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N-(4-hydroxyphenyl) retinamide (4HPR) enhances TRAIL-mediated apoptosis through enhancement of a mitochondrial-dependent amplification loop in ovarian cancer cell lines

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Abstract

The majority of ovarian cancer cells are resistant to apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Subtoxic concentrations of the semisynthetic retinoid *N*-(4-hydroxyphenyl)retinamide (4HPR) enhanced TRAIL-mediated apoptosis in ovarian cancer cell lines but not in immortalized nontumorigenic ovarian epithelial cells. The enhancement of TRAIL-mediated apoptosis by 4HPR was not due to changes in the levels of proteins known to modulate TRAIL sensitivity. The combination of 4HPR and TRAIL enhanced cleavage of multiple caspases in the death receptor pathway (including the two initiator caspases, caspase-8 and caspase-9). The 4HPR and TRAIL combination leads to mitochondrial permeability transition, significant increase in cytochrome *c* release, and increased caspase-9 activation. Caspase-9 may further activate caspase-8, generating an amplification loop. Stable overexpression of Bcl-xL abrogates the interaction between 4HPR and TRAIL at the mitochondrial level by blocking cytochrome *c* release. As a consequence, a decrease in activation of caspase-9, caspase-8, and TRAIL-mediated apoptosis occurs. These results indicate that the enhancement in TRAIL-mediated apoptosis induced by 4HPR is due to the increase in activation of multiple caspases involving an amplification loop via the mitochondrial-death pathway. These findings offer a promising and novel strategy for the treatment of ovarian cancer.
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Abbreviations: ATRA, all *trans*-retinoic acid; BID, BH3 interacting domain death agonist; $DiOC_6$, 3,3′-dihexyloxacarbocianine iodide; DISC, death-inducing signaling complex; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FLIP, FADD-like ICE inhibitory proteins; GST, gluthathione-*S*-transferase; IAPs, inhibitors of apoptosis; MAPK, mitogen-activated protein kinase; MPT, mitochondrial permeability transition; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]; 4HPR, *N*-(4-hydroxyphenyl) retinamide; RARs, retinoic acid receptors; RXRs, retinoid X receptos; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R1, TRAIL receptor 1; TRAIL-R2, TRAIL receptor 2; TRAIL-R3, TRAIL receptor 3; TRAIL-R4, TRAIL receptor 4; XIAP, X-linked inhibitor of apoptosis; Z-IETD-fmk, Z-Ile-Glu(OCH_3)-Thr-Asp(OCH_3)-fmk; Z-LEHD-fmk, Z-Leu-Glu(OCH_3)-His-Asp-(OCH_3)-fmk; Z-VAD-fmk, Z-Val-Ala-Asp (OCH_3)-fmk.

Introduction

Activation of the death receptors can induce apoptosis in a variety of epithelial malignancies.[1] Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) binds to the death receptors, TRAIL receptor 1 (TRAIL-R1; also called DR4) and TRAIL receptor 2 (TRAIL-R2; also called DR5), and induces caspase-mediated apoptosis.[2] TRAIL has been shown to induce apoptosis selectively in a variety of cancer cell lines but not in normal cells.[3,4] Also, animal studies have shown that TRAIL can induce regression of cancer xenografts without toxicity to normal tissues.[4,5] The selective induction of apoptosis in cancer, but not in normal cells, has prompted investigation into the use of TRAIL in cancer therapy.

We have shown previously that a majority of breast and ovarian cancer cell lines are resistant to TRAIL-mediated apoptosis.[6,7] Similar results have been reported in other cancer cell lines.[8,9] Several mechanisms have been described that regulate sensitivity to TRAIL-mediated apoptosis. These include the expression of decoy receptors that bind to TRAIL but do not activate the caspase cascade,[3] the expression of inhibitory downstream molecules such as survivin,[10] FADD-like ICE inhibitory proteins (FLIP),[11,12] and inhibitors of apoptosis (IAPs),[13] and the activation of antiapoptotic transcription factors such as NF-κB.[14,15] Studies in breast and ovarian cancer cell lines have failed to identify the major determinants of TRAIL sensitivity.[6,7] Recently, we reported that the combination of chemotherapy and TRAIL

enhances TRAIL-mediated apoptosis in breast and ovarian cancer cells.[6,7] Similar enhancement of TRAIL-mediated apoptosis by chemotherapy has been described in a variety of solid tumors.[9,16,17] However, the combination also resulted in an increase in toxicity to normal epithelial cells.[6] Therefore, finding strategies to overcome resistance to TRAIL, while avoiding toxicity in normal tissues, warrants further investigation.

N-(4-hydroxyphenyl) retinamide (4HPR; or fenretinide) is a semisynthetic retinoid that has cytotoxic activity in a wide variety of tumors both *in vitro* and *in vivo*.[18] *In vitro* studies in ovarian cancer cells have shown that 4HPR inhibits cell proliferation and induces apoptosis.[19,20] The mechanisms of action of 4HPR are not completely understood. Recent reports have indicated that 4HPR can induce apoptosis through RAR-dependent or -independent pathways.[20–22] The activation of the c-Jun N-terminal kinase, the activation of the mitochondrial pathway via generation of reactive oxygen species (ROS), or the induction of increased ceramide production have all been implicated in 4HPR-mediated apoptosis.[21,23,24] Recently, it was reported that 4HPR enhances the effect of chemotherapy in neuroblastoma and ovarian carcinomas.[25,26] The mechanism behind this interaction is not completely understood, but it is likely that mitochondrial cytochrome *c* release is important to the induction of apoptosis by 4HPR and chemotherapy.[21,27,28]

Previous work has shown that TRAIL induces direct caspase activation and that, in some cells, TRAIL also induces mitochondrial cytochrome *c* release and caspase activation.[29] Thus, we investigated if 4HPR could enhance TRAIL-mediated apoptosis in ovarian cancer cells, which are resistant to TRAIL-mediated apoptosis.

Results

4HPR enhances TRAIL-mediated toxicity in ovarian cancer cells

Nine ovarian cancer cell lines and two immortalized non-tumorigenic ovarian cell lines were assessed for sensitivity to TRAIL-mediated apoptosis in the presence or absence of 4HPR pretreatment (Figure 1a). As we have previously shown, the majority of the ovarian cancer cell lines are resistant to TRAIL-mediated toxicity.[7] Most of the ovarian cancer cell lines (seven of nine) were resistant when treated with 4HPR at 1 μM (Figure 1a). As has been previously

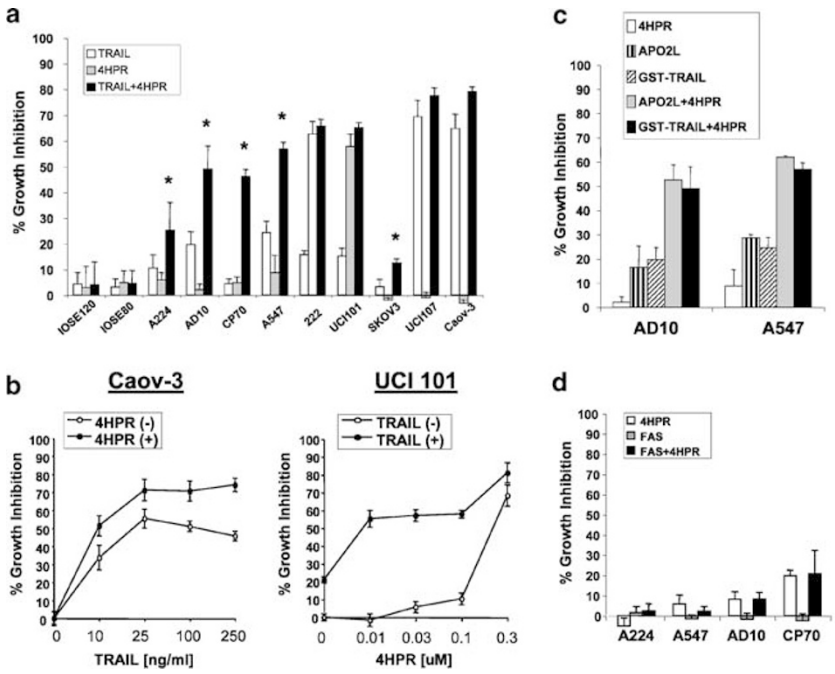


Figure 1 4HPR enhances TRAIL-mediated toxicity in ovarian cancer cells. **(a)** In total, 11 ovarian cell lines, two immortalized nontumorigenic (IOSE 80 and IOSE 120) and nine ovarian cancer cell lines (A224, AD10, CP70, A547, 222, UCI 101, SKOV3, UCI 107, and Caov-3), were incubated with TRAIL alone, 4HPR alone, or the combination. Cells were incubated with or without 4HPR (1 μM) for 96 h, and TRAIL was added to a final concentration of 250 ng/ml for the final 16 h in culture. Cell viability was measured by the MTS assay, and the data represent growth inhibition as a percentage of control cells. Data points show the mean $\pm$ S.E. for a minimum of three experiments for each cell line. The (*) indicates that synergism was demonstrated at these concentrations of 4HPR and TRAIL (see text for discussion). **(b)** Left panel, the TRAIL-sensitive cell line, Caov-3, was incubated with different concentrations of TRAIL (as indicated in the figure) with or without a fixed concentration of 4HPR (1 μM). Right panel, the 4HPR-sensitive cell line UCI 101 was incubated with different concentrations of 4HPR (as indicated in the figure) with or without a fixed concentration of TRAIL (250 ng/ml). Cell viability was measured as previously described. Data points show mean $\pm$ S.D. of a representative experiment. **(c)** Two ovarian cancer cell lines (AD10 and A547) were treated same as above but using two different forms of TRAIL (GST-TRAIL and recombinant Apo2L/TRAIL, indicated as Apo2L on figure). Cell viability was measured as previously described. Data points show mean $\pm$ S.D. of a representative experiment. **(d)** Four ovarian cell lines (A224, A547, AD10, and CP70) were incubated with 4HPR alone, anti-FAS antibody alone, or the combination. Cells were incubated with or without 4HPR (1 μM) for 96 h, and the anti-FAS mouse monoclonal antibody was added to a final concentration of 1 μg/ml for the final 16 h in culture. Cell viability was measured as previously described. Data points show mean $\pm$ S.D. of a representative experiment

shown,[20] higher concentrations of 4HPR could inhibit growth in the majority of the ovarian cancer cell lines tested (data not shown). Treatment with 4HPR at 1 μM significantly enhances TRAIL-mediated growth inhibition in five of the nine ovarian cancer cell lines, but not in the immortalized nontumorigenic ovarian cell lines, IOSE 80 and IOSE 120 (Figure 1a). The enhancement of TRAIL-mediated toxicity by 4HPR required pretreatment for at least 48 h. Cotreatment or shorter pretreatments did not result in enhanced TRAIL-mediated toxicity. The enhancement of TRAIL-mediated toxicity by 4HPR does not reflect a shift in the kinetics of TRAIL-mediated apoptosis. Longer incubation with TRAIL alone (i.e., 48 or 72 h) resulted only in slight increases in TRAIL-mediated toxicity. Pretreatment with 4HPR resulted in a significant increase of TRAIL-mediated toxicity when TRAIL was added for 24, 48, or 72 h. Similarly, no shift in kinetics was seen when the length of 4HPR preincubation was varied (data not shown).

