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Sonic-hedgehog pathway inhibition normalizes desmoplastic tumor microenvironment to improve chemo- and nanotherapy

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ABSTRACT

Targeting the rich extracellular matrix of desmoplastic tumors has been successfully shown to normalize collagen and hyaluronan levels and re-engineer intratumoral mechanical forces, improving tumor perfusion and chemotherapy. As far as targeting the abundant cancer-associated fibroblasts (CAFs) in desmoplastic tumors is concerned, while both pharmacologic inhibition of the sonic-hedgehog pathway and genetic depletion of fibroblasts have been employed in pancreatic cancers, the results between the two methods have been contradictory. In this study, we employed vismodegib to inhibit the sonic-hedgehog pathway with the aim to i) elucidate the mechanism of how CAFs depletion improves drug delivery, ii) extent and evaluate the potential use of sonic-hedgehog inhibitors to breast cancers, and iii) investigate whether sonic-hedgehog inhibition improves not only chemotherapy, but also the efficacy of the most commonly used breast cancer nanomedicines, namely Abraxane® and Doxil®. We found that treatment with vismodegib normalizes the tumor microenvironment by reducing the proliferative CAFs and in cases the levels of collagen and hyaluronan. These modulations re-engineered the solid and fluid stresses in the tumors, improving blood vessel functionality. As a result, the delivery and efficacy of chemotherapy was improved in two models of pancreatic cancer. Additionally, vismodegib treatment significantly improved the efficacy of both Abraxane and Doxil in xenograft breast tumors. Our results suggest the use of vismodegib, and sonic hedgehog inhibitors in general, to enhance cancer chemo- and nanotherapy.

1. Introduction

Inefficient delivery of cytotoxic drugs to solid tumors can dramatically reduce the efficacy of chemotherapy and nanotherapy and thus, negatively affect the quality of life and survival of cancer patients. This can explain in large part why standard therapies many times fail to treat desmoplastic cancers, *i.e.*, tumors abnormally rich in stromal components such as breast and pancreatic cancers, even though these agents are potent enough to eradicate cancer cells in *in vitro* systems. Effective delivery of drugs to solid tumors is hindered by abnormalities in the structure of the tumor vasculature, which can drastically reduce tumor perfusion and as a result the systemic delivery of the drug [1,2].

In desmoplastic tumors, in particular, mechanical interactions among rapidly proliferating cancer cells, cancer associated fibroblasts (CAFs), extracellular matrix (ECM) fibers, primarily collagen and hyaluronan, and the surrounding normal tissue lead to accumulation of intratumoral solid stresses, causing vessel compression and hypo-perfusion [2–6]. To improve blood vessel functionality and treatment efficacy, already approved drugs with anti-fibrotic properties have been repurposed (*e.g.*, losartan, tranilast, pirfenidone) and it has been shown that these drugs can normalize the microenvironment of breast and pancreatic tumors to improve the delivery of chemotherapy and nanomedicine [7–9]. Importantly, this strategy has already reached clinical trials (clinicaltrials.gov identifier: NCT01821729) and the first results of a phase-II trial for the use of losartan to enhance therapy in pancreatic cancer patients confirm the preclinical findings [10].

Apart from the use of anti-fibrotic drugs, pertinent studies have employed inhibitors of the Sonic Hedgehog (SHH) signaling pathway to achieve pharmacologic depletion of CAFs in pancreatic cancers. Inhibition of SHH signaling using saridegib was shown to reduce proliferation and number of CAFs in mouse models of pancreatic cancer, improve blood vessel functionality and eventually the efficacy of

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gemcitabine [11]. Despite these encouraging data, however, saridegib failed in a phase-II clinical trial for previously untreated patients with metastatic pancreatic cancer when combined with gemcitabine [12]. Similarly, more recent phase-II clinical trials in an unselected cohort of patients with metastatic pancreatic cancer indicated that, while modestly improved, combination of vismodegib and gemcitabine did not result in a statistically significant increase in overall survival compared to gemcitabine alone [13]. However, these results could be due to different reasons, such as intrinsic resistance to gemcitabine as increased delivery of a drug might not benefit patients if cancer cells are or become resistant to that drug, or these cancers are already at such an advanced stage for survival improvement to be significantly demonstrated. Moreover, random selection of patients could also affect clinical trial results, since one would expect vismodegib to be particularly beneficial for highly desmoplastic, fibroblast-rich pancreatic tumors.

Additionally, a series of recent *in vivo* studies have shown that deletion of CAFs by genetic manipulation in mouse models induces immunosuppression and promotes tumor progression in pancreatic cancers [14,15]. Therefore manipulation of CAFs has been shown to both promote and restrain tumor progression, but any comparison between genetic deletion and pharmacologic depletion should be viewed with caution and take into account that these methods significantly differ from each other as:

i. Genetic deletion is chronic and effects of genetic deletion are not reversible. On the other hand, pharmacologic depletion is acute and effects are reversible when the treatment stops.
ii. Pharmacological agents could be delivered specifically to the tumor site while genetic deletion might affect the entire body.
iii. Genetic deletion might improve drug delivery and increase hypoxia, whereas pharmacologic depletion improves drug delivery and decreases hypoxia.

