Inhibition of breast adenocarcinoma growth by intratumoral injection of lipophilic long-acting lathyrogens

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Prevention of the formation of crosslinks and/or disintegration of already formed collagen fibrils in the tumor by known lathyrogens, β-aminopropionitrile or D-penicillamine, may result in the weakening of tumor support, decreasing angiogenesis and promoting tumor regression. This paper reviews our studies with a single intratumoral injection of lipophilic lathyrogens and others, using a systemic administration to investigate the effect of both lathyrogens. Details of our experimental results are also given. ***Anti-Cancer Drugs*** **16:201–210 © 2005 Lippincott Williams & Wilkins.**

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Introduction

We formulated a hypothesis that local intratumoral injection of lathyrogens, such as β-aminopropionitrile (BAPN) or D-penicillamine (DPA), will cleave and inhibit the formation of covalent crosslinks between collagen fibrillar proteins forming the supporting stroma of the tumor tissue. Disintegration of collagen structure will result in weakening of the mechanical properties of the tumor stroma and the microvessels' pericapillary sheath or basement membrane. The collapse of tumor stroma and loss of blood supply should inhibit tumor growth. The basic principles are based on the knowledge of collagen chemistry and biology.

The growth of a malignant as well as benign tumor (or any tissue in general) depends on: (i) structural stability (integrity) of the supporting stroma consisting mainly of the fibrous collagen framework of the extracellular matrix (ECM), and (ii) growth and integrity of microvessels in the tumor (angiogenesis).

The structural stability of the tumor consists of highly organized fibrillar structures of polymerized collagen, forming the stroma or framework of a tumor. Prevention of collagen crosslinking and/or disintegration of already formed collagen fibrils results in faster degradation of collagen fragments by tumor metalloproteinases, loss of the mechanical properties of the tissue-supporting stroma, and inhibition of cell adhesion, propagation and viability—all of which have been recognized as essential for tumor growth.

Several methods for interfering with the various aspects of collagen metabolism were proposed and tested. Inhibition of collagen hydroxylation of proline residues by proline analogs such as azetidin-carboxylic acid, D-proline and *cis*-hydroxyproline was thoroughly studied utilizing tissue culture systems. In animal testing, however, these drugs were shown to be toxic and non-specific as they inhibited synthesis of other vital non-collagen proteins as well [1,2]. Halofuginone was documented to inhibit collagen synthesis and gene expression [3]. It is a low-molecular-weight coccidiostatic alkaloid used mainly in commercial poultry. In several animal models of collagen abnormalities (lung fibrosis, urethral strictures) as well in the growth of tumors, this drug was shown to inhibit the pathologies. Still, this drug was toxic [3].

An experimental review of the feasibility and safety of these various approaches led us to focus on the use of drugs which affect the crosslinking of the collagen structure. BAPN was found most specific in reducing the mechanical strength of collagen (as well as elastin) and, in local administration, it was non-toxic. The same applies to another lathyrogen, DPA, which has many effects on various targets of collagen metabolism (summarized later in Table 2). This drug has been used for more than 40 years in the oral treatment of various human disorders with definite toxic side-effects. In local administrations, no toxicity was reported [4] as the local effective dose is a few 100 times lower than that needed for the effectiveness of systemic administrations.

Role of ECM (collagen) stroma in tumor development

Several studies document that only the fibrillar collagen serves as the attachment for cells, their movement and

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proliferation. The role of ECM in the growth and differentiation of the cells during organogenesis is well established [5,6]. The important role of ECM integrins, various growth factors and cell–cell adhesion molecules has been described [5,7]. The importance of tensional forces in fibrillar ECM was recognized as a factor controlling directional capillary sprouting [8,9]. The formation of the connective tissue (mostly collagen of ECM) in the growing tumor follows the same principles as the growth of any organ. There exists almost a constant relation between the mass of tissue cells and the density of the supporting stroma represented mainly by collagen fibers. Meredith *et al.* [10] found that programmed cell death or apoptosis occurred through modifications of the ECM.

It has to be stressed that it is not the amount of collagen in the ECM that provides the mechanical support to the tumor tissue, capillaries or microvessels, but rather the structural stability of the collagen matrix, which is related to the number of covalent crosslinks within the collagen structures.

BAPN is a well-documented irreversible inhibitor of lysyl oxidase. By this very specific effect, BAPN affects the crosslinking and associated polymerization of collagen molecules into a hierarchy of filaments, fibrils and fibers. It affects all biological functions dependent upon the existence of fibrillar collagen. After BAPN treatment the supporting vessel structures (pericapillary sheath, basement membrane of microvessels) are weakened, and do not provide the necessary strength to resist blood pressure or to form the collagen network required for the attachment of endothelial cells and their further budding.

Effect on angiogenesis in tumor development

Angiogenesis is a complex process regulated by several soluble growth factors, copper and insoluble components of the ECM. As indicated by Ingber [11], 'the soluble factors act over large distances to initiate capillary growth whereas changes in ECM govern whether individual cells grow, differentiate, or involute in response to these stimuli in the local tissue microenvironment'. Sage and Vernon [12] see two regulatory pathways during angiogenesis: the early proliferative (corresponding to the soluble factors effect) and morphogenic pathway that 'depends on the synthesis and assembly of fibrillar type I collagen used as a template for endothelial cell migration and lumen formation'. They also indicate that 'endothelial cells interact with substrates of type I collagen [fibers] and form networks based on the establishment of traction centers' [12].

