

Efficacy and pharmacodynamic effects of bosutinib (SKI-606), a Src/Abl inhibitor, in freshly generated human pancreas cancer xenografts

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Abstract

Recently, Src tyrosine kinase has emerged as an attractive target for anticancer therapy, and Src is overexpressed in pancreatic cancer. The purpose of the study was to investigate the *in vivo* efficacy and pharmacodynamic effects of bosutinib (SKI-606), a Src/Abl inhibitor, using a panel of human pancreatic tumor xenografts. Surgically resected human pancreatic tumors were implanted into female nude mice and randomized to bosutinib versus control. Src and other pathways were analyzed by Western Blot, IHC, and Affymetrix U133 Plus 2.0 gene arrays. Of 15 patient tumors, 3 patient tumors were found to be sensitive to bosutinib, defined as tumor growth of <45% than that of control tumors. There were no definite differences between sensitive and resistant tumors in the baseline Src kinase pathway protein expression assessed by Western Blot. Caveolin-1 expression, as assessed by

reverse transcription-PCR and immunohistochemistry, was frequently higher in sensitive cases. In sensitive tumors, bosutinib resulted in increased apoptosis. Phosphorylation of key signaling molecules downstream of Src, signal transducers and activators of transcription 3, and signal transducers and activators of transcription 3, were significantly inhibited by bosutinib. K-Top Scoring Pairs analysis of gene arrays gave a six-gene classifier that predicted resistance versus sensitivity in six validation cases. These results may aid the clinical development of bosutinib and other Src inhibitors in pancreas cancer. [Mol Cancer Ther 2009;8(6):1484–93]

Introduction

Pancreatic adenocarcinoma is the fourth leading cause of cancer deaths annually. The estimated incidence for 2008 was 37,680 new cancers with an estimated mortality of 34,290 (1). Despite recent improved understanding in pancreatic cancer biology, the 5-year survival remains at 4% despite multimodality therapy (2). Gemcitabine has been the stalwart of chemotherapy but results in a median survival of only 5.65 months (3). Improvements in therapy have been modest with the addition of erlotinib or capecitabine to gemcitabine in combination, resulting in improved median survival on the order of weeks (4, 5). The continued poor survival despite the new understanding of pancreatic cancer biology and the incorporation of novel therapies shows an acute need for improvement in therapy. To this end, a patient-derived pancreatic adenocarcinoma explant xenograft model (PancXenoBank) has been produced to help develop novel therapies for pancreatic cancer (6).

One emerging cancer target is the nonreceptor tyrosine kinase, c-Src (7). This protein is one of at least nine members of the Src family kinases, which have significant homology. Src regulates multiple cascades that effect cellular adhesion, migration, and invasion, factors that when dysregulated enable tumor cells to disrupt their microenvironment, travel to distant sites, and invade host tissues independent of normal regulatory signals. Src TK activity is important in the epithelial to mesenchymal transition that occurs in the early stages of invasion of carcinoma cells (8). Src has also been implicated in angiogenesis and vascular permeability (9).

There are multiple lines of clinical and preclinical evidence implicating Src as a key player in pancreas cancer. Src tyrosine kinases are overexpressed in pancreatic carcinoma (10). The Src pathway is activated in preclinical models of regenerating pancreas epithelial cells (11) and in proliferative responses of pancreatic stellate cells (12). Most importantly, in an animal model of orthotopically implanted human pancreatic cancer cell lines, Src inhibition

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led to decreased proliferation, decreased microvessel density, and increased apoptosis, and significantly decreased metastases (13, 14). Thus, Src is a highly attractive target in pancreas cancer.

Several Src inhibitors are under clinical development, including dasatinib (BMS-354825), AZD0530, and bosutinib (SKI-606). All of these compounds also inhibit Abl, likely due to the similar conformation of Src homology domains between Src and Abl (15), and dasatinib is Food and Drug Administration–approved for resistant chronic myelogenous leukemia due to its Abl inhibition (16). These compounds have undergone phase I testing in solid tumors, and phase II studies are planned or under way. Interestingly, in the first-in-human clinical phase I study of bosutinib, a previously treated pancreas cancer patient experienced prolonged stable disease lasting >1 year (17).

Because Src inhibitors have limited single-agent cytotoxic activity, predictive (pharmacodiagnostic) or pharmacodynamic markers of activity will likely be necessary for the successful development of these compounds in solid tumors. One important preclinical study found a six-gene predictor for dasatinib susceptibility of breast cancer cell lines, and this was associated with a “triple-negative” [estrogen receptor (ER), progesterone receptor (PR), HER2] pattern in archival human breast cancer specimens (18). Another used a panel of prostate cancer cell lines to identify a five-gene set predictor, several of which were down-regulated after dasatinib treatment (19). We used a panel of 15 human tumor xenografts to explore the antitumor activity and search for pharmacodynamic and pharmacodiagnostic markers of efficacy to bosutinib in pancreas cancer.

Materials and Methods

In vivo Growth Inhibition Studies

The research protocol was approved by the Johns Hopkins University Animal Care and Use Committee and animals were maintained in accordance to guidelines of the American Association of Laboratory Animal Care. Six-week-old female athymic nude mice (Harlan) were used. Xenografts were generated according to methodology published elsewhere (6). Briefly, surgical nondiagnostic specimens of patients operated at the Johns Hopkins Hospital were reimplanted s.c. to one to two mice for each patient, with two small pieces per mouse (F1 generation). Tumors were allowed to grow to a size of 1.5 cm³, at which point they were harvested, divided, and transplanted to another five mice (F2 generation). After a second growth passage tumors were excised and propagated to cohorts of 20 mice or more, which constituted the treatment cohort (F3 generation). Tumors in the PancXenoBank are kept as a live bank that is expanded as required for drug testing and biological studies. Histology and most molecular markers remain consistent through passaging (6).

