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Adjuvant Trametinib Delays the Outgrowth of Occult Pancreatic Cancer in a Mouse Model of Patient-Derived Liver Metastasis

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Abstract

Purpose—Most patients with pancreatic ductal adenocarcinoma (PDAC) die within 5 years following resection plus adjuvant gemcitabine (Gem) from outgrowth of occult metastases. We hypothesized that inhibition of the KRAS pathway with the MEK inhibitor trametinib would inhibit the outgrowth of occult liver metastases in a preclinical model.

Methods—Liver metastases harvested from two patients with PDAC (Tumors 608, 366) were implanted orthotopically in mice. Tumor cell lines were derived and transduced with lentiviruses encoding luciferase and injected into spleens of mice generating microscopic liver metastases. Growth kinetics of liver metastases were measured with bioluminescent imaging and time-to-progression (TTP), progression-free survival (PFS), and overall survival (OS) were determined.

Results—Trametinib (0.3 mg/kg BID) significantly prolonged OS versus control (Tumor 608: 114 vs. 43 days, $p < 0.001$; Tumor 366: not reached vs. 167 days, $p = 0.0488$). In vivo target validation demonstrated trametinib significantly reduced phosphorylated-ERK and expression of the ERK-responsive gene *DUSP6*. In a randomized, preclinical trial, mice were randomized to: (1) control, (2) adjuvant Gem (100 mg/kg IP, Q3 days) × 7 days followed by surveillance, or (3) adjuvant Gem followed by trametinib. Sequential Gem-trametinib significantly decreased metastatic cell outgrowth and increased TTP and PFS.

Conclusions—Treatment of mice bearing micrometastases with trametinib significantly delayed tumor outgrowth by effectively inhibiting KRAS-MEK-ERK signaling. In a randomized, preclinical, murine trial adjuvant sequential Gem followed by trametinib inhibited occult metastatic cell outgrowth in the liver and increased PFS versus adjuvant Gem alone. An adjuvant trial of sequential Gem-trametinib is being planned in patients with resected PDAC.

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DISCLOSURE The authors disclose no potential conflicts of interest.

Patients who undergo a margin-negative resection of localized pancreatic ductal adenocarcinoma (PDAC) followed by adjuvant chemotherapy have a median survival of only 21 months and 80 % of patients will die within 5 years due to the development of metastases.[1,2] Most of these metastases occur in the liver, and thus, patients likely harbor occult metastatic PDAC in the liver at the time of surgery and these occult metastatic cells may be resistant to current adjuvant strategies. The fate of these metastatic cells is dependent on the complex interactions between the liver microenvironment and metastatic niche, local immune factors, and individual PDAC tumor biology.[3]

Given the prominent role of activating *KRAS* mutations in PDAC progression, effective inhibition of the RAS-RAF-MEK-ERK signaling cascade is an important target for therapy and has been identified as a priority in pancreatic cancer research.[4–8] This signaling is required for epithelial-to-mesenchymal transition (EMT) and increased cancer cellular motility and invasiveness, which are all required for metastasis.[9,10] Additionally, cancer cellular-microenvironment interactions are dependent on RAS pathway signaling, including angiogenesis and immune system evasion.[9,11,12] However, the role of RAS pathway signaling in cells within the metastatic microenvironment is largely unknown. We previously demonstrated that the MEK inhibitor trametinib significantly inhibited orthotopic tumor growth of patient-derived pancreatic cancer xenografts in mice.[13,14] Based on these results and the known role of MEK signaling in processes necessary for metastatic cell growth, we hypothesized that MEK inhibition would inhibit the outgrowth of occult liver metastases from PDAC.

Utilizing a patient-derived splenic injection model of PDAC metastatic to the liver, we demonstrate that trametinib decreased overall liver tumor burden, increased time to proliferative outgrowth of PDAC tumors, and significantly increased survival of mice. Moreover, we designed a rational preclinical trial using this model to reflect an adjuvant clinical trial in patients and show that gemcitabine therapy followed sequentially by maintenance trametinib therapy is superior to standard-of-care gemcitabine.

METHODS

Therapeutics

Trametinib is a potent and selective allosteric inhibitor of MEK1/2 and was obtained from GlaxoSmithKline (Brentford, United Kingdom).[15–17] Gemcitabine (2′,2′-difluoro-2′-deoxycytidine), a nucleoside, was obtained from the University of Virginia Clinical Pharmacy.

