Tumor-Priming Smoothened Inhibitor Enhances Deposition and Efficacy of Cytotoxic Nanoparticles in a Pancreatic Cancer Model

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Abstract

Most pancreatic adenocarcinoma patients present with unresectable disease and benefit little from chemotherapy. Poor tumor perfusion and vascular permeability limit drug deposition. Previous work showed that Smoothened inhibitors of hedgehog signaling (sHHI) promote neovascularization in spontaneous mouse models of pancreatic cancer (PaCa) and enhance tumor permeability to low-molecular weight compounds. Here, we tested the hypothesis that sHHI can enhance tumor deposition and efficacy of drug-containing nanoparticles consisting of 80 to 100 nm sterically-stabilized liposomes (SSL) containing doxorubicin (SSL-DXR). SCID mice bearing low-passage patient-derived PaCa xenografts (PDX) were pretreated p.o. for 10 days with 40 mg/kg/d NVP-LDE225 (erismodegib), followed by i.v. SSL-DXR. Microvessel density, permeability, perfusion, and morphology were compared with untreated controls, as was SSL deposition and therapeutic efficacy. The sHHI alone affected tumor growth minimally, but markedly increased extravasation of nanoparticles into adenocarcinoma cell–enriched regions of the tumor. Immunostaining showed that sHHI treatment decreased pericyte coverage (α-SMA+ vascular endothelium structures, and increased the abundance of endothelium-poor (CD31+) basement membrane structures (collagen IV+), suggesting increased immature microvessels. SSL-DXR (15 mg/kg) administered after sHHI pretreatment arrested tumor volume progression and decreased tumor perfusion/permeability, suggesting an initial vascular pruning response. Compared with controls, one cycle of 10-day sHHI pretreatment followed by 6 mg/kg SSL-DXR doubled median tumor progression time. Three cycles of treatment with sHHI and SSL-DXR, with a 10-day between-cycle drug holiday, nearly tripled median tumor progression time. Based upon these data, short-term sHHI treatment sequenced with nanoparticulate drug carriers constitutes a potential strategy to enhance efficacy of pancreatic cancer therapy.

Introduction

Pancreatic cancer (PaCa) afflicts approximately 46,000 annually and 5-year survival is just 6% (1). Advanced or disseminated disease at diagnosis precludes surgery with curative intent for 80% of patients, and neither first-line- nor newer approved agents offer sustained benefit to most patients (2, 3).

Advanced disease stage contributes considerably to the poor prognosis for PaCa, but additional factors hinder therapy. High interindividual pharmacodynamic variability is the result of prevalent genetic mutations; one small-scale transcriptional analysis identified >60 genetic alterations in PaCa that suggested derangement of approximately 12 signaling pathways in 70% to 100% of tumors (4). The diverse mutations enable tumors to adapt to chemotherapy, and molecularly targeted agents seldom control disease progression. Because of interindividual variability, standardized regimens have failed to yield significant improvement in survival, and treatment regimens are needed that address patient-specific tumor biology (5).

A major factor contributing to treatment resistance is inadequate tumor drug delivery arising from the low perfusion, permeability, and microvessel density that is characteristic of PaCa (6–8). Desmoplastic stroma is implicated in nurturing tumor cell progression and contributing to the drug delivery barrier (7, 9–11). Stroma consists of numerous cell types, including fibroblasts, pancreatic stellate, inflammatory, and vascular endothelial cells, the majority of which are non-neoplastic (6, 12). Stromal components collaborate with malignant cells in both autocrine and paracrine interactions (13–15). In contrast with other solid tumors, in which stromal fibroblasts assist in angiogenesis and drive tumor progression, some evidence suggests the amplified network of extracellular matrix and activated fibroblasts in desmoplastic stroma impedes the formation of functional vasculature in PaCa, resulting in sparse, inadequate microvessels that are physically distant from the cancer cells (10, 16–19). Given the potential role of stroma in supporting PaCa tumor growth, malignancy, metastasis, and treatment resistance (6, 9, 20), and
the fact that stromal cells do not share the high mutation frequency of the adenocarcinoma cells (6, 12), stroma represents a potential target for PaCA therapy (10). Multiple lines of evidence suggest that in PaCA, modulation of the tumor microenvironment may improve tumor perfusion and therefore delivery of chemotherapeutic agents (10, 14). Paracrine activation of hedgehog (HH) signaling in PaCA is implicated in inducing tumor-associated fibroblasts to produce tumor-promoting factors (21). At least 7 Smoothened (SOMO) inhibitors that interdict hedgehog signaling (sHHI) are in clinical trial or approved, and activity is observed in some patients (11, 15, 22–24). One preclinical report demonstrated that pharmacologic disruption of HH signaling decreased activated tumor fibroblasts, increased microvessel density and permeability to small-molecule drugs or contrast agents, and enhanced the therapeutic activity of gemcitabine in a spontaneous, genetically engineered mouse model of pancreatic adenocarcinoma (7).

