NOVEL THERAPEUTICS AND PRECLINICAL IMAGING FOR PANCREATIC CANCER – VIEW FROM THE LAB

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Abstract

Pancreatic cancer is a devastating disease with a five-year survival rate of 6%. A key driver of disease progression is the tumour microenvironment, which is characterised by fibrosis. A dynamic interplay between tumour cells, pro-fibrogenic pancreatic stellate cells and a dense extracellular matrix impedes effective drug delivery and promotes chemoresistance and metastases. In addition, mutations in pancreatic cancer are highly heterogeneous, making it difficult to effectively treat all patients with one approach. Thus, any effective pancreatic cancer treatment should consider targeting both pancreatic cancer cells and the stromal compartment. While basic research has provided promising new leads on therapeutic targets for this disease, many of them remain ‘undruggable’ by conventional approaches. Advances in nanoparticle technology and intravital preclinical imaging of live tumours is providing new insight into the behaviour of the disease in vivo and guiding how best to target this disease with higher specificity and lower off-target toxicity. Here, we describe in brief, key advancements in both rapidly emerging fields and highlight their current and future application in the treatment of pancreatic cancer.

Tumour microenvironment

By 2030, pancreatic cancer is predicted to become the second leading cause of cancer-related deaths in western nations.¹ The poor prognosis is due to late clinical presentation, metastasis and chemoresistance. A major driver of the aggressive nature of pancreatic cancer is the microenvironment.² Pancreatic cancer is characterised by extensive stromal reaction or fibrosis surrounding tumour elements.³ Fibrosis distorts the tumour vasculature, creating hypoxia and nutrient deprivation.⁴-⁶ The fibrosis also acts as a physical barrier to drug delivery.²,⁶ This environment drives chemoresistance and metastases of cancer cells. The stroma is complex and involves multiple cell types including immune, endothelial, fibroblasts and stellate cells.⁵ This review will focus on pro-fibrogenic stromal pancreatic stellate cells (PSCs). PSCs are activated by tumour cells, which causes them to proliferate and deposit excessive fibrotic proteins.⁶ While activated, PSCs promote tumour cell proliferation, invasion, metastasis and chemoresistance.⁷ This complex interaction between tumour cells, PSCs and the surrounding microenvironment is a major reason many therapeutic approaches have failed in pancreatic cancer and needs to be considered when designing new therapeutic approaches (figure 1).

Figure 1: Strategies to visualise and overcome barriers to therapeutics in pancreatic cancer.

There are currently several barriers to effective drug delivery in pancreatic tumours. 1. Pancreatic stellate cells are responsible for orchestrating fibrosis and feed pro-survival signals to tumour cells. Simultaneous targeting of both tumour and stromal pancreatic stellate cells is a potential strategy in pancreatic cancer treatment. 2. Chemoresistance and genetic heterogeneity of pancreatic cancer cells make it extremely difficult to treat all patients with a single approach. Multi-target approaches using RNA interference (RNAi) therapeutics can help overcome this problem. RNAi therapeutics can inhibit any target at the gene level. These can be delivered using nanoparticles, which can also be tailored to specifically target tumour cells/stromal cells. RNAi therapeutics can be combined with nanoparticle vehicles and advanced genomics to deliver personalised medicine based on the genetics of a patient’s tumour, with high efficacy and minimal off-target toxicity. 3. Fibrosis can act as a physical barrier to drug penetration. It distorts tumour vasculature, resulting in a hypoxic microenvironment, driving chemoresistance and metastases. To overcome this barrier, nanoparticles can be used to bypass fibrosis and reach tumour cells. Stromal remodelling strategies can also enhance
Current pancreatic cancer chemotherapies

Gemcitabine has long been the first line treatment for patients with unresectable pancreatic cancer. In recent years, gemcitabine and abraxane® (albumin-bound paclitaxel) combination therapy has become a standard of care for unresectable pancreatic cancer. More aggressive polychemotherapeutic regimens such as FOLFIRINOX (folinic acid, 5-fluorouracil, irinotecan, oxaliplatin) are also used in the clinic. The survival benefit of these new approaches is only in the vicinity of a few extra months (gemcitabine + Abraxane® extends median survival by eight weeks over gemcitabine; FOLFIRINOX extends median survival by 17 weeks over gemcitabine). Clearly, new therapeutic approaches are urgently needed.