The growth of capillaries is connected with the production of collagen by endothelial cells. Collagen is one of the important components of the ECM, contributing to the formation of the pericapillary sheath, which provides by its fibrillar components and associated macromolecules the means for cell adhesion, locomotion and strength for the capillary to sustain blood pressure. Angiogenesis, in its complexity, is essential for tumor growth and formation of metastases [1,8]. Sipos *et al.* suggested that 'it is becoming apparent that many modulators of collagen metabolism inhibit angiogenesis and may offer clinically useful anticancer treatments' [13].

In a study using the formation of capillaries in the developing chorioallantoic membrane of chicken embryos, Ingber and Folkman [14] tested the hypothesis that alteration of the ECM could be casually involved in angiogenesis. They studied inhibitors of collagen synthesis (proline analogs), collagen hydroxylation (2,2′)dipyridyl and collagen crosslinking BAPN. BAPN was inhibitory to angiogenesis in a dose-dependent manner. In 1978, Bear *et al.* [15], Diegelman and Cohen [16] and, later in 1979, Cohen *et al.* [17] studied the effect of pretreatment of rats with BAPN on collagen synthesis in capsules surrounding dimethylbenzanthracene-induced breast tumor. The authors found a 3-fold increase in collagen synthesis in the capsule around the tumor parenchyma and a 6-fold increase of collagen synthesis over the normal breast connective tissue. Pretreatment of animals with 1–2% BAPN, in the diet, caused 82% decrease in tumor formation and a significant reduction in tumor volume. They suggested that the apparent antitumor effect of BAPN may be due to immunostimulation and/or cytotoxic actions of the drug. Bankowski *et al.* [18] also studied the effect of BAPN on the collagen of methylcholanthrene-induced sarcoma of rats. The treatment reduced the collagen density from 17 to 12 mg collagen/g tissue and increased the solubility of collagen from 37 to 67%. The data reflects the inhibition of collagen crosslinking with an increased pool of 'soluble', non-polymerized collagen, which is degraded faster by metalloproteinases. Ogata *et al.* [19] report alternations in alveolar capillary formation in growing rat lung after BAPN feeding.

DPA is a multifunctional drug. First, DPA is a chelating agent that binds several bivalent cations. The stability constant of DPA with metals decreases in the following order: Hg > Ni > Cu > Zn > Cd > Pb. Ca and Mg are not bound by DPA. The binding of some of these metals may be the reason for the well-documented DPA toxicity after systemic administration. Of interest is the blocking of copper, which is considered an essential factor in angiogenesis [20], also inhibiting the function of lysyl oxidase Chelation of zinc, which is a cofactor in all metalloproteinases, inhibits their many functions. The second mechanism of the DPA effect is related to cleavage of the non-reduced double bonds in proteins,

mainly collagen. This effect may be especially important as the collagen in tumors is mainly crosslinked by Schiff base covalent crosslinks such as hydroxylysinonorleucine and histidinomerodesmosine that are still labile, and cleaved by acid pH, temperature above 45°C and some thiol compounds, such as cysteine and DPA [21]. Finally, the third mechanism of DPA action is the reaction of the SH groups of DPA with aldehydes formed by oxidative desamination of some Lys or Hyl residues of the collagen chain effected by the action of lysyl oxidase.

DPA has been used for more than 40 years in clinical practice in the treatment of rheumatoid arthritis, scleroderma, chronic active hepatitis, pulmonary fibrosis, multiple sclerosis and metal poisoning. However, the clinical use of DPA is still problematic due to the frequent occurrence of considerable, although generally reversible, toxic side-effects.

Nimni *et al.* [22,23] documented the three above-outlined effects of DPA on collagen metabolism. (i) At low dose, the drug blocks aldehyde groups in collagen. As a consequence, collagen remains in molecular form without formation of fibrils. (ii) With thiol and amino groups in adjacent positions [24], DPA cleaves non-reduced Schiff base crosslinks in the collagen structure which are labile due to the presence of a double bond that can be broken by various chemicals, of which thiol compounds are quite effective. (iii) Finally, at a higher dose of DPA, the copper is chelated and does not act as a cofactor in the function of lysyl oxidase or participate in angiogenesis [20]. Moreover, chelation of zinc blocks the function of tissue metalloproteinases.

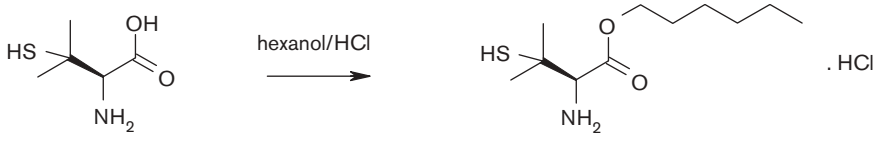
Already, in 1969, Hourami and Demopoulos [25] found that systemically administered DPA inhibits S-91 mouse melanoma metastasis and growth of tumors. The striking inhibition of tumor growth was achieved by a dose of 25 mg DPA/mouse/day, administered for 4 weeks. Assuming the body weight of the mice as 25 g, this dose corresponds to 1 g DPA/kg, which is a very high dose. Okuyama and Mishina [26] found 'a remarkable inhibition of tumor growth' after treatment with systemic DPA. Jong-Hong *et al.* [27] documented that administration of DPA to Long-Evans cinnamon rats with hereditary hepatitis prevented the development of hepatocellular carcinoma. They suggested that the effect was mediated by chelation of copper. Brem [20] documented that DPA is antiangiogenic. He and his coworkers believe this effect is related to chelation of copper because a copper-deficient diet produced the same inhibition of angiogenesis and related tumor growth. Brem *et al.* [28] stated that copper modulates angiogenesis and is an obligatory factor in endothelial cell propagation. Camphausen *et al.* [29] also found that copper-chelating agents have an anti-angiogenic effect. Yoshida *et al.* [30] found inhibition of angiogenesis and tumor growth by DPA in Fisher rats injected s.c. with gliosarcoma cells.

