Tumor-priming *Smoothened* inhibitor enhances deposition and efficacy of cytotoxic nanoparticles in a pancreatic cancer model

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### Abbreviations

<table>
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<th>Abbreviation</th>
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<tr>
<td>DiIC₁₈(5)-DS</td>
<td>Dioctadecyl-3,3',3',3'-tetramethylindocarbocyanine disulfonate</td>
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<td>DXR</td>
<td>Doxorubicin</td>
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<td>HH</td>
<td>Hedgehog signaling pathway</td>
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<tr>
<td>sHHI</td>
<td>SMO inhibitor of hedgehog signaling</td>
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<tr>
<td>SSL</td>
<td>Sterically stabilized liposomes</td>
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<tr>
<td>SSL-DXR</td>
<td>Sterically stabilized liposomes containing doxorubicin</td>
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<tr>
<td>SSL-DiI</td>
<td>Sterically stabilized liposomes labeled with fluorescent probe DiIC₁₈(5)-DS</td>
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<td>TVL</td>
<td>Tumor volume limit of animal protocol</td>
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Abstract

Most pancreatic adenocarcinoma (PaCA) 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 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 80-100nm 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/day 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+) of CD31+ 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 to controls, one cycle of 10d 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 10d 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 ~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 inter-individual pharmacodynamic variability is the result of prevalent genetic mutations; one small-scale transcriptional analysis identified >60 genetic alterations in PaCA that suggested derangement of ~12 signaling pathways in 70-100% of tumors (4). The diverse mutations enable tumors to adapt to chemotherapy, and molecularly targeted agents seldom control disease progression. Because of inter-individual 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 to 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 (SMO) 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 pharmacological 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 nanoparticles, drug carriers, and tested the hypothesis that sequencing sHHI with 80-100nm 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 7-10 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 inter-individual 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 iv-injected probes that included 80-100nm 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 (St. Louis, MO); NVP-LDE225: Novartis
(Basel, CH); phospholipids: Avanti Polar Lipids (Alabaster, AL); dioctadecyl-3,3,3',3'-
tetramethylindocarbocyanine disulfonate (DiIC<sub>18</sub>(5)-DS): Invitrogen (Carlsbad, CA);
monoclonal antibodies against α-SMA, NG2, CD31, collagen IV: Millipore-Chemicon (Billerica,
MA).

SSL Preparation

A dried film of 9:5:1 mol:mol:mol
distearoylphosphatidylcholine:cholesterol:polyethyleneglycol-derivatized
phosphatidylethanolamine was hydrated with 250mM ammonium sulfate to a lipid concentration
of 20mM, extruded through 80nm 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 80-100nm based upon dynamic light scattering. Encapsulation efficiency was ~99%.

Fluorescent SSL (SSL-DiI) were prepared as described above, except the phospholipids included 0.1 mole% of the non-exchangeable membrane label DiIC18(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, sc tumors (passage 6-7) were harvested from donor mice, cut into 2x2x2 mm blocks under RPMI 1640 medium, and implanted sc on the abdominal wall of anesthetized 18-20 gm C.B-Igh-1<sup>b</sup>/IcrTac-Prkdc<sup>scid</sup> mice.

Treatment was initiated 4-6 weeks after implantation, when tumors were 100-500 mm<sup>3</sup>. Immunohistochemistry and permeability/perfusion experiments employed ≥3 mice/group/time point, and therapeutic groups employed ≥6. Treatments included: vehicle controls, a 10-day course of 40 mg/kg/day NVP-LDE225 in 0.5% methylcellulose/0.5% Tween 80 (34) by oral gavage, a 10d NVP-LDE225 course followed by iv SSL-DXR on the 10<sup>th</sup> 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 iv. H33342 (15 mg/kg) was
administered iv 24h later, 20 min 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 non-perfused tumor areas, some tissue sections were counterstained with H33342 (2 µg/ml for 2 min) after acquisition of SSL-Dil fluorescence data.

**Immunofluorescence**

Tumor sections were fixed with ice-cold acetone, washed in Dulbecco’s PBS containing 0.5% Tween20, incubated in blocking buffer, and incubated 1h 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 (amino-methylcoumarin-acetic acid)-labeled anti-collagen IV, Cy5-labeled anti-α-SMA) for 30 min at 20°C, and then washed, dehydrated, and mounted with anti-fade medium.

