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SMAC Mimetic (JP1201) Sensitizes Non–Small Cell Lung Cancers to Multiple Chemotherapy Agents in an IAP-Dependent but TNF- α -Independent Manner

Rachel M. Greer¹, Michael Peyton¹, Jill E. Larsen¹, Luc Girard^{1,2}, Yang Xie⁴, Adi F. Gazdar¹, Patrick Harran⁷, Lai Wang⁶, Rolf A. Brekken^{1,2,7}, Xiaodong Wang⁸, and John D. Minna^{1,2,3}

Abstract

Inhibitors of apoptosis proteins (IAP) are key regulators of apoptosis and are inhibited by the second mitochondrial activator of caspases (SMAC). Previously, a small subset of TNF- α -expressing non–small cell lung cancers (NSCLC) was found to be sensitive to SMAC mimetics alone. In this study, we determined if a SMAC mimetic (JP1201) could sensitize nonresponsive NSCLC cell lines to standard chemotherapy. We found that JP1201 sensitized NSCLCs to doxorubicin, erlotinib, gemcitabine, paclitaxel, vinorelbine, and the combination of carboplatin with paclitaxel in a synergistic manner at clinically achievable drug concentrations. Sensitization did not occur with platinum alone. Furthermore, sensitization was specific for tumor compared with normal lung epithelial cells, increased in NSCLCs harvested after chemotherapy treatment, and did not induce TNF- α secretion. Sensitization also was enhanced *in vivo* with increased tumor inhibition and increased survival of mice carrying xenografts. These effects were accompanied by caspase 3, 4, and 9 activation, indicating that both mitochondrial and endoplasmic reticulum stress-induced apoptotic pathways are activated by the combination of vinorelbine and JP1201. Chemotherapies that induce cell death through the mitochondrial pathway required only inhibition of X-linked IAP (XIAP) for sensitization, whereas chemotherapies that induce cell death through multiple apoptotic pathways required inhibition of cIAP1, cIAP2, and XIAP. Therefore, the data suggest that IAP-targeted therapy using a SMAC mimetic provides a new therapeutic strategy for synergistic sensitization of NSCLCs to standard chemotherapy agents, which seems to occur independently of TNF- α secretion. *Cancer Res*; 71(24); 7640–8. ©2011 AACR.

Introduction

Non–small cell lung cancer (NSCLC) accounts for 80% of all lung cancers, has a poor prognosis, and often is clinically resistant to chemotherapy (1). Although therapy targeted at "oncogene addiction," such as mutant epidermal growth factor receptor (EGFR) or ALK fusion proteins, is an important approach, ways to improve tumor response to existing chemotherapies without affecting normal cells would be a major step forward.

Cancer cells avoid apoptosis by upregulation of antiapoptotic factors such as the inhibitors of apoptosis proteins (IAP), which bind caspases and sequester them or inhibit their protease activity (2). The human IAP family contains 8 proteins, most notably cIAP1, cIAP2, and X-chromosome-encoded IAP (XIAP). XIAP is the only IAP known to actively inhibit caspase activity (3). cIAP1 and cIAP2 were identified for their ability to interact with TRAF1 and TRAF2, localizing the cIAPs to the TNF receptor (TNFR; ref. 4). The recruitment of cIAP1 and cIAP2 to TNFR1 inhibits TNF- α -dependent, RIPK1-dependent activation of caspase 8 (4–9).

Authors' Affiliations: ¹Hamon Center for Therapeutic Oncology Research; Departments of ²Pharmacology, ³Internal Medicine, ⁴Clinical Sciences, and ⁵Division of Surgical Oncology, Department of Surgery, University of Texas Southwestern Medical Center; ⁶Joyant Pharmaceuticals Inc., Dallas, Texas; ⁷Department of Chemistry and Biochemistry, University of California, Los Angeles, California; and ⁸Zhongguancun Life Science Park, Beijing, China

Currently, there are 3 known apoptotic pathways, the intrinsic or mitochondrial, the extrinsic or death receptor, and the endoplasmic reticulum (ER) stress-induced pathway (10). These pathways differ by the upstream signals that activate them and the initiator caspase involved: caspase 9 in the mitochondrial pathway, caspase 8 or 10 in the death receptor pathway, and caspase 4 in the ER stress pathway (11).

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Corresponding Author: John D. Minna, 6000 Harry Hines Boulevard, Hamon Center for Therapeutic Oncology Research, UT-Southwestern Medical Center, Dallas, TX 75390. Phone: 214-648-4900; Fax: 214-648-4940; E-mail: john.minna@utsouthwestern.edu

Our previous work aimed to develop second mitochondrial activator of caspases (SMAC) mimetics to target cancer cell-specific alterations (6, 9, 12, 13). We hypothesized that inhibition of IAPs by a SMAC mimetic would prime NSCLCs to respond to apoptogenic chemotherapies. In the current work using an oncogenotype diverse panel of NSCLC cell lines resistant to single-agent SMAC mimetic (JP1201), we found JP1201 can

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sensitize tumor cells to several standard chemotherapy agents and erlotinib in *in vitro* studies. In xenograft models of NSCLC, we found the combination of JP1201 with gemcitabine or vinorelbine reduces *in vivo* tumor growth to a greater extent than the sum of each agent alone. The JP1201-induced sensitization showed specificity for tumor over normal epithelial cells, and was independent of TNF- α production, but dependent on 2 other apoptotic programs. Thus, SMAC mimetics are potent sensitizers of NSCLC to conventional chemotherapy and EGFR tyrosine kinase inhibitor (TKI)-targeted therapy and their IAP targets differ between chemotherapy regimens.

