Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Cooperates with Anticancer Drugs to Overcome Chemoresistance in Antiapoptotic Bcl-2 Family Members Expressing Jurkat Cells

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ABSTRACT

Purpose: **Overexpression of antiapoptotic Bcl-2 family members has recently been related to resistance to chemo/radiotherapy in several human malignancies, particularly lymphomas. Hence, innovative approaches bypassing this resistance mechanism are required in the therapeutic approach. This study evaluated whether chemoresistance associated with Bcl-2 and Bcl-x_L overexpression would be overcome by activating the death receptor pathway by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in the Jurkat cell model**

Experimental Design: **We made use of genetically modified Jurkat cells to evaluate the effect of Bcl-2 or Bcl-x_L overexpression on the cytotoxic effect produced by the anticancer drugs doxorubicin, etoposide, and oxaliplatin and TRAIL. Caspase activation was detected by cleavage of caspase-8 and -3. The mitochondrial transmambrane potential was assessed by staining with $DiOC_6$ and flow cytometry. Caspase activity was blocked by the broad-spectrum caspase inhibitor zVAD-fmk.**

Results: **Bcl-2 and Bcl-x_L overexpression but not lack of caspase-8 protects the Jurkat cells from the anticancer drug-induced cytolysis. However, Bcl-2/Bcl-x_L Jurkat cells retained some susceptibility to TRAIL-induced cytolysis. A highly synergistic cytotoxic effect of the combination of TRAIL with any of the antiblastic used in this study was detected in the chemoresistant cells. This effect was associated with mitochondrial disassemblage and dependent on caspase activation**

Conclusions: **The combination of TRAIL with conventional anticancer drugs may prove to be useful in the treatment of antiapoptotic Bcl-2 family proteins-expressing malignancies.**

INTRODUCTION

Bcl-2 family proteins are key regulators of apoptosis, which plays an essential role in controlling tissue homeostasis and proliferation. Bcl-2 itself was first identified in B-cell lymphomas carrying a translocation of the *Bcl*-2 gene to the control of the immunoglobulin promoter [t(14:18); Ref. 1]. The resulting Bcl-2 overexpression inhibits apoptosis that is required to maintain B-cell homeostasis, resulting in B-cell accumulation and follicular lymphoma (2). The Bcl-2 family includes both proapoptotic (Bcl-x_S, Bax, Bak, Bid, Bad, Bim, Noxa, and Puma) and antiapoptotic proteins (Bcl-2, Bcl-x_L, A1, and Mcl-1), which converge to control disassemblage of mitochondria in response to cell injuries like irradiation, anticancer drugs, oxidative stress, or membrane damage. At the mitochondrion level, these stimuli promote mitochondrial membrane permeabilization and the release of apoptotic cofactors, such as cytochrome *c* and Smac/DIABLO, that is blocked by Bcl-2 and Bcl-x_L (2–7). Once in the cytosol, released cytochrome *c* promotes assemblage of the apoptosome that consists of cytochrome *c*, Apaf-1, and caspase-9 (3). Thus, caspase-9 becomes activated and launches the apoptotic cascade mediated by effector caspases, the executioner of cell demise (8).

In addition to follicular lymphomas, Bcl-2 expression levels are elevated in several hematological malignancies and solid tumors, indicating that this molecule might have a role in raising the apoptotic threshold in a broad spectrum of cancerous disorders. *In vitro* studies demonstrated that overexpression of antiapoptotic Bcl-2 family members in tumors of different histology decreases apoptosis in response to anticancer drugs, irradiation, and hormone withdrawal (9, 10). In this context, expression of the Bcl-2 protein has been recognized as an independent adverse prognostic factor in large cell lymphomas, a tumor potentially curable with modern chemotherapy (11–14). In light of these data, apoptosis-modulating approaches capable to override chemoresistance associated with Bcl-2/Bcl-x_L overexpression may provide precious tools in the treatment of human malignancies.