There exist several reasons why lathyrogens must be delivered to the tumor tissue locally by intratumoral injection. Both BAPN and DPA are small molecules that are very soluble in water, and, as a result, are quickly excreted. Furthermore, both drugs are rapidly metabolized into inactive products. Also, the target enzyme in collagen crosslinking, lysyl oxidase, is re-synthesized within hours, thus requiring a continuous supply of the inhibitor. This is the reason why both drugs were administered systemically to animals and patients on a daily basis at relatively high doses for several weeks. Toxic effects were quite common. In order to overcome these problems and to obtain high tissue concentration of the lathyrogen, we documented that their topical or local administration was the optimal solution [4]. Still, the high water solubility of both lathyrogens required daily local administration. This was the reason we modified the molecule from a hydrophilic to a lipophilic substance. After several unsuccessful attempts, the esters of DPA proved to be effective for several days after a single injection. Lipophilic BAPN is commercially available as hexyl-3-(imino)propionitrile (Matrix Scientific, Columbia, OH).

Synthesis of lipophilic DPA esters

DPA or BAPN were modified by the synthesis of methyl-ester (Me)- or hexyl-ester (He)-DPA·HCl or using hexyl-3-(imino)propionitrile to render them lipophilic, thus allowing for longer effectiveness. In principle DPA was incubated for several days in methanol or hexanol in the presence of thionylchloride, as shown in Scheme 1.

Scheme 1



Molecular weight=149.21 Molecular weight =233.38

The yield for the methyl derivative was around 70% and it was less than 20% for the hexyl derivative, due to difficulties with isolation of the final product. The purity of the esters was controlled by NMR, showing only traces of disulfide. The esters were in the form of hydrochloride, soluble in water. In this form the esters are resistant to oxidation to inactive disulfides. By neutralization *in vitro* or *in vivo* by tissue fluid, an oily substance is formed.

Induction of mammary adenocarcinoma tumors

Adenocarcinoma cells (13 762NF; 1.5×10^6) were injected into the fourth lumbar (L4) mammary fat pad. Once the volume of the tumor reached 1–1.3 cm^3, a dose of 0.2 ml of the drug in water, in PEG 400 or in thermosensitive polymer (TSP) was infiltrated through the tumor. Tumor volume growth was determined 3 times a week by a caliper and the volume was calculated as (length $\times$ width^2)/2. Determination of body weight changes, observation for metastasis and histology were also performed. A single intratumoral injection of either Me-DPA·HCl or hexyl-3-(imino)propionitrile significantly inhibited the tumor growth. No metastases were observed. Many areas of apoptotic cells and several larger necrotic foci were found with condensed collagen accumulation. A dose–response effect to He-DPA·HCl was observed. Systemic administration of cyclophosphamide (CP) at low dose combined with intratumoral single injection of He-DPA·HCl was significantly inhibitory over the injection of the lathyrogen alone. A low dose of CP alone was ineffective. A synergistic effect of combined CP with He-DPA·HCl ester was found. Only in the experiment with combination treatment, metastases were found in the lung and liver in all groups with the exception of the DPA-treated group. The occurrence of metastases in a group of rats with combined treatment was observed later than in the other two groups. A detailed description in support of the above statements is presented below.

Effect of a single injection of two lipophilic lathyrogens on the growth of mammary carcinoma

This experiment tested the effectiveness of both lipophilic lathyrogens administered in PEG 400.

Fischer 344 inbred female rats were used. The initial body weight was 100 ± 7 g. The right flank area was clipped and rats were tattooed on the ear for identification. A mammary adenocarcinoma cell line, code 13762 NM, originated from Fisher 344 rats, 10×10^6 viable cells/0.1 ml of minimal essential medium, was injected s.c. in the right flank using a G24 needle under aseptic conditions. No anesthesia was needed. After 2 weeks, the tumors reached 2 cm^3 in volume. Animals were killed and the tumor dissected in a Petri dish with a 1-mm^2 matrix to allow cutting the tumor tissue into 1-mm^3 cubes. These were dispersed in sterile saline, mixed and one chunk injected into the flank region of the rats.

Once the tumor was detectable, the size of the tumor was measured by a caliper in perpendicular directions. There were six rats per group. The control group was not treated. Experimental groups were injected with Me-DPA·HCl or 3-hexyl(imino)propionitrile dissolved in 0.2 ml PEG 400. Growth of the tumor was measured every second day. When the tumor reached 6 cm^3 the animals were terminated by barbital overdose. Tumors were then dissected and fixed in formalin for morphological analysis.