Materials and Methods

Cell lines

With the exception of A549 [from the American Type Culture Collection, (ATCC)], all tumor cell lines were established in our laboratories (A.F. Gazdar and J.D. Minna) and are deposited at the ATCC (14, 15). Cancer cell lines were grown in RPMI-1640 medium (Life Technologies Inc.) supplemented with 5% FBS. Immortalized human bronchial epithelial cells (HBEC) were established by ectopically expressing CDK4 and hTERT, denoted by KT at the end of the cell line name, and were grown in Keratinocyte Serum-Free Medium (KFSM) supplemented with bovine pituitary extract and recombinant human EGF (Gibco; ref. 16). Defined oncogenic changes, Kras G12V and p53 short hairpin RNA, were introduced in HBEC3KT and tumorigenic clones (clone 1, clone 5) isolated by soft agar formation, which were further cultured in RPMI-1640 with 5% FBS (17). All cell lines were grown in a humidified atmosphere with 5% CO₂, at 37°C have been DNA fingerprinted for provenance using the PowerPlex 1.2 kit (Promega) and confirmed to be the same as the DNA fingerprint library maintained by ATCC and the Minna/Gazdar lab (the primary source of the lines), and were confirmed to be free of mycoplasma by the e-Myco kit (Boca Scientific).

MTS assay

Assays were done as described (12). Chemotherapies were given as 4-fold dilutions with a maximum dose of 1,000 nmol/L for paclitaxel (Bristol-Myers Squibb) and vinorelbine (Pierre Fabre Company), 2,000 nmol/L for gemcitabine (Eli Lilly and Company) and doxorubicin (Teva Parenteral), or 100 μ mol/L for erlotinib (OSI Pharmaceuticals), cisplatin (Teva Parenteral), or JP1201 (Joyant Pharmaceuticals) alone. A dose of 10 μ mol/L or 100 nmol/L of JP1201 was used in combination assays as indicated. Each experiment contained 8 replicates per concentration, and the entire assay was done in multiple replicates ($n \geq 4$). Drug sensitivity curves and IC₅₀s were calculated using in-house software (DIVISA).

Mouse xenograft treatment studies

Mouse work followed an International Animal Care and Usage Committee-approved protocol. NCI-H1395 and NCI-H157 cells were trypsinized, washed, and counted using trypan blue exclusion to assess viability. Cell suspensions with viability greater than 95% were used in animal studies. Subcutaneous tumors were established in nonobese diabetic/severe combined immunodeficient mice as described (6, 12). Briefly,

NCI-H1395 or NCI-H157 cells (1×10^6 in 100 μ L) were injected s.c. on the left flank. Animals were monitored 3 times a week and tumors measured with digital calipers. Tumor volumes were calculated using the formula ($D \times d^2 \times 0.52$), where D is the largest diameter and d is the shortest (18). At sacrifice, tumors were harvested and fixed in formalin. Treatment groups (8 mice per group) consisted of saline, 25 mg/kg gemcitabine, 2.4 mg/kg vinorelbine, 6 mg/kg JP1201, 6 mg/kg JP1201 + 25 mg/kg gemcitabine, or 6 mg/kg JP1201 + 2.4 mg/kg vinorelbine; each injection was given in a volume of 100 μ L administered intraperitoneally (12, 19). Saline, gemcitabine, and JP1201 were given 3 times a week, and vinorelbine was given twice a week to minimize toxicity (19).

siRNA-mediated MTS assay

siRNA transfections were done as a reverse transfection with 25 nmol/L as the final siRNA concentration at a 1:2 lipid to oligo ratio (12). RNA-lipid complexes were allowed to form during a 20- to 30-minute incubation. Then 8,000 cells were added to each well in RPMI with 5% FBS, total volume per well 50 μ L. Drugs were administered 24 hours after transfection as described above.

Quantitative PCR analysis of siRNA knockdown efficiency

Transfections were done as described above, except that RNA-lipid complexes were formed in 6-well plates in a volume of 1 mL for a 20-minute incubation period, and cells were plated at 70% confluency. Twenty-four hours later, cells were harvested and total RNA prepared (RNeasy Plus Mini Kit, Qiagen). cDNA was synthesized from 1 μ g total RNA using the iScript cDNA synthesis kit (BioRad). Gene-specific TaqMan probes (Applied Biosystems) were used to quantitate GAPDH, cIAP1, cIAP2, XIAP, Caspase 3, 4, 8, and 9, RIPK1, TNFR1, and TNF- α levels in biological duplicates and duplicate samples of siRNA transfected H1395 cells. The $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels (20).

Microscopy

Formalin-fixed tissues were embedded in paraffin, cut in 10- μ m sections, and stained with hematoxylin and eosin. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was done per manufacturer instructions (Promega) on paraffin-embedded tumor sections from 3 tumors per treatment group with data averaged from 3 images per slide, 3 slides per treatment group. Images of stained slides were taken using an Eclipse TE2000 epifluorescent microscope (Nikon). ImageJ (NIH) software was used to produce overlaid images of 4',6-diamidino-2-phenylindole and antibody staining.

