Losartan inhibits collagen I synthesis and improves the distribution and efficacy of nanotherapeutics in tumors

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Contributed by Rakesh K. Jain, December 21, 2010 (sent for review October 20, 2010)

The dense collagen network in tumors significantly reduces the penetration and efficacy of nanotherapeutics. We tested whether losartan—a clinically approved angiotensin II receptor antagonist with noted antifibrotic activity—can enhance the penetration and efficacy of nanomedicine. We found that losartan inhibited collagen I production by carcinoma-associated fibroblasts isolated from breast cancer biopsies. Additionally, it led to a dose-dependent reduction in stromal collagen in desmoplastic models of human breast, pancreatic, and skin tumors in mice. Furthermore, losartan improved the distribution and therapeutic efficacy of intratumorally injected oncolytic herpes simplex viruses. Finally, it also enhanced the efficacy of i.v. injected pegylated liposomal doxorubicin (Doxil). Thus, losartan has the potential to enhance the efficacy of nanotherapeutics in patients with desmoplastic tumors.

Although nanotherapeutics have offered new hope for cancer treatment, their clinical efficacy is modest (1–4). This is partly because their penetration is hindered, especially in fibrotic tumors, where the small interstitial space in the interstitial retards the movement of particles larger than 10 nm (5–8). Pegylated liposomal doxorubicin (Doxil), approved by the Food and Drug Administration, and oncolytic viruses, currently in multiple clinical trials, represent two nanotherapeutics whose size (∼100 nm) hinders their intratumoral distribution and therapeutic effectiveness (9). Matrix modifiers such as bacterial collagenase, relaxin, and matrix metalloproteinase-1 and -8 have been used to modify the collagen or proteoglycan network in tumors and have improved the efficacy of intratumorally (i.t.) injected oncolytic viruses (8, 10–13). However, these agents may produce normal tissue toxicity (e.g., bacterial collagenase) or increase the risk of tumor progression (e.g., relaxin, matrix metalloproteinases).

Losartan (14)—approved to control hypertension in patients—does not have many of these safety risks. Furthermore, in addition to its antihypertensive properties, losartan is also an antifibrotic agent that has been shown to reduce the incidence of cardiac and renal fibrosis (15, 16). The antifibrotic effects of losartan are caused, in part, by the suppression of active transforming growth factor-β1 (TGF-β1) levels via an angiotensin II type I receptor (AGTR1)-mediated down-regulation of TGF-β1 activators such as thrombospondin-1 (TSP-1) (15–19). Using a dose that has minimal effects on mean arterial blood pressure (MABP), we show that losartan reduces collagen I levels in four tumor models—a spontaneous mouse mammary carcinoma (FVB MMTV PyVT), an orthotopic pancreatic adenocarcinoma (L3.6pl), and s.c. implanted fibrosarcoma (HSTS26T and melanoma (MuS9). Losartan also improves the intratumoral penetration of nanoparticles injected i.t. or i.v.

Based on these results, we tested how losartan would affect the distribution and efficacy of oncolytic herpes simplex viruses (HSV) administered i.t.—a widely used method of administration in patients for gene therapy (20–22)—and the efficacy of i.v. administered Doxil. Losartan improved the efficacy of both i.t. injected oncolytic HSV and i.v. administered Doxil. The results from our intratumoral experiments show that losartan enhances nanoparticle penetration in the interstitial space by improving interstitial transport. Additionally, the results from our i.v. studies indicate that losartan improves the efficacy of systemically administered nanotherapeutics to highly fibrotic solid tumors, such as pancreatic adenocarcinomas. Altogether, these results suggest that losartan, a Food and Drug Administration–approved antihypertensive drug, could potentially be used to improve the efficacy of various nanotherapeutics in multiple tumor types.

Results

Losartan Inhibits Collagen I Synthesis by Carcinoma-Associated Fibroblasts. We first determined the effect of losartan on the expression and activation of TGF-β1 and collagen I production by mammary carcinoma-associated fibroblasts (CAFs) (Fig. 1). Losartan did not affect the levels of total TGF-β1, but significantly (P < 0.05) reduced active TGF-β1 levels by 90%. It also significantly decreased collagen I synthesis by 27% (P < 0.04). Because collagen in tumors is mostly produced by CAFs, we next determined how losartan would affect the collagen content in tumors.