New therapeutic targets

There are two main hurdles that make pancreatic cancer difficult to treat - the chemoresistant nature of the cancer cells and the extensive fibrosis. Any effective pancreatic cancer treatment should consider targeting both the pancreatic cancer cells and the stromal reaction (figure 1). Recent discoveries at the bench have identified novel therapeutic targets that hold promise for reprogramming the stroma, modulating fibrosis dynamics and enhancing our ability to kill pancreatic tumour cells.

Several studies have demonstrated the potential benefit of targeting aspects of the stromal reaction in tissue culture and mouse models of pancreatic cancer. For example, the anti-fibrotic drug pirfenidone, which is used to treat idiopathic pulmonary fibrosis, reduced fibrosis, tumour growth and metastatic spread, and improved the efficacy of gemcitabine in an orthotopic xenograft. Novel work showed that the vitamin D receptor on PSCs is a master regulator of their cancer-promoting phenotype. Administration of a vitamin D analog into a clinically relevant spontaneous pancreatic cancer mouse model reduced fibrosis, improved drug access and when combined with gemcitabine, improved survival. Enzymatic depletion of hyaluronic acid, which is an abundant component of pancreatic fibrosis, in a similar mouse model, improved drug access and efficacy. More recently, the texture of fibrosis can predict pancreatic cancer patient outcome. The group showed that by inhibiting lysyl oxidase, an enzyme that increases the stiffness of fibrosis, they could suppress tumourigenesis and metastatic spread and enhance gemcitabine efficacy. These studies highlight the need to reprogram the stroma in order to overcome a major barrier to pancreatic cancer treatment. However, caution must be taken when targeting the stroma, as studies have demonstrated some components can help contain pancreatic cancer, but this is dependent on the therapeutic target and the cells affected. Thus, it is important to examine the effects of targeting one stromal component on other cells in the stroma.

The growing understanding of pancreatic cancer cell biology has allowed scientists to focus on new and more effective molecular targets for pancreatic cancer. Major goals for researchers studying pancreatic cancer include to identify new molecular targets that can impair tumour growth and metastasis, reduce off-target toxicity compared to traditional therapies, and improve the efficacy of existing therapeutics. McCarroll et al recently showed that inhibition of III-tubulin, a cytoskeletal protein,
was able to halve tumour growth and metastatic spread in an orthotopic pancreatic cancer mouse model.\textsuperscript{16} Ideal therapeutic targets for pancreatic cancer are not just proteins. For example, microRNA-21, a small RNA sequence that downregulates tumour suppressors, is upregulated in pancreatic cancer.\textsuperscript{17,18} Inhibition of this target in a mouse model of pancreatic cancer was also able to reduce tumour growth and increase tumour sensitivity to gemcitabine.\textsuperscript{19} While targets like these hold great promise for pancreatic cancer treatment, their translation to the clinic is hindered by their ‘undruggable’ status, that is, there are currently no pharmacological inhibitors against them. Exciting new progress in the field of nanotechnology is set to challenge this perception.\textsuperscript{20}

