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17-Allylamino-17-demethoxygeldanamycin overcomes TRAIL resistance in colon cancer cell lines

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Abstract

Tumor necrosis factor related apoptosis-inducing ligand (TRAIL) is a promising candidate for treatment of cancer, but displays variable cytotoxicity in cell lines. The mechanisms of sensitivity and resistance have not been fully elucidated; both AKT and NF-κB pathways may modulate cytotoxic responses. We have shown that the Hsp90 inhibitor 17-AAG enhances the cytotoxicity of oxaliplatin in colon cancer cell lines through inhibition of NF-κB. We analyzed the effects of TRAIL and 17-AAG in combination in a series of nine colon cancer cell lines and characterized activation of the pathways to apoptosis. IC_{50} values for a 72 h exposure to TRAIL ranged from 30 to 4000 ng/ml. Cytotoxicity assays demonstrated additivity or synergism of the TRAIL/17-AAG combination in all cell lines, with combination indices at IC_{50} ranging from 0.53 to 1. The sensitizing effect of 17-AAG was greater in the TRAIL-resistant cell lines. In TRAIL-resistant cell lines, the combination of 17-AAG and TRAIL resulted in activation of both extrinsic and intrinsic apoptotic pathways, though with quantitative differences between HT29 and RKO cells: differential effects of 17-AAG on AKT and NF-κB characterized these cell lines. In both cell lines, the combination also led to down-regulation of X-linked inhibitor of apoptosis protein (XIAP) and enhanced activation of caspase-3. We conclude that either AKT or NF-κB may promote resistance to TRAIL in colon cancer cells, and that the ability of 17-AAG to target multiple putative determinants of TRAIL sensitivity warrants their further investigation in combination.

Keywords: TRAIL; 17-AAG; AKT; NF-κB; Apoptosis; Colon cancer

1. Introduction

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, also known as Apo2L) is a potent inducer of cell death, active mostly against cancer but not normal cells [1]. Apoptosis induced by TRAIL is initiated by its binding to death receptors (TRAIL-R1 or DR4 and TRAIL-R2 or DR5) followed by formation of the death-inducing signaling complex (DISC) upon recruitment of specific cytoplasmic proteins—Fas-associated death domain (FADD) and caspase-8 or -10. Caspases are then activated by proteolysis and in turn activate the effector caspase-3 either by direct processing (Type I cells) cells, or through cleavage of Bid by caspase-8, and engagement of mitochondrial apoptotic pathway, which involves release of cytochrome c, formation of the apoptosome and activation of caspase-9 (Type II cells) [2]. Specificity for tumor over normal cells makes TRAIL a promising candidate for cancer treatment alone or in combination, since the synergism of TRAIL with established chemotherapeutic drugs and radiation has been shown [3,4]. Numerous cell lines of different origin, however, demonstrate resistance to TRAIL: determinants of TRAIL sensitivity are reported to include the expression levels of DR4, FLICE inhibitor protein (FLIP) and Bcl-X_L [5,6], but such relationships are not observed in all cell lines [7]. TRAIL sensitivity in tumor cell lines can be modulated by p53, protein kinase C, MAP kinase, c-Myc and AKT [1,8,9]. The importance of PI3K/AKT activation in decreasing the TRAIL cytotoxicity has been shown [10,11]. Although the activation of

Abbreviations: hsp90, 90 kDa heat-shock protein; 17-AAG, 17-allylamino-17-demethoxygeldanamycin; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DR4 and DR5, death receptors 4 and 5, respectively; CI, combination index; DISC, death-inducing signaling complex; FLIP, FLICE inhibitory protein; c-IAP1/2, cellular inhibitors of apoptosis 1/2; XIAP, X-linked IAP; AIF, apoptosis-inducing factor; AP-1, activator-protein 1; NF-κB, nuclear factor kappa B; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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NF-κB by TRAIL is much less pronounced than that induced by TNFα, in many cancer cell lines this transcription factor is constitutively up-regulated, and this phenomenon could play a significant role in attenuating the effects of TRAIL through up-regulation of such anti-apoptotic NF-κB targets as c-IAP1 and c-IAP2, XIAP and Bcl-X_L [1,2]. Indeed, inhibition of NF-κB signaling by various agents has been shown to enhance TRAIL cytotoxicity in numerous cellular models [12–14].

The benzoquinone ansamycin geldanamycin and its analogue 17-AAG inhibit the molecular chaperone Hsp90 by binding to the ATP/ADP pocket of Hsp90 and causing the destabilization of its complexes with client proteins [15]. Dissociation of hsp90–protein complexes triggers the proteosomal degradation of a number of important signaling intermediates, including AKT, Raf-1, B-Raf, ErbB2 kinase, mutant p53 and others [16,17]. The inhibitory effect of geldanamycin on signaling through the AP-1 and NF-κB transcription factors has also been reported [18,19]. Inhibition of Hsp90 by 17-AAG directly affects activation of AKT through both protein degradation, since AKT is a client protein of Hsp90 [20,21], and inhibition of upstream activators, such as Her2/Neu and PDK1 [22,23]. Inhibition of NF-κB activation is a result of 17-AAG-induced destabilization of another Hsp90 target—IKK, the major upstream regulator of NF-κB [19]. Notably, AKT itself has been shown to phosphorylate IKK in response to various stimuli including TNFα [24], providing an additional mechanism of inhibitory effects of 17-AAG on NF-κB. It has been shown recently that 17-AAG preferentially targets Hsp90 of malignant but not normal cells by virtue of its greater affinity for the activated polymerized form of the molecular chaperone [25], which potentially increases its selectivity for cancer cells. 17-AAG has demonstrated antitumor activity as a single agent both in preclinical models and in Phase I trials [15]. We have shown recently that 17-AAG enhances the therapeutic effects of oxaliplatin in human colon adenocarcinoma cell lines in part through inhibition of NF-κB, resulting in down-regulation of c-IAP1 [26].

