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Preclinical pharmacodynamic evaluation of a new Src/FOSL1 inhibitor, LY-1816, in pancreatic ductal adenocarcinoma

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Despite tremendous efforts, the clinical prognosis of pancreatic ductal adenocarcinoma (PDAC) remains disappointing. There is an urgent need to develop more effective treatment strategies to improve the prognosis of patients with PDAC. In this study, we evaluate the anti-PDAC effects of LY-1816, a new multikinase inhibitor developed by us. In in vitro assays, LY-1816 showed significant inhibitory effects on the proliferation, migration, and invasion of human PDAC cells, and induced PDAC cell apoptosis. Western blot analysis revealed that LY-1816 markedly suppressed the Src signaling, and downregulated the expression of FOSL1; *FOSL1* is an oncogene vulnerability in KRAS-driven pancreatic cancer. In in vivo models of PDAC xenografts (Aspc-1 and Bxpc-3), LY-1816 showed more potent antitumor activity than dasatinib and gemcitabine. Moreover, mice treated with LY-1816 showed a much more significant survival advantage in a metastatic model of PDAC compared with those treated with vehicle, dasatinib, or gemcitabine. These results provide effective support for the subsequent clinical evaluation of LY-1816 in the treatment of PDAC.

KEYWORDS
FOSL1, KRAS, multikinase inhibitor, pancreatic cancer, Src

1 | INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal alimentary canal malignancies. It is the fourth most frequent cause of all cancer-related deaths in developed countries.[1] The median survival of PDAC is <6 months and the 5-year survival rate is between 3% and 5%.[2] Surgery remains the only chance of cure for PDAC.[3,4] However, because of the vague clinical symptoms of PDAC, patients are often diagnosed late with regional invasion or distant metastasis already evident, and only 15%-25% of patients present with resectable disease at the time of primary diagnosis.[4,5] Currently, gemcitabine, a chemotherapeutic drug, is the standard of systemic treatment for PDAC. Nevertheless, the clinical efficacy of gemcitabine is limited,[6] in addition to its high toxicity. Targeted drugs usually have low toxicity, and have shown excellent efficacy in many other cancer types.[7–9] Unfortunately, there are no targeted drugs approved for clinical use in the treatment of PDAC. Therefore, development of new potent targeted drugs is of great significance to the therapy of PDAC.

In the past decade, strenuous efforts have been undertaken on understanding the molecular and biological underpinnings of PDAC,[2,10,11] which revealed that mutations and/or the aberrant expression of specific protein tyrosine kinases (PTKs) were important factors responsible for the occurrence and development of PDAC.[12-14] Among these PTKs, Src is of particular importance. Activating mutations in the Src kinase are found in up to 70% of PDAC cases.[15] Moreover, aberrant Src activation is often correlated with poor clinical prognosis of PDAC patients.[15] Hence, Src inhibitors have been considered to be promising agents for the treatment of PDAC.

Wei Yang, Lingwei Meng, and Kai Chen contributed equally to this work.

Currently, a number of Src inhibitors have been developed,[16-20] and one of them, namely dasatinib (BMS-354825), has been in phase II clinical trials for treating metastatic PDAC.[21] Unfortunately, single use of dasatinib did not show a promising therapeutic effect.[21]

One of the main reasons why PDAC is difficult to treat is due to the notorious *KRAS* mutations, which were found in up to 90% of PDAC patients.[22,23] Many researchers have tried to identify KRAS inhibitors and a number have indeed been discovered.[24,25] However, the potencies of these inhibitors are not sufficient to achieve a good in vivo antitumor effect.[26,27] Recently, Vallejo et al[28] reported that *FOSL1* (also called *Fra-1*), which is a transcription factor, was a vulnerable oncogene in *KRAS*-driven pancreatic cancer, and genetic inhibition of *FOSL1* was able to block the growth of *KRAS*-driven tumor types. Furthermore, recent studies have indicated that *FOSL1* plays an important role in the regulation of epithelial-mesenchymal transition, which is associated with tumor metastasis.[29–33] Additionally, our data (Figure S1 and Table S4) and other published data[34] all showed that pancreatic cancer tissues have a slightly higher expression of FOSL1 compared with normal pancreatic tissues. All of these results imply that agents that can downregulate the expression of FOSL1 might have potential to be used for the treatment of PDAC.

Considering the unsatisfactory antitumor efficacy of sole inhibition of the Src kinase and the pathological role of *FOSL1* in *KRAS*-driven tumors, we hypothesized that agents that are able to inhibit the Src kinase, and concurrently downregulate the expression of FOSL1, might bring an improved clinical outcome in the treatment of PDAC. In a recent study,[35] we discovered a new drug candidate, *N*-(3-((4-amino-1-(4-hydroxycyclohexyl)-1*H*-pyrazolo[3,4-d]pyrimidin-3-yl)ethynyl)-4-methylphenyl)-4-methyl-3-(trifluoromethyl)benzamide, termed LY-1816 (Figure 1A). This compound showed excellent activity in inhibiting Src. In the same time, it could also significantly downregulate the expression of FOSL1. In this investigation, we evaluated the anti-PDAC activities of LY-1816 both in vitro and in vivo, and also explored the mechanisms of action of its antitumor potential.

2 | MATERIALS AND METHODS

2.1 | Compounds

LY-1816 (Figure 1A) was synthesized at the State Key Laboratory of Biotherapy, Sichuan University (Chengdu, China). Dasatinib and gemcitabine were acquired from commercial suppliers (Dalian Meilun Biotechnology, Liao Ning, China). Stock solutions of compounds for all in vitro assays were prepared in DMSO and then diluted in optimal medium. The final concentration of DMSO in the incubation mixture did not exceed 0.1% (v/v) in each experiment.

2.2 | Cell culture

Human pancreatic cancer cell lines were obtained from ATCC (Manassas, VA, USA). All tumor cell lines were maintained according to the ATCC guidelines for <6 months from the time they were received or thawed. No further authentication was done for tumor cell lines.

2.3 | In vitro kinase inhibition assay

The in vitro kinase enzymatic inhibition assays were carried out by the Kinase Profiling Services provided by Eurofins (Dundee, UK). The ATP concentration used was 10 μmol/L in all assays.

2.4 | Cell proliferation assay

Cell proliferation assays were carried out as previously reported.[36] A variety of human pancreatic cancer cell lines were seeded at an appropriate density in 96-well plates (1000-5000 cells per well) overnight. Then they were treated with indicated concentrations of LY-1816 or other agents. An MTT proliferation assay was carried out after 72 hours. Mean values were calculated from quadruplicate wells and plotted on log dose response curves as the mean percentage of the untreated controls. The IC_{50} values were then calculated by GraphPad Prism 6.04 software (GraphPad, San Diego, CA, USA). The highest concentration of DMSO (drug diluent) added to the cells had no effect on cell proliferation.

2.5 | Colony formation assay

Cells were seeded in 6-well plates at a density of 5000-8000 cells per well. The next day, indicated concentrations of LY-1816 or other agents were added. The medium containing vehicle or agents was replaced every 4 days. After 13-15 days of incubation, cells were fixed with methanol and stained with crystal violet. Colonies more than 50 cells) were counted under an inverted microscope.

2.6 | DNA synthesis assay

Cells were seeded overnight at a density of 5000-8000 cells per well in 96-well plates, and then treated with different concentrations of LY-1816 or other agents for 24 hours. Subsequently, the EdU incorporation assay was carried out on the cells according to the manufacturer's instructions (Guangzhou RiboBio, Guangdong, China).

2.7 | Apoptosis assay

The Annexin V-PI detection kit (Keygen Biotech, Jiangsu, China) was used for apoptosis analysis. Briefly, the cells were treated with different concentrations of LY-1816 or other agents for 24 hours, then washed with PBS. Annexin V-FITC and propidium iodide (PI) were then added according to the manufacturer's instructions, and the samples were incubated in the dark for 15 minutes. Pictures were taken by using an Olympus digital camera (Shinjuku, Tokyo, Japan) that was attached to a light microscope.

