A Novel Kinase Inhibitor of FADD Phosphorylation Chemosensitizes through the Inhibition of NF-κB

Katrina A. Schinske1,4, Shyam Nyati1,4, Amjad P. Khan4,5, Terence M. Williams1,4, Timothy D. Johnson3, Brian D. Ross2,3, Ricardo Pérez Tomás6, and Alnawaz Rehemtulla1,4

Abstract

Fas-associated protein with death domain (FADD) is a cytosolic adapter protein essential for mediating death receptor–induced apoptosis. It has also been implicated in a number of nonapoptotic activities including embryogenesis, cell-cycle progression, cell proliferation, and tumorigenesis. Our recent studies have shown that high levels of phosphorylated FADD (p-FADD) in tumor cells correlate with increased activation of the antiapoptotic transcription factor NF-κB and is a biomarker for aggressive disease and poor clinical outcome. These findings suggest that inhibition of FADD phosphorylation is a viable target for cancer therapy. A high-throughput screen using a cell-based assay for monitoring FADD-kinase activity identified NSC 47147 as a small molecule inhibitor of FADD phosphorylation. The compound was evaluated in live cells and mouse tumors for its efficacy as an inhibitor of FADD-kinase activity through the inhibition of casein kinase 1α. NSC 47147 was shown to decrease levels of p-FADD and NF-κB activity such that combination therapy leads to greater induction of apoptosis and enhanced tumor control than either agent alone. The studies described here show the utility of bioluminescent cell-based assays for the identification of active compounds and the validation of drug–target interaction in a living subject. In addition, the presented results provide proof-of-principle studies as to the validity of targeting FADD-kinase activity as a novel cancer therapy strategy. Mol Cancer Ther; 10(10); 1807–17. ©2011 AACR.

Introduction

Fas-associated protein with death domain (FADD) was first identified as a cytosolic adapter protein essential for mediating death receptor–induced apoptosis (1–3). It links to the cytoplasmic tail of active death receptors, such as Fas, DR4, and DR5, where it binds to procaspases-8 and -10, leading to the formation of the death-inducing signaling complex (DISC) at the cytosolic side of the cell membrane. DISC formation initiates intracellular processing and activation of procaspases, which, in turn, initiates cleavage of the downstream targets, such as caspase-3, -6, and -7, and subsequently, apoptosis (4–6). Besides its role in regulating death receptor–induced apoptosis, FADD is also implicated in a number of death receptor–induced nonapoptotic activities including embryogenesis, cell-cycle progression, cell proliferation, and tumorigenesis (6–10). Many of these nonapoptotic activities are determined by the level of phosphorylation of a specific C-terminal serine (Ser194) in a region distinct from the proapoptotic function related to the death domain (11, 12).

Recent studies have led to a better understanding of the FADD gene and its location on chromosome 11q13.3, a hot spot for chromosomal amplification in a number of human cancers including esophagus, lung, and head and neck carcinomas (13, 14). Our recent studies provide evidence of overexpression of FADD mRNA and protein in human lung adenocarcinoma and its correlation to NF-κB activation. We have also shown that high levels of phosphorylated FADD (p-FADD), predominantly localized to the nucleus in lung tumor cells, is a biomarker for aggressive disease as well as for poor clinical outcome (13). The molecular basis for this correlation stems from the role of p-FADD as a potent mediator of the nonapoptotic transcription factor NF-κB (13, 15), a known regulator of cell fate decisions such as resistance to programmed cell death and lack of proliferation control (16).

Phosphorylation of FADD at Ser194 has been shown to be mediated by casein kinase 1α (CK1α; 4) and FADD-interacting serine-threonine kinase/homeodomain-interacting protein kinase (FIST/HIPK3; refs. 3, 17), but the exact regulation and role of p-FADD in cancer are not well understood. In this study, we used a bioluminescent cell–based assay to characterize NSC 47147 as a potent
inhibitor of FADD phosphorylation and to evaluate its potential as a therapeutic agent in cancer treatment.