One possibility is represented by the activation of the other

Received 11/11/02; revised 10/2/03; accepted 10/16/03.
Grant support: Grant 9906114952, 1999 from Ministero dell'Università e della Ricerca Scientifica e Tecnologica, Progetto FIRB n. RBAU01THPL, and Università di Genova. A. Nencioni acknowledges a fellowship from the Anna Fuller Fund for Research in Molecular Oncology 2003.
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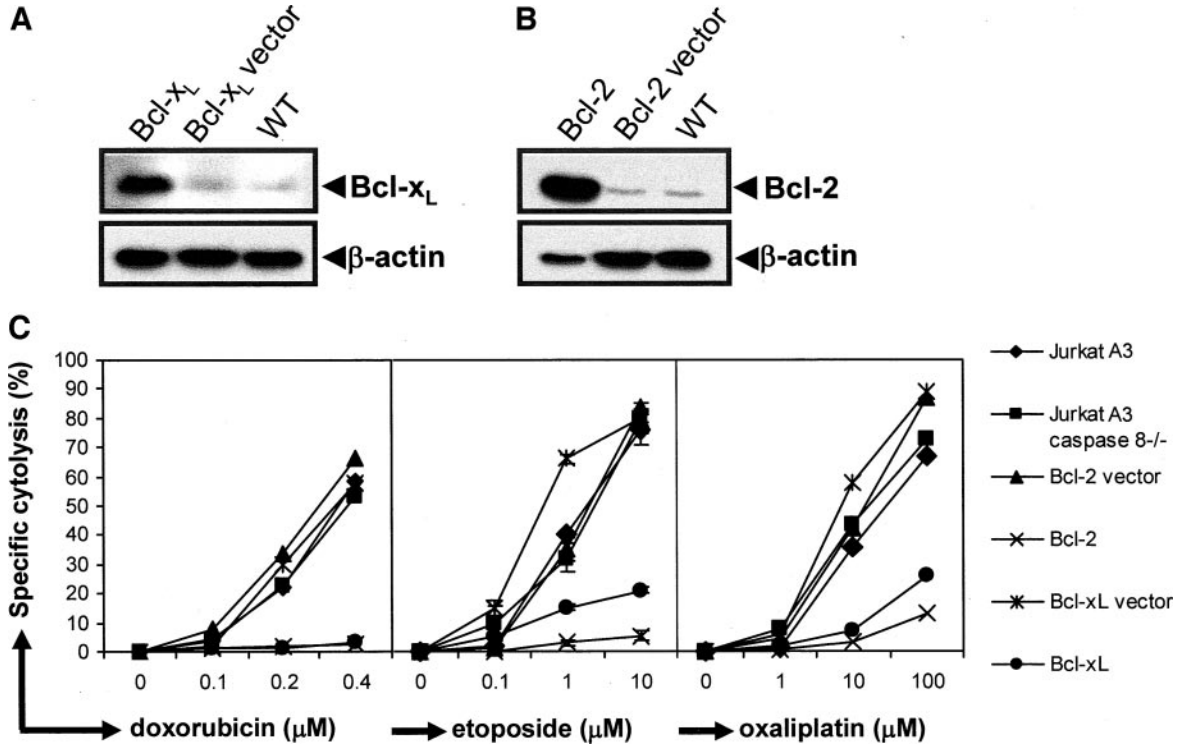

Fig. 1 Bcl-2 or Bcl-x_L overexpression but not caspase-8 deficiency inhibits the antitumor activity of doxorubicin, etoposide, and oxaliplatin. In *A*, lysates were prepared from 2×10^6 wild-type Jurkat cells or Jurkat cells stably transfected with Bcl-x_L or respective vector control cells. The levels of Bcl-x_L were determined by immunoblotting. β-actin detection was used as a protein loading control. In *B*, the levels of Bcl-2 were determined by immunoblotting in wild-type Jurkat and Jurkat cells stably transfected with Bcl-2 or vector control cells. In *C*, caspase-8-negative A3 Jurkat cells and the cognate wild-type cells, Jurkat cells stably transfected with Bcl-2 or Bcl-x_L, and cognate vector control cells were seeded at 5×10^4/well in 96-well plates and exposed for 24 h to the indicated concentrations of anticancer drugs. Thereafter, cells were harvested, and the dead cells were quantified by flow cytometry after staining with propidium iodide. Means of triplicates with SD are shown.

major apoptotic pathway, the death receptor pathway, which is triggered by members of the death receptor superfamily like CD95 (Fas, APO-1) or tumor necrosis factor receptor I (15, 16). Death receptor ligands like CD95 ligand or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL; Ref. 17) bind to the cognate receptor (CD95 and DR4/DR5, respectively) at the cell surface and induce caspase-8 oligomerization and proximity-induced autoproteolytic activation via adapter molecules, such as FADD/Mort1. Cleaved caspase-8 directly activates downstream effector caspases independent of mitochondria. Importantly, death receptor signaling also activates the apoptotic mitochondrial pathway via caspase-8-mediated cleavage of Bid, a proapoptotic Bcl-2 protein (18, 19). First attempts to exploit this approach using CD95L or tumor necrosis factor-α in animal tumor models discouraged application of these cytokines to humans given their extreme liver toxicity (20). More recently, the introduction of recombinant TRAIL (Apo-2L) has generated new enthusiasm because of the reported differential sensitivity to TRAIL-stimulated apoptosis between normal and cancerous cells. About 80% of human tumor cell lines including colon, lung, breast, kidney, skin, and brain tumors show some degree of sensitivity to TRAIL, whereas most normal cell types are resistant. The reasons for such different sensibility are still unclear and may be related to the up-regulated expression of TRAIL decoy receptor in normal cells (20, 21). Concerns related to TRAIL use were raised by a study by Jo *et al.* (22) reporting that TRAIL induces apoptosis in human hepatocytes, although this effect was described on relatively high TRAIL concentrations (200 and 400 ng/ml). In fact, administration of soluble recombinant TRAIL in experimental animals, including mice and primates, induced significant tumor regression without systemic toxicity (23–25).

