STAT3 Mediated Remodeling of the Tumor Microenvironment Results in Enhanced Tumor Drug Delivery in a Mouse Model of Pancreatic Cancer

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Supplemental Methods

Cell Lines/Culture and Reagents

Murine PanIN, primary PDAC (PDA) and liver metastasis (LMP) cell lines were derived from the *LSL-Kras$^{G12D/+}$; Pdx1$^{Cre/+}$* and *LSL-Kras$^{G12D/+}$; Trp53$^{R172H/+}$; Pdx1$^{Cre/+}$* mouse models of PDAC (18) (kindly provided by Dr. Andrew Lowy) and maintained as previously described (36). The immortalized human pancreatic ductal cell line HPDE6-E6E7 (H6c7) was kindly provided by Dr. M.S. Tsao (37) and was maintained in in keratinocyte growth media (Invitrogen) supplemented with human epidermal growth factor and bovine pituitary extract. Human pancreatic cancer cell lines MiaPaCa2, PANC1, SW1990, AsPC1, Capan2, Capan1, CFPAC, HPAC and BxPC3 were obtained from American Type Culture Collection (ATCC). Tumor cells were maintained according to the ATCC guidelines. Immortalized mPSCs and hPSCs were a gift from Dr. Anna Means (Vanderbilt University, Nashville, Tennessee, USA) and Dr. Ralf Jesnowski (German Cancer Research Center, Germany), respectively (38, 39) and PANC1-Luc cells from Dr. Thiru Arumugam (MD Anderson Cancer Center, Houston, TX). Stattic was purchased from Calbiochem. AZD1480 was provided by AstraZeneca, and gemcitabine was

purchased from Eli Lilly and Company. For *in vitro* experiments, AZD1480 was dissolved in 100% DMSO to prepare a 10 mM stock and stored at -20^{0}C. For *in vivo* experiments, AZD1480 was formulated daily in purified, sterile water supplemented with 0.5% hydroxypropyl methyl cellulose (Acros Organics) and 0.1% Tween 80 (Fisher Scientific).

Cell viability assay

Cells were treated with DMSO or AZD1480 (0-5000 nmol/L) for 48 hours and cell viability was determined by MTT (Sigma, St. Louis, MO, USA) assay according to the manufacturer's direction. IC_{50} was calculated using Prism software package. Each condition was assayed in triplicate.

Immunohistochemistry

Tissues were fixed and immunostained using antibodies against pSTAT3 (Tyr705), trichrome blue and collagen IV (Abcam), SPARC (Invitrogen), hyaluronic acid binding protein (Calbiochem) and CD31 (Dianova). Stained tissues were evaluated by an expert pathologist (C.S). Immunostained slides were scanned and staining was quantified using the Ariol SL-50 platform (Leica Camera AG). Digital slide images were adjusted to exclude areas containing obvious histologic artifacts, such as tissue folds or nonorganic material, from the digital image. Computer-based image analysis was performed with Ariol Review Software (Leica Camera AG). After threshold levels were established for each stain, the resulting color recognition algorithm was applied to all digital images. Calculated percentage positive cells stained relative to total area analyzed with a scale for relative intensity and reported as relative expression of protein staining.

Western Blot Analysis

Western blot analyses were performed using standard methods previously described (11, 44).

Xenograft Models

Subcutaneous tumors were established by injecting 2×10^6 PANC1 cells into the flank of 6-week-old Fox1-*nu/nu* mouse (n=5 in each group). Treatment was initiated when the subcutaneous tumors reached 75 – 100 mm^3 size. Orthotopic tumors were established by injecting a single cell suspension of 250,000 PANC1-Luc cells (infected with lentivirus-expressing luciferase) into the pancreas of 8-week-old Fox1-*nu/nu* mice (n=3 in each group). BLI of anesthetized animals was performed every three days starting 15 days after injection until the end of the study to assess tumor growth. Drug treatment was initiated at the same time point. AZD1480 (30 mg/kg/day) or vehicle (Hydroxypropyl methyl cellulose/Tween 80) was administered by oral gavage; gemcitabine (20 mg/kg/3 days) was administered intra-peritoneally (IP) for 50 (s.c.) or 40 (orthotopically) days. The subcutaneous tumor volume (V) was determined by caliper measurements obtained every two days and calculated by the equation $V = L \times W^2 \times 0.5$, where L is length and W is width of a tumor. The percent body weight change for each mouse was calculated with the following formula: $[(W_n - W_0)/W_0] \times 100\%$, where W_n is the mouse weight on day_n, and W_0 is the mouse weight at the start of treatment. Growth curves for tumors were plotted as the mean volume ± standard deviation (s.d.) of tumors of mice from each group. At the end of the study, animals were sacrificed and their primary tumors were removed for further analysis.