Losartan Decreases Collagen I in Tumors in a Dose-Dependent Manner. To determine the dose–response of losartan on intratumoral collagen levels, we injected 10, 20, and 60 mg kg−1 i.p. and performed second-harmonic generation (SHG) imaging of fibrillar collagen in HSTS26T tumors in dorsal skinfold chambers (Fig. 2) and collagen I immunostaining of tumor sections (Fig. 3). Collagen I and other fibril-forming collagens (e.g., collagen III, V) could contribute to SHG signal intensity. However, because collagen I is the predominant collagen type in most soft tissues (23), it is likely the main source of the SHG signal. Additionally, in human pancreatic tumors, collagen I is the main fibrillar collagen, with significantly lower levels of collagen V

Author contributions: B.D.-F., V.P.C., S.K., Y.B., and R.K.J. designed research; B.D.-F. and V.P.C. performed research; R.K.J. contributed new reagents/analytic tools; B.D.-F., V.P.C., Y.B., and R.K.J. wrote the paper.

Conflict of interest statement: R.K.J. received commercial research grants from Dyax, AstraZeneca, and Medimmune; consultant fees from AstraZeneca/Medimmune, Dyax, Astellas-Fibrogen, Regeneron, Genzyme, Morphosys, and Noxxon Pharma; and a speaker honorarium from Genzyme. R.K.J. owns stock in SynDevRx. No reagents or funding from these companies was used in these studies. There is no significant financial or other competing interest in the work.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1018892108/-/DCSupplemental.
Losartan doses of 20 and 60 mg·kg⁻¹·d⁻¹ significantly reduced intratumoral SHG signal intensity, whereas the lowest dose of 10 mg·kg⁻¹·d⁻¹ did not have a significant effect on SHG signal intensity (Fig. 2 A and B). The injection of losartan at 60 and 20 mg·kg⁻¹·d⁻¹ also significantly reduced the collagen I immunostaining in HSTS26T tumors by 65% and 42%, respectively (Fig. S1). Although treatment with the 60 mg·kg⁻¹·d⁻¹ dose led to the highest reduction in collagen I, we did not use this dose because it significantly reduced the MABP by 35 mmHg (P < 0.04; Fig. S2). Consequently, we chose the 20 mg·kg⁻¹·d⁻¹ dose for further study because after 2 wk of losartan treatment it only reduced the MABP by 10 mmHg (Fig. S2), thus maintaining the MABP within the normal range (70–95 mmHg) for severe combined immunodeficient mice (25). It also had no effect on mouse weight (average of 26 ± 1 g for treated vs. 26 ± 1 g for control). The 20 mg·kg⁻¹·d⁻¹ dose also decreased collagen I immunostaining in three other tumor types—FVB MMTV PyVT, L3.6pl, and Mu89—by 47% (P < 0.05), 50% (P < 0.03), and 20% (P < 0.02), respectively (Fig. 3 A–D).

Losartan Decreases TSP-1 Expression in Tumors. TSP-1 is a key regulator of TGF-β1 activation, and losartan has been shown to reduce TSP-1 expression and TGF-β1 activity in mouse models of Marfan’s syndrome and muscular dystrophy (19). The measurement of protein levels in homogenized HSTS26T tumors showed that losartan did not affect total TGF-β1 levels but significantly reduced TSP-1, active TGF-β1, and collagen I levels (Fig. S3). Losartan also decreased TSP-1 immunostaining in HSTS26T (73%, P < 0.04) and Mu89 (24%, P < 0.03) (Fig. S4).

In both Mu89 and HSTS26T tumors, the immunostaining patterns for TSP-1 and collagen I were closely matched (Fig. 3C; Fig. S4). We found high levels of TSP-1 and collagen I in the tumor margin, whereas losartan induced obvious reductions in TSP-1 and collagen I levels in the tumor center (Fig. 3C; Fig. S4). These data indicate that the reduction in collagen I levels could result in part from the decreased activation of TGF-β1 due to the losartan-induced reduction in TSP-1 expression.

Losartan Improves the Intratumoral Distribution of Nanoparticles and Nanotherapeutics. Based on our previous studies on the tumor interstitial matrix (6, 8), we hypothesized that a decrease in collagen content would improve the intratumoral distribution of nanoparticles. We therefore measured the intratumoral distribu-

![Fig. 1](image1.png) **Fig. 1.** Losartan reduces TGF-β1 activation and collagen I production in carcinosa-associated fibroblasts in vitro. Cells were treated with 10 μmol/L losartan for 24 h. Losartan reduced by 90% the active TGF-β1 levels, whereas total TGF-β1 levels were unaffected. There was a corresponding 27% decrease in collagen I levels. The reduction in active TGF-β1 and collagen I was statistically significant (Student’s t test, *P < 0.05).