In the present study, we demonstrate that the combination of TRAIL and anticancer drugs induces dissipation of mitochondrial transmembrane potential, thus overriding the mitochondrial block in antiapoptotic Bcl-2 family members expressing Jurkat cells. This results in a highly synergistic proapoptotic effect. This effect relies on caspase activation and is reproduced on low TRAIL concentrations.

MATERIALS AND METHODS

Cells and Reagents. Caspase-8-deficient Jurkat cells and the parental Jurkat cell line A3 were kindly provided by J. Blenis (Harvard Medical School, Boston, MA; Refs. 26–28). Stable transfectants of Jurkat cells overexpressing Bcl-2 and Bcl-x_L and their vector controls were a gift of C. Belka (University of Tübingen, Tübingen, Germany). The cells were cultured in RPMI 1640 supplemented with 10% inactivated FCS, 50 nM 2-mercaptoethanol, and antibiotics, all purchased from

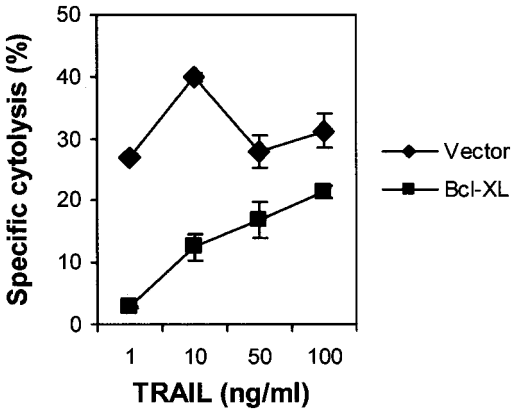

Fig. 2 Dose-response effect of tumor necrosis factor-related apoptosis-inducing ligand (*TRAIL*) in Bcl-x_L-overexpressing cells. Bcl-x_L-transfected Jurkat cells and respective vector control cells were stimulated with recombinant human TRAIL at the indicated concentrations for 24 h. Then, cell viability was assessed by propidium iodide staining and flow cytometry. Means of triplicates with SD are shown.

Life Technologies, Inc. (Grand Island, NY). Cells were grown at 37°C in a humidified 5% CO_2 atmosphere.

Oxaliplatin, a third-generation antiblastic platinum coordination complex of the 1,2-diaminocyclohexane family [*trans*-L-dach (1R, 2R-diaminocycloexane) oxalatoplatinum], was purchased from Sanofi Winthrop (Gentilly Cedex, France). Doxorubicin and etoposide were obtained from Sigma-Aldrich (St. Louis, MO). Recombinant, histidine-tagged human TRAIL was by Alexis Biochemicals (Lausen, Switzerland), and Benzyloxycarbonil-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) was purchased from Bachem Biochemica GmbH (Heidelberg, Germany).

Cytotoxicity Assay. For all assays, 5×10^4 cells were seeded in 96-well microtiter plates and cultured for the indicated times in the presence of different stimuli in a final volume of 200 μl. Cell death was determined by propidium iodide cell staining as described previously (26, 27). In brief, cells were incubated for 5 min in 2.5 μg/ml propidium iodide in PBS at 4°C in the dark. Propidium iodide-positive dead cells were enumerated by flow cytometry. Percentage of specific cytolysis was calculated as follows: 100 × [experimental sample (%) − spontaneous sample (%)/100% − spontaneous sample (%)]. Spontaneous cytolysis was required to be <20%.

Flow Cytometric Assay of Mitochondrial Transmembrane Potential ($\Delta\Psi_m$). Mitochondrial depolarization was determined by means of the cationic lipophilic fluorochrome $DiOC_6$, which accumulates in the mitochondrial matrix driven by the $\Delta\Psi_m$. For quantitation of cells with reduced $\Delta\Psi_m$, 2×10^6 cells/well were incubated in 0.5 ml of culture medium in 24-well plates in the presence of different stimuli. At different time points, cells were harvested, washed, and incubated for 15 min in culture medium containing 20 nm of $DiOC_6$ (Sigma-Aldrich). The percentage of $\Delta\Psi_m^{low}$ cells was determined by flow cytometry (29).

Immunoblotting. Cell lysates were prepared by resuspending 2×10^6 cells in lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 1% Triton X-100, 100 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin]. Proteins were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Proteins were visualized by probing the blots with the following antibodies: (*a*) anticaspase-3; (*b*) anticaspase-8 (both by Cell Signaling Technology, Beverly, MA); (*c*) anti-Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA); (*d*) anti-Bcl-x_L (R&D Systems, Inc., Minneapolis, MN); or (*e*) anti-β-actin (Sigma-Aldrich), followed by detection with matched horseradish peroxidase-conjugated secondary antibodies. Blots were developed using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ) and Kodak X-ray film (30).

