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Chemotherapy overcomes TRAIL-R4-mediated TRAIL resistance at the DISC level

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Abstract

Apo2L/TRAIL is a promising anti-cancer drug owing to its ability to trigger apoptosis by binding to TRAIL-R1 or TRAIL-R2, two membrane bound receptors that are often expressed by tumor cells. TRAIL can also bind non-functional receptors such as TRAIL-R4, but controversies still exist regarding their potential to inhibit TRAIL-induced apoptosis.

We show here that TRAIL-R4, expressed either endogenously or ectopically, inhibits TRAIL induced apoptosis. Interestingly, the combination of chemotherapeutic drugs with TRAIL restores tumor cell sensitivity to apoptosis in TRAIL-R4 expressing cells. This sensitization, which mainly occurs at the DISC level, through enhanced caspase-8 recruitment and activation, is compromised by c-FLIP expression and is independent of the mitochondria.

Importantly, TRAIL-R4 expression prevents TRAIL-induced tumor regression in nude mice, but tumor regression induced by TRAIL can be restored with chemotherapy.

Our results clearly support a negative regulatory function for TRAIL-R4 in controlling TRAIL signaling, and unveil TRAIL-R4's ability to cooperate with c-FLIP to inhibit TRAIL-induced cell death.

Author Keywords TRAIL ; chemotherapy ; cancer

Introduction

TRAIL (TNF-Related Apoptosis Inducing Ligand or Apo2L) is a promising tool for cancer therapy, owing to its ability to eradicate tumor cells while sparing normal cells 1 . TRAIL is a type II transmembrane protein, whose binding to its agonistic receptors namely TRAIL-R1 (DR4) and TRAIL-R2 (DR5, TRICK2 or KILLER) triggers apoptosis in a p53-independent manner. Engagement of TRAIL agonistic receptors induce the formation of a molecular platform called the DISC (Death-Inducing Signaling Complex) within minutes, through homotypic interactions 2 . This platform includes the adaptor protein FADD, and caspase-8, an initiator caspase that is activated and subsequently released from the DISC to the cytosol for the dismantling of the cells. The amount of caspase-8 generated within the DISC in type I cells is sufficient to trigger apoptosis through the direct activation of the effector caspase-3. Type II cells require the engagement of a mitochondrial amplification loop, which is activated by caspase-8-dependent cleavage of Bid, a BH3-only protein that targets the intrinsic pathway through Bax and Bak, allowing the formation of the apoptosome. However, enforced aggregation of TRAIL agonistic receptors in these cells enhances caspase-8 activation at the DISC level and overcomes mitochondrial checkpoints 3 . Likewise, enhanced caspase-8 recruitment and activation at the TRAIL DISC by chemotherapeutic drugs has been associated with the restoration of TRAIL sensitivity in hepatocellular and colon carcinomas 4 , 5 .

Cellular resistance to TRAIL-induced cell death arises from a large variety of events, ranging from defects in DISC formation, or inhibition of more distal events, including mitochondrial block 6 , 7 .

TRAIL-induced cell death can be specifically inhibited by two membrane bound antagonistic receptors, TRAIL-R3 (DcR1, LIT or TRID) or TRAIL-R4 (DcR2 or TRUNDD)2 . These receptors have been shown to be expressed and to prevent TRAIL-induced cell death

in various human primary tumor cells, including lymphomas, lung, breast and prostate carcinomas 8 –10 , but the inhibitory potential of this receptor still remain controversial 11 . While TRAIL-R3 is a GPI anchored receptor that sequesters TRAIL into lipid rafts, TRAIL-R4 interacts with TRAIL-R2 within the DISC, and impairs caspase-8 processing 12 , inhibiting thus TRAIL-induced apoptosis 13 , 14 .

The efficacy of recombinant hAPO2L/TRAIL in association with chemotherapy is evaluated in ongoing clinical trials 1 . It remains unknown whether TRAIL-R4 expression may compromise the efficacy of TRAIL.

We demonstrate here that TRAIL-R4 efficiently inhibits TRAIL, and that chemotherapeutic drugs can overcome this resistance. Restoration of apoptosis primarily occurs at the membrane level, irrespective of the mitochondria, through enhanced caspase-8 recruitment and activation at the TRAIL DISC. TRAIL-R4 expression also impairs TRAIL-induced tumor regression in vivo , but sequential treatments associating CDDP and TRAIL prevent tumor growth in nude mice. Altogether, our results demonstrate that TRAIL-R4 is a negative regulator of TRAIL, whose inhibitory function can be overcome by chemotherapy.

Results

TRAIL and chemotherapeutic drugs synergistically induce apoptosis in TRAIL-R4 expressing cells

We have demonstrated previously that ectopic expression of TRAIL-R4 impairs TRAIL-induced cell death through the formation of a heteromeric complex with TRAIL-R2, leading to the inhibition of caspase-8 activation within the TRAIL DISC 12 . Owing to TRAILR4's ability to inhibit TRAIL-induced cell death, we evaluated whether its expression may compromise combination therapies associating TRAIL with conventional chemotherapeutic drugs. To test this hypothesis, TRAIL-R4 was ectopically expressed using retroviruses in three TRAIL-sensitive tumoral cell lines, HeLa, Jurkat and SW480. Cell surface expression was assessed by flow cytometry (Fig. 1A and B). TRAIL-R4 inhibited TRAIL-induced apoptosis in these cells (Fig. 1C and D). Strikingly, TRAIL-R4 also inhibited death induced by chemotherapeutic drugs in some cell lines. Pretreatment with pharmacological concentrations of CDDP, VP16 for 3 hours or 5FU for 72 hours, however, restored TRAIL sensitivity in these cells (Fig. 1C and D). Similar results were obtained in the B lymphoma cell line VAL, which are poorly sensitive to TRAIL-induced cell death (Fig. 1E). VAL cells endogenously express TRAIL-R4 at the cell surface and high levels of Bcl-2, due to the t(14;18) chromosomal translocation (Fig. 1F and G). Cells sensitivity to TRAIL-induced cell death was restored in VAL cells after pretreatement with CDDP, VP16, or 5FU (Fig. 1E). siRNA-mediated downregulation of TRAIL-R4 or Bcl-2 expression in VAL cells also restored sensitivity to TRAIL (Fig. 1F and G), demonstrating that both TRAIL-R4 and Bcl-2 are functional in these cells.

Sequential chemotherapy and TRAIL treatments restore caspase activation

Chemotherapeutic drugs enhanced caspase activation upon TRAIL stimulation (Fig. 2A) without changing TRAIL receptor expression (Fig. S1). In HeLa control cells, TRAIL alone triggered the activation of caspase-8, caspase-9 and caspase-3 and induced Bid and PARP cleavage, as demonstrated by the disappearance of their proform or the appearance of cleaved fragments (Fig. 2A). However, in HeLa cells expressing TRAIL-R4 (H-TRAIL-R4 cells), TRAIL induced only a modest cleavage of caspase-8 and caspase-9, resulting in the poor activation of caspase-3 (Fig. 2A). Pretreatment of these cells with CDDP, VP16, or 5FU restored caspase-3 activation upon TRAIL stimulation, as demonstrated by the appearance of the caspase-3 p17 fragment and an increase in PARP cleavage (Fig. 2A). Restoration of caspase-3 activation by chemotherapeutic drugs in HeLa-TRAIL-R4 cells was associated with partial activation of both caspase-8 and caspase-9 (Fig. 2A).

