**Effective modulation of stromal signaling through ROCK inhibition: Is it all in the timing?**

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**ABSTRACT**

Our recent publication demonstrates that transient inhibition of Rho-associated kinase signaling within stroma, significantly decreased in vivo primary tumor growth, metastasis and improved response to standard-of-care therapy in pancreatic cancer. Automated analysis of collagen organization in patient tumors may present a promising tool to predict response to our proposed treatment.

Pancreatic ductal adenocarcinoma (PDA) is a lethal malignancy, with less than 6% of patients surviving 5 y post-diagnosis. Large-scale genomics efforts are providing new opportunities to improve current approaches to cancer therapy.1 Contribution of the intricate, dynamic tumor microenvironment to cancer progression, metastasis and chemoresistance is increasingly being realized, with depletion of stromal elements shown to restrain or promote tumor progression and mediate treatment resistance.2 In addition, the potential effect timing of administration of targeted agents and chemotherapies may have on the therapeutic benefit of a given combination represents a phenomenon as yet not explored in detail.

The Rho family of small GTPases regulates diverse cellular processes, including cytoskeletal dynamics, cell polarity, membrane transport and gene expression, which are integral in the growth and metastatic potential of cancers.4 Several inhibitors that target Rho GTPase or its downstream effectors including Rho-associated kinases (ROCK) have shown anti-tumor activity in preclinical models,5 with only one ROCK A, G and C family kinase inhibitor, AT13148, being assessed clinically as cancer therapy (NCT01585701). Fasudil is a ROCK inhibitor and an inexpensive, off-patent agent used in the clinical management of stroke, making it an attractive drug to potentially repurpose as a cancer therapeutic.5 Using a novel short-term or “priming” treatment approach, we recently demonstrated that transient inhibition of ROCK signaling within the extracellular matrix (ECM) and tumor-associated vascularization, increased tumor perfusion, decreased in vivo primary tumor growth, metastasis and improved response to standard-of-care therapy in pancreatic cancer.6 Using biosensor intravital imaging7 we monitored the effects of ROCK inhibition in a combination of 3-dimensional (3D) platforms and within live tissues from genetically engineered and patient-derived models of PDA.

Initially, we examined the effect of ROCK targeting on ECM integrity using fibroblast-driven contraction assays, which revealed a fasudil-mediated decrease in contraction. Upon investigating the ultrastructure, integrity, and stiffness of the ECM using Second Harmonic Generation (SHG) imaging, gray-level co-occurrence matrix, scanning electron and atomic force microscopy, we observed a significant disruption of matrix integrity in fasudil-treated plugs. These initial findings prompted us to examine the effect of varying the timing of fasudil administration on cancer cell invasion and gemcitabine/Abraxane response in a 3D organotypic model using primary PDA cells established from genetically engineered LSL-KrasG12D+/−;LSL-Trp53R172H+/−;Pdx-1-Cre (KPC) mice8 and patient-derived models. We examined 3 treatment schedules using fasudil: pre-treatment or “priming,” treatment during cellular invasion (“late”) or both (“priming” plus “late” treatment). Of note, the most significant inhibition of invasion and improvement in efficacy of standard-of-care chemotherapy, gemcitabine/Abraxane, measured as changes in CDK1 activity, proliferation and apoptosis in 3D, were observed when fasudil “priming” was applied, suggesting that long-term drug exposure, often associated with toxicity, is not beneficial.

In vivo, similar “priming” or transient ROCK inhibition significantly delayed PDA progression and increased sensitivity to treatment with gemcitabine/Abraxane at both primary and secondary sites, accompanied by modulation of key events in the metastatic process: increased sensitivity of circulating tumor cells to shear stress and impaired ability of these cells to...
extravasate from the circulation, remodel the host ECM and ultimately to establish a supportive growth environment at distant sites. Moreover, reduced ECM remodeling and tissue stiffness upon fasudil “priming” was accompanied by improved short-term tumor blood perfusion and, thereby, drug delivery, as assessed by the imaging of quantum dots diffusing from blood vessels and into surrounding tumor tissue. In agreement, the role of Rho/ROCK signaling in regulating vessel integrity and tumor perfusion in a pancreatic islet carcinoma model has been described. Recently, chronic administration of fasudil monotherapy has been shown to significantly improve survival and reduce tumor burden in KPC mice, simultaneously resulting in changes of ECM remodeling, mediated at least in part by reduced release of matrix metalloproteases in the microenvironment. In contrast, our transient “priming” approach with fasudil led to reduced ECM crosslinking and relaxation of tumor tissue in the KPC and selected patient-derived xenograft models, highlighting the intricacies of ROCK-induced ECM remodeling and moreover, the critical role timing of drug administration may play in these complex environments. Moreover, targeting ECM-dependent and ECM-independent components within cancer/stromal signaling may produce an important advantage of transient ROCK inhibition compared with chronic ECM targeting, particularly when combined with clinically-used chemo-therapeutics, and warrants further consideration.

Finally, we created an automated SHG imaging approach to monitor fibrosis levels in pancreatic tumors. By analyzing the patient tumor material and matched patient-derived xenografts from the genomically-sequenced Australian Pancreatic Cancer Genome Initiative cohort we observed a graded response to our ROCK “priming” treatment approach, which correlated with the level of cross-linked collagen in the patient-derived xenografts, with high cross-linked tumors responding significantly better to our transient stromal-targeting approach. Based on our in vivo findings on the blood vasculature relaxation and improved drug delivery with fasudil, markers such as CD31, along with collagen levels could present useful companion biomarkers to guide our proposed “priming” treatment strategy (Fig. 1).

Although fasudil monotherapy or combinations with selected cardiovascular agents have previously been safely used in numerous large-scale human studies, this agent has not been combined with chemotherapies in the clinic. Hence, a phase I clinical trial would be the next logical step to determine the feasibility of “priming” human pancreatic tumors with Fasudil before gemcitabine/Abraxane treatment. We envisage that fine-tuning the modulation of stromal components via ROCK inhibition or using other anti-stroma targeted therapies before standard-of-care treatment may lead to substantial improvements in therapeutic response in PDA, but also other malignancies defined by a significant stromal reaction.

Disclosure of potential conflicts of interest
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