Derivation of Cell Lines and Lentiviral Transduction

Patient-derived cell lines (Tumors 608, 366) were obtained under IRB protocol as previously described and have been extensively characterized.[13,14,18,19] Cells were transduced using firefly luciferase lentivirus (KeraFAST, Boston, MA). Cell lines were maintained in RPMI containing 10 % FBS and penicillin/streptomycin. Cells were thawed, propagated, and used for experiments every 4– 6 months (fewer than ten passages).

Splenic Injection Model of PDAC Growth in the Murine Liver

Patient-derived PDAC cell lines (Tumors 608, 366) were harvested by trypsinization and a suspension of 1×10^6 cells/50 μL was prepared in serum-free media for injection. Six-to-eight week old athymic nude mice (NCI, Frederick, MD) were anesthetized with 0.1 cc of ketamine (75 mg/kg) and dexmedetomidine (0.2 mg/kg), intraperitoneal for splenic injection. A 1.5-cm left flank incision was made and the spleen was exteriorized; then, cells were injected into the spleen. Tumor cells were allowed to circulate for 10 min, and then the spleen was resected. The peritoneum and skin were sutured, Atipamezole (2 mg/kg, subcutaneous) was used to reverse the anesthesia, and 100-μL subcutaneous injection of ketoprofen (1 mg/mL, provided by the University of Virginia Department of Comparative Medicine) was administered postoperatively for analgesia.

In Vivo Bioluminescent Imaging

After splenic injection of luciferase-transduced PDAC cell lines, hepatic tumor cell burden was monitored by serial in vivo bioluminescent imaging. Ten minutes before imaging, mice were anesthetized with aerosolized isoflurane and dosed with luciferin substrate (150 mg/kg i.p.). Mice were then imaged using the IVIS 100 imaging system (Xenogen, Alameda, CA). Luminescence was captured and quantified using a region of interest tool, as previously described.[20,21] Luminescence data were plotted relative to the initial bioluminescence level to generate tumor growth curves. Time to proliferative outgrowth, or time-to-progression (TTP), was defined as the time to develop relative average hepatic bioluminescence of 2.0. Progression-free survival (PFS) was defined as survival of mice without evidence of tumor progression (i.e., relative average hepatic bioluminescence <2.0).

Histologic Evaluation

Paraffin-embedded sections of murine livers pre- and post-splenic injection of PDAC cell lines were stained with H&E using standard methods. Immunohistochemical staining was performed using antibodies to human EpCAM (CD326) and human phosphorylated ERK1/2. Negative controls were performed omitting the primary antibody.

Quantitative Real-Time PCR

Isolation of tumor cells from the livers of mice was performed utilizing magnetic-activated cell sorting with bead-labeled antibodies to human EpCAM (CD326; Miltenyi Biotec Inc, San Diego, CA). RNA extraction, reverse transcription, and quantitative real-time PCR were performed as previously described.[19]

Conditions for PCR included 95 °C for 3 min, followed by 40 cycles of 15 s at 95 °C, 30 s at 56.7 °C, and 30 s at 72 °C for GAPDH and DUSP6. Relative expression levels of target sequences were determined by the standard curve method using cDNA from MPanc-96 pancreatic cancer cell line (American Type Culture Collection, Manassas, VA) using serial dilutions. Expression levels of *DUSP6* target sequences were normalized to *GAPDH* expression.

Statistical Analysis

The average hepatic radiance of individual mice during treatment was divided by starting radiance to calculate relative change in radiance. Student's *t* test was used to compare continuous variables. All group comparisons were unpaired. Continuous variables were expressed as means ± the standard error of the mean. All *p* values reported are two-tailed, and statistical significance was indicated by *p* values <0.05. All experiments were performed in triplicate. GraphPad Prism (Version 5.0b) software (La Jolla, CA) was used for all statistical analyses.

RESULTS

Splenic Injection of PDX Cell Lines

After splenic injection, both patient-derived cell lines developed liver metastases and followed reproducible phases of growth kinetics (Fig. 1a, b). Both cell lines experienced an initial rapid clearance phase corresponding with decay in hepatic bioluminescence, which led to a cell survival phase (the minimum measured hepatic bioluminescence). This phase of cellular survival was followed by proliferative outgrowth and the rapid and robust increase in bioluminescent signal from the murine livers (Fig. 1a, b).

