Single intratumoral injection of long-acting benzyl ester of D-penicillamine inhibits the growth of melanoma tumor in mice

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Using a murine model of melanoma we tested the effect of D-penicillamine administered in repetitive, daily injections, or as a single large dose injected either in saline or in a biodegradable polymer. We also studied the effect of a single intratumoral injection of benzyl-ester-D-penicillamine on the growth of the tumor. Daily injections of the drug or its administration in a polymer or benzyl-ester of D-penicillamine were all significantly inhibitory. The inhibitory effect manifested 4–5 days after injection. The inhibition lasted 8–10 days. There was no evidence of local or systemic toxicity and no changes in body weight. Several possible mechanisms for the inhibitory effect are presented.

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Introduction

In a recent study [1] we reported that a single injection of either 3-hexyl(imino)propionitrile or methyl- or hexyl-ester-D-penicillamine (ester-DPA) hydrochloride inhibited the growth of 13762 NM breast adenocarcinomas in Fisher rats. The effect was dose dependent and lasted on average 10–12 days. In one of the experiments, inhibition of the metastases occurred only in ester-DPA-treated rats. In other groups (control, cyclophosphamide alone or the combination of cyclophosphamide with ester-DPA) the incidence of tumor spread into the lung and liver was 50% of rats. As both β-Aminopropionitrile (BAPN) and ester DPA were equally effective, we concluded that the effectiveness is due to the alternation of collagen polymerization into mechanically strong fibers. Other effects, mainly of DPA, were also considered, especially the inhibitory effect on matrix metalloproteinases (MMPs).

The complexity of the effectiveness of both lathyrogens, mainly DPA, is shown in Figure 1 and is briefly summarized. β-Aminopropionitrile (BAPN) is a selective and irreversible inhibitor of lysyl oxidase (LOX). Other nitriles with a primary amino group were shown to inhibit the enzyme; however, BAPN was the most potent inhibitor [2]. The inhibition occurs when certain Lys and Hyl residues in collagen are not desaminated to form the corresponding aldehydes. Therefore, further non-enzymatic formation of covalent cross-links is prevented. As a consequence, the tensile strength and mechanical stability of collagen fibrils is compromised. Due to this effect, the interactions of various components of the extracellular matrix (ECM) are altered, as reflected in altered cell attachment, locomotion and propagation [3], sprouting of microvessels [4–6], formation of metastases [7], and regulation of MMPs activity [8].

DPA is a multifunctional drug affecting three different aspects of collagen structural stability, and having other effects related to its chelating capacity for copper and zinc. DPA inactivates LOX by binding copper at its active site. It acts by masking the aldehydes formed by LOX from defined Lys and Hyl collagen residues, thereby preventing polymerization into fibers. DPA also cleaves non-reduced Schiff base cross-links due to the presence of amino-thiol groups in adjacent positions. This effect is quite important in fast-growing tissues such as granulomas or tumors, where Schiff base cross-links mediate the dominant collagen fiber stabilizing force [1,9,10].

Several studies document the chelation of copper in the ceruloplasmin carrier and a copper decrease in tissues inhibits angiogenesis in various tumors [11–13]. Copper is also considered an obligatory factor for angiogenesis [11]. Conversely, zinc, an essential cofactor in all known MMPs, is sequestered by chelation from its histidine active site. MMPs are involved in remodeling and growth of tissues, tumors, angiogenesis, and metastases [14–17]. In addition, other effects of DPA have been proposed and documented (for details and references, see Fig. 1).