Statistical Analysis. The results of cytotoxicity experiments were reported as mean and SD of triplicate cultures. Mitochondrial depolarization experiments were performed at least three times with similar results. Representative experiments are shown. The drug interaction was assessed by calculating the combination index (CI) as reported by J-L. Fischel *et al.* (31). The CI indicates synergism when <0.8, antagonism when >1.2, and additive effect when located between 0.8 and 1.2.

RESULTS

Bcl-2 and Bc-x_L Overexpression Inhibit Cytotoxicity Mediated by Oxaliplatin, Doxorubicin, and Etoposide. In the first series of experiments, we made use of Jurkat cells genetically modified to overexpress Bcl-2 or Bcl-x_L (Fig. 1, *A* and *B*) or of caspase-8-negative Jurkat cells (26–28) to monitor the effect of these proteins in anticancer drug-mediated cytotoxicity. It is known that Bcl-2/Bcl-x_L overexpression inhibits the mitochondrial apoptosis pathway by preventing mitochondrial disassemblage in response to apoptotic stimuli. Conversely, the caspase-8-negative Jurkat are resistant to apoptosis initiated by engagement of surface death receptors (26, 27).

We chose for our experiments doxorubicin and etoposide, two drugs frequently used in lymphoma chemotherapy and known to induce cell apoptosis in a death receptor pathway-independent manner (26, 27), along with the new platinum analogue oxaliplatin that has a broad spectrum of activity in a wide range of human tumors *in vitro* and *in vivo*, including non-Hodgkin lymphomas (32–37).

Preliminary data indicated that oxaliplatin effectively inhibits Jurkat cell viability in a dose-dependent manner, being active in the range from 1 μM to 5 mM with a IC_{50} of 2–3 μM (data not shown). It is of note that this range includes the clinically achievable plasma concentration of 10 μM (38), which reduced the survival fraction of Jurkat cells to 17% (SD ± 6.25) of control.

We found that Bcl-2- and Bcl-x_L-overexpressing Jurkat cells were efficiently protected against cytotoxicity induced by doxorubicin, etoposide, and oxaliplatin, whereas caspase-8 deficiency did not show any effect (Fig. 1*C*). This indicates the central role played by the mitochondrial apoptotic pathway for all of these compounds.

TRAIL Overcomes Anticancer Drug Resistance in Bcl-2/Bcl-x_L-Overexpressing Jurkat Cell Clones. Because Bcl-2/Bcl-x_L-overexpressing Jurkat cells retain a functional death receptor signaling (26, 27), we investigated the cytotoxic efficacy of TRAIL. In interaction experiments with doxorubicin, etoposide, and oxaliplatin, we also tested the hypothesis that