**Image acquisition**

Dil 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/](http://imagej.nih.gov/ij/)), whereas microvessel counts were done on multiple individual images (N=5-12) comprising the panoramas. Statistical testing included Mantel-Cox and unpaired, one-tailed Student t-tests, implemented in Prism5.0d (Graphpad, La Jolla, CA).
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, 80-100nm 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 Fig. S1,S2A*) was selected for these studies based on its low microvessel density, which is comparable\(^a\) to 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) (35) permeability probes, consistent with histological characteristics.

**sHHI effects upon tumor perfusion and permeability to nanoparticles.** Our strategy was to employ 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/day was selected based upon published reports: (i) tumor vascular permeability compromise after 8-12 days’ dosing with sHHI (7), (ii) Gli1 suppression for 24h for this dose range (34,36), and (iii) the emergence of functional sHHI resistance after 13 days’ treatment (36). Quantitative-RT-PCR with species-specific probes verified >95% suppression of stromal (murine) Gli1 for 24h after a

\(^a\) Wang, J, Straubinger, RM, manuscript under preparation
single oral dose. Fluorescent 80nm liposomes (SSL-DiI) were injected iv to probe vascular permeability on d10 (the final sHHI dosing day) and d13. Twenty min before sacrifice at 24h, the peak time for SSL tumor deposition (26,37,38), Hoechst 33324 was injected iv as a perfusion marker.

Vehicle-treated control animals showed little deposition of H33324 or SSL-DiI, except for limited extravasation around the few vessels investing the tumor (Fig. 1A, Supplementary Fig. S3A). Higher magnification images showed vascular- or perivascular localization of SSL-DiI and limited interstitial diffusion of H33342 (Fig. 1B). In contrast, extensive SSL-DiI deposition was observed throughout tumors 1d and 4d (Fig. 1C) after completion of the shHI regimen, with no statistical difference in deposition observed between those days. Optical absorbance of the DiI probe was observable grossly upon necropsy because of the large amount of liposome deposition (Supplementary Fig. S3B). SSL-DiI deposition generally corresponded to regions of H33342 fluorescence, and was particularly intense in coronas (Fig. 1C) around the mucinous structures lined by adenocarcinoma cells (Supplementary Fig. S1B) that appear as 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 (3-4d post-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 7-10 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 iv. When tumor perfusion/permeability was probed 3d after SSL-DXR administration by injection of SSL-DiI, deposition of SSL-DiI in shHI-pretreated animals was reduced drastically (Fig. 1E,F). Blanching of the tumors and a lack of DiI dye absorbance was 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,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,D), tumor deposition of the SSL-DiI probe was doubled when administered 3d after completion of the shHI course (Fig. 2A; p<0.01). In contrast, animals pretreated with shHI followed by SSL-DXR (on d10) showed drastically reduced SSL-DiI deposition, equivalent to untreated controls (Fig. 2A), when probed 3d later. This finding was consistent with the reduction in tumor permeability/perfusion apparent in Fig. 1E,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 to 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 to vehicle controls.
sHHI effects upon tumor vasculature. Tissue-level responses were investigated immunohistologically to gain insight into mechanisms by which sHHI pretreatment altered tumor vascular permeability to nanoparticulates. Representative histology for all treatments is shown in Supplementary Fig. S2. CD31 was employed as a vascular endothelium marker, and α-SMA was used to identify pericytes associated with CD31+ structures in control (Fig. 3A) vs. 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 employed to address the non-specificity 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 to 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) vs. 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 to controls (Fig. 3C/left). Correlation of CD31+ with collagen IV+ structures in sHHI-treated animals showed a significant increase (Fig. 4E; p<0.05) in basement membrane-containing structures (collagen IV+) that were negative for vascular
endothelium (CD31⁺; Fig. 3D/right) compared to controls (Fig. 3C/right), which had a collagen IV⁺/CD31⁺ ratio of ~1.

The effect of SSL-DXR treatment upon tumor vascular markers also was evaluated. SSL-DXR following sHHI pretreatment reduced the number of CD31⁺ structures (Supplementary Fig. S4A) to below control values (Fig. 4A; p<0.01) and 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 3d after the sHHI/SSL-DXR sequence (cf. Fig. 1E,F). SSL-DXR without sHHI pretreatment did not exert significant effects (Figs. 4).

