17-Allylamino-17-Demethoxygeldanamycin Synergistically Potentiates Tumor Necrosis Factor–Induced Lung Cancer Cell Death by Blocking the Nuclear Factor-κB Pathway

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Abstract

Nuclear factor-κB (NF-κB), a survival signal induced by tumor necrosis factor (TNF), contributes substantially to the resistance to TNF-induced cell death. Previous studies suggest that heat shock protein 90 (Hsp90) regulates the stability and function of receptor-interaction proteins (RIP) and IκB kinase β (IKKβ), the key components of the TNF-induced NF-κB activation pathway. In this study, we showed that the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17AAG) was synergistic with TNF to induce apoptotic cell death in a panel of lung tumor-derived cell lines. Treatment with 17AAG caused degradation of RIP and IKKβ that, in turn, blocked TNF-induced NF-κB activation and antiapoptotic gene expression. The synergistic cytotoxicity was detected only when TNF treatment followed 17AAG preexposure. Importantly, the potentiation of cell death was abolished in NF-κB-disabled cells that express a nondegradable IκBα mutant (IκBαAA). These results suggest that the cytotoxicity seen with 17AAG and TNF treatment results from blocking TNF-induced NF-κB activation. The other components of the TNF receptor I signaling cascade were not altered, whereas TNF-induced c-Jun NH2-terminal kinase activation and apoptosis were potentiated. A similar synergism for inducing apoptosis was also observed in 17AAG-treated and TNF-related apoptosis-inducing ligand (TRAIL)-treated cancer cells. Our results suggest that NF-κB plays a key role in the resistance of lung cancer cells to TNF and TRAIL and that disabling this survival signal with 17AAG followed by TNF or TRAIL treatment could be an effective new therapeutic strategy for lung cancer. (Cancer Res 2006; 66(2): 1089-95)

Introduction

Tumor necrosis factor (TNF) plays an important role in a variety of cellular processes that include cell survival, proliferation, differentiation, and apoptosis (1). The ability of TNF to induce apoptosis in cancer cells makes it a potential therapeutic agent. However, most cancer cells are resistant to TNF-induced death (1, 2). Although the mechanism has not been well elucidated, it is believed that the survival signals induced by TNF may blunt the apoptotic pathway, which results in the resistance of cells to TNF-induced apoptosis (3). Therefore, interventions that inhibit TNF-induced survival signals may sensitize cancer cells to TNF-induced apoptosis.

Of the two TNF receptors, TNF receptor I (TNFRI) is the main receptor in most cell types. Following the binding of TNF to TNFRI, the TNF receptor–associated death domain protein (TRADD) is recruited and acts as a platform to recruit the receptor-interaction protein (RIP), TNF receptor–associated factor 2 (TRAF2), and Fas-associated death domain protein (FADD). This action leads to the activation of several pathways that mediate diverse biological responses (3). For example, the activation of the transcription factor nuclear factor-κB (NF-κB) through RIP and TRAF2 is critical for cell survival and proliferation (3–5). The role of c-Jun NH2-terminal kinase (JNK) activation through TRAF2 in cell death regulation is controversial (6–8), but recent studies using genetic disruption of JNK genes showed that transient JNK activation contributes to cell survival (9), whereas sustained JNK activation is proapoptotic (6, 8). The caspase cascade can be activated through FADD (10), independent of RIP and TRAF2, resulting in apoptotic cell death (11, 12). Therefore, the balance of TNF-induced survival and death signaling is pivotal in determining the fate of TNF-exposed cells.