![Fig. 2](image2.png) **Fig. 2.** Losartan reduces collagen production in tumors. (A) Over a period of 2 wk, there was a dose-dependent reduction in collagen levels assessed by SHG imaging in losartan-treated HSTS26T tumors (10, 20, and 60 mg·kg⁻¹·d⁻¹). (Scale bar, 200 μm.) (B) At the end of 15 d, losartan doses of 10, 20, and 60 mg·kg⁻¹·d⁻¹ decreased the SHG levels by 20%, 33%, and 67%, respectively. There was a statistically significant difference (*) between the control group and the two higher doses (20 and 60 mg·kg⁻¹·d⁻¹). There was also a statistically significant difference (†) between the 20 and 60 mg·kg⁻¹·d⁻¹ groups.

![Fig. 3](image3.png) **Fig. 3.** Losartan reduces collagen levels in tumors. (A) Collagen I (red) and nuclei (blue) immunostaining in tumor sections in L3.6pl and MMTV control and losartan (20 mg·kg⁻¹·d⁻¹)-treated tumors. (Scale bar, 100 μm.) (B) The 2-wk losartan treatment at 20 mg·kg⁻¹·d⁻¹ significantly reduced the collagen I immunostaining in L3.6pl and FVB MMTV PyVT by 50% (*P < 0.03) and 47% (*P < 0.05), respectively. (C) Collagen I (red) and nuclei (blue) immunostaining in tumor sections in HSTS26T and Mu89 control and losartan (20 mg·kg⁻¹·d⁻¹)-treated tumors. Note that there is no reduction in collagen I immunostaining at 200 μm from the edge of HSTS26T tumors. This phenomenon is less obvious in treated Mu89 tumors, where there is some persistent staining both at the edge and in central tumor areas. (Scale bar, 100 μm.) (D) Losartan significantly reduced the collagen I immunostaining in HSTS26T and Mu89 by 42% (*P < 0.02) and 20% (*P < 0.05), respectively.
tion of fluorescent polystyrene nanoparticles (100-nm diameter) in three different tumor types—HSTS26T, Mu89, and L3.6pl—after an i.t. or i.v. injection. In mice injected i.t. with nanoparticles, losartan improved nanoparticle accumulation and penetration in the tumor center (Fig. 4A) (HSTS26T, \( P < 0.001 \); Mu89, \( P < 0.001 \)). Conversely, there was little or no nanoparticle accumulation in the center of control tumors. Most of the injected nanoparticles in control tumors were found in the tumor margin and around the needle insertion point (Fig. 4A). We also determined the effects of losartan on the intratumoral distribution of oncolytic HSV. In both HSTS26T and Mu89, losartan significantly increased the intratumoral spread of HSV injected intratumorally (Fig. 4B). Whereas these data show that losartan increases the distribution of large nanoparticles, we also found that in HSTS26T it increased interstitial diffusion of IgG (Fig. S5) and the mean interstitial matrix pore radius from 9.91 ± 0.43 nm to 11.78 ± 0.41 nm, calculated based on IgG diffusion data (26).

We then tested the effect of losartan on blood vessel perfusion and the intratumoral distribution of i.t. injected nanoparticles in mice with orthotopic pancreatic tumors (L3.6pl). Losartan did not significantly change the fraction of perfused vessels in tumors (Fig. S6A). However, the intratumoral accumulation and penetration of beads away from blood vessels were significantly higher in losartan-treated tumors (Fig. 4C; Fig. S6B). These results indicate that losartan improves the transport and distribution of both i.t. and i.v. injected nanoparticles.

**Losartan Improves the Efficacy of Doxil and Oncolytic HSV.** We then determined whether losartan could improve the efficacy of i.t. injected oncolytic HSV and i.v. injected Doxil. The effect of losartan combined with the i.t. injection of HSV was determined in HSTS26T and Mu89 tumors. The administration of losartan alone did not affect the tumor growth rate (Fig. 5A and B). However, when animals were treated with losartan for 2 wk before i.t. injection of HSV, losartan significantly delayed (\( P < 0.001 \)) the growth in both Mu89 and HSTS26T tumors. Interestingly, the volume of HSTS26T tumors remained stable for up to 9 wk in 50% of mice treated with losartan and HSV. On the other hand, the growth delay in Mu89 tumors was only transient; 4 wk after the virus injection, all of the tumors were threefold larger than the starting treatment size.