Materials and Methods

Antibodies and reagents

NSC 47147 was synthesized as previously described (18). SP600125 was purchased from Calbiochem (EMD Chemicals), CKI-7 from Toronto Research Chemicals, and bortezomib from Sigma Aldrich. The NCI Diversity Chemicals), CKI-7 from Toronto Research Chemicals, and D-luciferin was purchased from Promega. Rabbit polyclonal antibodies to phospho-FADD (Ser194), phospho-c-Jun (Ser63), phospho-β-catenin (Ser45), phospho-IκBα (Ser32/36), β-catenin, IκBα, caspase-3, cleaved caspase-3 (Asp175), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and mouse monoclonal antibody to c-jun were purchased from Cell Signaling Technology. Goat polyclonal antibody to CK1α was purchased from Santa Cruz Biotechnology and mouse monoclonal antibody to FADD from BD Pharmingen.

Cell culture

A549 (lung epithelial carcinoma), Jurkat (T lymphocyte), and SW620 (colo-rectal adenocarcinoma) cells were purchased from the American Type Culture Collection (ATCC). Cell cultures were maintained in a humidified incubator at 37°C and 5% CO₂. A549 and Jurkat cells were grown in RPMI 1640 and SW620 cells in Leibovitz’s L-15 medium. Each cell culture was supplemented with 10% heat-inactivated FBS (Invitrogen) and 100 units/mL penicillin. ATCC cell lines were tested routinely for Mycoplasma and purity. All ATCC lines were expanded immediately upon receipt, and multiple vials of low-passage cells were maintained in liquid N₂. No vial of cells was cultured for more than 1 to 2 months. A549 and Jurkat cells were removed by centrifugation at 13,000 rpm for 15 minutes at 4°C. The supernatants were collected, and protein content was estimated with a detergent-compatible protein assay kit from Bio-Rad. Whole-cell lysates containing equal amounts of protein (10–20 μg) were separated by 12% Bis-Tris polyacrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride membranes. The membranes were probed against specific primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies and visualized with the Enhanced Chemiluminescence Plus Western Blotting System (GE Healthcare).

Bioluminescent FADD-kinase reporter assay

The bioluminescent FADD-kinase reporter (FKR) assay was executed as previously described (18). Briefly, A549 cells-expressing FKR cells were seeded (1 × 10⁵ cells/well) in opaque 96-well plates, 24 hours before assaying. Compound stocks were prepared in dimethyl sulfoxide (DMSO) and diluted 1:100 in PBS. Intermediate stocks (10 μL) were added to the assay plates using the Beckman Biomek NXP Laboratory Automation Workstation (Beckman Coulter). Unless otherwise noted, cells were incubated with test compound at 37°C, 5% CO₂ for 1 hour (CKI-7) and 6 hours (SP600125 and NSC 47147) at the indicated concentrations. Live cell luminescence imaging was read with an EnVision Xcite Multilabel Reader (PerkinElmer) 10 minutes after the addition of β-luciferin (100 μg/mL final concentration) to the assay medium. The percentage of change in FKR activity was calculated as (Acontrol/A samples) × 100.

CK1α inhibition assays

CK1α enzymatic activity was evaluated by the Lance Ultra CK2α/β Kinase Assay (PerkinElmer) according to the manufacturer’s instructions. Recombinant CK1α was purchased from ProQinase. Serial dilutions of NSC 47147 (1–100 μmol/L) and CKI-7 (1–300 μmol/L) were incubated with 25 nmol/L CK1α enzyme, 50 nmol/L ULight-Topo-Fkt (Thr1342) peptide, and 1 μmol/L ATP, and final concentrations of inhibitors were in 2% DMSO. Kinase reactions were terminated after 30 minutes by the addition of EDTA. Experiments were carried out in triplicate, and the data were derived from a minimum of 3 independent experiments. The percentage of inhibition was calculated as % inhibition = (rate with no inhibitor) – (rate with inhibitor)/(rate with no inhibitor) × 100.