When cells are exposed to TNF, NF-κB is rapidly activated through RIP-mediated activation of IκB kinase (IKK). IKK is a ternary protein complex consisting of IKK-α, IKK-β, and IKK-γ. During TNFRI signaling, IKK is recruited to the TNFRI signaling complex through TRAF2 and activated through a RIP-mediated mechanism that involves MEKK3 (13, 14). The activated IKKβ subunit of IKK then phosphorylates the NF-κB-bound IκBs, which retain NF-κB in the cytoplasm, at their regulatory region to trigger their rapid polyubiquitination followed by degradation in the 26S proteasome. This process releases NF-κB to allow its translocation into the nucleus and activation of its target genes (15). Several of the target genes of NF-κB, including A20, cIAP-1, cIAP-2, Bcl-xL, XIAP, IEX-1L, and manganese superoxide dismutase (MnSOD), have antiapoptotic properties (16–18).

Heat shock proteins are a group of chaperones that are important in maintaining stability and function of their client proteins. Heat shock proteins also function in renaturing or targeting unfolded proteins for degradation when cells are subjected to heat shock or other stresses (19). As one of the most abundant heat shock proteins in the cell, heat shock protein 90 (Hsp90) is distinct from other heat shock proteins because it does not participate in general protein folding. Instead, through regulating stability and function of several signal transduction proteins, Hsp90 plays an important role in biological processes that include hormone signaling, cell cycle control, and development (20). Hsp90 is a specific target of the antitumor drug geldanamycin and its derivatives, such as 17-allylamino-17-demethoxygeldanamycin (17AAG) and 17-dimethylamino-ethylamino-geldanamycin (21). Geldanamycin and its analogues induce disruption of the interaction between Hsp90 and its client proteins, such as the...
tyrosine kinase v-Src, the serine/threonine kinase Raf, mutant p53 proteins, the glucocorticoid receptor, and the androgen receptor HE2, resulting in protein destabilization and degradation, usually mediated by the proteasome (20). Compared with geldanamycin, 17AAG may be more efficacious in cancer therapy because it is well tolerated in most cancer patients as shown in several phase I clinical trials (22).

RIP and IKKβ, two essential components of the TNF-induced NF-κB activation pathway, have been identified as Hsp90 client proteins. Inhibition of Hsp90 activity in the human cervical cancer cell line HeLa and Hodgkin's lymphoma cells caused degradation of RIP and IKKβ, which in turn blocked TNF-induced NF-κB activation (23, 24). In the lung cancer cell line A549, IKKβ degradation and NF-κB inhibition induced by Hsp90 suppression was also observed (25). The purpose of this study was to determine whether an inhibitor of Hsp90 can sensitize the lung tumor cells to TNF-induced apoptosis by blocking NF-κB activation. Our results suggest that NF-κB plays a key role in the resistance of lung cancer cells to TNF and TNF-related apoptosis-inducing ligand (TRAIL), and the combination of 17AAG and TNF or TRAIL could be an effective therapeutic strategy that improves the anticancer potential of TNF and TRAIL.

Materials and Methods

**Plasmids and reagents.** Reporter plasmids 2×B-Luc, AP1-Luc, and pRSV-LacZ have been described previously (26, 27). HA-IκBαAA was a gift from Dr. Zheng-gang Liu (National Cancer Institute). 17AAG and human TNF were purchased from Sigma (St. Louis, MO) and R&D Systems (Minneapolis, MN), respectively. Glutathione S-transferase (GST) TRAIL was prepared as described previously (28, 29). Antibodies against cIAP-1, cIAP-2, TRADD, TRAF2, IκBα, JNK, and caspase-8 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-A20, anti-MnSOD, anti–Bcl-xL, anti–Bcl-2, and anti-RIP were from BD Biosciences (San Diego, CA). Anti-IκBα, anti-phospho-JNK, anti-β-actin, and anti–poly(ADP-ribose) polymerase (PARP) were purchased from Upstate (Chicago, IL), Biosource (Camarillo, CA), Sigma, and Biomol (Plymouth Meeting, PA), respectively. Antibodies for Bcl-xL, XIAP, and phospho-IκBα were from Cell Signaling (Beverly, MA).