As outlined in our recent paper [1], several studies document the inhibitory effect of either BAPN or DPA on growth, collagen content, metastasis and angiogenesis

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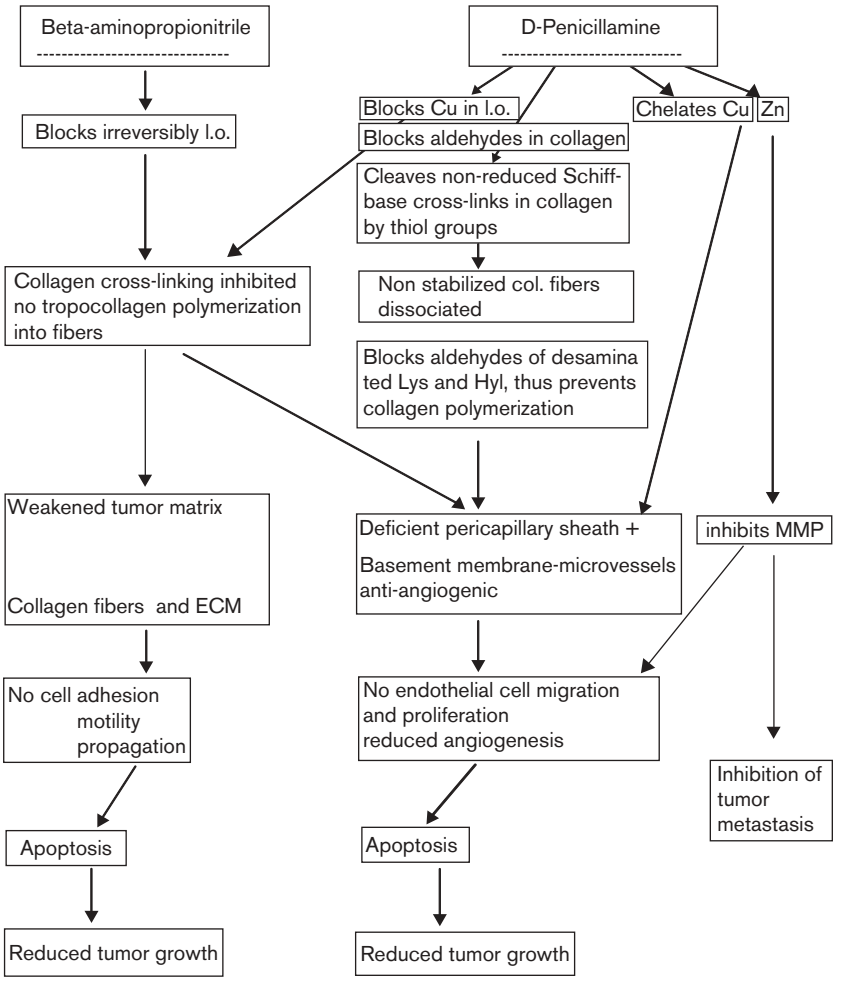
Fig. 1



Suggested mechanisms of action of two lathyrogens on tumor growth. The scheme does not include the reported effects on activation of angiostatin by SH groups of DPA [34], which may explain the angiosuppressive properties of DPA. Okuyama and Mishina [23] suggest that DPA inhibits the activity of superoxide dismutase, thus increasing the peroxidative damage to cells.

after systematic administration in the diet or parenteral injection [18–24]. The high water solubility of these drugs and their fast metabolism temper their efficacy due to the fast synthesis of LOX. This dictates the need for daily administration at relatively high doses [25]. Unfortunately, the incidence of toxic side-effects is quite common [26]. In order to overcome these problems we started to use either drug in topical formulations or local administration. The effective dose was 50 to 200 times lower that that needed in systemic administration [26]. However, daily continuous administration was still needed to achieve anti-tumor effectiveness [26].

In 1999 we initiated the development of long-acting DPA. The most promising and effective efforts involved the synthesis of various esters of DPA. We found that a single injection was effective for 8–12 days, at which time the tumor reduced in size and turned into a 'scab' form, or stopped growing, or started growing again. These observations were made on a model of breast adenocarcinoma in Fisher rats [1]. In this study we used a murine melanoma model and tested if this type of cancer is also inhibited by a single injection of the drug.