Injection of a single minced 1-mm^3 chunk of breast adenocarcinoma tumor into the flank region of Fisher rats was followed by rapid growth of the tumor in all but two rats, which did not grow the tumor and were, hence, eliminated from the experiment. After 9 days, most of the tumors reached a volume of 1 cm^3 and were injected intratumorally with 0.2 ml of the respective drugs. Two tumors from the Me-DPA·HCl group leaked the drug through the injection port. These two rats were followed separately and showed the same tumor growth pattern as the control group. One rat from the 3-hexyl(imino)propionitrile group also leaked the drug solution and showed the same tumor growth pattern as the Me-DPA·HCl-leaking rats.

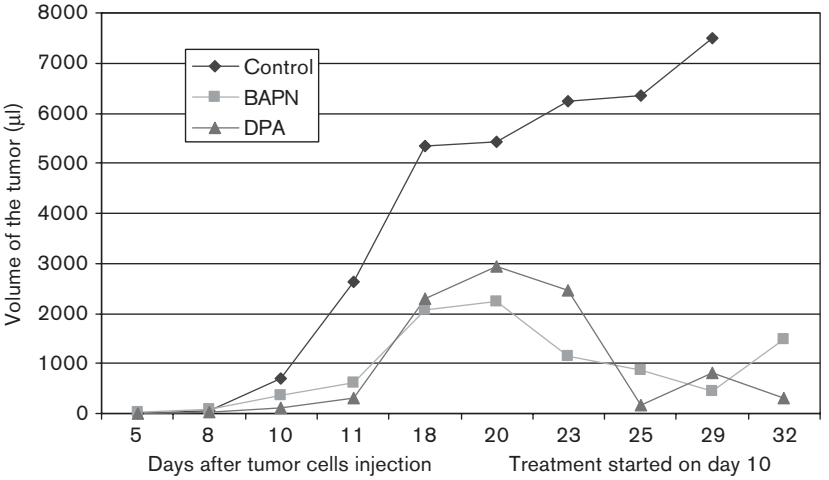
The effect of both treatments is shown in Figure 1. Despite great variability of the individual measurements, there was a statistically significant inhibition of tumor growth after either drug administration when compared to the controls. After 32 days, the study was terminated. The harvested control tumors were soft, juicy and brittle. The tumors from the treated group were characterized as 'scab'. No metastases were detected.

Effect of lipophilic DPA and BAPN and free DPA on the growth of breast cancer

In this experiment, we repeated the above-reported experiment using eight rats per group, and used a polymer with the hope to prevent the bothersome leaking and possibly prolong the effect of the drugs. We used a TSP which was kindly provided by Dr Anna Gutowska (Battel, Seattle, WA). TSP is a composite of polylacetate and polyglycolate trimers which at room temperature exists as sol and gels at body temperature. The sample we tested was biocompatible and biodegradable after approximately 14 days in water.

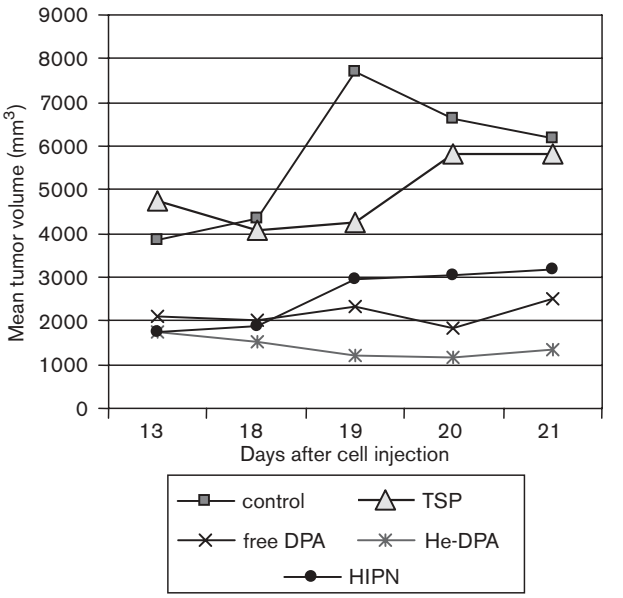
An injection of tumor chunks and an intratumoral injection of 0.2 ml of the drug were dispersed in a polymer. In addition, one group of rats received, intratumoral, He-DPA·HCl dissolved in water. There were five groups of animals consisting of eight rats

Fig. 1



Effect of a single intratumoral injection of two lathyrogens of mammary adenocarcinoma.

Fig. 2



Effect of various treatments on the growth of breast cancer in Fischer rats.

per group: (i) control, not treated, (ii) TSP alone, (iii) He-DPA·HCl in water, (iv) He-DPA·HCl in TSP and (v) 3-hexyl(imino)propionitrile in TSP.

In spite of using TSP, we encountered problems similar to those in the previous experiment. Some rats did not grow the tumors either at all or very poorly. Some injected tumors leaked the drug suspension through the injection porthole or through the lesion of the skin inflicted by clipping the hair. These outlying rats were eliminated from the final evaluation, leaving five to seven animals in each group. The results are shown in Figure 2.

No difference was found in tumor growth of controls and TSP-alone injected tumors. The other three groups documented significant inhibition of tumor growth. No difference was found among these three groups. It is important to note that the tumors injected with He-DPA·HCl in water were found to be as inhibited as the rats injected with the drug in polymer. There were no differences in the body weight changes among the five groups of rats, indicating no toxic effects of the treatment. No metastases were found.