The phases of rapid clearance and cellular survival were temporally different between Tumor 608 and 366, which led to a time to proliferative outgrowth (twofold increase in relative hepatic bioluminescence) that was characteristic and reproducible for each cell line examined (Tumor 608: 18 days, Fig. 1a vs. Tumor 366: 80 days, Fig. 1b).

Histologic Evaluation of Liver Microenvironment After Splenic Injection

Hematoxylin and eosin staining of livers at 48 h after injection of Tumor 608 cells demonstrated intact tumor cells within the distal hepatic portal venous structures with corresponding well-demarcated areas of regional hepatic injury and necrosis (Fig. 1c). The areas of hepatic injury were devoid of intact hepatic architecture and associated cells. Higher magnification revealed intact embolized PDAC tumor cells filling the hepatic portal venous structures within the area of injury. After 72 h post-injection, tumor cells were still evident within the vasculature and there was an increase of immune response cells surrounding the perimeter of injury and beginning to infiltrate the remodeling liver (Fig. 1c). Intact disordered glandular structures characteristic of adenocarcinoma were clearly developed by 21 days with associated stroma reflective of PDAC's characteristic desmoplastic reaction (Fig. 1c). Similar results were seen for Tumor 366 (data not shown).

Role of RAS-MEK-ERK Signaling on PDX Cell Proliferation in the Liver

Mice underwent splenic injection of either Tumor 608 or Tumor 366 cells then were randomized to either trametinib (0.3 mg/kg, oral gavage, daily) or vehicle control beginning 48 h post-splenic injection (Fig. 2). Mice harboring Tumor 608 and treated with control vehicle exhibited a steady decay of bioluminescence to a nadir at approximately 7–10 days post-splenic injection, whereas mice treated with trametinib reached a nadir in bioluminescence at approximately 14 days and at a level lower than that of the bioluminescence in the livers of control mice (Fig. 2a, b). Mice bearing Tumor 366 cells and

treated with vehicle control reached a nadir in hepatic bioluminescence at approximately 10 days (vs. 17 days for trametinib, Fig. 2c). Control mice harboring Tumor 608 cells reached proliferative outgrowth at approximately 16 days post-splenic injection (vs. 28 days with trametinib treatment, Fig. 2b). For mice harboring Tumor 366 cells in the liver, time to proliferative outgrowth with trametinib treatment was over 170 days, compared with approximately 60 days for control mice (Fig. 2c). Thus, trametinib therapy resulted in a more than twofold increase in time to proliferative outgrowth for both patient-derived PDAC cell lines.

In a survival study, mice with hepatic Tumor 608 treated with trametinib at 48 h post-injection had a median overall survival of 114 vs. 43 days for control ($p < 0.001$; Fig. 2d). Control mice harboring Tumor 366 had a median overall survival of 167 days. Trametinib significantly prolonged survival and median survival was not reached in the trametinib group by the end of the study as very few mice died ($p = 0.0488$; Fig. 2e). Trametinib therapy resulted in a dramatic increase in overall survival for mice harboring hepatic patient-derived PDAC tumors.

In Vivo Target Validation

We next investigated the degree that trametinib inhibits the RAS-RAF-MEK-ERK signaling axis in vivo. Mice harboring Tumor 608 and treated with either control or trametinib starting at 48 h post-injection were sacrificed after 22 days and histologic analyses were performed. Immunohistochemical staining of tumors for phosphorylated ERK (pERK1/2) revealed a significant decrease of this activated protein with trametinib treatment (Fig. 3a). RT-PCR for *DUSP6* from tumor cells harvested from the livers demonstrated decreased levels of transcripts of *DUSP6* in trametinib treated mice (Fig. 3b), consistent with *DUSP6*'s role as a negative feedback regulator of the RAS pathway and a biomarker of RAS pathway output.[22] These results demonstrated in vivo target validation that trametinib decreases RAS-RAF-MEK-ERK signaling.