To test whether losartan would increase the efficacy of a nanotherapeutic injected i.v., mice with orthotopic pancreatic tumors (L3.6pl) were treated with Doxil and losartan. Four weeks after tumor implantation and 2 wk after initiation of losartan treatment (20 mg·kg\(^{-1}\)·d\(^{-1}\)), we treated mice with a subtherapeutic dose of Doxil (4 mg·kg\(^{-1}\), i.v.). After 7 d, losartan or Doxil alone did not affect the mean tumor volume (Fig. 5C). However, in mice treated with losartan and Doxil, the tumors were significantly smaller (\( P < 0.001 \)) than in mice that received Doxil alone (Fig. 5C and D).

**Pattern of Collagen Distribution Governs the Effectiveness of Losartan.** To investigate the differences in response between HSTS26T and Mu89 to the losartan-HSV combination therapy,
we determined the HSV infection and necrosis patterns 21 d after the i.t. injection of HSV. First, we noticed striking differences between the collagen structure in Mu89 (Fig. 6A) and HSTS26T (Fig. 6B) tumors. These differences altered the virus propagation in the two tumor types. In Mu89 tumors, the collagen fiber network was well-organized and formed finger-like projections into the tumor (Figs. 6A and 7A). These projections divided the tumor into distinct compartments which could not be crossed by HSV particles, and thus the virus infection and resulting necrosis were restricted to the infected compartments (Fig. S7A). Losartan treatment disrupted the collagen projections to some extent but did not completely eliminate them (Fig. 6A). As a result, there was some cross-over of virus particles between compartments in losartan-treated Mu89 tumors. In contrast, in HSTS26T tumors, the dense collagen network was more diffuse, less fibrillar, and less compartmentalized (Figs. 6B and 7B). The dense collagen network seemed to slow down virus propagation but did not completely impede it, resulting in increased virus propagation and a more diffuse pattern of necrosis in this tumor (Fig. S7A).

Discussion

The renin-angiotensin-aldosterone system (RAAS) plays an important role in the regulation and production of extracellular matrix components (27–29). Angiotensin II in particular has been shown to stimulate collagen production via both TGF-β-dependent and -independent pathways (30). As a result, losartan and other RAAS inhibitors can reduce the levels of collagen I and III and basement membrane collagen IV in various experimental models of fibrosis (31, 32) and reverse renal and cardiac fibrosis in hypertensive patients (33, 34). Using four different tumor types, we show that losartan also inhibits collagen I production in tumors.

Other matrix modifiers, such as bacterial collagenase, relaxin, and matrix metalloproteinase-1 and -8, have been used to modify the collagen or proteoglycan network in tumors and have improved the efficacy of oncolytic virus injected intratumorally (8, 10–13). However, these agents may produce normal tissue toxicity (e.g., bacterial collagenase) or increase the risk of tumor progression (e.g., relaxin, matrix metalloproteinases). In contrast, losartan (14) has limited side effects and has been shown to reduce the incidence of metastasis in some tumor types (35).
Our findings strongly support the hypothesis that a reduction in collagen content by losartan improves interstitial transport and the intratumoral distribution of nanoparticles and nanotherapeutics. We also discovered that the organization of the collagen fibrillar network affects nanoparticle distribution. This was striking because of significant differences in the structural organization of fibrillar collagen I between Mu89 and HSTS26T. In Mu89 tumors, thick bundles of fibrillar collagen I surround the tumor margins and form finger-like projections which subdivide the tumor mass into isolated compartments and confine the viral infection to the injection site/isolated compartments (Fig. 7A). In contrast, HSTS26T tumors have a mesh-like collagen structure which hinders the virus spread but does not restrict viral particles to the injection site (Fig. 7B). The slower growth rate of HSTS26T compared with Mu89 tumors could also explain in part the enhanced efficacy of losartan combined with HSV in HSTS26T tumors. Our data also suggest that not only the collagen content but also the collagen network organization plays an important role in limiting the penetration of large therapeutics in tumors.

Pancreatic cancer patients treated with cytotoxic agents have a very high frequency of relapse with a 5-y survival of less than 5% (36). The poor vascular supply and increased fibrotic content of pancreatic tumors most likely play a significant role in limiting the delivery and efficacy of cytotoxics (37). We also show—in a mouse orthotopic model of human pancreatic cancer (L3.6pl)—that losartan increases both the intratumoral dispersion and extravascular penetration distance of i.v. injected nanoparticles. The increased distribution and extravasation of nanoparticles suggest that losartan might not only improve interstitial transport—as shown with the i.t. injections of nanoparticles and virus—but also transvascular transport. When used alone, losartan did not affect the growth of pancreatic tumors or the weight of treated mice. However, losartan combined with Doxil reduced the tumor sizes by 50% compared with Doxil treatment alone. These results suggest that losartan increased the tumor penetration and distribution and enhanced the efficacy of Doxil injected i.v. in orthotopic pancreatic carcinomas in mice.