Effect of three doses of He-DPA·HCl on the growth of breast cancer

This experiment studied the effect of He-DPA·HCl at three concentrations. Individual cancer cells, rather than a single chunk of tumor, were injected in order to limit the variability caused by the heterogeneity of the injected tumor chunk. The adenocarcinoma cells were grown in tissue culture and harvested in the log phase after the same passage number. The cells were trypsinized and dispersed in tissue culture medium, 5×10^6/0.1 ml. The viability of cells was determined. Cell suspension was injected into the flank region in the fourth lumbar fat pad (L4) [31].

In this experiment on the effect of three dosages of He-DPA·HCl on the growth of adenocarcinoma tumor, we initiated the tumor by injecting a suspension of tumor cells. We expected that variability in the same group would be reduced.

Nine days after cell injection, the tumor size was 1–1.5 cm^3. At this time, the rats were randomly stratified into four groups. We encountered several problems during the injection. In almost 50% of rats, the drug fluid leaked out from the tumor through the skin needle puncture. Although an effort was made to pinch the skin opening with tweezers, an unaccounted volume of drug was lost. This was recorded in the experimental notes. In two rats, the oozing of the drug was through the abrasion of the skin induced by close shaving of the tumor area with electric clippers.

A typical result, including all rats injected with a low dose of Me-DPA·HCl, is shown in Figure 3. The graph also indicates that four tumors in this group leaked the drug and all showed quick growth of the tumor, consistent with the control group results. A similar situation was found in other treated groups, which only reinforces our conclusion that the drug, when retained in the tissue, significantly inhibited the growth of the tumor. Justified elimination of the compromised rats from the final evaluation resulted in Figure 4. It shows dose–response relationship and the significance among the three treated groups is at $p < 0.05$. All treated groups were found to be statistically different from the control group at $p < 0.01$. Body weight growth was the same in all groups of rats. No metastases were found.

Combination treatment using single intratumoral He-DPA·HCl and systemic CP

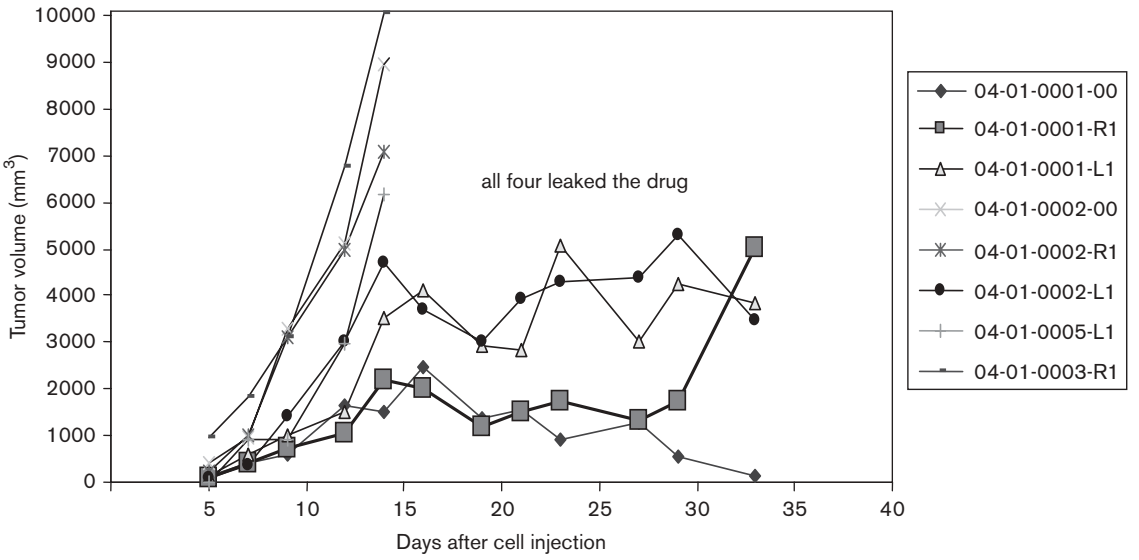
The fourth experiment studied the effect of the combination treatment with systemic CP and a single intratumoral injection of He-DPA·HCl on breast adenocarcinoma tumor development.

It was assumed that simultaneous administration of a lower, non-toxic dose of commonly used chemotherapeutics, CP, together with intratumoral injection of lipophilic DPA should work synergistically without the manifestation of an adverse effect characteristic of large therapeutic doses of CP. The literature indicates that the effective therapeutic dose for rats is 150 mg/kg body weight, which often results in toxic effects. For this reason, we selected the dose of 85 mg CP/kg body weight, which is considered to be safe, but possibly not effective enough to interfere with tumor growth.

A total of 24 Fisher female rats, body weight 130–140 g, were injected with 5×10^6 adenocarcinoma cells in 0.1 ml of tissue culture medium, which were harvested by trypsinization in their confluent state and dispersed in a tissue culture medium. After the tumor reached 1–1.2 cm^3 in volume, the rats were divided into four groups consisting of six rats per group: (i) control, not treated, (ii) CP, 85 mg/kg body weight, dispersed in distilled water injected in 0.1 ml i.p., (iii) He-DPA·HCl, 100 mg/0.2 ml distilled water, and (iv) a combination of the treatments described as (ii) and (iii).