Preclinical Trial of Gemcitabine Followed by Trametinib for Occult Metastatic PDAC

We next designed a preclinical adjuvant trial utilizing patient-derived PDAC cells in our splenic injection model. Currently, most patients with PDAC receive adjuvant gemcitabine following surgical resection.[23] We chose a study design of sequential therapy with gemcitabine followed by trametinib as in vivo studies demonstrated that simultaneous therapy was not superior to trametinib alone (data not shown). Given that both agents target proliferating cells, these results were not surprising and were consistent with results in human clinical trials.[24,25] Mice underwent splenic injection of patient-derived PDAC cells (Tumors 608, 366) and were then randomized to receive placebo (vehicle control), gemcitabine (100 mg/kg, IP, q3 days) for 7 days followed by placebo, or gemcitabine for 7 days followed by trametinib (0.3 mg/kg, oral, daily). A total of 7 days of gemcitabine was chosen to allow modest response to gemcitabine and initiation of trametinib during a period of occult-only disease to model an adjuvant setting in humans in whom there is occult-only disease.

The results of the preclinical trials are depicted in Fig. 4. Adjuvant gemcitabine followed sequentially by daily trametinib significantly prolonged TTP for Tumor 608 (Fig. 4a; 39 vs. 30 days, $p = 0.0384$) and for Tumor 366 (Fig. 4b; >132 vs. 53 days, $p = 0.00013$). The growth inhibition of occult liver metastases by sequential gemcitabine-trametinib translated to a significant increase in PFS in Tumor 608 (Fig. 4c) and Tumor 366 (Fig. 4d).

DISCUSSION

Patients diagnosed with PDAC have a prognosis that is among the worst of any solid organ malignancy and current therapy unfortunately leads to meager increases in survival after surgery.[1,2,26] The lack of significant improvement in patient survival following complete resection of localized PDAC can be attributed to the limited efficacy of current adjuvant therapies. This may be due in part to a lack of efficient, reproducible preclinical models of metastatic PDAC that are necessary to foster therapeutic development.[27] We have developed a reproducible patient-derived xenograft model that recapitulates many of the steps of metastatic PDAC to the liver and this model has been extensively internally validated (data not shown). The patient-derived cell lines used are from untreated patients (e.g., drug-naïve) and are sensitive to gemcitabine in vitro and in vivo.

This preclinical model has limitations in that nude mice lack T cells, and thus, any contribution of these cells to tumor growth and response to MEK inhibition cannot be assessed. However, nude mice do have an intact innate immunity (fibroblasts, macrophages, natural killer cells). Another limitation of this model, like any preclinical model, is the difficulty in translating drug dosing and scheduling from humans to mice. In these studies, we used previously published drug doses and schedules.

The RAS-RAF-MEK-ERK signaling cascade has been shown to be integral in the development and proliferation of cancers harboring activating *RAF* or *RAS* mutations, such as PDAC.[11,28] Inhibiting this growth and differentiation pathway via BRAF inhibitors has significantly improved the outcomes of patients with melanoma, and clinical trials have demonstrated the efficacy of combination therapy with BRAF inhibitor plus MEK inhibitor.[29,30] Recently, a phase II trial of concurrent gemcitabine plus trametinib in patients with previously untreated, radio-graphically detectable (non-occult) metastatic PDAC did not translate into any significant improvement in survival.[31] Our preclinical model of advanced PDAC (unresectable primary tumor and grossly visible metastatic disease) using patient-derived tumors corroborated the findings of this phase II trial, demonstrating that concurrent trametinib plus gemcitabine is ineffective in the setting of advanced PDAC (data not shown). In contrast, the studies described here demonstrate that micrometastatic PDAC cells in the liver are susceptible to RAS pathway signaling inhibition. Trametinib treatment significantly delayed time to metastatic cell outgrowth and this led to significant increase in survival. Sequential, rather than simultaneous, treatment with gemcitabine and trametinib may prove superior in patients because of the competing mechanisms of action of these drugs—gemcitabine is a nucleoside-analog that targets dividing cancer cells and similarly trametinib has been shown to induce cell-cycle arrest, thus inhibiting cell division. The archived tumor tissues generated from this work will allow for future investigation of the mechanism of resistance to MEK inhibition with trametinib (i.e., trametinib-treated tumors

in Figs. 2 and 4 that eventually grew out). This could lead to novel combination therapies employing MEK inhibition for PDAC.