The present study demonstrates that the TRAIL/17-AAG combination is additive or synergistic in the entire colon cancer cell lines analyzed, with particular effectiveness in cell lines more resistant to TRAIL as a single agent. Combination treatment leads to enhanced apoptosis through activation of caspase-3 in both TRAIL-sensitive and TRAIL-resistant cell lines. The sensitizing effect of 17-AAG in TRAIL-resistant cell lines is achieved in part through enhancement of both extrinsic and intrinsic apoptotic pathways and down-regulation of the X-linked inhibitor of apoptosis protein (XIAP). Although the resistant cell lines, HT29 and RKO, differ in their dependence upon AKT and NF-κB as survival pathways, the effective targeting of both by 17-AAG underlies TRAIL resistance reversal.

2. Materials and methods

2.1. Cell lines and reagents

Colon cancer cell lines HT29, HCT116, DLD1, LoVo, SW480, RKO and SK-CO-1 were purchased from American Type Culture Collection (Rockville, MD, USA), BE cells were a gift from Dr. A. Jaiswal (Baylor College of Medicine, Houston, TX, USA), and HCT116p53−/− cells were kindly provided by Dr. B. Vogelstein (John Hopkins Oncology Center, Baltimore, MD, USA). Cell lines were cultivated in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 U/ml) (Invitrogen, Carsbad, CA, USA). Cultures were maintained in a humidified incubator at 37 °C in 5%CO_2–95% air. TRAIL (soluble), LY294002 and BAY 11-7082 were purchased from Biomol (Plymouth Meeting, PA, USA), and 17-AAG was kindly provided by Dr. Edward Sausville (Developmental Therapeutics Program, NCI, Bethesda, MD, USA). Stock solutions of 17-AAG (1 mM), LY294002 (20 mM) and BAY 11-7082 (10 mM) were prepared in DMSO, aliquoted and stored at −20 °C. TRAIL (after adjustment to a final concentration of 200 μg/ml with sterile PBS containing 0.1 mM DTT) was aliquoted and stored at −70 °C. Caspase inhibitors Z-IETD-Fmk (caspase-8), Z-LEHD-Fmk (caspase-9) and Z-VAD-Fmk (general caspase inhibitor) were purchased from BD Pharmingen (San Diego, CA, USA), dissolved in DMSO (10 mM) and stored at −20 °C.

2.2. Cytotoxicity assays and calculation of combination indices

The IC_{50} concentrations of TRAIL and 17-AAG in colon cancer cell lines were established in MTT assays after continuous incubation with drugs for 72 h. For assessment of the cytotoxicity of TRAIL/17-AAG in combination (simultaneous addition), cells were plated in 96-well plates and TRAIL was then added in concentrations of 0, 3, 10, 30, 100 and 300 ng/ml (for TRAIL-sensitive cell lines) or 0, 50, 100, 250, 500 and 1000 ng/ml (for TRAIL-resistant cell lines) from left to the right side of the plate (each concentration of TRAIL in quadruplicate, two plates for each experiment). 17-AAG was added cross-wise in concentrations of 0–50 or 0–100 nM, depending on sensitivity of each cell line to 17-AAG. Results were quantified as described in [27], with the method of Chou and Tremblay [28] used to determine combination indices at IC_{50}. CI's values above 0.9 but less than 1.1 represent additive effect and ones less than 0.9 or above 1.1 represent synergism or antagonism, respectively.

2.3. Cell survival assays

To assess the cytotoxicity of the TRAIL/17-AAG combination cells were plated in 12-well plates at a density of

10,000 per well and after 24 h were treated overnight with 200 ng/ml of TRAIL alone or in combination with 17-AAG, used at the concentrations corresponding to 5× IC_{50} for each particular cell line. After addition of fresh media, cells were cultivated for an additional 5–7 days, fixed and stained with Coomassie Blue (Sigma). For colony-forming assays, HT29 and RKO cells were plated into six-well plates at the density of 300 cells per well (with 80% efficiency) and after 24 h treated with 100 ng/ml TRAIL or/and 17-AAG (75 nM for HT29, 200 nM for RKO) overnight. After exchange of media, cells were allowed to grow for 10–14 days, followed by staining with Coomassie Blue and manual count. Caspase inhibitors were added in the concentration of 5 μM 2 h prior to drug treatment.

2.4. Cell treatment, protein extract preparation and Western blotting

Cells were treated for 24 h with 0.01% DMSO (control), 100 ng/ml of TRAIL (unless noted otherwise) or 17-AAG alone (at the concentrations corresponding to 5× IC_{50}), and with combinations of both. Total protein extracts were prepared as described previously [18]. Mitochondrial and cytosolic extracts were prepared from cells treated with drugs for 6 h, using the cytochrome c Release Apoptosis Assay Kit (Sigma). Western blotting was carried out according to standard procedures, using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the ECL + Plus detection system (Amersham, Arlington Heights, IL, USA). The following antibodies were used: rabbit polyclonal antibodies against DR4 and DR5 (Imgenex, San Diego, CA, USA); rabbit polyclonal antibodies against phosphorylated forms of p65 and AKT from Cell Signaling Technology (Beverly, MA, USA); mouse monoclonal antibodies against caspase-8, -9 and -3 from Oncogene Research Products (Boston, MA, USA); mouse monoclonal antibodies against the mitochondrial marker COX IV from Molecular Probes (Eugene, OR, USA). All other antibodies were from Santa Cruz Biotechnology.

2.5. Caspase assay

ApoTarget caspase colorimetric protease assay kits were purchased from BioSource International Inc. (Camarillo, CA, USA). Cells were plated in 10 cm Petri dishes and subjected to drug treatment as described above. If needed, caspase inhibitors were added at concentration of 5 μm 2 h prior to drugs. After 20 h, cellular extracts were isolated and assays were carried out according to the manufacturer's recommendations as described [26].