Here, we investigated whether SMO inhibitors could increase PaCA tumor vascular permeability to nanoparticulate drug carriers, and tested the hypothesis that sequencing sHHI with 80 to 100 nm nanoparticles, consisting of sterically-stabilized liposomes (SSL) containing DXR (similar to FDA-approved Doxil), could improve treatment efficacy by enhancing tumor deposition of drug-loaded nanoparticles. Previously we demonstrated in a poorly permeable intracranial brain tumor model that SSL-DXR can establish an intra-tumor drug depot that persists for 7 to 10 days and mediates progressive antitumor and antivascular effects (25, 26). Therefore, we also tested whether repeated cycles of sHHI priming followed by nanoparticulate drug carriers could improve PaCA antitumor efficacy. Given the interindividual variability of pancreatic cancers, and the translational relevance of patient-derived tumors as models for the development of therapeutic regimens (5, 27, 28), we selected from a panel of engrafted, low-passage, patient-derived PaCa adenocarcinomas that resemble closely the clinically encountered disease in terms of stromal content, extracellular matrix architecture, microvessel density, and poor vascular permeability/perfusion, and retain these characteristics through multiple passages in SCID mice (29). Treatment-mediated alterations in perfusion and permeability were monitored using i-injected probes that included 80 to 100 nm nanoparticles. Vascular status was investigated by immunofluorescence, and tumor volume progression was the endpoint for efficacy by enhancing tumor deposition of drug-loaded nanoparticles. Previously we demonstrated in a poorly permeable intracranial brain tumor model that SSL-DXR can establish an intra-tumor drug depot that persists for 7 to 10 days and mediates progressive antitumor and antivascular effects (25, 26). Therefore, we also tested whether repeated cycles of sHHI priming followed by nanoparticulate drug carriers could improve PaCA antitumor efficacy. Given the interindividual variability of pancreatic cancers, and the translational relevance of patient-derived tumors as models for the development of therapeutic regimens (5, 27, 28), we selected from a panel of engrafted, low-passage, patient-derived PaCa adenocarcinomas that resemble closely the clinically encountered disease in terms of stromal content, extracellular matrix architecture, microvessel density, and poor vascular permeability/perfusion, and retain these characteristics through multiple passages in SCID mice (29). Treatment-mediated alterations in perfusion and permeability were monitored using i-injected probes that included 80 to 100 nm nanoparticles. Vascular status was investigated by immunofluorescence, and tumor volume progression was the endpoint for antitumor efficacy of single- and repeated cycles of sHHI sequenced with SSL-DXR.

Materials and Methods

Materials

Doxorubicin-HCl, cholesterol: Sigma-Aldrich; NVP-LDE225: Novartis; phospholipids: Avanti Polar Lipids; dioctadecyl-3,3’,3’-tetramethylindocarbocyanine disulphonate (DiIC8(5-DS)): Invitrogen; monoclonal antibodies against α-SMA, NG2, CD31, collagen IV; Millipore-Chemicom.

SSL Preparation

A dried film of 9:5:1 mol:mol:mol distearoylphosphatidylcholine:cholesterol:polyethyleneglycol-derivatized phosphatidylethanolamine was hydrated with 250 mmol/L ammonium sulfate to a lipid concentration of 20 mmol/L, extruded through 80 nm polycarbonate filters (25, 26, 30), and dialyzed against isotonic sucrose. The liposomes were remote-loaded (30, 31) with 10.5 mg/mL DXR (drug:lipid 0.25:1 mol:mol). After dialysis to remove traces of unencapsulated DXR, phospholipid was quantified by phosphorous assay (30) and encapsulated drug by absorbance. Liposome diameter was 80 to 100 nm based upon dynamic light scattering. Encapsulation efficiency was approximately 99%.

Fluorescent SSLs (SSL-DiI) were prepared as described above, except the phospholipids included 0.1 mole% of the nonexchangeable membrane label DiIC8(5)-DS (32, 33), lipid was hydrated with buffered saline, and drug loading was omitted.

