Molecular regulation of death receptor- and DNA damage-induced apoptosis

Maas, C.

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Radiation and anticancer drugs can facilitate mitochondrial bypass by CD95/Fas via c-FLIP downregulation

Inge Verbrugge, Chiel Maas, Marah Heijkoop, Marcel Verheij and Jannie Borst
Radiation and anti-cancer drugs can facilitate mitochondrial bypass by CD95/Fas via c-FLIP down-regulation

Inge Verbrugge¹-², Chiel Maas¹, Marah Heijkoop¹, Marcel Verheij³ and Jannie Borst¹-⁴

¹Division of Immunology, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands
²Current address: Cancer Therapeutics Program, The Peter MacCallum Cancer Centre, East Melbourne Victoria, Australia
³Division of Radiotherapy, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands
⁴Correspondence to Jannie Borst: j.borst@nki.nl

In many tumor cell types, ionizing radiation or DNA damaging anti-cancer drugs enhance sensitivity to death receptor-mediated apoptosis, which is of clinical interest. APO010, a form of CD95/Fas ligand is currently in a Phase I trial in patients with solid tumors. To investigate the potential of combined modality treatment with APO010, we used p53-mutant Jurkat T leukemic cells, in which the mitochondrial pathway was blocked by Bcl-2 overexpression. These cells were strongly sensitized to APO010 by pretreatment with ionizing- or UV radiation, etoposide, histone deacetylase- or proteasome inhibitors. These stimuli alone did not induce apoptosis in J16-Bcl-2 cells. Sensitization could not be explained by overruling of mitochondrial resistance imposed by Bcl-2, upregulation of Fas membrane levels, or modulation of Inhibitor of Apoptosis Proteins. Rather, the stimuli commonly downregulated c-FLIPₕ/s protein levels, which was causally related to the sensitization: Deliberate c-FLIPₕ/s downregulation by RNA interference largely overruled the capacity of the various stimuli to sensitize Jurkat-Bcl-2 cells to apoptotic execution by APO010. In p53 mutant, Bcl-2 overexpressing HCT-15 colon carcinoma cells, c-FLIP downregulation correlated with sensitization to APO010 for some, but not all stimuli. We conclude that c-FLIP downregulation represents a mechanism by which diverse anti-cancer regimens can facilitate tumor cell execution by Fas via the direct pathway of caspase activation.

Introduction

Inhibition of apoptotic pathways critically contributes to cancer development. Consequently, tumor cells are often ‘addicted’ to overexpression of anti-apoptotic proteins such as Bcl-2 for their survival. Targeting apoptosis regulators in cancer treatment is therefore interesting, as overcoming blocks in apoptosis may render cancer cells more susceptible to death than normal cells [1]. Ionizing radiation (IR) and DNA damaging chemotherapeutics are effective in cancer treatment, because they hamper clonogenicity of tumor cells in a variety of ways. The DNA damage they induce triggers an -often p53-dependent- cell cycle arrest allowing for DNA repair. Failing this, irreversible cell cycle arrest, death due to mitotic catastrophe, or apoptotic cell death may ensue [2]. Apoptosis induction by DNA damaging anti-cancer regimens is blocked by Bcl-2 overexpression, indicating that apoptosis signaling proceeds via the intrinsic, mitochondrial pathway [3]. In this pathway,
pro-apoptotic members of the Bcl-2 family induce mitochondrial permeabilization and the subsequent release of Cytochrome c (Cyt c) and other mediators allows for Caspase-9 and effector caspase activation [4]. In hematopoietic tumors, the apoptotic response to IR and DNA-damaging drugs is generally evident and apoptosis resistance can contribute to treatment resistance [5]. Solid tumors are less apoptosis prone when treated with conventional regimens. Therefore, the death receptor ligand TRAIL was of immediate clinical interest when it effectively induced apoptosis in many long-term established solid tumor cell lines. TRAIL proved non-toxic to normal tissue in animal models and moved rapidly towards the clinical testing phase [6]. Currently, TRAIL receptor agonists are in Phase I and Phase II clinical trials, either as single modality treatment or in combination with conventional or novel anti-cancer drugs [7]. Combined modality treatment with death receptor agonists and DNA damaging anti-cancer regimens was expected to be more effective than single agent treatment, given the distinct cell death pathways they engage. TRAIL-R1/2 and Fas death receptors induce apoptosis by directly recruiting and activating caspases [8]. Upon ligand binding, they recruit FADD, inducer Caspase-8 and/or -10 and c-FLIP molecules [8,9]. In the death-inducing signaling complex (DISC) thus formed at the cytoplasmic receptor tail, Caspase-8/10 are activated. Depending on their expression level, c-FLIP molecules can facilitate or inhibit this activation [10]. Upon self-processing by proteolysis, Caspase-8/10 are released into the cytosol, where they find effector caspases as their targets. In this way, death receptors can in principle execute apoptosis in a mitochondrion-independent manner. However, by cleaving and activating BH3-only protein Bid, Caspase-8/10 also connect death receptors to the mitochondrial pathway [4].

The efficacy of TRAIL receptors and Fas to kill cells via the direct pathway for effector caspase activation depends on the cell type. In so-called Type II cells, the mitochondrial contribution is required for apoptotic execution, whereas in Type I cells it is not, as ascertained by Bcl-2 overexpression. This relates to the efficacy of Caspase-8/10 activation in the DISC, which seems a cell-intrinsic phenomenon [11]. The mitochondrial bypass by death receptors is of interest, since it potentially overrules intrinsic apoptosis resistance in tumor cells, as e.g. imposed by Bcl-2 overexpression. Although TRAIL receptors and Fas induce apoptosis by highly similar mechanisms [8,9], Fas agonists were not pursued for cancer therapy, due to severe hepatotoxicity upon systemic administration in mice [12]. Recently however, a Phase I clinical trial has been initiated at TopoTarget (ClinicalTrials.gov identifier: NCT00437736) with APO010 (MegaFas Ligand). This is a fusion protein of the extracellular domain of Fas ligand and the collagen domain of the serum protein ACRP30. The dimer of Fas ligand trimers that is thus formed can crosslink two adjacent Fas trimers, rendering APO010 highly agonistic as compared to other available Fas agonists [13]. It is therefore envisioned that combined modality treatment with low concentrations of this agent is effective and has acceptable toxicity, especially in settings of local therapy, such as radiotherapy or local perfusion with anti-cancer drugs. We have recently shown that in p53 mutant Jurkat cells engineered to overexpress Bcl-2 (J16-Bcl-2), as well as in solid tumor types, APO010 and IR had a combined effect on cell death induction, both in short-term apoptosis assays and in clonogenic survival assays [14].