The interaction between 4HPR and TRAIL was synergistic as measured by the fractional inhibition method[30] in five of the nine ovarian cancer cells studied (denoted with asterisks in Figure 1a). To confirm this synergism, a dose–effect analysis and a combination index (CI) were calculated at different doses of TRAIL (0.1–2.5 μg/ml) and 4HPR (0.1–10 μM).[31] In all of the cell lines tested, the synergism was confirmed.

When the cells were relatively sensitive either to TRAIL at 250 ng/ml (i.e., UCI107 and Caov-3) or 4HPR at 1 μM (i.e., 222 and UCI101), the interaction was only additive or slightly more than additive, as observed in Figure 1a. To investigate the lack of synergy observed in cell lines sensitive to either TRAIL or 4HPR, we measured the toxicity of the combination using lower doses of the reagent to which the cell line was sensitive. When cell lines sensitive to TRAIL (i.e. Caov-3, UCI 107) were incubated with doses of TRAIL that induced submaximal toxicity, TRAIL and 4HPR were synergistic (Figure 1b left panel, and data not shown). Similarly when cell lines sensitive to 4HPR (i.e. 222, UCI 101) were incubated with doses of 4HPR that induced submaximal toxicity, TRAIL and 4HPR were synergistic (Figure 1b right panel, and data not shown). The enhancement in TRAIL-mediated toxicity was more dramatic in 4HPR-sensitive than in TRAIL-sensitive cell lines. These data suggest that when the cells are relatively sensitive to one of the reagents (TRAIL or 4HPR), the same level of growth inhibition can be obtained by combining lower doses of TRAIL and 4HPR.

To investigate if the effects observed with gluthathione-*S*-transferase (GST)-TRAIL were reproducible with the human nontagged homotrimeric recombinant soluble Apo2L/TRAIL,[32] we compared the two reagents in two of the ovarian cell lines (Figure 1c). 4HPR acts synergistically with GST-TRAIL and Apo2L/TRAIL. Dose–response curves were similar for the two forms of TRAIL when used alone or in combination (data not shown).

To investigate whether the increase in TRAIL-mediated toxicity induced by 4HPR was specific for TRAIL or could be a general phenomenon occurring with other death receptor pathways, we treated ovarian cancer cells with the combination of 4HPR and FAS ligand. All the four cell lines (A224, A547, AD10, and CP70) expressed the FAS receptor but they were resistant to FAS-mediated apoptosis (Figure 1d). Similar results have been reported for FAS-mediated apoptosis in ovarian cell lines.[33,34] In contrast to TRAIL-mediated apoptosis, FAS-mediated apoptosis in ovarian cancer cells was not enhanced by 4HPR pretreatment (Figure 1d).

The combination of 4HPR and TRAIL induces caspase-mediated apoptosis

To assess if growth inhibition by the combination of 4HPR and TRAIL was mediated by apoptosis, we quantitated the induction of apoptosis by measuring the fraction of cells with sub-G0/G1 DNA content by flow cytometry. 4HPR did not significantly increase the fraction of apoptotic cells in two of the three cell lines tested compared with control cells. TRAIL alone increased apoptosis in all three cell lines ($P \leqslant 0.05$). However, the combination of 4HPR and TRAIL resulted in a significant increase compared with TRAIL alone or the sum of 4HPR and TRAIL used alone (Figure 2a).

TRAIL induces apoptosis by a caspase-dependent mechanism.[2] To determine whether the increased apoptosis by the combination was due to triggering of the caspase cascade, the effect of the pan-caspase inhibitor Z-Val-Ala-Asp (OCH_3)-fmk (Z-VAD-fmk) was investigated. The addition of Z-VAD-fmk inhibited the toxicity of TRAIL either alone or in combination with 4HPR in all the cell lines tested (Figure 2b). No consistent effect of Z-VAD-fmk was observed on the toxicity of 4HPR alone. Together, the results in Figures 2a and b demonstrate that the growth inhibition by TRAIL alone and by the combination of 4HPR with TRAIL is due to caspase-mediated apoptosis.

4HPR enhances TRAIL-mediated apoptosis by a retinoic acid receptor (RAR)–retinoid X receptor (RXR)-independent pathway

Recent studies, including those in ovarian cancer cells, have suggested that apoptosis and growth inhibition by 4HPR might occur via activation of RAR-dependent or RAR-independent mechanisms.[19–22] In contrast to 4HPR, the effects induced by all *trans*-retinoic acid (ATRA) are clearly dependent on retinoid receptor activation.[19,21,26,35] To investigate whether 4HPR enhances TRAIL-mediated apoptosis in a retinoid receptor-dependent or independent mechanism, three cell lines (A224, A547, and AD10) were incubated with 4HPR or ATRA (Figure 3a). Two of the cell lines tested (A224, AD10) have been shown previously to express low or no RAR or RXR receptors and are resistant to 4HPR-induced apoptosis.[20] Upon treatment with ATRA, enhancement in TRAIL-mediated apoptosis was observed in only one of the three cell lines tested (A547) (Figure 3a, left panel). In contrast, 4HPR enhanced TRAIL-mediated apoptosis in all of the cell lines (Figure 3a, right panel). To investigate if the enhancement of TRAIL-mediated apoptosis requires retinoid receptor activation, two pan-antagonists, one blocking the RAR receptors (LE540) and the other blocking the RXR receptors (HX531), were tested in the A547 and AD10 cell lines.[36,37] Preincubation with these antagonists did not inhibit the enhancement in TRAIL-mediated apoptosis induced by 4HPR in the cell lines tested (A547 shown in Figure 3b and AD10 not shown). To

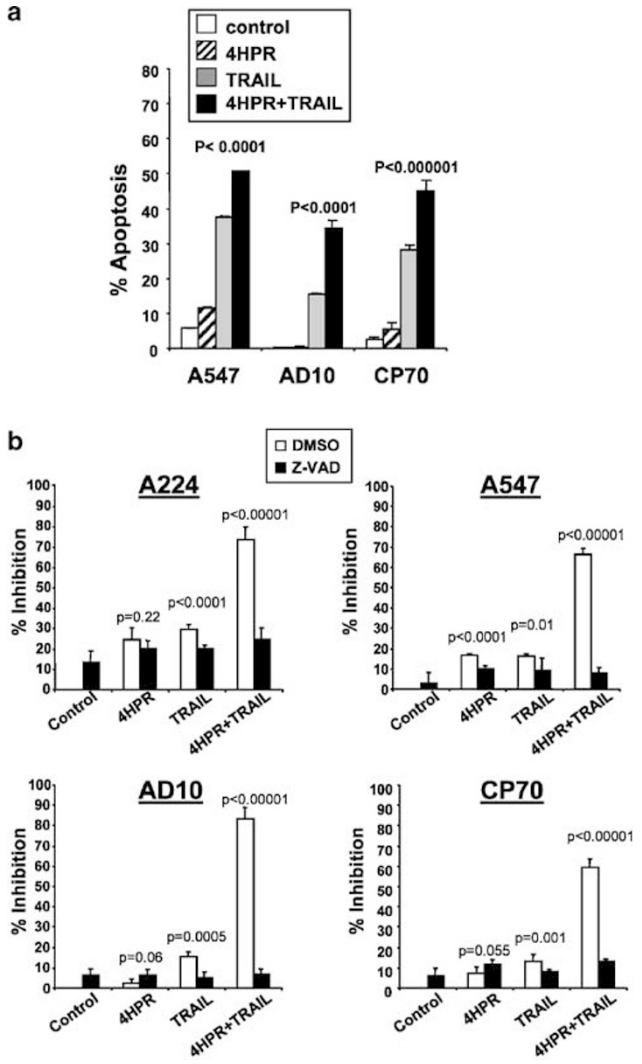


Figure 2 4HPR plus TRAIL induces caspase-mediated apoptosis. **(a)** Three ovarian cancer cell lines (A547, AD10, and CP70) were incubated with no addition, 4HPR alone, TRAIL alone, or the combination as described in Figure 1. Cells were collected and stained with propidium iodide. Apoptosis was measured as a percent of cells with sub-G0/G1 DNA content in the DNA histogram compared to the total number of cells present. Data points show mean $\pm$ S.E. for a minimum of three experiments. The *P*-values comparing the average of apoptosis with the combination of 4HPR plus TRAIL to the average of apoptosis from the sum of each treatment alone are shown above the bars. **(b)** The enhancement in TRAIL-mediated apoptosis induced by 4HPR is abrogated by a pan-anatagonist of caspases. Four ovarian cancer cell lines (A224, A547, AD10, and CP70) were incubated with no addition, 4HPR alone, TRAIL alone, or the combination. Cells were incubated with 4HPR (1 μM) for 96 h and TRAIL was added to a final concentration of 250 ng/ml for the final 16 h in culture. At 1 h before adding TRAIL, a caspase inhibitor, Z-VAD-fmk (indicated by Z-VAD in figure), at 50 μM in DMSO or DMSO alone was added to the culture. Cell viability was measured by the MTS assay. The data represent the growth inhibition, as a percentage of control cells. Data points show mean $\pm$ S.E. for a minimum of three experiments. The *P*-values comparing each treatment in the absence or presence of the inhibitor are shown above the bars

demonstrate that the inhibitors block RAR- or RXR-mediated effects, we treated the A547 cell line with ATRA plus TRAIL in the presence or absence of both inhibitors. In this cell line, an increase in TRAIL-mediated toxicity was observed upon incubation with ATRA (Figure 3a, left panel). Preincubation