MALDI-MS

Tumors from animals were snap-frozen in liquid N_2 and stored at -80°C. Frozen tissues were cut into 12-µm thick sections on a cryostat (Leica Microsystems). These sections were transferred and thawed onto gold-coated stainless-steel MALDI target plates. For imaging applications, matrix solution (2,5-dihydroxybenzoic acid, DHB, 30 mg/ml in 1:1 acetonitrile:water with 0.1% trifluoroacetic acid) was manually spray-coated over the tissue using a glass nebulizer until there was a relatively homogeneous layer of matrix crystals over the surface as detailed before (46). MALDI mass spectra were acquired on a LTQ XL mass spectrometer (Thermo Scientific) in MS/MS mode. MS/MS was performed on the protonated parent ions and full product ion spectra were obtained, allowing AZD1480 and gemcitabine to be effectively analyzed by selected reaction monitoring. The following transitions were used for each compound: for AZD1480, m/z 349/351→ m/z 225/227 and for gemcitabine, m/z 264 → m/z 112 (Supplementary Figure 8). Spectra were acquired over each tissue section at 150 or 200 µm spatial resolution. Images were created in ImageQuest software (Thermo Scientific) by plotting the intensity of the main fragment ions as a function of position over the tissue surface.

MRI

Mice were anesthetized via inhalation of 2%/98% isoflurane/oxygen. Animals were secured in a prone position and placed in a 38-mm inner diameter radiofrequency (RF) coil. A rigid bite-bar and head restraint were used to secure the animal's head, as well as to reduce motion-induced artifacts in the images. Animals were then placed in a Varian 7T horizontal bore imaging system

(Varian) for data collection. Respiration rate and internal body temperature were continuously monitored, and a constant body temperature of 37° C was maintained using heated airflow.

For each animal, multislice scout images were collected in all three imaging planes (axial, sagittal, and coronal) using a gradient echo sequence with repetition time (TR) = 75ms, echo time (TE) = 5ms, slice thickness = 2mm, flip angle = 35 °, and an average of 4 acquisitions. Additional parameters include field of view (FOV) = 50mm x 50mm and data matrix = 128 x 128. Following localization of the pancreas, T2-weighted fast-spin echo images were acquired over 22 slices in the axial and coronal planes, with field of view (FOV) = 25.6mm x 25.6mm, slice thickness = 1.0mm, and data matrix = 256 x 256. Additional parameters included repetition time (TR) = 2 seconds, effective echo time (TE) = 36ms, echo train length = 8, echo spacing = 9ms, and number of experiments (NEX) = 12. Additionally, magnetization transfer ratio (MTR) data was acquired using a multislice spoiled gradient echo sequence over the same field of view and axial slices as the anatomical (fast spin-echo) images acquired earlier. Additional parameters included TR = 500ms, TE = 3.2ms, matrix = 256 x 256, flip angle = 30 degrees, NEX = 6, and off-resonance frequency = 1500 Hz. An additional scan, without MT saturation, was also acquired.

Following acquisition, data were transferred to Matlab 2013a (MathWorks), and voxel-wise MTR maps were calculated as follows: $100 \times (1 - M_{sat}/M_0)$, where M_{sat} represents the signal intensity of the image following application of the off-resonance MT pulse, and M_0 is the signal intensity without MT saturation. MTR data matrices were then written to ASCII-delimited files for further analysis in ImageJ (NIH). Once the data was imported into ImageJ, a region of interest (ROI) circumscribing the pancreas was determined for each imaging slice, and the mean MTR within the ROI and volume were calculated and reported.