We demonstrate here that pre-treatment with IR, but also with UV radiation and various anti-cancer drugs sensitizes J16-Bcl-2 cells to APO010-mediated apoptosis, without breaking mitochondrial resistance imposed by Bcl-2 or upregulating Fas membrane levels. The underlying mechanism concerns downregulation of c-FLIP long (L) and short (S) isoforms, which enables apoptotic execution by Fas via the direct pathway for caspase activation. Since Jurkat cells are classified as Type II cells [15], this implies a conversion from Type II to Type I signaling by Fas.
Results

Combining APO010 with chemotherapy overcomes apoptosis resistance imposed by Bcl-2

The wild-type Jurkat clone J16 readily undergoes apoptosis upon single treatment with the death ligands Fas ligand or TRAIL, or with the DNA damaging anti-cancer regimens IR or etoposide [16]. The response of J16 cells to each of these stimuli was strongly impaired upon Bcl-2 overexpression [14,16]. J16-Bcl-2 cells therefore provided an excellent cellular system to examine whether combined treatment with APO010 and anti-cancer regimens might overrule resistance imposed by Bcl-2. APO010 and IR were previously shown to have a combined effect on the apoptotic response of J16-Bcl-2 cells [14]. To investigate whether this also held true for DNA damaging drugs, we examined combined effects of APO010 and etoposide, which is a topoisomerase inhibitor that - like IR - induces double strand DNA breaks. Cells were stimulated with different doses of APO010 in the presence or absence of etoposide and apoptosis was read out 48h later by nuclear fragmentation. J16-Bcl-2 cells were fully resistant to treatment with 2 µg/ml etoposide alone (Figure 1a). As expected from the Type II nature of Jurkat cells [15], J16-Bcl-2 cells also showed low sensitivity to APO010 alone. In agreement with our previous work [16] and studies by others [17,18], Bcl-2 overexpression did not fully abrogate the apoptotic response of J16 (Jurkat) cells to Fas stimulation, as evident at high doses of

![Figure 1. Combined effects of etoposide and APO010 on apoptosis induction in J16-Bcl-2 cells.](image-url)

(a) J16-Bcl-2 cells were stimulated with the indicated doses of APO010 in the absence (Control) or presence of 2 µg/ml etoposide (Etop). Apoptosis was read out by nuclear fragmentation after 48h. Results shown are means ± SD of 2 independent experiments with duplicate samples. Statistically significant differences between values of Control and Etop samples are indicated for **P<0.001. (b) J16-Bcl-2 cells were left untreated (J16-Bcl-2) or incubated with 2 µg/ml etoposide (J16-Bcl-2 Etop). After 15h, cells were stimulated with 0.4 ng/ml APO010. Empty vector transduced J16 cells (J16) treated with APO010 alone served as a control. Active Caspase-3 content was analyzed at the indicated time points by flow cytometry. Results shown are means ± SD of 3 independent experiments. Statistically significant differences between values of J16-Bcl-2 and J16-Bcl-2 Etop samples are indicated for **P<0.01 and ***P<0.001. (c) J16-Bcl-2 cells were left untreated (-) or stimulated with 2 µg/ml etoposide (+). After 15h (t = 0), cells were stimulated with 0.4 ng/ml APO010 and harvested at the indicated time points. After separation by SDS-PAGE, protein levels of the p53/55 Caspase-8 proform and its p41/43 and p18 cleavage products and the PARP p116 proform and its p85 cleavage product were determined by immunoblotting. Actin served as a loading control. Numbers indicate integrated density values of the Caspase-8 p41/43, Caspase-8 p18 and PARP p85 fragments, as quantified from autoradiograms and normalized to Actin signals in arbitrary units.
Type II to Type I switch by anticancer drugs in Jurkat cells

APO010 (Figure 1a). Upon combined treatment with etoposide, however, J16-Bcl-2 cells were significantly more sensitive to APO010 and underwent apoptosis at very low doses (Figure 1a). Wild-type J16 cells were highly sensitive to treatment with 2 µg/ml etoposide alone, which precluded a read out for sensitization to APO010 (Supplementary Figure 1). To investigate whether etoposide pre-conditioned J16-Bcl-2 cells to more efficiently activate effector caspases upon APO010 stimulation, cells were first incubated with etoposide and 15h later stimulated with APO010. Whereas APO010 alone readily induced Caspase-3 activation in parental J16 cells, J16-Bcl-2 cells were relatively resistant. In etoposide-pretreated J16-Bcl-2 cells, Caspase-3 activation was significantly enhanced, approximating the response seen in J16 cells (Figure 1b). Caspase-3 activation correlated with the cell death response as measured by nuclear fragmentation and propidium iodide (PI) uptake (Supplementary Figure 2).

Since effector caspases are directly activated by Caspase-8/10 in the mitochondrion-independent death receptor pathway, we investigated whether pre-treatment with etoposide improved the capacity of APO010 to activate Caspase-8. In etoposide-treated J16-Bcl-2 cells, Caspase-8 activation -as read out by appearance of the p41/p43 and p18 cleavage products- was first observed at 2h after APO010 stimulation (Figure 1c). Caspase-3 activity was also apparent by proteolytic processing of its substrate PARP. At each subsequent time point, Caspase-8 activation and PARP processing were more pronounced in cells incubated with etoposide than in untreated cells.

We conclude that etoposide treatment conditioned J16-Bcl-2 cells to more efficiently activate inducer and effector caspases upon APO010 stimulation.