2.6. Statistical analysis

Data presented were analyzed with unpaired Student's *t*-test; p values of less than 0.05 were accepted as a statistically significant difference compared to controls. In figure legends, $^{*}p < 0.05$; $^{**}p < 0.01$.

3. Results

3.1. Variation in TRAIL sensitivity among colon cancer cell lines

We assessed the effects of Hsp90 inhibition on TRAIL cytotoxicity in a representative panel of nine human colon cancer cell lines, which differ in their p53 status, growth characteristics, apoptotic pathways induced by death ligands (Type I and II) and sensitivity to 17-AAG as single agent. Results of cytotoxicity assays (MTT) revealed a broad range in sensitivity of cell lines to TRAIL, with IC_{50} ranging from 30–45 ng/ml in TRAIL-sensitive cell lines to 1000–4000 ng/ml for TRAIL-resistant lines (Fig. 1A). We wished to know if such variation in sensitivity to TRAIL is based on the expression of TRAIL receptors and other proteins relevant to TRAIL sensitivity. Results of western blot analysis of untreated cell lines (Fig. 1B) show a poor correlation between sensitivity to TRAIL and level of various putative determinants of activity. The most sensitive cell lines were characterized by high expression of DR4, but at lower levels of expression sensitivity was variable. Similarly, the most resistant cells had high basal activity of NF-κB and AKT, but these were also variable in the other cell lines. DR5, FLIP, Bcl-X_L and IAP expression did not clearly correlate with sensitivity. However, in one of the most resistant cell lines, RKO, up-regulation or activation of several relevant proteins was demonstrated (Bcl-X_L, c-IAP1, XIAP, phospho-AKT and phospho-p65/Rel), suggesting that resistance to TRAIL is multifactorial and could correlate with overexpression of one or more anti-apoptotic proteins.

3.2. 17-AAG enhances cytotoxicity of TRAIL in colon cancer cell lines through enhanced activation of apoptosis

The TRAIL/17-AAG combination, as is evident from combination indices determined in MTT assays, was synergistic in the majority of cell lines tested (Table 1). Notably, the synergism of the combination was more pronounced in TRAIL resistant cell lines, whereas sensitivity to 17-AAG did not appear to correlate with the combination's cytotoxicity (Fig. 2A). Synergism of TRAIL and 17-AAG was also evident in cytotoxicity assays (Fig. 2B), where the combination caused a significant increase in cell death compared to that with each drug alone. Previous studies of the interactions between TRAIL and therapeutic agents or signaling inhibitors showed that enhanced cytotoxicity occurred predominantly through enhancement of apoptosis [1,2]. Therefore, we began by assessing the activation of caspases following treatment with the TRAIL/17-AAG

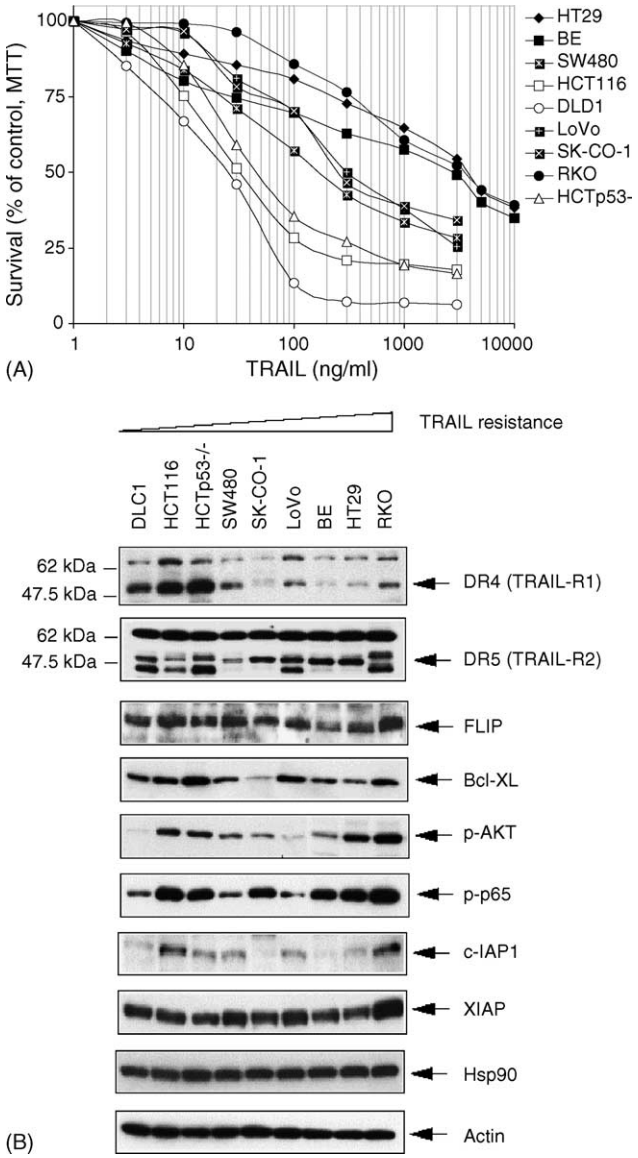

Fig. 1. Colon cancer cell lines display a wide range of sensitivity to TRAIL with no evident correlation to the expression of cellular determinants of TRAIL resistance. (A) Survival curves for colon cancer cell lines treated with TRAIL (derived from MTT assays), graph represents the average of values from three independent experiments in quadruplicates; usual error magnitude for each point did not exceed 4%, but error bars are omitted to enhance viewing of the graph; (B) protein extracts were prepared from untreated cells and the expression level of proteins relevant to TRAIL cytotoxicity was assessed by Western blot as described in Section 2.