A bioluminescent reporter cell line expressing a construct designed to monitor changes in CK1α activity recently described by our laboratory (SW620 BGCR) was used to provide additional evidence that NSC 47147 inhibits FADD phosphorylation through inhibition of CK1α (20). In brief, SW620-BGCR cells were plated in 12-well plates and treated with 250 μmol/L CKI-7 and 3 μmol/L NSC 47147 for 3 hours. Bioluminescence was acquired on an IVIS 200 imaging platform (Caliper Life Science) after adding 100 μg/mL β-luciferin (Xenogen). Region of interest values were calculated for each
exposure and analyzed. Bioluminescence measurements were carried out in triplicate.

**Cell viability assay**

A549 cells were monitored for viability based on ATP levels 24 hours post–NSC 47147 treatment. NSC 47147/bortezomib and cisplatin sensitization experiments were preincubated with cisplatin for 24 hours, followed by the addition of various doses of NSC 47147 or bortezomib for 24 hours. The percentage of viability was measured by the CellTiter-Glo Luminescent Cell Viability Assay (Promega) and measured with the EnVision Xcite Multilabel Reader (PerkinElmer). The percent of viability was calculated as \((A_{\text{sample}}/A_{\text{control}}) \times 100\).

**Phospho-IκB functional assay**

Phospho-IĸB levels were monitored in whole-cell lysates using AlphaScreen SureFire p-IκB (p-Ser32/Ser36) Assay Kit (PerkinElmer), a homogenous bead-based assay designed to measure the phosphorylation of endogenous p-IκB (Ser32/Ser36) in cell lysates, according to the manufacturer's protocol. TNFα-mediated IκB phosphorylation was measured in cells treated with 10 ng/mL TNFα (Invitrogen) for 0, 5, 15, and 30 minutes following 6-hour pretreatment with 6 μmol/L NSC 47147.

**Flow cytometry**

A549 cells were pretreated with cisplatin (10 μmol/L) or control vehicle for 18 hours, followed by treatment with 6 μmol/L NSC 47147 for 6 hours after which cells were trypsinized, counted, and pelleted at 1 × 10⁶ cells per sample. The pellet was resuspended in PBS, fixed by dropwise addition of an equivalent volume of ice-cold 100% ethanol. The samples were placed on ice for 20 minutes and stored at 4°C until day of analysis. On the day of analysis, the cells were pelleted, ethanol decanted, and pellet resuspended in propidium iodide/RNase solution and analyzed at the Flow Cytometry Core Facility at the University of Michigan Cancer Center.

**In vivo studies**

Tumor xenografts expressing FKR were established by implanting 2 × 10⁶ stably transfected A549-FKR cells onto both flanks of nu/nu CD-1 male nude mice (Charles River Laboratories). When tumors reached a volume of approximately 100 to 150 mm³, treatment was initiated. All mouse experiments were approved by the University Committee on the Use and Care of Animals of the University of Michigan.

**In vivo bioluminescence imaging and tumor growth studies**

For bioluminescence imaging, mice bearing A549-FKR xenografts were given a single intraperitoneal injection of 150 mg/kg α-luciferin in PBS. Bioluminescent images were acquired beginning 5 minutes after luciferin injection and at designated times posttreatment. Relative luminescence was calculated as the ratio of bioluminescence at each time point over bioluminescence pretreatment.

For the in vivo tumor growth studies, tumor-bearing mice were randomized into 4 groups as follows: vehicle (DMSO), cisplatin (2 mg/kg), NSC 47147 (3 mg/kg), and a combination of cisplatin and NSC 47147 (2 and 3 mg/kg, respectively). Cisplatin was dissolved in water and NSC 47147 in 20% DMSO/water. Both agents were administered via intraperitoneal injection. NSC 47147 was given daily for 8 days either alone or coadministered in combination with cisplatin. Cisplatin was given on days 1 and 7 in all cisplatin-containing regimens. Tumor volume was calculated according to the equation for a prolate spheroid as follows: tumor volume = \((p/6) \times (L \times W)^2\), where L and W represent the longer and shorter dimensions of the tumor, respectively. Data are expressed as the ratio of tumor volume at various times posttreatment compared with tumor volume on day 1. Mice were monitored daily, and tumor size and animal weight were measured every 2 or 3 days until day 13.