Method

Melanoma model in mice

We used a well-established model of murine melanoma in our laboratories [27,28].

Female mice, C57/Black, 20–25 g body weight, were injected s.c. in the right flank region with 0.1 ml containing 10^6 B16 melanoma cells. The cells are harvested at the end of the phase growth in tissue cultures using RPMI 1640 medium with 10% FBS enriched with L-glutamine, penicillin and streptomycin. Cells were harvested by trypsinization, counted and

suspended in sterile phosphate-buffered saline (PBS) for injection into the flank region.

It has been established that these cells and the amount injected resulted in a tumor volume of 250 mm^3 within 7 days post-injection. At this time the tumors were injected by a 28G needle. Control mice were injected with the same volume of sterile saline or a polymer used as a vehicle for the tested drugs. The polymer, Atrigel-30, is a biocompatible and biodegradable hydrogel. It is a copolymer of polyglycolic and polylactic acid of defined molecular weights and proportions, dissolved in *N*-methylpyrolidone. The polymer solution in contact with aqueous media gels which releases the incorporated drug by diffusion and corrosion of the gel.

In the last reported experiment we used B16-FO mice and injected s.c. the right flank region with 5×10^5 C57BI/J6 mouse melanoma tumor cells obtained from the ATCC (National Cancer Institute). A single injection in 0.1 ml distilled water was administered once the tumors reached 200 mm^3. According to the protocol approved by the Animal Experimentation Committee at the University of Arizona, the mice had to be terminated when tumors were larger than 2 cm^3.

The benzyl ester of DPA hydrochloride (Fig. 2) was dissolved in distilled water. The weight of the tumors was monitored 3 times per week using a caliper (Mituyo Digital; Small Parts, Phoenix, Arizona, USA) in two perpendicular directions and once per week for body weight. Tumor volume in cubic millimeters was calculated by the formula: (length $\times$ (width^2))/2. Individual animals were terminated if the tumor volume exceeded 20% of body weight or if the body weight loss exceeded 20% of the starting body weight.

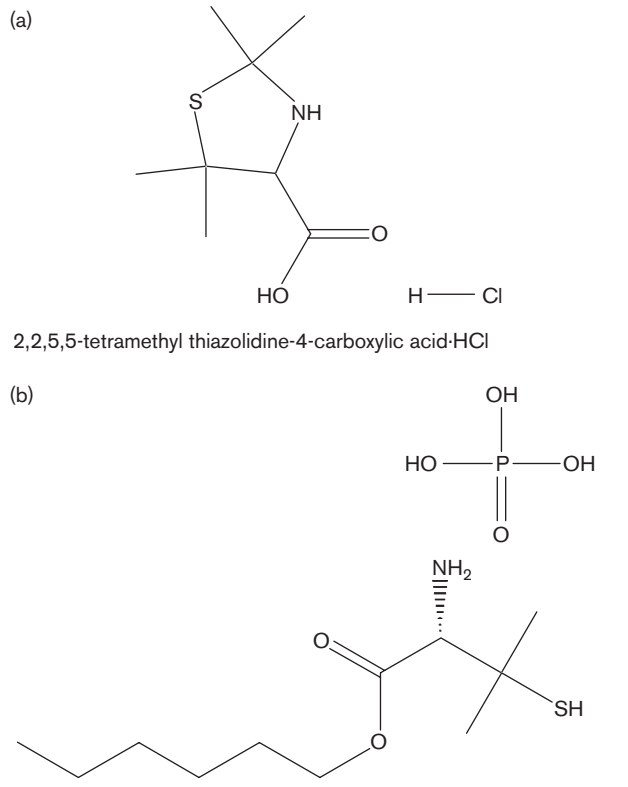
Tested drugs and their synthesis

DPA was purchased from Sigma (St Louis, Missouri, USA). 3-(Imino)propionitrile was obtained from Matrix Scientific (Columbia, South Carolina, USA). Atrigel-30 was obtained courtesy of Dr Richard Dunn (Atrix, Ft Collins, Colorado, USA). As mentioned, Atrigel is a hydrophilic biodegradable copolymer of polylactate and polyglycolate, the molecular size and their proportion regulating the rate of biodegradation. Atrigel-30 should be dissolved within 30 days in biological systems [29].