\textbf{Nanoparticle therapeutics: targeting the ‘undruggable’}

Nanoparticles are delivery vehicles ideally between 10-100 nanometres in diameter.\textsuperscript{21,22} They are capable of carrying a drug or RNA interference (RNAi) therapeutics. RNAi makes it possible to inhibit any target gene, with high specificity. Nanoparticle technology is already in use in the clinic, including for example albumin-bound paclitaxel (table 1) and in clinical trials for a variety of cancers (table 2). More recently, Boyer et al published a first-generation nanoparticle for delivery to pancreatic tumours and demonstrated that it was capable of delivering and releasing RNAi therapeutics into pancreatic cancer cells in vitro.\textsuperscript{23}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Composition} & \textbf{Trade name} & \textbf{Disease} & \textbf{Administration} & \textbf{Reference} \\
\hline
Liposomal doxorubicin & Myocet & Combination therapy with cyclophosphamide in metastatic breast cancer & Intravenous & 58 \\
\hline
Liposomal-PEG doxorubicin\# & Doxil/Caelyx & HIV-related Kaposi’s sarcoma, metastatic breast cancer, metastatic ovarian cancer & Intramuscular & 59-61 \\
\hline
Albumin-bound paclitaxel & Abraxane\#* & Metastatic breast cancer, metastatic pancreatic cancer & Intravenous & 62, 63 \\
\hline
Methoxy-PEG-poly(D,L-lactide) taxol & Genexol-PM & Metastatic breast cancer & Intravenous & 64 \\
\hline
PEG-L-asparaginase & Oncaspar & Acute lymphoblastic leukaemia & Intravenous, intramuscular & 65 \\
\hline
\end{tabular}
\caption{Nanoparticle-based therapies in clinical use for cancer.}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{Composition} & \textbf{Trade Name} & \textbf{Disease} & \textbf{Administration} & \textbf{Status} & \textbf{Reference} \\
\hline
Liposomal doxorubicin & Doxil & Soft tissue sarcoma & Intravenous & Phase 1/2 & 66 \textsuperscript{NCT00949325} \\
\hline
Polyglutamate paclitaxel & Xyotax & Metastatic breast cancer & Intravenous & Phase 2 & 67 \textsuperscript{NCT00265733} \\
\hline
PEG-camptothecin\# & MAG-CPT (PNU 166148) & Advanced solid cancers & Intravenous & Phase 1 & 68 \textsuperscript{NCT00004076} \\
\hline
Liposomal irinotecan & MM-398 & Metastatic pancreatic cancer & Intravenous & Phase 3 & 69 \textsuperscript{NCT01494506} \\
\hline
\end{tabular}
\caption{Current clinical trials testing nanoparticle therapies in cancer.}
\end{table}

\#PEG, Polyethylene glycol.

\textsuperscript{*The field of nanotherapies has exploded in the last five years, resulting in the use of nanoparticle-based therapies in the treatment of several malignancies. Albumin-bound paclitaxel is currently employed as a therapy for pancreatic cancer. There is ongoing work by several groups towards establishing the ideal nanoparticle for use in the treatment of pancreatic cancer. \#PEG, Polyethylene glycol.
Nanoparticles must be stable in the bloodstream and able to deliver their cargo to tumour cells if they are to be used in a therapeutic setting. They are often charged to enable them to bind their cargo. However, this charge can trigger immune responses and bind proteins in the blood that hinder their function. One way to improve the stability of nanoparticles is by addition of neutral charged polymers to the surface of nanoparticles. For example, the addition of polyethylene glycol has been used to shield nanoparticles in the bloodstream. An appealing feature of nanoparticles is the ability to target them to specific cell types by attaching targeting moieties. This reduces off-target toxicity commonly associated with conventional chemotherapy. For example, studies have employed vitamin A-conjugated nanoparticles to deliver RNAi therapy to hepatic stellate cells and PSCs in mouse models of hepatic and pancreatic fibrosis. The group demonstrated that they could effectively deliver RNAi therapy to inhibit a protein involved in production of fibrosis, specifically in stellate cells. Notably, these nanoparticles/RNAi therapies were able to resolve pancreatic and hepatic fibrosis.

Nanoparticles therefore have the potential to transform treatment for pancreatic cancer, especially in the context of recent advances in pancreatic cancer genomics. We now know that there are only a few common mutations in pancreatic cancer, making personalised medicine essential. Using nanoparticles/RNAi therapies and advanced genomics, clinicians could eventually be able to administer a specific cocktail of RNAi therapeutics based on the genetics of a patient’s tumour, with minimal off-target toxicity and high efficacy. In addition, nanoparticles can be applied in combination therapies, to package and deliver enzymes or drugs such as Abraxane® (table 1) to specific cell types, thus avoiding off-target toxicity and enhancing tumour penetration.

