Distinct TRAIL Resistance Mechanisms Can Be Overcome by Proteasome Inhibition but not Generally by Synergizing Agents

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**Abstract**

One impediment to the use of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor-targeted agents as antitumor drugs is the evolution of resistance, a common problem in cancer. On the contrary, many different kinds of drugs synergize with TRAIL in TRAIL-sensitive tumor cells, raising the question whether one can overcome resistance with the same drugs producing synergy. This is an important question, because recent clinical trials suggest that combination treatments with cytotoxic drugs and TRAIL receptor-targeted agents do not provide additional benefit compared with cytotoxic agents on their own. Such results might be expected if drug combinations that synergize in sensitive tumor cells but cannot overcome TRAIL resistance are used in patients whose tumors were not selected for retention of TRAIL sensitivity. We tested this idea by creating isogenic tumor cells with acquired TRAIL resistance or defined mechanisms of resistance that occur in human tumors and then comparing them to the TRAIL-sensitive parental cell line. Although diverse classes of anticancer drugs were all able to synergize with TRAIL in sensitive cells, most agents were unable to overcome resistance and there was no relationship between the amount of synergy seen with a particular agent and its ability to overcome acquired resistance. An important exception was proteasome inhibitors, which were, however, able to overcome diverse resistance mechanisms. Our findings suggest that one should select drugs for TRAIL receptor agonist combination therapy based not just on their ability to synergize, but rather on their ability to overcome resistance as well as synergize.

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**Introduction**

Cancer therapy is often hampered because the efficiency with which susceptible tumor cells are killed is too low and tumors evolve such that they either present with primary resistance or acquire resistance over time. To address the first issue, it is useful to identify agents that synergize when they are combined. To address the second issue, it is necessary to find new treatments that avoid the resistance mechanisms or combine with agents that allow the resistance to be overcome.

It is often assumed that if a drug synergizes with another drug, then the combination will provide a better way to treat cancer and that more synergy is better. However, obtaining synergy and overcoming resistance is not necessarily the same thing. Just because a drug combination can synergize in susceptible tumor cells, it does not necessarily follow that the same combination will be able to overcome resistance.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor-targeted drugs are an interesting type of anticancer treatment with which to test these ideas, because these drugs directly activate the apoptosis machinery. There are many different ways that tumor cells can become resistant to TRAIL R agonists, and there are a large number of other agents that can synergize with TRAIL in susceptible tumor cells (1, 2).

TRAIL R agonists bind to 2 receptors (DR4 and DR5, also known as TRAIL R1 and TRAIL R2, and TNFRSF10a and TNFRSF10b) to cause the recruitment of an adaptor protein called FADD, which in turn recruits caspase-8 to form a platform called the DISC that leads to the activation of caspase-8 (3). Active caspase-8 induces apoptosis by directly activating the effector caspase-3 or, more commonly, by cleavage of the BH3 protein Bid, which leads to release of...
cytochrome c and activation of the mitochondrial apoptosis machinery. Several TRAIL R activating drugs are in clinical trials and others are in preclinical development (1, 2); in addition, some other agents may work indirectly through activation of DR4 and DR5.

Many tumor cell lines are TRAIL resistant (4) and resistance varies in primary human tumor cells. For example, it has been reported that primary colon cancer cells are usually sensitive to TRAIL (5), whereas primary astrocytoma cells (5) and B cell chronic lymphocytic leukemia cells (6) are not. Other tumor types, for example, ovarian cancers, show more variable responses (7). Because TRAIL signaling is an important part of host mechanisms to suppress tumor formation and metastasis (1, 2), it is to be expected that advanced cancers would often evolve TRAIL resistance. Diverse mechanisms that confer selective TRAIL resistance have been identified in human tumors (8). For example, somatic mutations in TRAIL receptors, downregulation of DR4 or DR5 and overexpression of decoy receptors DcR1 and DcR2, and expression of a tumor-related homeobox transcription factor called Six1 can all confer selective inhibition to TRAIL. In addition, more general antiapoptotic mechanisms like increased expression of Bcl-2 can also cause TRAIL resistance.

