Low concentrations of doxorubicin sensitizes human solid cancer cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-receptor (R) 2-mediated apoptosis by inducing TRAIL-R2 expression

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There is accumulating evidence suggesting that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-receptor (R) 2 is a promising molecular target for cancer therapy. Therefore, we investigated the effect of chemotherapeutic agents on TRAIL-R2-mediated apoptosis and cytotoxicity in various human solid cancer cells. Treatment of the ACHN human renal cell carcinoma (RCC) cell line with agonistic TRAIL-R2 antibody (lexatumumab) in combination with 5-fluorouracil, vinblastine, paclitaxel, or docetaxel did not overcome resistance to these agents. However, treatment with lexatumumab in combination with doxorubicin had a synergistic cytotoxicity. Synergy was also achieved in two other human RCC cell lines, Caki-1 and Caki-2, and in eight primary RCC cell cultures. Sequential treatment with doxorubicin followed by lexatumumab induced significantly more cytotoxicity than reverse treatment or simultaneous treatment. Low concentrations of doxorubicin (0.1 and 1 μg/mL) significantly increased TRAIL-R2 expression at both the mRNA and protein levels. Furthermore, the combination of doxorubicin and lexatumumab significantly enhanced caspase 8 activity, Bid cleavage, Bcl-xL decrease, release of cytochrome *c*, and caspase 9 and caspase 3 activity, and induced synergistic apoptosis. The activation of caspases and apoptosis induced with lexatumumab and doxorubicin was blocked by the human recombinant DR5:Fc chimeric protein. In addition, synergistic cytotoxicity was also observed in human prostate, bladder, and lung cancer cells, but was inhibited by the DR5:Fc chimeric protein. These findings suggest that doxorubicin sensitizes solid cancer cells to TRAIL-R2-mediated apoptosis by inducing TRAIL-R2 expression, and that the combination treatment with lexatumumab and doxorubicin might be a promising targeted therapy for cancers, including RCC, prostate, bladder, and lung cancers. (*Cancer Sci* 2007; 98: 1969–1976)

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is potentially an effective anticancer agent because it selectively induces apoptosis in a variety of tumor cells, yet it is relatively non-toxic to normal cells.[1,2] TRAIL triggers apoptosis by binding to two receptors: TRAIL-receptor (R) 1 and TRAIL-R2.[3] Activation of these receptors results in a signal transduction cascade that initiates intrinsic and extrinsic apoptotic pathways.[4] In addition, TRAIL binds to two other receptors, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), which lack a functional cytoplasmic death domain, and to the secreted tumor necrosis factor receptor homolog osteoprotegerin.[3,5] These receptors have been proposed to inhibit TRAIL-induced apoptosis. Potentially, the degree of TRAIL-R1- and TRAIL-R2-mediated apoptosis induced by TRAIL might be lowered in the presence of DcR1 and DcR2 activation. Therefore, using a specific activator of TRAIL-R1 or TRAIL-R2 is preferable to exclude interference from competition with DcR.

It was reported that mouse or rabbit monoclonal antibodies (mAb) to human TRAIL-R1 or TRAIL-R2 have antitumor activities *in vitro* and *in vivo*.[6,7] These agonistic antibodies work by activating TRAIL-mediated apoptotic pathways in a manner similar to TRAIL, as agonistic TRAIL-R1 antibody induces poly(adenosine diphosphate-ribose) polymerase cleavage and the agonistic TRAIL-R2 antibody induces activation of caspases and c-Jun *N*-terminal kinase and p38 in tumor cells.[8] It has also been reported that mapatumumab, a fully human agonistic mAb specific for TRAIL-R1, reduces the viability of multiple types of tumor cells *in vitro* and inhibits tumor growth *in vivo*.[9] We recently reported that lexatumumab, a human agonistic TRAIL-R2 mAb, induces apoptotic cell death in renal cell carcinoma (RCC) cells. However, it requires cross-linking with IgG:Fc to exert apoptotic activity as a single agent.[10] Developing ways to optimize the effects of lexatumumab, particularly through combinations with chemotherapy agents, is warranted.

The initial results of phase 1 studies with mapatumumab and lexatumumab showed good compatibility and only mild non-specific toxicity in a patient with solid malignancies.[11] The use of fully human antibodies would be preferable for clinical applications to avoid the immunogenicity associated with mouse and rabbit antibodies.[12]

We have previously demonstrated a synergistic effect of TRAIL and doxorubicin (ADR) in RCC cells without relevant cytotoxicity to normal cells.[13] We hypothesized that lexatumumab combined with chemotherapeutic agents might represent an alternative and possibly more specific therapy for cancers. Several reports have shown that combination treatment with agonistic TRAIL-R2 mAb and chemotherapeutic agents has synergistic apoptotic effects in some tumor cell lines, such as lymphoma, breast cancer, and colorectal cancer.[11,14,15] However, the synergistic cytotoxicity of agonistic TRAIL-R2 antibody and chemotherapeutic agents in RCC and bladder cancer cells has not been reported up to now. Furthermore, the synergistic mechanisms by which chemotherapeutic agents enhance TRAIL-R2-mediated apoptosis are not fully understood.