The effects of losartan are not limited to the interstitial space. Modifications to the RAAS can also inhibit angiogenesis (38) or alter tumor blood flow (39, 40). Losartan blockade of AGTR1 can also reduce the production of vascular endothelial growth factor (VEGF) by cancer cells and the expression of VEGFR1 in endothelial cells, and inhibit tumor angiogenesis and growth (41, 42). In the present study, losartan did not affect tumor growth or the vascular density in HSTS26T tumors. Losartan can also reduce the proliferation of tumor cells expressing AGTR1 (43). We did not find a decrease in cancer cell proliferation (Fig S8) or tumor size in the human melanoma Mu89, which expresses AGTR1 (Fig. S9). The difference between our study and other studies might be due to differences in dosage. For example, in previous studies, the dose of losartan was up to 15-fold higher than in our experiments (41). We believe that a low dose of losartan will allow for a more clinically translatable protocol and avoid hypotensive complications.

Patients receiving RAAS antagonists have reduced incidence of breast and lung cancer (44). Several mechanisms have been proposed to explain the antitumor properties of RAAS antagonists (45–48). AGTR1 signaling has been shown to increase the proliferation of stromal and tumor cells and the transcription of inflammatory cytokines and chemokines that promote cancer cell migration and dissemination (49). The reduction in active TGF-β1 levels by RAAS antagonists could also reduce metastasis (50). Consequently, in addition to improving the delivery of antitumor agents, losartan may also inhibit tumor progression and metastasis.

To use losartan as an adjunct in the treatment of cancer patients, it is important to consider dosing and treatment schedules along with potential side effects. Results from our dose- and time-dependent studies suggest a minimum of 2 wk of losartan administration before antitumor treatment. To obtain maximum effects in patients, it might be prudent to initiate losartan treatment 2 wk before and continue it during the entire antitumor treatment schedule. Because long-term losartan therapy in hypertensive patients has been shown to have limited and manageable side effects and many antitumor agents (e.g., anti-VEGF drugs) have been shown to increase blood pressure (45), extended losartan cotherapy could be beneficial to cancer patients. For clinical studies, we suggest treating patients with a dose of 2 mg·kg·d−1, which is similar to that used for the treatment of patients with Marfan’s syndrome (51).

Although losartan and angiotensin II receptor blockers have limited side effects, losartan therapy is not recommended for patients with known renal disease. Losartan can induce renal insufficiency in patients with renal microvascular or macrovascular disease or congestive heart failure (52). Hyperkalemia can also occur in patients with poor renal function or patients who are concomitantly receiving potassium supplements or potassium-sparing diuretics. Finally, angioedema caused by high levels of circulating angiotensin II can occur in patients treated with losartan (52).

It is also important to consider tumor resistance to losartan therapy after extended treatment. Tumor drug resistance is thought to occur at many levels, including increased drug efflux, drug inactivation, evasion from apoptosis, and alterations in target pathways (53). Because losartan is not an antitumor agent, any potential resistance may result from other mechanisms. Given that TGF-β1 activation is induced by different agents such as matrix metalloproteinases and integrins in addition to TSP-1, tumor resistance to losartan could result from changes in TGF-β1 activation and signaling. Fortunately, long-term losartan therapy after myocardial infarction is not associated with a reduction in antifibrotic properties (54). It will be important to determine whether these results can be reproduced in tumors.

In conclusion, we show that losartan reduces the stromal collagen content in tumors and improves the penetration and therapeutic efficacy of nanoparticles (Doxil, HSV) delivered both intratumorally and intravenously. Losartan also exhibits vasoactive and antimetastatic properties that could increase its clinical application. Furthermore, because losartan is already approved for clinical use, it represents a safe and effective adjunct for improving the efficacy of nanotherapeutics in cancer patients.

Materials and Methods

A more detailed description of techniques is presented in SI Materials and Methods. Briefly, CAFs isolated from human breast cancer biopsies were treated with losartan for 24 h before measurement of collagen and cytokine levels. Protein assays were done with commercial ELISA kits. All animal experiments were done with the approval of the Institutional Animal Care and Use Committee of Massachusetts General Hospital. Losartan was administered i.p. at concentrations of 10, 20, or 60 mg·kg·d−1 for up to 2 wk. Mice were treated with HSV (i.t.) and Doxil (i.v. via tail vein) after 2 wk of losartan treatment. Excised tumors were either snap-frozen for biochemical analysis or fixed in paraformaldehyde and embedded in paraffin or optimum cutting temperature compound for immunohistochemistry.