Mitochondrial resistance imposed by Bcl-2 is not broken upon combined treatment

It was possible that in etoposide-treated cells, APO010 evoked effector caspase activation by overruling the mitochondrial resistance imposed by Bcl-2. Therefore, we monitored Cyt c release in parental J16 cells and in J16-Bcl-2 cells that were pretreated with etoposide or not. J16 cells showed Cyt c release upon APO010 treatment,
but J16-Bcl-2 cells did not throughout the 8h time course, showing the effectiveness of Bcl-2 in preventing mitochondrial permeabilization (Figure 2a). The primary data depicted in Figure 2b illustrate this point. Importantly, J16-Bcl-2 cells that were pretreated with etoposide showed no significant increase in Cyt c release after APO010 stimulation, even after 8h (Figure 2b). These were the same samples analyzed for Caspase-3 activation in Figure 1b, indicating that effector caspase activation in etoposide-treated J16-Bcl-2 cells was not a consequence of breaking Bcl-2-mediated mitochondrial resistance. We conclude that after etoposide treatment, APO010 more efficiently activated effector caspases via the direct, mitochondrion-independent pathway.

**Etoposide downregulates c-FLIP L/S and improves Caspase-8 activation in the DISC**

Since Caspase-8 is activated in the DISC, we investigated whether DISC formation was improved in etoposide-treated cells. After 15h of incubation in the presence or absence of etoposide, J16-Bcl-2 cells were stimulated with crosslinked Fas ligand. The combined treatment effect in this setting was verified by enhanced PARP processing in corresponding total cell lysates (Figure 3a). The DISCs isolated from etoposide pretreated cells showed enhanced Caspase-8 activation as compared to the DISCs from control cells, which was most apparent at the 4h time point (Figure 3a). In the same samples, the DISCs from etoposide-treated cells showed a decrease in processed c-FLIP L levels. Immuno-blotting of total cellular lysates of control and etoposide-treated cells for c-FLIP levels showed that etoposide treatment reduced protein expression of the FLIP L and FLIP S isoforms (Figure 3b). This was a post-translational effect, as c-FLIP mRNA levels were not reduced after etoposide treatment (Supplementary Figure 3).

These results suggest that downregulation of c-FLIP protein levels by etoposide treatment enhanced the ability of Fas ligand to activate Caspase-8 in the DISC.

**Figure 3. Etoposide promotes Fas/CD95 ligand-induced Caspase-8 activation in the DISC and reduces c-FLIP levels.**

(a) J16-Bcl-2 cells were incubated with 2 μg/ml etoposide (+) or left untreated (-). After 15h, cells were stimulated with 20 ng/ml FLAG-Fas/CD95 ligand pre-coupled to 100 ng/ml biotinylated anti-FLAG antibody for the indicated periods of time. Fas/CD95 ligand-bound receptor complexes were isolated using streptavidin-conjugated sepharose beads. Caspase-8 and c-FLIP in the Fas/CD95 DISC were detected by immunoblotting. The Caspase-8 proforms (p53/55) and their cleavage products (p41/43, p18), as well as the c-FLIP proform (p53) and its cleavage product (p41) are indicated. Combined effects in this setting were confirmed by immunoblotting, corresponding cell lysates for PARP processing (conversion of p116 to p85) were used as a measure for effector caspase activity. Actin served as a loading control. (b) Protein levels of c-FLIP L/S in total lysates of J16-Bcl-2 cells were detected by immunoblotting at 15h after incubation in the absence (Control) or presence of 2 μg/ml etoposide (Etop). Actin served as a loading control.
**JNK inhibition reveals relation between c-FLIP downregulation and sensitization to APO010**

It was demonstrated previously that activation of the Jun N-terminal Kinase (JNK) pathway through activation of the E3 ubiquitin ligase Itch leads to c-FLIP downregulation [19]. Since etoposide is known to activate the JNK pathway [20], we tested whether JNK inhibition - using the chemical inhibitor SP600125 [21] or the cell permeable L-JNKi peptide [22] - reversed etoposide-induced c-FLIP downregulation and APO010 sensitivity. Etoposide activated JNK in J16-Bcl-2 cells, since both JNK and its target protein c-Jun were phosphorylated upon treatment (Supplementary Figure 4). JNK activity induced by etoposide was strongly reduced by SP600125 and to a lesser extent by L-JNKi peptide, validating the compounds as JNK inhibitors. In the same experiment, etoposide treatment downregulated c-FLIP\_L protein levels. SP600125 and L-JNKi peptide restored c-FLIP protein levels to an extent that was correlated with their inhibitory effect on JNK activity. Next, we examined the effect of SP600125 on etoposide-mediated sensitization of J16-Bcl-2 cells to Fas ligand. J16-Bcl-2 cells were pre-treated with etoposide or not, in the presence or absence of the JNK inhibitor. In total cellular lysates and in the Fas DISC, c-FLIP\_L was downregulated by etoposide-treatment. However, co-treatment with SP600125 abrogated this effect, as seen at t=0 and at each successive time point after Fas ligand stimulation in total cell lysates and at 1h and 4h in the DISC (Figure 4a). Concomitantly, SP600125 reversed etoposide-induced sensitization to Fas ligand, since the increment in Caspase-8 activation and effector caspase activity (PARP cleavage) seen in etoposide-treated cells did not occur in cells that had been treated with etoposide and SP600125.

The effect of the JNK inhibitors on etoposide-mediated sensitization of J16-Bcl-2 cells to APO010-induced Caspase-3 activation was validated by flow cytometry throughout an 8h time course. Etoposide strongly sensitized J16-Bcl-2 cells to APO010-induced Caspase-3 activation.

**Figure 4. Inhibiting JNK signaling restores c-FLIP levels and overrules sensitization to APO010-induced apoptosis by etoposide.** (a) J16-Bcl-2 cells were left untreated (-) or incubated for 30 min with 10 μM SP600125 (+). Next, cells were incubated with (+) or without (-) etoposide (Etop) for 15h and subsequently stimulated with 20 ng/ml FLAG-CD95L pre-coupled to biotinylated anti-FLAG antibody for the indicated periods of time. DISCs were isolated and c-FLIP protein levels in DISCs and total cell lysates were determined by immunoblotting. Caspase-8 (p18) and PARP (p85) processing were also determined in total cell lysates. Actin served as a loading control. (b) J16-Bcl-2 cells were incubated in the presence or absence of 10 μM SP600125 (30 min) or 10 μM L-JNKi peptide (24 h) prior to treatment with 2 μg/ml etoposide. After 15h, cells were stimulated with 0.4 ng/ml APO010 and harvested for analysis of Caspase-3 activation by flow cytometry at the indicated time points. Values represent mean ± SD of 3 independent experiments. Statistically significant differences between values of Etop and Etop + L-JNKi, or Etop + SP samples are indicated for *P<0.05 and **P<0.01.
activation, which was efficiently reversed by SP600125 and also partially by L-JNKi peptide (Figure 4b). The ability of SP600125 and L-JNKi to reverse etoposide-mediated sensitization to APO010 correlated with their ability to inhibit c-Jun phosphorylation and to restore c-FLIP levels (Supplementary Figure 4).