Activation of the mitochondrial intrinsic pathway is not required to restore sensitivity to TRAIL in response to chemotherapy

Since most chemotherapeutic drugs engage the mitochondrial pathway to trigger apoptosis, we next analyzed its contribution with regard to chemotherapy-mediated sensitization to TRAIL-induced cell death. TRAIL stimulation in control HeLa cells triggered the activation of the intrinsic pathway, as evidenced by the disappearance of Bid (Fig. 2A), the release of cytochrome c, Smac/DIABLO and omi to the cytosol (Fig. 2B), and by the activation of Bax (Fig. 2D and E). Release of cytochrome c, Smac/DIABLO and omi were much weaker in H-TRAIL-R4 cells as compared to control cells (Fig. 2C), however, chemotherapy combined with TRAIL nearly completely restored Bax activation in these cells (Fig. 2D and E). Overexpression of Bcl-2 or Bcl-xL in H-TRAIL-R4 failed to protect cells from TRAIL-induced apoptosis after chemotherapy (Fig. 3A and B). These results are consistent with the demonstration that chemotherapeutic drugs can restore TRAIL sensitivity in VAL cells, despite large amounts of Bcl-2 expression (Fig. 1F). To determine the role of Bax in drug-mediated sensitization to TRAIL-induced cell death, we performed the same experiments in the Bax-deficient or parental wt HCT116 cells engineered to express TRAIL-R4 (Fig. 3C). TRAIL-mediated apoptosis in HCT116 cells was shown to rely on Bax- but not Bak-activation 15 . According to these findings, TRAIL alone, or simultaneous combinations of TRAIL and 5FU, failed to induce apoptosis in Bax-deficient cells (Fig. 3D and S2). Nevertheless, pretreatment for 72 h with 5FU before adding TRAIL efficiently induced cell death in these cells (Fig. 3D). Likewise, treating Bax-deficient cells sequentially for 3 h with CDDP or VP16, and stimulating with TRAIL, 48 h after the onset of the treatment in drug-free medium (see material and methods) restored TRAIL-induced apoptosis (Fig. 3D

). As in HeLa cells, sequential use of chemotherapy and TRAIL afforded sensitization to TRAIL-induced cell death in Bax-proficient cells expressing TRAIL-R4 ectopically (Fig. 3D). However, in the absence of Bax, TRAIL-R4 overexpression induced resistance to TRAIL after CDDP or VP16 pretreatment, but not upon 5FU stimulation (Fig. 3D).

Sensitization to TRAIL-induced cell death by 5FU has previously been described to involve the deregulation of c-FLIP 4 , 16 . We therefore analyzed c-FLIP expression after chemotherapy at the time when the cells were exposed to TRAIL stimulation. Contrary to our expectations, we found that 5FU poorly induced c-FLIP deregulation in our settings (Fig. 3E), but that CDDP and VP16 induced c-FLIP up-regulation in these cells (Fig. 3E). Consistent with these findings, expression of c-FLIP abrogated sensitization to TRAIL after 5FU treatment, irrespective of TRAIL-R4 or Bax expression in HCT116 cells (Fig 3F), indicating that the mere up-regulation of c-FLIP is probably sufficient to impair the synergy irrespective of the mitochondria. In agreement with this finding, the caspase-9 inhibitor z-LEHD-fmk failed to protect TRAIL-R4 expressing cells from TRAIL-induced cell death after chemotherapy, while the pan-caspase inhibitor z-VAD-fmk completely abrogated the synergy (Fig. S3).

Chemotherapeutic drugs enhance caspase-8 recruitment and activation within the TRAIL DISC

To determine the contribution of TRAIL DISC formation and caspase-8 activation in the synergy, c-FLIP was co-expressed in H-TRAIL-R4 cells and cells were stimulated with TRAIL after chemotherapy. Like TRAIL-R4, expression of c-FLIP (Fig. 3G) reduced cell sensitivity to TRAIL, but alone failed to block apoptosis induced by TRAIL upon chemotherapy (Fig. 3H). However, combined expression of TRAIL-R4 and c-FLIP severely impaired TRAIL-induced apoptosis by chemotherapeutic drugs, indicating that activation of caspase-8 per se plays a prominent role in the synergy (Fig. 3H). In agreement with these findings, DISC analysis in cells subjected to chemotherapy and TRAIL treatments revealed that chemotherapeutic drugs enhanced caspase-8 recruitment and activation within the TRAIL DISC in HeLa control cells (Fig. 4A) but, probably more importantly, also in HeLa expressing TRAIL-R4 (Fig 4B) and VAL cells (Fig. 4C), which express TRAIL-R4 endogenously. Altogether, these results clearly demonstrate that TRAIL-R4 inhibits TRAIL-induced cell death, but that chemotherapy can restore tumor cell sensitivity to apoptosis, mainly through the restoration of caspase-8 recruitment and activation within the DISC.

TRAIL-R4 inhibits TRAIL-induced cell death but not chemotherapy induced sensitization to TRAIL in vivo

TRAIL-R4's ability to prevent TRAIL-induced tumor regression, combined or not with chemotherapy, was next evaluated in nude mice using xenografts of HCT116 cells expressing TRAIL-R4. Mice were implanted, in both flanks, with HCT116-Ctl cells (right flank) and HCT116-TRAIL-R4 (left flank). When the tumor volume reached 20 mm^3 , mice were treated with PBS, CDDP, recombinant TRAIL or treated sequentially with CDDP and TRAIL as described in the material and methods section. Compared to PBS-treated mice, HCT116-Ctl tumor growth was inhibited in mice receiving injections of TRAIL, CDDP and by the combined treatment (Fig. 5A). However, TRAIL, and to a lesser extent CDDP, failed to induce tumor regression in TRAIL-R4 expressing cells (Fig. 5B), but combined treatments induced a marked inhibition of the tumor growth of HCT116 cells expressing TRAIL-R4 (Fig. 5B), with statistically significant P values <0.001 as compared to PBS-treated mice. These results demonstrate that TRAIL-R4 efficiently inhibits TRAIL-induced cell death not only in vitro , but also in vivo . However, chemotherapeutic drugs, including CDDP, can overcome TRAIL-R4 mediated resistance, highlighting the potential therapeutic value of these combined therapies for cancer.

Discussion

TRAIL-based combinatorial therapies are emerging paradigms for cancer treatment since synergistic activation of TRAIL-induced apoptosis by chemotherapeutic drugs generally affords to overcome tumor cell resistance, while monotherapies are most of the time poorly successful. Preclinical studies and clinical trials are giving promising results, supporting the potential of these combining approaches 17 , 18 .