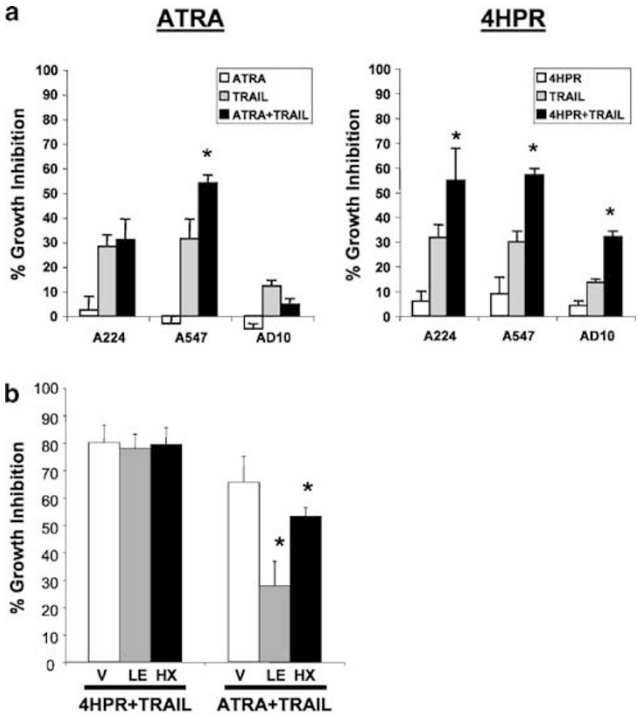


Figure 3 4HPR enhances TRAIL-mediated apoptosis by a RAR/RXR-independent pathway. (**a**) Three ovarian cancer cells (A224, A547, AD10) were incubated with ATRA or 4HPR alone, TRAIL alone, or the combination of ATRA or 4HPR and TRAIL. In the left panel, the effects on cells incubated with ATRA alone, TRAIL alone, or the combination of ATRA plus TRAIL are shown. In the right panel, the effects on cells incubated with 4HPR alone, TRAIL alone, or the combination of 4HPR plus TRAIL are shown. Cells were incubated with 4HPR (1 μM) or ATRA (1 μM) for a total of 96 h and TRAIL (250 ng/ml) was added for the final 16 h in culture. Cell viability was measured by the MTS assay and the data represent growth inhibition as a percent of control cells. Data points show mean $\pm$ S.E. for a minimum of three experiments. The (*) indicates synergism. (**b**) The A547 cell line was treated with 4HPR or ATRA and TRAIL as above in the presence or absence of two pan-antagonists of retinoid receptors ((the pan-antagonist of the RAR receptors, LE 540 (LE), and the pan-antagonist of RXR receptors, HX 531 (HX)]. Both pan-antagonists at 2.5 μM (LE or HX) or vehicle (V) were added 1 h before adding 4HPR. Data points show mean $\pm$ S.D. of a representative experiment. The (*) indicates when differences were statistically significant comparing each treatment in the absence or presence of the inhibitor

with both inhibitors significantly decreased the enhancement in TRAIL-mediated toxicity induced by ATRA (Figure 3b). Pretreatment with the inhibitors did not affect the toxicity induced by TRAIL alone. 4HPR and ATRA by themselves had only minimal toxicity and the inhibitors did not significantly affect this (data not shown). These data suggest that 4HPR enhances TRAIL-mediated apoptosis predominantly by a RAR–RXR-independent pathway.

4HPR does not change levels of proteins known to modulate TRAIL sensitivity

Different therapies have been shown to enhance TRAIL-mediated apoptosis in epithelial tumors including chemotherapeutic drugs, radiotherapy, and immunotherapy (i.e., trastuzumab).[6,7,9,16,17,38,39] The explanation for the increases in TRAIL-mediated apoptosis induced by these therapies has included the increase in expression of proapoptotic death receptors TRAIL-R1 and TRAIL-R2, downregulation of Akt

kinase activity, synergistic caspase activation, and a decrease in inhibitory proteins such as FLIP.[11,39–41]

To assess if the enhancement of TRAIL-mediated apoptosis by 4HPR is due to changes in proteins known to modulate TRAIL sensitivity, we investigated the expression of different proteins involved in the death receptor pathway upon treatment with 4HPR. The protein levels of TRAIL receptors and their surface expression were evaluated (Figure 4). 4HPR did not induce increases in protein expression of the death-inducing TRAIL receptors, TRAIL-R1 and TRAIL-R2 (Figure 4a). To assess the specificity of the anti-TRAIL-R1 and anti-TRAIL-R2 antibodies, lysates from 293T cells transfected with TRAIL-R1 or TRAIL-R2 were included on the immunoblot. In the lysates from 293T cells transfected with the TRAIL-R1 cDNA, the predominant species of TRAIL-R1 protein migrates at ~45 kDa and a second protein species migrates at ~51 kDa (Figure 4a, top panel, heavy arrow and light arrow, respectively). The antibody specifically detected TRAIL-R1 and did not crossreact with TRAIL-R2. TRAIL-R1 was expressed in both AD10 and A224 and treatment with 4HPR did not induce increased expression of any form of the TRAIL-R1 protein. In lysates from 293T cells transfected with the TRAIL-R2 cDNA, the protein migrates at ~49 kDa (Figure 4a, middle panel, heavy arrow). This protein was detected in both AD10 and A224. A second form of the TRAIL receptor migrating at ~43 was detected in the AD10 cells and weakly in the A224 cells (Figure 4a, middle panel, light arrow). This was not seen in the 293T cells transfected with the TRAIL-R2 cDNA. The antibody to TRAIL-R2 crossreacted with TRAIL-R1 when the protein was highly overexpressed in the 293T cells, but this cross-reactivity was not observed in cells expressing endogenous levels of TRAIL-R2. Treatment with 4HPR did not induce increased expression of any form of the TRAIL-R2 protein. There was also no change in the levels of the TRAIL decoy receptor TRAIL receptor 4 (TRAIL-R4) (Figure 4c). The decoy receptor TRAIL receptor 3 (TRAIL-R3) was not expressed in these cell lines (data not shown).

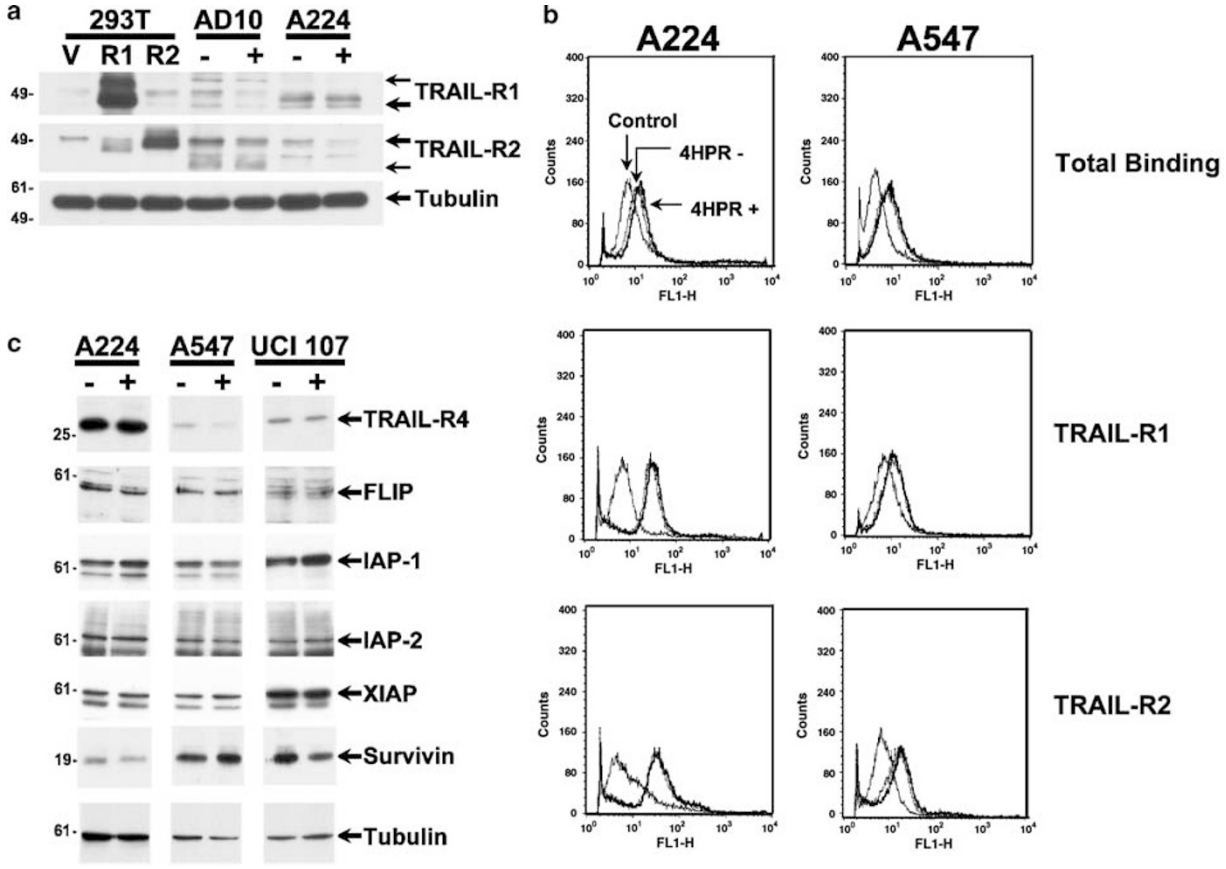


Figure 4 4HPR does not change the levels of proteins known to modulate TRAIL-mediated apoptosis. **(a)** Cell lines were incubated in medium supplemented with (+) or without (−) 4HPR at 1 μM for 96 h. Cell lysates were prepared and immunoblotted for TRAIL-R1 and TRAIL-R2 as indicated to the right of the panel. To control for specificity, lysates from 293T cells transfected with vector (V), TRAIL-R1 (R1), or TRAIL-R2 (R2) were included on the immunoblots. Tubulin is shown as a loading control for each paired sample. The heavy arrows indicate the positions of the predominant species of TRAIL-R1 or TRAIL-R2. The light arrows indicate the position of alternative protein species (see text for discussion). **(b)** The A224 and A547 cell lines were incubated as above. The total surface TRAIL binding was determined by measuring GST-TRAIL or GST binding with an fluorescein isothiocyanate (FITC)-conjugated anti-GST antibody by flow cytometry. The surface expression of TRAIL-R1 and TRAIL-R2 was determined by measuring the binding of anti-TRAIL-R1, anti-TRAIL-R2, or isotype-matched IgG antibody (as a control) with an FITC-conjugated antibody by flow cytometry. The upper, middle, and bottom panels represent the total surface expression, the TRAIL-R1 and TRAIL-R2 surface expression, respectively. The curves represent the control and the surface expression upon medium and 4HPR incubation. **(c)** Cell lysates from cells incubated in medium supplemented with (+) or without (−) 4HPR at 1 μM for 96 h were immunobloted for TRAIL-R3, TRAIL-R4, FLIP, IAP-1, IAP-2, XIAP, and Survivin as indicated to the right of the panel. Tubulin is shown as a loading control for each paired sample. TRAIL-R3 was not detected in these cell lines and so is not shown. Molecular weights in kDa are shown to the left of the panels. Arrows indicate positions of each protein