Tumors from this treatment cohort were allowed to grow until reaching ~200 mm³, at which time mice were randomized in the following two treatment groups, with 5 to 6 mice (10 evaluable tumors) in each group: (a) control and (b) bo-

sutinib 100 mg/Kg/d by oral gavage (provided by Wyeth Research). Treatment was given for 28 d. Mice were monitored daily for signs of toxicity and were weighed thrice per week. Tumor size was evaluated twice per week by caliper measurements using the following formula: tumor volume = (length × width²)/2. Relative tumor growth inhibition was calculated by relative tumor growth of treated mice (T) divided by relative tumor growth of control mice (C) because the initiation of therapy (T/C). Cases with a T/C of ≤45% were considered sensitive, and those with a T/C between 46% and 60% of intermediate sensitivity.

Western Blot and Immunohistochemical Studies

Frozen tumor tissues (50–75 mg/mouse) from control and experimental mice were minced and incubated on ice for 30 min in ice-cold cell lysis buffer (Cell Signaling Technology) in the presence of EDTA-free protease and phosphatase inhibitors (Roche Molecular Biochemicals). The minced tissue was homogenized using a Dounce homogenizer and centrifuged at 16,000 × g at 4°C for 10 min. Protein concentrations were measured, and 40 µg proteins were electrophoresed on 4% to 12% Bis-Tris precast gels (Bio-Rad Laboratories, Inc.). After electrotransfer to Immobilon-P membranes (Millipore), membranes were blocked at room temperature with TBS [10 mmol/L Tris-HCl (pH 7.5), 0.5 mol/L NaCl, and 0.1% (v/v) Tween 20] containing 5% nonfat milk (Pierce) for 1 h. Primary antibodies for Src kinase pathway proteins and apoptotic markers (Cell Signaling Technology) were diluted at 1:1,000 in TBS containing 5% protease-free bovine serum albumin (Sigma-Aldrich), and the membranes were incubated with primary antibodies overnight at 4°C. After washing thrice with TBS, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies, rabbit, or mouse IgG-horseradish peroxidase (Santa Cruz Biotechnology) at a final dilution of 1:3,000. After washing thrice with TBS, antibody binding was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). Western blots were quantitated by densitometry, and the results were shown as percentage of control.

For immunohistochemistry (IHC), formalin-fixed, paraffin-embedded tissues for tissue microarray were constructed. Five-micron sections were used for staining of Src, epidermal growth factor receptor (EGFR), FAK, mitogen-activated protein kinase (MAPK), p-Akt, signal transducers and activators of transcription (STAT)-3, STAT-5, Cleaved PARP, XIAP, and Caveolin-1 (CAV-1; Cell Signaling Technology) were done as per the manufacturer's instructions.

Microarray Gene Expression

Baseline, untreated tumors were profiled using Affymetrix U133 Plus 2.0 gene arrays in duplicate. Sample preparation and processing procedure was done as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Inc.). Raw files are available as Supplementary Data. Gene expression levels were converted to a rank-based matrix and standardized (mean, 0; SD, 1) for each microarray. To identify differentially expressed genes between sensitive and resistant groups, we used to signal-to-noise measurement as previously described (20).

Gene Set Enrichment Analysis

Gene Set analysis was done using the gene set enrichment analysis software (21) Version 2.0.1 obtained from the Broad Institute.⁷ Genes represented by more than one probe were collapsed using the Collapse Probes utility to the probe with the maximum value. The gene sets database was compiled from the KEGG database (May 29, 2007 version; ref. 22). The KEGG gene sets database contains 197 human pathways that include metabolism, genetic information processing, environmental information processing, cellular processes, and human diseases. One hundred sixty-five gene sets passed the gene set size filter criteria (min, 10; max, 500). *P* values for the gene sets were computed by permuting the genes 500 times in this study.

Gene Expression Classifiers

The K-Top Scoring Pairs (k-TSP) classifier was done as previously described (23). For each pair of genes, the algorithm computes a binary comparison *indicator* variable, $Z_{ij} = I\{X_i^{(mRNA)} > X_j^{(mRNA)}\}$, whose value is 1 if $\{X_i^{(mRNA)} > X_j^{(mRNA)}\}$ and is 0 otherwise. In the comparison-based approach, classifiers are based on small collections of such indicator variables, which are selected by estimating their conditional class distributions from the training data. A score (Δ_{ij}) is computed for each pair of genes to reflect the degree to which of the distribution of Z_{ij} changes from class 1 to class 2. Gene pairs with high score represent "informative" markers for the classification task. The classification of a new sample is based on majority voting by pairs $(X_i^{(mRNA)}, X_j^{(mRNA)})$, where the pair $(X_i^{(mRNA)}, X_j^{(mRNA)})$ votes for the class for which the actual observed value of Z_{ij} is most likely.

Quantitative Real-Time Reverse Transcription-PCR Analysis

Total RNA was extracted from 15 baseline tumors using the RNeasy Mini kit (Qiagen). cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad), following the manufacturer's instructions. cDNA (50 ng) from each sample was amplified with specific primers for CAV-1 and housekeeper gene ubiquitin from Applied Biosystems Taqman probes using an iCycler iQ real-time PCR detection system (Bio-Rad). Accumulation of the specific PCR products was detected as an increase in fluorescence that was plotted against cycle number to determine the CT values. Relative expression of the mRNA analyzed was estimated using the formula: $2^{-\Delta CT}$, where $\Delta CT = C_T(\text{mRNA}) - C_T(\text{Housekeeper})$. Samples were analyzed in a blinded manner.

CAV-1 Knockdown in Pancreatic Cancer Cells

MiaPaCa-2 and PANC-1 cells were purchased from American Type Culture Collection and were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (Invitrogen). Cells were trypsinized, seeded at 5×10^3 per well in 96-well plate, and allowed to grow overnight. Cells were transfected with siRNA specific CAV-1 (Dharmacon, Inc.) using transfection reagent on the next day and

kept for 48 h before SKI-606 (1 and 10 $\mu\text{mol}/\text{L}$) treatment. After a 72-h period of exposure, cell viability was determined by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) to formazan. Briefly, 20 μL MTT solutions (5 mg/mL in PBS) were added to each well and the plates were incubated for 3 h at 37°C. The medium was replaced with 200 μL DMSO per well. Plates were shaken and the absorbance was measured at 595 nm using a multiwell plate reader (Model 550; Bio-Rad, Inc.).