Synthesis of hexanal-DPA adduct

We followed the procedure of Weigert *et al.* [30]. To DPA dissolved in hexanol we added hexanal (Sigma) in a 2:1 molar ratio and heated the mixture in a water bath at 70°C for 2–3 h. After cooling, excess hexanol was removed by a vacuum. The oily substance formed remains fluid at room temperature. The structure and chemical formula of the adduct are shown Fig. 2(a).

Fig. 2



(a) Hexanal-DPA adduct. (b) Hexyl-ester-DPA phosphate.

Synthesis of benzyl-ester-DPA hydrochloride

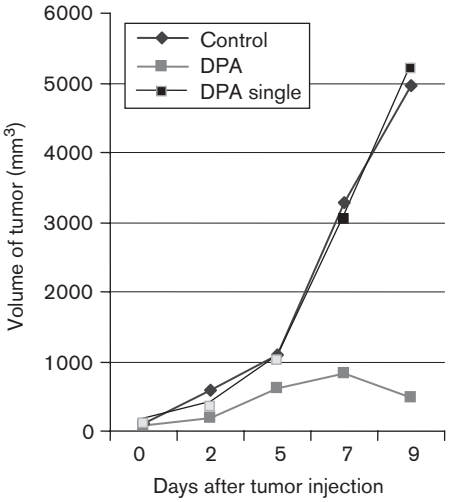
The compound was synthesized by F. Kielar and F. Liska in the Department of Organic Chemistry, Prague Institute of Chemical Technology (Czech Republic). DPA was heated for 6 h at 110°C in benzyl alcohol in the presence of polyphosphoric acid. The ester-DPA formed was then alkalized to pH 9.5 and extracted into an organic solvent. After evaporation of the solvent, the residue was dissolved in diethylether. Ester-DPA hydrochloride was obtained by introducing a hydrochloride gas into the ether solution. A suspension of white crystals formed and these were separated by filtration. The sediment was then washed repeatedly with ether. The yield was 37%. The chemical purity was checked by nuclear magnetic resonance analysis and was almost 100%. The formula is shown in Fig. 2(b). The ester in the form of hydrochloride is freely soluble in water. After neutralization of hydrochloride with PBS or tissue fluid, an emulsion is formed which is then slowly released into the tissue.

Results

Effect of single and daily injections of DPA on the growth of melanoma tumor

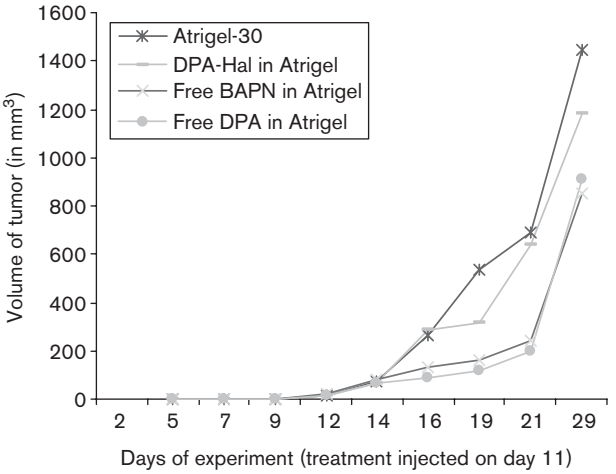
Seven days after injecting melanoma cells into the right flank area the tumors reached a mean volume of 1 cm^3.

Fig. 3



Effect of single or daily injections of DPA into melanoma tumor.

Fig. 4



Effect of DPA and BAPN on the growth of melanoma tumor.

