SUPPLEMENTAL MATERIAL

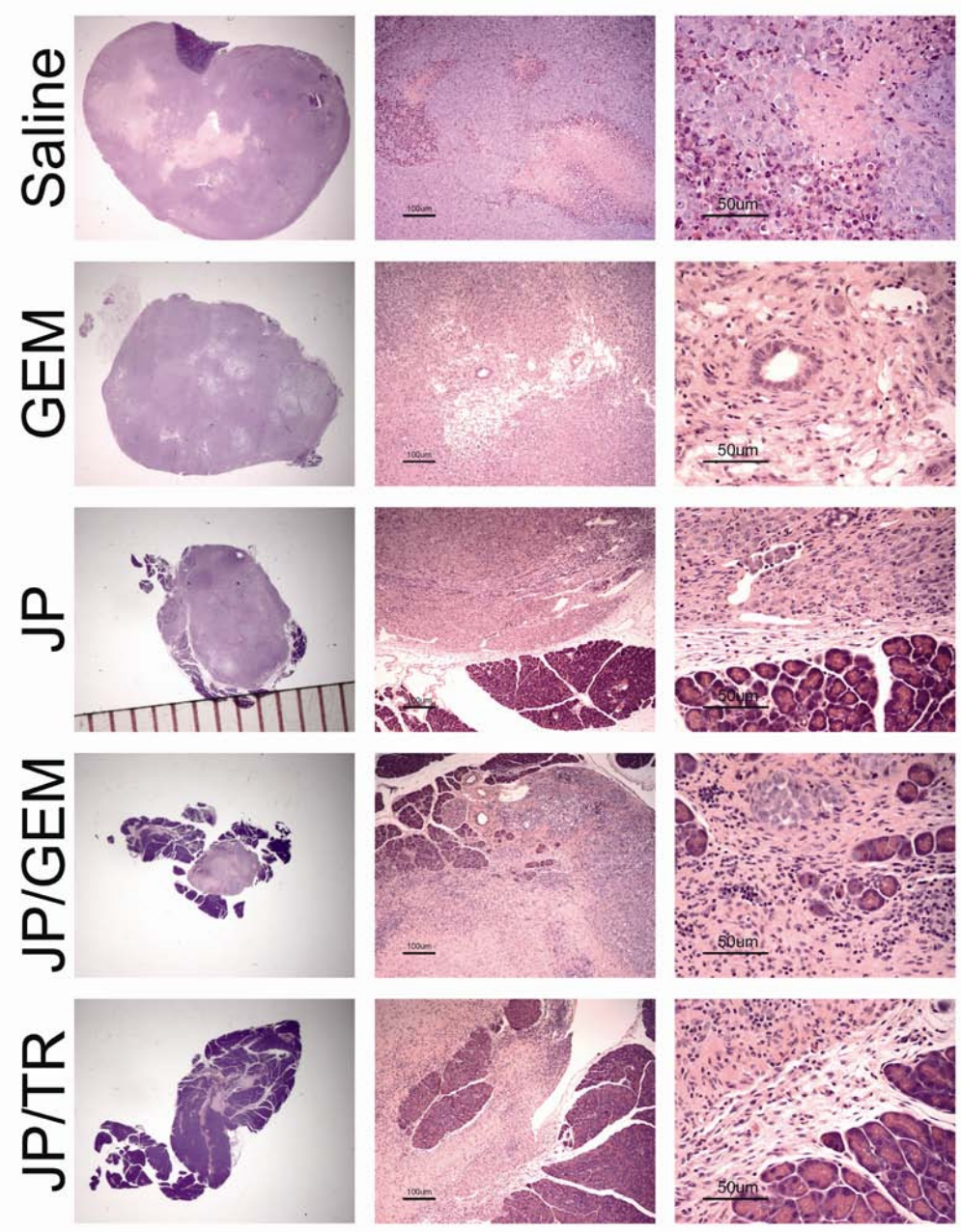
Dineen, SP et al., Smac mimetic increases chemotherapy response and survival in mice with pancreatic cancer

Pharmacokinetics. Tumor samples were weighed and snap frozen in liquid nitrogen. Plasma collection was done in the presence of 100 µg/ml tetrahydrouridine (THU) to prevent ex vivo metabolism of GEM (2',2'-difluoro-2'-deoxycytidine, dFdC) to the inactive metabolite, 2'-2'-difluorodeoxyuridine (dFdU). Tumor samples were homogenized in a 3-fold (3X weight) volume of PBS containing 100 µg/ml THU and were stored long term at -80C.