Statistical Analysis

The comparisons between means and proportions and the correlations obtained from the biological studies were done using Student's *t* test, χ^2 method, and Spearman's test, using a *P* value of 0.05 as the cutoff for significance.

Results

Effects of Bosutinib Administration on Xenografts

Bosutinib exhibited a range of *in vivo* tumor growth inhibition (T/C) between 32% to 140% in the 15 human pancreas xenografts (Fig. 1A). Using 45% as a cutoff, there were three sensitive cases: P281, P247, and P294. As with most drugs tested in this model, bosutinib did not result in tumor regression even in the most sensitive cases. Rather, the established tumors showed decreased growth compared with control in sensitive cases (Fig. 1B). This is consistent with previously published studies of bosutinib in colorectal xenografts (24) and breast xenografts (25) as well as other Src inhibitors in pancreas cancer mouse models (14).

Assessment of Src Signaling in Baseline (Untreated Tumors)

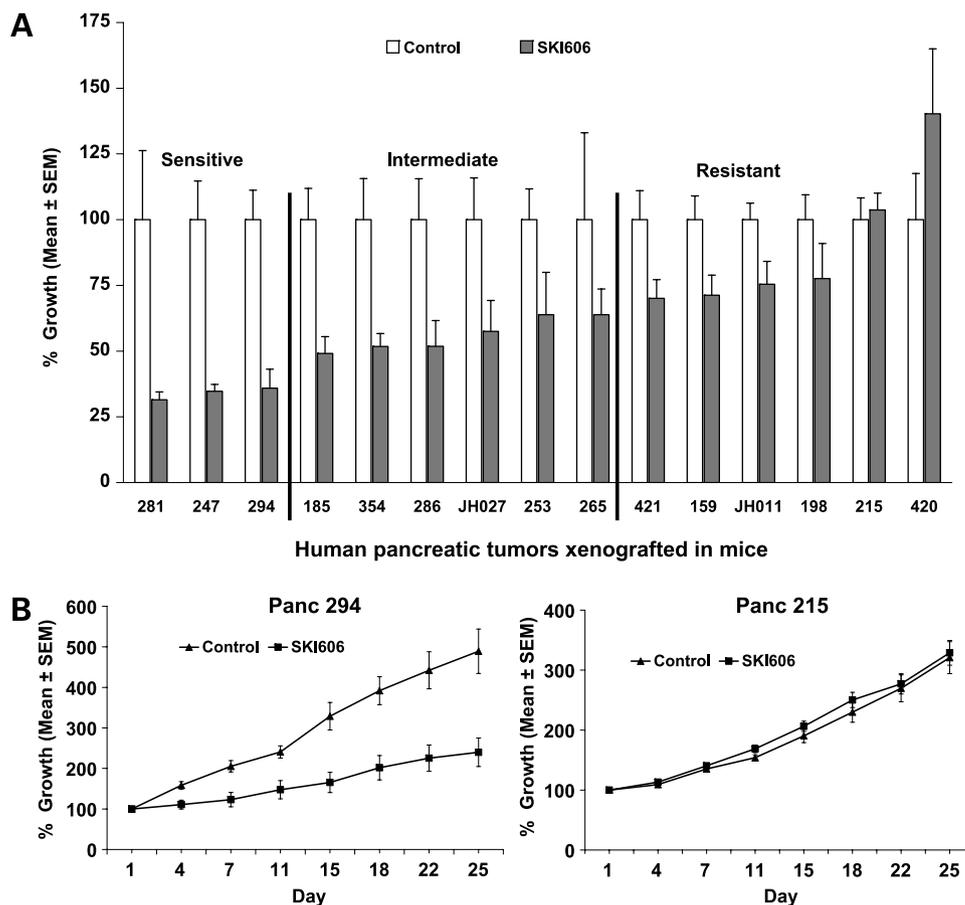
Eight tumors were assessed for baseline expression of Src pathway proteins by Western blotting, including 3 sensitive (defined as having a T/C of $\leq 45\%$: P281, P294, and P247), 3 intermediate (T/C, 46–60%: P185, P354, and P253) and 2 resistant tumors (T/C, $> 60\%$: P215 and P420). Correlation between duplicate samples was good. There were no clear differences or patterns with regard to bosutinib sensitivity and baseline expression of Src, p-Src (Tyr416), Akt, p-Akt (Ser473), STAT-3, p-STAT-3 (Tyr705), STAT-5, p-STAT-5 (Tyr694), FAK, p-FAK (Tyr925), Paxillin, p-Paxillin (Tyr118), p44/42 MAPK, p-p44/42 MAPK (Thr202/Tyr204), EGFR, PTRF, and IGFBP-2 (Fig. 2A). IHC on a tissue microarray of these tumors for total Src and p-Src, Akt and p-Akt, and MAPK and p-MAPK also failed to show any clear patterns between sensitive and resistant tumors (data not shown).

Assessment of Src Signaling and Markers of Apoptosis in Bosutinib-Treated Tumors

Two xenografts, one sensitive (P281) and one resistant (P420), were chosen to assess pharmacodynamic changes by Western blot after treatment with bosutinib (Fig. 2B). Four different samples were analyzed from P281 and P420, and optical scanning was done. The sensitive xenograft P281 showed decreased posttreatment Src, STAT-5, p-STAT-5, p-Akt, MAPK, and EGFR (Fig. 2C). Bosutinib-treated P281 showed a marked increase in cleaved PARP

⁷ <http://www.broad.mit.edu/gsea>

Figure 1. Effect of bosutinib (SKI606) on growth of human pancreas tumor xenografts. **A**, fifteen cases treated with SKI606 for 28 d; *white bars*, control group ($n = 10$ tumors implanted into 5 mice); *shaded bars*, treated group ($n = 10$); *bars*, SE. **B**, growth curves of representative sensitive (294) and resistant (215) cases (*bars*, SE).



compared with control and the resistant xenograft P420, indicating that bosutinib exerts apoptotic effects in sensitive tumors (Fig. 2C).