There were 18 mice in this experiment, divided into three groups. Control mice received injections of 0.1 ml PBS into the tumor; DPA-treated tumors received either 5 mg of the drug in 0.1 ml PBS daily for the next 9 days or a single injection of 35 mg.

Significant inhibition of the melanoma growth at $p < 0.01$ was recorded 7 days after the first injection of DPA. There was no difference in tumor growth between controls and mice injected with a single dose of DPA (Fig. 3).

Effect of a single injection of hexanal-DPA adduct and DPA, both injected in Atrigel hydrogel

Atrigel solution, 0.1 ml, containing 35 mg of DPA was injected intratumorally as such or with 45 mg of the hexanal-DPA adduct.

Figure 4 shows that there was no difference between the growth of control tumors and those in hexanal-DPA adduct-treated mice. A significant effect ($p < 0.05$) of DPA administered in Atrigel was first recorded 5 days after the single injection and significant inhibition lasted only 5 days. After this time, the tumors started to grow and reached the values of the Atrigel- or hexanal-DPA-treated tumors.

The morphologic analysis (not shown) showed round melanoma cells with large melanin-containing vacuoles in Atrigel-treated tumors. Fine collagen septa surrounding the islands of cells were present all through the section. The hexanal-DPA adduct was identified in the tumor as numerous vacuoles without any effect on cell morphology. This contrasted with DPA-Atrigel-treated tumors: melanoma vacuoles were mostly released from the cells, which showed signs of degradation. Condensation of collagen structures within the tumor stroma was a prominent feature.

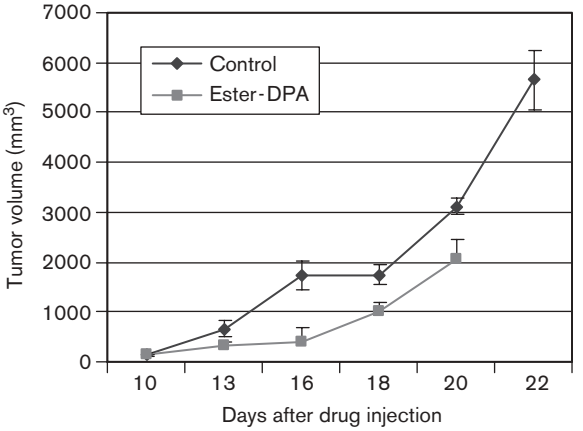
Growth of melanoma after a single intratumoral injection of ester-DPA hydrochloride

Nine days after cell inoculation the tumors reached a mean size of 200 mm^3. They were then injected with 50 µl of benzyl ester dissolved in distilled water. The control mice were injected intratumorally with the same volume of distilled water. The results are shown in Fig. 5. Inhibition of tumor growth was noticed 4 days after drug injections and became significantly different for the next 7 days. There were 12 mice in each group. Half of the ester-DPA-treated mice died 4 days after intratumoral injection. This indicated that the dose of 35 mg/tumor, calculated to be continuously released for 10–12 days, was high in the animals with a body weight of 20 g. The data also indicate that the inhibitory effect lasted at least 10 days.

Discussion

This study extends the evidence for the inhibitory effects of systemically or locally administered DPA on the growth of various tumors, i.e. breast adenocarcinoma [1], to melanoma in the SCID mouse model. Repetitive daily injections of this drug into the tumor were inhibitory to the growth of the tumors and no toxic side-effects were noticed. However, a single larger dose of DPA was not effective. This is not surprising as there are studies indicating that DPA is quickly metabolized and excreted from the body [25,31]. However, the most important finding is that a single injection of long-acting ester-DPA hydrochloride significantly inhibited the growth of the

Fig. 5



Effect of benzyl-ester DPA on the growth of melanoma. DPA injected on day 9 and 19.

tumor. This inhibition became significant 5 days after injecting the drug.