combination. Fig. 2C demonstrates the differential effects of the combination on processing of caspase-3 in four cell lines, the TRAIL-sensitive HCT116 and DLD1 cells, and the TRAIL-resistant HT29 and RKO cells. In this experiment, TRAIL concentration (200 ng/ml) was toxic for sensitive cell lines and sub-toxic for resistant cell lines. Cells were treated with each drug alone and with the combination for 24 h. In HCT116 and DLD1 cells, 17-AAG only marginally increased processing of caspase-3 as compared to TRAIL alone, while in HT29 and RKO cell lines the combination treatment resulted in a significant

Table 1
Synergistic effect of TRAIL and 17-AAG combination in human colon cancer cell lines

Cell line	p53	Sensitivity (IC_{50})		MTT
		TRAIL (ng/ml)	17-AAG (nM)	CI at IC_{50}
DLD1	–	30.66 ± 3.2	51.4 ± 4.6	1.03 ± 0.05
HCT116	+	32.5 ± 2.3	47 ± 4.7	0.92 ± 0.07
HCT116p53–/–	–	47.7 ± 3.5	48 ± 3.5	0.85 ± 0.03
SW480	–	216.6 ± 14.3	53.6 ± 5.4	0.79 ± 0.11
SK-CO-1	+	242.2 ± 19.8	23.3 ± 2.8	0.76 ± 0.09
LoVo	+	216.7 ± 17.3	56.5 ± 4.2	0.76 ± 0.19
BE	–	2900 ± 59.5	18.2 ± 1.9	0.53 ± 0.08
HT29	–	4000 ± 100.5	12.1 ± 2.2	0.68 ± 0.07
RKO	+	3900 ± 90.2	69.2 ± 8.1	0.53 ± 0.09

Sensitivity of colon cancer cell lines to TRAIL and 17-AAG as single agents or in combination were determined by MTT assays after continuous treatment for 72 h. Combination indices (CI) were calculated, with the values below 0.9 representing synergism.

increase in procaspase-3 processing. In the sensitive cell lines (HCT116 and DLD1), activation of both caspase-9 and (to a lesser extent) caspase-8 is observed without differences between TRAIL alone and TRAIL/17-AAG combination; in the resistant lines (HT29 and RKO), TRAIL/17-AAG combination shows an increase in activation of both caspases over TRAIL alone. Notably, 17-AAG alone at the relatively high concentration of 500 nM did not cause visible proteolysis of procaspases in any of the cell lines, consistent with our previous data [27,29]. These findings supported a detailed analysis of the pathways to caspase-3 activation.

3.3. 17-AAG overcomes TRAIL resistance in a cell line-dependent manner through enhancement of both extrinsic and intrinsic apoptotic pathways

Since the mechanisms of 17-AAG-mediated sensitization of resistant cells to TRAIL were of primary interest to us, we focused our further study on the HT29 and RKO cell lines. We treated cells with non-toxic concentrations of TRAIL (100 ng/ml, corresponding to 0.03–0.05× IC_{50} established in MTT assays) and increasing amounts of 17-AAG for 24 h (equitoxic concentrations were ranging from 1 to 5× IC_{50} established in MTT assay for each cell line). Western blot analysis of isolated extracts (Fig. 3A) demonstrated concentration-dependent increase of caspase-3 activation, with visible processing of procaspase-3 when 17-AAG was used at the concentrations of 30 or 100 nM in HT29 and RKO cell lines, respectively. Although, the combination of TRAIL and 17-AAG resulted in a dramatic increase in caspase-3 processing in both cell lines, more pronounced processing of caspase-9 was observed in the RKO cell line. More sensitive colorimetric caspase activity assays showed at least three-folds activation of caspase-8 and -9 in both cell lines (data not shown), suggesting an enhancement of mitochondrial apoptosis. We attempted to further delineate the input of particular caspase cascades by assessing the activation