2.8 | Western blot analysis

Western blot analyses were carried out using standard methods. Cells were grown in complete media overnight and then treated with LY-1816 or other agents as required in each assay. Cells were lysed with RIPA buffer and protein concentrations were determined. Cell

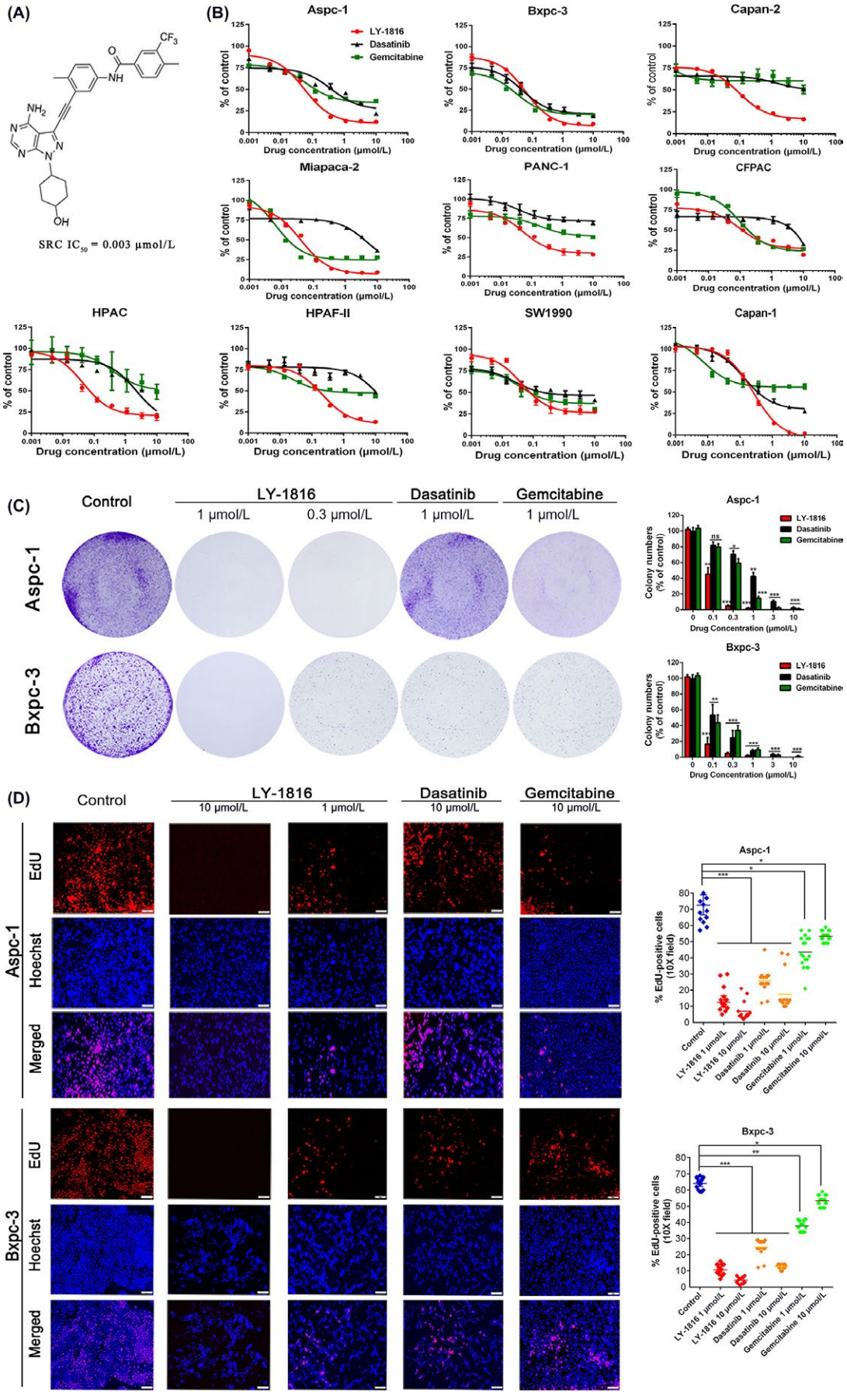

(A)
SRC IC50 = 0.003 μmol/L
(B)
Aspc-1
Bxpc-3
Capan-2
Miapaca-2
PANC-1
CFPAC
HPAC
HPAF-II
SW1990
Capan-1
LY-1816
Dasatinib
Gemcitabine
% of control
Drug concentration (μmol/L)
(C)
Control
LY-1816
1 μmol/L
0.3 μmol/L
Dasatinib
1 μmol/L
Gemcitabine
1 μmol/L
Aspc-1
Bxpc-3
Colony numbers (% of control)
Drug Concentration (μmol/L)
(D)
Control
LY-1816
10 μmol/L
1 μmol/L
Dasatinib
10 μmol/L
Gemcitabine
10 μmol/L
EdU
Hoechst
Merged
% EdU-positive cells (10X field)
Control
LY-1816 1 μmol/L
LY-1816 10 μmol/L
Dasatinib 1 μmol/L
Dasatinib 10 μmol/L
Gemcitabine 1 μmol/L
Gemcitabine 10 μmol/L

FIGURE 1 Chemical structure of LY-1816 and its antiviability/proliferative activities against pancreatic ductal adenocarcinoma (PDAC) cells in vitro. A, Chemical structure of LY-1816. B, Antiviability activities of LY-1816, dasatinib, and gemcitabine against various PDAC cell lines. Every experiment was carried out in triplicate. Points, mean value; bars, SD. C, Antiproliferation activities of LY-1816 against PDAC cells by colony formation assays. Aspc-1 and Bxpc-3 cells were incubated with agents in indicated concentrations for 14 days (Aspc-1) or 18 days (Bxpc-3). Cells were stained with crystal violet and quantified. Left panels, representative images; right panels, percentages of colony numbers. Columns, mean (n = 3); bars, SD.*P < .05; **P < .01, ***P < .001, ns, not significant. D, Antiproliferation activities of LY-1816 against PDAC cells by EdU incorporation assays. Aspc-1 and Bxpc-3 cells were treated with agents in indicated concentrations for 24 hours, followed by incubation with EdU and Hoechst in sequence. The fluorescence of EdU (red) and Hoechst (blue) represent proliferating cells and cell nuclei, respectively. Scale bar = 50 mm for micrographs (left). Percentages of EdU-positive cells are presented (right). Columns, mean (n = 3); bars, SD. *P < .05; **P < .01; ***P < .001

lysates were subjected to SDS-PAGE and then transferred to PVDF membranes (Millipore, Boston, MA, USA), blocked in TBS-T and 5% non-fat dry milk for 2 hours, and subsequently washed and incubated with TBS-T and the specific Abs (Cell Signaling Technology, Boston, MA, USA) including anti-Src, anti-pSrc^{Tyr416}, anti-signal transducer and activator of transcription 3 (STAT3), anti-pSTAT3^{Tyr705}, anti-ERK, anti-pERK$^{Thr202/Tyr204}$, anti-AKT, anti-pAKT^{Ser473}, anti-FOSL-1, anti-pFOSL^{Ser265}, and anti-β-actin. Specific proteins were detected using the Enhanced Chemoluminescene System (Millipore).

2.9 | Wound healing assay

Wound healing assays were done following the method reported previously.[37] Cells were plated in 6-well plates at a seeding density of 6×10^5 cells per well. The confluent monolayers were scraped with a sterile 200 μL pipette tip the next day. After that, cells were cultured with various concentrations of LY-1816 or other agents in normal growth media. Images were taken using an Olympus inverted microscope after 18 hours. Three representative areas of each treatment group were scored, and the migrated cells were quantified by manual counting.

