma cells when the cells were treated with NDGA before their i.v. injection. In conclusion, taking into account all literature data and our findings presented here, it can be summarized that LOX inhibitors, namely NDGA and esculetin, suppress the proliferation of G5:113 cells *in vitro* but are not suitable for *in vivo* treatment of solid fibrosarcoma tumor in mice.

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