Gene Array Profiling and Gene Set Enrichment Analysis of Untreated Tumors

Affymetrix U133 Plus 2.0 gene arrays were done in duplicate on baseline tumor samples. To determine whether gene expression profiles could be used to distinguish sensitive and resistant tumors, we selected the top 200 differentially expressed genes between these groups. Interestingly, there was a clear difference between sensitive and resistant tumors (Fig. 3A).

We next sought to determine pathways enriched in the sensitive and resistant tumors following the CAV-1 expression seen earlier. We used the baseline expression profiles of the four sensitive tumors that expressed CAV1 (P281, P247, P163, and P410) and four resistant tumors that have low expression of CAV1 (P420, P215, P374, and JH011) for this analysis. Using gene set enrichment analysis, pathways related to focal adhesion, ECM-receptor interaction, cytokines interactions, and cell communication were enriched in the sensitive cases (Table 1). Notably, CAV1 is a core gene member in the focal adhesion pathway per KEGG annotation and represents an upstream component of the Src pathway. Conversely, metabolic pathways were enriched in the resistant tumors (Table 1).

k-TSP Classifier to Predict Bosutinib Sensitivity

We sought to build a classifier from baseline gene expression profiles to predict bosutinib susceptibility in the tumors. We used the 15 tumors as the training set and used k-TSP algorithm to these tumors. When k-TSP was done, a six-gene predictor emerged with the following decision rules: GPR116>LQK1, CEACAM7>SPFH2, and ALPK3>GUF1 indicating sensitivity versus resistance (Table 2; Fig. 3B). The leave-one-out cross-validation accuracy for the k-TSP classifier was 81%. We used an independent test set to validate the predictive power of the classifier. The k-TSP classifier was used to predict sensitivity of 15 other pancreas cancer xenografts in the PancXenoBank with independent baseline gene expression profiles. Three cases (P163, P219, and P410) were predicted as sensitive (Fig. 3C), and the other 12 cases were predicted as resistant. Six of the 15 xenografts were available for testing, and they were treated with bosutinib for 28 days in exactly the same manner as the test set. On the last measurement, the TGI values for the three predicted sensitive cases were 43%, 45%, and 48% for P219, P163, and P410, respectively (Fig. 4). The three predicted resistant cases had TGI's of 71% (JH015), 84% (P374), and 90% (JH010). Although the dynamic range of the xenograft model is limited, and several of these cases were in an intermediate range of sensitivity, this indicates the k-TSP classifier achieved reasonable predictive accuracy on the six validated cases.

CAV-1 Expression in Tumor Xenografts by Reverse Transcription-PCR, Microarray, and IHC

CAV-1 has been reported to negatively regulate the downstream activation of many signaling molecules, including the EGFR, Src family tyrosine kinases, and components of the Ras/MAP kinase pathway (26, 27). There was a higher IHC expression of baseline CAV-1 staining in the tumors (P281 and P247) sensitive to bosutinib compared with resistant tumors (P215 and P420), and bosutinib treatment augmented the CAV-1 expression in sensitive cases compared with untreated tumors (Fig. 5A). Note that nuclear staining is considered negative. We also conducted

Affymetrix gene array and reverse transcription-PCR (RT-PCR) of 15 baseline tumors in which efficacy data were obtained to assess the CAV-1 levels. There was a correlation, albeit not statistically significant, between bosutinib sensitivity and CAV-1 gene expression (relative to ubuitin) as shown by microarray and RT-PCR data. Four of the six sensitive or predicted sensitive cases (all having TGI of <48%) showed high CAV-1 expression versus 2 of 6 intermediate and 2 of 9 resistant (Fig. 5B and C). This relationship is also supported by IHC (Fig. 5A), where bosutinib treatment increased the CAV-1 expression in sensitive cases compared with untreated tumors. To further explore the role of