Luciferase reporter assay

Pathway analysis was done using the Stress and Toxicity Signal Finder 10 pathway reporter array (SA Biosciences) following the manufacturer's instructions. Sixteen hours after cells were reverse transfected, they were treated with 1 μ mol/L vinorelbine with 10 μ mol/L JP1201 or 10 μ mol/L JP1201 as control, then incubated for an additional 24 hours. Luciferase activity was measured using the Dual Luciferase Assay system

(Promega) on a FLUOstar omega (BMG Biotech). Firefly luciferase was the experimental reporter, and *Renilla* luciferase was the normalizing reporter; samples were further normalized to nontransfected control cells.

Statistics

Combination indices (CI) for the drug sensitivity data were calculated using the Chou–Talay method (21). SDs were calculated based on drug sensitivity data. Data from the xenograft studies were analyzed using GraphPad software (GraphPad Prism version 5.02, www.graphpad.com). Results are expressed as mean \pm SEM. Data were analyzed by *t* test or ANOVA and results are considered significant at $P < 0.05$.

Results

SMAC mimetic synergizes with conventional chemotherapy in NSCLC cell lines *in vitro*

An agent with minimal side effects that specifically sensitizes tumor cells to standard available chemotherapies would provide an important new therapeutic approach. SMAC mimetics could be such an agent, and thus we evaluated the ability of the SMAC mimetic, JP1201, to sensitize NSCLCs to chemotherapies that have been used to treat NSCLC in the clinic. Using a panel of 16 NSCLC cell lines resistant to JP1201 monotherapy, 2 immor-

talized human bronchial epithelial cells (HBEC), and 2 genetically modified malignant HBEC lines, we examined the effects of combinations of 10 μ mol/L JP1201 with single-agent cisplatin, doxorubicin, erlotinib, gemcitabine, paclitaxel, vinorelbine, and the combination of paclitaxel with carboplatin and found JP1201 induced sensitization in most cases (Table 1, Supplementary Tables S1 and S2; refs. 1–3). All but one NSCLC line were sensitized to the combination of JP1201 + vinorelbine, whereas almost no cell lines were sensitized to the combination of JP1201 + cisplatin. In addition, the pattern of response across the NSCLC panel was unique for each drug combination. Of importance, 3 NSCLCs with wild-type EGFR were sensitized to the EGFR TKI, erlotinib. CIs were calculated and revealed that most combinations resulted in synergy (Supplementary Table S3; ref. 4). Furthermore, we confirmed the range of sensitization seen in mass culture MTS assays with similar results in colony formation assays (Supplementary Table S4). Thus, we found JP1201 showed synergistic sensitization to available chemotherapy agents and EGFR TKI-targeted therapy.

JP1201 exhibits great selectivity in chemotherapy sensitization in isogenic cell line pairs

Included in this panel are several types of isogenic cell line pairs: a tumor/normal lung epithelial cell pair, HCC4017, and HBEC30KT established from the same patient;

Table 1. JP1201 Sensitizes NSCLC to doxorubicin, erlotinib, gemcitabine, paclitaxel, vinorelbine, and paclitaxel/carboplatin *in vitro*

Cell line	Absolute fold decrease in IC ₅₀ of chemotherapy agents when combined with JP1201						Paclitaxel/ Carboplatin
	Vinorelbine	Paclitaxel	Doxorubicin	Gemcitabine	Erlotinib	Cisplatin	
H2073	31,000	1	1	1	1	0.4	1
H1819	4,400	5,600	1	6	4	0.7	18
H2887	3,600	22	7,726	23	1	1	20
H1993	270	6	1	2	0.7	0.4	—
H358	397	17	13	4	6	1	12
H441	181	3	4	16	4	0.5	2.8
A549	100	4	5	2	18	0.7	4.6
H2882	73	11	1	21	0.7	0.6	10
H460	36	2	3	1	10	1.2	1.8
H1395	33	43	10	134	0.4	1	23
H1355	22	10	1	1	1	0.7	—
H157	16	32	3	2	111	1	15
H1693	15	6	1	7	—	0.6	—
H2009	12	4	1	3	1	1	—
H2087	1	4	4	1	1	1.4	3.6
HCC4017	25	10	6	15	1	0.7	—
HBEC30KT	1	1	1	1	1	1	—
HBEC3KT	1	1	1	1	s1	1	—
HBEC3KT-RL53-s1	20	12	3	7	—	—	—
HBEC3KT-RL53-s5	20	15	2	9	—	—	—

NOTE: — Indicates where data were not obtained. Fold change is determined by dividing the median IC₅₀ ($n \geq 4$) for each chemotherapy as a single agent by the median IC₅₀ ($n \geq 3$) for each chemotherapy in combination with 10 μ mol/L JP1201. Histology and oncogenotype of cell lines are given in Supplementary Table S1.

nontransformed HBEC/transformed HBEC pair, HBEC3KT where defined oncogenic changes, Kras G12V and p53 shRNA, were introduced and tumorigenic clones (clone 1, clone 5) isolated by soft agar formation; and 2 sets of tumor lines that were each established from the same patient. These pairs were isolated before and after treatment of the same patients with cisplatin and etoposide: NCI-H1693 (before) and NCI-H1819 (after); NCI-H1993 (before) and NCI-H2073 (after; refs. 1–3). JP1201 sensitizes HCC4017 but not HBEC30KT to chemotherapy (Fig. 1A, Table 1). HBEC3KT tumorigenic clones 1 and 5 are sensitized 10- to 20-fold to vinorelbine (20-fold) or paclitaxel (12–15-fold), whereas the parental HBEC3KT cells are not sensitized to chemotherapy (Fig. 1B, Table 1). Surprisingly, H1819 and H2073, which were started from residual NSCLC cells harvested after neoadjuvant platinum and etoposide chemotherapy, were sensitized approximately 200-fold greater to chemotherapy than H1693 and H1993, which were started from NSCLC samples from each respective patient prior to treatment (Table 1). Thus, we found specificity of JP1201 sensitization for tumor compared with normal cells and even in tumor cells after neoadjuvant chemotherapy.