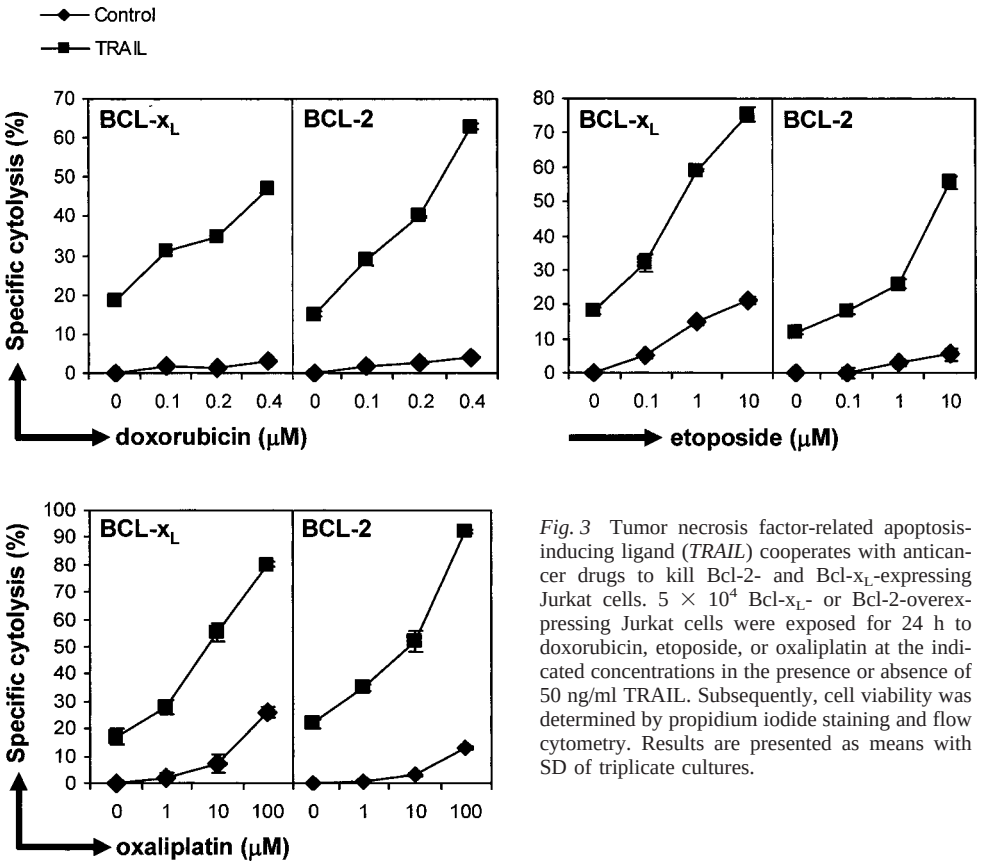

Fig. 3 Tumor necrosis factor-related apoptosis-inducing ligand (*TRAIL*) cooperates with anticancer drugs to kill Bcl-2- and Bcl-x_L-expressing Jurkat cells. 5×10^4 Bcl-x_L- or Bcl-2-overexpressing Jurkat cells were exposed for 24 h to doxorubicin, etoposide, or oxaliplatin at the indicated concentrations in the presence or absence of 50 ng/ml TRAIL. Subsequently, cell viability was determined by propidium iodide staining and flow cytometry. Results are presented as means with SD of triplicate cultures.

TRAIL may enhance anticancer drug-induced cytotoxicity and overcome chemoresitance.

Preliminary experiments demonstrated that TRAIL was able to induce dose-dependent cytolysis of both parental and Bcl-2 family members overexpressing Jurkat clones. However, as shown for the Bcl-x_L-overexpressing cells, the cytolytic efficiency was lower as compared with the wild-type cells, the reduction being more evident on exposure to 1 and 10 ng/ml rather than 50–100 ng/ml TRAIL (Fig. 2).

In interaction experiments, the anticancer drug resistant cell clones were treated with each drug alone and drug combinations. The doses of anticancer drugs were in the range of clinically achievable plasma concentrations (38–40). Fig. 3 shows the positive interaction produced by the combination of 50 ng/ml TRAIL with doxorubicin (CI of 0.78 ± 0.06 in Bcl-xL cells and CI of 0.68 ± 0.12 in Bcl-2 cells), etoposide (CI of 0.61 ± 0.14 in Bcl-xL cells and CI of 0.78 ± 0.12 in Bcl-2 cells), and oxaliplatin (CI of 0.61 ± 0.08 in Bcl-xL cells and CI of 0.56 ± 0.32 in Bcl-2 cells). Similar interaction effects were also observed in cell growth experiments with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (data not shown).

As demonstrated in the combination with oxaliplatin, TRAIL

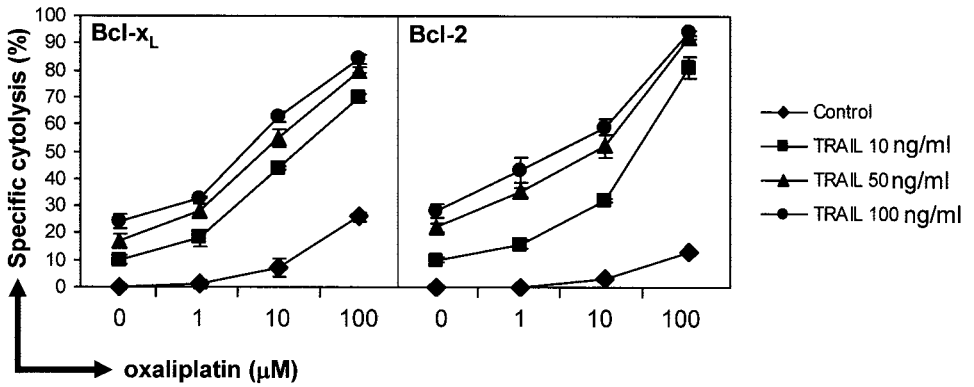

Fig. 4 Response to different doses of TRAIL and oxaliplatin of Bcl-x_L- and Bcl-2-overexpressing Jurkat cells. Bcl-x_L- and Bcl-2-overexpressing Jurkat cells were exposed to TRAIL, oxaliplatin, or the combination of them at the indicated concentrations for 24 h. Thereafter, dead cells were quantified by propidium iodide staining and subsequent flow cytometric analysis. Results are presented as means with SD of triplicate cultures.