Preclinical imaging in pancreatic cancer

High resolution preclinical laboratory imaging technologies are being employed to unravel the biological events in pancreatic cancer. These approaches have shed light on the spatio-temporal regulation of events driving pancreatic cancer at the single cell and subcellular levels. Here, we describe how complementary preclinical imaging approaches offer insight into the molecular basis of pancreatic cancer and facilitate the development of new therapies.

Imaging the tumour microenvironment

Pancreatic cancer progression occurs in a complex three-dimensional microenvironment with reciprocal feedback from the surrounding host tissue. In vitro models combined with immunohistochemical analysis of patient tissues have been used to characterise the pancreatic tumour microenvironment. While these approaches give insights into the interactions between cancer cells and their surrounding stroma, they are rather static and therefore do not fully recapitulate the intricacy of pancreatic cancer biology. However, direct imaging of stromal components has provided insight into the complexity of tissue structures and functions during disease development.

Second harmonic generation (SHG) imaging, a label-free technique, is used to characterise the extracellular matrix texture and organisation in pancreatic cancer fibrosis. SHG imaging assessed collagen remodelling following dual treatment with gemcitabine and signal transducer and activator of transcription 3 (STAT3) inhibitors in a mouse model of pancreatic cancer, while gemcitabine delivery upon stromal intervention/reduction was monitored using dual SHG and fluorescence doxorubicin imaging. Recent SHG imaging of a human pancreatic tissue microarray (>80 patients) revealed a positive correlation between collagen abundance, tumour stage and resistance to chemotherapy.

The metabolic activity of cancer can be observed using fluorescence lifetime imaging of cellular Nicotinamide adenine dinucleotide (NADH) and FADH fluorescence (ratio of free to bound NADH) and supported the hypothesis that enhancing tumour vasculature patency may improve drug penetration in pancreatic cancer tissue. Engineering stromal and cancer cells to express fluorescent reporters has been employed to visualise the cross-talk between cancer cells and stroma, and implicated PSCs in the onset of angiogenesis and in colonisation of distant organs. Yang et al implanted RFP-pancreatic cancer cells in a green fluorescent protein-expressing host to directly visualise tumour-stroma interactions and drug response of both cancer and stromal cells. Imaging the tumour microenvironment allows us to understand the complexity of pancreatic tumours and fine-tune how to best modulate the pancreatic tumour-associated stroma.

Live imaging of biosensors to monitor tumour cell signalling

The development of fluorescent biosensors has enabled us to dissect the dynamics of molecular events and has
provided insights into their spatio-temporal regulation. As such, imaging of biosensors has shed light on mechanisms occurring in pancreatic cancer in vivo, such as changes in cell proliferation, survival, invasion, metastasis and response to chemotherapy. For example, live imaging of the prototypical RhoGTPases, RhoA and Rac-1, which are known to drive cancer cell migration, has been achieved using Förster Resonance Energy Transfer (FRET) biosensors and revealed a subcellular regulation of the small GTPases at the leading edge of invading cells in vitro and in vivo.\textsuperscript{40,41} Similarly, live monitoring of cell-cell adhesion dynamics upon anti-migratory drug treatment was recently assessed using a fluorescence recovery after photobleaching biosensor to monitor E-cadherin stability in pancreatic cancer.\textsuperscript{42}