Analytical methods were developed to detect GEM (dFdC), JP1201, and the GEM inactive metabolite, dFdU using an Applied Biosystems/MDS Sciex 3200 QTRAP mass spectrometer coupled to a Shimadzu Prominence LC. GEM and dFdU were detected as a singly charged species with two daughter ions each, while JP1201 was detected as a doubly charged species with two daughter ions. Only one daughter ion was used for quantitation. Chromatography was performed using a Phenomenex C8 column (Luna C8, 5 micron, 100 X 4.6 mm).

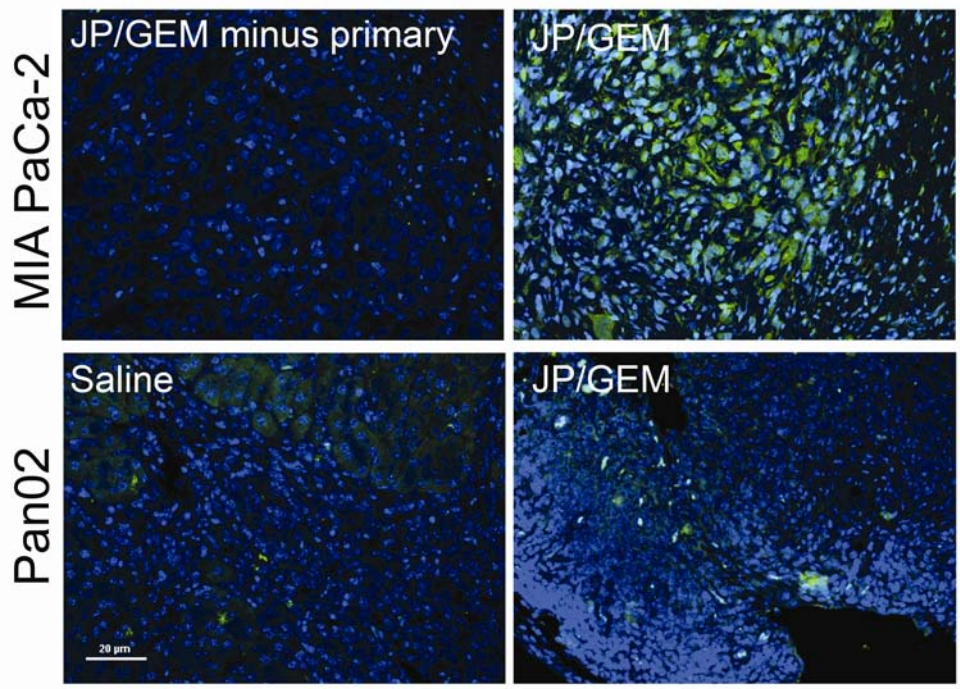
Standard curves were prepared using blank plasma or tumor homogenate spiked with known concentrations of GEM, JP1201, and authentic dFdU metabolite (Toronto Research Chemicals, Cat# D445740). Blank plasma or tumor homogenate was used to establish the limit of detection (LOD) as 3 times the signal seen in these samples.

Calculation of tumor drug concentrations was performed by multiplying the concentration of drug in the tumor homogenate by the total volume of homogenate. This total drug amount was then divided by the total tumor weight to give a drug concentration in ng/g of tumor tissue. Pharmacokinetic analysis was carried out using the Pharsight WinNonLin software package. Noncompartmental modeling with sparse sampling was utilized.