In Vivo *Bioluminescence Imaging (BLI)*

Mice were injected once intraperitoneally with 150 mg/kg (10 mg/mL in PBS) VivoGlo luciferin (Promega)(48). Mice were anesthetized with 1.5% isoflurane and placed in the imaging chamber of an IVIS 200 CCD camera (Caliper Life Sciences). Bioluminescence images were captured with 1 min integration time for 10 min after the luciferin injection. Images and measurements of bioluminescent signals were acquired and analyzed using Living Image software (Xenogen). Image data are displayed in photons/sec/cm^2/sr. Camera settings, such as integration time, binning, f/stop, and field of view were kept constant during all measurements. Mice were initially imaged before treatment and thereafter imaged weekly.

STAT3 Gene Knockdown by Shrna

Lentiviral pSIH1-puro-STAT3 shRNA (#26596) and pSIH1-puro-control shRNA (#26597) were obtained from Addgene. Transfection was performed as previously described (49). Lentiviral particles were prepared by co-transfecting three plasmids into 293T cells, including pMD2.G, psPAX2 and lentivectors. Supernatants were collected every 12 hours between 36 to 96 hours after transfection, pooled together and were concentrated using ultracentrifugation. PANC1 cells were transduced with lentiviral vectors at an MOI (multiplicity of infection) of 20 supplemented with polybrene (6 μg/ml) for 18 hours, and GFP expression was confirmed by FACS Caliber flowcytometer (BD Biosciences). The cells were then selected for 7 days with puromycin (1.5 μg/ml), and when cultures reached near confluency, cells were trypsinized and processed by FACS analysis to separate cells with highest GFP expression. To generate stable

knockdown clones, these cells were plated at high dilutions in 10 cm petri dishes and colonies obtained from single cells were screened for the expression of STAT3 by Western blot analysis.

Orthotopic Injections

Mice were anesthetized via inhalation of 2%/98% isoflurane/oxygen. Animals were secured in a supine position on a 37° C heating pad and the abdomen was sterilized with a 10% povidone iodine solution. All animals were given analgesia prior to surgery. Sterile surgical instruments were utilized to make a 1.5 cm transverse incision in the left upper quadrant of the abdomen. The tail of the pancreas was isolated and a 29-gauge 0.3 ml syringe was used to inject 250,000 cells in 25 μl of PBS. After injection the site was monitored to ensure no leakage or active bleeding. The spleen and pancreas were then internalized and the abdominal wall was closed in two layers with absorbable suture. Mice were monitored every 15 minutes for 2 hours, and then every 12 hours over 48 hours. 48 hours of post-operative analgesia was provided to the mice.

Soft agar assays

$5x10^4$ cells were suspended in media containing 0.33% Select Agar (Invitrogen, Carlsbad, CA) and plated on a bottom layer of media containing 0.5% Select Agar. Plates were incubated at 37^0C for 2-3 weeks prior to imaging. Colonies were photographed and quantified using ImageJ and analysis was performed with Prism software (Graphpad Software Inc., La Jolla, CA).

Supplemental Figure Legends

Supplemental Figure 1. Effect of STAT3 inhibition on PDAC cell lines. (*A*) Immunoblot of total and pSTAT3 expression in PANC1 and MiaPaCa2 cells treated with AZD1480 (0-500 nmol/L) for 4 hours shows a dose-dependent decrease in pSTAT3. (*B*) Immunoblot of total and pSTAT3 expression in PANC1 cells treated with 100 nmol/L AZD1480 for up to 8 hours shows a time-dependent decrease in pSTAT3. (*C*) Immunoblot of total and pSTAT3 expression in MiaPaCa2 and LMP cells treated with Stattic (0-100 μmol) for 4 hours shows a dose-dependent decrease in pSTAT3. (*D*) Immunoblot of SPARC expression in MiaPaCa2 and PANC1 cells treated with Stattic.