**Cell culture.** H460, H23, H2009, H358, and H1568 cells were obtained from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 supplemented with 10% fetal bovine serum, 1 mmol/L glutamate, 100 units/mL penicillin, and 100 μg/mL streptomycin. HCCBE-2 human bronchial epithelial cells immortalized by insertion of cyclin-dependent kinase 4 and p53 were obtained from Dr. Jerry Shay and John Minna (University of Texas Southwestern Medical Center) and cultured in keratinocyte-serum-free medium on collagen-coated plates.

**Transfection.** Transfections were done with LipofectAMINE PLUS reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Stable integration of IκBαAA was obtained by cotransfection of H460 cells with HA-IκBαAA and pcDNA3.HisB and selected with RPMI 1640 with 200 μg/mL G418. Clones stably expressing IκBαAA were identified by Western blot with an anti-IκBα antibody and maintained in RPMI 1640 with 200 μg/mL G418.

**Cytotoxicity assay.** A cytotoxicity assay based on the release of lactate dehydrogenase (LDH) was conducted using cytotoxicity detection kit (Roche, Penzberg, Germany). Cells were seeded in 24-well plates at 70% to 80% confluence. After overnight culture, cells were treated as indicated in each figure legend. Culture medium from each well was collected and transferred into 96-well flat-bottomed plates. LDH activity was determined by adding equal volumes of reaction mixture to each well and incubating for up to 30 minutes. The absorbance of the samples was measured at 490 nm using a plate reader. All the experiments were repeated three to five times and the average is shown in each figure. Cell death was calculated using the formula:

\[
\text{Cytotoxicity (\%) = \left( \frac{E_{\text{experimental}} - E_{\text{spontaneous}}}{E_{\text{maximum}} - E_{\text{spontaneous}}} \right) \times 100}
\]

**Results**

**Synergistic cytotoxicity of 17AAG and TNF treatment in human lung cancer cell lines.** The effect of 17AAG treatment on TNF-induced cell death in human lung cancer cells was investigated by initially treating H460 cells with 17AAG (50 nmol/L) for 10 hours followed by exposing the cells to various concentrations of human TNF for an additional 24 hours. Cell death was observed microscopically and detected quantitatively by release of LDH. H460 cells were resistant to toxicity at the highest dose of TNF (40 ng/mL) evaluated (Fig. 1A). Treatment with 17AAG (50 nmol/L) caused a moderate amount of cytotoxicity (~15% cell death). In contrast, when cells were treated with both 17AAG and TNF, a synergistic cytotoxicity that killed ~50% of the cells was detected. The synergistic response was detected with concentrations of TNF as low as 1 ng/mL. At this concentration, TNF alone caused no detectable cell death (Fig. 1A). A dose-dependent synergistic effect was detected with increasing TNF concentration. A similar synergism was also found with a fixed TNF dose (10 ng/mL) and increasing concentrations of 17AAG (Fig. 1B). To determine the commonality for inducing the cytotoxicity, additional lung cancer cell lines were tested under similar experimental conditions. Among these cells, H23 is resistant to TNF, whereas H2009, H358, and H1568 are partially sensitive to TNF (Fig. 1C). When TNF treatment followed preexposure to 17AAG, a synergistic cytotoxicity was detected in all cell lines (Fig. 1C). These results suggested that the cotreatment of 17AAG and TNF could enhance the cytotoxicity in lung cancer cells. Interestingly, among the tested cell lines, H460 and H1568 contain a wild-type p53, whereas p53 is mutated in H23 and H2009 (31). The above results suggested that the synergistic cytotoxicity of 17AAG and TNF does not depend on the wild-type status of p53. We further addressed whether the combined treatment of 17AAG and TNF would be toxic to nontransformed lung epithelial cells. HCCBE-2 and HCCBE-3, immortalized human bronchial epithelial cell lines, were treated under the same condition as shown in Fig. 1C. No detectable cytotoxicity was observed in these immortalized bronchial epithelial cells (Fig. 1D; data not shown), suggesting that the combination of 17AAG and TNF may selectively kill malignant cells.