Anti-tumor 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 10d with 40 mg/kg/day NVP-LDE225, a dose that showed little single-agent efficacy (Fig. 5A,C). In dose ranging experiments, 15 mg/kg SSL-DXR (~85% of maximum tolerated dose reported for immunocompetent mice (38)) was administered on d10 of sHHI dosing, and tumor volume regressed within 4d 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 10d of
sHHI priming resulted in sustained (~20d) arrest of tumor progression and increased survival compared to SSL-DXR- or sHHI-alone (Supplementary Table ST1). Notably, the sHHI pretreatment did not exacerbate body weight loss or other toxicity compared to 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 10d, 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 sHHI priming followed by SSL-DXR treatment. Fig. 5A shows mean tumor volume progression for all treatment groups after one treatment cycle. Mean plots terminate when the 2\(^{nd}\) of N=7 animals per group surpassed the protocol tumor volume limit (TVL) of 2000 mm\(^3\), to avoid bias of the mean when the largest tumors are eliminated. Body weight loss was mild and transient. Whereas tumor volume doubled over 10 days in control- or sHHI-treated animals (Fig. 5A), it increased only 30% in sHHI-primed animals that received SSL-DXR. Single-agent SSL-DXR reduced tumor progression significantly compared to controls (p<0.05) but was less effective than the sHHI/SSL-DXR combination as early as 14d after initiation of the treatment cycle (p<0.05). The slight reduction in tumor progression mediated by sHHI-alone was not significant.

Survival time to 2000 mm\(^3\) (TVL) was evaluated for each treatment group (Fig. 5B). The median was 29d for control- and 34d for SSL-DXR-alone groups, whereas the median time to TVL for the sHHI/SSL-DXR sequence was 56d, nearly double that of controls (significant at p<0.05; Table 1).
Repeated cycles of sHHI/SSL-DXR were investigated to determine whether the sequence of sHHI 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 10d. Fig. 5C shows mean tumor progression, with curves terminating when the 2nd animal surpassed TVL. Over 3 cycles and ≥50 days, sustained inhibition of tumor progression was achieved with sHHI/SSL-DXR, and efficacy of this sequence exceeded that of all other treatments.

Median survival to TVL for the 3-cycle treatment (Fig. 5D) was 29d for controls, 41d for SSL-DXR-alone, 50d for sHHI-alone, and 78d for the sHHI/SSL-DXR sequence. Thus the sHHI priming/nanoparticle sequence nearly tripled survival relative to controls, which was significant (p<0.005; Table 1). For all animals receiving the sHHI/SSL-DXR sequence, 3 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 inter-individual 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 to normal pancreatic tissue (8,43).

However, the roles played by the numerous factors in tumor-stroma crosstalk are complex. For example, whereas 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 employed low sHHI doses that were sufficient for sustained Gli1 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 to 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 pharmacological 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 10d treatment of patient-derived PaCA tumors with low-dose sHhI enhanced extravasation of 80-100nm nanoparticles. Markedly elevated microvessel permeability persisted for at least 3-4 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 employed was associated with a more significant overall increase in CD31+ structures lacking pericyte coverage (α-SMA−). An additional, striking response to sHHI treatment was increased collagen IV+ structures; many had a vascular morphology but lacked co-localized 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 80-100nm 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 pharmacological action. A single sHHI priming cycle followed by SSL-DXR suppressed tumor volume progression effectively, nearly doubling median survival to TVL compared to 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 10d permitted recovery of mild SSL-DXR effects on body weight prior to 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 SMO, and SMO-activating mutations, have been observed clinically and preclinically with long-term treatment (23,36,52), and activation of compensatory growth and survival signaling pathways contributing to sHHi resistance was reported after just 13 days’ sHHi treatment in vitro (36). Furthermore, transient, intermittent sHHi treatment might maintain tumors in a state of disequilibrium, staving off the apparently deleterious effects of tumor adaptation to long-term hedgehog inhibition or stromal fibroblast depletion, such as increased tumor malignancy and progression (17-19). Although treatment resistance was not investigated here, the sHHi/SSL-DXR sequence was more effective than any other, and repeated cycles of the sHHi/SSL-DXR sequence delayed tumor progression significantly compared to 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 pruning 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 sHH 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 SSL-DXR alone, but responsive after 11d 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 80-100nm
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.

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References


Tables

Table 1. Effect of treatment regimens on median time to tumor volume limit

<table>
<thead>
<tr>
<th>Cycles</th>
<th>Control</th>
<th>sHHI</th>
<th>SSL-DXR</th>
<th>sHHI/SSL-DXR</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Time to TVL (days)</td>
<td>% Increase</td>
<td>Time to TVL</td>
<td>% Increase</td>
</tr>
<tr>
<td>1</td>
<td>29 §§ -</td>
<td>-</td>
<td>n.d. ‡</td>
<td>34</td>
</tr>
<tr>
<td>3</td>
<td>29 *** -</td>
<td>-</td>
<td>49.5</td>
<td>70%</td>
</tr>
</tbody>
</table>