Combination of SMAC mimetic (JP1201) and chemotherapy control NSCLC xenograft growth

To determine *in vivo* efficacy, we used s.c. NCI-H1395 xenografts to examine the effects of JP1201 in combination with gemcitabine or vinorelbine. Tumors in mice treated with saline, gemcitabine, or JP1201 alone continued to grow exponentially during the 3-week treatment period. Tumor growth was partially controlled by vinorelbine, and well controlled in the JP1201 with gemcitabine (JP + Gem) and JP1201 with vinorelbine (JP + Vin)-treated groups (Fig. 2A). Tumors from the JP + Vin group showed a 70% decrease in tumor burden as compared with the vinorelbine group. Similarly, tumors from the JP + Gem group showed a 60% decrease in tumor burden as compared with the tumors treated with gemcitabine (Fig. 2B). Induction of apoptosis was explored using TUNEL assays and revealed a significant increase in TUNEL staining in the JP + Gem and JP + Vin groups compared with single agent and vehicle-treated tumors (Fig. 2C). In the very aggressive NCI-H157 NSCLC xenograft model, we saw an increase in survival time with treatment of JP + Vin compared with JP1201, vinorelbine, or control (Fig. 3). Thus, combination therapy was effective *in vivo*.

Chemotherapy agents require targeting of different IAPs for sensitization

To determine which IAPs are involved in the sensitization to each chemotherapy, siRNAs targeting cIAP1, cIAP2, and XIAP were used in place of JP1201 treatment in combination with gemcitabine, vinorelbine, and paclitaxel in 4 NSCLC lines (NCI-H1395, NCI-H358, NCI-H2887, and NCI-H1819) and knockdowns were validated by quantitative PCR analysis (Fig. 4, Supplementary Fig. S2, Supplementary Table S5). Knockdown of XIAP alone mimics JP1201 treatment with gemcitabine, whereas knockdown of either cIAP1 or cIAP2 had no effect (Fig. 4B). By contrast, knockdown of cIAP1, cIAP2, and XIAP increasingly sensitized

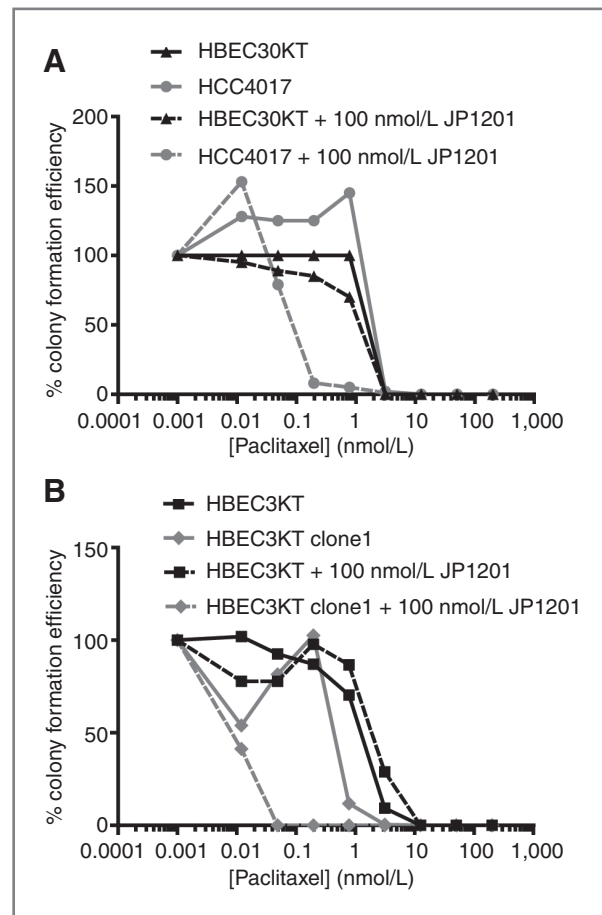


Figure 1. JP1201 specifically sensitizes tumorigenic cell lines in isogenic systems. A, colony forming efficiency expressed as a percentage of untreated HCC4017 (circles) and HBEC30KT (triangles) in the presence of paclitaxel alone (solid line), or in combination with 100 nmol/L JP1201 (dashed line); data are representative of 2 independent assays. B, colony forming efficiency expressed as a percentage of untreated HBEC3KT RL53 clone1 (diamonds) and HBEC3KT (squares) in the presence of paclitaxel alone (solid line), or in combination with 100 nmol/L JP1201 (dashed line); data are representative of 2 independent assays.

the cells to paclitaxel and vinorelbine (Fig. 4C and D). We conclude that different classes of chemotherapy require inhibition of different sets of IAPs for sensitization, and in general these targets are similar among different NSCLCs.