Cell surface expression of TRAIL agonistic receptors is the first requirement in order to trigger the TRAIL apoptotic machinery but, to date, the expression of TRAIL receptors in primary tumors remains poorly studied and the anti-apoptotic function of TRAIL-R4 remains controversial. It was found however in a few studies that primary lymphomas could express functional TRAIL antagonistic receptors at the cell surface 10 . In solid tumors, analysis of TRAIL receptor expression was often performed by immunohistochemistry, and although this method does not provide the information whether the receptors are expressed at the cell surface, these studies indicate that the extent of expression of the antagonistic receptors TRAIL-R3 and TRAIL-R4 is probably underestimated 9 , 19 –21 .

Engagement of apoptosis upon TRAIL stimulation in a given tissue type, primary tumour or cell line, relies on the contribution of multiple players, including proapoptotic and prosurvival factors, which ultimately determine cell fate. It has recently been demonstrated that naturally occurring differences in the levels or states of proteins regulating TRAIL signaling are the primary causes of cell-to-cell variability 22 . The large variety of cellular changes in protein levels or status induced by chemotherapeutic drugs may explain why these

drugs, restore TRAIL sensitivity in resistant cells, albeit targeting different signaling pathways. Sensitization to TRAIL by chemotherapeutic drugs, has been attributed to multiple molecular mechanisms including the up-regulation of TRAIL-R2 23 , activation of the mitochondrial pathway 24 , 25 , inhibition of c-FLIP expression 26 or enhanced caspase-8 recruitment to the TRAIL DISC 4 , 5 .

We demonstrate here that chemotherapy overcomes TRAIL resistance induced by TRAIL-R4 at the level of the DISC, and provide strong evidence that the mitochondrial pathway is dispensable for the restoration of TRAIL sensitivity by chemotherapeutic drugs (Fig. 6). Chemotherapeutic drugs afforded sensitization to TRAIL of aggressive B-cell follicular lymphomas such as VAL cells, despite endogenous expression of functional TRAIL-R4 and Bcl-2. Likewise, these compounds restored TRAIL sensitivity of epithelial derived tumor cell lines harboring either a deficiency for Bax expression, or engineered to express Bcl-2 or Bcl-xL, irrespective of TRAIL-R4 expression levels. Bax deficiency however, may be detrimental to some chemotherapeutic drugs in cells that express TRAIL-R4 and low but significant levels of c-FLIP. Accordingly, restoration of TRAIL sensitivity in HCT116 Bax-deficient cells expressing TRAIL-R4 was only observed with 5FU, but not CDDP or VP16 due to their ability to induce c-FLIP expression. These results could explain some discrepancies regarding the lack of correlation regarding TRAIL-R4 expression and cell sensitivity to TRAIL-induced cell death. In particular, c-FLIP expression levels have scarcely been taken into consideration in these studies.

Our results demonstrate that TRAIL-R4 can inhibit TRAIL-induced cell death both in vitro and in vivo and cooperate with c-FLIP to inhibit chemotherapy-mediated sensitization to TRAIL-induced apoptosis (Fig. 6). These findings have important implications for the understanding of the molecular mechanisms involved in the regulation of TRAIL signaling, but also for therapeutic approaches aiming at utilizing recombinant TRAIL to cure patients suffering from cancer.

The physiological function and relevance of TRAIL-R4 is still unclear. Our study is probably the first demonstration that TRAIL-R4 can confer TRAIL resistance in vivo , as we demonstrate that ectopic expression of this receptor in the colon carcinoma cell line HCT116 efficiently impairs TRAIL-induced tumour killing in nude mice. At the physiological level, TRAIL-R4 could protect cells selectively from TRAIL-induced cell death. Noteworthy, it has been demonstrated that NK and CD8+ T cells are induced to express TRAIL, TRAIL-R2, TRAIL-R4 and c-FLIP upon activation 27 . Despite high expression levels of TRAIL, these cells are resistant to TRAIL, but selective inhibition of c-FLIP expression induced TRAIL sensitivity 27 . It should be noted however that selective TRAIL-R4 downregulation was not assessed in this study, therefore, it is conceivable that TRAIL-R4 may also play a role in protecting these cells from TRAIL-induced cell death. While the function of TRAIL-R4 remains to be determined in a physiological context, our results indicate that this receptor in pathological conditions, such as overexpression in primary tumor cells, could represent a problem in oncology. Our results clearly support TRAIL-R4's inhibitory potential and, in agreement with previous studies 4 , 5 , sustain the demonstration that chemotherapy sensitize tumor cells to TRAIL mainly through the regulation of caspase-8 activation at the DISC level.

The molecular mechanisms leading to the restoration of caspase-8 recruitment and enhancement of caspase-8 activation within the TRAIL DISC after chemotherapy remains an open question. Some reports indicate that chemotherapeutic drugs could enhance TRAIL receptor clustering at the cell surface, through ceramide production and receptor partitioning into lipid rafts 28 , 29 . Work is currently in progress to address this question in our laboratory.

Remarkably, like c-FLIP 30 , 31 , ectopic expression of TRAIL-R4 induced cross-resistance to some chemotherapeutic drugs in vitro and in vivo . How TRAIL-R4 impairs chemotherapy-induced apoptosis remain to be determined. Some reports point to the observation that forced aggregation of some death receptors of the TNF family including Fas, or downstream effectors like Bid, may contribute to genotoxic drug-induced apoptosis in a ligand independent manner 31 –33 . Combined expression of TRAIL-R4 and c-FLIP may therefore not only impair TRAIL-induced cell death after chemotherapy, but may also alter chemotherapy itself. In line with this hypothesis it has been demonstrated that c-FLIP and TRAIL-R4 are overexpressed in a growing number of primary tumors that their expression levels could be associated with has recently been defined as a poor prognostic marker in colorectal 34 and prostate cancer patients 35 .

Altogether our results clearly demonstrate that TRAIL-R4 is a negative regulator of TRAIL whose inhibitory function can be overcome using chemotherapy to restore TRAIL-induced cell death. However, we also demonstrate that TRAIL-R4 cooperates with c-FLIP to inhibit TRAIL-induced apoptosis after chemotherapy. Their ability to cooperate and to efficiently inhibit TRAIL-induced apoptosis needs to be taken into consideration both in vitro and in future clinical trials to assess the efficacy of combinatorial treatments associating recombinant TRAIL with chemotherapy. It is anticipated that patients expressing both TRAIL-R4 and c-FLIP may respond better to alternative therapeutic approaches, including non-conventional chemotherapeutic drugs, TRAIL derivatives, targeting specifically TRAIL-R2 specifically, or to strategies aiming at inhibiting c-FLIP expression or blocking TRAIL-R4.