A randomized, phase II, clinical trial is being planned to evaluate the efficacy and tolerability of adjuvant sequential gemcitabine followed by trametinib for patients with resected PDAC (Fig. 5). Following surgical resection of pathologically confirmed PDAC patients will be randomized to adjuvant gemcitabine followed by placebo versus gemcitabine followed by trametinib. Patients will be monitored for disease progression with CT every 3 months. The primary endpoint of the study will be DFS, and the secondary endpoint will be OS. Correlative studies on pancreatic resection specimens will include *KRAS* status and staining for ERK and phosphorylated-ERK.

CONCLUSIONS

In a preclinical model we have shown that the MEK inhibitor trametinib is effective at increasing TTP and survival of mice harboring occult patient-derived PDAC in the liver. Furthermore, we demonstrated that sequential treatment with adjuvant gemcitabine followed by trametinib is superior to gemcitabine alone in a preclinical trial of PDAC metastatic to the liver. These promising preclinical results support the conduct of a clinical trial (currently in development) of a MEK inhibitor following standard-of-care gemcitabine for patients with resected PDAC.

Acknowledgments

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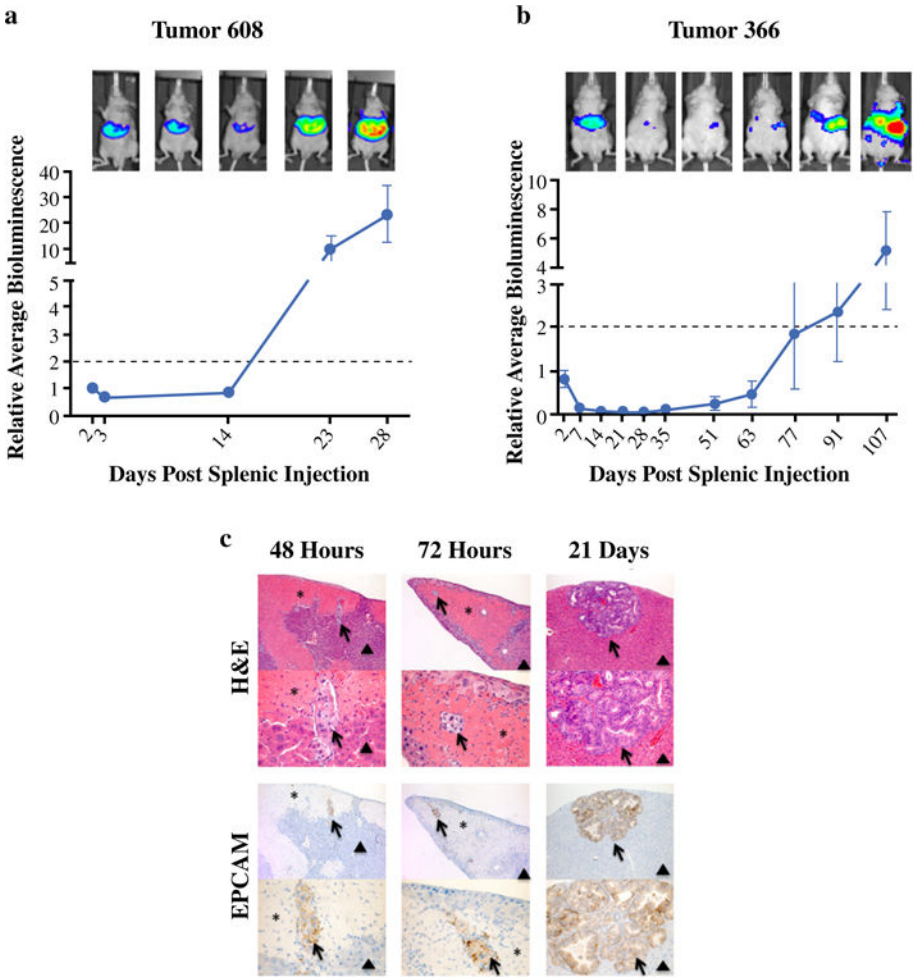

FIG. 1.