A myriad of anticancer drugs including antimetabolites, DNA-damaging agents, microtubule-targeted drugs, protein kinase inhibitors, proteasome inhibitors, targeted toxins, deacetylase inhibitors, Bcl-2 antagonists, and antibody-based therapeutics have been reported to synergize with TRAIL receptor-targeted drugs (1, 2, 8, 9). This broad ability to synergize along with limited evidence of efficacy as single agents has led to the view that optimal use of TRAIL R-targeted drugs will be in combination with cytotoxic chemotherapy or signal transduction pathway-targeted agents. Indeed, clinical trials with TRAIL or antibodies against DR4 or DR5 and overexpression of decoy receptors DcR1 and DcR2, and expression of a tumor-related homeobox transcription factor called Six1 can all confer selective inhibition to TRAIL. Thus, it is necessary to choose the correct agent for the combination because most synergizing drugs do not overcome acquired resistance and the most synergistic drugs are not necessarily the best ones to use in combination therapy if the objective is to try to both increase tumor killing in sensitive cells and overcome TRAIL resistance.

To answer this question we examined isogenic BJAB tumor cell lines. We tested if the parental cells, which are sensitive to all TRAIL receptor-targeted therapeutics, could synergize with anticancer drugs that work through different mechanisms. We developed an isogenic line with acquired TRAIL resistance through continued exposure to increasing doses of TRAIL receptor-activating antibody and asked if these cells, or cells that were made resistant using defined molecular mechanisms that occur in human tumors, could be made sensitive to TRAIL by combining with drugs that synergize. Surprisingly, we found that most synergizing drugs could not overcome resistance and that more synergy does not make it more likely that the drug will overcome resistance. The most effective agents at overcoming resistance were proteasome inhibitors and this ability applied to different, but not all, TRAIL resistance mechanisms. These data show that it is possible to both obtain synergy and overcome TRAIL resistance by combining TRAIL receptor-targeted drugs with other agents. However, it is necessary to choose the correct agent for the combination because most synergizing drugs do not overcome acquired resistance and the most synergistic drugs are not necessarily the best ones to use in combination therapy if the objective is to try to both increase tumor killing in sensitive cells and overcome TRAIL resistance.

Materials and Methods

Cell lines
Parental BJAB cells, which are an Epstein-Barr virus-negative B lymphoma cell line, were provided previously by Marcus Peter (University of Chicago, Chicago, Illinois). The cells were most recently fingerprinted using the ABI Identifier kit in January 2010, and were distinct from other cells in the merged DSMZ, ATCC, JCRB, and Riken databases of DNA profiles of cell lines that are available at DSMZ web site (14) and are therefore not contaminated with any other cell line. BJAB cells expressing mutant DR5 in place of the wild-type protein were described previously (15), the various resistant cells expressing DcR1, DcR2, FADD-DD, and Six1 were made by stably expressing the respective cDNA in pcDNA3 puro (16). DR5 and XIAP knockdowns were achieved by stably expressing a lentivirus expressing a DR5- or XIAP-targeted shRNA (Open Biosystems), whereas BJAB<sup>Lex<sup>R</sup></sup> cells were made by gradually increasing lexatumumab concentrations and selecting the cells capable of continued growth. All cell clones were derived from representative single clone isolated by limiting dilution. Cells were grown in RPMI 1640 with 10% FBS, sodium bicarbonate, and glucose in a 5% CO₂ humidified atmosphere at 37°C.

MTS cell viability

Cells were plated in 96-well format at 40,000 cells per well. Lexatumumab (Human Genome Sciences) was cross-linked with antihuman IgG Fc (Sigma) for 30 minutes before serial dilution. TRAIL (R&D Systems) and SuperFas ligand (Enzo Life Sciences) were prepared according to manufacturer's
instructions prior to addition to cells. Two hours prior to the end of the experiment, cells were treated with 20 μL of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; Promega] inner salt according to manufacturer’s instructions. The plate was incubated at 37°C for 2 hours. A Bio-Rad Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories) was used to measure absorbance of samples at 490 nm.

**Immunoblotting**

One million cells were harvested and lysates were prepared by boiling in SDS buffer 5 minutes prior to gel electrophoresis. Lysates were resolved on 12% SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P transfer membrane (Millipore Corporation). Blots were blocked with 5% milk in TBST and incubated with antibodies that recognize PARP, X-linked inhibitor of apoptosis protein (XIAP), Bid, caspase-3, caspase-8 (Cell Signaling Technologies), Fas associated protein with death domain (FADD; BD Biosciences), and β-actin (Sigma). Blots were then incubated with antirabbit or antimouse horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technologies). Detection was done using chemiluminescent ECL reagent (Millipore Corporation) and developed on blue X-ray film (Life Science Products).