TNF- α signaling is not required for sensitization to chemotherapy by JP1201

Autocrine TNF- α secretion is a strong predictor of sensitivity to JP1201 as a single agent in a subset of NSCLCs (6–8). Thus, we explored whether the mechanism of sensitization involved the induction of TNF- α after chemotherapy treatment in 8 NSCLC lines. No TNF- α secretion was found under any conditions tested (Supplementary Fig. S3). When we added recombinant TNF- α to the media (10 pg/mL) no sensitization to gemcitabine or vinorelbine was seen. Finally, the combination of TNF- α with gemcitabine or vinorelbine in the presence of XIAP knockdown showed

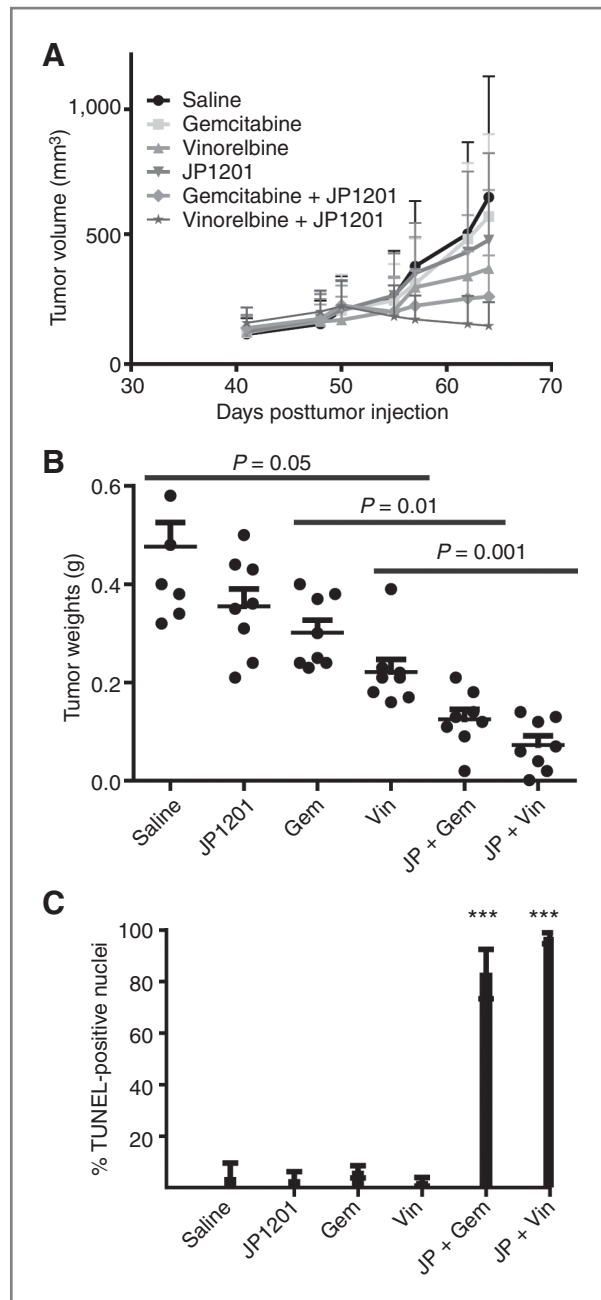


Figure 2. JP1201 in combination with gemcitabine or vinorelbine is effective in controlling NCI-H1395 xenograft growth by inducing apoptosis. A, tumor growth curves during the course of treatment; treatment began on day 41; saline, 25 mg/kg gemcitabine, and 6 mg/kg JP1201 were given 3 times weekly, and 2.4 mg/kg vinorelbine was given twice weekly. B, tumor weights of xenografts were harvested 24 hours after the final treatments. C, induction of apoptosis was analyzed using TUNEL immunofluorescence. Data from a minimum of 3 tumors per treatment group were normalized to the saline group and are representative of at least 2 independent assays. ***, $P < 0.0001$.

no sensitization (Supplementary Table S6). NCI-H1395 cells were transfected with siRNAs against luciferase, RIPK1, caspase 8, TNFR1, and caspase 3, then treated with JP1201 + vinorelbine (JP + Vin). The knockdown of caspase 8,

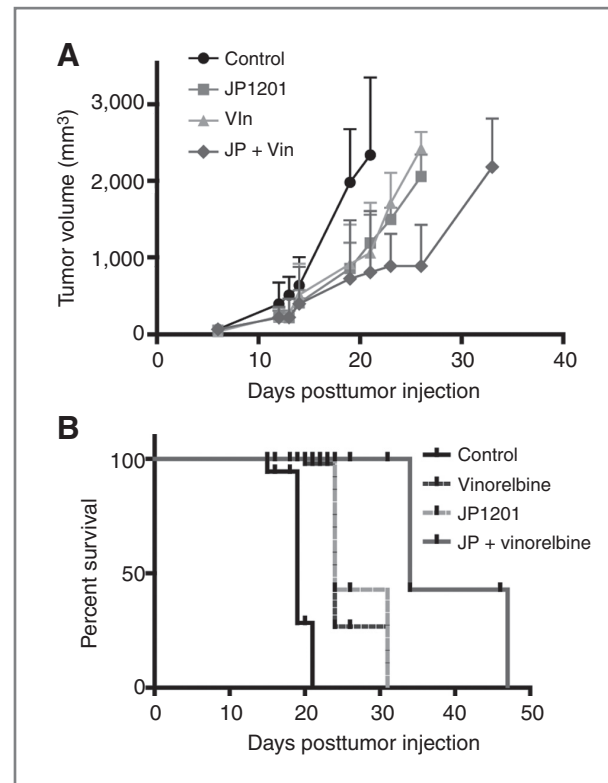


Figure 3. JP1201 in combination with vinorelbine is effective in controlling NCI-H157 xenograft growth and extends survival time. A, tumor growth curves for xenografts; animals were sacrificed throughout the study due to tumor burden. Treatment began on day 14; saline and 6 mg/kg JP1201 were given 3 times weekly, and 2.4 mg/kg vinorelbine was given twice weekly. B, Kaplan-Meier survival curves. Animals had median survival times of 19, 24, 24, and 35 days, respectively for saline, vinorelbine, JP1201, and JP1201 with vinorelbine.