Tumor implantation and treatment

Patient-derived pancreatic adenocarcinoma #18269, established in SCID mice at Roswell Park Cancer Institute (Buffalo, NY), was maintained as described (29). Briefly, s.c. tumors (passage 6–7) were harvested from donor mice, cut into 2 × 2 × 2 mm blocks under RPMI-1640 medium, and implanted s.c. on the abdominal wall of anesthetized 18 to 20 gm C.B-lgh-1/TcrTac-Prkdc<sup>scid</sup> mice.

Treatments was initiated 4 to 6 weeks after implantation, when tumors were 100 to 500 mm<sup>3</sup>. Immunohistochemistry (IHC) and permeability/perfusion experiments used ≥3 mice/group/time point, and therapeutic groups used ≥6. Treatments included: vehicle controls, a 10-day course of 40 mg/kg/d NVP-LDE225 in 0.5% methylcellulose/0.5% Tween 80 (34) by oral gavage, a 10-day NVP-LDE225 course followed by i.v. SSL-DXR on the 10th day (d10), and SSL-DXR alone administered on the equivalent of d10.

Vascular permeability/perfusion assessment

Hoechst 33342 (H33342) and SSL-DiI were used as low- and high-mass fluorescent probes of tumor vascular perfusion and permeability (30). At intervals following the sHHI pretreatment (±SSL-DXR on d10, the final day), 1 μmole SSL-DiI was injected i.v. H33342 (15 mg/kg) was administered i.v. 24 hours later, 20 minutes before sacrifice. The tumor was excised rapidly and bisected, with half embedded in mounting medium and frozen in liquid nitrogen, and half formalin fixed and paraffin embedded. To identify regions of cellularity in nonperfused tumor areas, some tissue sections were counterstained with H33342 (2 μg/mL for 2 minutes) after acquisition of SSL-DiI fluorescence data.

Immunofluorescence

Tumor sections were fixed with ice-cold acetone, washed in Dulbecco PBS containing 0.5% Tween20, incubated in blocking buffer, and incubated 1 hour at 20°C with primary antibodies (diluted 1:100). After washing thrice in PBS/Tween, sections were incubated with secondary antibodies [fluorescein-labeled anti-CD31, AMCA (aminomethylcoumarin-acetic acid)-labeled anti-collagen IV, Cy5-labeled anti-α-SMA] for 30 minutes at 20°C, and then washed, dehydrated, and mounted with anti-fade medium.

Image acquisition

DiI and Cy5 were imaged using Zeiss filter set #50 on an Axiovert 200M fluorescence microscope. Standard FITC and DAPI filter sets were used for fluorescein and AMCA/H33342. Panoramas encompassing the entire tumor were acquired using a 20X/0.75 lens under constant exposure conditions for all sections. Intensity was quantified from whole tumor panoramas using ImageJ (NIH, Bethesda, MD; http://imagej.nih.gov/ij/), whereas microvessel counts were done on multiple individual images (N = 5–12) comprising the panoramas. Statistical testing.
Results

Oral dosing with SMO inhibitors was reported to increase tumor microvessel density, vascular perfusion/permeability, and deposition of small-molecule chemotherapeutic agents in a hypovascular transgenic PaCA model (7). Here, we investigated sHHI treatment effects on tumor vascular morphology and permeability, and tested the hypothesis that sHHI treatment could promote extravasation of long-circulating, 80 to 100 nm nanoparticulate drug carriers in a hypovascular PaCA model, thereby enhancing tumor drug deposition, exposure, and efficacy.

PaCA tumor characteristics

The low-passage, histopathologically verified patient-derived pancreatic ductal adenocarcinoma #18269 (Supplementary Figs. S1 and S2A) was selected for these studies based on its low microvessel density, which is comparable with human PaCA tumors (7), abundant stroma, and retention of those characteristics through passage in mice (29). Tumor vascular permeability/perfusion was exceedingly low, as evaluated in preliminary experiments by dynamic contrast-enhanced MR imaging: virtually no enhancement was observed with low-(Gd-DTPA) or high-mass (Gd-albumin; ref. 35) permeability probes, consistent with histologic characteristics.

sHHI effects on tumor perfusion and permeability to nanoparticles

Our strategy was to use a short-term sHHI pretreatment at the lowest daily dose necessary to suppress Gli1 expression continuously. Ten days’ dosing with NVP-LDE225 at 40 mg/kg/d was selected based upon published reports of (i) tumor vascular permeability compromise after 8 to 12 days’ dosing with sHHI (7), (ii) Gli1 suppression for 24 hours for this dose range (34, 36), and (iii) the emergence of functional sHHI resistance after 13 days’ treatment (36). qRT-PCR with species-specific probes verified >95% suppression of stromal (murine) Gli1 for 24 hours after a single oral dose. Fluorescent 80 nm liposomes (SSL-DiI) were injected i.v. to probe vascular permeability on d10 (the final sHHI dosing day) and d13. Twenty minutes before sacrifice at 24 hours, the peak time for SSL tumor deposition (26, 37, 38), Hoechst 33324 was injected i.v. as a perfusion marker.