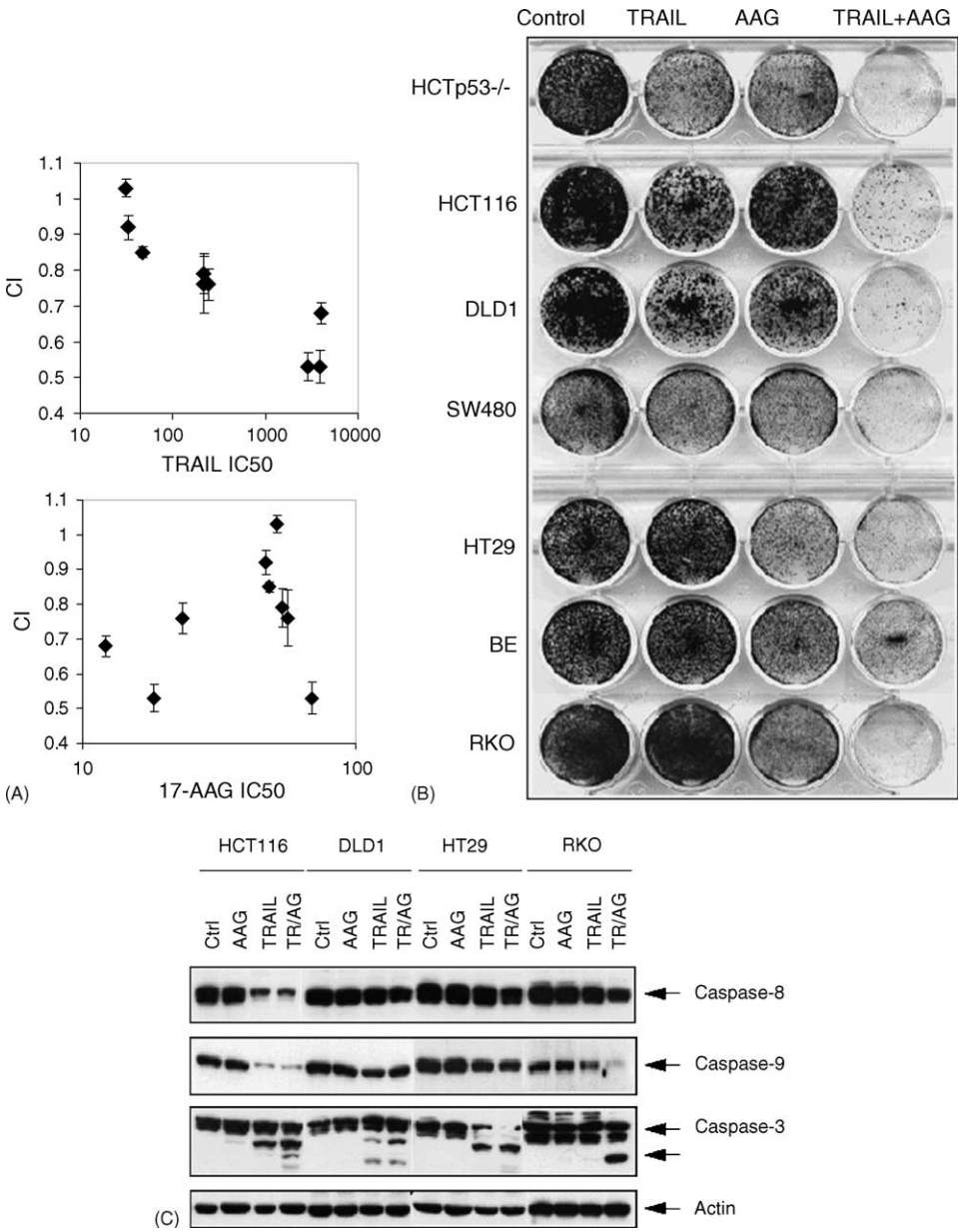

Fig. 2. Effect of the TRAIL/17-AAG combination is synergistic in the majority of colon cancer cell lines tested. (A) Synergism of TRAIL and 17-AAG in combination correlates with sensitivity of cell lines to TRAIL, but not to 17-AAG, as single agent. Combination indices for each cell line were plotted against the corresponding IC_{50} concentrations of TRAIL (upper panel) or 17-AAG (lower panel) as single agents; bars represent S.D.; (B) high cytotoxicity of TRAIL/17-AAG combination demonstrated in survivability assays: cells were plated in 12-well plates and treated as described in Section 2; (C) 17-AAG enhances TRAIL cytotoxicity in colon cancer cell lines through differential induction of initiator caspases resulting in activation of caspase-3. Cells were treated with TRAIL (200 ng/ml) and 17-AAG (500 nM) alone or in combination for 24 h, protein extracts were prepared and subjected to Western blot to analyze the processing of caspases.

of caspase-3 in HT29 and RKO cells pre-incubated with different inhibitors of caspases prior to TRAIL/17-AAG treatment. Fig. 3B demonstrates that inhibition of either caspase-8 or -9 prevents the activation of caspase-3 by combination treatment, proving the necessity of the intrinsic apoptotic pathway in induction of caspase-3 activation after treatment with the combination in both cell lines. To validate these findings we measured the release of mitochondrial factors into the cytosol of both cell lines in response to combined treatment. Fig. 3C shows that treatment with the TRAIL/17-AAG combination for 6 h led to depletion of Bax, Bid and XIAP in cytosolic fractions of both cell lines, demonstrating the activation of the apoptotic pathways up-stream and down-stream of mitochondria. The use of the sub-toxic concentrations of both drugs and short treatment time could explain the absence of significant release of cytochrome c and Smac into cytosol of HT29 cells; however, the release of AIF reflected the loss of mitochondrial integrity in both cell lines. Notably, release of AIF is more pronounced in samples treated with 17-AAG, but not TRAIL alone, which suggests a role for it in 17-AAG-induced cell death even in the absence of