RIPK1, or TNFR1 did not protect the cells from JP + Vin, whereas the knockdown of caspase 3 offered some protection against JP + Vin (Fig. 5A). These results suggest the effects of JP + Vin are dependent on an apoptotic pathway different from the TNF- α -RIPK1 pathway, indicating JP1201 sensitization to standard chemotherapies occurs by a TNF- α -independent mechanism.

ER stress-induced apoptosis mediated through caspase 4 as mechanism for JP1201 + chemotherapy sensitization

To better understand how NSCLCs are sensitized to the combination of JP1201 with vinorelbine, we used the Stress and Toxicity Signal Finder 10-pathway reporter array. HCC4017, HBEC30KT, H1395, and H157 were reverse transfected with a set of pathway-specific reporter constructs, and cells were treated with JP1201 or the combination of JP1201 + vinorelbine for 24 hours after transfection. The activity of the indicated pathway was determined by firefly luciferase activity relative to control *Renilla* luciferase activity (Table 2). Only the ER stress pathway was activated by combination therapy in all 3 NSCLC lines but not in normal cells (Table 2). Caspase 4 is the putative initiator caspase for the ER stress-induced apoptotic pathway (8, 9). Knockdown of caspase 4 protects H1395 cells against

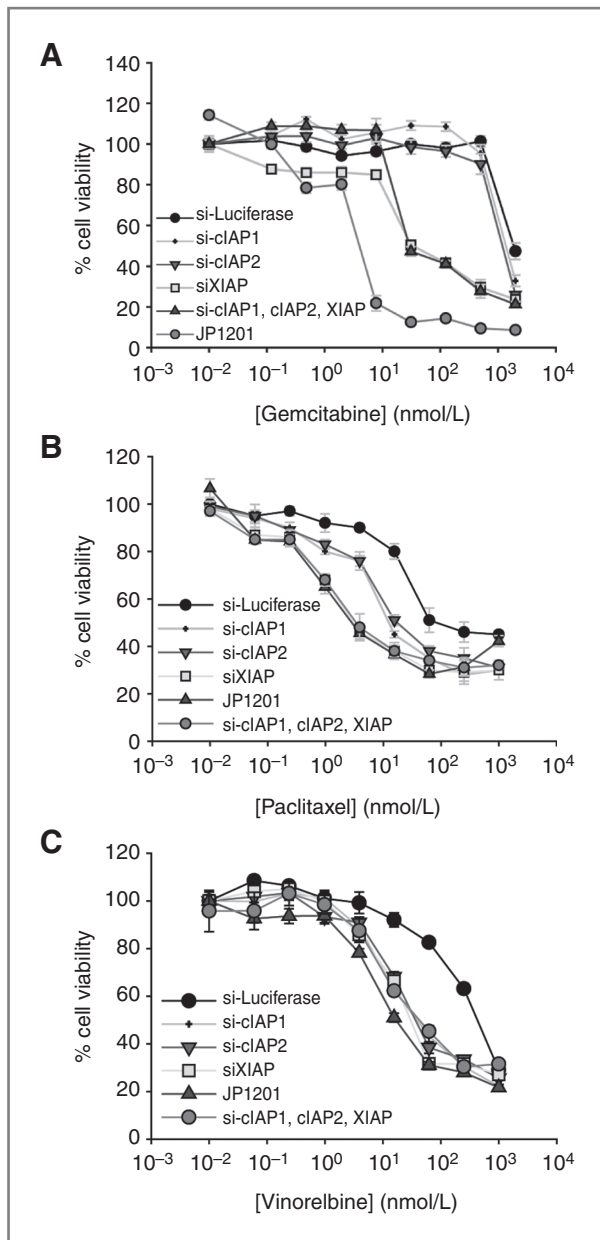


Figure 4. siRNA-mediated knockdown of XIAP can mimic JP1201 when given as part of cotreatment with gemcitabine, whereas cIAP1, cIAP2, and XIAP knockdowns are all required to mimic JP1201 when given as cotreatment with vinorelbine or paclitaxel. A, knockdown of XIAP is sufficient and necessary to mimic combination of JP1201 with gemcitabine. B, knockdown of cIAP1, cIAP2, or XIAP mimics the sensitization to paclitaxel seen with JP1201. A–C, on day 0 H1395 cells were transfected in 96-well format with firefly luciferase siRNA, cIAP1 siRNA, cIAP2 siRNA, XIAP siRNA, or cIAP1, cIAP2, and XIAP siRNAs as indicated and on day 1 treated with increasing amounts of gemcitabine (A), paclitaxel (B), or vinorelbine (C).

the effect to a greater extent than knockdown of caspase 3 or 9 (Fig. 5B). In addition, using an antibody that only recognizes procaspase 4 but not activated caspase 4, we found that vinorelbine treatment resulted in activation of caspase 4 over