Supplemental Figure 1. Histology of tumors from late intervention trial. MIA PaCa-2 tumors harvested from mice treated with saline, gemcitabine (GEM), JP1201 (JP), JP1201 combined with GEM (JP/GEM), or TRAIL (JP/TR) in the late interventions scheme (see Fig. 3A) were formalin-fixed, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). A representative example of H&E staining from treatment group is shown. The left column shows a low magnification (total magnification, 5x) picture of a section from the center of the tumor. The middle column displays an image from the same

tumor at 100x (scale bar, 100 µm), while the right column is an image at 400x (scale bar, 50 µm). Note the relative lack of residual normal pancreas (darker purple) in tissue from the saline and GEM treated animals while the mice treated with combination therapy show reduced tumor burden and an increase in residual normal pancreas.



Supplemental Figure 2. TNFα immunohistochemistry controls. Formalin-fixed sections of MIA PaCa-2 and Pan02 tumor tissue from mice in the indicated treatment groups were incubated with an anti-human TNFα antibody. Note the lack of TNFα signal (green) in MIA PaCa-2 tumor tissue incubated with secondary alone and the abundant signal in the tumor from mice treated with JP1201 and gemcitabine (JP/GEM). In contrast there is only minimal reactivity in Pan02 tumor tissue regardless of therapy. Scale bar, 20 µm

Table S1. Pharmacokinetic parameters of gemcitabine and JP-1201 in mice bearing orthotopic MIA PaCa-2 tumors

		Plasma Parameters					Tumor Parameters		
	Treatment group	Half-life (min)	Cmax (ng/ml)	AUC (min*ng/ml)	Vd (ml)	Cl (ml/min)	Half-life (min)	Cmax (ng/g)	AUC (min*ng/g)
GEM	GEM	61	21400	678038	258.9	2.9	215.8	91467	6767280
	JP/GEM	85.6	13367	814747	303.2	2.5	172	95200	9669760
JP-1201	JP1201	322.9	8300	307263	174.6	0.37	926.4	3639	2022433
	JP/GEM	394.5	7133	377469	174.1	0.31	981.7	2872	1654077
dFdU	GEM	583.5	24500	7069073	204.2	0.24	358.4	29260	11291170
	JP/GEM	411	15097	3695378	297.9	0.5	525.7	37400	10866020

Mice bearing established orthotopic tumors were treated with gemcitabine (100 mg/kg), JP1201 (6 mg/kg), or the combination and then blood and tumor tissue were collected in a time course (5 min, 30 min, 2, 8, 12, 24 hr) post injection. Plasma and tumor lysates were prepared in the presence of tetrahydrouridine (THU) to prevent ex vivo metabolism of gemcitabine to the inactive metabolite 2'-2'-diflurodeoxyuridine (dFdU). Levels of gemcitabine, JP1201, and dFdU were determined by LC/MS/MS analysis. Pharmacokinetic analysis was carried out using the Pharsight WinNonLin software package. Noncompartmental modeling with sparse sampling was utilized.

Cmax, maximum concentration of analyte; AUC, area under curve (drug exposure); Vd, volume of distribution; Cl, clearance.

Table S2. TNFα levels after treatment with JP1201 or siRNA-mediated knock-down of XIAP

	0 hr	12 hr			48 hr			96 hr		
Cell Line	**NT**	**NT**	**JP1201**	**XIAP KD**	**NT**	**JP1201**	**XIAP KD**	**NT**	**JP1201**	**XIAP KD**
AsPC-1	nd	nd	6.5	15	nd	nd	nd	28	9	nd
BxPC3	nd	nd	8	nd	nd	15.4	nd	17	nd	nd
MIA PaCa-2	nd	13	6	28.6	9.5	14	nd	15.4	20	8.3
PANC-1	15	28	27.2	49	13.5	28	nd	21	15	21.2

ELISA analysis of TNFα in conditioned media from the indicated cell lines. Cells were treated in duplicate with JP1201 (100 nM) or siRNA specific for XIAP (XIAP KD) in serum-free media as described in the methods. Conditioned media (100 µl) was harvested at the indicated time points.
Values are expressed as pg/ml.
NT, no treatment; nd, not detected