**Immunohistochemistry**

A549-FKR tumor-bearing mice were treated each day for 4 days with vehicle, cisplatin, NSC 47147, or cisplatin with NSC 47147. On the final day, mice were sacrificed 2 hours after injection and tumors were excised and fixed in 10% neutral-buffered formalin for 48 hours (n = 6 tumors for each of the 4 groups). Tumor slices (5 μm) were prepared according to standard procedures and stained with hematoxylin–eosin. Level of apoptosis was detected with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Inc.), which stains for apoptosis by labeling and detecting DNA strand breaks by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) method. Representative images were taken on an Olympus Bx-51 microscope with ×20 magnification.

**Statistical analysis**

All data were expressed as the mean ± SEM from at least 3 independent experiments. GraphPad Prism v5 (GraphPad Software) nonlinear regression analyses and sigmoidal dose–response (variable slope) was used to generate the IC₅₀ values. For tumor growth analysis, ANOVA was done on the proportional change of volume from baseline at each time point. If there was a significant difference, all pairwise comparisons were run adjusting for the multiple comparisons using Tukey’s HSD test. Differences between groups were considered significant at \(P \leq 0.05\).
Results

Identification of a FADD-kinase inhibitor, NSC 47147

Using the A549-FKR assay to screen an NCI compound diversity set, we identified NSC 47147, a tripyrrole alkaloid compound that inhibits FADD phosphorylation (Fig. 1). The compound increases FKR activity in a concentration-dependent manner with an IC₅₀ value of 2.0 μmol/L (Fig. 2A). Comparatively, the c-jun-NH₂-kinase inhibitor (SP600125), known to downregulate FIST/HIPK3 activity, and the CK1α inhibitor (CKI-7) also show a concentration-dependent increase in FKR bioluminescence with IC₅₀ values of 22 and 143 μmol/L, respectively (Fig. 2A). These results are supported by a concomitant decrease in p-FADD expression following treatment with SP600125 and CKI-7 (Fig. 2B). Inhibition of CK1α and JNK activity was validated based on decrease in p-β-catenin and p-c-jun levels, respectively. Changes in expression levels were not due to differences in protein loading as shown by expression of GAPDH. It is important to note that in response to inhibition of FADD phosphorylation, corresponding levels of total FADD also show a decrease. We have previously shown that inhibition of FADD phosphorylation results in its ubiquitin-dependent degradation. Using MG132, a proteasome inhibitor, we confirmed that a decrease in total FADD levels in response to FADD dephosphorylation (i.e., in the presence of a FADD-kinase inhibitor) can be reversed by inhibiting proteasomal degradation (19).

To delineate possible FADD-kinase targets of NSC 47147, we examined whether the compound inhibited phosphorylation of β-catenin, a downstream target of CK1α. Western blot analysis of A549 and Jurkat cells following 6-hour treatment with NSC 47147 shows a decrease in phosphorylated β-catenin (p-β-catenin) protein (Fig. 2C). These data show a trend similar to the inhibition of p-β-catenin expression following treatment with CKI-7 (Fig. 2B).

Three-hour treatment of 250 μmol/L CKI-7 and 3 μmol/L NSC 47147 of our recently described CK1α reporter cell line (SW620-BGCR) resulted in 10-fold induction in bioluminescence activity, indicative of CK1α inhibition (Fig 3A). In this assay, CKI-7 resulted in a 7.5-fold increase in activity. To examine further the effect of NSC 47147 on CK1α inhibition, we directly evaluated CK1α enzymatic activity in response to the compound (Fig. 3B). It is worth mentioning that maximal inhibition by NSC 47147 was not observed because of its limited aqueous solubility. However, the resulting IC₅₀ value for NSC 47147, 6 μmol/L, supports the ability of NSC 47147 to inhibit CK1α. Taken together, these results substantiate our finding that NSC 47147 inhibits phosphorylation of FADD through inhibition of FADD-kinase CK1α.

Effect of NSC 47147 on cell viability

To examine the correlation of NSC 47147 activation of the FKR and corresponding decrease in p-FADD on cell viability, A549 lung carcinoma cells were treated with increasing concentrations of NSC 47147 and ATP levels were measured at 24 hours posttreatment. The increase in FKR activity directly correlates with a decrease in the percentage of cell viability. Results show similar IC₅₀ values (2 μmol/L) for both inhibition of p-FADD, as indicated by increased reporter activity, and corresponding decrease in cell viability (Fig. 4).