Material and methods

Ligand production and antibodies

Flag-tagged recombinant soluble human TRAIL, his-tagged TRAIL and FasL were produced and used as described previously 36 . Anti-Flag (M2) and staurosporin were from Sigma-Aldrich (Lyon, France). For western blot analysis, anti-TRAIL-R1, anti-TRAIL-R2,

anti-TRAIL-R3 and anti-TRAIL-R4 antibodies were purchased from Chemicon (Millipore, Molsheim, France), anti-FADD was obtained from Transduction Laboratories (BD biosciences, Le Pont de Claix, France), anti-caspase-8 and -10 were from Medical & Biological Laboratories (Clinisciences, Montrouge, France). Antibodies against active cleaved fragment of caspase-3, and PARP were from Cell Signaling (Millipore, Molsheim, France), anti-GFP, Bcl-2, cytochrome c, Bax (N-20) and HSC-70 from Santa Cruz Biotechnology (Tebu-bio, Le Perray en Yvelines, France) and anti-caspase-9 was from Upstate (Millipore, Molsheim, France). Anti-Bid, anti-Bcl-xL and anti-FLIP (NF6) antibodies were purchased from BD Pharmingen, Transduction Lab (BD biosciences, Le Pont de Claix, France), Calbiochem (VWR, Fontenay-sous-Bois, France) and Alexis (Coger, Paris, France) respectively. Anti-CoxII, anti-Smac/DIABLO and anti-Omi/HtrA2 were respectively from Molecular probes (Invitrogen, Cergy Pontoise, France), Proscience (Coger, Paris, France) and R&D systems (Lille, France). For flow cytometry experiments, the anti-TRAIL-R1, anti-TRAIL-R2, anti-TRAIL-R3 and anti-TRAIL-R4, (clones wB-K32, B-L27, wB-B44 and wB-P30 respectively), were kindly provided by Diaclone (Besançon, France). The secondary antibody was an Alexa-488 coupled-goat anti-mouse from Molecular Probes (Invitrogen, Cergy Pontoise, France). The pan caspase inhibitor (z-VAD-fmk) and caspase-9 inhibitor (z-LEHD-fmk) were purchased from Alexis (Coger, Paris, France).

Cell culture

The HeLa (human cervix carcinoma) and SW480 (human colon adenocarcinoma) cell lines were cultured with high glucose Dulbecco's modified Eagle's medium medium (Lonza, Levallois-Perret, France) supplemented with 10% fetal bovine serum (Lonza, Levallois-Perret, France) and penicillin/streptomycin (100 mg/ml of each). The Jurkat (human T lymphoma) cells, VAL (human B lymphoma), HCT116 human colon adenocarcinoma cell lines were cultured in RPMI 1640 medium (Lonza, Levallois-Perret, France) containing 10% fetal bovine serum and penicillin/streptomycin. All these cell lines were grown in 5% CO_2 at 37°C. HCT116 Bax+/− or Bax−/− are kind gifts of Dr. Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD).

Retrovirus production and cell transduction

The retroviral vector pMSCV-puro for TRAIL-R4 expression and generation of viruses has been previously described 37 . Cells were transduced for 16 hours with viral supernatants containing polybrene (8 mg/ml), washed in phosphate-buffered saline, and cultured in complete medium containing puromycin (2.5 mg/ml). EGFP, $FLIP_L$ and Bcl-2 were cloned into pBabe-Blasticidin. Transduced cells were then selected with blasticidin (2.5 μg/ml). pMIG-Bcl-xL expression vector38 was purchased from addgene (plasmid 8790, Cambridge, MA, USA). pMIG-$FLIP_L$ was obtained as previously described14 . After transduction cells were sorted using a cell sorter Coulter Epics Elite ESP.

Treatments with chemotherapy and TRAIL

For sequential treatments, cells were treated for 3 hours with CDDP (20 μM) or VP16 (10 μM) in serum free medium and then washed. Cells were cultured 48 hours in complete medium before being treated for 6 hours with His-TRAIL (500 ng/ml). 5FU was added in complete medium 72 hours before TRAIL treatments and the Hoechst analysis.

Hoechst analysis

Apoptosis was assessed by Hoechst staining and determination of the percentage of condensed and fragmented nuclei from at least 300 cells per conditions. Experiments were repeated at least 3 times.

Bcl-2 and TRAIL-R4 gene silencing by siRNA

TRAIL-R4 siRNA #1 (5′-UCCUUAAGUUCGUCGUCUU-3′), TRAIL-R4 siRNA #2 (5′-UCACUACCUUAUCAUCAUA-3′) and TRAIL-R4 siRNA #3 (5′-GGGUGUGGAUUACACCAUU -3′) were purchased from Eurogentec (Angers, France). Bcl-2 siRNA was purchased from Invitrogen (Cergy Pontoise, France). Cells were transfected with a scramble, Bcl-2 or TRAIL-R4 targeting siRNAs using Amaxa cell line nucleofector kit V (Lonza, Levallois-Perret, France) with transfection program N016. 48 hours post transfection Bcl-2 and TRAIL-R4 expression were monitored either by western blotting or by flow cytometry and sensitivity to TRAIL was assessed by Hoechst.

Bax activation by flow cytometry analysis

Cells, treated or untreated with His-TRAIL and/or chemotherapy were fixed with 4% PFA, permeabilized (PBS, BSA 1%, saponin 0.1 %) for 10 minutes at room temperature and stained with an anti-Bax antibody which recognizes the active N-terminal form of Bax (clone 6A7, Tebu-bio, Le Perray en Yvelines, France). 10 000 events were analysed using a LSR2 flow cytometer (BD Biosciences, Le Pont de Claix, France).

Digitonin permeabilisation

After treatment, cells were washed in PBS and lysed in buffer containing 75 mM KCl, 1mM NaH_2PO_4, 8 mM Na_2HPO_4 and 250 mM Sucrose containing 400 μg/ml digitonin. Cells were kept on ice upon to reach 90–95% of trypan blue permeabilized cells. After 5 minutes

at 16 000g supernatants were collected as the cytosolic fraction. Pellets were then lysed in buffer containing 1% Triton-X100. After centrifugation for 20 minutes at 16 000 g, supernatants were collected.

Immunoprecipitations

For DISC analysis, 10^8 cells in 1 ml of medium were stimulated with 5 μg Flag-TRAIL cross-linked with 10 μg of M2 antibody for the indicated times at 37°C. Cells were then washed with cold phosphate saline buffer, lysed in 1 ml of lysis buffer containing 1 % NP40, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 10 % glycerol. Lysates were precleared with Sepharose 6B (Sigma-Aldrich), and immunoprecipitated overnight at 4°C with G protein Sepharose beads (Amersham Biosciences, Les Ullis, France). Beads were then washed four times with the respective detergent, and immunoprecipitates were eluted in lysis buffer (Tris-HCl 63 mM; SDS 2 %; phenol red 0.03 %; glycerol 10 %; DTT 100 mM; pH 6.8), boiled for 5 minutes and processed for immunobloting.

Western blotting

Immunoprecipitates or cell lysats were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific binding sites were blocked by incubation in PBS containing 0.05% of Tween 20 and 5 % of milk powder. Immunoblots were then incubated with specific primary antibody followed by HRP-conjugated secondary antibody and were developed by the enhanced chemiluminescence method according to the manufacturer's protocol (Pierce, Rockford, IL).