Vehicle-treated control animals showed little deposition of H33324 or SSL-DiI, except for limited extravasation around the tumor; bar, 500 μm. Right column, higher-magnification composite images showing 3 to 4 mm² field of the tumor; bar, 500 μm. Results included Mantel–Cox and unpaired, one-tailed Student t tests, implemented in Prism5.0d (GraphPad).

Figure 1.
sHHI effects on tumor vascular permeability to nanoparticles. Mice (n = 6) bearing patient-derived pancreatic adenocarcinoma #18269 received NVP-LDE225 (40 mg/kg/d) or vehicle p.o. for 10 days. On d10, n = 3 control- and sHHI-treated animals were administered SSL-DXR i.v. (15 mg/kg). Three days later, all received fluorescent 80 nm SSL-DiI i.v. as a vascular permeability probe. H33342 (15 mg/kg) was injected i.v. as a perfusion probe 24 hours later, 20 minutes before sacrifice. Tumors were flash-frozen, sectioned, and panoramas encompassing the tumor were acquired using constant exposure conditions. Left column, low-magnification composite images showing 3 to 4 mm² field of the tumor; bar, 500 μm. Right column, higher-magnification image of tumor; bar, 50 μm. A and B, representative vehicle control tumor; probe deposition was low, occurring primarily near the superficial veins on the surface. B, SSL-DiI remained vascular/perivascular, whereas Hoechst diffused somewhat into tumor. C and D, tumor from sHHI-only animal; crescents of SSL-DiI deposition correspond to regions of columnar tumor cells organized around glandular structures (dark voids). E and F, tumor from sHHI-treated animal (same as C and D) except SSL-DXR was administered 4 days before, on d10 of sHHI treatment. Deposition of the high-mass permeability probe was reduced drastically. G and H, tumor from animal treated with SSL-DXR alone on the equivalent of d10 for sHHI-treated animals, showing little extravasation of 80 nm SSL-DiI despite some Hoechst permeation.
voids in fluorescence images. Higher-magnification images showed little organization of the SSL-DiI fluorescence (Fig. 1D), suggesting intra-tumor diffusion of extravasated SSL-DiI in sHHI-treated animals.

Previously we reported that extravasation of SSL-DXR in a low-permeability intracranial brain tumor model can establish a drug depot proximal to tumor microvessels that persists for more than 7 days (26). The initial tumor response (3–4 days after dose) was a profound reduction in chaotic microvessel density, vascular endothelial cells, perfusion, and nanoparticle deposition (30), which progressed to increased tumor vascular permeability to nanoparticles over the following 7 to 10 days (25, 26). Here, we investigated whether similar temporal effects were mediated by SSL-DXR in PaCa tumors. On the 10th day of sHHI pretreatment, groups were administered 15 mg/kg SSL-DXR i.v. When tumor perfusion/permeability was probed 3 days after SSL-DXR administration by injection of SSL-DiI, deposition of SSL-DiI in sHHI-pretreated animals was reduced drastically (Fig. 1E and F). Blanching of the tumors and a lack of Dil dye absorbance were observed upon necropsy (Supplementary Fig. S3C). Higher magnification images suggested some residual permeability to the low-molecular weight H33342 probe (Fig. 1D). In animals receiving SSL-DXR without sHHI pretreatment (Fig. 1G and H), there was little difference in SSL-DiI deposition relative to untreated controls, confirming that only the sHHI/SSL-DXR sequence exerted the observed effects upon tumor permeability/perfusion.

Treatment-mediated changes in permeability/perfusion were quantified from tissue sections. In sHHI-treated animals (cf. Fig. 1C and D), tumor deposition of the SSL-DiI probe was doubled when administered 3 days after completion of the sHHI course (Fig. 2A; P < 0.01). In contrast, animals pretreated with sHHI followed by SSL-DXR (on day 10) showed drastically reduced SSL-DiI deposition, equivalent to untreated controls (Fig. 2A), when probed 3 days later. This finding was consistent with the reduction in tumor permeability/perfusion apparent in Fig. 1E and F. Treatment with SSL-DXR alone did not alter deposition of a subsequent SSL-DiI probe dose (Fig. 2A).