Supplemental Figure 2. STAT3 knockdown decreases number of colonies. Sh-Scrambled (Sh-Scram) and Sh-STAT3 PANC1 cells were analyzed for colonies as detailed in Materials and Methods. Each experiment was performed in triplicate. *** – $P < 0.001$

Supplemental Figure 3. Mouse weight during therapeutic intervention**. (***A***)** Orthotopic pancreatic injections of luciferase tagged PANC1 cells in Fox1-nu/nu mice were treated as indicated. Weights were obtained weekly, and combination treated mice actually had significantly more weight gain during therapy than mice treated with gemcitabine or vehicle (Two-way ANOVA with Tukey's multiple comparison test). (*B***)** PKT mice were treated as indicated and weights were recorded weekly. There was no significant difference between the weights of any treatment group. ** – $P < 0.01$.

Supplemental Figure 4. Bioluminescence imaging (BLI) of orthotopic xenograft tumors. Luciferase tagged PANC1 cells (PANC1-Luc) were injected orthotopically into the pancreata of Fox1-*nu/nu* mice. BLI was performed weekly for 6 weeks after initial injections. Representative mice from each treatment group (n=2 for control, AZD1480, gemcitabine; n=3 for combination) are shown in the upper panels, with measured mean photon emission of the corresponding mouse in the lower panel. There was a trend towards decreased mean photon emission with AZD1480 and the combination therapy when compared with Gemcitabine treatment ($p = 0.073$, 0.016 respectively; Benjamini-Hochberg corrected significance level $q = 0.014$). Mean photon intensity was expressed in units of photons/sec/cm^2/steradian (p/sec/cm^2/sr).

Supplemental Figure 5. Immunohistochemical analysis of flank tumor xenografts. Athymic nude mice were injected with PANC1 cells, and harvested tumors were analyzed for (*A*) SPARC and (*B*) CD31 expression. SPARC expression was significantly decreased with AZD1480 treatment and AZD1480/Gem treatment compared with Gem or vehicle treatment. CD31 expression was significantly increased with AZD1480/Gem treatment compared to all other treatments. * – $P < 0.05$; ** – $P < 0.01$; *** – $P < 0.001$.

Supplemental Figure 6. Overall survival of PKT mice. AZD1480/Gem treatment led to significantly longer OS compared with vehicle treated controls ($p = 0.033$, log rank test). There was no significant difference in OS compared with Gem or AZD1480 monotherapy. * – $P < 0.05$.

Supplemental Figure 7. MALDI-IMS detection of therapeutic compounds. (*A***)** Structure of AZD1480 and the resulting spectra of standard AZD1480 in MS and MS/MS mode, using 2,5-dihydroxybenzoic acid as matrix. The fragment ions at m/z 225/227 likely result from cleavage of the amine bond as shown in the figure. (*B***)** Structure of Gemcitabine and the resulting spectra in MS/MS mode, using 2', 4', 6'-trihydroxyacetophenone (THAP) as matrix.

Supplemental Figure 8. Quantification of MALDI-MS signal intensity. (*A***)** Quantification of average signal intensity over the middle region of each flank tumor xenograft (n=3) demonstrates significantly enhanced delivery of AZD1480 and Gem in only the combination therapy group. (*B***)** The same process demonstrates that the combination therapy enhances delivery of both AZD1480 and Gem in orthotopic tumor xenografts (n=2). *** – $P < 0.001$.

Supplemental Figure 9. STAT3 knockdown in PDAC cells and expression of Cda. (A) Multiple Sh-STAT3 PANC1 clones were generated, and western blot confirmed attenuated expression of total and pSTAT3 compared with sh-Scrambled (Sh-Scram) controls. (B) Sh-STAT3 PANC1 cell lysate was analyzed for Cda expression by immunoblot.