These results indicate that in J16-Bcl-2 cells, etoposide-mediated activation of the JNK pathway is (partly) responsible for c-FLIP downregulation. Moreover, they suggest that c-FLIP downregulation by etoposide is causally related to the drug’s ability to sensitize J16-Bcl-2 cells to APO010-induced apoptosis.

**Downregulation of c-FLIP by RNA interference sensitizes J16-Bcl-2 cells to APO010**

To test whether c-FLIP downregulation was sufficient to sensitize J16-Bcl-2 cells to APO010-induced apoptosis, we stably downregulated c-FLIP levels in these cells by RNA interference (RNAi), using retroviral constructs. The different short interfering (si) RNA constructs tested downregulated c-FLIP isoforms to a variable degree. The FLIP#1 construct strongly reduced expression of c-FLIP<sub>L</sub> and FLIP<sub>S</sub>, whereas e.g. the FLIP#4 construct had a clear, but more modest effect (Figure 5a).

**Figure 5. Downregulating c-FLIP levels facilitates the direct pathway for apoptotic execution by APO010 and significantly impairs sensitization of J16-Bcl-2 cells by etoposide.** (a) c-FLIP protein levels determined by immunoblotting in total lysates of empty vector (EV)-transduced J16-Bcl-2 cells and in J16-Bcl-2 cells expressing RNAi constructs for c-FLIP (FLIP#1-5). Actin served as a loading control. (b) EV, FLIP#1 and FLIP#4 J16-Bcl-2 cells were stimulated with 0.4 ng/ml APO010 and analyzed for active Caspase-3 by flow cytometry at the indicated time points. (c) EV-transduced J16 cells (J16) or FLIP#1 transduced J16-Bcl-2 cells (FLIP#1) were stimulated with 0.4 ng/ml APO010 for 8h or left unstimulated (control). Active Caspase-3 content and Cyt c release were determined by flow cytometry. Flow cytometric plots in left panels are data from one representative experiment. Numbers in the panels represent percentage of events in the indicated gate. F1 = fluorescence intensity. The bar diagram (right panel) shows means ± SD of 3 independent experiments. (d) EV-, FLIP#1- or FLIP#4-transduced J16-Bcl-2 cells were incubated for 15h in the absence (Control) or presence of etoposide (Etop) at the indicated concentrations. Next, cells were stimulated with APO010 for 8 h and analyzed for active Caspase-3 content. Values in left panel are mean ± SD of 3-4 independent experiments. Background apoptosis at t = 0, prior to stimulation with APO010 was subtracted (values: Control < 1%, 1 μg/ml etoposide 2.4 - 3.1%, 2 μg/ml etoposide 2.7 - 5.8%). Fold sensitization depicted in the right panel was extrapolated from Caspase-3 activation data in the left panel and indicates the fold enhancement of APO010-induced Caspase-3 activation by pretreatment of J16-Bcl-2 cells with etoposide. Statistically significant differences between values of EV versus FLIP#1 or FLIP#4 samples are indicated for *P<0.05, **P<0.01 and ***P<0.001.
Upon treatment of FLIP#1 and FLIP#4 cell lines with APO010, Caspase-3 activation was enhanced as compared to empty vector-transduced cells to a degree that correlated with the extent of c-FLIP downregulation (Figure 5b). As tested in the FLIP#1-transduced cells, Caspase-3 activation at 8h was not accompanied by Cyt c release, indicating that mitochondrial resistance was not broken (Figure 5c).

We next investigated to which extent etoposide treatment could (further) sensitize control, FLIP#1- or FLIP#4-transduced J16-Bcl-2 cells to APO010-induced apoptosis. After pre-treatment with etoposide, cells were stimulated for 8h with APO010. Control cells were efficiently sensitized to APO010 by etoposide in a dose-dependent manner. FLIP#1-transduced cells were very sensitive to treatment with APO010 alone and etoposide treatment incremented the response to a limited degree. FLIP#4-transduced cells were sensitized to lesser extent by c-FLIP siRNA, allowing us to quantify the added effect of etoposide pretreatment (Figure 5d). For this purpose, Caspase-3 activation upon combined treatment was normalized to Caspase-3 activation by APO010 alone and expressed as fold sensitization. This analysis revealed that etoposide-mediated sensitization to APO010 in FLIP#4-transduced cells was significantly reduced as compared to control cells (Figure 5d).

In summary, c-FLIP downregulation by RNAi greatly facilitated the mitochondrion-independent pathway for effector caspase activation and largely overruled the capability of etoposide to increment apoptotic execution.

Figure 6. Various stimuli - that sensitize J16-Bcl-2 cells to APO010 - downregulate c-FLIP protein levels, without upregulating Fas membrane levels. (a-d) J16-Bcl-2 cells were left untreated (Control) or stimulated with (a) 30 Gy IR, (b) 60 J/m² UV, (c) 300 nM TSA or (d) 6.4 mM VPA. Directly after stimulation (UV) or 15h later (IR, TSA, VPA), cells were stimulated with 0.4 ng/ml APO010 for the indicated periods of time and analyzed for active Caspase-3 content by flow cytometry. Data represent means ± SD of at least 3 independent experiments. Statistically significant differences between values of Control and IR, UV, VPA or TSA treated cells are indicated for *P<0.05, **P<0.01 and ***P<0.001. (e) J16-Bcl-2 cells were harvested at 15h after stimulation with 2 μg/ml etoposide (Etop), 10 or 30 Gy IR, 300 nM TSA, 10 μM lactacystin (Lacta), 6.4 mM VPA, 5 μM MG132 or at 3h after irradiation with 60 J/m² UV. Total c-FLIP protein levels were determined in cell lysates by immunoblotting. Open and filled triangles indicate the c-FLIP S and c-FLIP L isoforms, respectively. Actin served as a loading control. Brackets indicate samples derived from the same experiment, each with its own control. Data are representative of multiple experiments. (f) J16-Bcl-2 cells were stimulated with the indicated regimes as outlined in (e) and Fas membrane levels of live cells were determined by flow cytometry. Values of mean fluorescence intensity (MFI) are represented as mean ± SD of at least 4 independent experiments, normalized to Fas MFI of untreated cells. Statistically significant differences between values of Control and VPA, TSA or IR treated samples are indicated for *P<0.05 and ***P<0.001.
These data indicate that downregulation of c-FLIP protein expression is a major mechanism by which etoposide sensitizes J16-Bcl-2 cells to APO010-induced apoptosis.