‡ Median time to protocol tumor volume limit (TVL) of 2000 mm³.
† Percent increase in median TVL relative to control animals
‡ Not Done; no significant difference vs. control was observed in 3 experiments; animals here allocated to triple cycle group on d20

Mantel-Cox Statistics:
§§ Control vs. sHHI/SSL-DXR sequence, single treatment: differ at p<0.05
¥¥ SSL-DXR vs. sHHI/SSL-DXR, single treatment: differ at p<0.05
*** Control vs. sHHI/SSL-DXR sequence, multiple treatment: differ at p<0.005
†† sHHI/SSL-DXR single vs. multiple treatments: differ at p<0.05
Figure Legends

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/day) or vehicle po for 10d. On d10, n=3 control- and sHHI-treated animals were administered SSL-DXR iv (15 mg/kg). Three days later, all received fluorescent 80nm SSL-DiI iv as a vascular permeability probe. H33342 (15 mg/kg) was injected iv as a perfusion probe 24h later, 20 min 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 3-4 mm² field of the tumor; bar: 500 μm. **Right column**: higher-magnification image of tumor; bar: 50 μm. (A,B): Representative vehicle control tumor; probe deposition was low, occurring primarily near the few superficial veins on the surface. (B), SSL-DiI remained vascular/perivascular, whereas Hoechst diffused somewhat into tumor. (C,D): Tumor from sHHI-only animal; crescents of SSL-DiI deposition correspond to regions of columnar tumor cells organized around glandular structures (dark voids). (D), higher magnification shows SSL-DiI nanoparticles distributed randomly among Hoechst-stained areas, suggesting diffusion of SSL-DiI away from afferent microvessels. (E,F): Tumor from sHHI-treated animal (same as C,D) except SSL-DXR was administered 4d before, on d10 of sHHI treatment. Deposition of the high-mass permeability probe was reduced drastically. (G,H): Tumor from animal treated with SSL-DXR alone on the equivalent of d10 for sHHI-treated animals, showing little extravasation of 80nm SSL-DiI despite some Hoechst permeation.
Figure 2. Quantification of intratumor probe deposition and distribution.

Intensity of SSL-DiI probe deposition and fractional area of tumor accessible to H33342 were quantified for treatment groups of Fig. 1. Multiple (n≥3) tissue sections acquired at the mid-axis of tumors from n=3 animals/treatment group/time point were imaged and fluorophore deposition in the entire section was quantified. (A) Average deposition of SSL-DiI based on fluorescence intensity; the ~2.5 fold increase in sHHI-treated animals was significant (**, p<0.01). Deposition of SSL-DiI in sHHI-pretreated animals administered SSL-DXR on d10 of sHHI treatment was reduced to control levels 4d later. SSL-DXR alone did not alter SSL-DiI deposition significantly. (B) Tumor area accessible to H33342 was 3-fold greater in sHHI-pretreated animals compared to controls (*, p<0.05); in animals receiving the sHHI/SSL-DXR sequence, accessible tumor area was reduced to control values. SSL-DXR alone did not alter tumor area accessible to H33342.

Figure 3. sHHI treatment effects upon microvessel density and maturation.

Tumor-bearing mice (n=3/treatment group) were sacrificed 1d after a 10d treatment course with NVP-LDE225 (40 mg/kg/day) or vehicle. Tumor sections were dual-labeled for endothelial cells (CD31) and either vessel-associated pericytes (α-SMA) or basement membrane (collagen IV). Images were acquired from 8-14 regions of each tissue section from each animal, thresholded and masked to exclude background fluorescence and the lighter-staining collagen IV meshwork, and masks were compared for correlation of features in the two images. Rows A,B: α-SMA⁺, CD31⁺ staining for control- (Row A) and sHHI-treated (Row B) animals. Bar: 50 µm. Left panels/red: α-SMA⁺ features. Middle panels/green: CD31⁺ features. Right panels: composite of CD31⁺/α-SMA⁺ masks. Rows C,D: Collagen IV⁺, CD31⁺ stained tissues for control- (Row C) and sHHI-treated (Row D) animals. Left panels/red: Collagen IV⁺ features. Middle panels/green: CD31⁺ features. Right panels: composite of CD31⁺/Collagen IV⁺ masks.
Figure 4. Quantification of sHHI effects upon tumor microvessels