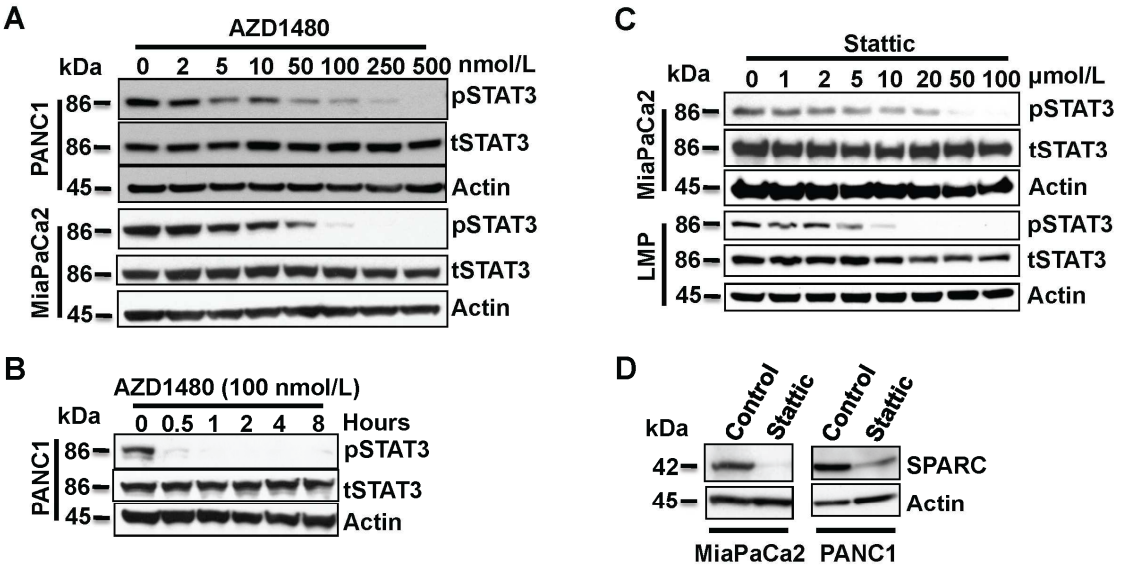
Supplemental Table 1. Patient information.

		Low STAT3	High STAT3	p Value
n		25	53	
Median age		62.1	65.8	0.1525
Sex				
	Female	13	31	0.5952
	Male	12	22	
Ethnicity				
	White	25	51	0.3269
	Other	0	2	
T Stage				
	p1	0	1	0.0408
	p2	0	5	
	p3	24	45	
	p4	1	0	
Positive Lymph Node				
	No	12	26	0.9317
	Yes	13	27	
Stage				
	1A	0	1	0.7103
	1B	0	1	
	2A	10	22	
	2B	13	25	
	3	1	0	
	4	1	4	
Margin status				
	Negative	19	41	0.6641
	Positive	6	10	
Recurrence Site				
	Local	4	3	0.1645
	Liver	1	12	
	Peritoneal	0	4	
	Other	2	9	
Grade				
	Well-Differentiated	13	2	< 0.0001
	Moderately-Differentiated	8	23	
	Poorly-Differentiated	4	28	

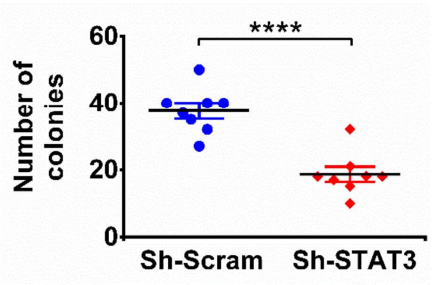
Supplemental Table 2. Pancreas cancer cell lines characteristics and their IC50 values for AZD1480.

Cell Line	***Derivation***	***Genetic background***	***IC50 (nmol/L)***
			AZD1480
MiaPaCa2	Primary	Mutant K-*ras*, mutant p*53*, wt *smad4*, wt *EGFR*	472.5
CFPAC	Metastasis (liver)	Mutant K-*ras*, wt p*53*, mutant *smad4*, ND *EGFR*	274.5
PANC1	Primary	Mutant K-*ras*, mutant p*53*, wt *smad4*, mutant *EGFR*	148.8
Capan1	Metastasis (liver)	Mutant K-*ras*, ND p*53*, mutant *smad4*, mutant *EGFR*	132.4
Capan2	Primary	Mutant K-*ras*, wt p*53*, wt *smad4*, ND *EGFR*	74.8
BxPC3	Primary	Wt K-ras, mutant p53, mutant smad4, wt EGFR	58.3
HPAC	Primary	Mutant K-*ras*, wt p*53*, wt *smad4*, mutant *EGFR*	38.3
SW1990	Metastasis (spleen)	Mutant K-*ras*, wt p*53*, ND *smad4*, ND *EGFR*	26.4
AsPC1	Metastasis (ascites)	Mutant K-*ras*, wt p*53*, wt *smad4*, wt *EGFR*	21.7