In this study, we revisited the use of SHH inhibitors to target CAFs with the aim to i) elucidate the mechanism of how CAFs depletion improves drug delivery, ii) extent and evaluate the potential use of SHH inhibitors to breast cancers, and iii) investigate whether SHH inhibition can improve not only chemotherapy, but also the efficacy of the most commonly used breast cancer nanomedicines, namely Abraxane® and Doxil®. To achieve our aims, we employed vismodegib (Erivedge®) in mouse tumor models for pancreatic and breast cancers to explore its ability to normalize the tumor microenvironment, decrease solid stress levels and improve tumor perfusion and therapeutic outcomes.

Vismodegib is the first oral medication approved by the US Food and Drug Administration in 2012 for adults with metastatic or locally advanced Basal Cell Carcinoma that has recurred after surgery or for patients who are not candidates for surgery or radiation. Previous studies have shown that administration of vismodegib in a mouse model of medulloblastoma and in xenograft models of primary human tumors, including colorectal and pancreatic carcinoma, inhibits SHH pathway and exerts antitumor activity [16–19].

To elucidate the mechanism by which depletion of CAFs improves drug delivery, we initially hypothesized that depleting stromal cells in primary pancreatic tumors will reduce solid stresses and improve the functionality of tumor blood vessels. To investigate this, we employed two human pancreatic cancer cell lines, namely MiaPaCa2 and BxPC3 to develop xenograft tumor models in immunodeficient mice. We show that vismodegib reduces solid stresses, decreases interstitial fluid pressure (IFP), improves perfusion, increases delivery of chemotherapy and improves therapeutic outcomes. Furthermore, we show that these observations are not only due to the reduction in the activity of CAFs but also due to the decrease in collagen and hyaluronan tumor content due to SHH signaling pathway inhibition and downregulation of downstream key effector genes Gli1 and Gli2. Finally, we developed an orthotopic xenograft breast tumor model, using the human breast cancer cell line MCF10CA1a, to study the effect of combining vismodegib with two clinically approved nanoparticles of different sizes, Abraxane (10 nm) and Doxil (100 nm), and show that vismodegib can also enhance the efficacy of these common cancer nanomedicines.

2. Materials and methods

2.1. Cell culture

MiaPaCa2 human pancreatic cancer cell line and BxPC3 human primary pancreatic adenocarcinoma cell line were purchased from ATCC and were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotics. MCF10CA1a human breast cancer cell line was obtained from the Karmanos Cancer Institute (Detroit, MI, USA) and maintained as previously described [20].

2.2. Drugs and reagents

Vismodegib (GDC-0449, Erivedge) was purchased from Selleckchem and the compound was formulated in a 10 mg/ml suspension in MCT (0.5% methylcellulose, 0.2% Tween 80), as previously described [16]. Gemzar (gemcitabine, Lilly) was dissolved in 0.9% NaCl (12.5 mg/ml stock). Doxil (pegylated liposomal doxorubicin, Janssen Pharmaceuticals) was purchased as already made solution (2 mg/ml) and Abraxane (albumin-bound paclitaxel, Celgene) was solubilized in 0.9% NaCl in final stock concentration of 5 mg/ml.

2.3. Animal tumor models and treatment protocols

Xenograft pancreatic tumor models were generated by subcutaneous implantation of 2×10^6 MiaPaCa2 or BxPC3 cells resuspended in 40 μl of serum-free medium into 6-week old male NOD/SCID mice. Vismodegib was administered orally once a day at different doses (40 mg/Kg or 100 mg/Kg, as indicated) from day 35 in MiaPaCa2 and day 4 in BxPC3 post-implantation, 10 days before the initiation of chemotherapy. Gemcitabine (50 mg/kg) was administered by intraperitoneal (i.p.) injection when tumors reached an average size of $\sim 200\ mm^3$; from day 45 in MiaPaCa2 and day 13 in BxPC3 post-implantation, every 72 h. Orthotopic xenograft breast tumors were generated by implantation of 5×10^5 MCF10CA1a cells resuspended in 40 μl of serum-free medium into the mammary fat pad of 6-week old female CD1 nude immunodeficient mice. Vismodegib (40 mg/kg) was administered orally from day 4 post-implantation. In both models, Doxil (3 mg/kg) and Abraxane (20 mg/kg) were administered by intravenous (i.v.) injections on day 14 and 21 post-implantation [8,21–23]. During the course of each experiment, tumor growth was monitored daily and the planar dimensions (x, y) were measured using a digital caliper. Tumor volume was calculated using the volume of an ellipsoid and assuming that the third dimension, z, is equal to $\sqrt{xy}$. All *in vivo* experiments were conducted in accordance with the animal welfare regulations and guidelines of the Republic of Cyprus and the European Union under a license acquired by the Cyprus Veterinary Services (No CY/EXP/PR.L1/2014), the Cyprus national authority for monitoring animal research.

To study alterations in the tumor microenvironment, right before the end of each treatment protocol, animals were anesthetized by i.p. injection of Avertin (200 mg/kg) and interstitial fluid pressure was measure using the wick-in-needle technique [3,24,25]. Next, mice were injected intracardially with 100 μl biotinylated lectin (1 mg/ml, Vector Labs), which was allowed to distribute throughout the body for 7 min [7–9]. Finally, mice were sacrificed *via* CO_2 inhalation and tumors were excised for measurement of mechanical properties and/or histological analysis.