Patient-derived PDAC cell lines exhibit characteristic and reproducible growth kinetics in the murine liver microenvironment following splenic injection. Sequential in vivo bioluminescent imaging following splenic injection of 10^6 tumor cells reveals both Tumor 608 (**a**) and Tumor 366 (**b**) cell lines share conserved phases of growth but have differential growth kinetics. *Dotted line* signifies a twofold increase in average hepatic bioluminescence from 48 h post-splenic injection and represents time to proliferative outgrowth (days). Patient-derived PDAC cell lines develop into mature PDAC tumors (**c**) and recapitulate metastatic disease in the liver of patients. Livers were harvested from mice harboring Tumor 608 cells 48, 72 h, and 21 days post-splenic injection and prepared for histologic review. Hematoxylin and eosin staining reveals tumor architecture and corresponding areas were prepared for IHC utilizing an anti-EpCAM (CD326) antibody recognizing human epithelial cells and, thus, patient-derived PDAC cells. *Arrows*, metastatic tumor cells. *Asterisks*, areas of peritumoral hepatic injury and necrosis. *Arrow heads*, normal liver

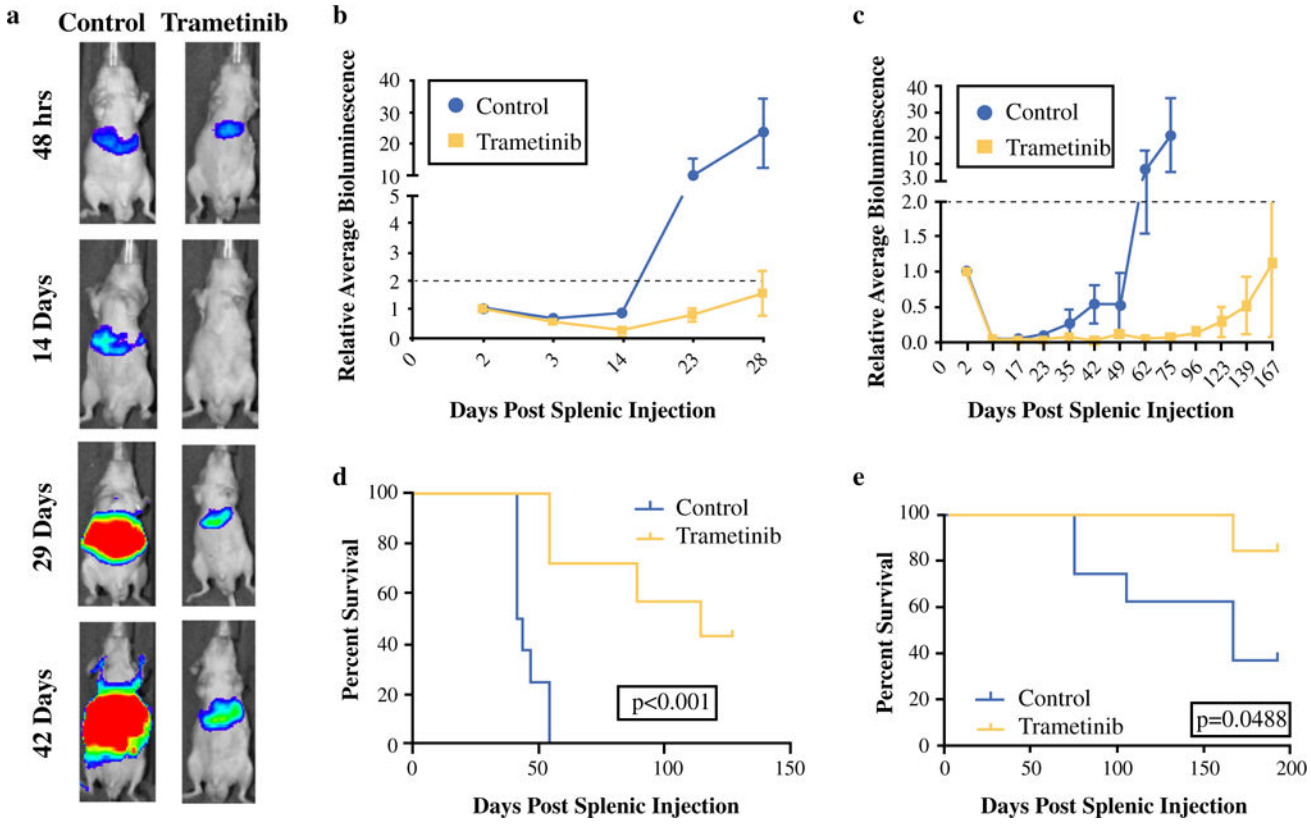

FIG. 2.