The effect of sHHI pretreatment on the tumor area accessible to the H33342 perfusion marker was quantified. In sHHI-pretreated animals, H33342 penetrated a 3-fold greater area compared with control animals (P < 0.05; Fig. 2B). Consistent with an initial perfusion reduction response to the SSL-DXR, sequential sHHI/SSL-DXR treatment reduced H33342 access to control values. SSL-DXR alone had no discernable effect upon H33342 dye penetration compared with vehicle controls.

**sHHI effects on tumor vasculature**

Tissue-level responses were investigated immunohistochemically to gain insight into mechanisms by which sHHI pretreatment altered tumor vascular permeability to nanoparticles. Representative histology for all treatments is shown in Supplementary Fig. S2. CD31 was used as a vascular endothelium marker, and α-SMA was used to identify pericytes associated with CD31+ structures in control (Fig. 3A) versus sHHI-treated (Fig. 3B) animals. Because activated fibroblasts also express α-SMA (39), only α-SMA-positive structures associated with a lumen or obvious vascular track were quantified. In initial analysis, NG2, a second pericyte marker, was also used to address the nonspecificity of most pericyte markers (39). NG2 and α-SMA showed nearly complete overlap, but because α-SMA is associated with mature vessels (40), only α-SMA was quantified in the full analysis. Based on CD31 staining, tumors of sHHI-treated animals (Fig. 3B, middle) showed a significant but small increase in microvessel density (Fig. 4A; P < 0.05) relative to controls (Fig. 3A, middle). In contrast, the number of structures that were α-SMA+ (Fig. 4B) was similar in control (Fig. 3A, left) and sHHI-treated animals (Fig. 3B, left). However, correlation of each CD31+ structure with α-SMA+ objects revealed a significant preponderance (P < 0.001; Fig. 4D) of immature microvessels lacking associated pericytes (CD31+/α-SMA−) in sHHI-treated animals (Fig. 3B, right) compared with controls (Fig. 3A, right).

Correlation of CD31+ with collagen IV+ structures, which is associated with vascular basement membrane (41), also was compared in control (Fig. 3C) versus sHHI-treated (Fig. 3D) animals. The sHHI mediated a striking and significant elevation (P < 0.001; Fig. 4C) of collagen IV+ structures (Fig. 3D, left).
compared with controls (Fig. 3C, left). Correlation of CD31+ with collagen IV structures in sHHI-treated animals showed a significant increase (Fig. 3D, right), which had a collagen IV/CD31 ratio of approximately 1.

The effect of SSL-DXR treatment upon tumor vascular markers also was evaluated. SSL-DXR following sHHI pretreatment reduced the number of CD31+/α-SMA− structures (Supplementary Fig. S4A) to below control values (Fig. 4B, P < 0.01) and caused retraction of the vascular front (Supplementary Fig. S2C), analogous to the loss of vascular endothelium mediated by extravasated SSL-DXR that we observed when treating intracranial brain tumors (30). However, the number of α-SMA− structures was unchanged (Fig. 4B). Thus, SSL-DXR treatment reversed the sHHI-mediated elevation in CD31+/α-SMA− ratios to nearly control values within 3 days (Fig. 4D). SSL-DXR also reduced drastically the number of collagen IV+ structures in sHHI-treated animals (Fig. 4C, P < 0.001), reversing the sHHI-mediated formation of collagen IV+/CD31− structures to control values (Fig. 4E, P < 0.05), consistent with the reduced perfusion/liposome deposition observed 3 days after the sHHI/SSL-DXR sequence (cf. Fig. 1E and F). SSL-DXR without sHHI pretreatment did not exert significant effects (Fig. 4).