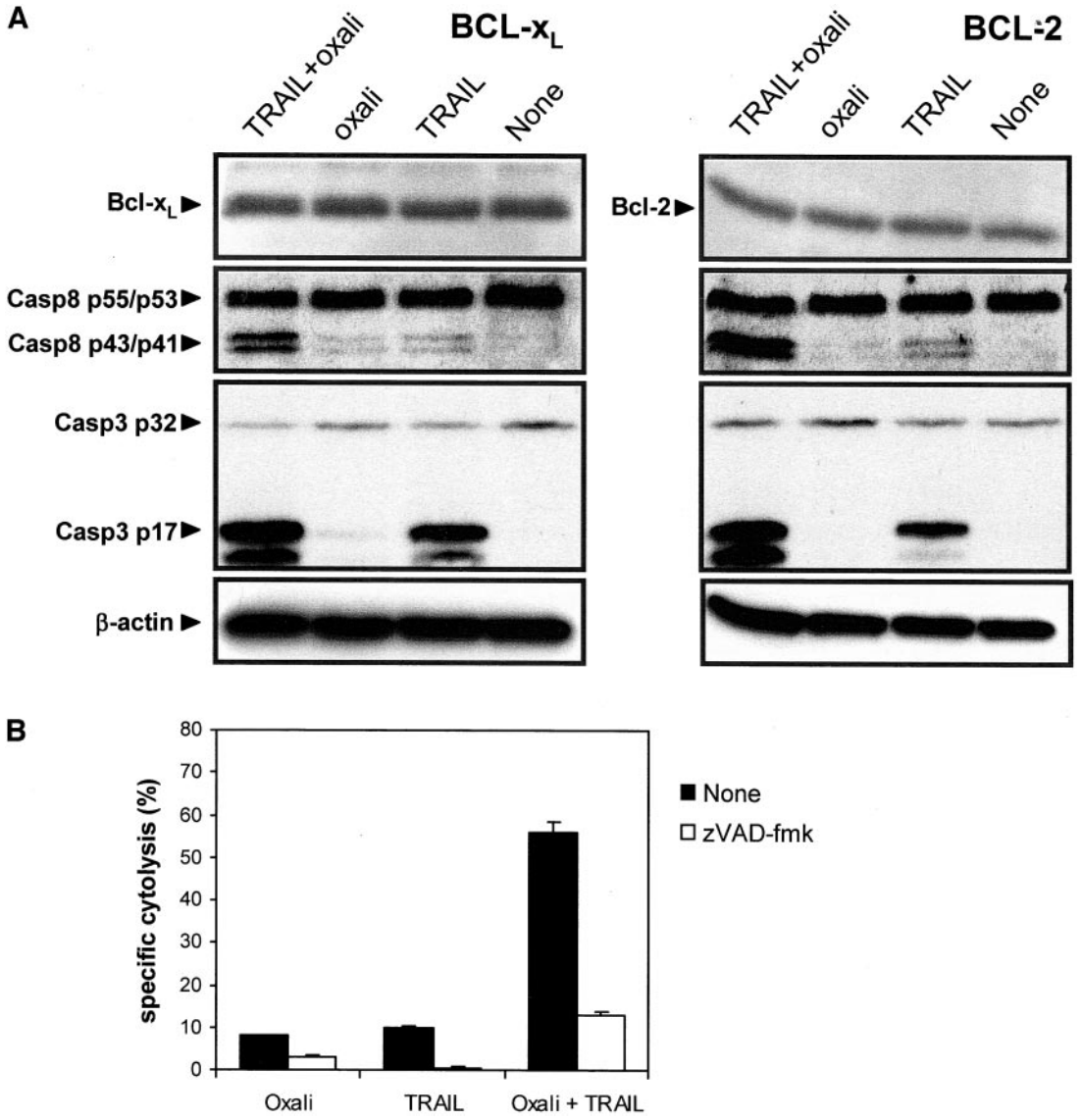

Fig. 5 Caspase activation is required for the synergistic killing of Bcl-x_L-overexpressing cells by tumor necrosis factor-related apoptosis-inducing ligand (*TRAIL*) and oxaliplatin. In *A*, lysates were prepared from 2×10^6 Jurkat cells after an 8-h treatment with medium alone (*None*), 50 ng/ml TRAIL, 100 μM oxaliplatin, or combined TRAIL and oxaliplatin. Cellular proteins were resolved by SDS-PAGE. Caspase activity was detected by cleavage of caspase-8 and -3 using immunoblot analysis. Similarly, the levels of Bcl-x_L, Bcl-2, and β-actin were detected by immunoblotting. In *B*, 5×10^4 Bcl-x_L-transfected Jurkat cells were preincubated for 1 h in the presence (*white bars*) or absence (*black bars*) of zVAD-fmk (100 μM) and thereafter were exposed to TRAIL (10 ng/ml), oxaliplatin (10 μM), or the combination of them for 24 h. Subsequently, cell death was quantified by flow cytometric analysis of propidium iodide-stained cells. Mean values of triplicate cultures with SD are presented.

enhances drug-induced cytolysis in a concentration-dependent fashion (Fig. 4). However, a TRAIL concentration of 10 ng/ml still produces a synergistic effect with the anticancer drug.

TRAIL Promotes Caspase Activation in Bcl-2/Bcl-x_L-Overexpressing Jurkat Cells. Caspase activation via proteolytic cleavage plays a central role in cytotoxicity mediated by the apoptotic machinery. Hence, we monitored cleavage of caspase-3 and -8 in response to oxaliplatin and TRAIL, used alone or in combination. We found that TRAIL but not oxaliplatin induces caspase cleavage in the Jurkat cells overexpressing Bcl-2 or Bc-x_L, and this effect was strongly enhanced when cells were treated with the combination of the two compounds (Fig. 5*A*). Consistent with a central role for caspase activation in the observed cooperation, the cytotoxicty produced by the combination of oxaliplatin and TRAIL was strongly reduced by z-VAD-fmk, a broad spectrum caspase inhibitor (Fig. 5*B*). Same results were obtained with doxorubicin and etoposide (data not shown).