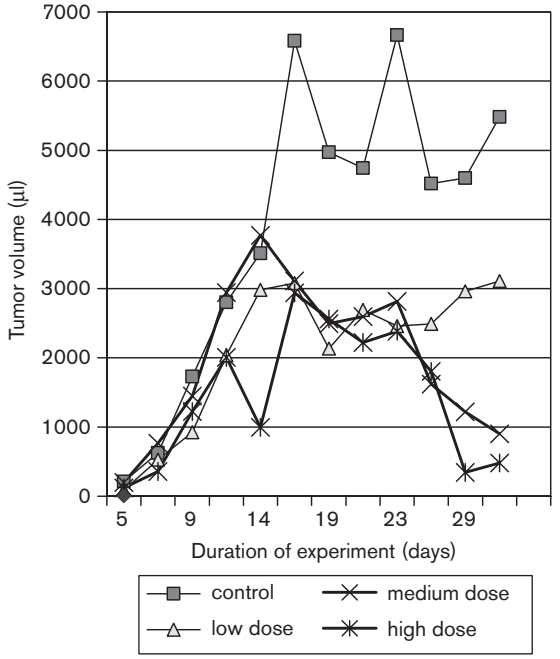
The volume of the tumor was measured 3 times per week by a caliper. Body weight, signs of toxicity such as hemorrhagic cystitis, unkempt hair, and body weight were recorded. Liver and lung tissue was inspected for metastases. All harvested tumors, lung and liver tissue were processed for histology. Staining was done with

Fig. 3



Variability in the same group related to leaking drug.

Fig. 4



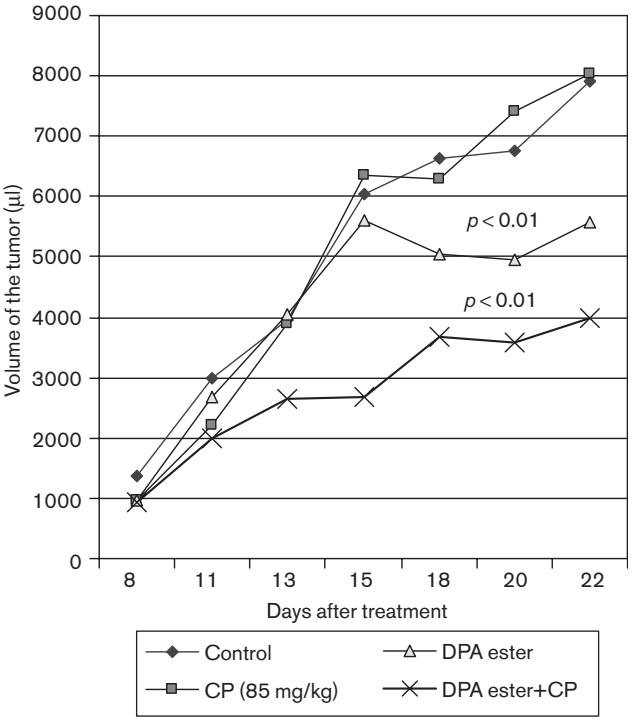
The effect three doses of He-DPA·HCl on the growth of adenocarcinoma tumor.

hematoxylin & eosin and Masson trichrom. The slides were evaluated at maximum ($\times 400$) magnification using a Nikon Eclipse 400 microscope with an attached Sony Exwave HAD camera. The software used was ImagePro (Media Cybernetics, Silver Spring, MD). Statistical evaluation was done by analysis of variance and Student's pair *t*-test.

In this experiment on the combination treatment using systemic CP and intratumoral He-DPA·HCl, a total of 24 rats were included in four groups. In the control group, there were only five rats due to one rat having died from undetermined causes. The statistics of the tumor volumes from day 8 to 22 did not significantly differ from the control group of the previous experiment and, therefore, both groups were combined for the final statistical evaluation. In the CP group, the statistics showed no difference in the tumor growth from the control group. In the group injected with He-DPA·HCl, one rat was eliminated due to leakage of the injected drug. The statistics of the remaining five rats were significantly lower than the control and CP groups at $p < 0.01$. Finally, the group of rats receiving the combined treatment showed the growth of the tumors significantly inhibited when compared with DPA-treated tumors ($p < 0.01$, $t = 4.05$). These results are presented in Figure 5. There was no effect of any treatment on the body growth weight.

In all experiments conducted with either adenocarcinoma tumor chunks or cell injections and involving more that 200 Fisher rats, we did not detect any metastases even though the provider of tumor cells stated that metastases are common in this model (R. Camalier, National Cancer Institute, Frederick, MD, pers. commun.). In the last reported experiment, we noticed the presence of metastases in the lung and liver in all groups with the exception of the He-DPA·HCl-treated group. There were no metastases in any of the DPA ester-treated rats. In all the other groups, the metastases appeared in 40–50% of rats. These were single tumors on the organ surface. The incidence of tumors, as related to individual group and days of tumor identification, is shown in Table 1. Although based on a rather limited number of animals, there are two interesting observations. In two groups, control and CP-treated rats, the metastases were detected on the 11th, 13th and 20th days following tumor inoculation, while in the group with a combined treatment, the tumors appeared at day 27. It has to be stressed that the lipophilic drug, according to our findings, disappears from the tumor tissue after 10–14 days and we speculate this was the reason for the delay in the appearance of the metastases in the group utilizing a combined treatment. Morphologic evaluation of selected slides, stained with hematoxylin & eosin and Mason trichrom, was performed in every reported experiment.

Fig. 5



Combination treatment of breast adenocarcinoma in Fisher rats by intratumoral DPA ester and systemic CP.