**Luciferase assay.** Cells cultured in six-well plates were transfected with 0.8 μg p2x-B-Luc or pAP1-Luc and 0.2 μg pRSV-LacZ. Twenty-four hours after transfection, cells were treated with 17AAG (100 nmol/L) for 10 hours. Cells then were treated with 20 ng/mL TNF for an additional 12 hours and collected for luciferase assay as described (29). Luciferase activity in these cells was measured using a luciferase assay kit (Promega, Madison, WI) and normalized to the β-galactosidase activity of each sample. All the experiments were repeated thrice and the average is shown in each figure.

**Immunoblotting analysis.** Cells were collected and lysed in M2 buffer [20 mmol/L Tris-HCl (pH 7.6), 0.5% NP40, 250 mmol/L NaCl, 3 mmol/L EDTA, 3 mmol/L EGTA, 2 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L β-glycerophosphate, 1 mmol/L sodium vanadate, and 1 μg/mL leupeptin]. Cell extracts (~ 50 μg) were resolved by 15% SDS-PAGE and analyzed by Western blot. The proteins were seen by enhanced chemiluminescence following the manufacturer’s instruction (Amersham, Piscataway, NJ). Each experiment was repeated at least thrice and representative results are shown in each figure.

**Statistical analysis.** Data are expressed as mean ± SD. Statistical significance was examined by two-way ANOVA pairwise comparison. In all analyses, P < 0.05 was considered statistically significant.
Role of RIP in the cytotoxic response of cells to 17AAG and TNF. It was reported previously that inhibition of Hsp90 with geldanamycin caused degradation of RIP and suppressed TNF-induced NF-κB activation in HeLa cells (23). Therefore, we addressed whether this mechanism is also responsible for the cytotoxicity induced in lung cancer cells by cotreatment with 17AAG. Expression of the adaptors of the TNFRI signaling complex was examined in H460 cells after treatment with 17AAG for 10 hours. As expected, the expression level of RIP declined dramatically after treatment presumably through degradation of RIP (23). The expression of other factors, TRADD, TRAF2, and FADD, remained unchanged (Fig. 2A).

Dose-response studies revealed that 50 nmol/L 17AAG caused a significant decrease of RIP, whereas 10 nmol/L 17AAG caused partial RIP degradation. The decline in RIP levels began at 4 hours and reached 70% of maximal response at 10 hours after 17AAG treatment (Fig. 2B). Because RIP plays an indispensable role in TNF-induced NF-κB activation, we propose that RIP degradation induced by 17AAG contributes substantially to the synergistic cytotoxicity of 17AAG and TNF cotreatment. To test this hypothesis, H460 cells were either pretreated with 17AAG followed by TNF treatment or simultaneously exposed to 17AAG and TNF. RIP degradation induced by 17AAG occurred over several hours (Fig. 2B), whereas the effect of RIP on NF-κB activation mediated by TNFRI occurred within minutes (13). Therefore, if the synergistic cytotoxicity requires the disruption of RIP function, this response would be lost with simultaneous treatment with 17AAG and TNF. Our results strongly support this hypothesis, because the synergistic cytotoxicity was only detected when pretreatment with 17AAG was conducted (Fig. 2C), and suggest that blocking the RIP-mediated NF-κB pathway by 17AAG may be crucial for this response (see below).

17AAG pretreatment blocks TNF-induced activation of the NF-κB pathway. The effect of 17AAG on the TNF-induced NF-κB activation pathway was examined in H460 cells treated with 17AAG for 10 hours followed by treatment with TNF for different times. Western blot was used to examine components of the TNF-induced NF-κB activation pathway. The 17AAG treatment markedly decreased the expression of IKKβ and RIP in H460 cells (Fig. 3A), and the phosphorylation and degradation of IκBα, a hallmark of TNF-induced NF-κB activation, was subsequently blocked (Fig. 3A and B). Similar observations also were made in H23 lung cancer cells (data not shown). The effect of 17AAG on the NF-κB pathway...
MnSOD, Bcl-xL, cIAP-1, and cIAP-2 were detected by Western blot. H460 cells were treated with 17AAG (100 nmol/L) for 10 hours. Then, the cells were pretreated with 17AAG for 10 hours followed by TNF treatment for indicated times. RIP, IKKβ, and IkBα were detected by Western blot. 