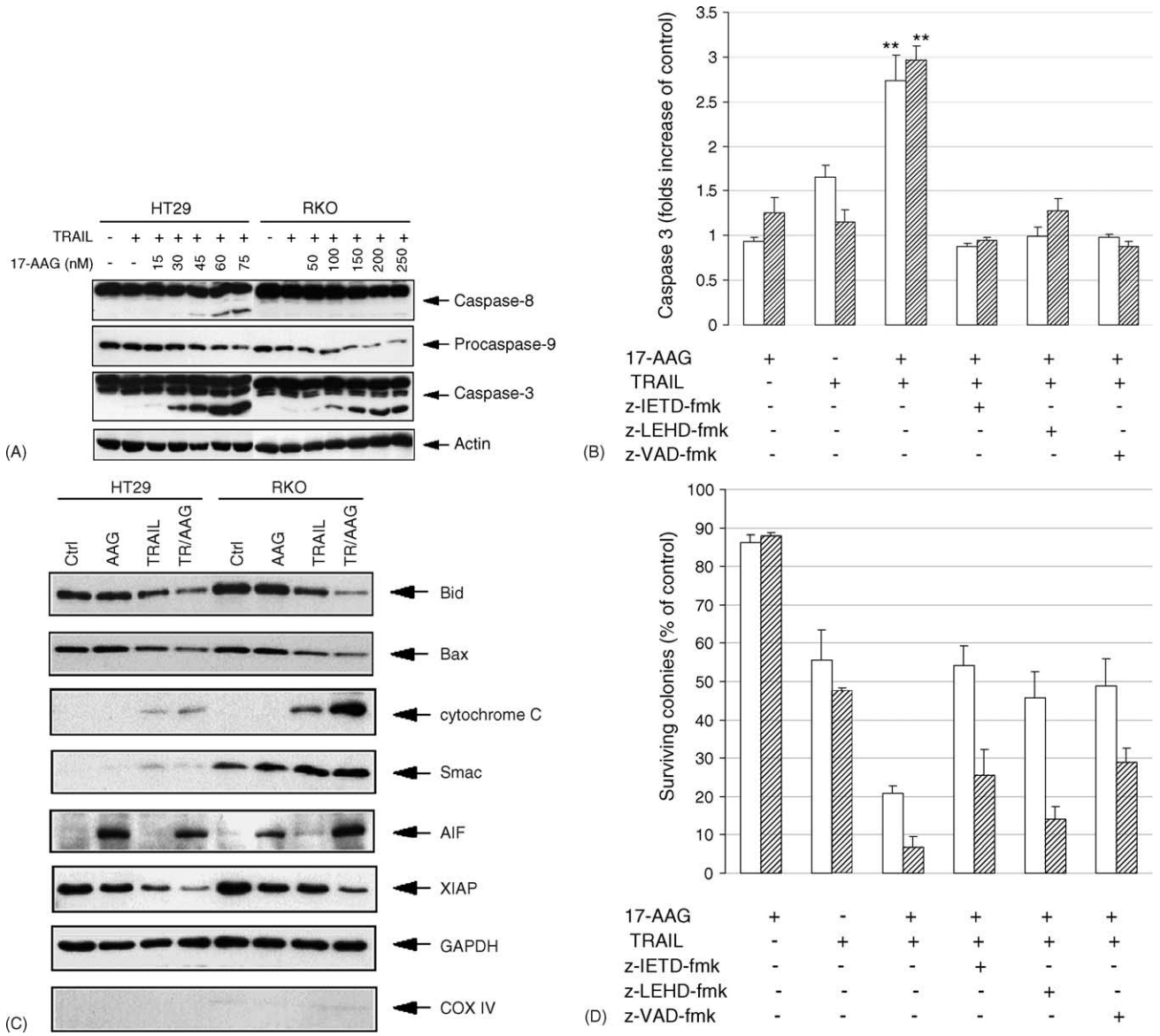

Fig. 3. 17-AAG sensitizes resistant cell lines to TRAIL through diverse molecular mechanisms, including the induction of both extrinsic and intrinsic apoptotic pathways. (A) 17-AAG concentration-dependent induction of caspases in TRAIL-resistant cell lines, HT29 and RKO. Cells were treated with 100 ng/ml of TRAIL in the presence of increasing amounts of 17-AAG for 24 h, and protein extracts isolated and subjected to Western blot analysis as described in Section 2; (B) Inhibition of either caspase-8 or -9 effectively leads to abrogation of caspase-3 activation by the TRAIL/17-AAG combination. Shown are the results of colorimetric caspase-3 activity assays in HT29 (open columns) and RKO (hatched columns) cells pretreated with inhibitors of caspase-8 (Z-IETD-Fmk), caspase-9 (Z-LEHD-Fmk) or general caspase inhibitor (Z-VAD-Fmk). Cells were treated as described in Section 2, graph represents averages of values from three independent experiments in duplicate, and bars represent standard deviation, $p < 0.01$ (combination versus TRAIL alone); (C) truncation of Bid and release of cytochrome c, Smac/Diablo and AIF into cytosol of HT29 and RKO cells are indicative of induction of intrinsic apoptotic pathway, and associated with depletion of XIAP in both cell lines. Cells were treated with TRAIL/17-AAG combination for 6 h, followed by cytosolic extract preparation as described in Section 2, and Western blot analysis. Picture shown is representative of two independent experiments; (D) inhibition of caspases attenuates cytotoxicity of TRAIL/17-AAG combination. Shown are the results of colony forming assays in HT29 (open columns) and RKO (hatched columns) cells pretreated with inhibitors of caspase-8 (Z-IETD-Fmk), caspase-9 (Z-LEHD-Fmk) or general caspase inhibitor (Z-VAD-Fmk) prior to incubation with TRAIL or/and 17-AAG as described in Section 2; graph represents averages of values from three independent experiments in duplicate, and bars represent S.D.

significant activation of caspases by this drug. It also provides an additional possible mechanism for the synergism of the combination through induction of caspase-independent apoptotic events. We also carried out colony-forming assays to establish if the pattern of cell death induced by the combination correlates with the activation of apoptosis through caspase-3. Fig. 3D demonstrates that the inhibition of either of the initiator caspases was able to rescue HT29 cells from TRAIL-induced (but not 17-AAG-induced) cell death, while in the RKO cell line inhibition of caspase-9 alone was less effective in reversing cell death after combination treatment. These observations suggest the contribution of both extrinsic and intrinsic apoptotic pathways, as well as caspase-3-independent cell death, to