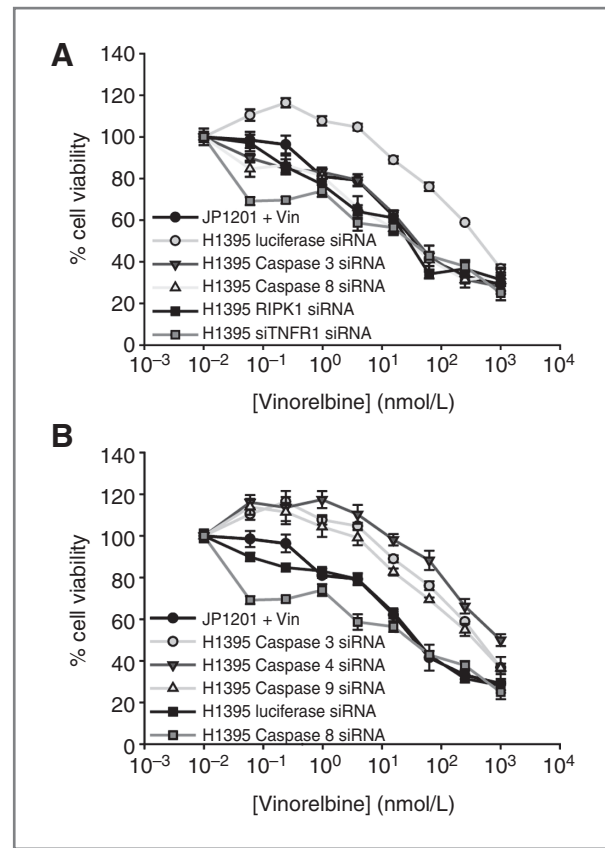


Figure 5. Synergy of vinorelbine and JP1201 does not act through TNF- α /RIPK1-dependent pathway. A, knockdown of caspase 3 or 9 protects H1395 cells from the effects of JP1201 + vinorelbine. H1395 cells were transfected with siRNAs that are specific for the indicated target in 96-well plates and treated with vinorelbine + 10 μ mol/L JP1201; the MTS assay was used to produce drug response curves for each transfection. B, determination of which caspases are involved in cell kill induced by vinorelbine + JP1201. H1395 cells were transfected with siRNAs that are specific for the indicated target in a 96-well plate format and treated with vinorelbine + 10 μ mol/L JP1201; the MTS assay was used to produce drug response curves for each transfection.

time, similar to an agent known to activate the ER stress pathway, thapsigargin (Supplementary Fig. S4). We conclude that JP1201 sensitizes NSCLCs to multiple chemotherapy agents, and this sensitization is not dependent on TNF- α signaling but is dependent on ER stress and mitochondrial-induced apoptotic pathways.

Discussion

Evaluation of the apoptotic machinery in tumors has led to the development of therapeutic agents targeting this machinery, including SMAC mimetics (13, 22–27). We addressed the utility of SMAC mimetic JP1201 in NSCLCs that do not express TNF- α and are resistant to SMAC mimetic monotherapy. In a panel of NSCLC lines of various histotypes and oncogenotypes, JP1201 frequently synergized with available chemotherapy agents, with considerable intertumor heterogeneity in NSCLC responses between drug combinations. In NSCLC xenografts,

Table 2. Analysis of pathways activated by JP1201 + chemotherapy using Signal finder assay system (SABiosciences)

Pathway ^a	Fold change in relative luciferase activity of treated H1395 cells compared with untreated control				Fold change in relative luciferase activity of cells treated with JP1201 + vinorelbine compared with JP1201 alone			
	JP1201	Gemcitabine	Vinorelbine	Vinorelbine + JP1201	H1395	H157	HCC4017	HBEC30KT
1	0.9	1.1	0.8	1.3	<u>3.4</u>	0.7	1.1	0.4
2	0.8	<u>2.6</u> ^b	0.7	1.1	<u>1.7</u>	<u>2.6</u>	0.4	0.4
3	0.8	0.9	0.9	1.1	2.2	0.9	1.1	1.5
4	0.9	0.8	1.1	1.1	<u>2.5</u>	0.8	0.6	0.7
5	1.4	1.7	0.8	<u>3.5</u>	<u>4.4</u>	<u>2.1</u>	<u>3.8</u>	0.5
6	0.6	0.8	1.1	1.1	<u>1.7</u>	<u>2.8</u>	0.4	0.4
7	0.8	1.3	1	1	<u>1.7</u>	<u>3.5</u>	0.7	0.5
8	1.1	0.8	1.4	1.1	0.9	1.3	0.3	0.4
9	1.3	0.9	0.8	<u>2.5</u>	<u>4.7</u>	1.1	0.5	0.9
10	1.2	1.3	0.7	1.3	0.8	1.3	0.7	0.7

NOTE: Left, the combination of JP1201 + vinorelbine selectively activates the ER stress pathway. H1395 cells were reverse transfected with firefly luciferase reporter constructs that contain response elements for the indicated pathways and control *Renilla* luciferase constructs, then treated with 10 $\mu\text{mol/L}$ JP1201, 1 $\mu\text{mol/L}$ gemcitabine, 1 $\mu\text{mol/L}$ vinorelbine, or JP1201 + vinorelbine for 24 hours; luciferase activity was assayed, and firefly luciferase activity was normalized to *Renilla* luciferase activity for each sample and normalized to untransfected cells. Right, ER stress pathway is activated by the combination of vinorelbine + JP1201. H1395, H157, HCC4017, and HBEC30KT cells were reverse transfected with the indicated firefly luciferase reporter construct, treated with JP1201 + vinorelbine. Firefly luciferase activity was normalized to *Renilla* luciferase for each sample, and to JP1201-treated cells.

^aPathways are (1) oxidative stress, (2) p53, (3) NF- κ B, (4) hypoxia, (5) ER stress, (6) heavy metals, (7) heat shock, (8) the glucocorticoid receptor, (9) the JNK pathway, or (10) the xenobiotic receptor.