2.10 | Transwell assay

Transwell assays were carried out as described previously.[38] Transwell chambers (Corning, Fisher Scientific, Loughborough, UK) precoated with polymerized collagen type I (Becton Dickinson, Lake Franklin, NJ, USA) were inserted in 24-well plates. Cells were seeded on the bottom of Transwell inserts in serum-free medium containing serial dilutions of agents or vehicle. Medium supplemented with 10% FBS was added to the lower chamber. One day after seeding, invading cells were stained with Calcein-AM (Invitrogen, Paisley, UK) and visualized using an inverted microscope. Serial optical sections were captured at 10-μm intervals and quantified using ImageJ software (Bethesda, MD, USA) using the area analysis module. Invasion was calculated as the number of cells that had moved more than 20 μm into the collagen.

2.11 | Subcutaneous xenograft models

All animal experiments were approved by the Animal Care and Use Committee of Sichuan University. Six-week-old female NOD-SCID mice were purchased from HFK Bio Technology Company (Beijing, China). Tumor xenograft models were established by s.c. injecting 100 μL tumor cell suspension (between 5×10^6 and 1×10^7) into the right flank of animals. Mice were randomized into groups of 6-7 when tumors sizes reached a volume of 100-200 mm^3. LY-1816 was dissolved in 25% (v/v) PEG400 plus 5% DMSO in deionized water. Dasatinib was suspended in a mixture of propylene glycol/water (50:50). Gemcitabine was dissolved in normal saline. Animals were given LY-1816 (20 and 40 mg/kg), dasatinib (40 mg/kg), or vehicle once daily by oral gavage. Gemcitabine was given i.p. once a week (80 mg/kg). Tumors were measured every 3 days using calipers, and the volume was calculated using the following formula: length × width^2 × 0.5.

2.12 | Immunohistochemistry

Paraffin-embedded sections of tumors were stained with H&E using standard methods. Immunohistochemical staining was carried out using Abs from Cell Signaling Technology or Abcam (Cambridge, UK). Staining was undertaken using frozen sections of tissue embedded at an optimal temperature. The images were captured with a Carl Zeiss digital camera (Stuttgart, Germany) attached to a light microscope. The number of Ki-67- and cleaved caspase-3-positive cells was quantified.

2.13 | Experimental metastasis assay

For the metastasis study, Miapaca-2 cells (5×10^6) were injection into 5- to 6-week-old female NOD-SCID mice into the tail vein and the mice were immediately randomized into 5 groups (6 mice per group): LY-1816 (20 and 40 mg/kg, p.o.), dasatinib (40 mg/kg, p.o.), gemcitabine (80 mg/kg, i.p.) and vehicle control. Survival was determined by observation. The tumor burdens in the lungs were examined by H&E staining. Survival data were analyzed using the Kaplan–Meier method, and statistical significance was evaluated with a log rank test by comparing the survival time of each treatment group with the control group.

2.14 | Statistical analysis

The IC_{50} values were calculated with GraphPad Prism version 6.04 software. The statistical significance was determined by Student's t test and ANOVA. P < .05 was considered statistically significant.

3 | RESULTS

3.1 | Enzymatic activities of LY-1816 against recombinant human protein kinases

Kinase inhibitory activities of LY-1816 against a panel of recombinant human protein kinases were measured by the "gold

standard" radiometric kinase assay approach (also see Zhang et al[35]). First, activities of LY-1816 at a single fixed concentration of 10 μmol/L were tested against a panel of 335 kinases, and the results are presented in Table S1. Kinases that had a higher inhibition rate at 10 μmol/L were then selected for further testing for their half-maximal inhibitory concentrations (IC_{50}); the results are summarized in Table S2. Obviously, LY-1816 is a multikinase inhibitor with high potencies against Src (IC_{50} = 0.003 μmol/L), Yes (IC_{50} = 0.001 μmol/L), Hck (IC_{50} = 0.003 μmol/L), Lyn (IC_{50} = 0.004 μmol/L), Fyn (IC_{50} = 0.006 μmol/L), Lck (IC_{50} = 0.014

TABLE 1 Antiviability activities of LY-1816 against various cell lines

Cell line	Tumor type	IC_{50} (μmol/L)[a]
Aspc-1	Pancreatic ductal adenocarcinoma	0.054 ± 0.008
Bxpc-3	Pancreatic ductal adenocarcinoma	0.058 ± 0.011
Capan-2	Pancreatic ductal adenocarcinoma	0.097 ± 0.014
Miapaca-2	Pancreatic ductal adenocarcinoma	0.040 ± 0.005
PANC-1	Pancreatic ductal adenocarcinoma	0.123 ± 0.015
CFPAC	Pancreatic ductal adenocarcinoma	0.096 ± 0.060
HPAC	Pancreatic ductal adenocarcinoma	0.045 ± 0.004
HPAF-II	Pancreatic ductal adenocarcinoma	0.215 ± 0.031
SW1990	Pancreatic ductal adenocarcinoma	0.042 ± 0.053
Capan-1	Pancreatic ductal adenocarcinoma	0.255 ± 0.026
MDA-MB-231	Triple negative breast cancer	0.030 ± 0.002[b]
MDA-MB-435	Triple negative breast cancer	0.008 ± 0.001[b]
Hs 578T	Triple negative breast cancer	0.032 ± 0.012[b]
HCC1937	Triple negative breast cancer	0.455 ± 0.087[b]
BT474	Breast cancer	>10[b]
MDA-MB-415	Breast cancer	~10[b]
MDA-MB-436	Breast cancer	>10
ZR-75-1	Breast cancer	~10[b]
MCF-7	Breast cancer	>10
H1437	Lung cancer	2.340 ± 0.480[b]
PC-9	Lung cancer	0.152 ± 0.041[b]
A375	Melanoma	0.134 ± 0.002
Malme-3M	Melanoma	0.008 ± 0.010
HCT116	Colorectal carcinoma	3.890 ± 0.054[b]
HT29	Colorectal carcinoma	0.231 ± 0.012[b]
COLO-205	Colorectal carcinoma	0.017 ± 0.001
NCI-N87	Gastric carcinoma	0.147 ± 0.044
HepG2	Hepatocarcinoma	>10[b]
plc/prf/5	Hepatocarcinoma	~10[b]
SMMC7721	Hepatocarcinoma	>10[b]
RAMOS	Lymphoma	~10[b]
MV4-11	Leukemia	0.138 ± 0.015[b]
KG-1a	Acute myeloid leukemia	>10
HL-60	Acute myeloid leukemia	0.031 ± 0.012[b]
Hela	Cervical cancer	6.300 ± 0.073[b]
H4	Neuroglioma	0.670 ± 0.012[b]
DU145	Prostate carcinoma	>10[b]
L929	Mouse fibroblast	>10[b]
LO2	Human hepatic cells	>10[b]

[a]Each cell was tested in triplicate; the data are presented as the mean ± SD.
[b]Data reproduced from Zhang et al (2016).[35]