ACKNOWLEDGMENTS. We acknowledge Drs. N. Kirkpatrick, D. Lacorre, and R. Guang for help in planning experiments, Dr. T. Stylianopoulos for help with manuscript preparation, and Dr. L. Fisher (National Institute of Dental Research) for kindly providing the LF-67 antibody. We thank Eve Smith and Sylvie Roberge for their technical assistance. This work was supported by US National Cancer Institute grants to R.K.J. (R01CA80124 and R01CA85140) and Y.B. (R01CA88706) and a Department of Defense Breast Cancer research fellowship to B.D.-F. (W91ZQF1010607) and innovator award to R.K.J. (WB81XWH-10-1-0016).
Supporting Information

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SI Materials and Methods

Cell Culture. Carcinoma-associated fibroblasts (CAFs) were isolated from human breast cancer biopsies using a previously described protocol (1). CAFs were plated in 24-well plates at a concentration of 0.5 million cells per well. Cells were allowed 24 h to adhere to the plates before the addition of losartan at 10 μmol/L for 24 h (2). Treatment was done in low serum to reduce background collagen levels. Conditioned medium was collected at the end of the 24-h treatment period and analyzed for collagen levels.

Protein Assays. Collagen I measurements were done with a type I C-terminal collagen propeptide ELISA kit (Quidel) and the Sircol soluble collagen assay (Biocolor). Transforming growth factor-β1 (TGF-β1) assays were performed with a human TGF-β1 ELISA kit (R&D Systems). The assay only measures the free form of mature TGF-β1. To measure total levels of TGF-β1, the latent form of TGF-β1 was activated with 1 N HCl. Thrombospondin-1 (TSP-1) assays were performed with a human TSP-1 ELISA kit (R&D Systems).

Mice and Tumor Models. All experiments were done with the approval of the Institutional Animal Care and Use Committee of Massachusetts General Hospital. Human soft-tissue sarcoma (HSTS26T) and human melanoma (Mu89) tumors were grown s.c. in the legs and dorsal skin fold chamber of severe combined immunodeficient (SCID) mice (3). Human pancreatic adenocarcinoma cells (L3.6pl) were grown orthotopically in the pancreas of SCID mice. L3.6pl tumors were induced with a subcapsular injection of 1 million cells in the tail of the pancreas. Tumor sizes were monitored in spontaneous FVB/N-Tg (MMTV-PyVT) 634MU1/J mice and tumors were selected for treatment when they reached a size of 4–6 mm in diameter (4).

Losartan Preparation and Treatment. Cozaar (losartan potassium) tablets were ground using a mortar and pestle. The powder was then dissolved in water to obtain a concentration of 2.5 mg/mL. Losartan was administered by daily i.p. injections at a concentration of 405 nm were used. SCID mice bearing HSTS26T tumors in dorsal chambers were either treated with losartan (10, 20, or 60 mg·kg⁻¹·d⁻¹) or saline for the duration of the dose–response experiment (15 d). Vascular markers were used to locate four regions of interest in each mouse and periodically returned to the same region of SHG imaging. SHG images were analyzed with a custom-built Matlab (The MathWorks) code. The fraction of the region of interest that was positive for the SHG signal was normalized to the amount of SHG signal obtained on day 1 of the dose–response study (before initiation of losartan or saline treatment).

Analysis of Herpes Simplex Virus Infection and Nanoparticle Distribution. Intratumoral injection. Nanoparticles and oncolytic herpes simplex viruses (HSV) were infused with a syringe pump (Standard Pump 22; Harvard Apparatus) at a flow rate of 4 μL/min. We injected 10 μL of HSV (2.5 × 10⁵ transducing units) expressing green fluorescent protein (GFP) or 10 μL of fluorescent nanoparticles (diameter of 100 μm; concentration of 1 × 10¹³ nanoparticles per mL). The injected tumors were resected 30 min after the nanoparticle injection and 24 h after the HSV infusion. Resected tumors were bisected at an angle perpendicular to the needle track, fixed in parafomaldehyde, and frozen in OCT. All tumor sections were obtained perpendicular to the angle of the needle track. The entire tumor section was imaged with a confocal microscope (BX61W1; Olympus) at 2× magnification and images were reconstituted as mosaics. The nanoparticle distribution and GFP-positive areas (HSV-infected cells) correspond to the fraction of pixels brighter than the background signal.