**Drug synergy**

Cells were plated in duplicate wells at a density of 40,000 cells per well in a 96-well plate and allowed to sit 24 hours prior to treatment. TRAIL was used alone and in combination with the following drugs: MG132 (EMD Biosciences), doxorubicin hydrochloride, 5-fluorouracil (5-FU), etoposide, oxamflatin, staurosporine, and β-sorbitol (Sigma). Bortezomib (NSC 681239), sorafenib (NSC 747971), and azacitidine (NSC 102816) were obtained from The NCI/DTP Open Chemical Repository. MTS assays were done after 24 hours incubation and combination index (CI) values were obtained using Calcusyn, which determines synergy using the median effect principle comparing the sensitivity to each drug on its own and together with TRAIL. Synergy was scored as CI values 0.0 to 0.3 (++++), 0.3 to 0.7 (++), and 0.7 to 1.0 (+). For the data shown in Table 1, the doses used for each drug combination were as follows:

1. **Bortezomib** D1: 7.5 ng/mL TRAIL, 5 nmol/L bortez; D2: 15 ng/mL TRAIL, 10 nmol/L bortez.
2. **MG132** D1: 2 ng/mL TRAIL, 105 μmol/L MG132; D2: 4 ng/mL TRAIL, 210 μmol/L MG132.
3. **Doxorubicin** D1: 2 ng/mL TRAIL, 0.875 μg/mL doxo; D2: 4 ng/mL TRAIL, 1.75 μg/mL doxo.
4. **5-FU** D1: 2 ng/mL TRAIL, 25 nmol/L 5-FU; D2: 8 ng/mL TRAIL, 100 nmol/L 5-FU.
5. **Etoposide** D1: 3.125 ng/mL TRAIL, 4 μmol/L etoposide; D2: 6.25 ng/mL TRAIL, 8 μmol/L etoposide.
6. **Oxamflatin** D1: 4 ng/mL TRAIL, 1 μg/mL oxam; D2: 8 ng/mL TRAIL, 2 μg/mL oxam.
7. **Sorafenib** D1: 7.5 ng/mL TRAIL, 5 nmol/L sorafenib; D2: 15 ng/mL TRAIL, 10 nmol/L sorafenib.

**Table 1.** Many different drugs can synergize with TRAIL

<table>
<thead>
<tr>
<th>Drug</th>
<th>Type of drug</th>
<th>Doses</th>
<th>CI</th>
<th>Synergy</th>
</tr>
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<tr>
<td>Bortezomib</td>
<td>Proteasome inhibitor</td>
<td>1</td>
<td>0.049</td>
<td>+++</td>
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<td></td>
<td></td>
<td>2</td>
<td>0.123</td>
<td>+++</td>
</tr>
<tr>
<td>MG132</td>
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<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.299</td>
<td>+++</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Topoisomerase inhibitor</td>
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<td>0.087</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.092</td>
<td>+++</td>
</tr>
<tr>
<td>5-FU</td>
<td>Inhibitor of thymidine synthesis</td>
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<td>0.463</td>
<td>++</td>
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<td></td>
<td></td>
<td>2</td>
<td>0.366</td>
<td>++</td>
</tr>
<tr>
<td>Etoposide</td>
<td>Topoisomerase II inhibitor</td>
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<td>0.065</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.068</td>
<td>+++</td>
</tr>
<tr>
<td>Oxamflatin</td>
<td>HDAC inhibitor</td>
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<td>0.195</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.287</td>
<td>+++</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>Selective kinase inhibitor</td>
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<td>0.795</td>
<td>+</td>
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<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.316</td>
<td>++</td>
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<tr>
<td>Azacitidine</td>
<td>DNA/RNA methyltransferase inhibitor</td>
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<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.189</td>
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</tr>
<tr>
<td>Sorbitol</td>
<td>Hyperosmotic agent</td>
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<td>0.089</td>
<td>+++</td>
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<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.002</td>
<td>+++</td>
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<tr>
<td>Staurosporine</td>
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<tr>
<td></td>
<td></td>
<td>2</td>
<td>0.175</td>
<td>+++</td>
</tr>
</tbody>
</table>

**NOTE.** Drugs were combined with TRAIL in different dose combinations (see Materials and Methods) and synergy determined using the Calcusyn program. All drugs displayed synergy; however, some drugs were significantly more synergistic than others.
Azacitidine D1: 5 ng/mL TRAIL, 6.25 nmol/L aza; D2: 10 ng/mL TRAIL, 12.5 nmol/L aza.