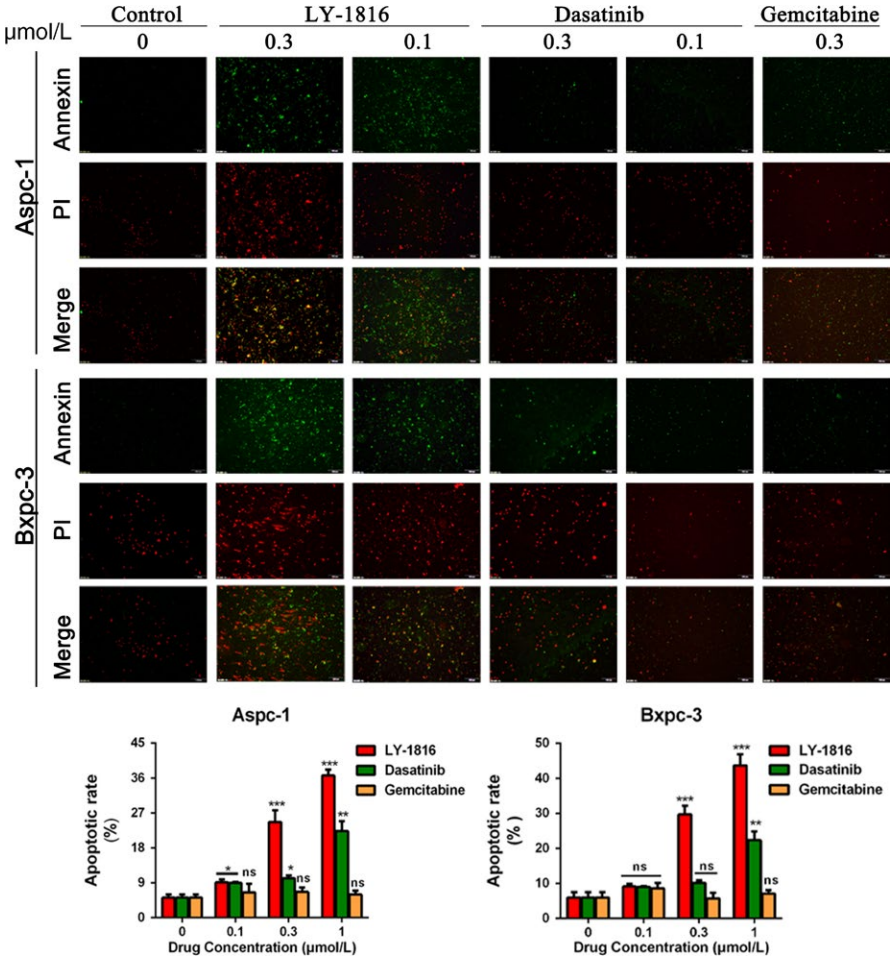


FIGURE 2 LY-1816 induced apoptosis in Aspc-1 and Bxpc-3 cells. Aspc-1 and Bxpc-3 cells were treated with different concentrations of LY-1816, dasatinib, or gemcitabine for 24 hours and stained with annexin V (green) and propidium iodide (PI) (red). Top panels, green and red colors represent early and late apoptotic cells, respectively. Scale bar = 50 mm for micrographs. Bottom panels, percentages of early and late apoptotic cells. Columns, mean (n = 3); bars, SD. **P* < .05; ***P* < .01; ****P* < .001. ns, not significant

μmol/L), Blk (IC_{50} = 0.016 μmol/L) and PTK5 (IC_{50} = 0.019 μmol/L); all of them belong to the Src family kinases. LY-1816 also showed activities against other kinases including Abl, Arg, Ret and Txk. Despite the potencies of LY-1816 to the kinases mentioned above, it did not show activity against 205 other kinases (IC_{50} > 10 μmol/L), indicating some selectivity.

3.2 | Antiviability activities of LY-1816 against PDAC cells and other cells in vitro

We used MTT assays to examine the activities of LY-1816 against tumor cell. Ten human PDAC cell lines, Aspc-1, Bxpc-3, Capan-2, Miapaca-2, PANC-1, CFPAC, HPAC, HPAF-II, SW1990, and Capan-1, were selected. The results showed that LY-1816 potently inhibited the viability of PDAC cell lines Aspc-1, Bxpc-3, Capan-2, Miapaca-2, CFPAC, HPAC, and SW1990 with IC_{50} values of 0.054 μmol/L, 0.058 μmol/L, 0.097 μmol/L, 0.040 μmol/L, 0.096 μmol/L, 0.045 μmol/L, and 0.042 μmol/L, respectively (Table 1 and Figure 1B). It also moderately inhibited the viability of PDAC cell lines PANC-1 (IC_{50}, 0.123 μmol/L), HPAF-II (IC_{50}, 0.215 μmol/L), and Capan-1(IC_{50}, 0.255 μmol/L) (Table 1 and Figure 1B). The inhibitory potencies of LY-1816 against these PDAC cells are superior or at least comparable to those of dasatinib or gemcitabine (Table S3). For comparison, the inhibitory activities of LY-1816 against 27 cell lines of other cancer types (including breast cancer, lung cancer, melanoma, colorectal carcinoma, gastric carcinoma, hepatocarcinoma, lymphoma, leukemia, cervical cancer, neuroglioma, and prostate carcinoma) as well as two normal cell lines (mouse fibroblast and human hepatic cells) are also presented in Table 1. We noticed that LY-1816 showed high potency against some of these cancer cell lines, including MDA-MB-231 (triple negative breast cancer [TNBC]; IC_{50}, 0.030 μmol/L), MDA-MB-435 (TNBC; IC_{50}, 0.008 μmol/L), Hs 578T (TNBC; IC_{50}, 0.032 μmol/L), PC-9 (lung cancer; IC_{50}, 0.152 μmol/L), A375 (melanoma; IC_{50}, 0.134 μmol/L), Malme-3M (melanoma; IC_{50}, 0.008 μmol/L), COLO-205 (colorectal cancer; IC_{50}, 0.017 μmol/L), and HL-60 (leukemia; IC_{50}, 0.031 μmol/L). But for 11 cancer cell lines, including BT474, MDA-MB-415, MDA-MB-436, ZR-75-1, MCF-7, HepG2, Plc/prf/5, SMMC7721, RAMOS, KG-1a, and DU145, as well as 2 normal cell lines (L929 and LO2), LY-1816 showed very weak or no activity (Table 1), indicating that LY-1816 is not a nonselective cytotoxic agent.

3.3 | Antiproliferation activities of LY-1816 against PDAC cells

We next examined the antiproliferation activity of LY-1816 in 2 representative PDAC cell lines Aspc-1 and Bxpc-3 by colony formation assays. Dasatinib and gemcitabine were also tested for