NSC 47147 inhibits NF-κB activation in A549 lung carcinoma cells

Our previous studies have shown a close correlation between levels of p-FADD and NF-κB activation (13). On the basis of this evidence, we sought to examine the effect of NSC 47147 on the inhibition of both endogenous and TNFα-induced levels of phosphorylated IκBα (p-IκBα), critical to the transcriptional activity of NF-κB (16). Treatment of A549 cells with 1, 3, and 6 μmol/L NSC 47147 resulted in 31%, 63%, and 76% decrease in p-IκBα activity, respectively, along with a corresponding decrease in protein expression of both p-IκBα and total IκBα (Fig. 5A and B). A549 cells stimulated with TNFα, following pretreatment with NSC 47147, show that the inhibitor also blocks TNFα-induced IκBα phosphorylation (Fig. 5C). Western blot analysis shows that NSC 47147 inhibits TNFα-induced expression of p-IκBα at 5 minutes poststimulation (Fig. 5D) as well as the continued phosphorylation and turnover of newly synthesized IκBα 30 minutes after TNFα stimulation (Fig. 5D; ref. 21).

NSC 47147 as a chemosensitizing agent

We hypothesized that p-FADD–mediated NF-κB inhibition by NSC 47147 would sensitize tumor cells to chemotherapeutic agents and enhance tumor cell death. To test this hypothesis, A549 cells were treated with NSC 47147 or bortezomib, a small-molecule NF-κB inhibitor, in combination with cisplatin, a chemotherapeutic agent known to induce apoptosis. The cells were pretreated with cisplatin (10 μmol/L) for 18 hours followed by 24-hour treatment with NSC 47147 or bortezomib following which, ATP levels were measured. As shown in Fig. 6A,
Figure 2. Identification of NSC 47147 as a FADD-kinase inhibitor. A, A549-FKR cells were treated with NSC 47147 and SP600125 for 6 hours and CKI-7 for 1 hour at the indicated concentrations followed by bioluminescent measurement. NSC 47147 blocks phosphorylation of FADD with greater potency than the FADD-kinase inhibitors SP600125 and CKI-7. B, A549-FKR cells were treated as described for FKR assay and lysates were analyzed with specific antibodies by Western blotting. Treatment with the FADD-kinase inhibitors CKI-7 and SP600125 shows dose-dependent decrease in p-FADD protein that correlates with FKR activity. Total and p-β-catenin and p-c-jun, respective targets of CKI-7 and SP600125, have been shown for comparison. C, A549 and Jurkat cells were treated for 6 hours with NSC 47147. Western blot analysis reveals that NSC 47147 leads to a decrease in p-FADD and p-β-catenin levels.
NSC 47147 treatment in combination with cisplatin leads to an increase in cell death (*, \( P < 0.05 \)) as compared with NSC 47147 treatment alone. As shown in Fig. 6B, treatment of A549 cells with bortezomib and cisplatin also resulted in a marked (*, \( P < 0.05 \)) decrease in cell viability. These data suggest that the enhanced sensitivity to cisplatin in response to NSC 47147 or bortezomib treatment is due to inhibition of NF-\( \kappa \)B.

To further confirm the cooperative effects of cisplatin and NSC 47147, A549 cells pretreated with cisplatin (10 \( \mu \)mol/L) for 18 hours followed by 6-hour treatment with NSC 47147 (6 \( \mu \)mol/L) were subjected to cell-cycle analysis. Figure 6C shows 12.3% of cells in sub-G1 phase in response to NSC 47147/cisplatin combination treatment as compared with vehicle control (1.8%), NSC 47147 (1.6%), and cisplatin (5.2%) alone. Western blot analysis on similarly treated cells resulted in an increase in caspase-3 cleavage with cisplatin, no effect with NSC 47147 alone, and a marked increase in cleaved caspase-3 levels (17- and 19-kDa bands) with combined NSC 47147 and cisplatin treatment (Fig. 6D), thus supporting the capacity of NSC 47147 to augment cisplatin-induced apoptosis.