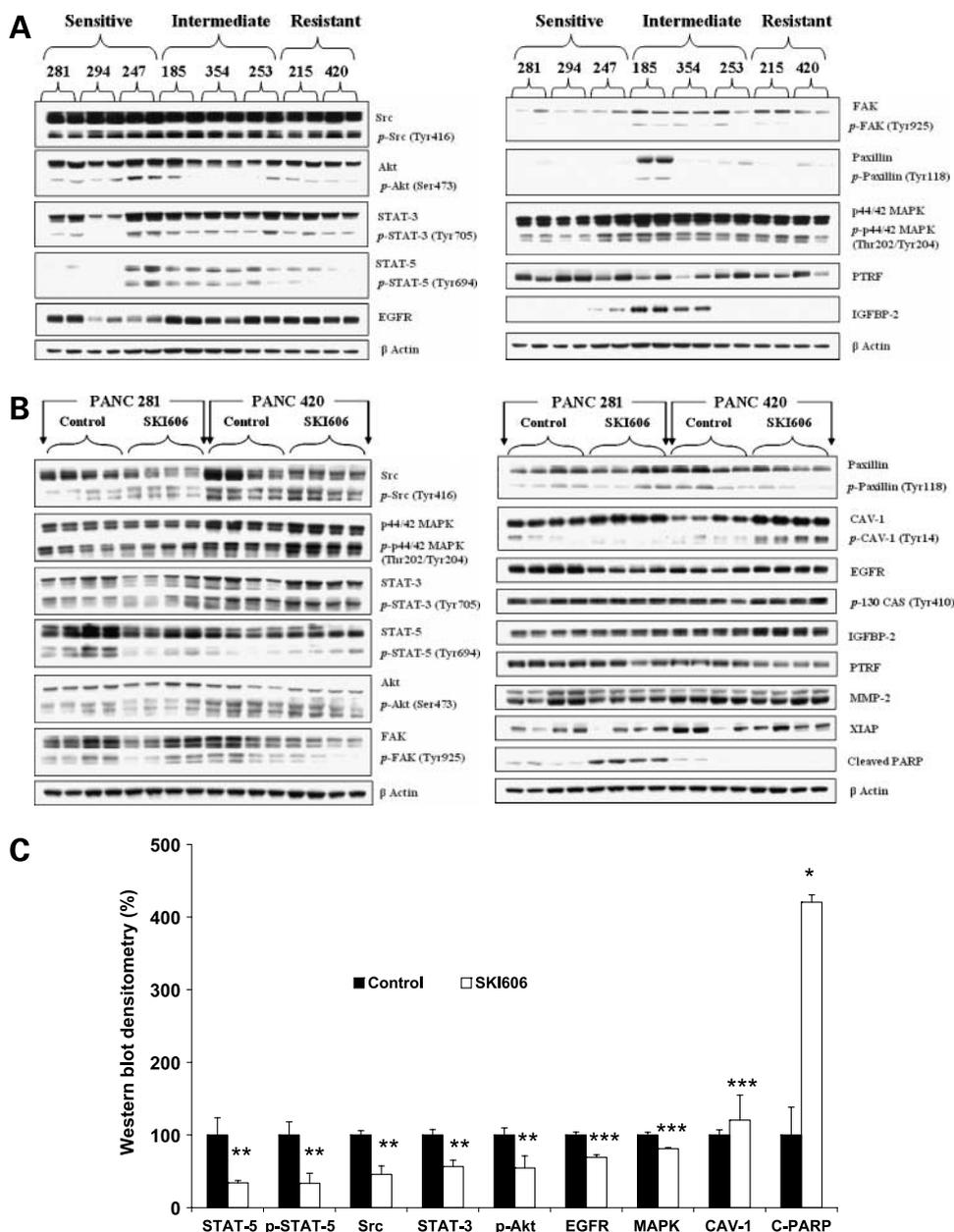


Figure 2. A, Western blot analysis of Src pathway proteins in eight pretreatment tumors (in duplicate) grouped by sensitivity. B, Western blot analysis of Src and apoptotic pathway proteins in control and SKI606 treated xenografts (Panc 281; TGI, 32%) and resistant (Panc 420; TGI, 140%). C, densitometry quantification of Src and apoptosis pathway proteins adjusted to β actin in control and bosutinib (Panc 281). *, $P \leq 0.001$; **, $P \leq 0.01$; ***, $P \leq 0.05$ compared with control

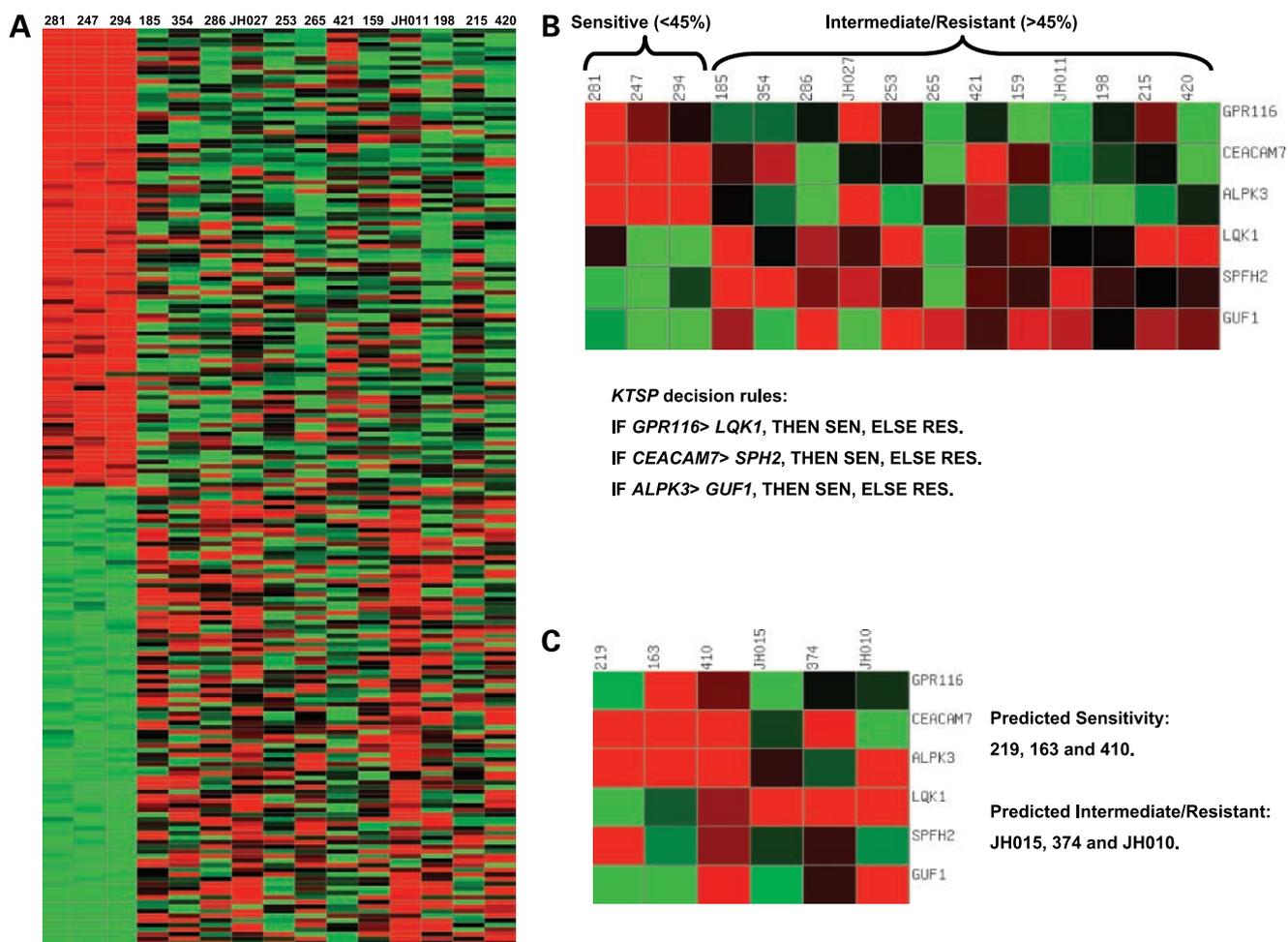


Figure 3. **A**, heatmap of the top 200 differentially expressed genes for 15 baseline pancreas xenografts arranged in order of tumor growth inhibition. The three most sensitive (281, 247, and 294) have a different profile from the intermediate and resistant tumors. **B**, heatmap of genes in the kTSP classifier. High (red) and low (green) gene expression are indicated. **C**, Heatmap of six cases predicted for SKI606 sensitivity (219, 163, and 410 were predicted sensitive; JH015, 374, and JH010 were predicted resistant).