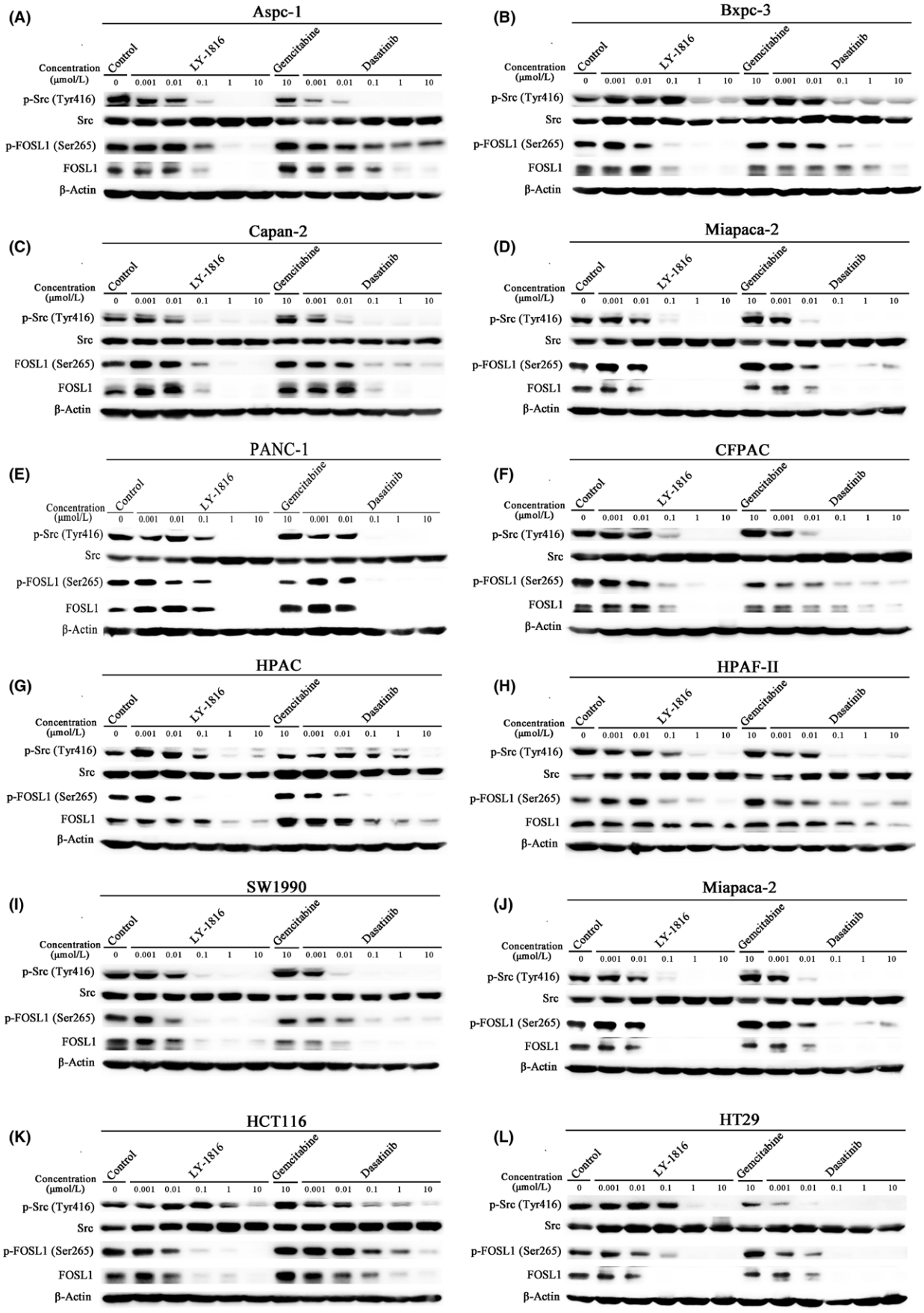


FIGURE 3 Effects of LY-1816, gemcitabine, and dasatinib on Src activation and FOSL1 expression in intact cells. Cells were treated with LY-1816, dasatinib, or gemcitabine for 12 hours and then lysed for western blot assays

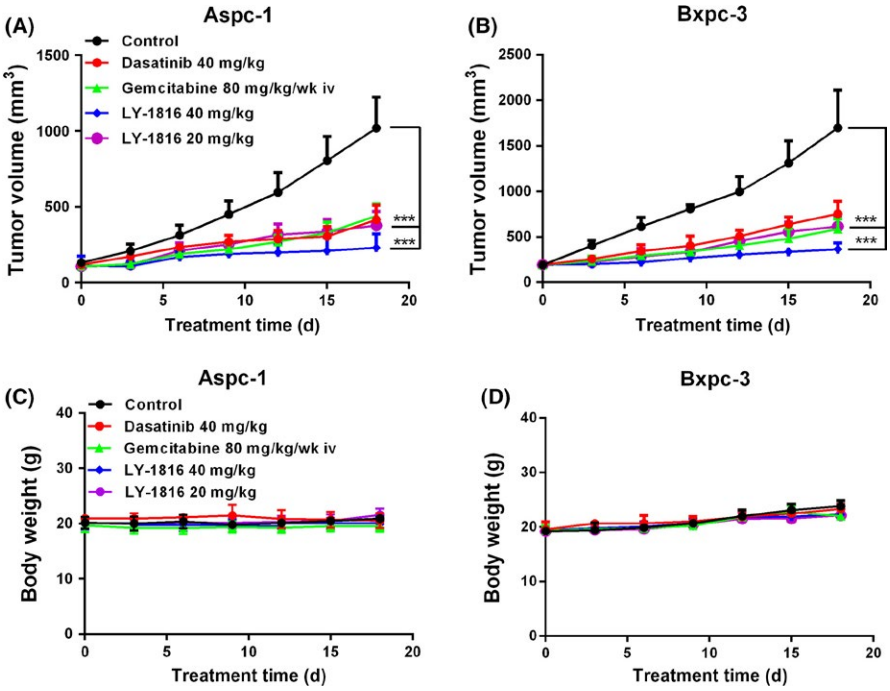


FIGURE 4 In vivo antitumor activities. A, Tumor growth curves in an Aspc-1-induced nude mice (NOD-SCID) xenograft model after different treatments. B, Tumor growth curves in a Bxpc-3-induced nude mice (NOD-SCID) xenograft model after different treatments. C,D, Average body weights for treated mice in the Aspc-1 (C) and Bxpc-3 (D) xenograft models. Mice implanted with Aspc-1 or Bxpc-3 cells were treated when the tumor grew to approximately 200 mm^3. Animals (6 per group) were treated with solvent control, LY-1816 (20 or 40 mg/kg/d, p.o.), dasatinib (40 mg/kg/d, p.o.), or gemcitabine (80 mg/kg/wk, i.p.). Points indicate mean tumor volume (mm^3) or mean body weight (g); bars indicate SD. ***$P < .001$

comparison. As shown in Figure 1C, LY-1816 significantly inhibited the colony growth of both cell lines in a dose-dependent manner. At a concentration of 1 μmol/L, the cytoreductive activity of LY-1816 was slightly superior to that of the other agents, especially compared with dasatinib on Aspc-1 cells. Moreover, EdU cell proliferation assays were carried out to further assess the antiproliferation activity of LY-1816. The results showed that LY-1816 also could dose-dependently suppress the DNA replication of both cell lines (Figure 1D).

3.4 | LY-1816 induced apoptosis of PDAC cells in vitro

Annexin V/PI staining was used to investigate the pro-apoptotic ability of LY-1816. As shown in Figure 2, LY-1816 treatment significantly increased the annexin V and PI-positive populations in cultured Aspc-1 and Bxpc-3 cells in a dose-dependent manner, indicating a strong pro-apoptotic effect. Dasatinib and gemcitabine also could induce apoptosis in both cell lines. However, the potency was weaker than that of LY-1816.

3.5 | LY-1816 inhibited Src activation and FOSL1 expression in intact PDAC cells

Western blot analysis was used to assess the ability of LY-1816 to inhibit Src activation and FOSL1 expression in intact PDAC cells. As shown in Figure 3, LY-1816 potently inhibited the phosphorylation of Src and downregulated the FOSL1 expression in a dose-dependent manner in 10 PDAC cell lines. It also suppressed the Src phosphorylation and the FOSL1 expression in HT29 and HCT116 cells but with relatively weak potency (Figure 3). Dasatinib showed a similar effect on the phosphorylation of Src, and a slight influence on FOSL1 expression. As a cytotoxic drug, gemcitabine had little inhibitory effect against Src and FOSL1 expression.

In addition, we examined the influence of LY-1816 on the MAPK and PI3K/AKT signaling pathways. Aspc-1 and Bxpc-3 cell lines were selected. As shown in Figure S2, LY-1816 showed very weak or no impact on the phosphorylation of ERK and AKT, indicating a slight or no effect on the MAPK and PI3K/AKT signaling pathways. Additionally, it has been reported that a lack of inhibition of activated STAT3 signaling could be one of the important reasons leading to the poor efficacy of dasatinib as first-line therapy in patients with metastatic PDAC.[21] We thus examined the influence of LY-1816 and dasatinib on STAT3 signaling. The results showed that dasatinib indeed showed very weak or no impact on the phosphorylation of STAT3. Interestingly, LY-1816 could significantly inhibit STAT3 phosphorylation, indicating inactivation of STAT3 signaling (Figure S2).

3.6 | In vivo antitumor activity and mechanisms of action of LY-1816 in PDAC xenograft models

The in vivo antitumor activities of LY-1816 were evaluated with mouse s.c. xenograft models of Aspc-1 and Bxpc-3 (NOD-SCID mice were used). LY-1816 was given daily, orally, for 18 days at a dose of 20 or 40 mg/kg. Dasatinib (40 mg/kg/d given orally and once daily) and gemcitabine (i.v. injection at a dose of 80 mg/kg once a week) were used as positive controls. The tumor volumes were measured every 3 days. In both Aspc-1 and Bxpc-3 models, LY-1816 showed dose-dependent tumor inhibitory activities and the 40 mg/kg dose showed a tumor-inhibition rate of >90% (Figure 4A,B). Dasatinib and gemcitabine also showed antitumor activities in both models. However, the potencies of these 2 agents were obviously weaker than that of LY-1816. In addition, during the period of LY-1816 treatment, there was no significant decrease in body weight (Figure 4C,D), implying a low toxicity.