Sorbitol D1: 4 ng/mL TRAIL, 50 mmol/L sorbitol; D2: 8 ng/mL TRAIL, 100 mmol/L sorbitol.

Staurosporine D1: 2 ng/mL TRAIL, 0.375 μmol/L stauro; D2: 4 ng/mL TRAIL, 0.75 μmol/L stauro.

Cell death assays

Cells were plated at 40,000 cells per well in triplicate in a 96-well plate and allowed to sit for 24 hours prior to treatment. Drug was added at the indicated values and an MTS assay was done after 24 hours. For sensitization experiments, cells were plated at 40,000 cells per well in triplicate in a 96-well plate and allowed to sit for 4 hours. The sensitization drug was added to all wells to a concentration to kill less than 35% of the cells on its own. After 24 hours, TRAIL was added in a dose-dependent manner. MTS cell viability assay was done 24 hours later.

DISC IP

Cells (1 × 10^7) were suspended in 10 mL of culture medium, incubated with 1 μg/mL lexatumumab at 4°C for 30 minutes, transferred to 37°C for another 1 hour, washed in PBS three times, and then lysed in IP buffer (150 mmol/L NaCl and 20 mmol/L Tris-Cl, pH 7.5/1% Triton X-100) supplemented with complete protease inhibitors (Roche Applied Science). After the lysates were centrifuged (15 minutes at 12,000 xg), antibodies were precipitated at 4°C overnight. The beads were washed three times with IP buffer supplemented with 0.5 mol/L NaCl, and samples were subjected to Western blotting analysis.
DNA fragmentation assay

Cells were pretreated with MG132 (0.35 μmol/L) for 24 hours. Cells (5 × 10⁶) were then suspended in 10 mL of culture medium and treated with 150 ng/mL TRAIL for 4 hours. After treatment, cells were washed with ice-cold PBS and lysis buffer (20 mmol/L EDTA, 10 mmol/L Tris at pH 8.0, 200 mmol/L NaCl, 0.2% Triton-X 100 and RNase A 0.5 mg/mL) was added for 2 hours at 37°C. Proteinase K (5 mg/mL) was added and incubated at 56°C overnight. DNA was precipitated and run on 1.6% agarose gel for 3 hours at 75 V.

Results

TRAIL synergizes with multiple agents in TRAIL-sensitive BJAB cells

Although many different kinds of drugs have been reported to synergize with TRAIL (1, 2, 9), there are few reports in which the same tumor cells have been treated with different classes of anticancer drug along with TRAIL to compare the extent of synergy between different kinds of agents. To do this, we compared the sensitivity of BJAB cells to TRAIL and different

Figure 2. LexR cells have functional DISC formation and proximal signaling events. A, PARP Western blot analysis of parental BJAB cells and BJAB LexR cells treated with TRAIL after pretreatment with MG132 (0.35 μmol/L). B, DNA fragmentation assay of parental BJAB cells and BJAB LexR cells. TRAIL was added at a concentration of 150 ng/mL after pretreatment with MG132 (0.35 μmol/L) and treated for 4 hours to assess apoptosis. C, DISC IP using lexatumumab in BJAB and BJAB LexR cells; the Western blot was probed with anti-FADD and anti-caspase-8, indicating no apparent difference in FADD and caspase-8 recruitment. D, TRAIL R signaling events in parental BJAB cells and BJAB LexR cells treated with TRAIL after pretreatment with MG132 (0.35 μmol/L) or etoposide (0.75 μmol/L). TRAIL was added at 150 ng/mL for the indicated times before harvesting in RIPA buffer. These data show that proximal TRAIL R signaling events are functional in the LexR cells with acquired TRAIL resistance and that the block in signaling lies between caspase-8 and caspase-3.
kinds of anticancer drugs alone and in combination. To determine the extent of synergy, we used different dose combinations and calculated synergy using the CalcuSyn program, which assesses synergy using the median effect principal to calculate the CI in which CI equal to 1 indicates additivity, CI less than 1 indicates synergy, and CI more than 1 indicates antagonism. Table 1 shows CI values for different dose combinations for various anticancer drugs including proteasome inhibitors, DNA-damaging agents, antimitabolites, deactylase inhibitors, a selective protein kinase inhibitor, and an inhibitor of methyltransferases. In addition, we compared two general apoptotic stimuli, sorbitol, which induces hyperosmolar stress, and staurosporine, which is a broad kinase inhibitor. All these agents caused synergy with TRAIL as indicated by CI values less than 1. For example, the topoisomerase inhibitor etoposide led to very high synergy (CI < 0.1) whereas the proteasome inhibitor MG132 produced strong synergy (CI ~ 0.3), and the kinase inhibitor sorafenib was somewhat less effective (CI = 0.3–0.7). These data indicate that in the same tumor cells, different anticancer agents, and even general proapoptotic stimuli, all synergize with TRAIL although to different extents.