Antitumor efficacy of sHHI/nanoparticle sequence

Given that sHHI pretreatment enhanced deposition of subsequently administered nanoparticles, we tested the hypothesis that a short sequence of sHHI priming followed by SSL-DXR would enhance antitumor efficacy. As above, animals were treated for 10 days with 40 mg/kg/d NVP-LDE225, a dose that showed little single-agent efficacy (Fig. 5A and C). In dose ranging experiments, 15 mg/kg SSL-DXR (~85% of MTD reported for immunocompetent mice; ref. 38) was administered on d10 of sHHI dosing, and tumor volume regressed within 4 days in the sHHI/SSL-DXR group, but not in the single-agent sHHI- nor SSL-DXR groups. However, weight loss was significant for SSL-DXR-treated mice, and doses were de-escalated. A single dose of 8 mg/kg SSL-DXR after 10 days of sHHI priming resulted in sustained (~20 days) arrest of tumor progression and increased survival compared with SSL-DXR- or sHHI-alone (Supplementary Table ST1). Notably, the sHHI pretreatment did not exacerbate body weight loss or other toxicity compared with treatment with SSL-DXR alone, suggesting the sHHI did not increase SSL-DXR deposition in other tissues (Supplementary Fig. S5). Free DXR at the same dose exerted uniform, lethal toxicity (Supplementary Fig. S5), and was discontinued from comparisons. Although 8 mg/kg/week SSL-DXR was tolerated in immunocompetent mice (38), weight loss of ≤20%, which resolved after 10 days, was observed here in SCID mice (Supplementary Fig. S5).
The SSL-DXR dose was further de-escalated to 6 mg/kg to permit comparison of efficacy for single and multiple cycles of shHH priming followed by SSL-DXR treatment. Figure 5A shows mean tumor volume progression for all treatment groups after one treatment cycle. Mean plots terminate when the 2nd of N = 7 animals per group surpassed the protocol tumor volume limit (TVL) of 2,000 mm³, to avoid bias of the mean when the largest tumors are eliminated. Body weight loss was mild and transient. Although tumor volume doubled over 10 days in control- or shHH-treated animals (Fig. 5A), it increased only 30% in shHH-primed animals that received SSL-DXR. Single-agent SSL-DXR reduced tumor progression significantly compared with controls (P < 0.05) but was less effective than the shHH/SSL-DXR combination as early as 14 days after initiation of the treatment cycle (P < 0.05). The slight reduction in tumor progression mediated by shHH-alone was not significant.

Survival time to 2,000 mm³ (TVL) was evaluated for each treatment group (Fig. 5B). The median was 29 days for control- and 34 days for SSL-DXR-alone groups, whereas the median time to TVL for the shHH/SSL-DXR sequence was 56d, nearly double that of controls (significant at P < 0.05; Table 1).

Repeated cycles of shHH/SSL-DXR were investigated to determine whether the sequence of shHH priming followed by SSL-DXR nanoparticle administration could sustain tumor volume suppression for longer periods with acceptable toxicity. The between-cycle interval, based upon the time course of weight recovery in SSL-DXR groups (Supplementary Fig. S5), was 10 days. Figure 5C shows mean tumor progression, with curves terminating when the second animal surpassed TVL. Over three cycles and ≥50 days, sustained inhibition of tumor progression was achieved with shHH/SSL-DXR, and efficacy of this sequence exceeded that of all other treatments.

Median survival to TVL for the three-cycle treatment (Fig. 5D) was 29 days for controls, 41 days for SSL-DXR-alone, and 50 days for shHH-alone, and 78 days for the shHH/SSL-DXR sequence. Thus, the shHH priming/nanoparticle sequence nearly tripled survival relative to controls, which was significant (P < 0.005; Table 1). For all animals receiving the shHH/SSL-DXR sequence, three cycles and ≥50 days sustained inhibition of tumor progression.

Figure 4.
Quantification of shHH effects on tumor microvessels. Abundance and correlation of vascular components (cf. Fig. 3) were quantified. For all panels, symbol above bar signifies comparison with vehicle controls, horizontal lines indicate comparisons between groups other than control: *, P < 0.05; **, P < 0.01; ***, P < 0.001. A, number of microvessels (CD31⁺) in shHH-treated animals was elevated slightly but significantly (**) relative to controls. SSL-DXR following shHH pretreatment reduced CD31⁺ features significantly below shHH-alone animals (****) and controls (**). SSL-DXR alone reduced CD31⁺ features slightly relative to controls (*). B, the number of α-SMA⁺ features did not change significantly with any treatment. C, the number of collagen IV⁺ (basement membrane) structures increased drastically with shHH pretreatment compared with controls (**), whereas SSL-DXR after shHH pretreatment halved collagen IV⁺ structures (**), to values significantly below controls (*). D, fraction of CD31⁺ structures containing α-SMA⁺ in shHH-treated animals, CD31⁺ features that were α-SMA⁺ increased significantly (***) compared with controls, whereas SSL-DXR after shHH pretreatment reduced the CD31⁺/α-SMA⁺ features significantly (**), to levels below control values (*), suggesting denuding of endothelium. SSL-DXR alone did not alter CD31⁺/α-SMA⁺ ratios. E, fraction of Collagen IV⁺ features that were CD31⁺ was increased significantly in shHH-treated animals compared with controls (*), and SSL-DXR after shHH pretreatment reduced the fraction of collagen IV⁺/CD31⁺ structures significantly (**), to values slightly below controls (*).