Abundance and correlation of vascular components (cf. Fig. 3) were quantified. For all panels, symbol above bar signifies comparison to 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\textsuperscript{+}) in sHHI-treated animals was elevated slightly but significantly (*) relative to controls. SSL-DXR following sHHI pretreatment reduced CD31\textsuperscript{+} features significantly below sHHI-alone animals (***), and controls (**). SSL-DXR alone reduced CD31\textsuperscript{+} features slightly relative to controls (*). (B) The number of \(\alpha\)-SMA\textsuperscript{+} features did not change significantly with any treatment. (C) The number of collagen IV\textsuperscript{+} (basement membrane) structures increased drastically with sHHI pretreatment compared to controls (***,), whereas SSL-DXR after sHHI pretreatment halved collagen IV\textsuperscript{+} structures (***,), to values significantly below controls (*). (D) Fraction of CD31\textsuperscript{+} features co-staining with \(\alpha\)-SMA: in sHHI-treated animals, CD31\textsuperscript{+} features that were \(\alpha\)-SMA\textsuperscript{-} increased significantly (***) compared to controls, whereas SSL-DXR after sHHI pretreatment reduced the CD31\textsuperscript{+}/\(\alpha\)-SMA\textsuperscript{-} features significantly (**), to levels below control values (*), suggesting denuding of endothelium. SSL-DXR alone did not alter CD31\textsuperscript{+}/\(\alpha\)-SMA\textsuperscript{+} ratios. (E) Fraction of Collagen IV\textsuperscript{+} features that were CD31\textsuperscript{-} was increased significantly in sHHI-treated animals compared to controls (*), and SSL-DXR after sHHI pretreatment reduced the fraction of collagen IV\textsuperscript{+}/CD31\textsuperscript{-} structures significantly (**), to values slightly below controls (*).

Figure 5. Inhibition of tumor progression by sHHI/SSL-DXR sequence.

Tumor-bearing mice received NVP-LDE225 (40 mg/kg/day) or vehicle po for 10d (grey shading). Half (n=7) the animals from control- and sHHI-pretreatment groups received 6 mg/kg SSL-DXR iv on d10 (vertical dashed line). (A) Mean tumor volume progression with single
treatment cycle. (* signifies p<0.05 in all panels). Symbols terminate on d20 for sHHI-alone group (green triangles), when all animals were allocated to the multiple cycle arm (panel C); mean volume did not differ significantly from controls (inverted black diamonds) and prior experiments showed no significant antitumor effect for this sHHI dose. For all other groups, symbols terminate when 2\textsuperscript{nd} animal reached protocol tumor volume limit (TVL). Four days after SSL-DXR administration (box, d14), tumor volume for sHHI/SSL-DXR group (red circles) differed significantly from all other groups. SSL-DXR-alone group (orange squares) did not differ from controls until d17. Boxes, d27, d29: tumor volumes of the sHHI/SSL-DXR group differed significantly from SSL-DXR-alone group. (B) Plot of time to TVL for treatment groups shown in (A). For control and sHHI/SSL-DXR groups, dashed lines show uncensored data. Solid lines show censored data for groups from which animals were sacrificed for reasons other than tumor volume progression or treatment toxicity. Time to TVL was ~90% greater for sHHI/SSL-DXR group compared to controls. Reasons for censoring include one animal with a tumor that failed to grow, two animals having 500-1000 mm\textsuperscript{3} tumors in which large mucinous vacuoles collapsed, and skin integrity was compromised, and a long-term survivor in the sHHI/SSL-DXR treatment group, sacrificed on d87 because of swollen lymph nodes and spleen, that subsequently was histopathologically classified as consistent with lymphoma. (C) Effect of three treatment cycles. Shading: 10d sHHI treatment period; vertical dashed lines: SSL-DXR administration. Between-cycle interval was 10d. Boxes: sHHI/SSL-DXR group differed significantly from all others including SSL-DXR-alone. (D) Plot of time to TVL for the treatment groups shown in (C). Solid lines show censored data excluding animals removed for reasons other than tumor volume or toxicity; dashed lines show uncensored data. Time to TVL was ~200% greater for
sHHI/SSL-DXR group compared to controls. Three cycles extended time to TVL significantly (p<0.05) compared to 1 treatment cycle.
Figure 1
Figure 2

A

Average Fluorescent Intensity

sHII | Control | sHII+SSLDXR | SSLDXR

**

B

Percent Area

sHII | Control | sHII+SSLDXR | SSLDXR

*

Figure 2
Figure 3
Figure 4
Figure 5
Molecular Cancer Therapeutics

Tumor-priming Smoothened inhibitor enhances deposition and efficacy of cytotoxic nanoparticles in a pancreatic cancer model

Tista Roy Chaudhuri, Ninfa L Straubinger, Rosemarie F. Pitoniak, et al.

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