Table 1 Metastases from breast adenocarcinoma in Fisher rats with various treatments

Treatment	Rats/ group	Metastases (days when detected)	Incidence (%)
Control	5	lung (11 days), lung (20 days)	40
Cyclophosphamide	6	lung (11 days), lung (20 days), liver (13 days)	50
He-DPA·HCl	5	none for 41 days of the study duration	0
He-DPA + CP	6	lung (27 days), lung (27 days), liver (27 days)	50

Due to similarities of the observations, the morphologic analysis of all four experiments is presented as one.

In the tumors harvested from the control rats, the morphology depended on the age/volume of the tumor. In tumors up to 2–3 cm^3 thick, a collagenous capsule and the presence of continuous collagen stroma was found throughout the tumor tissue. The tumor cells looked viable and closely packed. Most of the analyzed tumors were of large size (6 cm^3 or above). The thick outer collagen capsule was present and underneath it was a rim of intact, malignant cells. The center was occupied by necrotic, disintegrated cells forming 50–70% of the whole tumor mass. The collagen stroma was absent in the necrotic zone. Fluid was often present.

Tumors treated with lathyrogens showed a different picture. Those tumors labeled macroscopically as 'scab' had a thick, collagen capsule. Through the tumor tissue, there were several smaller areas of necrotic tissue not containing collagen structures. In between these foci were residual malignant cell islands, many of which displayed apoptotic cells on one side with either pycnotic nuclei or without nuclei. There was evidence of condensed collagen structures staining mostly blue, but, in some areas, the staining of collagen was red. In those tumors that grew to a larger volume, the staining was not as dramatic when more malignant cell areas were present. The change of collagen stainability is considered as evidence of collagen denaturation, during which process acidic glycosaminoglycans are detached, resulting in loss of the characteristic blue stain of the remaining collagen structures [32].

We encountered several problems during the course of the reported experiments. Utilizing a single tumor chunk to produce a growing tumor mass was effective, yet non-uniform in terms of reproducibility. This is understandable in view of the morphologic heterogeneity of the tumor. In spite of the large variability of the results, the inhibitory effect of both lipophilic lathyrogens was still evident due to the pronounced magnitude of the inhibition. The second problem was the leakage of the drug from the tumor tissue which is known for its high osmotic pressure. Our effort to mechanically pinch the injection port or to inject the drug through an intradermal tunnel was somewhat successful. We believe that once we identify the right polymer which will gel or solidify in the tissue shortly after injecting the drug, the problem of leakage will be solved. The breast adenocarcinoma tumor is closely attached to the skin and we noticed that any mechanical injury to the skin overlaying the tumor may allow additional leakage of the drug. Skin necrosis was a common occurrence in tumors from any group once their volume reached 5–6 cm^3.

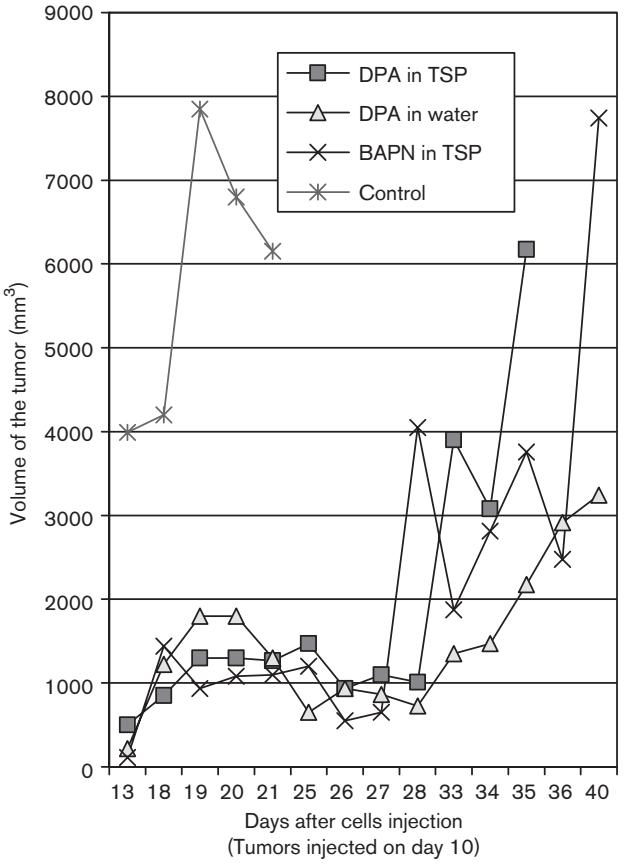
As shown in Figure 3, the growth of tumors in the rats with leakage of the lathyrogenic drugs was the same in control and treatment groups, and those retaining the treatment were all markedly inhibited. This finding reinforced our conclusion about the effective inhibition of the tumor by long-acting lathyrogens.