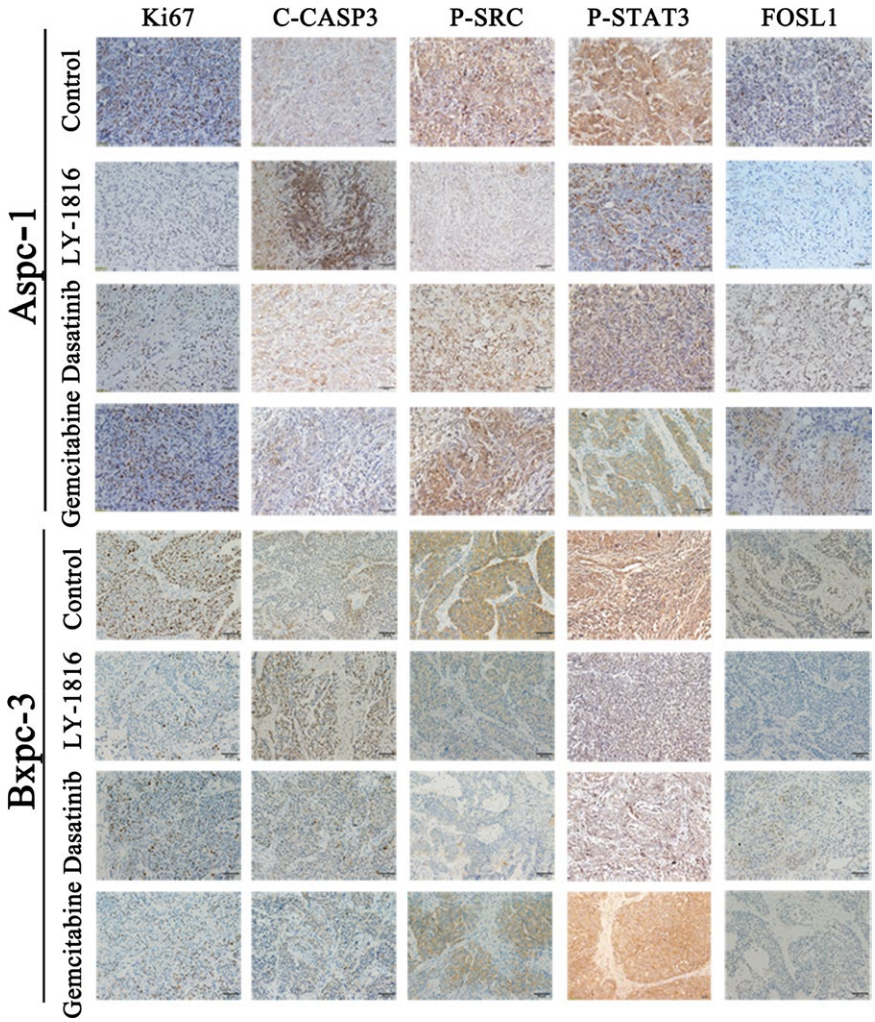


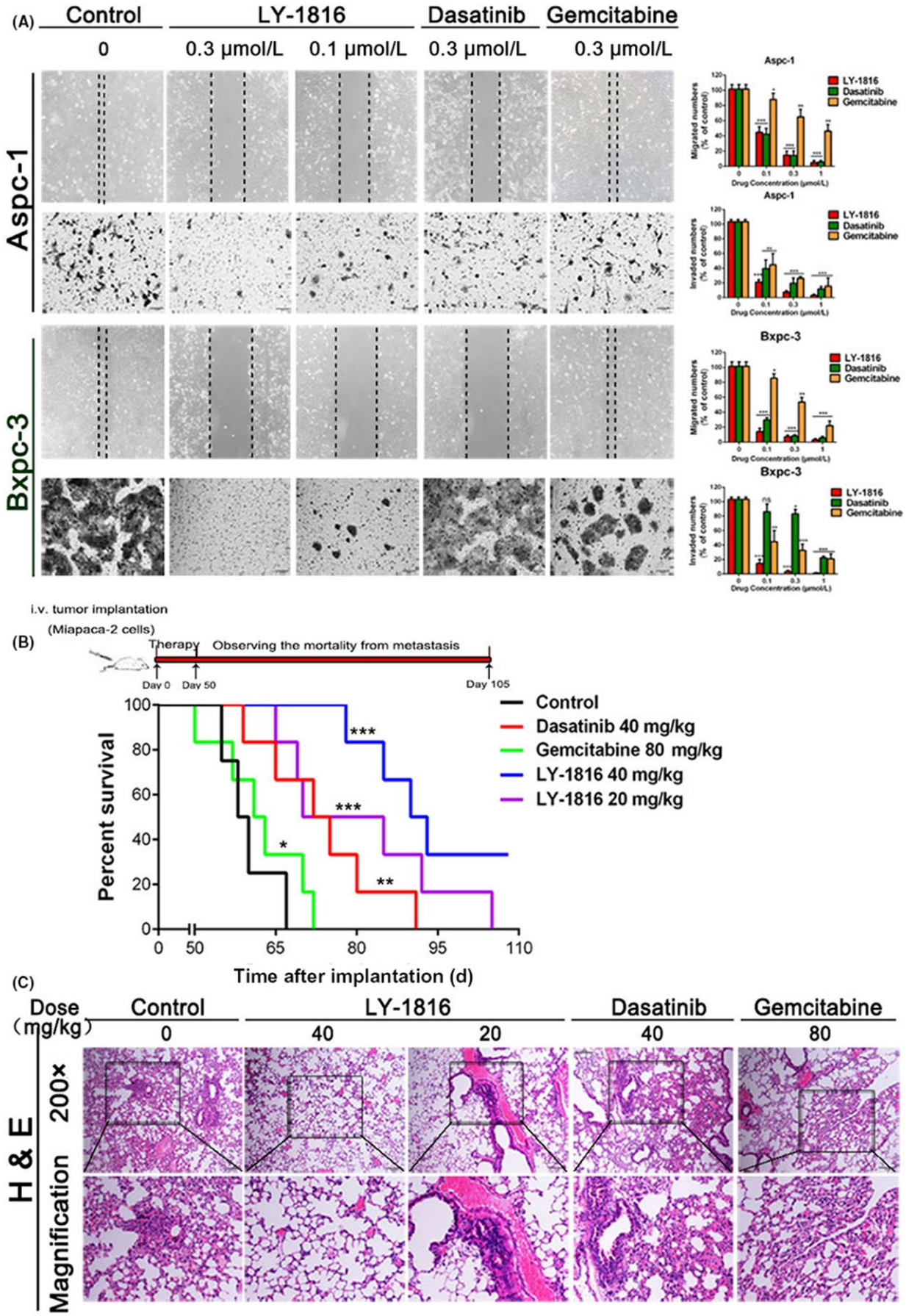
FIGURE 5 Mechanisms of action of LY-1816 in pancreatic ductal adenocarcinoma xenograft models. Mice implanted with Bxpc-3 or Aspc-1 cells were treated with solvent control, LY-1816 (40 mg/kg/d, p.o.), dasatinib (40 mg/kg/d, p.o.), or gemcitabine (80 mg/kg/wk, i.v.) when tumors grew to approximately 200 mm^3. Tumor tissues were removed for immunohistochemical staining at the end of experiments. Scale bar = 50 mm for micrographs. C-CASP3, cleaved caspase-3

To understand the antitumor mechanisms of action of LY-1816, immunohistochemical analyses of tumor tissues resected from experimental animals were carried out. As shown in Figure 5, LY-1816 evidently reduced the phosphorylation of Src and STAT3, as well as the expression of FOSL1, in both Aspc-1 and Bxpc-3 models, which are consistent with the in vitro results. The percentage of Ki-67-positive cells in viable tumor tissue was markedly lower in the LY-1816 treatment group compared with that of vehicle control, indicating a strong antiproliferation activity of LY-1816. Furthermore, the results of cleaved caspase-3 stain indicated that LY-1816 also had a considerable ability to induce apoptosis in tumor tissues. By contrast, dasatinib also downregulated the phosphorylation of Src, but had very weak or no influence on the phosphorylation of STAT3 or the expression of FOSL1. Gemcitabine did not evidently inhibit the activities of Src or STAT3, nor FOSL1 expression in either model, although it led to a moderate decrease of Ki-67 expression and increase in the number of cleaved caspase-3-positive cells.