Acquired TRAIL resistance in BJAB cells is overcome by only some synergizing agents

To test if acquired TRAIL resistance could be overcome by combining with the other agents, we selected BJAB cells for resistance to TRAIL-targeted therapeutics by treating with increasing doses of the TRAIL R2/DR5 agonistic antibody lexatumumab. Figure 1A shows that these cells (named BJABLexR) displayed essentially complete resistance to lexatumumab. Figure 1A shows that these cells (named BJABLexR) displayed essentially complete resistance to lexatumumab. To examine the kinetics of these responses in parental BJABs and the BJABLexR cells with and without MG132 (Fig. 2D, top) or etoposide (Fig. 2D, bottom). In the resistant cells with MG132 or etoposide, TRAIL R signaling leads to similar cleavage of full-length Bid and XIAP with relatively small differences in the extent or kinetics of these proximal signaling events in the presence or absence of MG132 or etoposide. As expected, there was a marked difference in caspase-3 cleavage.

To determine if the sensitivity caused by proteasome inhibition was associated with altered signaling downstream of the TRAIL receptors, we analyzed proximal events in TRAIL R signaling in matched wild type and BJABLexR cells. Parental BJAB cells were similarly sensitive to TRAIL whether or not Bid was knocked down (Supplementary Fig. S2) indicating that these cells are Type I cells that do not require amplification through the mitochondrial pathway; however, Bid cleavage (Fig. 2D) and cytochrome c release (data not shown) occurs after TRAIL R activation in the parental cells, meaning that Bid cleavage is a useful way to monitor activation of the caspase-8. Figure 2A shows PARP cleavage in control parental cells and the BJABLexR cells after treatment with TRAIL, with or without MG132. In the BJABLexR cells, PARP cleavage only occurred when MG132 was present but was reduced compared with parental cells. Similarly, apoptotic cleavage of DNA was only observed in the BJABLexR cells when MG132 was added (Fig. 2B). TRAIL R stimulation leads to DISC formation, caspase-8 activation and cleavage leading to Bid cleavage, XIAP degradation, and caspase-3 cleavage. We therefore examined the kinetics of these responses in parental BJABs and the BJABLexR cells with and without MG132 (Fig. 2D, top) or etoposide (Fig. 2D, bottom). In the resistant cells with MG132 or etoposide, TRAIL R signaling leads to similar cleavage of full-length Bid and XIAP with relatively small differences in the extent or kinetics of these proximal signaling events in the presence or absence of MG132 or etoposide. As expected, there was a marked difference in caspase-3 cleavage.

Figure 3. XIAP contributes but does not fully explain TRAIL resistance. Western blot analysis of parental BJAB cells and BJABLexR cells treated with TRAIL after pretreatment with MG132 (0.35 μmol/L). XIAP was knocked down in BJABLexR cells using pGIPZ lentiviral shRNA. MTS assay quantifying cell viability of BJAB parental and BJABLexR cells after treatment with TRAIL.
with efficient cleavage in control cells but only MG132-treated BJAB

LexR cells displaying extensive caspase-3 cleavage. In addition, both BJAB and BJAB

LexR cells displayed similar DISC formation as determined by recruitment of FADD and cas-

pase-8 to precipitated DR5 (Fig. 2C). These data indicate that the proximal events in TRAIL R signaling are intact in the resistant BJAB LexR cells and the mechanism by which protea-

some inhibition overcomes resistance is downstream of DISC formation and initial signaling events but upstream of cas-

pase-3 cleavage.