Effect of NSC 47147 in tumor xenograft models

Having established the efficacy of NSC 47147 toward suppression of FADD phosphorylation and subsequent NF-\( \kappa \)B inhibition in cells, we next sought to investigate the effects of the compound in a mouse tumor model. To establish tumors, we implanted A549-FKR cells into the flanks of nude mice. When tumors reached a volume between 100 and 150 mm\(^3\), we monitored bioluminescence over time in mice treated with NSC 47147 or vehicle control. Bioluminescence imaging shows an increase in FKR activity following treatment with NSC 47147; maximum reporter activity was reached within 6 hours followed by sustained, although lower, activity up to 36 hours (Fig. 7A). Representative images of NSC 47147–treated mice are shown in Fig. 7B.

The chemosensitizing effects of NSC 47147 on tumor progression were evaluated in A549-FKR mouse xenografts treated with NSC 47147 (3 mg/kg) once daily for 8 days with or without cisplatin (2 mg/kg) intraperitoneal injections on days 1 and 7. Significant reduction \( (P < 0.05) \) in tumor volume was observed at day 13 for the NSC 47147/cisplatin combination therapy as compared with control or either chemical agent alone (Fig. 7C). Notably, mice treated with the combination therapy had no increase in tumor volume through the
duration of the study, whereas mice treated with cisplatin or NSC 47147 alone resumed tumor growth at day 13 and day 7, respectively.

On the basis of these results, we evaluated the degree of apoptosis in the tumor xenografts following treatment with NSC 47147 alone or in combination with cisplatin. Immunohistologic staining for apoptosis shows areas of cell death within tumors of cisplatin-treated mice, a small amount of apoptosis in tumors of NSC 47147–treated mice, and a pronounced increase in apoptosis throughout the tumors of mice receiving the combination of cisplatin and NSC 47147 (Fig. 7D).

Discussion

Although FADD was originally identified as a key mediator of apoptosis, it has recently been identified as a modulator of a number of death receptor–dependent and -independent nonapoptotic activities including embryogenesis, cell-cycle progression, cell proliferation, and tumorigenesis (6, 8, 9). Previous research from our laboratory has revealed p-FADD as a biomarker for poor clinical outcome in human lung adenocarcinoma. Our studies have shown a strong correlation between high levels of p-FADD and elevated NF-κB activity, the anti-apoptotic actions of which lead to the formation of aggressive phenotypes, resistance to chemotherapeutic agents, and poor clinical outcome (13, 15, 22). In this study, we provide evidence that abrogation of NF-κB signaling through small-molecule inhibition of FADD phosphorylation is a novel and viable approach for cancer therapy.

Comprehensive studies recently conducted in A549 and Jurkat cell lines have provided insight into the mechanistic basis for FADD and its involvement in NF-κB activation and tumorigenesis (13, 23, 24). In addition, it was in Jurkat cells that CK1ε was identified as the kinase that phosphorylates FADD (4). Research from our laboratory has shown these lines to express high levels of p-FADD protein, thereby providing the impetus toward selecting these models for the identification and evaluation of a potential FADD-kinase inhibitor. Using A549 lung cancer cells expressing FKR (19), we conducted a high-throughput screen with an NCI diversity
compound collection and identified NSC 47147 as a potent inhibitor of FADD phosphorylation. NSC 47147 is a prodigiosin, a family of natural red pigments synthesized by a variety of microorganisms. Prodigiosins have been shown to possess antineoplastic properties, showing an ability to initiate cell-cycle arrest and apoptosis (25, 26).