3.7 | Antitumor metastasis ability of LY-1816 in vitro and in vivo

Wound healing assays were adopted to assess the in vitro antimetastatic effect of LY-1816. Aspc-1 and Bxpc-3 cell lines were used.

FIGURE 6 Antitumor metastatic ability of LY-1816 in vitro and in vivo. A, Wound healing assay (upper rows) and Transwell assay (bottom rows). Aspc-1 or Bxpc-3 cells were treated with agents in indicated concentrations. Left panels, representative images; right panels, percentages of invasive and migrated cells. Columns, mean (n = 3); bars, SD. *P < .05; **P < .01; ***P < .001. ns, not significant. B, Survival tests. Kaplan–Meier survival curve for every treatment group. *P < .05; **P < .01; ***P < .001. C, Representative H&E staining images of intact lung tissue taken from mice at the end of experiments. Higher magnification images of boxed regions are also shown. Scale bar = 100 mm and 50 mm for low and high magnification, respectively


(A)
Control
LY-1816
Dasatinib
Gemcitabine
0
0.3 μmol/L
0.1 μmol/L
0.3 μmol/L
0.3 μmol/L
Aspc-1
Bxpc-3
Migrated numbers (% of control)
Invaded numbers (% of control)
Drug Concentration (μmol/L)
LY-1816
Dasatinib
Gemcitabine
i.v. tumor implantation
(Miapaca-2 cells)
(B)
Therapy
Observing the mortality from metastasis
Day 0
Day 50
Day 105
Percent survival
Time after implantation (d)
Control
Dasatinib 40 mg/kg
Gemcitabine 80 mg/kg
LY-1816 40 mg/kg
LY-1816 20 mg/kg
(C)
Dose (mg/kg)
Control
LY-1816
Dasatinib
Gemcitabine
0
40
20
40
80
H & E
200×
Magnification

As shown in Figure 6A, treatment with LY-1816 or dasatinib dose-dependently reduced cell migration in both cell lines. However, gemcitabine showed very weak activity in blocking cell migration. Transwell assays were then used to examine the ability of LY-1816 to inhibit cell invasion. The results showed that LY-1816 significantly suppressed the invasion of Aspc-1 and Bxpc-3 cells. By contrast, both dasatinib and gemcitabine showed poor ability to block cell invasion.

We further evaluated the in vivo antimetastatic effect of LY-1816 in the Miapaca-2 metastatic model. As shown in Figure 6B, the median survival time (MST) for the control group was 60 days. In the 20 mg/kg/d LY-1861 treatment group, LY-1816 significantly prolonged the MST to 90 days, which was much longer than those of dasatinib and gemcitabine treated groups. In the 40 mg/kg/d LY-1861 treatment group, there were still 33% mice alive at day 105, when mice were killed (Table S5).

To understand whether there is some association between prolonged MST and reduction in lung metastases, host mice were killed at day 50 after cell injection and receiving the same treatment as above. Intact lungs from mice were examined by H&E staining. Mice treated with LY-1816 showed much more significant reduction in the size of metastatic lung nodules compared with those treated with vehicle, dasatinib, or gemcitabine (Figure 6C).

4 | DISCUSSION

Pancreatic ductal adenocarcinoma is a highly heterogeneous malignant disease with multiple gene or signaling pathways involved in the tumorigenesis and development.[39,40] Among these genes, of particular importance are the Src kinase and *KRAS*. Various evidence has proved that dysregulation of Src contributes to the tumorigenesis and development of PDAC.[15,41] Moreover, mutated *KRAS* often plays a driver role in tumorigenesis and is the most common gene mutation in PDAC.[39,42–44] Despite great efforts in the discovery of agents targeting *KRAS*, there are no effective KRAS inhibitors at present. An alternative way to tackle the *KRAS* issue is to target the vulnerability of other oncogenes in *KRAS*-driven cancers. *FOSL1* is such an oncogene in *KRAS*-driven PDAC. Additionally, *FOSL1* has been reported to be a key regulator of epithelial-mesenchymal transition,[32] which is an important factor responsible for tumor metastasis.[33] We developed LY-1816 as a multitarget drug candidate. It potently inhibits Src and can also significantly inhibit the expression of FOSL1, even at low concentrations. In cell viability assays, LY-1816 showed excellent activity against PDAC cell lines harboring *KRAS* mutations (see Table S3). Of note, this compound also displayed potent activity against the WT *KRAS* PDAC cell line Bxpc-3. A possible explanation could be that *FOSL1* is also highly expressed in Bxpc-3; alternatively, LY-1816 might play its role against cell viability by strongly blocking Src, and possibly other kinases, because LY-1816 is a multikinase inhibitor. In addition, it has been reported that single use of dasatinib has shown limited efficacy in the treatment of PDAC, which was attributed to a lack of inhibition of activated STAT3 signaling.[21] LY-1816 remedies this defect of dasatinib; it is able to efficiently inhibit the phosphorylation of STAT3. Therefore, it is not surprising that LY-1816 showed more potent anti-PDAC activity than the Src inhibitor dasatinib.

Collectively, we carried out a comprehensive preclinical pharmacodynamic evaluation of LY-1816 in the treatment of PDAC. LY-1816 showed excellent anti-PDAC activities both in vitro and in vivo. Mechanisms of action studies indicated that LY-1816 inhibited Src signaling and FOSL1 expression as well as the activation of STAT3. Moreover, it showed considerable capacity to suppress tumor metastasis in metastasis models of PDAC. Overall, all data presented here suggest that LY-1816 could be a promising drug candidate for the treatment of PDAC. Even so, it is still necessary to mention that there are some aspects needed further investigation, for example, the mechanism underlying the LY-1816-mediated downregulation of FOSL1, and the contribution of FOSL1 downregulation to the antitumor effect. Additionally, LY-1816 is a multikinase inhibitor; it can potently inhibit a number of other kinases such as kinase insert domain receptor and epidermal growth factor receptor, in addition to Src. Whether and how much the inactivation of these kinases contributes to the antitumor effect have not been answered in this investigation. Further in-depth studies are required.

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CONFLICTS OF INTEREST

The authors have no conflict of interest.

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REFERENCES

1. Kamisawa T, Wood LD, Itoi T, Takaori K. Pancreatic cancer. *Lancet*. 2016;388:73-85.
2. Hezel AF, Kimmelman AC, Stanger BZ, Bardeesy N, DePinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. *Gene Dev*. 2006;20:1218-1249.
3. Fokas E, O'Neill E, Gordon-Weeks A, Mukherjee S, McKenna WG, Muschel RJ. Pancreatic ductal adenocarcinoma: from genetics to biology to radiobiology to oncoimmunology and all the way back to the clinic. *Biochim Biophys Acta*. 2015;1855:61-82.
4. Tsai S, Evans DB. Therapeutic advances in localized pancreatic cancer. *JAMA Surg*. 2016;151:862-868.
5. Pylayeva-Gupta Y, Grabocka E, Bar-Sagi D. RAS oncogenes: weaving a tumorigenic web. *Nat Rev Cancer*. 2011;11:761-774.
6. Burris HA, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol*. 1997;15:2403-2413.