Blocking the RAS-RAF-MEK-ERK signaling pathway results in significant inhibition of PDAC growth in the liver and prolongs survival of mice harboring hepatic PDAC. Mice received either the MEK inhibitor trametinib (0.3 mg/kg, oral gavage, daily) or vehicle control beginning 48 h post-splenic injection. **a** Representative images of hepatic bioluminescence from mice harboring Tumor 608 over the course of therapy (quantified linearly in **b**). Daily trametinib beginning 48 h post-splenic injection significantly decreased hepatic tumor burden (**a**) and prolonged time to proliferative outgrowth for both Tumor 608 (**b**) and Tumor 366 (**c**). Moreover, trametinib significantly prolonged survival of mice harboring hepatic Tumor 608 (**d**) and Tumor 366 (**e**)

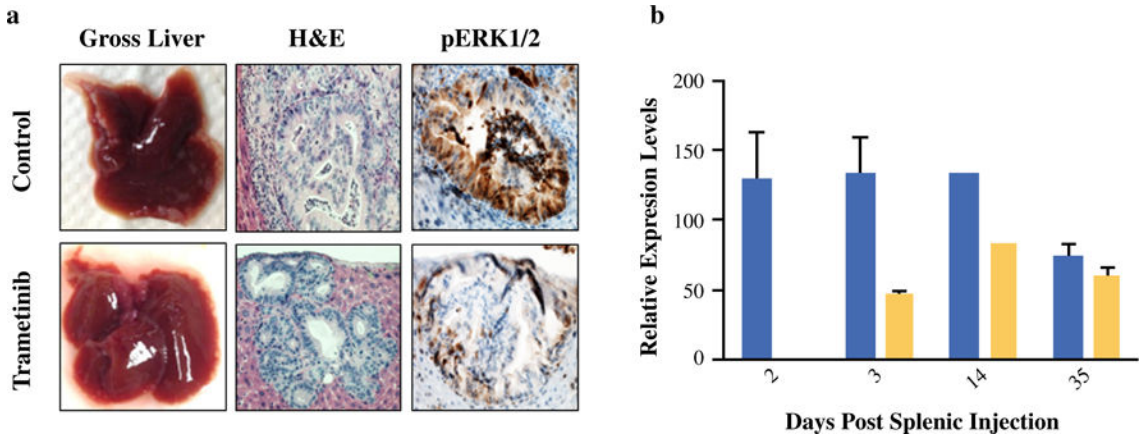

FIG. 3.

Trametinib decreases RAS pathway signaling output in PDAC tumors in the liver microenvironment. **a** Gross and histologic review of livers harvested from mice harboring Tumor 608 21 days post-splenic injection. Mice were treated with either vehicle control or trametinib beginning at 48 h post-splenic injection. Immunohistochemistry utilizing an antibody recognizing phosphorylated human ERK1/2 revealed decreased levels of activated ERK1/2 in mice receiving trametinib. **b** RT-PCR for *DUSP6* from RNA isolated from PDAC cells isolated from the livers of mice harboring Tumor 608 at various time points after splenic injection revealed decreased levels of transcripts of *DUSP6* in mice treated with trametinib (*yellow bars*) versus mice that received vehicle control (*blue bars*). Expression levels of *DUSP6* were normalized to expression levels of a housekeeping gene, *GAPDH*