Intravenous injection. A total volume of 10 μL at a concentration of 3.6 × 10¹³ nanoparticles per mL was injected via the tail vein. Twenty-four hours later, 50 μL of FITC-lectin was injected to identify functional vessels. Five minutes after the lectin injection, tumors were resected, fixed in parafomaldehyde, and embedded in OCT. Tumors were then sectioned before confocal imaging and analysis. The extent of nanoparticle distribution was determined by measuring the fraction of pixels brighter than the background signal. Nanosphere penetration was determined by drawing contours around perfused vessels and recording the fraction of pixels positive for nanospheres in each contour. Contours extended out to 30 μm for each perfused vessel. Using a previously described algorithm (8), we fit the plot of nanosphere fraction and distance away from the vessel to an exponential and obtained a relative penetration depth of nanospheres from each vessel.

Diffusion Measurements by Fluorescence Recovery After Photo-bleaching. Mice with HSTS26T tumors implanted in a dorsal skin fold chamber were treated with i.p. injections of losartan (40 mg·kg⁻¹·d⁻¹) for 1 wk. Fluorescence recovery after photo-bleaching (FRAP) measurements were done with a custom-built multiphoton microscope based on a previously described
All our animal experiments were conducted with at least 121:335. All statistical analyses involving RNA was extracted using an RNeasy Mini Kit and spatial Fourier analysis FRAP (SFA-FRAP) about 10 min after the injection. Matrix pore sizes were calculated using the SFA-FRAP data, using the equation \( D = \frac{1 - 2 \ln(1 + \frac{2 \lambda D}{\ln(1 + \frac{2 \lambda D}{\lambda})}) - 1.706 \lambda^{2} + 0.72D^{2}}{\lambda^{2}} \), where \( D \) is the diffusion coefficient for the probe molecule in the tumor, \( D_{0} \) is its diffusion coefficient in water, and \( \lambda \) is the ratio of the probe hydrodynamic radius to the pore radius (10).

Analysis of HSV Infection, Necrosis, and Collagen Structure. To determine the relationship between virus infection, necrosis, and collagen structure 21 d after HSV injection, consecutive paraffin sections were stained with either a polyclonal HSV-1 antibody (Dako). The slides were then treated with 0.05% trypsin before antigen retrieval with Target Retrieval Solution (pH 9). The entire tumor section was imaged at 20× magnification. The fraction of Ki67-positive cells was determined by counting Ki67-stained nuclei in each tumor. The fraction of Ki67-positive cells in each region was determined by manual count.

Doxil Treatment and Tumor Growth Delay. Two weeks after the implantation of orthotopic pancreatic L3.6pl tumors, mice were randomly selected for losartan or saline treatment. A subtherapeutic dose of Doxil (4 mg/kg) was infused i.v. via the tail vein after 2 wk of losartan treatment (20 mg·kg\(^{-1}\)·d\(^{-1}\)). One week after the Doxil injection, the tumors were resected and measured.

Virus Treatment and Tumor Growth Delay. SCID mice bearing s.c. HSTS26T and Mu89 tumors were randomly divided into control and losartan-treated groups. Each arm (control and treated) was subsequently divided into HSV-treated and non-HSV-treated groups. Tumors that had reached 60 mm\(^{2}\) after 2 wk were selected for i.t. HSV injections. Tumors were treated with 10 μL i.t. injections of either PBS or 2.5 × 10\(^{6}\) transducing units of oncolytic HSV MGH2 (gift from E. Antonio Chiocca, Ohio State University, Columbus, OH). Two i.t. injections of oncolytic HSV separated by 24 h were administered. The injections were done with a Harvard Apparatus Standard Pump 22 infusion/withdraw syringe pump system at a flow rate of 4 μL/min. Tumors were measured every 2–3 d. Tumor volume was estimated as \( V = \frac{A \cdot B}{2} \), where \( V \) is the tumor volume, and \( A \) and \( B \) are the maximum and minimum diameters of the tumor as measured with calipers, respectively.

Statistics. All our animal experiments were conducted with at least six mice in each treatment arm. The tumor growth delay studies in HSTS26T and Mu89 tumors were done with at least eight mice in each group. The rationale for the number of mice used was based on power calculations in our previous studies (11, 12), which showed that we needed at least eight mice in each group to reach statistical significance (\( P < 0.05 \)). All statistical analyses involving two groups were done using a Student’s \( t \) test. A \( P \) value lower than 0.05 was considered significant. For multiple groups, a one-way ANOVA test followed by a Tukey’s post hoc test was used to determine statistical significance between groups. Statistical significance in the figures is identified by an asterisk.