It is likely that their repression by 17AAG contributes to the sensitization of TNF-induced apoptosis.

Synergistic cytotoxicity of 17AAG and TNF requires blockage of the TNF-induced NF-κB activation pathway. We hypothesized that if the blockage of the TNF-induced NF-κB activation pathway by 17AAG is the main mechanism for cytotoxicity induced by 17AAG and TNF, this effect should be abolished by disabling the NF-κB activation pathway. This hypothesis was tested by establishing cell lines that stably express a mutant IκBα (the two serine residues at the IKK phosphorylation site are substituted with alanine, IκBαAA), designated IκBαAA-L3 and IκBαAA-L8. As expected, the mutant IκBα was resistant to TNF-induced degradation (Fig. 4A, top band), whereas the degradation of endogenous IκBα was observed after 20 minutes of TNF treatment (bottom band). The recovery of IκBα, which occurred following NF-κB activation, was abolished in the IκBαAA stable expression clones (Fig. 4A, compare H460 and H460A-2 clones treated with TNF for 60 and 120 minutes). These results suggest that the TNF-induced NF-κB activation pathway was blocked in these two IκBαAA stable expression clones, which was confirmed by using a luciferase assay with a NF-κB-responsive reporter. TNF treatment caused a robust increase in luciferase activity, which was abrogated when cells were pretreated with 17AAG (Fig. 3C). These results suggested that, through the down-regulation of RIP and IKKβ, 17AAG treatment blocks the activation of NF-κB induced by TNF.

Several genes downstream of NF-κB, including cIAP-1, cIAP-2, XIAP, Bcl-xL, A20, and MnSOD, encode antiapoptotic factors (17, 18). We examined whether 17AAG treatment affected the expression of these genes and found that TNF markedly induced the expression of XIAP, A20, and MnSOD in H460 cells. However, this effect was completely blocked by pretreatment with 17AAG (Fig. 3D). TNF in H460 cells did not increase the expression of cIAP-1, cIAP-2, and Bcl-xL, suggesting that these factors may not be involved in TNF-induced apoptosis in H460 cells. Because XIAP, A20, and MnSOD are important antiapoptotic molecules, it is likely that their repression by 17AAG contributes to the sensitization of TNF-induced apoptosis.

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is further supported by the luciferase reporter assay (Fig. 4B). The effect of 17AAG and TNF was compared between these two cell clones and the wild-type H460 cells. The two IκBαAA stable clones were more sensitive to TNF treatment. However, the synergistic cytotoxicity seen with 17AAG and TNF was largely abrogated (Fig. 4C), although the 17AAG-induced degradation of RIP and IKKβ was not affected by IκBαAA expression (Fig. 4D). These results suggest that blocking the TNF-induced NF-κB activation pathway by 17AAG is obligatory for the synergistic cytotoxicity of 17AAG and TNF.

17AAG treatment potentiates the JNK activation pathway. Having established that the blockage of NF-κB activation by 17AAG contributes to the synergistic cytotoxicity of 17AAG and TNF, we next investigated the effect of 17AAG on the TNF-induced JNK activation pathway. JNK activation was detected by Western blot with an antibody that recognizes the phosphorylated form of JNK. In H460 cells, exposure to TNF or 17AAG resulted in only minimal activation of JNK. In contrast to NF-κB, TNF-induced JNK activation was significantly enhanced and prolonged in 17AAG-treated cells (Fig. 5A). Consistent with this effect, a luciferase assay with an AP1-responsive reporter revealed that 17AAG treatment potentiates TNF-induced JNK activation pathway (Fig. 5B). The results also suggest that blocking the TNF-induced NF-κB activation pathway by 17AAG is obligatory for the synergistic cytotoxicity of 17AAG and TNF.