In addition, 4HPR did not change the surface expression of the death-inducing receptors (TRAIL-R1 and TRAIL-R2) (Figure 4b). There was also no change in total TRAIL binding. Since there was no change in total TRAIL binding, it indicated that there was also no change in the surface levels of the decoy receptors (TRAIL-R3 and TRAIL-R4). Similar results were obtained in other cell lines such as AD10 and CP70 (data not shown). We also investigated the expression of inhibitory proteins such as FLIP, IAPs, XIAP, and survivin (Figure 4c). No change was observed in the expression of these proteins upon treatment with 4HPR. Furthermore, 4HPR did not change the expression of the Bcl-2 family members Bcl-2, Bclx-L, and Bclx-s, Bax, and Bad (data not shown). Finally, no changes were observed in the expression or activity of kinases involved in cell proliferation and cell survival, including MAP kinases (i.e., erk, p38, and JNK kinases) and Akt (data not shown). Thus, alterations in expression or activity of different components of the TRAIL pathway or other apoptosis-modulating proteins by 4HPR do not account for the increase in TRAIL-mediated apoptosis.

Recently, it was reported that apoptosis induced by ATRA in leukemia cells is mediated by an increase in the expression of TRAIL ligand.[42] We investigated if the increase in apoptosis induced by the combination of 4HPR plus TRAIL was due to the induction of TRAIL or FAS ligand by 4HPR. Both TRAIL and FAS ligand are barely expressed in the ovarian cancer cells and no increase in their expression is observed upon incubation with 4HPR (data not shown). These data suggest that the increase in apoptosis is not due to induction of a paracrine effect either by FAS ligand or TRAIL ligand.

4HPR enhances TRAIL-mediated apoptosis by increasing caspase cleavage in a mitochondrial-dependent amplification loop

Since the addition of the pan-antagonist of caspases Z-VAD-fmk abrogated the synergism induced by the combination of 4HPR and TRAIL, we decided to investigate if 4HPR alters the pattern of caspase activation when combined with TRAIL. Activation of caspases (caspase-8, caspase-10, caspase-9, caspase-7, and caspase-3) was investigated in the AD10 cell line treated with each reagent alone or the combination (Figure 5). Caspases exist as inactive proenzymes and are activated by proteolytic cleavage after death stimuli.[43] TRAIL alone induced cleavage of caspase-8, caspase-9, and caspase-3 as indicated by the appearance of cleavage products of these caspases (Figure 5a). 4HPR treatment alone did not result in any cleavage of these caspases. The combination of 4HPR and TRAIL resulted in an obvious increase in the activation of caspase-8, -9, -3, and -7 as

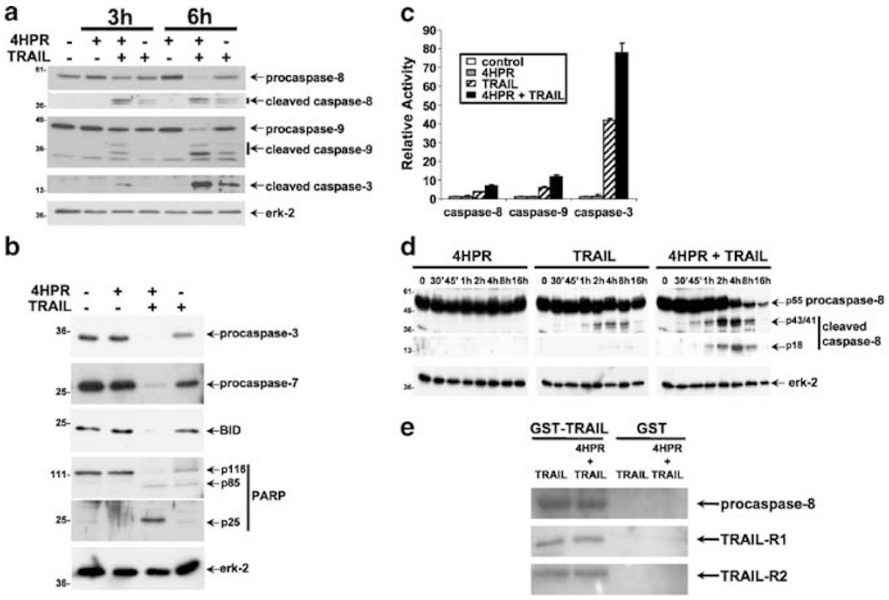


Figure 5 Combination of 4HPR and TRAIL activates caspase-8 and -9. **(a)** The AD10 cell line was incubated with or without 4HRP (1 μM) for a total of 48 h and TRAIL (250 ng/ml) was added for the indicated times. Cell lysates were prepared and immunoblotted with caspase-8, caspase-9, cleaved caspase-3, and erk-2 as indicated to the right of the panels. The procaspases and their cleaved products are indicated by the arrows to the right of the panels. The panels showing procaspase-8 and its cleavage product are from a short and long exposure of the same filter, respectively. Erk-2 is shown as a loading control. **(b)** The AD10 cell line was incubated with 4HRP (1 μM) for a total of 48 h and TRAIL (250 ng/ml) was added for the final 16 h in culture. Cell lysates were prepared and immunoblotted with caspase-3, caspase-7, caspase-3, BID, and PARP as indicated to the right of the panels. The procaspases and the full-length form of PARP (p116) are indicated by arrows to the right of the panels. p85 and p25 are the cleaved PARP products. Erk-2 is shown as a loading control. Molecular weights in kDa is show to the right of the panels. **(c)** Caspase assays were performed on the cell lysates treated with TRAIL for 6 h in panel (**a**) with the indicated substrates. Activity for each substrate was normalized to the activity in the untreated cells and the data represent the mean $\pm$ S.D. for triplicate measurements from a representative experiment. **(d)** The AD10 cell line was incubated with 4HRP (1 μM) for a total of 48 h and TRAIL (250 ng/ml) was added for the indicated times. Cell lysates were prepared and immunoblotted with caspase-8 and erk-2 as indicated to the right of the panels. Procaspase-8 (p55) and its cleaved products are indicated by the arrows to the right of the panels. The panel showing the 18 kDa cleavage product of caspase-8 is from a longer exposure of the same filter shown in the upper panel. Erk-2 is shown as a loading control. **(e)** Lysates for AD10 cells treated with either TRAIL or the combination of TRAIL and 4HPR for 3 h were precipitated using GST-TRAIL or GST as indicated, and the lysates were evaluated for the presence of TRAIL-R1, TRAIL-R2, and procaspase-8 by immunoblotting as indicated

evidenced both by an increase in the amount of cleaved caspase and/or the disappearance of the procaspase (Figure 5a and b). Neither reagent alone nor the combination activated caspase-10 (data not shown). TRAIL alone induced measurable PARP cleavage while 4HPR alone did not induce detectable PARP cleavage (Figure 5b). The combination of 4HPR plus TRAIL resulted in a significant increase of PARP cleavage as detected by a marked disappearance of the proenzyme form (p116) and the increase in the cleaved forms (p85 and p25 forms) (Figure 5b). To quantitate the activation of caspases, caspase activity in lysates was assessed using colorimetric peptide substrates specific for caspases-8, caspase-9, or caspase-3 (Figure 5c). TRAIL alone induced a 4-6-fold increase in the activity of caspase-8 and -9 over the untreated cells and approximately a 40-fold increase in the activity of caspase-3 (Figure 5c, striped bars). The caspase activity in lysates from cells treated with TRAIL for all three caspases was significantly greater than the activity seen in lysates from untreated cells or lysates from cells treated with 4HPR alone ($P<0.001$ for each caspase). The greater relative increase in caspase-3 activity is consistent with amplification of the signal by activation of multiple upstream caspases (i.e., caspase-8 and -9). 4HPR alone caused no increase in caspase activity (Figure 5c, gray bars). The combination of TRAIL and 4HPR resulted in approximately two-fold increase in the activity of all three caspases compared to TRAIL alone (Figure 5c, black bars). The caspase activity in lysates from cells treated with the combination of 4HPR and TRAIL for all three caspases was significantly greater than the activity seen in lysates from cells treated with TRAIL alone ($P<0.001$ for each caspase). The activity of the combination of 4HPR and TRAIL was also significantly greater than the sum of the activities of the two agents used separately ($P<0.001$ for each caspase).