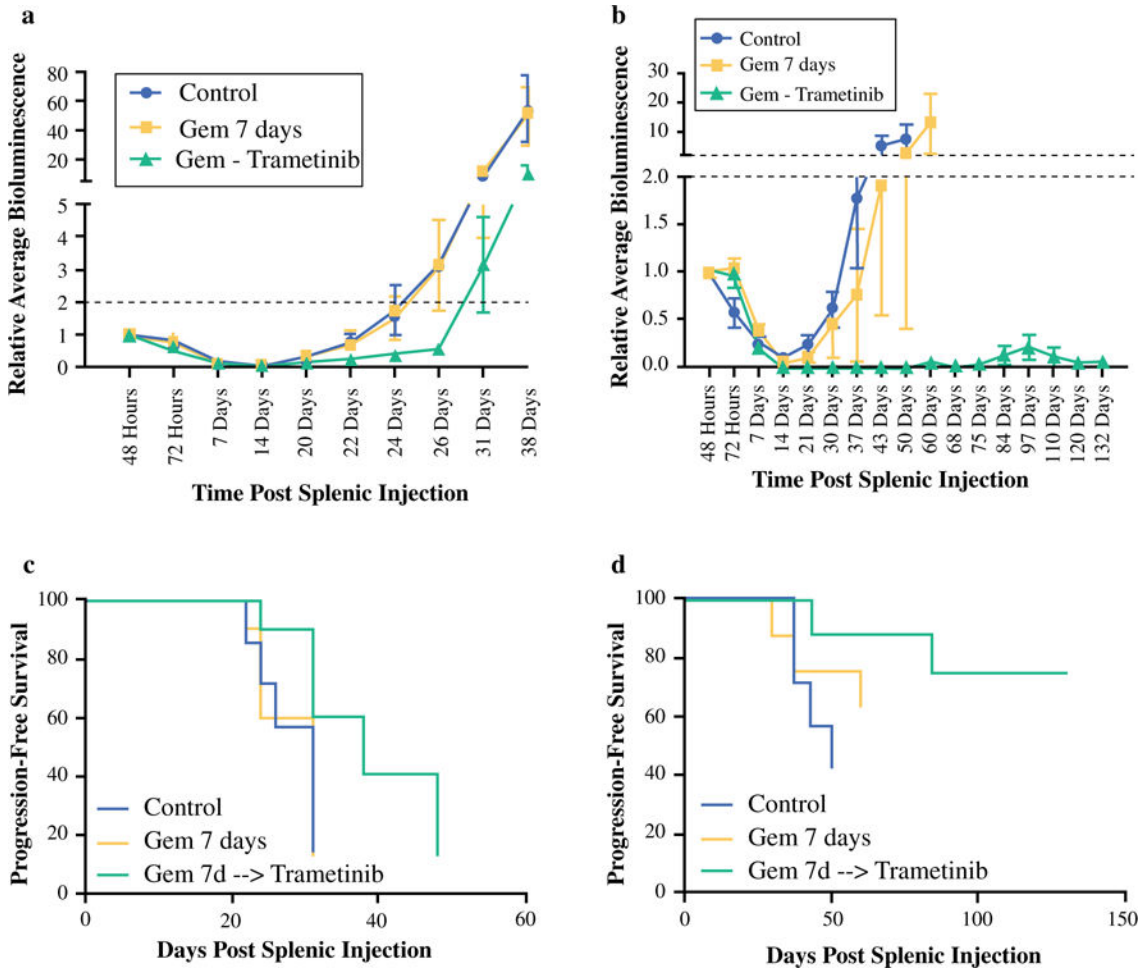

FIG. 4.

Preclinical trial of gemcitabine followed by trametinib for occult metastatic PDAC. Adjuvant gemcitabine followed sequentially by daily trametinib therapy resulted in significantly increased time to proliferative outgrowth and decreased metastatic tumor burden at conclusion of the trial in the livers of mice harboring Tumor 608 (**a**, $p = 0.0262$) and Tumor 366 (**b**, $p = 0.0007$) compared with vehicle control or gemcitabine alone. This adjuvant regimen resulted in increased 35-day PFS ($p = 0.0108$) in Tumor 608 (**c**) and improved median PFS ($p = 0.0367$) and 100-day PFS ($p = 0.007$) in tumor 366 (**d**)

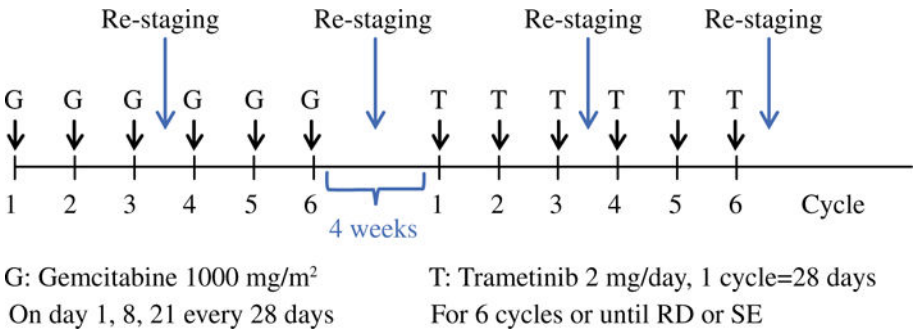

FIG. 5.

Planned clinical trial schema. A randomized, phase II, clinical trial is being planned to evaluate the efficacy and tolerability of adjuvant sequential gemcitabine followed by trametinib for patients with resected PDAC. *RD* recurrent disease, *SE* side effects