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treatment cycles mediated sustained arrest or regression of tumors and were significantly more effective than one cycle ($P < 0.05$; Table 1).

**Discussion**

Pancreatic cancer represents an intractable clinical problem for most patients because of the advanced, disseminated stage of disease at diagnosis, significant interindividual variability in tumor responsiveness to specific chemotherapy agents, and inadequate tumor drug delivery. These pharmacodynamic and biodispositional factors conspire to present a difficult therapeutic challenge, because the drug delivery barrier may render even active drugs inefficacious for the individual patient.

Desmoplastic stroma, which is characteristic of PaCA, has been implicated as a contributor to tumor growth, malignancy,
metastasis, and treatment resistance (6, 9, 20). Although the majority of stromal cells are non-neoplastic (6, 12), they collaborate with malignant cells in both autocrine and paracrine interactions (13, 14, 42). The stroma provides a microenvironment that supports tumor cells under hypoxic and low-glucose conditions, and contributes to poor tumor perfusion compared with normal pancreatic tissue (8, 43).

However, the roles played by the numerous factors in tumor–stroma crosstalk are complex. For example, although hedgehog signaling drives tumor establishment through neovascularization and capillary morphogenesis (44, 45), shorter term hedgehog inhibition in a hypovascular model of established primary PaCA resulted in increased microvessel density, stromal thinning, and a temporal window of elevated tumor vascular permeability and drug deposition (7). Furthermore, recent reports indicate that constitutive, genetic abrogation of hedgehog signaling, or depletion of tumor-associated fibroblasts, promotes a poorly-differentiated, malignant, and metastatic phenotype, leading to the conclusion that sHH-associated desmoplasia restrains PaCA progression (17–19). Thus, the role of sHH inhibitors is complex and poorly understood.

Here, we tested the hypothesis that modulation of tumor vascular perfusion and permeability by HH inhibitors could be exploited to create a window of opportunity within which to establish an intra-tumor nanoparticulate drug carrier depot, thereby enhancing PaCA therapy. The treatment strategy used low sHHI doses that were sufficient for sustained GliI suppression, but which caused little tumor inhibition or microvessel density change. The dosing period was limited to a 10-day window, based on the treatment duration reported to increase vascular permeability/microvessel density in a genetically engineered PaCA model (7) and the exposure duration that was reported to induce functional sHHI resistance in vitro (36).

Nanoparticles were chosen as the follow-on chemotherapeutic, with the rationale that sHH inhibitors may mediate an enhanced permeability and retention (EPR) phenomenon, which favors passive tumor deposition of macromolecules and nanoparticles over conventional small molecule drugs (46, 47). Under EPR conditions, bolus injection of small-molecule drugs can establish transient, high tumor drug concentrations, but once blood concentrations fall, clearance into the systemic circulation usually is rapid because of elevated tumor perfusion during the tumor–priming window. For larger molecules, extravasation and tumor clearance is molecular-mass dependent, with those in the megadalton range accumulating in, and clearing from tumors more slowly than those in the kilodalton range (47) or smaller. Nanoparticulate drug carriers have been shown in numerous reports to mediate greater tumor drug deposition compared with free drugs, and the delivered drug can persist for days at concentrations that greatly exceed peak tumor concentrations achieved with free drug (26). Furthermore, intratumor drug depots established by sustained-release nanoparticle carriers have been shown, in other model systems, to exert a progressive pharmacologic degradation of the tumor vascular barrier (25, 30, 48). Thus, residual drug could potentially sustain compromise of the drug delivery barrier without continued sHHI priming, and possibly reverse the tumor microenvironment changes reported after long-term inhibition of sHH signaling and depletion of stromal fibroblasts, which have been implicated in enhancing metastatic potential (17–19, 49).

The size dependence of extravasation from microvessels rendered permeable by sHH inhibitors has not been reported. Here, we demonstrate that 10-day treatment of patient-derived PaCA tumors with low-dose sHHI enhanced extravasation of 80 to 100 nm nanoparticles. Markedly elevated microvessel permeability persisted for at least 3 to 4 days after cessation of sHHI dosing. Notably, extravasation was greatest proximal to tumor cells, and the pattern of deposition suggested diffusion of the nanoparticles away from the afferent microvessels, consistent with elevated intra-tumor interstitial mobility.