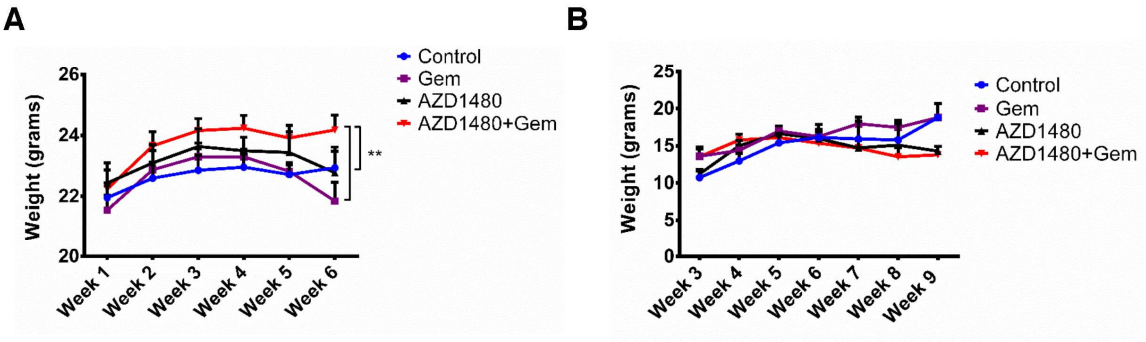
wt: wild type; ND: not determined;



Supplemental Figure 1



Supplemental Figure 2



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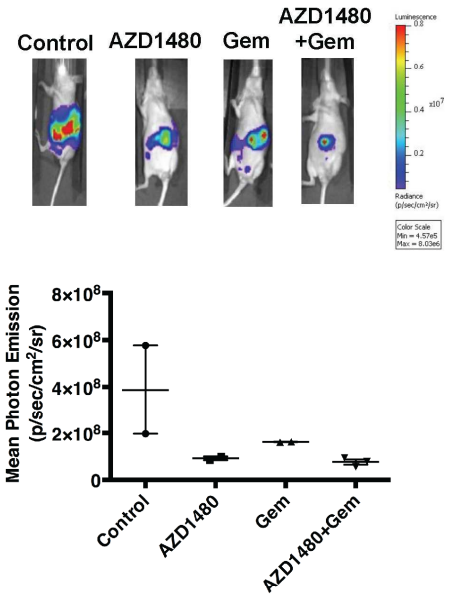
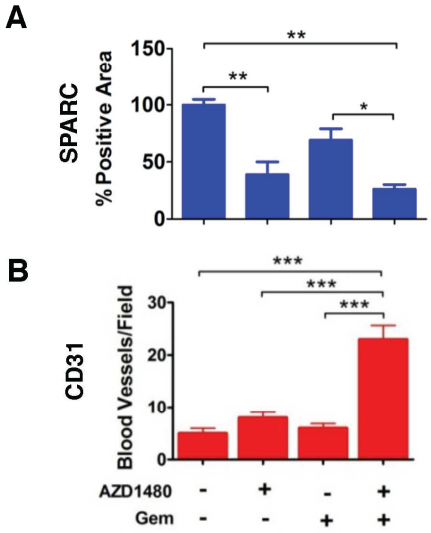


Supplemental Figure 4



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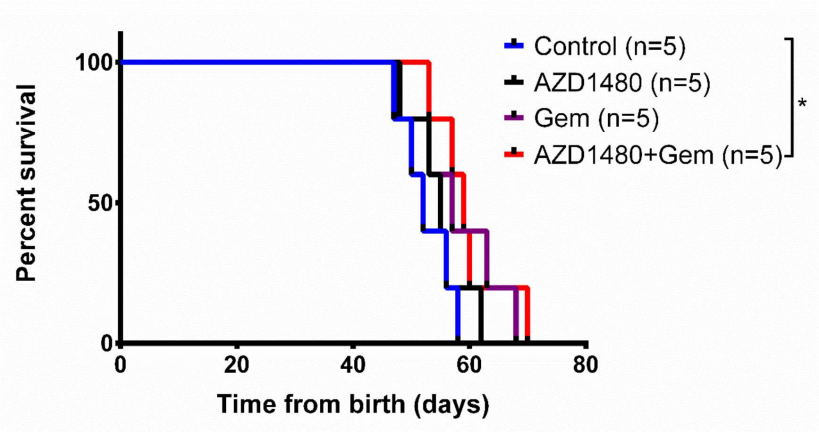