The determination of the effective dose for either lipophilic lathyrogen was based on the following considerations: Genovese *et al.* [33] reported that the effective dose for β-aminopropionitrile fumarate in the treatment of partial rupture of the superficial flexor tendon in a horse in local injection is 5 mg of the drug per lesion. Speer *et al.* [34] infused BAPN-free base into the stiff joint (related to increased crosslinking of collagen) at the daily dose of 3 mg/lesion. Nimni [22,23] found that the above-mentioned three effects of DPA on collagen structural stability depend on the actual concentration of the drug with the highest dose needed to chelate copper. As a result, we proposed the use of 3–7 mg of free lipophilic drug per day. After a single injection of the free lipophilic drug, we found an indication that it would remain locally effective in the tissue for 8–12 days. In order to document this statement, we present Figure 6. It shows tumor growth in selected rats from each treatment group. There is a regrowth of tumors 14 days after injecting a single dose of the drug. In some inhibited tumors, however, the growth was permanently inhibited and these tumors looked macroscopically as 'scab'. This discrepancy seems to relate to the actual volume of the injected drug retained inside the tumor. Therefore, a larger dose of 10–12 times higher than the recommended daily amount was administered through that injection. We took into consideration the whole structure of ester-DPA·HCl or 3-hexyl(imino)propionitrile where, in the first substance, free DPA represents almost 60% of the molecule, while free BAPN forms 45%. Based on these calculations, we had to prepare highly concentrated solutions or dispersions containing 30–200 mg of the lipophilic drug in 0.2 ml of the solvent. When we used the TSP as a carrier, its loading capacity was far exceeded, which could change the characteristics of the mixture with regard to the gelling and biodegradation.

Our study evaluated some basic parameters of tumor growth, appearance, morphology, metastases and

evidence of toxicity. The various proposed effects of DPA, as outlined in Table 2, can only be assumed. Still, the effectiveness of both drugs tested was evident and the inhibition lasted an average 2 weeks. The findings that HIPN produced the same inhibition as Me-DPA·HCl and BAPN, known as a very specific inhibitor of lysyl oxidase, led us to conclude that interaction with collagen crosslinking is the important mechanism for the inhibition of tumor growth for both lipophilic lathyrogens.

Fig. 6



Tumor growth in four representative rats.

The results of the combination treatment with dosages of CP, not inflicting any toxic effects, are most interesting and suggest possible clinical application.

In the last reported experiment the presence of metastases in all groups but those rats treated with a single injection of He-DPA·HCl and the later appearance of metastases in the group with combined treatment is most unexpected and definitely demands a more thorough study. We have no explanation why in several previous experiments with this cancer model we have not noticed any metastases even though the same methodology was utilized. Fast-growing tumors spread into lungs or liver sooner, already after 11–13 days. In the group with combined treatment the metastases appeared first on day 27. We can only speculate why He-DPA·HCl did not block the metastases in this group as when injected alone. It may be that CP at this low dose is not sufficiently cytotoxic, but only stimulates tumor cells and enhances the metabolism of the lipophilic drug. Such stimulation may also render the malignant cells more susceptible to the effect of DPA ester. It may be that CP as a strong immunosuppressive agent may cause increased metastatic manifestation of growing tumors.

A logical explanation for the absence of metastases in the DPA group would involve the inhibition of tumor metalloproteinases by chelation of zinc with DPA. Another explanation is that chelation of copper by DPA inhibits angiogenesis which is essential for formation of metastases. This view is supported by studies on tetrathiomolybdate, a new copper-chelating agent which successfully suppressed tumor recurrence and metastasis [36,37]. Other copper chelators, metabolites of disulfiram [38], are considered 'potential inhibitors of metastatic cells invasion and angiogenesis' [39]. It remains to be tested which of these two mechanisms (angiogenesis or metalloproteinases) or both are affected by these drugs to inhibit the spreading of the tumor.

Both lipophilic lathyrogens, BAPN and DPA, proved inhibitory to growing breast adenocarcinomas. This indicates the interference with collagenous structures, because of the specific inhibition of lysyl oxidase by

Table 2 Proposed mechanisms of antitumor activity of DPA [20,35–39]

Chelates copper as cofactor of lysyl oxidase, inhibits the enzyme responsible for the crosslinking of collagen. As a consequence, it blocks formation of collagen fibers (polymerization) and weakens mechanical strength of collagen structures (tumor stroma, pericapillary sheath, basement membrane of microvessels). By blocking copper, it inhibits angiogenesis.

By SH and NH_2 groups in adjacent position *cleaves non-reduced Schiff base crosslinks* in collagen, thus dissociating already formed, but not stabilized, collagen fibers.

Blocks existing aldehydes in collagen chains, formed by oxidative desamination of lysyl and hydroxylysyl residues in collagen by the effect of lysyl oxidase. By this mechanism, it also prevents polymerization of collagen molecules into fiber form.

Chelates zinc, which is an essential cofactor in all tumor metalloproteinases. These enzymes are involved in tumor metastases and angiogenesis.

By its SH groups *activates angiostatin*, which may explain another aspect of angiosuppressive properties of penicillamine. Angiostatin is an endogenous inhibitor of angiogenesis and tumor growth, inducing apoptosis of endothelial cells.

Inhibits urokinase-type plasminogen activator which is responsible for endothelial and tumor cells invasion [20]

As *an antioxidant*, decreases free radicals due to the presence of SH groups.

BAPN. Still, our preference is with the DPA esters due to the broad spectrum of functions of this drug.

The clinical application will concern all solid and solitary tumors, malignant or benign, which are reached by transcutaneous injection. This should not be a problem with today's quality of diagnostic radiology. The same treatment with long-acting lathyrogen can also be applied to any fibrotic pathology such as tendon adhesions, strictures or stenosis of esophagus, trachea, contractures of hypertrophic scars, where the crosslinking of collagen is the basis for the lesion. The effectiveness of both lathyrogens has been already documented by several laboratories as well as in clinical studies, when the drugs were given daily in systemic administration. Several toxic side-effects complicated this administration. In local administration of long-acting lathyrogen, no toxicity was observed as the dose of the drug is substantially lower and its local concentration high.

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