The small increase in CD31+ microvessels mediated by the sHHI dose used was associated with a more significant overall increase in CD31+ structures lacking pericyte coverage (ex-SMA−). An additional, striking response to sHHI treatment was increased collagen IV structures; many had a vascular morphology but lacked colocalized endothelial cells, a phenomenon observed previously in poorly differentiated tumors having high angiogenic drive, such as Gliomas (50), and a hallmark of neovascularization (51). Collagen IV is implicated in PaCA cell survival and migration (49), and data reported here demonstrate that SSL-DXR treatment reversed the marked sHHI-mediated increase in tumor-associated collagen IV. Reduced pericyte coverage of microvessels with sHHI treatment, and production of basement membrane lacking endothelium, would be consistent with deranged angiogenesis, resulting in elevated vascular permeability, and with increased tumor angiogenesis observed with genetic sHH deletion (19).

SSL-DXR, an 80 to 100nm nanoparticulate formulation similar to FDA-approved Doxil, was selected to test proof-of-principle as to whether nanoparticles deposited by the EPR effect during sHHI priming could exert sustained pharmacologic action. A single sHHI priming cycle followed by SSL-DXR suppression of tumor volume progression effectively, nearly doubling median survival to TVL compared with controls. SSL-DXR alone had some therapeutic effect, but median survival was not significantly greater than for control groups or those treated with sHHI alone. Free DXR was highly toxic at doses of SSL-DXR that were efficacious and well tolerated.

Multiple cycles of the sHHI/SSL-DXR sequence exerted long-term suppression of tumor progression with minimal toxicity. A between-cycle gap of 10 days permitted recovery of mild SSL-DXR effects on body weight before the next treatment cycle. The sHHI was discontinued at the time of SSL-DXR treatment with the reasoning that a drug holiday could delay emergence of functional resistance to the sHHI. Mutation-based loss of drug binding to sHHI/SSL-DXR sequence delayed tumor progression significantly compared with one cycle, and for an extended duration.

The initial effects of SSL-DXR administration after sHHI priming included drastically reduced tumor permeability/perfusion and probe nanoparticle deposition, and reversal of the immature microvessels induced by sHHI pretreatment. This was consistent...
with initial tumor antivascular responses to extravasated SSL-DXR that we observed previously in other tumor models (30), in which the reduced vascular function was associated with transient, immunohistologically verified plugging of chaotic microvessels. These antivascular effects have therapeutic implications that merit future investigation. They could initially cause reduction in perfusion-mediated clearance of tumor-deposited, drug-containing nanoparticles, thereby enhancing tumor drug exposure and cytotoxicity. Alternatively, deposition of subsequently administered chemotherapeutic agents might be reduced in the period immediately following the SSL-DXR dose, with important implications for drug scheduling.

Results reported here were obtained with patient-derived xenograft #18269, which was selected for abundant desmoplasia and verified to respond to sHHI inhibitors with stromal suppression of transcription factor Gli1. A broader survey of PDX tumors (29) is ongoing to determine inter-individual responsiveness to the sHHI/nanoparticle treatment sequence. Ongoing experiments with a second PDX tumor having similarly marked desmoplasia, #18254, show that tumor is unresponsive to SLS-DXR alone, but responsive after 11-day pretreatment with 80 and 120 mg/kg NVP-LDE225. Thus, optimization of the sHHI/nanoparticle sequence for a broader range of patient tumor phenotypes may enhance nanoparticle deposition and efficacy.

Finally, numerous drug-containing nanoparticulate formulations are under development for cancer therapy, including PaCA. It is reasonably expected that efficacy of these next-generation formulations may also benefit from tumor-priming strategies. In conclusion, the tumor drug delivery barrier is unquestionably a major factor in treatment resistance of PaCA. In a spontaneous PaCA model, sHHI administration caused barrier compromise and improved gemcitabine response (7), but initial clinical testing of this strategy was disappointing (15). Notably, recent reports associate deleterious effects on tumor progression with continuous, long-term inhibition of hedgehog signaling or stromal fibroblast depletion (17–19). A strategy of transient PaCA vascular barrier compromise mediated by short-term stromal modulation using SMO inhibitors, followed by nanoparticulate drug carrier administration to promote enhanced drug deposition and sustained antitumor effects, is efficacious in a patient-derived tumor model. The tumor vascular barrier to 80 to 100 nm nanoparticles clearly was breached by pretreatment with low doses of sHHI that were sufficient for sustained suppression of stromal Gli1, and a nanoparticle carrier similar to the FDA-approved product Doxil was pharmacologically active when administered to sHHI-primed tumors. Combination chemotherapy sequences that exploit enhanced nanoparticle drug delivery in conjunction with short-term stromal modulation by molecularly targeted agents merit further investigation as a means to increase the efficacy of pancreatic cancer therapy, and may constitute a strategy to circumvent potential negative consequences of long-term hedgehog- or stromal inhibition.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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