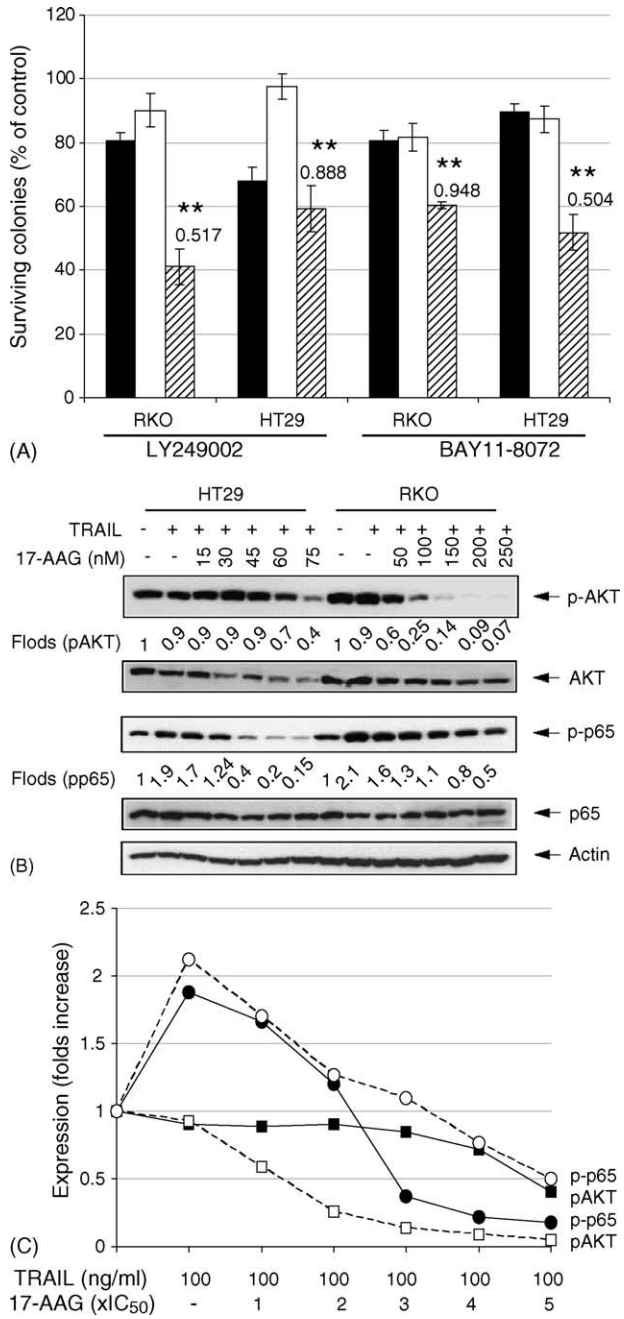

Fig. 4. Differential reliance on AKT and NF-κB characterizes the TRAIL-resistant cell lines. (A) Inhibition of PI3K/AKT and NF-κB is more effective in enhancing TRAIL cytotoxicity in RKO and HT29 cells, respectively. Graph shows the portion of surviving colonies (as per cent of untreated control) after treatment with TRAIL (closed columns) and inhibitor (open columns) alone or in combination (hatched columns). Cells were pretreated with 10 μM of LY249002 or 75 nM of BAY 11-8072 for 2 h prior to incubation with 100 ng/ml TRAIL for 24 h. Presented are averages of values from three independent experiments in triplicate; bars represent S.D. ($p < 0.05$, combination vs. TRAIL plus 17-AAG as single agents); (B) 17-AAG causes concentration-dependent inhibition of pro-survival signaling through AKT and NF-κB in TRAIL resistant cell lines, HT29 and RKO. Cells were treated as described in Fig. 3A, protein extracts were isolated and subjected to Western blot analysis as described in Section 2, picture of representative experiment is shown; (C) graphic representation of calculated results from two independent experiments (as in Fig. 4B) demonstrates that 17-AAG at equitoxic concentrations exerts differential effects of AKT and NF-κB activation. Expression levels of relevant proteins were quantified by densitometry and values normalized to β-actin content. Shown are results for phospho-p65 (circles) and phospho-AKT (squares) content in RKO (open symbols) and HT29 (closed symbols) cell lines. Concentrations of TRAIL (100 ng/ml) and of 17-AAG (in IC_{50} increments) were as shown in Fig. 4B. Graph represents the average of values from two independent experiments.

the synergism of the TRAIL/17-AAG combination in cell lines tested.

3.4. Differential reliance on AKT and NF-κB characterizes the TRAIL-resistant cell lines HT29 and RKO, and may underlie effectiveness of inhibition by the TRAIL/17-AAG combination

Since both HT29 and RKO demonstrate elevated basal activity of AKT and NF-κB pathways, we wished to establish the relative significance of each pathway in determining TRAIL cytotoxicity in these cell lines. We carried out colony-forming assays (three independent experiments in triplicate) after treatment of cells with 100 ng/ml of TRAIL in the presence or absence of LY294002 or BAY11-7082, specific inhibitors of PI3K/AKT and NF-κB activation, respectively. Cells were pretreated for 2 h before addition of TRAIL with 10 μM of LY294002 or 75 nM of BAY11-7082 (the concentrations of inhibitors used caused on average 15% reduction in numbers of colonies for a single agent and were established in separate series of colony-forming assays). The results, shown in Fig. 4A, demonstrate the higher synergism of TRAIL and the AKT inhibitor in RKO cells, whereas inhibition of NF-κB was more effective in enhancing TRAIL cytotoxicity in HT29 cell line. Numerical data labels reflect the ratio of cumulative cell death induced by each drug alone to the cell death induced by the combination. Use of the twice higher concentrations of inhibitors diminished the differences in these ratios, but was accompanied by higher toxicity of LY294002 in RKO cells and of BAY11-7082 in HT29 cells (the number of surviving colonies was reduced to 55% of control, not shown). Since inhibition of both AKT and NF-κB pathways are characteristics of 17-AAG, we wished to assess the effects of 17-AAG on these pathways in TRAIL-treated HT29 and RKO cell lines, and find out if differential responses to 17-AAG could account for the particular pathways to cell death induced by the combination. We treated cells with the same sub-toxic concentration of TRAIL (100 ng/ml) and increasing concentrations of 17-AAG as described above (ranging from 1 to 5× IC_{50} for each cell line, derived from MTT assays, and presented in nM). Western blot analysis of isolated extracts (Fig. 4B) shows concentration-dependent inhibition of activation (assessed as phosphorylation) of AKT and NF-κB by 17-AAG with quantitative differences between cell lines: at equitoxic concentrations, 17-AAG inhibited AKT more effectively in RKO cells, whereas in HT29 cells inhibition of NF-κB, assessed by phosphorylation of p65, was more profound (Fig. 4C).

4. Discussion

TRAIL is a potent inducer of apoptosis in malignant, rather than normal cells, and has been shown to sensitize cancer cell lines to chemotherapy and radiation [4]. The anti-neoplastic specificity of TRAIL warrants interest in the use of this compound in the clinic, however, in vitro resistance of some cancer cell lines might predict a limited role for TRAIL as a single agent. Hence, an active search for novel therapeutics directed against diverse molecular targets to overcome resistance of tumors to TRAIL is ongoing. We investigated the combination of TRAIL and 17-AAG, an inhibitor of signaling through both AKT and NF-κB pathways in colon cancer cell lines, since inhibitory effects of these pathways on TRAIL-mediated apoptosis have been observed [1,2,8,12,13].