One potential mechanism by which the BJAB

LexR cells could become resistant to TRAIL is through increased levels of XIAP, which were higher in the BJAB

LexR cells as shown by comparison of the 0 hour time point in Fig. 2D and which was degraded more slowly in the BJAB

LexR cells. XIAP can determine both the kinetics and extent of apoptosis after TRAIL R signaling (17) and this has been identified as a potential mechanism through which protea-

some inhibition overcomes resistance is downstream of DISC formation and initial signaling events but upstream of cas-

pase-3 cleavage.

Different methods of TRAIL resistance can be overcome by proteasome inhibition

Because reduction of the elevated XIAP levels only partly explains the ability of proteasome inhibitors to overcome resistance in the BJAB

LexR cells, we hypothesized that by targeting the proteasome, it may be possible to overcome mechanistically distinct ways of conferring TRAIL resistance. To test this hypothesis, we tested various different TRAIL resistance mechanisms that have been found in human tumors (8). We tested a variety of different mechanisms including an inactivating mutation in DR5, E338K, which acts as a dominant negative mutant, increased expression of two TRAIL decoy receptors DcR1 and DcR2, reduced expression of parental BJAB cells. Treatment with MG132 caused a small reduction in the amount of XIAP, but this was still above that in the parental cells. Knockdown of XIAP in the BJAB

LexR cells to levels equal to those in the parental line led to increased sensitivity to TRAIL that was further increased if MG132 was added as well. These data indicate that XIAP elevation may contribute to but does not fully explain the resistance, and that the ability to overcome resistance by the proteasome inhibitor involves but is not fully explained by reduction in XIAP.

Figure 4. Different mechanisms can confer TRAIL resistance. Matched cell lines BJAB

DCR1, BJAB

DCR2, BJAB

FADD-DN, BJAB

DR5E338K, BJAB

ORS K/D, and BJAB

Six1 were treated with TRAIL, lexatumumab, or Fas ligand for 24 hours and viability determined by MTS assay. All the resistance mechanisms conferred TRAIL resistance to various degrees.

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DR5, and expression of the transcription factor Six1. We also expressed a dominant negative FADD molecule (FADD-DD) that blocks all TRAIL receptor signaling. Fig. 4 shows that each of these manipulations resulted in TRAIL resistance (albeit to different extents). To test if the various resistance mechanisms could be overcome, we compared proteasome inhibition and etoposide treatment (Fig. 5). MG132 treatment caused TRAIL sensitivity equivalent to the parental BJAB cells for all resistant cells except the BJAB\textsuperscript{FADD-DN} and BJAB\textsuperscript{DR5 E338K} lines. Etoposide treatment failed to overcome TRAIL resistance for any of the resistant cell lines.

Figure 5. Sensitization experiments with TRAIL resistant cell lines. Parental BJAB cells and matched cell lines with different mechanisms of TRAIL resistance were pretreated with MG132 or etoposide 24 hours prior to TRAIL treatment and then treated with increasing doses of TRAIL and viability determined by MTS assay after a further 24 hours. MG132 caused TRAIL sensitivity equivalent to the parental BJAB cells for all resistant cells except the BJAB\textsuperscript{FADD-DN} and BJAB\textsuperscript{DR5 E338K} lines. Etoposide treatment failed to overcome TRAIL resistance for any of the resistant cell lines.
etoposide treatment did not. The failure of proteasome inhibitors to overcome resistance by FADD-DD is expected because this mutant prevents recruitment of endogenous FADD to the DISC and thus blocks all signaling. Additionally, it has been reported that dominant negative FADD expressing cells are resistant to proteasome inhibitors in the absence of exogenous TRAIL (20). Failure to overcome resistance in the BJABTRD123SK cells is also expected because we previously showed that this mutant inhibits TRAIL signaling by DR4 through competition for TRAIL binding (15). Decoy receptor-induced resistance was overcome, presumably because despite their name, these receptors work not just by competing for TRAIL binding but instead by forming heteromorphic complexes with the signaling receptors and altering their downstream signaling capacity (21). These data show that distinct mechanisms of TRAIL resistance can be overcome by proteasome inhibition but not etoposide treatment and that this extends to mechanisms that act at the receptor and those that activate downstream of the DISC.