All the prior experiments described with breast adenocarcinoma [1] showed that the significant effect of DPA manifests itself only after 4–5 days. The reasons for this delay are not clear and only speculation can be offered. First, it may be that the inhibitory effect depends on reaching an effective concentration of 'free' drug in the tumor. Nimni [32,33] showed that the effects of DPA on the three collagen-related structure, polymerization are dose related. The highest dose is needed to block copper in LOX. Second, it may be that in order to manifest the effect of slower growth of the tumor, a definite degree of 'collagen deterioration' must be achieved. In this respect it is of interest that the same delay in the effect of DPA on collagen structure was reported by Friedrich and Zimmermann [31]. They found that rats receiving 320 mg DPA/kg body weight achieved a significant reduction in the breaking strength of skin after 7 days. After discontinuation of the treatment, the effect was reversible. Third, it may be that the rate of drug diffusion from the Atrigel depot is effect limiting. It was shown that the release of the drug from Atrigel occurs by diffusion through the solid hydrogel and by simultaneous biodegradation of the polymer [29]. This does not explain the presence of a lag period in using the ester-DPA in water. Finally, in the case of the benzyl-DPA ester, we assume that the change from a hydrophilic nature to an insoluble and slowly released drug or oily substance is formed after neutralization of the ester-DPA hydrochloride by tissue fluid buffering capacity. This mechanism is based on the following findings. (i) Determination of the partition coefficient for hexyl-DPA ester and benzyl-DPA ester between octanol/water showed high lipophilicity for both substances, hexyl-ester being more lipophilic. DPA was strongly hydrophilic. (ii) Paper chromatography of both esters of DPA hydrochloride eluted with PBS (pH 7.4, 0.15 M) showed 5 times slower mobility of esters when compared with DPA alone. (iii) Solutions of DPA esters as hydrochlorides after addition of PBS form a whitish solution emulsion with the presence of oily spots on the surface of the buffer solution. Based on these observations, we propose the following hypothesis for the long-acting effect of DPA esters. After injecting aqueous solutions of ester-DPA hydrochloride into the tumor tissue, the buffering capacity of tissue fluid (related mostly to phosphates, carbonates and proteins) transforms the esters into insoluble and oily emulsion forms. DPA is then slowly released from these depots, reaching the effective inhibitory effects within few days. At this time this seems to us plausible, although still very speculative.

The strongest argument that the effect of inhibition of tumor growth is related to interference with collagen metabolism and structure is supported by the finding that both tested drugs inhibited tumor growth [1]. BAPN is a very specific inhibitor of LOX, binding directly to the copper at the active site. This results in the inhibition of the formation of aldehydes derived from the oxidative desamination of some Lys and Hyl residues in collagen. In the case of the ester-DPA, the relationship to LOX inhibition is not clear. Several studies document hypocupremia after systemic DPA administration. This would limit the availability of copper for synthesis of LOX [34,35]. In our system, DPA was administered locally and unless DPA blocks copper carriers present in the tumor tissue, a direct effect of the drug on the active site copper could not be postulated. This remains to be tested.

In the case of DPA, several other mechanisms are possible due to the many functions of this drug. Future use of biochemical or histochemical parameters will allow for a more focused analysis of the various possible effects of DPA in the tumor tissue.

Murine models of melanoma are not adequate to test a single injection of drugs administered in a dose projected for delivery for 8–12 days. The weight of the mice brings the dose of the agent close to their LD_{50}. This was also the reason that in the last reported experiment we reduced the dose of ester-DPA. The increased mortality of mice after a single high-dose injection indicates that the kinetics of drug release may not be linear, but rather exponential, as the peak of the death incidence occurred 3 days after drug injection.

Our method of tumor treatment addresses the most universal aspect of tissue growth as related to the role of the ECM, especially its fibrillar components, in providing the scaffold for cells and vessels. In this respect the

intratumoral injection of long-acting drugs interfering with collagen structure and functions should be broadly applicable to any localized solid tumor.

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