We noted that the cleaved caspase-8 products after 3 and 6 h of TRAIL treatment were only a small fraction of the total caspase-8 protein (in Figure 5a, the panel with the cleaved products is a longer exposure of the same filter showing the procaspase-8). To investigate this further, we performed a kinetic analysis of caspase-8 activation (Figure 5d). Again, the combination of 4HPR and TRAIL enhanced the amount of cleavage product observed compared to TRAIL alone (Figure 5d compare the middle and right panels). 4HPR alone did not cause detectable cleavage of caspase-8 (Figure 5d left panel). The total amount of cleavage product was always a small fraction of the total caspase protein. Interestingly, in spite of the relatively small amount of cleavage products seen, by 4 h of incubation with the combination of 4HPR and TRAIL, there was a readily observable decrease in the amount of procaspase-8. Upon longer incubation (i.e., 8 and 16 h), both the precursor and the cleaved products decreased markedly. Similar decreases in the procaspase and cleavage products were observed for caspase-3 and caspase-9 upon longer incubations with the combination of 4HPR and TRAIL (data not shown). This result suggests that the cleaved, active caspases have a short half life, and that the steady production of more cleaved caspases leads to depletion of the procaspases in the cells that are undergoing the greatest amounts of apoptosis (e.g., the cells treated with the combination of 4HPR and TRAIL).

Binding of TRAIL to its receptors, TRAIL-R1 and -R2, results in the formation of the death-inducing signaling complex (DISC), which is formed by association of proteins such as FADD and procaspase-8 with the cytoplasmic portion of the receptors.[44] We precipitated the TRAIL receptors from lysates of cells that had been treated with TRAIL or 4HPR and TRAIL at a time when the proenzymes levels had not yet begun to decrease (e.g., 3 h) and evaluated the precipitates for the presence of TRAIL-R1 and -R2 and procaspase-8 (Figure 5e). There was no difference in the amount of receptor or procaspase-8 precipitated from either lysate. Thus, the increase in caspase-8 activation by the combination of 4HPR and TRAIL is not due to an increase in the amount of procaspase-8 recruited to the DISC.

The combination of 4HPR and TRAIL resulted in an increase of cleavage in two caspases considered initiators, caspase-8 and caspase-9. TRAIL can induce apoptosis through mitochondrial-independent and mitochondrial-dependent pathways.[29] TRAIL activates the mitochondrial-death pathway through cleavage and translocation of BH3 interacting domain death agonist (BID) into the mitochondrial membrane. The BID translocation causes mitochondrial permeability transition (MPT), leading to cytochrome *c* release and caspase-9 activation.[29] 4HPR has been characterized to induce apoptosis through the mitochondrial pathway.[21,27,28] However, in these cells, 4HPR alone did not induce detectable BID or caspase-9 activation (Figure 5a–c). TRAIL alone induced some caspase-9 activation (Figure 5a and c); however, we were unable to detect BID cleavage (Figure 5b). In contrast, the combination of 4HPR and TRAIL resulted in a marked BID cleavage and caspase-9 activation, suggesting enhanced activation of the mitochondrial pathway.

To further characterize the role of the different caspases, selective caspase inhibitors were used in three cell lines (A224, AD10, and CP70) (Figure 6a). The addition of either a caspase-8 or caspase-9 inhibitor resulted in significant inhibition of toxicity induced by the combination of TRAIL and 4HPR (Figure 6a). The toxicity of TRAIL alone was inhibited by both inhibitors in two of the three cell lines (A224 and AD10). In the third cell line (CP70), TRAIL alone induced little toxicity and the inhibitors had no significant effect. The toxicity of 4HPR alone was inhibited by the caspase-9 inhibitor, but not by the caspase-8 inhibitor.

Since apoptosis induced by the combination of 4HPR and TRAIL was inhibited with both inhibitors, this suggested that both caspases, caspase-8 and -9, were important in determining the synergism between 4HPR and TRAIL. Immunoblotting of lysates of the AD10 cell line in presence of the caspase-8 inhibitor (Z-Ile-Glu(OCH_3)-Thr-Asp(OCH_3)-fmk (Z-IETD-fmk)) or the caspase-9 inhibitor (Z-Leu-Glu(OCH_3)-His-Asp-(OCH_3)-fmk (Z-LEHD-fmk)) resulted in significant inhibition of cleavage of caspase-8, -9, and PARP induced by the combination of 4HPR and TRAIL (Figure 6b). There was still detectable cleavage of caspase-3, BID, and PARP when the cells were incubated with the caspase-9 inhibitor (Figure 6b). This is consistent with the results in Figure 6a, which demonstrate that the caspase-8 inhibitor was more effective in inhibiting apoptosis than the caspase-9 inhibitor in this cell line (Figure 6a). Caspase-9 cleavage was completely blocked by Z-LEHD-fmk. The activity of caspases in these lysates from

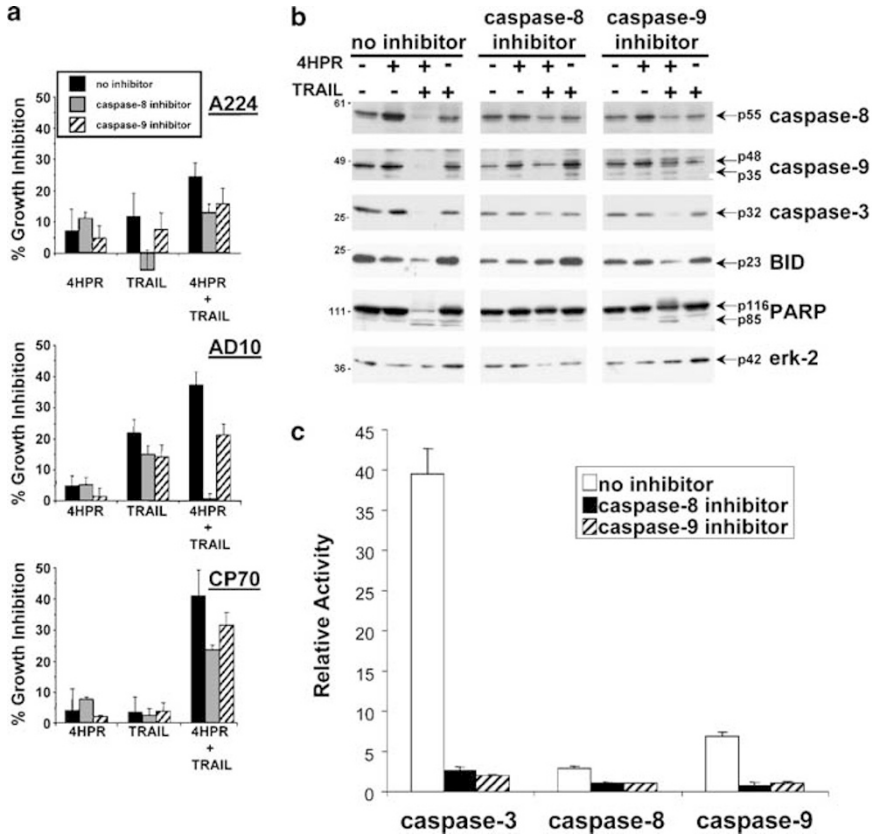


Figure 6 (**a**) Effects of caspase-8 and -9 inhibitors on TRAIL-mediated apoptosis induced by each condition. Three ovarian cancer cells (A224, AD10, and CP70) were incubated with 4HPR alone, TRAIL alone, or the combination as described above. The caspase-8 inhibitor Z-IETD-fmk (at 20 μM), the caspase-9 inhibitor Z-LEHD-fmk (at 20 μM), or DMSO were added at the time of 4HPR addition. Cell viability was measured by the MTS assay. The data represent the growth inhibition, as a percentage of control cells. Data points show mean $\pm$ S.E. for a minimum of three experiments. **(b)** Cell lysates from AD10 treated as above were prepared and immunoblotted with caspase-8, BID, caspase-9, caspase-3, and PARP as indicated to the right of the panels. Erk-2 is shown as a loading control. Molecular weights in kDa are shown to the left of the panels. **(c)** Caspases assays were performed with the indicated substrates using lysates from cells treated as in Figure 6b. Activity for each substrate was normalized to the activity in the untreated cells and the data represent the mean $\pm$ S.D. for triplicate measurements from a representative experiment

cells treated with the combination of 4HPR and TRAIL in the presence or absence of the specific caspase inhibitors was quantitated using colorimetric peptide substrates specific for caspases-8, caspase-9 or caspase-3 (Figure 6c). Both caspase-8 and caspase-9 inhibitors reduced the activation of caspases-3, -8, and -9 significantly. The reduction of both caspase-8 and caspase-9 cleavage and activity by each inhibitor suggests activation of each caspase by the other.

Since the activation of caspase-9 requires MPT and the release of cytochrome *c*,[45] we evaluated the effects of each reagent alone or in combination on the MPT and on cytochrome *c* release. We measured the mitochondrial inner transmembrane potential ($\Delta\psi_m$) as a measurement of MPT. To assess the $\Delta\psi_m$, the fluorescent dye 3,3′-dihexyloxacarbocianine iodide ($DiOC_6$)(3) was used. $DiOC_6$(3) localizes to the mitochondria and the MPT reduces the accumulation of $DiOC_6$(3) as a consequence of the loss in $\Delta\psi_m$. Treatment with 4HPR alone resulted in only slight changes in $\Delta\psi_m$ in two different cell lines (AD10 and A224) (Figure 7a and data not shown). TRAIL alone induced greater loss of $\Delta\psi_m$ than 4HPR. In contrast, the combination of 4HPR and TRAIL resulted in a marked loss of $\Delta\psi_m$. The loss of $\Delta\psi_m$ induced by the combination of 4HPR and TRAIL was statistically greater than the sum of loss of $\Delta\psi_m$ induced by each reagent alone (AD10 $P=0.01$, A224 $P=0.02$). The measurement of cytosolic cytochrome *c* and Smac/DIABLO demonstrated that 4HPR alone resulted in little or no release of these proteins from the mitochondria, whereas TRAIL alone did induce cytochrome C and Smac/DIABLO release from the mitochondria (Figure 7b). The combination of 4HPR and TRAIL resulted in greater release of these proteins than TRAIL alone.