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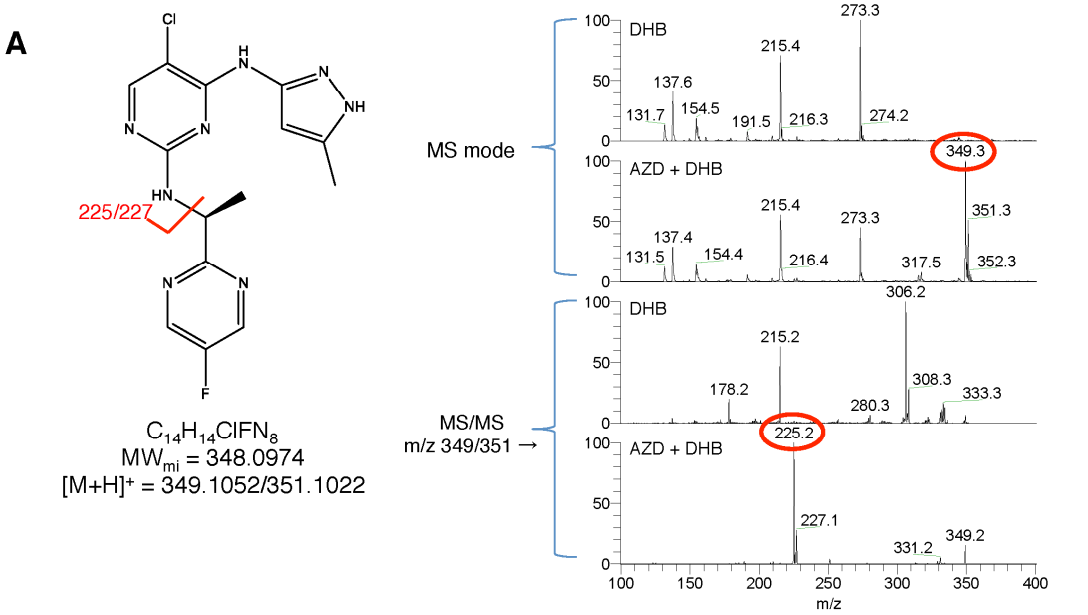
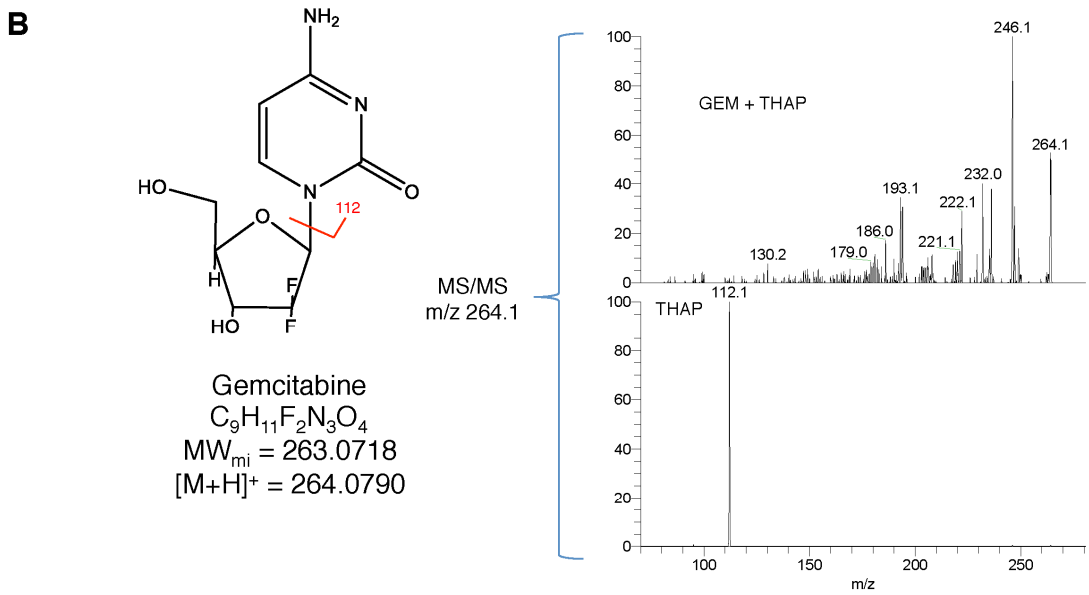