In the present study, we found that TRAIL displays a wide range of cytotoxicity in colon cancer cell lines which did not strongly correlate with the level of expression of several putative determinants of TRAIL resistance, including death receptors and their interacting proteins (Fig. 1B), consistent with the previous findings of Ashkenazi and colleagues [7]. 17-AAG was able to enhance TRAIL cytotoxicity in all of the colon cancer cell lines tested and importantly, the greatest synergistic effects of the TRAIL/17-AAG combination were observed in TRAIL-resistant cells (Table 1). The more muted effect of the combination in TRAIL-sensitive cells is in accord with the report of Tillman et al., who studied the sensitization of colon cancer cell lines to TRAIL by inhibitors of PKC isoforms. In that study, the sensitizing effect of rottlerin was more evident in TRAIL-resistant cell lines, RKO and HCT8, whereas cell line most sensitive to TRAIL, GC_3/c1, could not be further sensitized to TRAIL [30].

A burgeoning literature demonstrates that combining TRAIL with chemotherapeutic or certain signaling inhibitors results in robust enhancement of apoptosis, albeit via different mechanisms. For example, cisplatin, doxorubicin and 5-fluouracil synergize with TRAIL in HT29 cells through enhanced DISC assembly and activation of the mitochondria-dependent death pathway [31]. Inhibition of AKT by pharmacological inhibitors reduced resistance to TRAIL in human leukemic and gastric cancer cells through down-regulation of FLIP, and decreased Bad phosphorylation on Ser 136 [32,33]. Inhibition of NF-κB has been shown to underlie the synergism of TRAIL with flavopiridol [34] and curcumin [35] in human colon and prostate cancer cell lines, respectively.

In our study, the TRAIL/17-AAG combination resulted in differential induction of apoptotic pathways, ultimately leading to enhanced activation of caspase-3 and cell death. Correlation of observed quantitative differences in responses to the TRAIL/17-AAG combination and the combination of TRAIL with specific inhibitors of PI3K/AKT and NF-κB in TRAIL-resistant cell lines suggested that a cell line specific effect of 17-AAG on NF-κB in HT29 cell line, and AKT in RKO cells, exerted a pro-apoptotic effect. To what extent variable sensitivity to inhibition of AKT and NF-κB by 17-AAG in HT29 and RKO cells accounts for differential induction of particular apoptotic pathways remains to be investigated further. The full elucidation of these mechanisms could yield molecular markers for prediction of clinical efficacy of combination therapies involving TRAIL. The correlation of sensitivity to 17-AAG with the level of expression of Hsp90 and loss of AKT protein upon drug treatment was suggested for thyroid cancer cell lines [36]. We did not observe the same correlation in our study in regards to Hsp90, since the basal levels of Hsp90 were very close in all of cell lines tested (Fig. 1B). The ability of 17-AAG to down-regulate both AKT and NF-κB pathways in HT29 and RKO cell lines is highly advantageous for the outcome of the TRAIL/17-AAG treatment, since it ultimately leads to increased cytotoxicity through both enhancement of mitochondrial apoptosis and depletion of XIAP, transcriptional target of NF-κB, recently identified as a key modulator of TRAIL sensitivity [37–39]. Aside from enhancing TRAIL cytotoxicity through inhibition of the important Hsp90-dependent components of pro-survival pathways, 17-AAG might effect apoptosis through specific inhibition of Hsp90 itself, in instances where Hsp90 serves as an inhibitor of signaling. For example, the enhanced cleavage of Bid in the cytosol of both HT29 and RKO cell lines, a result of caspase-8 activation, could also be a consequence of direct inhibition of Hsp90 function by 17-AAG, since Hsp90 has been shown to interfere with Bid cleavage by caspase-8 [40]. In addition, enhancement of mitochondria-dependent apoptosis in the presence of 17-AAG might occur through abrogation of an inhibitory effect of Hsp90 on apoptosome formation and activation [41]. That would explain efficient activation of mitochondria-dependent apoptosis by TRAIL/17-AAG combination in HT29 cells, despite the low levels of cytochrome c and Smac in the cytosol of HT29 cells. This effect of 17-AAG on the apoptosome, together with higher activation of caspase-8 and depletion of XIAP may result in the induction of a positive loop of caspase-3 activation in HT29 cells. Such activation may occur when apoptosome-independent cleavage of caspase-9 by activated caspase-3 in turn leads to further activation of caspase-3 by both caspase-8 and -9 [42]. In support of this broader scenario, the results of clonogenic assays in the presence of caspase inhibitors indicate the input of caspase-independent cytotoxic effects of 17-AAG in the cytotoxicity of the combination (Fig. 3D), either through AIF release or induction of other forms of cell death. A more detailed analysis of the mechanisms of 17-AAG-mediated sensitization to TRAIL with a primary focus on the role of Hsp90 and AIF in this phenomenon is in progress.

In summary, we have demonstrated a synergistic interaction of TRAIL and 17-AAG in a panel of colon cancer cell lines that display a wide range of sensitivity to TRAIL as a single agent. Synergism of the combination was manifested through enhanced cell death in both TRAIL-resistant

and TRAIL-sensitive cell lines, and was most evident in the former. The molecular mechanisms involved include inhibition of pro-survival signaling via AKT and NF-κB, enhanced induction of intrinsic apoptotic pathway, and depletion of inhibitor of apoptosis protein (XIAP). The cell line-specific effects of 17-AAG on these mechanisms suggest that TRAIL combinations could be designed to target one or other more specifically with a view to greater tumor selectivity of the combination in TRAIL-resistant tumors.

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