**Various stimuli that sensitize J16-Bcl-2 cells to APO010 downregulate c-FLIP**

Next, we examined whether other stress stimuli could also facilitate APO010-induced apoptosis in J16-Bcl-2 cells. For this purpose, cells were pre-treated with IR, UV radiation, the HDAC inhibitors Trichostatin A (TSA) and Valproic Acid (VPA), or the proteasome inhibitors MG132 and lactacystin. In all cases, pretreatment significantly enhanced APO010-induced apoptosis (Figure 6a-d). In the time-frame of pre-sensitization, IR slightly increased Fas membrane levels (Figure 6f), but etoposide and UV had no effect, while VPA and TSA significantly downregulated Fas. This ruled out Fas upregulation at the plasma membrane as the mechanism for increased APO010 sensitivity.

In addition, we found no evidence that modulation of Inhibitor of Apoptosis Protein (IAP) expression was instrumental in the sensitization to APO010-induced apoptosis. Firstly, treatment with etoposide or IR did not alter c-IAP-1, c-IAP-2 or XIAP mRNA or protein expression in J16-Bcl-2 cells [16]. Secondly, stable expression of a validated cytosolic SMAC mutant (J16-Bcl-2-Δ55 SMAC) [16] did not abrogate the capacity of etoposide to sensitize J16-Bcl-2 cells to APO010-induced apoptosis (Supplementary Figure 5).

Treatment of J16-Bcl-2 cells with each of the stimuli did, however, downregulate total cellular c-FLIP protein levels (Figure 6e). In case of IR, UV and VPA, SP600125 could not overrule sensitization (Supplementary Figure 6), which correlated with an inability to restore c-FLIP levels (Supplementary Figure 7 and data not shown).

We conclude that in addition to etoposide, also IR, UV radiation, HDAC inhibitors and proteasome inhibitors sensitized J16-Bcl-2 cells to APO010 apoptosis, which was correlated with c-FLIP downregulation.

**Downregulation of c-FLIP by RNAi impairs the ability of different stimuli to sensitize to APO010**

Subsequently, we used c-FLIP RNAi to test whether c-FLIP downregulation was a common mechanism by which the various stress stimuli sensitized J16-Bcl-2 cells to APO010-induced apoptosis. Control or FLIP#4-transduced J16-Bcl-2 cells were pretreated with IR, UV, TSA, VPA, MG132, or lactacystin and next stimulated with APO010. After 8h, cells were harvested and analyzed for active Caspase-3 content by flow cytometry. All stimuli strongly sensitized control cells to APO010-induced Caspase-3 activation (Figure 7a,b). However, in FLIP#4 transduced cells, that had intermediate c-FLIP levels (Figure 5a), sensitization was significantly reduced (Figure 7b).

These results indicate that c-FLIP downregulation is a common mechanism by which J16-Bcl-2 cells are sensitized to Fas-mediated apoptosis by a wide range of stimuli.

**FLIP downregulation and sensitization to APO010-induced apoptosis in a solid tumor cell line**

To investigate the generality of this mechanism, we examined its occurrence in HCT-15 colon carcinoma cells, which are p53 mutant [23] and have been documented as Type II cells [24]. HCT-15 cells were retrovirally transduced with Bcl-2 to block the mitochondrial pathway for caspase activation (Supplementary Figure 8b). Cells were not pretreated (Control) and subsequently stimulated with APO010, or pre-treated with etoposide, IR, UV radiation, or HDAC inhibitor VPA and subsequently stimulated with APO010. These sensitizing stimuli alone did not induce Caspase-3 activation (or apoptosis) in HCT-15-Bcl-2 cells (Supplementary Figure 8a; 0h APO010). Also, in agreement with the documented Type II nature of HCT-15 cells, APO010 alone induced only very low level Caspase-3 activation in HCT-15-Bcl-2 cells. Sensitization of HCT-15-Bcl-2 cells to APO010-induced apoptosis by UV radiation and VPA was very strong (P<0.0001 at t=8h) (Supplementary Figure 8a) and these stimuli
concomitantly downregulated c-FLIP protein levels (Supplementary Figure 8c). IR gave a very low level of sensitization of HCT-15-Bcl-2 cells to APO010-induced apoptosis (P=0.015 at t=8h) and did not measurably downregulate c-FLIP protein levels. Therefore, in these cases, a correlation between sensitization to APO010 and c-FLIP downregulation was apparent. Etoposide, however, sensitized HCT-15-Bcl-2 cells to APO010-induced apoptosis at intermediate levels (P=0.001 at t=8h) (Supplementary Figure 8a), yet did not detectably downregulate c-FLIP protein levels (Supplementary figure 8c). Sensitization to any of these stimuli was not correlated with downregulation of XIAP, c-IAP1 or c-IAP2 protein levels (Supplementary figure 8d). These results indicate that c-FLIP downregulation may contribute to, but is not the only mechanism, by which Type II tumor cells can be sensitized to APO010-induced apoptosis.

**Discussion**

Many reports describe combined effects of conventional or targeted anti-cancer regimens and death receptor agonists in tumor cell killing. For TRAIL receptor agonists in particular, combination treatment proved effective in a variety of tumor types in vitro and in vivo [6]. The mechanisms of action of death receptors and conventional anti-cancer regimens predict additivity in combined treatment. Moreover, upregulation of death receptors at the plasma membrane in response to DNA damaging regimens may lead to synergy, in particular in p53-proficient cells [6]. However, in various cell lines, such as Jurkat leukemia [16], HT-29 colon carcinoma [25] and Hep3b hepatocellular carcinoma [26], combined effects of TRAIL and DNA damaging regimens could not be explained by changes in receptor membrane expression, raising the question of the underlying molecular mechanism.