In vivo *evaluation of the combination of CDDP and TRAIL*

5 weeks old athymic female mice (NMRI nu/nu) were obtained from Janvier (Le Genest Saint-Isle, France). This protocol was approved by the local Animal Ethical committee (Université de Bourgogne, Dijon, France). Mice were subcutaneously xenografted with 4×10^6 HCT116-Ctl cells in the right flank and 4×10^6 HCT116-TRAIL-R4 in the left flank. Mice were weighed and tumor volume was evaluated every two days by caliper measurement using the following formula: $(l \times l \times L)/2$, with l the lower and L the higher dimension. When the tumor volume reached 20 mm^3, mice were divided randomly into 4 groups with 4 mice per group (day 0). The first group served as a control and received 0.2 ml PBS as vehicle at days 0 and 8 and 0.1 mL PBS containing 10 mM β-mercaptoethanol at days 2, 3, 4, 5 and 10, 11, 12, 13. The second group was injected as group 1, but received 4 mg/kg CDDP at day 0 and day 8. The third group received 8 mg/kg recombinant His-TRAIL at days 2, 3, 4, 5 and days 10, 11, 12, 13 and PBS at days 0 and 8. The fourth group received 4 mg/kg CDDP at day 0 and day 8 and 8 mg/kg recombinant His-TRAIL at days 2, 3, 4, 5 and days 10, 11, 12, 13. All administrations were done intraperitoneally. The initial value for each group (day 0) was arbitrarily established as 100, and all subsequent changes in tumor volume for each group were expressed as a percentage change in comparison with the starting tumor volume [(Tumor volume day 1) × 100/(Tumor volume at day 0)], and are referred as arbitrary tumor volume.

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Abbreviation list

5FU : 5-Fluorouracil
CDDP : cisplatin
DISC : Death-Inducing Signaling Complex
TRAIL-R : TRAIL Receptor
VP16 : etoposide

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Figure 1

Chemotherapeutic drugs restore TRAIL induced cell death inTRAIL-R4 expressing cells

(A) and (B), HeLa, Jurkat or SW480 cancer cell lines were infected with empty vector (H-Ctl, J-Ctl or SW-Ctl) or with a vector encoding TRAIL-R4 (H-TRAIL-R4, J-TRAIL- R4 or SW-TRAIL-R4). TRAIL receptors expression was analyzed by flow cytometry (grey line) against an isotype control (filled curve). (C) and (D), control cells or TRAIL-R4 expressing cells were stimulated with His-TRAIL (500 ng/ml, 6 hours), Cisplain (CDDP, 20 μM, 3 hours), etoposide (VP16, 10 μM, 3 hours) or 5-fluorouracil (5FU, 1 μg/ml, 72 hours). Apoptosis was evaluated after 6 hours (TRAIL), 48 hours (CDDP or VP16) or 72 hours (5FU) by Hoechst staining in HeLa (white), Jurkat (grey) or SW480 (black). Sequential stimulation with chemotherapeutic drugs and TRAIL was performed as follows. Cells were pretreated with CDDP or VP16 for 3 hours, in serum free medium, then washed and allowed to recover at 37°C in complete medium for 48 hours before stimulation with His-TRAIL (500 ng/ml) for an additional 6 hours. Alternatively, cells were stimulated for 72 hours with 5FU, then His- TRAIL for 6 hours. (E) VAL cell sensitivity to His-TRAIL, chemotherapy or sequential treatments was analyzed as described above. (F) Deregulation of TRAIL-R4 expression in VAL cells using three different siRNAs (scramble siRNA, Src; TRAIL-R4 siRNA, #1, #2 and #3) as analyzed by Facs for TRAIL-R4 expression using an anti-TRAIL-R4 antibody (grey line) or a control isotype (filled curved). The effect of TRAIL-R4 downregulation was assessed by Hoechst staining 6 hours after His-TRAIL treatment (500 ng/ml), scramble (white) and TRAIL-R4 siRNA (# 1 grey; #2 dashed and #3 black). (G) Bcl-2 expression in VAL cells after transfection with the scramble siRNA (Src) or the Bcl-2 siRNA (Bcl-2) and corresponding Hoechst staining 6 hours after His-TRAIL treatment (500 ng/ml), Bcl-2 siRNA (in black) or a scramble siRNA (in white). These results are representative of at least 3 independent experiments. Mean percentage of apoptotic cells and SD shown (mean ± SD). Differences between selected groups were compared by nonparametric analysis of variance (ANOVA) with Bonferroni post hoc multiple comparison test, ***P<0.001. Molecular size markers are shown on the right in kDa.