7. Cao ZX, Liu JJ, Zheng RL, et al. SKLB1028, a novel oral multikinase inhibitor of EGFR, FLT3 and Abl, displays exceptional activity in models of FLT3-driven AML and considerable potency in models of CML harboring Abl mutants. *Leukemia*. 2012;26:1892.
8. Zheng MW, Zhang CH, Chen K, et al. Preclinical evaluation of a novel orally available SRC/Raf/VEGFR2 inhibitor, SKLB646, in the treatment of triple-negative breast cancer. *Mol Cancer Ther*. 2016;15:366-378.
9. Zhong L, Fu X-Y, Zou C, et al. A preclinical evaluation of a novel multikinase inhibitor, SKLB-329, as a therapeutic agent against hepatocellular carcinoma. *Int J Cancer*. 2014;135:2972-2983.
10. Chakraborty S, Baine MJ, Sasson AR, Batra SK. Current status of molecular markers for early detection of sporadic pancreatic cancer. *Bba-Rev Cancer*. 2011;1815:44-64.
11. Pan Y, Zheng M, Zhong L, et al. A preclinical evaluation of SKLB261, a multikinase inhibitor of EGFR/Src/VEGFR2, as a therapeutic agent against pancreatic cancer. *Mol Cancer Ther*. 2015;14:407.
12. Nagaraj NS, Washington MK, Merchant NB. Combined blockade of Src kinase and epidermal growth factor receptor with gemcitabine overcomes STAT3-mediated resistance of inhibition of pancreatic tumor growth. *Clin Cancer Res*. 2011;17:483-493.
13. Sclabas GM, Fujioka S, Schmidt C, et al. Overexpression of tropomysin-related kinase B in metastatic human pancreatic cancer cells. *Clin Cancer Res*. 2005;11:440-449.
14. Scotti ML, Bamlet WR, Smyrk TC, Fields AP, Murray NR. Protein kinase Cι is required for pancreatic cancer cell transformed growth and tumorigenesis. *Cancer Res*. 2010;70(5):2064-2074.
15. Nagathihalli NS, Merchant NB. Src-mediated regulation of E-cadherin and EMT in pancreatic cancer. *Front Biosci-Landmrk*. 2012;17:2059-2069.
16. Araujo J, Logothetis C. Dasatinib: a potent SRC inhibitor in clinical development for the treatment of solid tumors. *Cancer Treat Rev*. 2010;36:492-500.
17. Green TP, Fennell M, Whittaker R, et al. Preclinical anticancer activity of the potent, oral Src inhibitor AZD0530. *Mol Oncol*. 2009;3:248-261.
18. Gucalp A, Sparano JA, Caravelli J, et al. Phase II trial of saracatinib (AZD0530), an oral SRC-inhibitor for the treatment of patients with hormone receptor-negative metastatic breast cancer. *Clin Breast Cancer*. 2011;11:306-311.
19. Karni R, Mizrachi S, Reiss-Sklan E, Gazit A, Livnah O, Levitzki A. The pp60c-Src inhibitor PP1 is non-competitive against ATP. *FEBS Lett*. 2003;537:47-52.
20. Li Z, Cai T, Tian J, et al. NaKtide, a Na/K-ATPase-derived peptide Src inhibitor, antagonizes ouabain-activated signal transduction in cultured cells. *The Journal of Biological Chemistry*. 2009;284:21066-21076.
21. Chee CE, Krishnamurthi S, Nock CJ, et al. Phase II study of dasatinib (BMS-354825) in patients with metastatic adenocarcinoma of the pancreas. *Oncologist*. 2013;18:1091-1092.
22. Bryant KL, Mancias JD, Kimmelman AC, Der CJ. KRAS: feeding pancreatic cancer proliferation. *Trends Biochem Sci*. 2014;39:91-100.
23. Ying HQ, Kimmelman AC, Lyssiotis CA, et al. Oncogenic kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. *Cell*. 2012;149:656-670.
24. Lito P, Solomon M, Li LS, Hansen R, Rosen N. Allele-specific inhibitors inactivate mutant KRAS G12C by a trapping mechanism. *Science*. 2016;351:604-608.
25. Ostrem JML, Shokat KM. Direct small-molecule inhibitors of KRAS: from structural insights to mechanism-based design. *Nat Rev Drug Discovery*. 2016;15:771.
26. Bollag G, Zhang C. Drug discovery: pocket of opportunity. *Nature*. 2013;503:475-476.
27. Patricelli MP, Janes MR, Li LS, et al. Selective inhibition of oncogenic KRAS output with small molecules targeting the inactive state. *Cancer Discov*. 2016;6:316-329.
28. Vallejo A, Perurena N, Guruceaga E, et al. An integrative approach unveils FOSL1 as an oncogene vulnerability in KRAS-driven lung and pancreatic cancer. *Nat Commun*. 2017;8:14294.
29. Blick T, Hugo H, Widodo E, et al. Epithelial mesenchymal transition traits in human breast cancer cell lines parallel the CD44hi/CD24lo/- stem cell phenotype in human breast cancer. *J Mammary Gland Biol Neoplasia*. 2010;15:235-252.
30. Caramel J, Papadogeorgakis E, Hill L, et al. A switch in the expression of embryonic EMT-inducers drives the development of malignant melanoma. *Cancer Cell*. 2013;24:466-480.
31. Lombaerts M, van Wezel T, Philippo K, et al. E-cadherin transcriptional downregulation by promoter methylation but not mutation is related to epithelial-to-mesenchymal transition in breast cancer cell lines. *Br J Cancer*. 2006;94:661.
32. Tam WL, Weinberg RA. The epigenetics of epithelial-mesenchymal plasticity in cancer. *Nat Med*. 2013;19:1438.
33. Luo YP, Zhou H, Krueger J, et al. The role of proto-oncogene Fra-1 in remodeling the tumor microenvironment in support of breast tumor cell invasion and progression. *Oncogene*. 2009;29:662.
34. Nimmakayala RK, Seshacharyulu P, Lakshmanan I, et al. Cigarette smoke induces stem cell features of pancreatic cancer cells via PAF1. *Gastroenterology*. 2018;155(3):892-908.e6.
35. Zhang C-H, Chen K, Jiao Y, et al. From lead to drug candidate: optimization of 3-(phenylethynyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine derivatives as agents for the treatment of triple negative breast cancer. *J Med Chem*. 2016;59:9788-9805.
36. Ma S, Yang L-L, Niu T, et al. SKLB-677, an FLT3 and Wnt/β-catenin signaling inhibitor, displays potent activity in models of FLT3-driven AML. *Sci Rep*. 2015;5:15646.
37. Gao Y, Jia Z, Kong X, et al. Combining betulinic acid and mithramycin a effectively suppresses pancreatic cancer by inhibiting proliferation, invasion, and angiogenesis. *Cancer Res*. 2011;71:5182.
38. Pan Y, Xu Y, Feng S, et al. SKLB1206, a novel orally available multikinase inhibitor targeting EGFR activating and T790M mutants, ErbB2, ErbB4, and VEGFR2, displays potent antitumor activity both in vitro and in vivo. *Mol Cancer Ther*. 2012;11:952.
39. Corcoran RB, Contino G, Deshpande V, et al. STAT3 plays a critical role in KRAS-induced pancreatic tumorigenesis. *Cancer Res*. 2011;71:5020-5029.
40. Morris JP, Wang SC, Hebrok M. KRAS, hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma. *Nat Rev Cancer*. 2010;10:683-695.
41. Kelber JA, Reno T, Kaushal S, et al. KRas induces a Src/PEAK1/ErbB2 kinase amplification loop that drives metastatic growth and therapy resistance in pancreatic cancer. *Cancer Res*. 2012;72:2554-2564.
42. Carriere C, Young AL, Gunn JR, Longnecker DS, Korc M. Acute pancreatitis markedly accelerates pancreatic cancer progression in mice expressing oncogenic Kras. *Biochem Biophys Res Comm*. 2009;382:561-565.
43. Son J, Lyssiotis CA, Ying H, et al. Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. *Nature*. 2013;496:101.
44. Zimmermann G, Papke B, Ismail S, et al. Small molecule inhibition of the KRAS–PDEδ interaction impairs oncogenic KRAS signalling. *Nature*. 2013;497:638.

SUPPORTING INFORMATION

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