We show here that pretreatment with conventional anti-cancer regimens IR and etoposide, but also with UV radiation, HDAC inhibitors and proteasome inhibitors sensitized J16-Bcl-2 cells to APO010-induced apoptosis, in the absence of Fas upregulation and without breaking mitochondrial resistance. In the time frame in which Caspase-3 activation was observed - as read out by occurrence of its cleaved form and cleavage of its substrate PARP - Cyt c release did not take place. In agreement with this, we previously found that downregulation of Bak and Bax by RNAi in J16-Bcl-2 cells did not alter the combined response to IR and TRAIL [16]. Sensitization resulted in improved Caspase-8 activation by Fas and execution via the direct pathway for effector

**Figure 7.** c-FLIP downregulation by RNAi impairs sensitization to APO010 by various stimuli. Empty vector (EV)- and FLIP#4 transduced J16-Bcl-2 cells were stimulated with 60 J/m² UV, or incubated for 15h after stimulation with 30 GY IR, 300 nM TSA, 5 μM MG132 or 10 μM lactacystin (Lacta). Next, cells were stimulated with 0.4 ng/ml APO010 and analyzed 8 h later for active Caspase-3 content by flow cytometry. (a) Values of Caspase-3 activation represented as means ± SD of 3-4 independent experiments. Background apoptosis at t = 0 prior to stimulation with APO010 was subtracted (values: Control <1%, IR 2.2 - 5.1%, TSA 3.5 - 5%, MG132 15.6 - 19.1%, lactacystin 9.3 - 13%, UV 3.3 - 4.9%). (b) Fold sensitization as extrapolated from Caspase-3 activation data in (a). It indicates the fold enhancement of APO010-induced Caspase-3 activation by pre-treatment of J16-Bcl-2 cells with the indicated stimuli. Statistically significant differences between the indicated values are indicated for *P<0.05, **P<0.01 and ***P<0.001.
caspase activation, which could be explained by c-FLIP downregulation. Most likely, the reduction in c-FLIP protein levels induced by the different stimuli examined here allowed fewer c-FLIP molecules to be recruited into the Fas DISC, thereby altering the c-FLIP-to Caspase-8/10 ratio. c-FLIP plays a dual role in the DISC, since it inhibits Caspase-8/10 activation when present at high levels, but can promote Caspase-8/10 activation when present at low levels [10]. We found that c-FLIP downregulation facilitated Caspase-8/10 activation in the Fas DISC. This agrees with other studies: In SHEP neuroblastoma cells, cycloheximide or actinomycin D treatment reduced cellular c-FLIP protein levels and participation in the Fas DISC and incremented inducer caspase activation [27]. Prostate carcinoma [28] and Hodgkin/Reed-Sternberg cells [29] were sensitized to Fas-mediated apoptosis by c-FLIP RNAi. Moreover, melanoma cells were sensitized to both Fas- and TRAIL receptor-induced apoptosis by c-FLIP RNAi [30]. On basis of current data, downregulation of c-FLIP -which is overexpressed in several tumor types- was recently proposed as a rational strategy to sensitize tumor cells to death ligand therapy [31]. Future studies should pinpoint whether downregulation of the long or short isoform of c-FLIP is the critical determinant in sensitizing Jurkat-Bcl-2 cells to APO010-induced apoptosis. Both isoforms can be recruited to the DISC [32]. Downregulation of c-FLIP, was most consistently observed, but c-FLIP<sub>L</sub> levels were also affected. To resolve this issue, an isoform-specific RNAi approach is required.

We previously found that pretreatment of J16-Bcl-2 cells with IR improved Caspase-8/10 activation in the DISC and thereby allowed cellular execution by TRAIL via the direct pathway for effector caspase activation [16]. Now we show that in the same cells, c-FLIP downregulation by various stress stimuli allowed APO010 to bypass the mitochondrial pathway for apoptotic execution. These findings imply that the stress stimuli promote a ‘switch’ from Type II to Type I death receptor signaling in J16-Bcl-2 cells. Also in Type II CEM leukemia cells, cycloheximide pretreatment allowed Fas-mediated apoptosis via the direct pathway [33]. It is important to determine the generality of the mechanism defined here for J16-Bcl-2 cells and in particular whether it operates also in solid tumors. We have examined the case of HCT-15 colon carcinoma cells, since these have been well defined in terms of p53 and Type II status. UV and VPA could sensitize these cells to APO010-induced apoptosis, but IR was less effective. With these stimuli, a correlation between c-FLIP downregulation and sensitization to APO010-induced apoptosis was found. However, the correlation was not evident in the case of etoposide, which sensitized HCT-15-Bcl-2 cells to APO010 in the absence of c-FLIP downregulation. In SHEP neuroblastoma cells overexpressing Bcl-2 [27], we found a correlation between FLIP downregulation and sensitization by Bcl-2, but not with the other stimuli (data not shown). Therefore, cells may be sensitized by other mechanisms to undergo an apparent Type II to Type I switch. In addition, in other Type II cells (mesothelioma and carcinoma cell lines), an intact mitochondrial amplification loop was required for apoptotic execution upon combined treatment with DNA damaging regimens and TRAIL, even though inducer caspase activity was enhanced [34-36]. Therefore, enhanced inducer caspase activation may be sufficient in some cell types for execution via the direct pathway, while in other cell types -perhaps particularly in solid tumors- the mitochondrial pathway may still be required.