4HPR has been described to induce MPT through different mechanisms, which included both ROS and ceramide generation.[46–48] TRAIL induces changes in mitochondrial permeability through a different mechanism which involves translocation of the cleaved p15 form of BID into the mitochondrial membrane. As a marker of ROS generation, measurement of DCFH-DA by flow cytometry was used. 4HPR alone, but not TRAIL, resulted in slight increase in ROS generation and the combination of 4HPR and TRAIL did not increase this (data not shown). Since ROS generation and ceramide production mediated by 4HPR can induce MPT, we used vitamin C (at 100 μM) and SPP-1 (at 10 μM) as inhibitors, respectively.[47,49] Neither vitamin C nor SPP-1 inhibited apoptosis induced by the combination of 4HPR and TRAIL (data not shown). These data suggested that the ROS

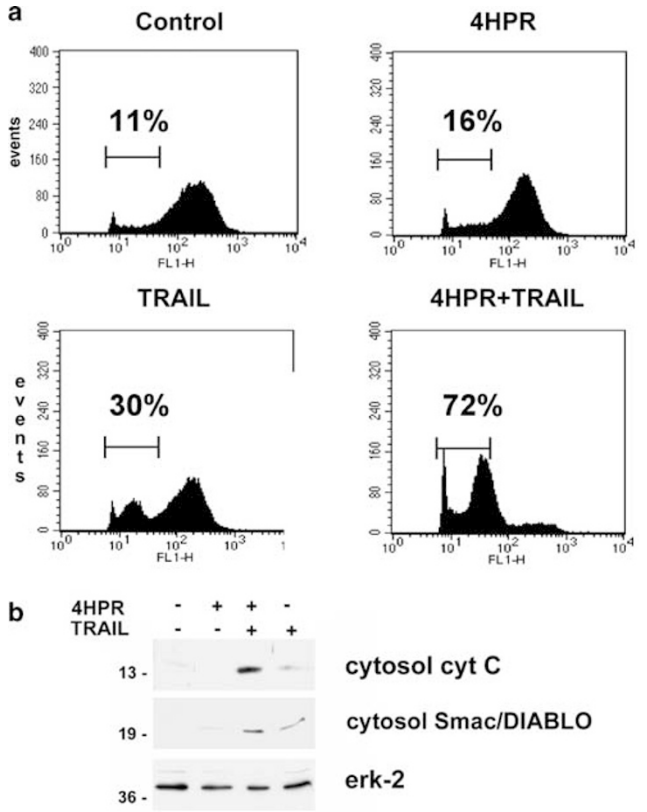


Figure 7 The combination of 4HPR and TRAIL induces mitochondrial permeability transition (measured by loss of $\Delta\psi_m$) and cytochrome *c* release. **(a)** Representative experiment of the changes in $\Delta\psi_m$ observed upon each treatment. The AD10 cell line was incubated with no addition (control), 4HPR alone, TRAIL alone, or the combination of 4HPR plus TRAIL. The cells were incubated with 4HPR (1 μM) for a total of 96 h and TRAIL (100 ng/ml) was added for the final 16 h in culture. Loss of $\Delta\psi_m$ (indicated as a percentage in each panel) was measured using $DiOC_6(3)$ staining and flow cytometry. **(b)** Cytosolic extracts from the AD10 cell line, treated as above, were prepared and immunoblotted with cytochrome *c* (cytosol cyt C) as indicated to the right of the panels. Erk-2 is shown as a loading control. Molecular weights in kDa are shown to the left of the panels

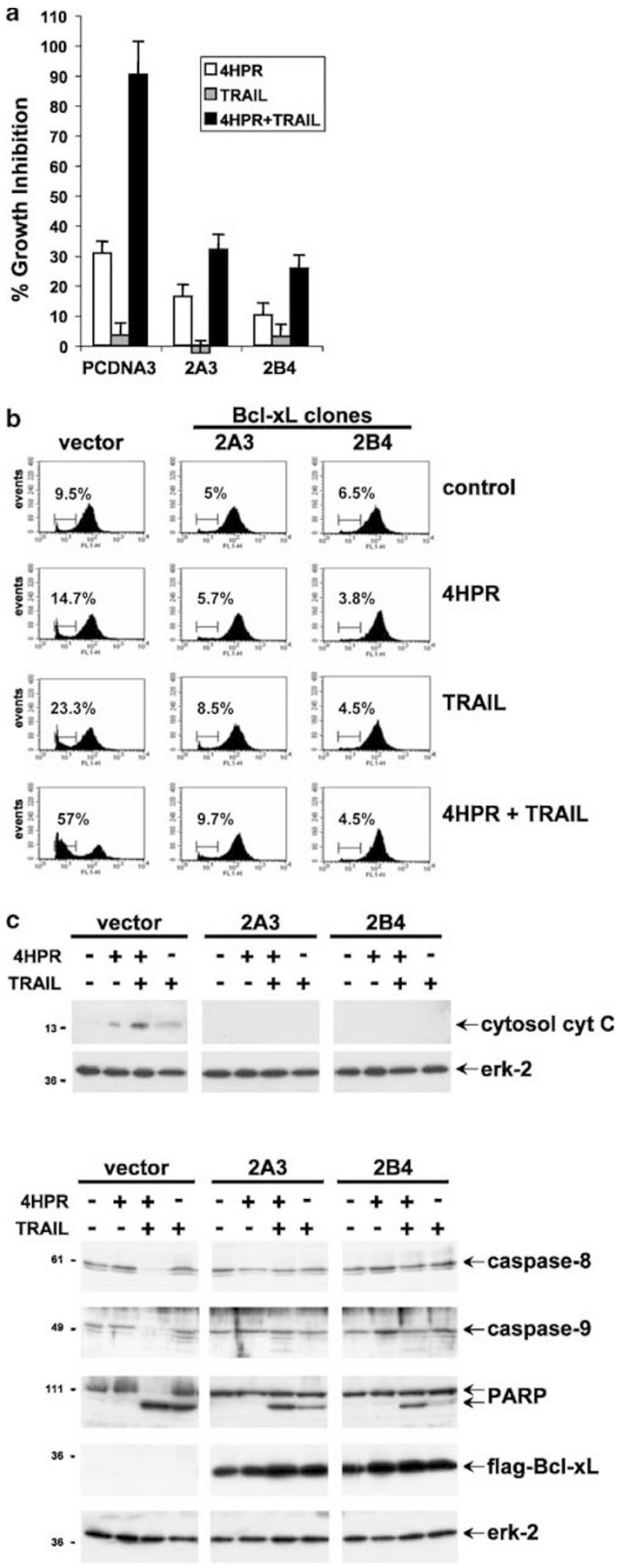
generation and ceramide production are not essential in determining the MPT induced by the combination of 4HPR and TRAIL.

The data above suggest that the combination of 4HPR and TRAIL induces increased MPT and cytochrome *c* release, resulting in increased caspase-9 activation. The inhibitor data would further suggest that the increase in caspase-8 activation is secondary to increased caspase-9 activation and *vice versa*. To demonstrate such an amplification loop, we used stable clones of the A2780 cell line overexpressing Bcl-xL.[50] Bcl-xL and Bcl-2 localize to the outer mitochondrial membrane and exert antiapoptotic effects by preventing the efflux of cytochrome *c* from the mitochondria.[51–53] Overexpression of Bcl-xL resulted in a significant reduction of toxicity induced by 4HPR alone or by the combination of 4HPR and TRAIL (Figure 8a). The measurement of $\Delta\psi_m$, using $DiOC_6(3)$ as a marker of MPT, demonstrated that the overexpression of Bcl-xL abrogated the MPT induced by each reagent alone and by the combination of 4HPR and TRAIL (Figure 8b). The overexpression of Bcl-xL also blocked the cytochrome *c* release (Figure 8c, top). Finally, immunoblotting of lysates treated under the same conditions demonstrated marked reduction of both caspase-8 and caspase-9 cleavage (as measured by the disappearance of the proenzymes) in cells treated with the combination of 4HPR and TRAIL (Figure 8c, bottom). TRAIL alone, and the combination of 4HPR and TRAIL induced some PARP cleavage in the Bcl-xL clones. However, this was reduced compared to the cleavage seen in the vector controls. Since Bcl-xL blocks the mitochondrial pathway[51–53] the inhibition of caspase-8 cleavage by the overexpression of Bcl-xL demonstrates that caspase-8 activation is occurring downstream of the mitochondria.

Discussion

Here we demonstrate that the combination of 4HPR and TRAIL results in enhanced induction of cell death in ovarian cancer cells (Figures 1a to c, 2a). Importantly, no toxicity was induced by the combination of 4HPR and TRAIL on immortalized nontumorigenic ovarian epithelial cells (Figure 1a). This enhancement in TRAIL-mediated apoptosis was specific for TRAIL since 4HPR did not increase FAS-mediated apoptosis (Figure 1d). The selective enhancement of TRAIL-mediated apoptosis by 4HPR is important because of the toxicity that FAS ligand could have on normal tissues.[54] Of note, the combination of 4HPR and TRAIL was effective at inducing cell death in cell lines which are relatively resistant to chemotherapeutic agents that are routinely used in the treatment of ovarian cancer. For example, the CP70 cell line, which is relatively resistant to cisplatinum and paclitaxel,[55,56] was effectively killed by the combination of 4HPR and TRAIL (Figure 1a). Thus, this combination may prove useful for the treatment of chemotherapy-resistant ovarian cancer.