Discussion

Our experiments and data from other groups show that TRAIL can synergize with various different kinds of anticancer treatments when tested in tumor cells that are TRAIL sensitive. Indeed, we find strong synergy even with general apoptotic stimuli such as increased hyperosmolar stress. The ability of a wide variety of agents to synergize with TRAIL suggests that there is no common underlying mechanism such as increased receptor expression; instead, these data are more consistent with synergy being caused by the addition of distinct apoptotic stimuli moving the tumor cells closer to their apoptotic threshold thus making it easier for the added signal coming from TRAIL receptor activation to push the cell over the threshold.

However, many tumor cells display resistance to TRAIL and, as shown here, susceptible tumor cells can easily acquire resistance. Thus, given the wide variety of agents that can synergize with TRAIL in sensitive cells, the question arises whether these agents can also work effectively with TRAIL in resistant cells. Our data show that achieving synergy in susceptible tumor cells and sensitizing resistant tumor cells are not the same, and it may be better to design combination therapies with agents like the proteasome inhibitors that are able to do both rather than develop combinations with drugs like etoposide that although very good at synergizing with TRAIL, are not good at overcoming resistance. Proteasome inhibitors may be able to do this because in addition to moving the tumor cells closer to their apoptotic threshold, inhibition of the proteasome affects multiple apoptosis regulators and thus increases the likelihood that any specific resistance mechanism can be bypassed. The fact that proteasome inhibition can overcome resistance in matched cells that occur both downstream of the DISC (e.g., with BJABSix1 and BJABLexR cells) and at the DISC (e.g., with BJABPRC1 and BJABP43 cells) supports this idea. Additional support comes from the fact that other studies of the ability of proteasome inhibitors to synergize with TRAIL have identified different alterations in the TRAIL signaling pathway associated with proteasome inhibition including reduced degradation of effector caspases (17), altered Bcl-XL levels (22), increased expression of receptors (23), and increased caspase activity (19). It has also been reported that a group of primary glioma cells from different patients can all be sensitized irrespective of the heterogeneity of the tumors (5). Together, these results from diverse tumor types along with our experiments in matched cells suggest that proteasome inhibitors are not only good at synergizing with, but are also good at overcoming TRAIL resistance. This is due to their ability to target different resistance mechanisms at the same time, thus making it more likely that any given mechanism will be affected by the drug combination.

Recent results of randomized phase II trials of TRAIL R-targeted drugs reported at the 2010 American Society of Clinical Oncology meeting were not encouraging; combinations with cytotoxic chemotherapy with or without bevacizumab did not show additional benefit from the TRAIL R-targeted drug in non-small cell lung cancer patients (10–12). However, these combinations were not chosen because the other drugs were able to overcome TRAIL resistance, rather the origination of these treatments was because (in cells that are susceptible to TRAIL) the cytotoxic agents could synergize and the drugs were already used to treat non-small cell lung cancer. Our data therefore show the need to understand the underlying reason for resistance if one wants to use "targeted" agents like TRAIL R agonists. For example, one resistance mechanism that has been found in human tumors (somatic mutation in DR5) was not overcome with the proteasome inhibitors, however, we previously showed that the inhibitory effect of the DR5 mutation could be bypassed through the use of a TRAIL receptor agonist (mapatumumab) that is targeted only to DR4 (15). However, the practical importance of this kind of approach may be limited because such TRAIL receptor mutations are rare in human tumors (24–27). On the contrary, another resistance mechanism (Six1 overexpression) that can be overcome by combination with proteasome inhibitors is common, being found in more than 60% of metastatic ovarian cancers (28) and up to 90% of metastatic breast cancers (29, 30), and associated with poor clinical outcomes in lymphoma and other cancer types (31). These data suggest that optimal use of TRAIL R-targeted drugs will require that we identify resistance mechanisms in a person’s tumor and use strategies like selective targeting of DR4 in DR5 mutant tumors when they are justified, but that in more common cases (such as with Six1 overexpressing tumors), we maximize both the amount of tumor killing and the ability to overcome resistance by combining TRAIL R-targeted drugs with agents that not only synergize but are also able to overcome resistance and, especially, resistance that is driven by more than one molecular mechanism if possible. Proteasome inhibitors may be a good place to start with this kind of approach.

Disclosure of Potential Conflicts of Interest

H. Ford, K. Behbakht, and A. Thorburn hold a patent on the use of Six1 to identify TRAIL–sensitive tumor cells.
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Distinct TRAIL Resistance Mechanisms Can Be Overcome by Proteasome Inhibition but not Generally by Synergizing Agents

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