CAV-1 expression in bosutinib activity, we measured the activity of the agent in pancreas cancer cell lines. As shown in Fig 5D, bosutinib induced cytotoxicity to MiaPaca-2 and PANC-1 cells in a concentration-dependent manner. Cell viability was reduced to 76% (MiaPaca-2) and 50% (Panc-1) with exposure of 10 $\mu\text{mol/L}$ bosutinib compared with control (100%). However, bosutinib exposure to MiaPaca-2 and Panc-1 cells after the knockdown of CAV-1 abolished bosutinib-induced cytotoxicity. Panc-1 cells showed 21% increase in proliferation compared with untreated cells (Fig 5D).

Discussion

Most anticancer agents tested in pancreatic cancer clinical trials have failed. It can be argued that more extensive pre-clinical studies in relevant models could help in selecting clinical candidates. In addition, studies oriented to determine which tumors are more likely to respond to drug

could facilitate patient selection strategies in clinical trials. To address these issues in pancreatic cancer, we developed the PancXenoBank, a collection of freshly generated pancreatic xenografts obtained from patients with pancreatic cancer at the time of surgical resection. These xenografts maintain the main genotypic features as the originator tumors and represent an interesting platform for preclinical drug screening and biomarker development (6). In this work, we have used the PancXenoBank to explore the activity and discover biomarkers of bosutinib, a Src kinase inhibitor. The results shown that bosutinib inhibited the growth of 3 (20%) of 15 subcutaneous xenografts. Importantly, bosutinib did not cause tumor regression, which is not surprising given the limited antiproliferative effects of Src inhibitors in multiple preclinical models and suggest that tumor growth inhibition, rather than tumor response, is the most likely effect of this drug in the clinic (28). Bosutinib inhibited Src signaling and induced apoptosis in

Table 1. Gene set enrichment analysis on the four most bosutinib-sensitive cases with high expression of CAV-1 versus four most resistant cases with low CAV-1

Top 10 pathways enriched in the sensitive cases, sorted by nominal <i>P</i>				
Name	KEGG Pathway	Size	NES	<i>P</i>
HSA03030	DNA polymerase	24	1.71	0.007
HSA00532	Chondroitin sulfate biosynthesis	18	1.75	0.011
HSA04510	Focal adhesion	194	1.36	0.011
HSA04512	ECM-receptor interaction	85	1.43	0.012
HSA00790	Folate biosynthesis	37	1.47	0.024
HSA04060	Cytokine-cytokine receptor interaction	247	1.30	0.027
HSA05222	Small cell lung cancer	86	1.33	0.028
HSA00760	Nicotinate and nicotinamide metabolism	39	1.44	0.034
HSA04916	Melanogenesis	101	1.35	0.037
HSA01430	Cell Communication	117	1.31	0.071
Top 10 pathways enriched in the resistant cases, sorted by nominal <i>P</i>				
HSA03010	Ribosome	54	-2.90	0.000
HSA00071	Fatty acid metabolism	50	-1.95	0.000
HSA00340	Histidine metabolism	40	-1.85	0.000
HSA00350	Tyrosine metabolism	55	-1.72	0.000
HSA00650	Butanoate metabolism	44	-1.59	0.005
HSA00591	Linoleic acid metabolism	35	-1.67	0.009
HSA00565	Ether lipid metabolism	29	-1.64	0.014
HSA00980	Metabolism of xenobiotics by cytochrome P450	60	-1.39	0.022
HSA00360	Phenylalanine metabolism	29	-1.53	0.023
HSA00960	Alkaloid biosynthesis II	19	-1.54	0.026

susceptible tumors. The agent also modulated the expression of CAV-1, which may be a mediator of this drug activity. Using the TSP approach, we developed a classifier that correctly predicted the drug activity in a different set of tumors and that could be used to enrich clinical trials with the agent.

One issue that will likely be critical for developing this class of compounds (as well as multiple other targets in oncology) is the identification of biomarkers that will help choose the patients most likely to benefit. Baseline expression of activated Src (p-Src) is one obvious possibility but neither Western blotting nor IHC showed differences between susceptible and resistant tumors. In fact, despite looking for multiple Src pathway proteins (both total and activated) by Western blotting, no clear patterns emerged.

Posttreatment changes in many of these pathways were also examined, and significant down-regulation were seen in STAT5, p-STAT-5, STAT-3, Src, p-Akt, EGFR, and MAPK and up-regulation CAV-1 in a susceptible tumor (P281), but it is not clear whether the changes are due to general apoptotic effects of bosutinib (as manifested by the 4-fold increase in cleaved PARP) or specifically to Src pathway blockade. The induction of cleaved PARP is consistent with other preclinical studies showing apoptotic effects of Src inhibitors in sarcoma (29), mesothelioma (30), squamous cell carcinoma (31), and pancreas cancer (13).