To overcome resistance to TRAIL-mediated apoptosis, different approaches have been used. Multiple reports have shown that chemotherapy enhances TRAIL-mediated apoptosis in different tumor cell types.[6,7,9,16,17,41] We recently reported that trastuzumab could also overcome resistance to TRAIL in cell lines that overexpress the erbB-2 receptor.[39] The mechanisms behind these interactions have been explained by upregulation of the death receptors TRAIL-R1 and TRAIL-R2, decreased activity of prosurvival kinases such as Akt kinase, and/or increased caspase activation.[6,7,11,39–41] Recently, it has been shown that another retinoid, CD437, enhances TRAIL-mediated apoptosis in prostate and lung cancer cells.[57,58] The increase in TRAIL-mediated apoptosis induced by the retinoid CD437 has been explained by upregulation of TRAIL-R1 and TRAIL-R2 in lung cancer cells in a p53-dependent manner.[57] However, in prostate cancer cells, there is no clear correlation between upregulation of the death receptors and the increase in TRAIL-mediated toxicity induced by CD437.[58] Using 4HPR, we investigated multiple proteins known to modulate TRAIL sensitivity. No changes were detected in the expression of any of them, including TRAIL death receptors, FLIPs, IAPs, survivin, and different kinases involved in cell proliferation and survival (Figure 4). Also, in contrast to CD437, the interaction between 4HPR and TRAIL is likely to be p53 independent. 4HPR enhancement of TRAIL-mediated apoptosis was observed in p53 wild type



(A2780), p53 mutant (Caov-3) and p53 null (SKOV3) ovarian cancer cells.[59,60]

Treatment with the combination of 4HPR and TRAIL resulted in a significant increase in cleavage of multiple caspases involved in the death receptor pathway (Figure 5). The addition of a pan-caspase inhibitor abrogated the interaction between 4HPR and TRAIL, suggesting that the synergism is due to an increase in caspase activation (Figure 2b). The combination augmented the activation of two caspases considered initiators, caspase-8 and caspase-9 (Figure 5a and c). Recent reports have shown that both caspase-8 and -9 can be activated downstream of each other in both drug-induced and death receptor-induced apoptosis.[45,61–64] In apoptosis induced by death receptors, caspase-8 becomes active at the DISC.[44] The active caspase-8 can directly activate downstream caspases such as caspase-3 and -7. When proapoptotic stimuli act through the mitochondria, the activation of the mitochondrial-death pathway leads to MPT, release of proapoptotic proteins such as cytochrome *c* and Smac/Diablo, and activation of caspase-9.[65] Caspase-9 has been shown to activate caspase-8 in a caspase-3-dependent manner.[45,66] Caspase-8 may also activate the mitochondrial-death pathway and caspase-9 through cleavage and translocation of BID to the mitochondria.[29] Our data show that TRAIL alone activates the mitochondrial pathway, but that the combination of 4HPR and TRAIL results in a more profound activation of this pathway, as measured both by MPT, the release of proapoptotic proteins from the mitochondria, and the activation of caspase-9 (Figures 5 and 7). The use of specific caspase-8 or caspase-9 inhibitors or overexpression of Bcl-xL resulted in decreased apoptosis induced by the combination of 4HPR and TRAIL (Figures 6a and 8). These results support the idea that both caspases are important in determining the synergism, and that the activation of the mitochondrial pathway is critical to TRAIL-induced apoptosis in ovarian cancer cells. TRAIL has been shown to induce apoptosis in cells by mitochondrial-independent and -dependent pathways – so-called type I and type II cells, respectively.[29] Thus, ovarian cancer cells are type II cells. Interestingly, the caspase-8 inhibitor resulted in a decrease of caspase-9 activation and the caspase-9 inhibitor resulted in a decrease of caspase-8 activation (Figure 6b and c). Similarly, inhibition of mitochondrial activation by overexpression of Bcl-xL inhibited caspase-8 and caspase-9 activation (Figure 8). These data suggest a role for caspase-9 in caspase-8

Figure 8 Bcl-xL overexpression blocks MPT, cytochrome *c* release, and inhibits the apoptosis induced by 4HPR and TRAIL. **(a)** Stable clones overexpressing PCDNA3/FLAG-Bcl-xL (2A3 and 2B4) or the PCDNA3 vector were incubated with 4HPR alone, TRAIL alone, or the combination as described in Figure 1 above. Cell viability was measured by the MTS assay. The data represent the growth inhibition, as a percentage of control cells. Data points show mean $\pm$S.E. for a minimum of three experiments. **(b)** Measurement of loss of $\Delta\psi_m$ (indicated as percentage in each panel) in the same clones, treated as above, using $DiOC_6(3)$ staining and flow cytometry. **(c)** Top panel, cytosolic extracts from the above clones were prepared and immunoblotted with cytochrome *c* (cytosol cyt C) as indicated to the right of the panels. Lower panel, cell lysates treated as above were prepared and immunoblotted with caspase-8, caspase-9, PARP, and FLAG as indicated to the right of the panels. Erk-2 is shown as a loading control. Molecular weights in kDa are shown to the left of the panels

activation and *vice versa*. The activation of caspase-8 both upstream (at the DISC) and downsteam of the mitochondria is consistent with a mitochondrial-dependent amplification loop in the induction of apoptosis in ovarian cancer cells.

How 4HPR cooperates with TRAIL in the enhancement of caspase activation is unclear. There was no appreciable increased association of caspase-8 with the DISC in cells treated with 4HPR and TRAIL, which could account for this enhancement (Figure 5e). Inhibition of either mitochondrial activation by Bcl-xL (Figure 8) or of caspase-9 activity with peptide inhibitors (Figure 6) resulted in decreased activation of caspase-8. At higher concentrations, 4HPR alone can induce apoptosis and the MPT is important to the ability of 4HPR to kill cells.[47] It is possible that 4HPR is lowering the threshold for MPT at the mitochondria, and that this could account for the enhanced apoptosis induced by the combination of 4HPR and TRAIL. 4HPR has been described to induce MPT through different mechanisms, which included both ROS and ceramide generation.[46–48] However, inhibition of ROS or ceramide production in our cells did not block the apoptosis induced by 4HPR and TRAIL.

Interestingly, the enhancement in TRAIL-mediated apoptosis induced by 4HPR at 1 μM required preincubation with 4HPR at least for 48 h. These data suggest that the activation or inhibition of a transcriptional process is required to induce the interaction between 4HPR and TRAIL. Previous studies have shown that higher concentrations of 4HPR by itself induced apoptosis in ovarian cancer cells.[20,67] In those studies, apoptosis was detected after 24 h of incubation with 4HPR and the expression of RARβ appeared to be associated with this effect. Retinoic acid (RA) and several of the synthetic retinoids bind the ligand-activated transcription factors RAR and RXR, thereby regulating genes involved in many retinoid-dependent processes.[35] The data presented here (Figure 3) indicate that 4HPR enhances TRAIL-mediated apoptosis independently of RAR or RXR receptor pathways. First, ATRA failed to enhance TRAIL-mediated apoptosis in two of three ovarian cancer cell lines (Figure 3a). Second, RAR- and RXR-pan-antagonists did not inhibit the synergism (Figure 3b). These data together support a retinoid receptor-independent mechanism. To further characterize the dependence or independence on new protein synthesis, we attempted experiments with the protein synthesis inhibitor cycloheximide. However, the addition of cycloheximide resulted in a marked enhancement of TRAIL toxicity both alone or in combination with 4HPR. Thus, we could not evaluate the effects of cycloheximide on the enhancement in TRAIL-mediated apoptosis induced by 4HPR. It is possible that 4HPR, like cycloheximide, inhibits the expression of proteins modulating TRAIL resistance. It is also possible that 4HPR has a direct effect on the mitochondria. Studies in which purified mitochondria were treated *in vitro* with 4HPR did not demonstrate cytochrome *c* release or changes in mitochondrial respiration (unpublished observations). However, isolated mitochondria can be used only for a few hours and in our experiments cells required prolonged incubation with 4HPR to see the optimal enhancement of TRAIL-induced apoptosis. Thus, we cannot rule out a direct effect of 4HPR on the mitochondria or on mitochondrial gene expression that requires a prolonged incubation. Further studies are required to elucidate the mechanisms by which 4HPR enhances TRAIL-mediated apoptosis.

In summary, this work shows that the combination of 4HPR enhances TRAIL-mediated apoptosis in ovarian cancer cells. The concentrations of 4HPR used in these experiments are within the clinically achievable range.[68] Similarly, TRAIL concentrations of 0.1–0.25 μg/ml have been achieved in animal studies.[4] These *in vitro* experiments with cancer cell lines suggest that the efficacy and specificity of the combination of 4HPR and TRAIL should be tested in animal models, and that this combination may provide a promising therapy for ovarian cancer.

Materials and Methods

Cell lines

Seven ovarian cancer cell lines (222, A224, A547, AD10, CP70, UCI 101, and UCI 107) were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100-U/ml penicillin G, and 100-mg/ml streptomycin sulfate. Dr. G Scott Rose (University of California, CA, USA) provided the cell lines AD10, UCI 101, UCI 107, and 222. Dr. E Reed (National Cancer Institute, Bethesda, MD, USA) provided the CP70 cell line. The A224 cell line was obtained from Dr. J De Greve (Oncologisch Centrum, Brussels, Belgium). Dr. M Birrer (National Cancer Institute, Bethesda, MD, USA) provided the A547 ovarian cancer cell line and Dr. N Auersperg (University of British Columbia, Vancouver, Canada) provided the two immortalized nontumorigenic ovarian epithelial cell lines (IOSE 80 and IOSE 120).[69] The ovarian cancer cell lines, SKOV3 and Caov-3, were grown as recommended by supplier (ATCC, Manassas, VA, USA). The IOSE 80 and IOSE 120 cell lines were grown in medium containing 50% medium 199, 50% MCDB 105 (Sigma Chemical Co., St. Louis, MO, USA), 2% FBS, 2 mM glutamine, and 25 μg/ml gentamycin sulfate. The stable clones expressing pcDNA3-Flag human Bcl-xL or control pcDNA3 plasmid have been previously described[50] and were grown in DMEM supplemented with 10% FBS, 100-U/ml penicillin G, and 100 mg/ml streptomycin sulfate and 500 μg/ml G418 antibiotic. 293T cells were maintained in culture in DMEM supplemented with 10% FCS and 1% penicillin–streptomycin and were transfected with various constructs using calcium phosphate (Edge Biosystems, Gaithersburg, MD, USA), according to the protocol included with the reagents. All tissue culture reagents were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA).

Reagents

4HPR (provided by RW Johnson Pharmaceutical Research Institute, Raritan, NJ, USA) and ATRA (Sigma-Aldrich Co., St. Louis, MO, USA) were reconstituted in absolute ethanol (Warner-Graham Co., Cockeysville, MD, USA) at concentrations of 5 and 10 mM, respectively. The stock solutions were stored protected from light at -20°C. All experiments using 4HPR or ATRA were carried out under light-free conditions. The RAR pan-antagonist, LE540, and the RXR pan-antagonist, HX531 (a gift from Dr. H Kagechika, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan) were reconstituted in DMSO to a concentration of 20 mM, and the stock solution was stored at -20°C. The GST-TRAIL construct and the isolation of the GST-TRAIL fusion protein have been previously described.[6] The nontagged homotrimeric recombinant soluble Apo2L/TRAIL was generously provided by Genentech®/Immunex (San Francisco, CA, USA and Seattle, WA, USA).[32,70] The Apo2L/TRAIL (1 mg/ml) was stored frozen at -70°C in aliquots until used. The

expression vectors for full-length TRAIL-R1 and TRAIL-R2 in pCMV-Sport6 were obtained from Open Biosystems (Huntsville, AL, USA).