^bBold and underlined values have $P < 0.001$ by 2-way ANOVA.

where JP1201 alone had little effect on tumor growth, the combination of vinorelbine or gemcitabine with JP1201 decreased tumor growth, which translated to an increase in survival of these mice. The frequent occurrence of synergy (particularly with paclitaxel and vinorelbine) suggests potential for JP1201 or similar drugs as a part of combination chemotherapy for NSCLC. This agrees with the findings of Dean and colleagues (28), who studied 2 NSCLC lines and found synergy with vinorelbine in combination with a different IAP antagonist that targeted XIAP.

JP1201 sensitized many NSCLC lines to concentrations of vinorelbine (10–15 nmol/L) and paclitaxel (~ 100 nmol/L) that are achievable in humans as steady-state plasma levels (Supplementary Table S2; refs. 29, 30). Also, 3 NSCLC lines, all wild type for EGFR, were sensitized to the EGFR TKI, erlotinib, at clinically achievable concentrations (31). Studying a large panel of NSCLC lines, including the 2 investigated by Dean and colleagues, we found no synergy with JP1201 + cisplatin, whereas Dean and colleagues, using the BIR2-binding XIAP antagonist XAC 1296-11, saw synergy with cisplatin (28). The reason for this discrepancy is not yet understood but suggests mechanistic specificity of the different SMAC mimetics. The lack of sensitization to cisplatin seen in this study correlates with p53 mutational status, indicating that p53 mutations may prevent cisplatin-induced apoptotic signaling (Supplementary Table S7).

Our panel includes 3 types of genetically paired cell lines, NSCLC lines started before (H1693 and H1993) and after

etoposide/cisplatin treatment (H1819 and H2073); an isogenic HBEC3KT-based system where oncogenic *Kras* and knockdown of p53 were introduced and tumorigenic clones were isolated from soft agar clones; and a tumor (HCC4017) and normal HBEC pair. Using these, we found sensitization by JP1201 to chemotherapy is specific for the malignant phenotype, and NSCLC lines derived from samples after chemotherapy could be sensitized to chemotherapy more than NSCLCs that were chemo-naïve. Thus, SMAC mimetic-based combinations with chemotherapy may be effective in tumor cells resistant to chemotherapy alone.

XIAP is the only IAP with the ability to inhibit the enzymatic activity of an active caspase (3). Thus, it was reasonable to predict that stimulation of the intrinsic pathway of apoptosis would only require the inhibition of XIAP for apoptosis to occur (2), as was found in pancreatic cancer cell lines treated with JP1201 + gemcitabine (12). When we combined siRNA against cIAP1, cIAP2, or XIAP with gemcitabine, we saw that the knockdown of XIAP mimics the combination of JP1201 + gemcitabine. By contrast, we found knockdown of each cIAP1, cIAP2, or XIAP mimics to different degrees the effect seen when JP1201 is combined with paclitaxel or vinorelbine, suggesting unique roles for each IAP in sensitizing NSCLCs to antimetabolites.

Inhibition of tubulin dynamics, for example with vinorelbine, can inhibit ER to golgi transport, a trigger for ER stress-induced apoptosis (10, 32). Analysis of pathways activated by the

combination of JP1201 + vinorelbine in multiple cell lines revealed that the ER stress pathway is activated. Furthermore, Western blotting and siRNA-mediated rescue experiments also showed that caspase 4, the putative ER stress-induced caspase, is activated after vinorelbine treatment, and knockdown of caspase 4 was better at protecting NSCLCs from treatment of JP1201 + vinorelbine than was knockdown of the executioner caspase, caspase 3. There are reports that suggest cIAP1 is involved in a complex at the ER membrane that is responsible for activating apoptosis; however, further studies are needed to characterize the role of the ER stress pathway in sensitizing NSCLCs to chemotherapy by SMAC mimetics (11, 33, 34).

We found that JP1201-resistant NSCLC lines do not induce or secrete TNF- α after chemotherapy, indicating the synergy seen between doxorubicin, gemcitabine, paclitaxel, or vinorelbine with JP1201 is TNF- α independent. Studies adding recombinant TNF- α with gemcitabine or vinorelbine also suggest that TNF- α is not involved in the JP1201-induced sensitization to chemotherapy. This is further strengthened by the lack of additional sensitization when the combination of TNF- α and gemcitabine or vinorelbine is given to cells where XIAP expression has been knocked down. The lack of protection of combinations of JP1201 + vinorelbine by the knockdown of caspase 8, RIPK1, or TNFR1 further suggests JP1201 is promoting apoptosis in a TNF- α -independent fashion (6–8).

In conclusion, we find that a SMAC mimetic significantly sensitizes NSCLC lines to chemotherapy and EGFR-targeted

therapy; the sensitization varies between NSCLCs and between chemotherapeutic agents; the sensitization is tumor specific and is stronger in NSCLCs that have progressed after neoadjuvant therapy; the required IAP target to be inhibited varied between chemotherapeutic agents; and the sensitization seemed to be TNF- α independent, but dependent on 2 complementary apoptotic pathways, mitochondrial and ER stress. These data strongly suggest the need to explore SMAC mimetics in combination with standard doublet chemotherapy as a new treatment approach for NSCLC patients.

Disclosure of Potential Conflicts of Interest

L. Wang was employed with Joyant Pharmaceuticals. R. Brekken has received commercial research grant from Joyant Pharmaceuticals. X. Wang has ownership interest in Joyant Pharmaceuticals. P. Harran has ownership interest in Joyant Pharmaceuticals.

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