17AAG treatment potentiates the TNF-induced apoptosis pathway. Cell death induced by 17AAG and TNF cotreatment is mainly apoptotic. Typical apoptotic features, such as cell shrinkage, cell membrane blebbing, and nuclear condensation, were detected microscopically in most acridine orange/ethidium bromide (AO/EB)-stained dead cells (data not shown). Flow cytometric analysis showed induction of sub-G1 fraction in 17AAG/TNF-treated samples (data not shown). Additionally, the cleavage of the precursor (55 kDa) and generation of the active form of caspase-8 (20 kDa) was increased in 17AAG/TNF-treated cells (Fig. 6A). Because caspase-8 is the initiator caspase that is recruited to the TNFR1 complex to initiate the apoptosis pathway, this result suggested that the synergistic cytotoxicity likely occurs through the TNF-induced apoptosis pathway. The cleavage of the caspase-3 substrate PARP (115 kDa) and generation of the 89-kDa fragment, a hallmark of apoptosis (32), was also significantly enhanced in 17AAG/TNF-treated cells (Fig. 6B). Collectively, these results suggest that the TNF-induced apoptosis pathway is stimulated in 17AAG-pretreated cells.

Synergistic cytotoxicity of 17AAG and TRAIL in lung cancer cells. Because of its selective toxicity to tumor cells, TRAIL is a promising anticancer agent in the TNF family (33). Previous studies have established that RIP is essential for TRAIL-induced NF-κB activation, and NF-κB negatively regulates TRAIL-induced apoptosis (29, 34). Therefore, we extended our study to examine the effect of 17AAG on the TRAIL-induced NF-κB activation pathway and cell death in lung cancer cells. Treating H460 cells with TRAIL caused a partial degradation of IκBα, which was abrogated when RIP was degraded in response to 17AAG pretreatment. This suggests that 17AAG blocks TRAIL-induced NF-κB activation (Fig. 7A). In addition, a panel of lung cancer cell lines, including H460, H23, H209B, H358, and H1568, were used to test the cytotoxicity of the 17AAG and TRAIL cotreatment. Synergistic cytotoxicity was achieved when cells were pretreated with 17AAG and followed by TRAIL exposure (Fig. 7B). The cell death induced by the 17AAG and TRAIL cotreatment is mainly apoptotic as detected microscopically in most AO/EB-stained dead cells (data not shown). These results suggest that 17AAG potentiates TRAIL-induced apoptosis by blocking the NF-κB pathway.

Discussion

This study shows that modulation of Hsp90 activity can be used to sensitize tumor cells to undergo apoptosis by blocking
These results suggest that NF-κB function (Fig. 7) potentiates cell death and sensitizes cancer cells to TNF and TRAIL and that 17AAG-induced degradation of RIP and IKK, suppresses TNF-induced apoptosis. Suppression of the NF-κB pathway by 17AAG through targeting RIP and IKK sensitizes cancer cells to TNF-induced apoptosis.

The death domain kinase RIP is a key component of the TNF-induced NF-κB activation pathway. Eliminating RIP by gene targeting significantly sensitizes cells to TNF-induced apoptosis, suggesting a central role for RIP in maintaining cell survival and resistance to TNF (11, 12). During the initial phase of TNF-induced apoptosis, RIP is cleaved to disable the NF-κB pathway and potentiate apoptosis (26, 45). RIP cleavage was observed also in TRAIL-mediated cell death that was enhanced by doxorubicin, cisplatin, etoposide, or paclitaxel in neuroblastoma cells (46), supporting the premise that RIP has a critical role in the regulation of tumor cell survival and resistance to TRAIL. In this study, 17AAG treatment significantly decreased RIP expression in lung cancer cells followed by a dramatic blockage of NF-κB activation and potentiation of apoptotic cell death. Our results support previous studies that suggest RIP plays a critical role in TNF-induced survival signaling and RIP may be a good target for abrogating the resistance of cancer cells to TNF- or TRAIL-induced apoptosis (26, 45).