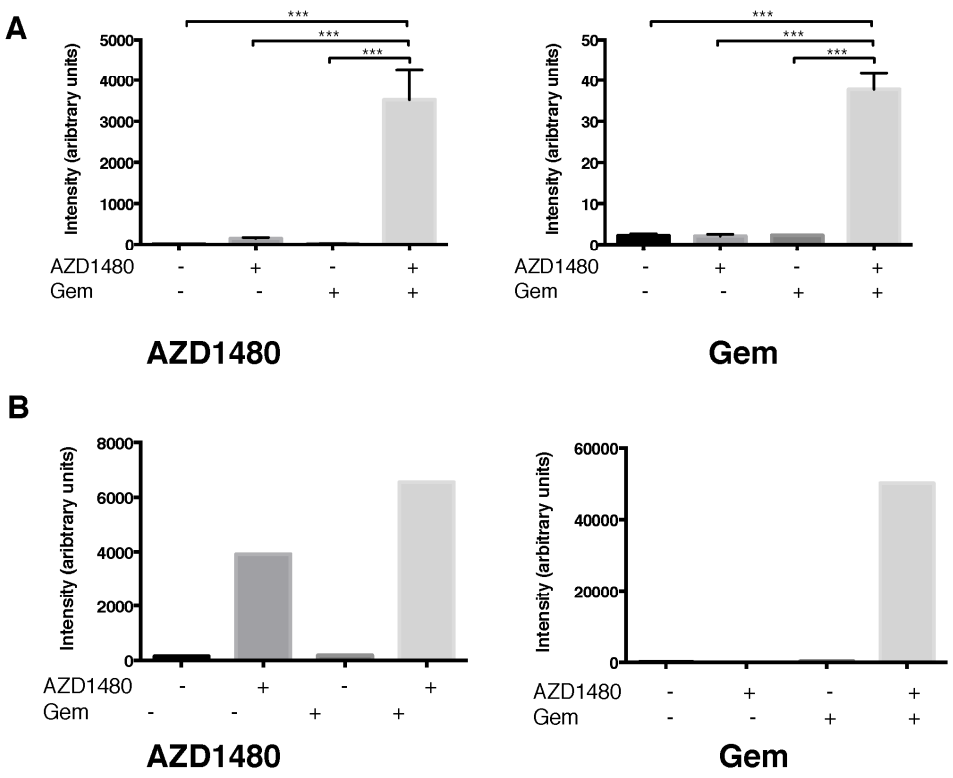
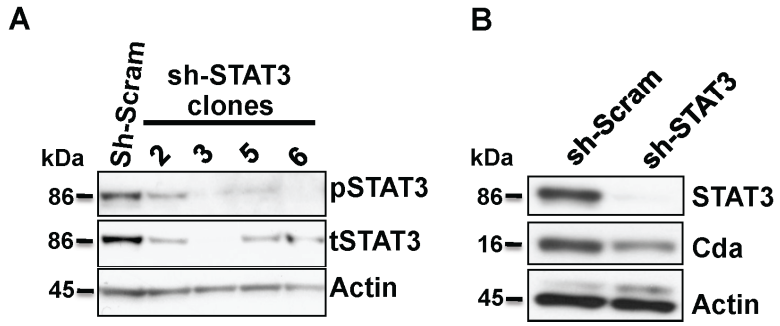




Supplemental Figure 7



Supplemental Figure 8



Supplemental Figure 9