Gene array profiling of the 15 tumors at baseline showed impressive differences between susceptible and resistant human pancreas xenografts. A signature of 200 genes was identified, and this was further reduced to a "k-TSP" prediction

Table 2. KTSP genes from analysis of 21 (15 training, 6 validation) human pancreas tumors *in vivo*

Up-regulated in sensitive tumors			
Name	KEGG	Chromosome	GeneCard no.
GPR116	G protein-coupled receptor 116	6p12.3	06M046928
CEACAM7	CEA-related cell adhesion molecule 7	19q13.2	19M04686
ALPK3	α-kinase 3 protein	15q25.2	15P083161
Down-regulated in resistant tumors			
LQK1	LQK1 hypothetical protein short isoform	1q32.3	01U901588
SPFH2	(ER) lipid raft associated 2 protein,	8p11.2	08P037715
GUF1	GUF1 GTPase homologue	4p13	04P044375

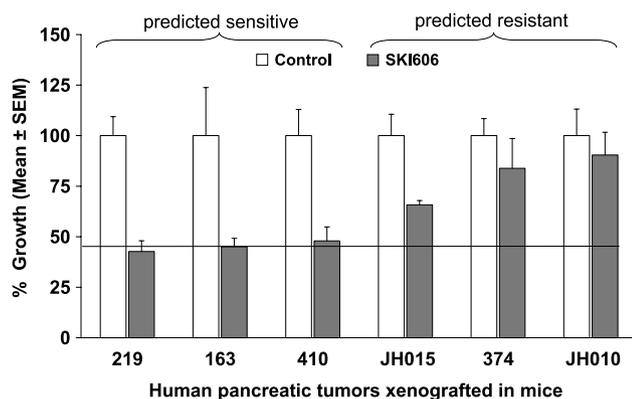


Figure 4. Prospective validation of efficacy predicted by KTSP classifier. The three xenografts predicted to be sensitive, 219, 163, and 410, had sensitive and intermediate sensitivity (T/C of 43%, 45%, and 48%, respectively). Three cases predicted to be resistant, JH015, 374, and JH010, had T/C of 66%, 84%, and 90%, respectively.

rule consisting of 6 genes. The k-TSP showed reasonable predictive accuracy in that the three predicted sensitive cases all had tumor growth 43% to 48% of control, and the three predicted resistant cases had growth 71% to 90% of control. Having a limited number of genes in a prediction rule is highly advantageous because RT-PCR could be used on limited patient samples (often fine needle aspiration in pancreas cancer) to ascertain whether a Src inhibitor is likely to have antitumor effects in a particular patient. In addition, a baseline pharmacodiagnostic sample would be simpler to obtain from a routine diagnostic biopsy, for example, as opposed to posttreatment (pharmacodynamic) signatures where a separate biopsy procedure would be required.

When comparing the pancreas cancer susceptibility gene signature for bosutinib described here to that for breast cancer (18) and prostate cancer (19) with dasatinib, we did not find shared genes. There are several possibilities to explain this difference. The most obvious include different biology of breast and prostate cancer versus pancreas cancer, and cell lines versus cancer tissue that was explanted directly from the operating room into mice. In addition, it should be noted that comparisons between the breast and prostate cell line gene profiling data with dasatinib yielded a relatively small subset of shared genes, with only one (EphA2) shared in the limited gene set predictive model (19). Finally, although dasatinib and bosutinib share Src/Abl as a target, they have different chemical structures and inhibitory activity against the various Src family members (32, 33).

The PancXenoBank model has limitations, many of which have been described previously (6). First, the model has a limited dynamic range, whereby small differences between cases are unlikely to be biologically meaningful and cutoffs are chosen somewhat arbitrarily. For this reason, we choose cases at the extremes of sensitivity for follow-up studies. Second, although there do not seem to be alterations in status of principal genes mutated in pancreas cancer, there are some variations in gene expression as a tumor is passaged

in mice. Moreover, because this is a subcutaneous xenograft rather than orthotopic, stromal interactions that may be extremely important in pancreas cancer are likely lost. This may be particularly important with Src inhibitors, where a nonmetastatic subcutaneous model may not pick up important anti-invasive or antimetastatic properties of a drug. Finally, the altered immunologic environment of a nude mouse obscures immunologic properties of these compounds, which may be important for Src inhibitors because STAT signaling regulates immune tolerance in animal models (34). Although the model can be used as a platform to screen novel drugs for therapeutic potential and evaluate biomarkers of response/resistance preclinically, it is also being evaluated as a way to select drugs for specific patients in an ongoing clinical protocol at Johns Hopkins (35). This will provide a rare opportunity for clinical validation of a pre-clinical model.

CAV-1 is a known Src substrate and has been implicated to act as both a tumor suppressor and an oncogene, depending on the tissue of origin and stage of disease (36). CAV-1 regulates multiple cancer-associated processes including cellular transformation, tumor growth, cell migration and metastasis, cell death and survival, multidrug resistance, and angiogenesis (37). Furthermore, CAV-1 levels are positively correlated with tumor stage and grade in numerous cancer types (38). Panc-1 cells have been reported to have higher levels (4.9-fold) of CAV-1 compared with MiaPaca-2 (39). In the present study, we showed that Panc-1 cells are more sensitive to bosutinib than MiaPaca-2 cells (Fig. 5D). However, bosutinib exposure to MiaPaca and Panc-1 cells after the knockdown of CAV-1 abolished SKI-606-induced cytotoxicity and induced cell proliferation (Fig. 5D). These results are in agreement with the high expression of CAV-1 in bosutinib sensitive cases as shown by Affimetrix data, RT-PCR, and IHC (Fig. 5A, B, and C), although a few resistant cases had high expression as well.

The clinical effects of CAV-1 expression in pancreatic carcinoma remain unknown. However, overexpression of CAV-1 in pancreatic carcinoma has been reported to contribute to tumor progression and has been a negative prognostic predictor following surgery (40). In addition, overexpression of CAV-1 in human pancreatic tumor cells confers resistance to ionizing radiation and CAV-1 knockdown leads to sensitivity to radiotherapy (39). In this context, it is interesting to note that bosutinib was effective in reducing tumor burden in cases that have high expression of CAV-1 and potentially could be used as predictive marker to select patients could benefit from bosutinib treatment. It can be speculated that high CAV-1 expression indicates a general activation and/or dependence on Src-dependent pathways, making these tumors more sensitive to bosutinib.