Treatment of A549-FKR cells with NSC 47147 showed a concentration-dependent increase in reporter activity, with 1 to 2 logs greater potency than the previously described FADD-kinase inhibitors SP600125 and CK1-7 (IC_{50} = 2.22, and 150 μmol/L, respectively). The increase in reporter bioluminescence following treatment with inhibitors of FADD-kinases, FIST/HIPK3, and CK1a suggests that the mechanism of action of NSC 47147 could be, in part, due to its inhibition of either of these kinases. Western blot analysis of A549 and Jurkat cells treated with NSC 47147 shows a dose-dependent depletion of p-FADD with a concomitant decrease in p-β-catenin, also a CK1α substrate (27). No decrease in c-jun

Figure 6. NSC 47147 chemosensitizes A549 lung cancer cells to cisplatin-induced apoptosis. A and B, A549 cells were preincubated with 10 μmol/L cisplatin for 24 hours followed by increasing concentrations of NSC 47147 or bortezomib for 24 hours. ATP levels following treatment were quantified with Promega CellTiter-Glo. Data show that inhibition of NF-κB by NSC 47147 or bortezomib in combination with cisplatin leads to greater cell death than NSC 47147 or bortezomib alone (*, P < 0.05). C, A549 lung cancer cells treated with NSC 47147 in the absence or presence of cisplatin were subjected to cell-cycle analysis. Data show more cells in sub-G1 with NSC 47147/cisplatin combination therapy (12.3%) than with control (1.8%), NSC 47147 (1.6%), and cisplatin alone (5.2%). D, A549 cells treated with NSC 47147, cisplatin, or both agents were evaluated for inhibition of p-FADD levels and induction of apoptosis by assessing the levels of cleaved caspase-3.
activation was apparent, indicating that the compound obtrudes CK1α activity but does not influence FIST/HIPK3 (data not shown). The dose-dependent decrease in p-FADD and p-β-catenin proteins in both cell lines suggests that NSC 47147 may affect p-FADD levels by either direct inhibition of CK1α or by impinging on an upstream signaling event. The results presented here reveal inhibitory activity in a CK1α cell–based assay as well as direct inhibition in a CK1α biochemical assay, thereby providing mechanistic evidence that NSC 47147 is a CK1α inhibitor. CK1α regulates multiple oncogenic pathways, in addition to its proposed involvement in

Figure 7. NSC 47147 inhibits FADD phosphorylation in an A549-FKR xenograft model and sensitizes tumors to an apoptotic stimulus. A, athymic nude mice bearing A549-FKR–expressing xenografts were treated with vehicle control (DMSO) or NSC 47147 (0.5 mg/kg) by intraperitoneal injection. Mice were imaged for bioluminescence at the indicated times. Relative luminescence was calculated as the ratio of bioluminescence at each time point to the basal bioluminescence before treatment. Data points represent mean ± SEM relative luminescence. B, representative bioluminescence images of tumor-bearing mice pretreatment (basal) and 3, 6, and 18 hours posttreatment with NSC 47147 (0.5 mg/kg). C, tumor-bearing mice were treated with 3 mg/kg NSC 47147 once daily for 8 days and/or 2 mg/kg cisplatin on days 1 and 7 by intraperitoneal injection, and tumor volume was calculated by day 13. Data are plotted as mean ± SEM relative tumor volume. D, immunohistochemical staining of A549-FKR xenografts after once daily treatment for 4 days with 3 mg/kg NSC 47147 and/or 2 mg/kg cisplatin. Tumors were harvested and fixed on day 4; sections were stained for apoptosis as described in Materials and Methods. Figure shows duplicate images of representative fields.
p-FADD–mediated NF-κB regulation, namely, the β-catenin/Wnt signaling axis (27, 28), making it a rational target for drug therapy.

A decrease in p-FADD levels, as detected by an increase in FKR expression, directly correlated with a reduction in cell viability, suggesting that the inhibition of FADD phosphorylation and increase in cytotoxicity are a consequence of the same biological event. It has been proposed that the molecular basis for the correlation between p-FADD levels and cell death stems from its role as a potent activator of NF-κB, an antiapoptotic transcription factor (13). Previous research has shown that overexpression of FADD stimulates NF-κB promoter activity (15, 29) and that FADD-deficient cells are more susceptible to viral infection due to defects in NF-κB activation (30). Furthermore, FADD plays an integral role in the recently described TRADDosome complex, a central mediator of NF-κB signaling (31). Although it was previously unappreciated, our published work has shown that increased levels of p-FADD result in elevated levels of NF-κB activation (13). The data presented in this article provide further evidence that the phosphorylated form of FADD rather than FADD is a key component of the NF-κB activating complex.