Coadministration of TRAIL and Oxaliplatin Induces Dissipation of $\Delta\Psi_m$ in Bcl-2/Bcl-x_L-Overexpressing Jurkat Cells, an Effect Dependent on Caspases. We further asked whether the molecular mechanisms induced by TRAIL and anticancer drugs would converge at the level of the mitochon-

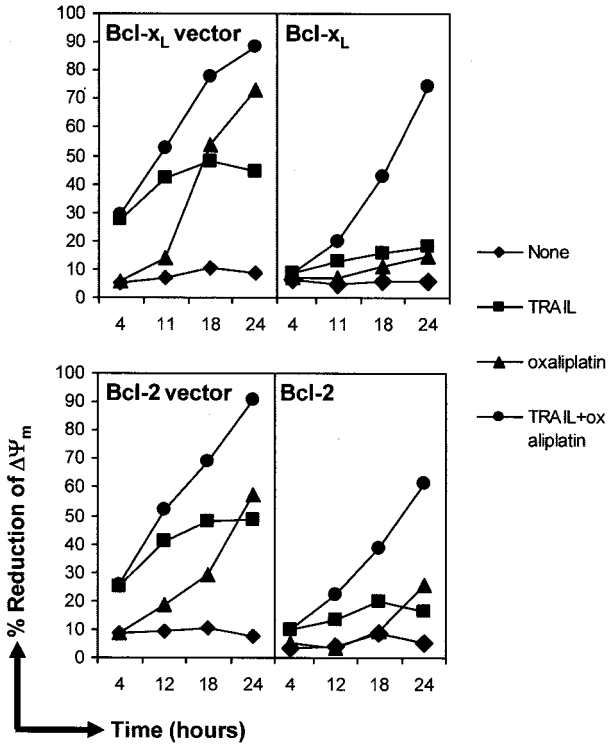

Fig. 6 Bcl-x_L and Bcl-2 inhibit the mitochondrial depolarization in response to tumor necrosis factor-related apoptosis-inducing ligand (*TRAIL*) and oxaliplatin but not to the combination of both. Bcl-x_L- and Bcl-2-overxpressing Jurkat cells and vector control cells were seeded at 2×10^6 cells/well in 24-well plates and treated with or without (*None*) oxaliplatin (100 μM), TRAIL (50 ng/ml), or the combination of both stimuli. Cells were harvested at different time points, washed, and stained with 20 nm of $DiOC_6$. $\Delta\Psi_m^{low}$ cells were enumerated by flow cytometry.

dria and thus overcome the block mediated by antiapoptotic Bcl-2 family proteins. To verify this hypothesis, we measured the loss of $\Delta\Psi_m$. Fig. 6 shows the results of a time course experiment with Bcl-x_L and Bcl-2 cells and respective vector controls exposed to oxaliplatin and TRAIL. In the vector control cells, both TRAIL and oxaliplatin were effective to induce mitochondrial perturbation, although the effect of TRAIL was detectable at an earlier time point than that of oxaliplatin, being already evident after 4 h (Fig. 6). Loss of $\Delta\Psi_m$ via oxaliplatin or TRAIL was strongly inhibited by Bcl-x_L or Bcl-2 overexpression. However, when the two drugs were used in combination, a high degree of depolarization was detected with a kinetics resembling that of oxaliplatin in the parental cells (Fig. 6). These data mirror the results of cytotoxicity experiments and suggest that the mitochondrial depolarization is a critical event in the synergistic effect observed.

The role of caspases in the effects on $\Delta\Psi_m$ observed on stimulation with TRAIL, oxaliplatin, or a combination of them was evaluated by means of the broad spectrum caspase inhibitor zVAD-fmk. In the Bcl-x_L vector cells, zVAD-fmk completely abolished the $\Delta\Psi_m$ loss induced via TRAIL and reduced the depolarization produced by oxaliplatin and the combination of TRAIL plus oxaliplatin (Fig. 7). In the Bcl-x_L clones, the $\Delta\Psi_m$ loss produced by oxaliplatin and TRAIL, used alone or in combination, was completely inhibited in the presence of zVAD-fmk.

DISCUSSION

In the present study, we have explored in the model of Bcl-2- and Bcl-x_L-overexpressing Jurkat cells a possible strategy to overcome drug resistance and induce cytolysis. To this purpose, we have tested the effectiveness of a death receptor pathway-activating agent, such as TRAIL, assuming that the resistant cell lines retain at least a partially functional extrinsic apoptotic pathway (26, 27).