Live imaging of fluorescent biosensors is an emerging preclinical tool for cancer research. For instance, various probes such as Fucci sensors, CDK1-FRET biosensor and photo-marking H2B-Dendra reporter are used to elucidate the dynamics of cell proliferation and give insights on the efficacy of anti-proliferative drugs.\textsuperscript{43-45} Similarly, the use of Akt, Erk and PAK-FRET biosensors have helped us untangle the molecular mechanisms governing cell survival and signal transduction in vivo.\textsuperscript{46,47} More recent developments may provide further insights into signalling pathways in pancreatic cancer. For example, polarisation resolved imaging of homo-FRET between identical fluorophores can visualise clustering of molecules commonly deregulated in cancer.\textsuperscript{48,49} Simultaneous imaging of several FRET biosensors in a single cell using spectral unmixing or homo-FRET based biosensors can help us probe the spatio-temporal dynamics of intertwined signalling events which often involve complex molecular feedback loops.\textsuperscript{50} This information can allow us to circumvent regulation loops that may lead to chemoresistance. For a list of biosensors and fluorescent techniques used to image cancer figure 2 see eg. of FRET imaging to monitor Src activity in live tumours).\textsuperscript{51} We suggest that using these tools for future pancreatic cancer research will rapidly expand our understanding of the molecular events occurring during pancreatic cancer progression and therapeutic intervention.

**Multi-modal imaging**

Simultaneous imaging of different aspects of pancreatic cancer provides a detailed picture of cancer response to preclinical strategies and may facilitate therapeutic discovery.\textsuperscript{30,44} A fluorophore-labelled lectin antibody was administered in mice bearing pancreatic tumours and used in combination with immunohistochemistry to image the effect of combination therapy on tumour vasculature.\textsuperscript{11} Likewise, Wang et al designed a gemcitabine-loaded magnetic albumin nanosphere to conduct simultaneous targeted chemotherapy and magnetic resonance imaging of drug delivery.\textsuperscript{52} These approaches allow us to assess the level of drug penetration into cancer tissue. Integrating multi-modal imaging technologies has also been employed to monitor drug targeting in a dynamic, context-dependent and subcellular level. For example, intravital imaging has been used to monitor the intracellular pharmacokinetics of PARP-1 and microtubule inhibitors.\textsuperscript{53,54} Longitudinal imaging using surgically implanted imaging windows allows us to integrate the spatio-temporal and contextual complexity of cancer progression. In particular, this technique was used to characterise the formation of a metastatic niche during

![Figure 2](image_url): Live intravital imaging of pancreatic cancer signalling: monitoring Src kinase activity using FRET imaging. FRET imaging of a Src biosensor in a xenograft model of pancreatic cancer with or without dasatinib treatment. Representative lifetime maps of ‘ON’ cells (top panel, control treatment) and ‘OFF’ cells (bottom panel, dasatinib treatment).\textsuperscript{37} FRET lifetime scale is shown on the right of each panel, from 1 nanosecond (1ns) to 3 nanoseconds (3ns).
liver colonisation by cancer cells, as well as monitor live events within the abdominal body cavity including in situ pancreatic biology in real-time.\textsuperscript{55} Importantly, this new approach will enable us to monitor drug kinetics in real-time within the same mouse (pre- and post-dosing) and is set to provide a reliable new tool for future therapeutic intervention studies in pancreatic cancer (figure 1).

Application of imaging technologies in the clinic

The use of sensitive imaging technologies may also improve the management of pancreatic cancer in the clinic. One example developed by VisEn Medical Inc is the injection of a proteolytically activated fluorophore coupled with fibre-optic confocal microscopy, which allows highly sensitive characterisation of tumour stage, lymph node status and fluorescence-guided surgery.\textsuperscript{56} Lastly, a surface-enhanced resonance Raman scattering nanoparticle has been used to detect macroscopic pancreatic lesions to identify tumour margins, and represents a tool for pancreatic resection.\textsuperscript{57}

Conclusion

The tumour microenvironment is highly complex and future therapies will likely require multi-cellular and multi-gene targeting approaches. Nanotechnology has potential to enhance both the delivery and specific targeting of pancreatic cancer, while state-of-the-art imaging technologies increase our understanding of the biology of the disease and facilitate the discovery of new treatments. In conclusion, marriage of nanoparticle delivery with advanced molecular imaging is set to rapidly improve the management and future treatment of pancreatic cancer.

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References