The role of NF-κB in tumorigenesis has been well established and is based on its action as a regulator of cell fate decisions. It is often dysregulated in cancer cells leading to uncontrolled cell proliferation and resistance to therapeutic intervention (16). These data show that A549 lung carcinoma cells treated with NSC 47147 yield a decrease in NF-κB activation, as measured by the phosphorylation status of IκBα. Phosphorylation of IκBα by IKK requires recruitment of the TRADDosome complex wherein IKK plays an essential role (31). These results confirm previous reports that TNFα–induced NF-κB activation depends on levels of FADD expression (15, 32, 33) and provide further evidence of NSC 47147 as an inhibitor of FADD phosphorylation.

Tumor cells with constitutively active NF-κB are known to be resistant to chemotherapeutic agents, a resistance that can be alleviated by inhibition of NF-κB signaling (16). We hypothesized that pretreatment with NSC 47147 would chemosensitize A549 lung cancer cells toward an apoptotic stimulus leading to enhanced cell death. Results from our cell-cycle analysis show a higher percentage of cells in the sub-G1 population with a parallel increase in caspase-3 activity, thereby providing evidence that NSC 47147 sensitizes cells to cisplatin leading to decreased cell viability. We believe the mechanistic basis for the proposed synergy is that NSC 47147, by inhibiting FADD phosphorylation, abates TRADDosome-mediated NF-κB activation. In the absence of NF-κB signaling, tumor cells become sensitive to apoptotic stimuli (i.e., in response to cisplatin treatment). In this regard, our data show that bortezomib-induced NF-κB inhibition in combination with cisplatin also results in a decrease in cell viability. The ability of NSC 47147 to exhibit a similar outcome when used in combination with cisplatin provides additional mechanistic evidence that the cytotoxic effects of NSC 47147 are at least in part due to its inhibition of NF-κB.

Studies from our laboratory, as well as others, have also shown a role of p-FADD in G2–M progression (24, 34). Therefore, we expected NSC 47147 to arrest cells at the G2–M phase of the cell cycle consistent with its proposed mechanism as an inhibitor of p-FADD levels. As expected, the fraction of cells in G2–M phase following NSC 47147 treatment was 19.1% compared with 11.4% for control cells, thereby providing additional support that NSC 47147 inhibits FADD phosphorylation leading to cell-cycle arrest and cell death.

Having shown the in vitro effect of NSC 47147 alone and in combination with cisplatin, we next examined the compounds efficacy as a chemotherapeutic agent in a mouse tumor model. Mouse tumor xenografts expressing the FKR showed an increase in bioluminescence following treatment with NSC 47147, showing the ability of the compound to inhibit activation of FADD in an animal model. The combined NSC 47147 and cisplatin therapy resulted in a decrease in tumor volume greater than the effect of either compound alone. Consistent with the reduction in tumor volume, TUNEL staining revealed enhanced apoptotic activity in mouse tumors treated with combination drug therapy compared with either agent alone. Treatment of mice with 3 mg/kg NSC 47147 did not result in overt toxicity in our treatment groups as well as by other research groups (35, 36). This, combined with the capacity of NSC 47147 to control tumor burden when used in combination with a known chemotherapy agent, provides validation for the investigation of more efficacious and biologically available inhibitors of FADD phosphorylation. The identification of compounds that, when coadministered with chemotherapeutic drugs, increase their efficacy is of significant clinical importance.

In summary, our previous research has identified p-FADD as a prognostic biomarker for poor clinical outcome. The mechanistic basis for this biomarker is the ability of p-FADD to promote the antiapoptotic actions of NF-κB. The results presented here identify a small molecule inhibitor of FADD phosphorylation that induces cell death through abrogation of NF-κB activity. We have provided proof-of-principle studies showing that inhibition of FADD phosphorylation through CK1α may be a viable target for anticancer therapy as a single agent, but more interestingly, in combination with clinically approved chemotherapeutic agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Katrina A. Schinske, Shyam Nyati, Amjad P. Khan, et al.


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