Concerning sensitization to TRAIL-induced apoptosis, a number of studies report a correlation between c-FLIP downregulation by cellular stressors and increased sensitivity to apoptosis [31]. In colon [25] and hepatocellular [26] carcinoma, c-FLIP downregulation by 5-FU was correlated with reduced levels of c-FLIP in the TRAIL DISC and with enhanced sensitivity to TRAIL-mediated apoptosis. However, we found that 10 Gy IR did not detectably alter c-FLIP levels in J16-Bcl-2 cells and did not sensitize them to APO010-induced apoptosis.
Type II to Type I switch by anticancer drugs in Jurkat cells

In J16-Bcl-2 cells, IR, UV radiation, etoposide, HDAC inhibitors and proteasome inhibitors all downregulated c-FLIP protein levels. c-FLIP expression is tightly controlled, both at the transcriptional and at the protein level. The NF-κB pathway can induce c-FLIP transcription, while the ubiquitin-proteasome pathway mediates c-FLIP degradation [37,38]. c-FLIP molecules have a short half life and are subject to proteasomal degradation at the steady state. In addition, they are ubiquitinated in response to death receptor stimulation, via a defined ubiquitin ligase that is under control of the JNK pathway [19,37]. In agreement with this, short-term incubation with proteasome inhibitor rescued the disappearance of c-FLIP after addition of the stress stimuli, but upon prolonged incubation, c-FLIP was still downregulated (results not shown). This can be explained by the fact that proteasome activity is required for NF-κB activation, which in turn directs c-FLIP transcription.

In conclusion, c-FLIP downregulation is a common mechanism by which various stress inputs sensitize J16-Bcl-2 cells to Fas-mediated apoptosis via the direct pathway for caspase activation. The apparent ability of death receptors to ‘switch’ from Type II to Type I signaling following c-FLIP downregulation may be of more general relevance for sensitizing tumor cells to death receptor-mediated apoptosis. It will be of interest to determine in which primary tumor types this is the case.

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Materials and methods

Cell lines and reagents

The J16 Jurkat clone stably overexpressing Bcl-2 and luciferase (J16-Bcl-2) was generated by retroviral transduction as described [39]. J16 cells are heterozygously mutant for p53 and the DNA damage-induced apoptosis pathway is p53-independent [39]. J16-Bcl-2 cells and derivatives were cultured in Iscove’s modified Dulbecco’s medium with 8% fetal bovine serum (FBS) and antibiotics. APO010 in native and biotinylated form were kindly provided by TopoTarget, produced as described [14] and kept frozen in storage buffer at -80°C. SP600125 was from ENZO Life Sciences International Inc. The cell permeable L-JNKi peptide (sequence NGRKKRRQRRPRPVRPPTTLNLFPQVPRSD with carboxy-terminal amide) was produced at the Division of Cell Biology at the Netherlands Cancer Institute, according to standard procedures. The product was purified by HPLC and its sequence was verified by mass spectrometry. TSA, VPA, anti-Caspase-8 monoclonal antibody (mAb, clone C15), anti-caspase-8 monoclonal antibody (mAb, clone C15), anti-FLIP mAb (clone NF6), and soluble human FLAG-tagged Fas ligand were obtained from Alexis Biochemicals (Lausen, Switzerland). Biotinylated anti-FLAG mAb (clone M2), etoposide, puromycin and blasticidin S were from Sigma-Aldrich (St Louis, MO, USA). Anti-Cyt c mAb (clone 6H2.84), anti- XIAP mAb and rabbit anti-active Caspase-3 antibody were from BD Biosciences (San Jose, CA, USA), anti-actin mAb (clone C4) was from Chemicon International (Temecula, CA, USA) and rabbit anti-cIAP1 and anti-cIAP2 were from R&D Systems. AlexaFluor 647-
 conjugated goat anti-mouse Ig and goat anti-rabbit Ig were from Molecular Probes (Leiden, The Netherlands). Lactacystin and MG132 were from Calbiochem (Darmstadt, Germany). Horseradish peroxidase-conjugated rabbit anti-mouse Ig and swine anti-rabbit Ig were from Dako A/S (Glostrup, Denmark) and the enhanced chemiluminescence kit was from Pierce Biotechnology (Rockford, IL, USA). Rabbit anti-PARP polyclonal Ab (pAb) 9542 was from Cell Signaling Technologies (Danvers, MA, USA). Streptavidin-conjugated sepharose beads were from Zymed (San Francisco, CA, USA).

**Retrosirval gene transduction**

Stable knock down of c-FLIP in J16-Bcl-2 cells with siRNA (complementary sense and antisense oligonucleotides FLIP#1: 5'-GAATAGACCTGAGACAAA-3', FLIP#2: 5'-GACATACAGATGGAGCAA-3', FLIP#3: 5'-GCATGAAGTCCAGAAATT-3', FLIP#4: 5'-GGAGCAGGACAGTTACA-3', FLIP#5: 5'-GCAGGAGGAGAGATTCCT-3') was performed with the pRsc retroviral vector that is a modification of pRetroSuper [40] with a puromycin resistance cassette. Packaging of the (dideoxynucleotide sequence verified) constructs in the HT1080 packaging cell line FLY and transduction of J16-Bcl-2 cells with freshly harvested virus supernatant was carried out as described [16]. Cells were selected after 3 days with 1 μg/ml puromycin.

**Apoptosis assays**

For apoptosis assays, cells were stimulated with the indicated doses of APO010, etoposide, IR, TSA, VPA, MG132 or lactacystin in their normal medium. After addition of stimulus, cells were incubated for the indicated time periods at 37°C, 5% CO₂, IR was delivered using a 137Cs source (415 Ci; Von Gahlen Netherlands, B.V.) at an absorbed dose rate of approximately 0.6 Gy/min. For UV irradiation, 5 x 10⁵ cells were exposed in 500 μl culture medium and transferred into 12-well plate. After allowing cells to sink to the bottom of the plate for at least 2400 s. In some experiments cells were incubated (prior to sensitization) with 10 μM SP600125 for 30 min or 10 μM L-JNKi peptide for 24 h at 37°C, 5% CO₂. Nuclear fragmentation was determined by flow cytometric analysis of propidium iodide (PI) stained nuclei as described [16]. Data were analyzed using FCS Express. Mean Fluorescence Intensity (MFI) values of stimulated cells were normalized to those of unstimulated (control) cells.

**Statistics**

Differences between treatment groups were analyzed with unpaired Student’s t-tests and were considered significant when P<0.05. Analyses were performed using GraphPad Prism version 4 for Windows (Graph Pad Software, San Diego, CA, USA).