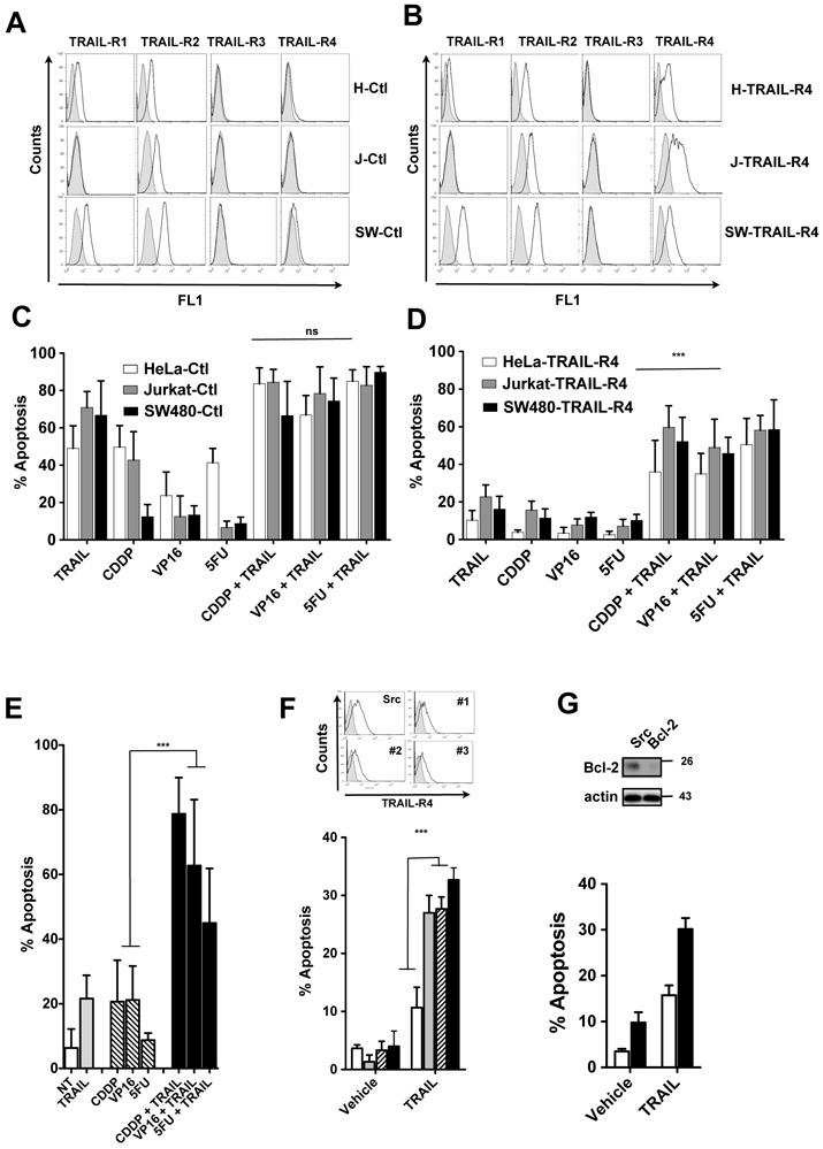


Figure 2

Chemotherapeutic drugs activate the mitochondrial apoptotic pathway

(A) Western blot analysis of caspase-8, -9, -3, PARP, Bid, Bcl-2, Bcl-xL and hsc70 in control HeLa cells (H-Ctl) or cells expressing TRAIL-R4 (H-TRAIL-R4) after stimulation with His-TRAIL (T) and/or chemotherapeutic pretreatments with cisplatin (C), etoposide (V) or 5FU (5). White arrows indicate cleavage fragments. Molecular size markers are shown on the left in kDa. These results are representative of at least 3 independent experiments. (B) and (C) A digitonin based permeabilisation experiment followed by western blot analysis of the different fractions (cytosolic or pellet) was performed to analyse the release of cytochrome c, Smac and Omi from the mitochondria. CoxII antibody was used as a control for efficient subcellular fractionation and the actin was probed for normalization. Control HeLa cells and H-TRAIL-R4 cells were treated as previously with cisplatin (C), etoposide (V) or 5-fluorouracil (5) plus or minus His-TRAIL (T). Molecular size markers are shown on the left in kDa. (D) Control HeLa cells and H-TRAIL-R4 cells were pretreated as above with cisplatin (CDDP), etoposide (VP16) or 5-fluorouracil (5FU) then subsequently stimulated or not with His-TRAIL (TRAIL), as in figure 1 . After treatment, cells were permeabilised, and stained with an antibody recognizing active Bax and analyzed by flow cytometry. (E) The percentage of cells containing active Bax was determined by FACS in control HeLa cells (H-Ctl, white bars) or TRAIL-R4 expressing cells (H-TRAIL-R4, black bars). These results are representative of at least 3 independent experiments. Mean %Active Bax values and SD are shown (mean ± SD).

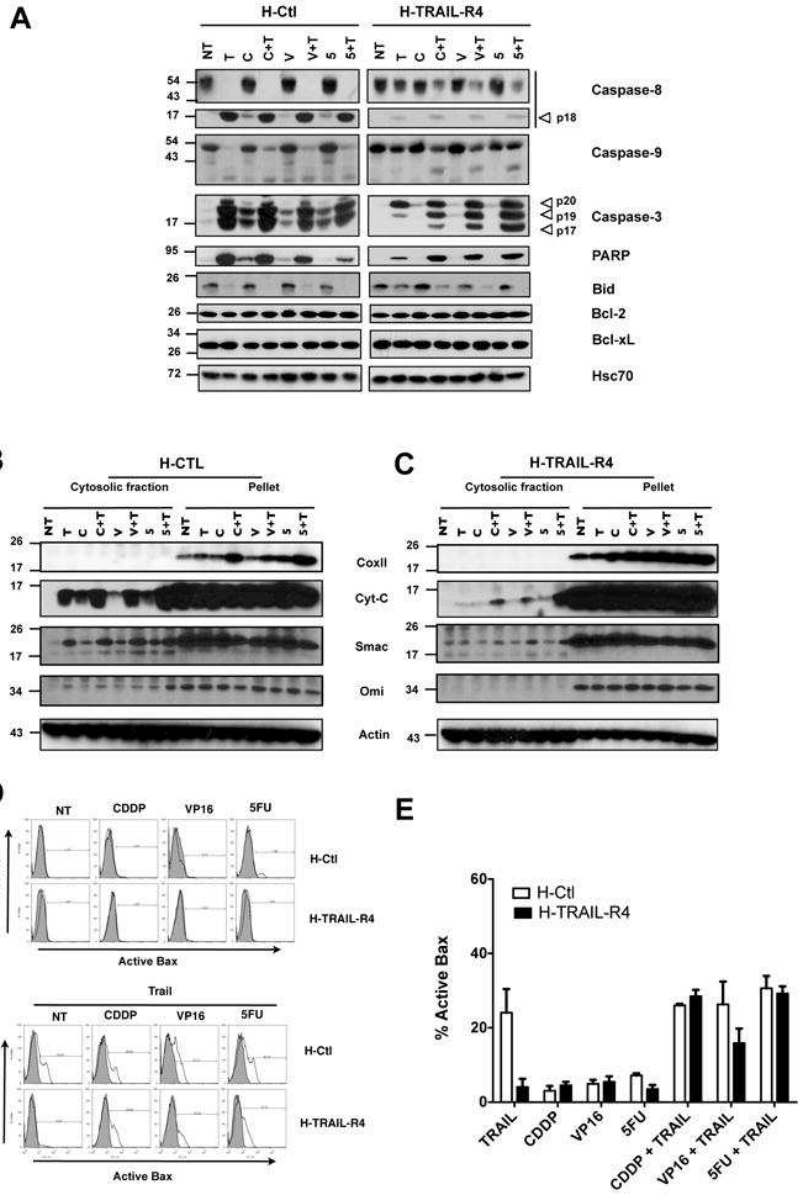


Figure 3

The mitochondrial pathway is dispensable for the synergy in TRAIL-R4 expressing HeLa cells

(A) H-TRAIL-R4 cells were infected using the pBabe-blasticidin retroviral vector encoding EGFP or Bcl-2. The expression of the different transgenes was checked by western blot using an anti-Bcl-2 or anti-GFP antibody. Hsc70 was used as a loading control. Molecular size markers are shown on the right in kDa. TRAIL-R4 HeLa cells overexpressing EGFP (EGFP, in white) or Bcl-2 (Bcl-2, in black) were pretreated with the chemotherapeutic drugs as described in Figure 1 and sequentially treated with His-TRAIL (500 ng/ml for 6 hours). Apoptosis was quantified by Hoechst staining. (B) H-TRAIL-R4 cells were infected with pMIG empty vector (EGFP) or pMIG-Bcl-xL and analyzed by western blot. Molecular size markers are shown on the right in kDa. Sensitivity to apoptosis induced by His-TRAIL, chemotherapy or sequential treatments (H-TRAIL-R4-EGFP, white; H-TRAIL-R4-Bcl-xL, black) was assessed by Hoechst staining. (C) HCT116 parental (HCT116 wt) and HCT116 Bax −/− cells were infected with an empty pMSCV-vector (HCT116 wt Ctl and HCT116 Bax−/− Ctl) or with pMSCV-vector encoding TRAIL-R4 (HCT116 wt TRAIL-R4 and HCT116 Bax−/− TRAIL-R4). TRAIL receptors expression was analyzed by flow cytometry. (D) Apoptosis induced by His-TRAIL (500 ng/ml, 6 hours) after chemotherapeutic treatment was measured by Hoechst staining in HCT116 parental Bax wt Ctl (white), HCT116 wt overexpressing TRAIL-R4 (HCT116 wt TRAIL-R4, dashed), HCT116 Bax −/− Ctl (grey) and HCT116 Bax −/− overexpressing TRAIL-R4 cells (HCT116 Bax −/− TRAIL-R4, black). These results are representative of three independent experiments performed in triplicate. Mean percentage of apoptotic cells values and SD are shown (mean ± SD). Differences between selected groups were compared by nonparametric analysis of variance (ANOVA) with Bonferroni post hoc multiple comparison test, ***P<0.001, compared with TRAIL stimulation alone in HCT116 Bax-deficient or HCT116 Bax-deficient expressing TRAIL-R4 cells, ns (not statistically significant). (E) Cells were stimulated as above for 3 hours with treatments CDDP or VP16 or 72 hours with 5FU, and c-FLIP or TRAIL-R4 expression was analyzed by western blotting 48 hours or immediately after stimulation, respectively. Molecular size markers are shown on the left in kDa. (F) HCT116 Bax wt and Bax −/− control (Ctl) or TRAIL-R4 (TRAIL-R4) were infected with pMIG-FLIP$_L$ (FLIP) or an empty vector (EGFP), and sorted by flow cytometry based on GFP positivity. Sensitivity to TRAIL-induced apoptosis after a 72 h pretreatment with 5FU was measured by Hoechst staining 6 h after His-TRAIL (500 ng/mL) treatment. (G) HeLa control (H-Ctl) and HeLa overexpressing TRAIL-R4 (H-TRAIL-R4) were infected with pBabe-EGFP or pBabe-FLIP. Expression of the different transgenes was checked by western blot. (H) Cells overexpressing EGFP (H-Ctl-GFP in white bars and H-TRAIL-R4-GFP in grey bars) or FLIP (H-Ctl-FLIP dashed bars and H-TRAIL-R4-FLIP in black bars) were stimulated with the chemotherapeutic agents, as described previously, and sequentially treated with His-TRAIL (500 ng/ml) for 6 hours. Apoptotic cells were counted after Hoechst staining. These results are representative of at least 3 independent experiments. Mean percentage of apoptotic cells and SD are shown (mean ± SD). Differences between selected groups were compared by nonparametric analysis of variance (ANOVA) with Bonferroni post hoc multiple comparison test. ***P<0.001, H-TRAIL-R4-FLIP compared with H-Ctl-Mock, H-Ctl-FLIP or H-TRAIL-R4- Mock.