**Fig. S1.** Dose-response of losartan versus collagen content in HSTS26T tumors. Losartan treatment at 20 and 60 mg \text{kg}^{-1} \text{d}^{-1} led to a 42\% and 63\% reduction in collagen I staining, respectively. The staining in each treatment group was compared with a control group that received saline.

**Fig. S2.** Losartan decreased the mean arterial blood pressure (MABP) in mice in a dose-dependent manner. Although 20 mg \text{kg}^{-1} \text{d}^{-1} decreased MABP by 10 mmHg (**P < 0.04), the MABP remained within the normal range for SCID mice (70–95 mmHg) (1). Conversely, when animals were treated with 60 mg \text{kg}^{-1} \text{d}^{-1}, the 35-mmHg (**P < 0.04) drop in MABP was lower than the normal-range MABP in SCID mice.


**Fig. S3.** Losartan effects on TSP-1, TGF-\(\beta\)1, and collagen I in HSTS26T tumors. Treated animals received losartan (15 mg \text{kg}^{-1} \text{d}^{-1}) in drinking water. Tumors were excised after 2 wk of treatment, homogenized, and analyzed for total and activated TGF-\(\beta\)1 levels by ELISA. Note a 3.5-fold reduction in TSP-1, a 4-fold reduction in active TGF-\(\beta\)1, and a 2-fold reduction in collagen I after losartan treatment (**P < 0.05).
Fig. S5. Changes in diffusion coefficient in HSTS26T tumors after losartan treatment. The diffusion coefficient of IgG was measured in HSTS26T tumors implanted in the dorsal window of SCID mice. Treated animals received (40 mg kg−1·d−1) losartan by i.p. injection, whereas control animals received saline. The results show a significant increase (*P < 0.04) in diffusion coefficient as measured by multiphoton FRAP.

Fig. S4. (A) Losartan decreases tumor TSP-1 immunostaining in both Mu89 and HSTS26T tumors. In HSTS26T tumors, the changes in TSP-1 after losartan treatment correspond to changes in collagen I immunostaining. TSP-1 levels decrease in the tumor center but remain high within 200 μm from the edge of the tumor. The TSP-1 margin was larger (500 μm from the edge) in Mu89 tumors. (Scale bar, 100 μm.) (B) Losartan treatment significantly reduced TSP-1 immunostaining in HSTS26T and Mu89 tumors by 73% (*P < 0.04) and 24% (*P < 0.03), respectively.
Fig. S6. (A) Losartan does not increase the total number of perfused/functional vessels. (B) Penetration depth of nanospheres as a function of the distance from a tumor vessel. The nanosphere penetration depth was analyzed in frozen sections from tumors resected 24 h after the i.v. nanosphere injection. The mean characteristic penetration length increased from 18 ± 5 μm (mean ± SE) in control to 37 ± 6 μm in losartan-treated tumors. Ten areas per tumor were analyzed in six control and six treated tumors.

Fig. S7. (A) Virus infection (HSV immunostaining) and necrosis 21 d after HSV injection in HSTS26T and Mu89. The blue, white and pinkish, and brown colors correspond to hematoxylin staining of intact tumor areas, necrosis, and HSV immunostaining, respectively. Necrotic regions are also indicated by black arrowheads. Even though there was no difference in necrotic area between HSTS26T and Mu89, necrosis is confined to specific regions in Mu89, whereas there is necrotic tissue (bounded by HSV immunostaining) throughout HSTS26T tumors. (Scale bar, 2 mm.) Contrast was adjusted to highlight necrotic areas. (B) There is a twofold increase (*, **P < 0.05) in necrosis in tumors (both HSTS26T and Mu89) that received losartan before HSV injection.
In vivo proliferation rates for HSTS26T and Mu89 after losartan treatment. Tumors were resected and stained for Ki67 to assess proliferation. There was no statistically significant difference in Ki67-positive cells after losartan treatment in HSTS26T and Mu89 tumors. There was, however, a significant difference in proliferation between the two tumor types: The number of Ki67-positive cells was threefold higher in HSTS26T tumors.

PCR analysis of AGTR1 expression in CAF, Mu89, and HSTS26T cells. Mu89 cells and CAFs express AGTR1, whereas HSTS26T cells do not. Human umbilical vein endothelial cells were used as a positive control. GAPDH levels revealed that all three samples had roughly the same amount of cDNA.