Most tumor cells are resistant to TNF- or TRAIL-induced apoptosis; however, the underlying mechanism has not been well elucidated. Although the TRAIL decoy receptors, DcR1 and DcR2, can block TRAIL signaling to apoptosis, their expression does not correlate well with TRAIL resistance in cancer cells (47). However, a role of DcR2 in TRAIL resistance was reported recently in MCF7 breast cancer cells (48). Although reduction of TRAIL receptors and downstream factors, such as ASK and caspase-8, by promoter hypermethylation has been observed, most cancer cells still retain all or some of the TRAIL-induced pathways (44). The basal and induced NF-κB activity has been regarded widely as a major determinant of TNF or TRAIL resistance in cancer cells (44, 49). Our current study provides further support for a combined strategy that includes 17AAG and the application of TNF family cytokines in tumor therapy. Because TRAIL selectively kills tumor cells (44), the combination of 17AAG and TRAIL at doses that cause minimal systemic toxicity could be highly effective cancer therapies. Because the synergistic cytotoxicity of these agents is highly dependent on blocking the NF-κB pathway by preexposure to 17AAG, a dosing strategy that allows enough time to eliminate RIP and IKKβ by 17AAG before TNF or TRAIL treatment is essential in achieving optimal anticancer efficacy.

This strategy has improved the therapeutic value of both TNF or TRAIL and the combined agents by allowing the use of lower, subtoxic doses to achieve effective cancer cell killing. A similar strategy was also explored in evaluating 17AAG as an anticancer agent. Both synergistic and additive effects were observed when 17AAG was combined with other agents, such as cisplatin, oxaliplatin, and a histone deacetylase inhibitor, against colon cancer and acute myeloid leukemia cell lines (41–43). Our current study provides further support for a combination therapy that includes 17AAG and the application of TNF family cytokines in tumor therapy. Because TRAIL selectively kills tumor cells (44), the combination of 17AAG and TRAIL at doses that cause minimal systemic toxicity could be highly effective cancer therapies. Because the synergistic cytotoxicity of these agents is highly dependent on blocking the NF-κB pathway by preexposure to 17AAG, a dosing strategy that allows enough time to eliminate RIP and IKKβ by 17AAG before TNF or TRAIL treatment is essential in achieving optimal anticancer efficacy.

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survival pathway. Additionally, suppression of Hsp90 by 17AAG inhibits multiple survival factors, such as AKT (20) and survivin (50). These factors also may contribute to the synergistic cytotoxicity of 17AAG and TNF or TRAIL by lowering the apoptotic threshold (51).

The synergistic cytotoxicity of 17AAG and TNF or TRAIL was detected in lung cancer cell lines with both wild-type (H460) and mutated p53 (H23 and H2009). Notably, one advantage of the TNF family of cytokines in cancer therapy is that they induce cell death independent of p53 status in the target cells. This advantage is highly relevant because p53 is mutated in many types of tumors and conventional anticancer drugs function through DNA damage–induced apoptosis that is often mediated by wild-type p53 (52). The combination of 17AAG and TNF or TRAIL could be particularly efficacious in therapy for p53–inactivated tumors and, overall, provides a new treatment paradigm against this deadly malignancy.

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References


20. Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV. Inhibiting multiple survival factors, such as AKT and survivin, by Akt family kinase RIP to the TNF receptor-1 signaling complex. Immunity 1996;4:387–96.


26. Krammer PH. Induction of apoptosis by monoclonal antibody 17-AAG Potentiates TNF-Induced Cancer Cell Death


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