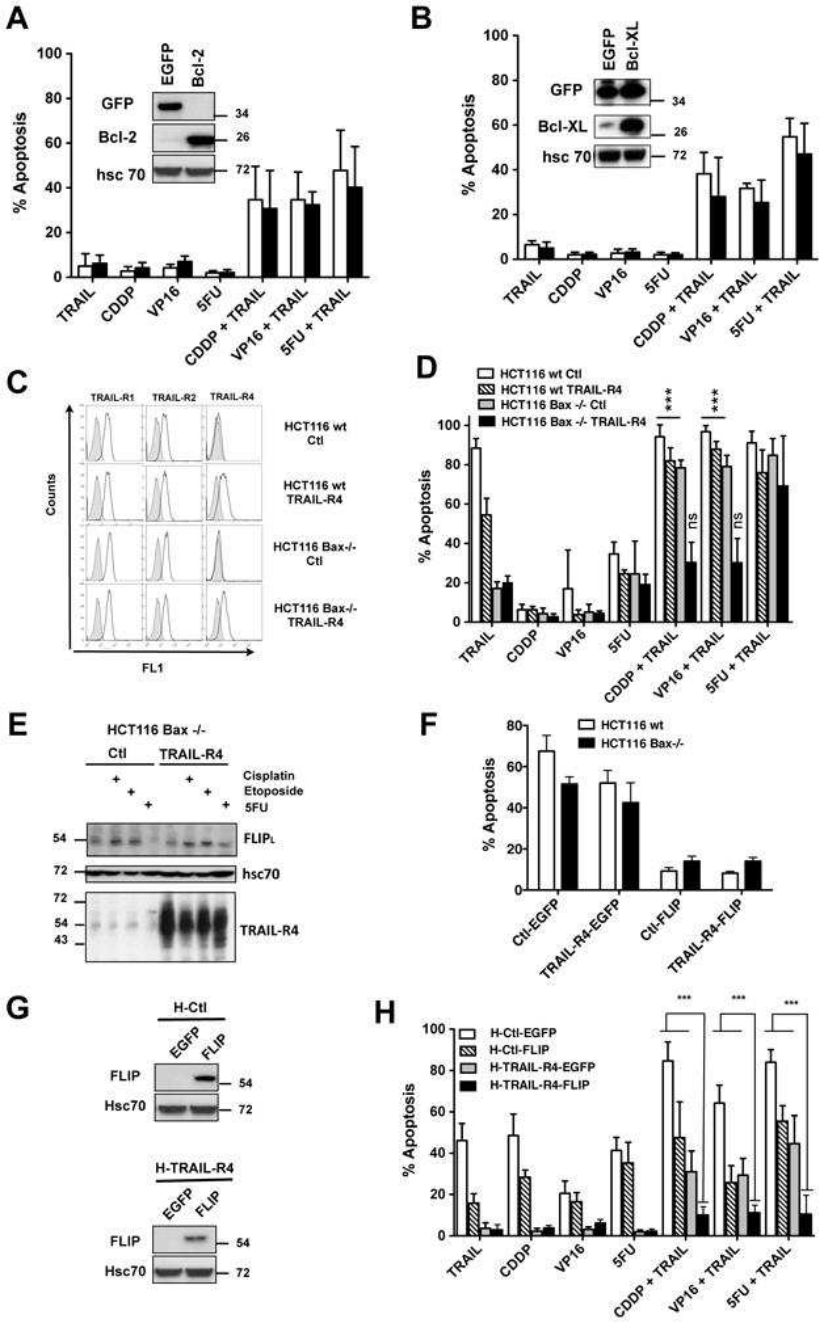

A
% Apoptosis
EGFP
Bcl-2
GFP
Bcl-2
hsc 70
34
26
72
TRAIL
CDDP
VP16
5FU
CDDP + TRAIL
VP16 + TRAIL
5FU + TRAIL
B
% Apoptosis
EGFP
Bcl-XL
GFP
Bcl-XL
hsc 70
34
26
72
C
TRAIL-R1
TRAIL-R2
TRAIL-R4
Counts
FL1
HCT116 wt Ctl
HCT116 wt TRAIL-R4
HCT116 Bax-/- Ctl
HCT116 Bax-/- TRAIL-R4
D
HCT116 wt Ctl
HCT116 wt TRAIL-R4
HCT116 Bax -/- Ctl
HCT116 Bax -/- TRAIL-R4
% Apoptosis
***
ns
E
HCT116 Bax -/-
Ctl
TRAIL-R4
Cisplatin
Etoposide
5FU
54
FLIPL
72
hsc70
72
54
43
TRAIL-R4
F
HCT116 wt
HCT116 Bax-/-
% Apoptosis
Ctl-EGFP
TRAIL-R4-EGFP
Ctl-FLIP
TRAIL-R4-FLIP
G
H-Ctl
EGFP
FLIP
FLIP
Hsc70
54
72
H-TRAIL-R4
EGFP
FLIP
FLIP
Hsc70
54
72
H
H-Ctl-EGFP
H-Ctl-FLIP
H-TRAIL-R4-EGFP
H-TRAIL-R4-FLIP
% Apoptosis
***

Figure 4

Chemotherapeutic drugs restore TRAIL sensitivity at the DISC level

(A) control HeLa cells (H-Ctl), (B) TRAIL-R4 expressing HeLa cells (H-TRAIL-R4) or (C) VAL cells were pretreated with CDDP, VP16 or 5FU or left untreated as described in figure 1 , then stimulated with TRAIL after for the indicated time. TRAIL DISC was immunoprecipitated (see material and method section) and analyzed by western blot. Molecular size markers are shown on the right in kDa.