**References**

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Supplementary information

Materials and Methods

Cell lines and reagents
HCT-15 colon carcinoma cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 8% fetal bovine serum (FBS), antibiotics and glutamine. They were retrovirally transduced to stably overexpress Bcl-2, in the same manner as J16 cells [39]. Transduced cells were selected with 20 μg/ml blasticidin S. Bcl-2 overexpression was validated by immunoblotting. Rabbit anti-phospho-JNK pAb 9251 and rabbit anti-phospho-c-Jun pAb 9261 were from Cell Signaling Technologies (Danvers, MA). Rabbit anti-c-Jun pAb (sc-1694) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Supplementary Figures

Supplementary Figure 1. Combined effects of etoposide and APO010 on apoptosis induction in J16 cells. J16 cells transduced with an empty vector (EV) were stimulated with 2 μg/ml etoposide (Etop) or left untreated (control). After 15h, cells were stimulated with 0.4 ng/ml APO010. Active Caspase-3 content was analyzed at the indicated time points by flow cytometry.
Supplementary Figure 2. Correlation of cell death induction measured in multiple ways in Jurkat-Bcl-2 cells. (a) Jurkat-Bcl-2 cells were left untreated (Control) or incubated with etoposide (Etop) for 15 h. Next, cells were stimulated with 0.4 ng/ml APO010, harvested at the indicated time periods and processed for cell death analysis by PI exclusion (PI), nuclear fragmentation according to the Nicoletti method (Nic) [16] or Caspase-3 activation (Casp-3), which were all read out by flow cytometry. % cells PI positive, % cells with sub-G1 DNA content or % cells with active Caspase-3 respectively were scored as ‘cell death’. (b) Primary flow cytometric data of the samples depicted in (a) at t=10 h after APO010 treatment.

Supplementary Figure 3. c-FLIP downregulation by etoposide is post-transcriptionally regulated. J16-Bcl-2 cells were either left unstimulated (Control) or treated with 2 μg/ml etoposide (Etop) for 15h. mRNA levels of Actin, GAPDH, PGK (controls), and c-FLIP (test) were determined in duplicate by quantitative real-time (qRT)-PCR. Values obtained were normalized to those of Actin, GAPDH and PGK in control samples. c-FLIP levels of control- and etoposide treated samples were normalized to the average expression levels of Actin, GAPDH and PGK. c-FLIP protein levels as detected by immunoblotting in total cell lysates of the same samples that were used for qRT-PCR are shown in Figure 3b.

Supplementary Figure 4. Inhibiting etoposide-induced JNK signaling restores c-FLIP protein levels. J16-Bcl-2 cells were incubated with 10 μM L-JNKi peptide for 24h or with 10 μM SP600125 for 30 min at 37˚C, 5% CO₂ and next stimulated with 2 μg/ml etoposide (Etop). 15h later, c-FLIP, p-cJun and p-JNK protein levels were determined in total cellular lysates by immunoblotting. Actin and total JNK levels served as a loading controls.

Supplementary Figure 5. Combined responses in J16-Bcl-2 cells are not due to effector caspase regulation by IAPs. J16-Bcl-2 cells transduced with a validated cytosolic SMAC mutant (∆55 SMAC) [16] were incubated with 2 μg/ml etoposide (Etop) or left untreated (Control). After 15h, cells were stimulated with 0.4 ng/ml APO010 and active Caspase-3 content in the cells was analyzed at the indicated time points. Data represent means ± SD of 3 independent experiments.
Supplementary Figure 6. JNK inhibition only effectively reverses sensitivity to APO010 in etoposide-treated cells. J16-Bcl-2 cells were incubated in the presence or absence of 10 μM SP600125 (SP) for 30 min, or 10 μM L-JNKi peptide for 24h at 37°C, 5% CO2. Subsequently, these cells were stimulated with (a) etoposide (Etop, 2 μg/ml), (b) UV (60 J/m²), (c) IR (30 Gy) or (d) VPA (6.4 mM). Directly after stimulation (UV) or after 15h incubation at 37°C, 5% CO2 (Etop, IR and VPA), cells were stimulated for the indicated time periods with 0.4 ng/ml APO010 and analyzed for Caspase-3 activation by flow cytometry. Data represent mean ± SD of at least 3 independent experiments. Statistically significant differences between values for 'sensitized' cells in the presence or absence of JNK inhibitors are indicated for *P<0.05 and **P<0.01. Note that (a) is the same graph as Figure 4b and is shown here only for comparison.

Supplementary Figure 7. JNK inhibition only effectively restores c-FLIP levels in etoposide-treated cells. J16-Bcl-2 cells were incubated with with 10 μM SP600125 for 30 min or with 10 μM L-JNKi peptide for 24h, after which cells were stimulated with etoposide (Etop, 2 μg/ml), 30 Gy IR or 60 J/m² UV (UV). 15h after treatment with Etop and IR or 2 and 4 h after treatment with UV, c-FLIP, p-cJun and p-JNK protein levels were determined in total cellular lysates by immunoblotting. Actin, total cJun and total JNK levels served as controls.
Supplementary Figure 8. Sensitization to APO010-mediated apoptosis and c-FLIP downregulation in HCT-15 colon carcinoma cells stably expressing Bcl-2. (a) Apoptosis assay. HCT-15-Bcl-2 cells were stimulated with 15 µg/ml etoposide (Etop), 30 Gy IR, 60 J/m² UV, 6 mM VPA or left untreated (Control). After 15h, or immediate after in case of UV, cells were stimulated with 4 ng/ml APO010 for the indicated periods of time and analyzed for active Caspase-3 content by flow cytometry. Data shown are representative of 2 independent experiments. (b) Bcl-2 expression. Validation of Bcl-2 overexpression in Bcl-2 transduced HCT-15 cells as compared to empty vector (EV) transduced cells by immunoblotting. (c) FLIP expression. After stimulation as indicated in (a), HCT-15-Bcl-2 cells were harvested and total c-FLIP protein levels were determined in cell lysates by immunoblotting. Actin served as a loading control. Open and filled triangles indicate the c-FLIP_S and c-FLIP_L isoforms respectively. (d) IAP expression, the same blot as in (b) was re-probed for cIAP1 expression. Additional lysates of cells that had been stimulated as indicated were probed for XIAP and cIAP2 protein expression, with Actin serving as loading control.