In this context, the ability of Bcl-2 protein overexpression to protect against TRAIL-mediated apoptosis is debated. Several reports indicate that in lymphoid cells, Bcl-2 overexpression does not protect against TRAIL-induced apoptosis (41–43). Conversely, Bcl-2 proteins may inhibit apoptosis by TRAIL in solid tumors (44, 45). In our experimental system, we found that TRAIL stimulation of Jurkat cells promotes apoptosis-dependent cytolysis. However, Bcl-x_L and Bcl-2 overexpression sig-

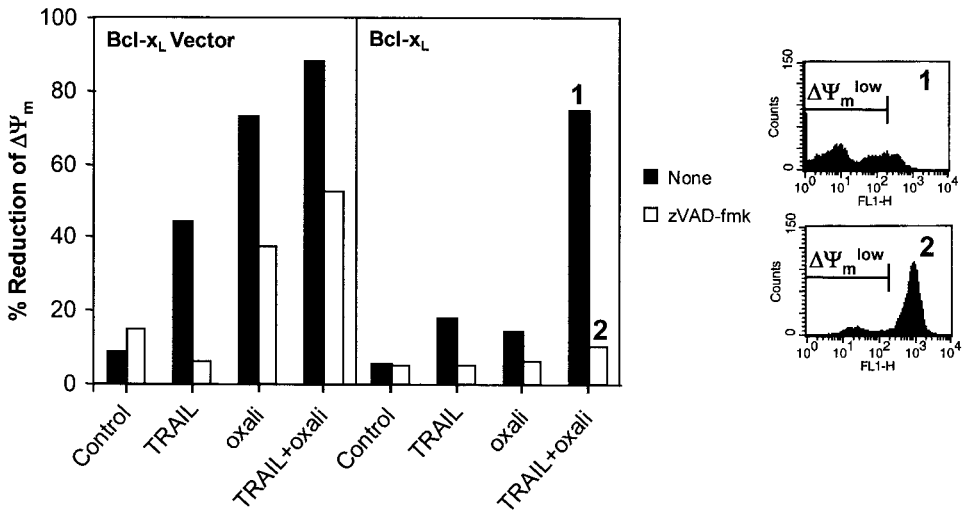

Fig. 7 Effect of caspase inhibition on the mitochondrial depolarization induced by tumor necrosis factor-related apoptosis-inducing ligand (*TRAIL*), oxaliplatin, and TRAIL plus oxaliplatin. Bcl-x_L-overxpressing Jurkat and vector control cells were seeded at 2×10^6 cells/well in 24-well plates and preincubated for 1 h with (*white bars*) or without (*black bars*) 100 μM zVAD-fmk. Thereafter, cells were treated with or without (*None*) oxaliplatin (100 μM), TRAIL (50 ng/ml), or the combination of both stimuli. Cells were harvested 24 h later, stained with 20 nm $DiOC_6$, and analyzed by flow cytometry.

nificantly reduced apoptosis induction, particularly on low TRAIL concentrations.

In interaction experiments, Bcl-x_L- and Bcl-2-overexpressing cells treated with TRAIL combined with an anticancer drug were induced to undergo cytolysis in a superadditive fashion. Importantly, this effect could be detected on concentrations of anticancer drugs achievable *in vivo* and low TRAIL doses. Coupling anticancer drugs with TRAIL has been reported to strongly enhance cytotoxicity in different human tumor models (46–52). The mechanism for this synergy remains elusive, although up-regulation of TRAIL receptor DR5 (52, 53) and Bak (54, 55) by anticancer drugs has been indicated as a possible cause for this effect. In our experiments, we demonstrate that the antiblastic oxaliplatin and TRAIL cooperate to promote caspase activation in the Bcl-x_L- and Bcl-2-overexpressing cells. Interestingly, the combination of the two agents also enhances caspase-8 cleavage, which can be mediated by caspase-3 as suggested previously (27). Moreover, combining the two stimuli overcomes the mitochondrial threshold raised by Bcl-x_L and produces dissipation of the $\Delta\Psi_m$. The effect of TRAIL on cell viability and $\Delta\Psi_m$ was inhibited by zVAD-fmk, as expected for the inhibition of caspase-8 (26, 27), which is known to be required for TRAIL-mediated proapoptotic signaling (27, 43).

Interestingly, in addition, oxaliplatin-mediated mitochondrial perturbation partially depended on caspases, because zVAD-fmk delayed (data not shown) and reduced the mitochondrial damage in response to oxaliplatin in wild-type Jurkat cells. This effect could be explained in the light of recent data indicating that some casapses, *i.e.*, caspase-2, may act upstream of mitochondria to induce cytochrome *c* release during etoposide or stress-induced apoptosis (56, 57). An alternative explanation might be the block of mitochondrial amplification loops by inhibition of caspase-dependent Bid cleavage (27).

In conclusion, our data indicate in the enhanced mitochondrial injury and caspase activation a novel mechanism for the cytotoxic synergy between anticancer drugs and TRAIL. The combination of antiblastic with activators of the extrinsic apoptotic pathway like TRAIL may help to overcome chemoresistance in Bcl-2-positive human lymphomas and, possibly, other malignancies.

ACKNOWLEDGMENTS

We thank Drs. C. Belka, J. Blenis, and K. Schulze-Osthoff for providing many valuable cell lines and reagents.

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Clin Cancer Res 2004;10:1463-1470.