Cell culture

To assess TRAIL-mediated cytotoxicity upon 4HPR or ATRA treatment, cells were plated at 1×10^4 cells/well in 96-well microtiter plates and allowed to adhere to the plates overnight. The adherent cells were incubated in the presence or absence of 4HPR or ATRA for 96 h. Freshly eluted GST-TRAIL or Apo2L/TRAIL was added for the last 16 h at the indicated concentrations. Cell viability was assessed by the MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]) dye reduction assay (Cell Titer 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega). All MTS measurements were carried out simultaneously in six wells and each experiment was carried out at least three times. Results of representative experiments are given as the mean $\pm$ the standard deviation (S.D.) and of multiple experiments as the mean $\pm$ the standard error of the mean (S.E.).

The tetrapeptide caspase inhibitor Z-VAD-fmk (Biomol Research Labs. Inc.) was resuspended in DMSO (Sigma Chemical Co.) at a concentration of 1 mM. Z-VAD-fmk was added to cells at a final concentration of 50 μM 72 h and 1 h prior to TRAIL treatment. The caspase-8 inhibitor Z-IETD-fmk, and the caspase-9 inhibitor Z-LEHD-fmk (both from Enzyme Systems Products, Livermore, CA, USA) were resuspended in DMSO at a concentration of 10 mM. These inhibitors were added to the cells at a final concentration of 20 μM 72 and 1 h prior to drug treatment. Control cells were incubated with DMSO at the same concentration as tested cells. Cell viability was analyzed by the MTS assay after a 16 h incubation with TRAIL. Concurrently, cells (8×10^5) were plated onto 10-cm-diameter dishes, treated under same conditions and harvested for immunoblotting.

Flow cytometry

The total TRAIL surface binding was determined using flow cytometry by measuring the binding of GST-TRAIL or GST alone as previously described.[39] The specific surface expression of TRAIL receptors 1 and 2 was determined as previously described by measuring the binding of a mouse anti-TRAIL-R1 antibody (Santa Cruz Biotechnology) or a goat anti-TRAIL-R2 antibody (Chemicon International, Temecula, CA, USA), respectively.[39] As isotype-matched antibodies, a purified mouse IgG (BD Pharmingen) and a goat IgG were used (Santa Cruz Biotechnology), respectively. The expression of FAS death receptor was determined by measuring the binding of a mouse anti-FAS antibody (clone CH-11; Upstate Biotechnology). As a control, a nonspecific isotype-matched monoclonal antibody was used.

To assess apoptosis, cells were plated at 5×10^5 in 100-mm tissue culture dishes, allowed to adhere overnight and then treated using the same conditions described in the MTS assay. The cells were trypsinized, washed with PBS, fixed in ice-cold methanol and stored at -20°C overnight. Fixed cells were washed twice with PBS and allowed to incubate with DNAse-free RNAse (1 U/ml) (Roche Molecular Biochemicals, Indianapolis, IN, USA) for 30 min at 37°C. Following incubation, nuclei were stained with propidium iodide at 50 μg/ml (Roche Molecular Biochemicals). Stained cells were stored at 4°C and protected from light until flow cytometric analysis. Cells undergoing apoptosis were determined as a percent of cells with sub-G0/G1 DNA content in the DNA histogram compared to the total number of cells present using FACSort system (Becton Dickinson, Mansfield, MA, USA) and Cell Quest™ Software (Becton Dickinson, San Jose, CA, USA).

For determination of mitochondrial inner transmembrane potential ($\Delta\psi_m$), the cells were harvested and incubated in medium supplemented with 40 nM $DiOC_6$ (Molecular Probes, Eugene, OR, USA) for 15 min at 37°C (protected from light), following the indicated treatment conditions. The cells were analyzed by flow cytometry using the FACSort system. At least, 20 000 events were acquired per sample and computed with the Cell Quest™ Software.

Isolation and analysis of protein lysates

Cells (8×10^5) were plated onto 10-cm diameter tissue culture dishes and allowed to adhere overnight. After the cells were treated with each condition as described in the MTS assay, both floating and attached cells were harvested using PBS containing 2.5 mM EDTA or alternatively PBS supplemented with 0.2 mM sodium vanadate. Protein was extracted from cells by detergent lysis (1% Triton-X 100, 10 mM Tris-HCl (pH7.5), 150 mM NaCl, 5 mM EDTA, 10% glycerol, 2 mM sodium vanadate, and protease inhibitors (Mini tabs®, Roche Molecular Biochemicals)). Protein lysates were cleared of debris by centrifugation at $15\,000 \times g$ for 10 min at 4°C and the concentration was assessed by the Biorad colorometric assay (Bio-Rad). Protein samples were boiled in an equal volume of sample buffer (20% glycerol, 4% SDS, 0.2% bromophenol blue, 125 mM Tris-HCl (pH 7.5), 640 mM β-mercaptoethanol), fractionated by 10–12% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Rabbit polyclonal anti-caspase 3 specific for the procaspase 3 or for the 18 kDa cleavage product (1 μg/ml; BD PharMingen, San Diego, CA, USA), anti-caspase 9 (1 μg/ml; Cell Signalling, Beverly, MA, USA), anti-PARP antibody (1 μg/ml; H-250, Santa Cruz Biotechnology), anti-TRAIL-R1 antibody (1 μg/ml; BD PharMingen, San Diego, CA, , USA), anti-TRAIL-R2 antibody (1 μg/ml; Imgenex, San Diego, CA, USA), anti-TRAIL-R3 antibody (1 μg/ml; Affinity bioreagents, Golden, CO, USA), anti-TRAIL-R4 antibody (1 μg/ml; Oncogene Research Products, Cambridge, MA, USA), anti-IAP-1 antibody (1 μg/ml; R&D Systems Inc., Minneapolis, MN, USA), anti-IAP-2 antibody (1.5 μg/ml; R&D Systems Inc.), anti-FLIP antibody (2 μg/ml; Calbiochem), anti-survivin antibody (1 μg/ml; R&D Systems Inc.), anti-erk-2 antibody (1 μg/ml; C-14, Santa Cruz Biotechnology), anti Smac/DIABLO (1 μg/ml; Imgenex), mouse monoclonal anti-caspase 7 (1 μg/ml; BD PharMingen), anti-caspase 8 (1 μg/ml; clone 5F7, Upstate Biotechnology, Lake Placid, NY, USA), anti-XIAP antibody (2 μg/ml; BD PharMingen), anti-cytochrome *c* antibody (1 μg/ml; BD PharMingen), anti-FLAG (10 μg/ml; clone M5, Sigma Chemical Co.), anti-α-tubulin antibody (0.5 μg/ml; Sigma Chemical Co.), goat polyclonal anti-caspase 10 (1 μg/ml; C-16, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-BID antibody (1 μg/ml; C-20, Santa Cruz Biotechnology) were used for immunoblotting. Horseradish peroxidase-conjugated donkey anti-rabbit and anti-mouse (1 : 5000 dilution; Amersham Pharmacia Biotech Inc.) and rabbit anti-goat (1 : 10 000 dilution; Santa Cruz Biotechnology) antibodies were used to visualize immunoreactive proteins using SuperSignal detection reagent (Pierce, Rockford, IL, USA).

To analyze cytosolic cytochrome *c* release, cells were treated as described above and cytosolic extracts were prepared as previously described.[71] Briefly, cell pellets were washed once with PBS and once with buffer A (0.25 M sucrose, 30 mM Tris-HCl (pH 7.9), and 1 mM EDTA). After a brief centrifugation at $750 \times g$ for 5 min at 4°C, the cells were resuspended in buffer B (buffer A plus protease inhibitors (mini tabs®, Roche Molecular Biochemicals)) in an 1.5 ml microcentrifuge tube, incubated 30 min on ice, and homogenized with a glass dounce homogenizer and a B pestle (35–40 strokes) (LABGLASS, Vineland, NJ, USA). Unlysed cells and nuclei were removed by centrifugation at

750 × *g* for 10 min at 4°C in a microcentrifuge. Supernatants were collected and centrifuged at 16 000 × *g* for 45 min at 4°C. The supernatant from this final centrifugation represented the cytosolic fraction and was stored at −70°C.

DISC analysis was performed by incubating cells with TRAIL with our without 4HPR for 2–3 h and then lysates were prepared as above. Lysates were incubated in solution with 5 μg of purified GST-fusion proteins prebound to agarose–GSH beads (Pharmacia) for 1 h with tumbling at 4°C, washed five times, boiled in sample buffer, and fractionated by 10% SDS-PAGE. Samples were immunoblotted for TRAIL-R1, -R2, and procaspase-8.

Caspase activity

Protein lysates were prepared as described above. Caspase activity was assayed in these protein lysates using colorimetric peptide substrates for caspase-3, caspase-8/10, and caspase-9 (Ac-DEVD-pNA, Ac-IETD-pNA, Ac-LEHD-pNA, respectively) under conditions recommended by the manufacturer (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA, USA). Experiments were performed in triplicate and the activity for each caspase was normalized to the activity of the untreated cells.

Statistical analysis

Statistical comparison of mean values was performed using the paired and unpaired Student's *t*-tests. All *P*-values are two tailed. Interactions between TRAIL and 4HPR were classified using the Fractional Inhibition Method as follows: when expressed as the fractional inhibition of cell viability, additive inhibition produced by both inhibitors (i) occurs when $i_{1,2} = i_1 + i_2$; synergism when $i_{1,2} > i_1 + i_2$; and antagonism when $i_{1,2} < i_1 + i_2$.[30] Synergism was confirmed by dose–effect analysis and the combination index using CalcuSyn software (Biosoft, Cambridge, UK).[31] By this method, a combination index equal to 1 indicates an additive effect, a combination index less than 1 indicates synergy, and a combination index greater than 1 indicates antagonism. The combination index was calculated at different 'effect levels' or 'fraction affected' levels (LC_{50} (i.e., concentration lethal to 50% of the cells) to LC_{99}). The mutually nonexclusive assumption was used in these analyses.

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