In conclusion, we have identified a subset of human pancreas cancer xenografts that are susceptible to bosutinib, and identified a gene array profile and k-TSP classifier associated with drug sensitivity in this model. The classifier correctly discriminated between sensitive/intermediately sensitive xenografts, versus resistant xenografts, when tested prospectively. High CAV-1 expression was

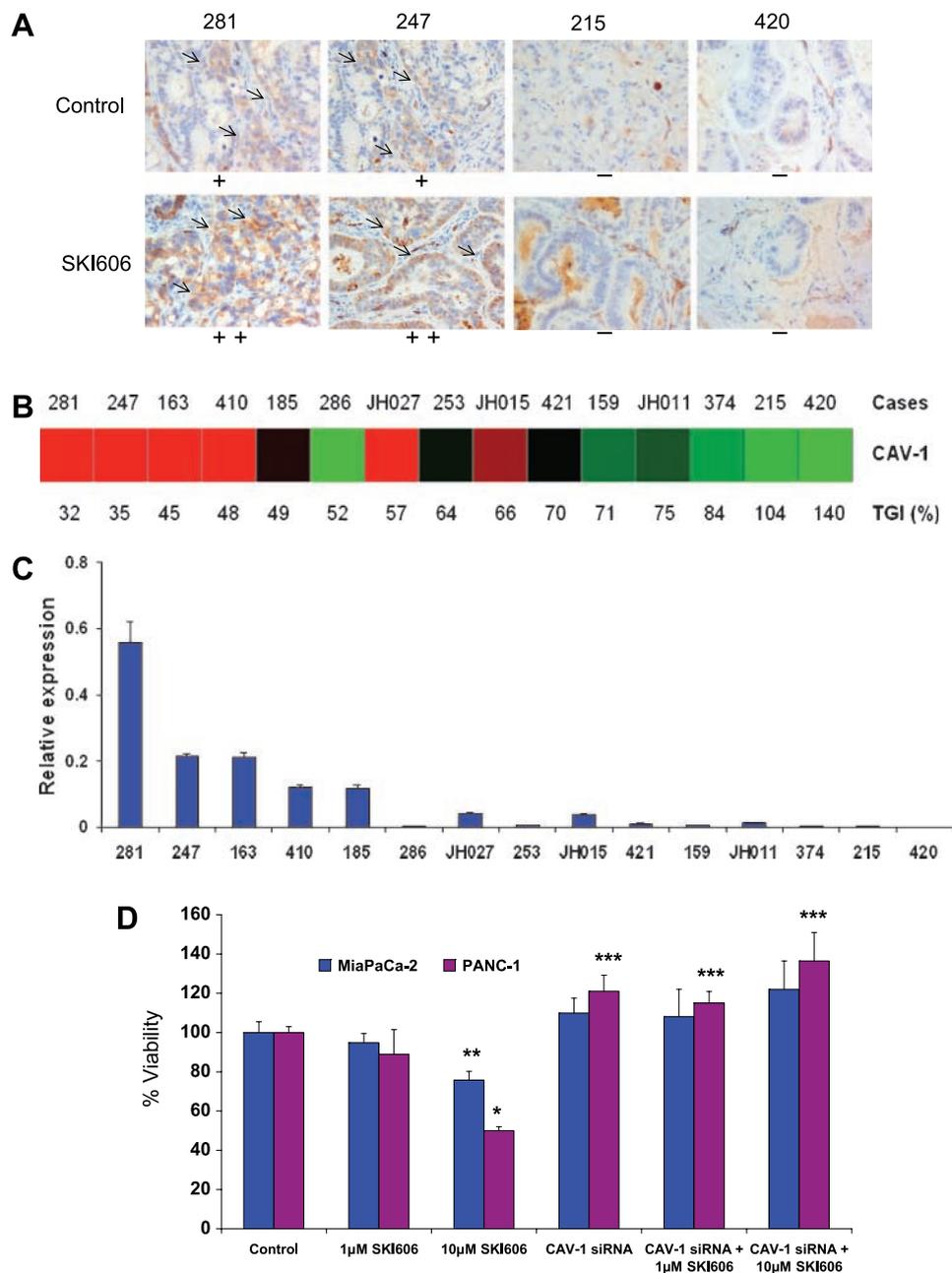
1492 *Effects of Bosutinib in Pancreas Cancer Xenografts*

Figure 5. **A**, tumor CAV-1 IHC of 2 sensitive (281 and 247) and 2 resistant (215 and 410) cases to SKI606 treatment. Positive controls were used to define positive staining distribution of cytoplasmic and membranous CAV-1 expression; nuclear staining only is considered negative. Grade: -, negative staining; +, weak positivity; ++, moderate positivity. *Arrows*, areas of positive staining. Bosutinib resulted in the up-regulation of CAV-1 in 281 and 247. **B**, heatmap of the CAV-1 expression of 15 baseline pancreas xenografts arranged in order of tumor growth inhibition as observed from the microarray. High (red) and low (green) gene expression are shown. **C**, relative expression of CAV-1 (to ubiquitin) by RT-PCR validation of 15 baseline pancreas xenografts arranged in order of tumor growth inhibition. Four of the six sensitive or predicted sensitive cases (all having TGI of $\leq 48\%$) showed high CAV-1 expression versus 2 of 6 intermediate and 2 of 9 resistant. **D**, cell viability of pancreatic cancer cells after the knockdown of CAV-1 by MTT assay. MiaPaCa-2 and PANC-1 cells were sensitive to bosutinib (10 μ M/L) exposure for 72 h compared with control. CAV-1 knockdown by siRNA leads to significant increase in cell proliferation of Panc-1 cells compared with control. Bosutinib was found to be ineffective in reducing cell proliferation after the knockdown of CAV-1. (*, $P \leq 0.001$; **, $P \leq 0.01$; ***, $P \leq 0.05$ compared with control).

also associated with sensitivity. These data could be useful in the clinical development of this agent in pancreatic cancer to help target a subpopulation of patients who will benefit.

Disclosure of Potential Conflicts of Interest

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