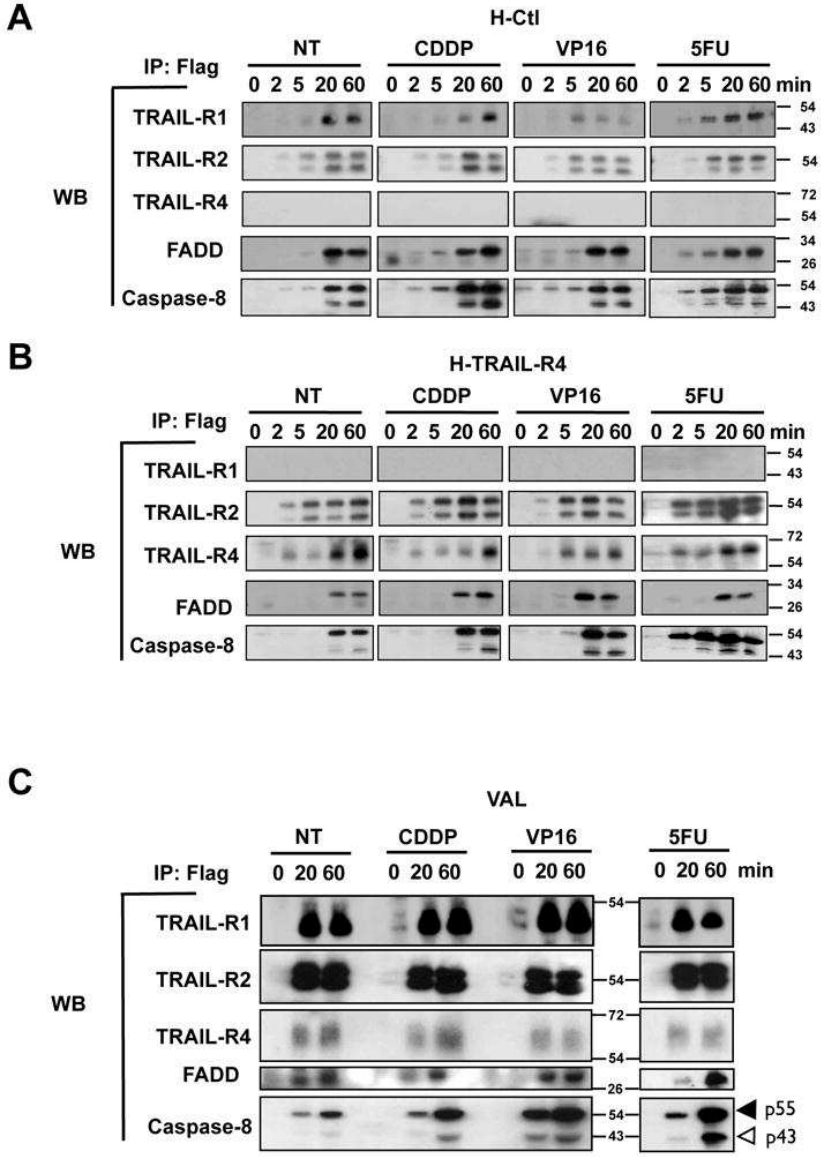


Figure 5

Chemotherapeutic drugs restore TRAIL sensitivity in vivo

(A) and (B), HCT116-Ctl or HCT116-TRAIL-R4 cells were implanted into NMRI nu/nu mice and allowed to reach 20 mm^3 . After randomization (day 0), mice were either injected with PBS (white squares), His-TRAIL alone at 8 mg/kg (grey squares), CDDP at 4 mg/kg (black squares) or sequentially with CDDP and two days later with His-TRAIL 8 mg/kg (white circle). Mice were subjected to two treatments spaced within two days. Tumors were measured every two days using a caliper. The combination was found statistically different from single treatments (*** $p<0.001$) at days 14, 16, 18 and 20 as analyzed by ANOVA, two-sided. These results represent mean tumor volume in arbitrary units ± SD of nine to ten mice per group from 3 independent experiments.

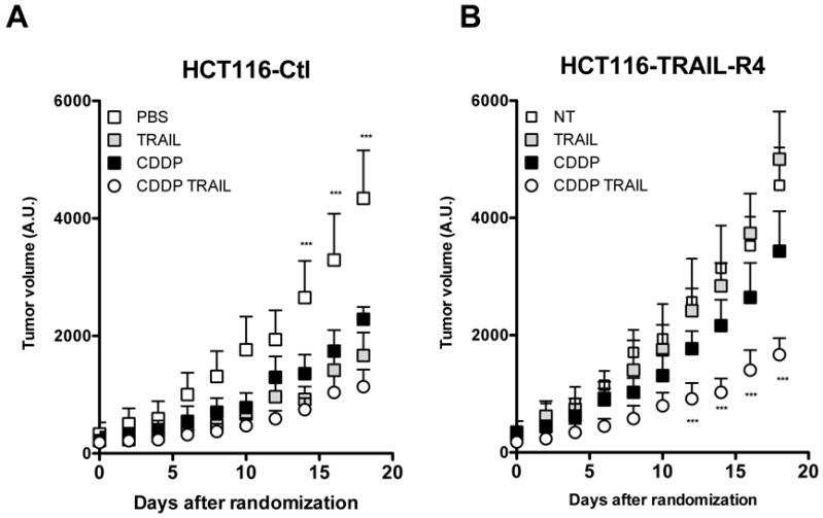


Figure 6

Proposed model of TRAIL-induced cell death regulation

(A) Direct activation of caspase-8 by TRAIL in type I cells. (B) A mitochondrial amplification loop of caspase activation in type II cells is required due to reduced caspase-8 activation upon TRAIL engagement. (C) Overexpression of TRAIL-R4, FLIP-L or mitochondrial block, protects type II cells from TRAIL-induced cell death. TRAIL-R4 and c-FLIP-L limit caspase-8 activation within the TRAIL DISC, which impairs mitochondrial activation, leading to low caspase-3 activation and survival. Mitochondrial block in type II cells, induced by Bcl-2 or Bcl-xL overexpression or Bax-deficiency inhibit amplification of the signal. Caspase-8 is activated but much less efficiently than in type I cells, leading to low caspase-3 activation and survival. (D) Chemotherapeutic drugs restore TRAIL sensitivity mainly through enhanced capase-8 recruitment to and activation at the DISC. Thus, the threshold of active caspase-8 required to induce direct caspase-3 activation can be reached and cells undergo apoptosis, overcoming TRAIL-R4- and c-FLIP-mediated inhibition of caspase-8, but also inhibition induced by Bcl-2 or Bcl-xL overexpression or Bax-deficiency. (E) Inhibition of the mitochondrial pathway by Bcl-2 or Bcl-xL overexpression in TRAIL-R4 expressing cells fails to compromise chemotherapy-induced sensitization to TRAIL. (F) Forced inhibition of caspase-8 activation in TRAIL-R4 and c-FLIP-L expressing cells abrogates apoptosis induced by TRAIL after chemotherapy. (G) Figure legends.