Our data are the first to demonstrate that low concentrations of ADR (0.1 and 1 μg/mL) enhance TRAIL-R2-mediated apoptosis and cytotoxicity by inducing TRAIL-R2 expression in various human solid cancer cells, including RCC, prostate cancer, bladder cancer, and lung cancer cells.

Materials and Methods

Reagents. Lexatumumab, a human TRAIL-R2 mAb, was kindly provided by Human Genome Sciences (Rockville, MD, USA).

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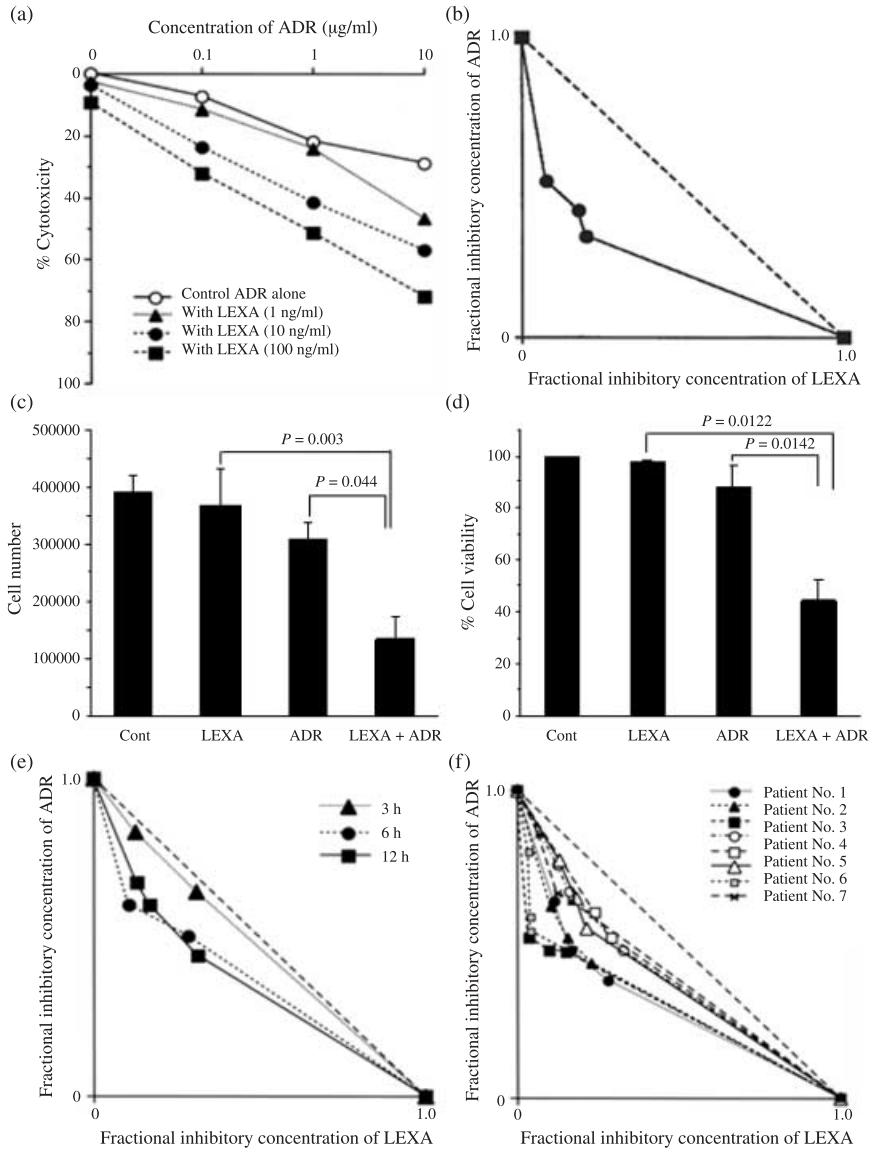

Fig. 1. Synergistic cytotoxicity of lexatumumab (LEXA) and doxorubicin (ADR) in renal cell carcinoma (RCC) cells. ACHN cells were treated 24 h with LEXA (1–100 ng/mL) alone, ADR (0.1–10 μg/mL) alone, or a combination of the two. (a) Cytotoxicity was measured by3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (b) Synergy was assessed by isobolographic analysis. ACHN cells were treated for 24 h with 100 ng/mL LEXA and/or 1 μg/mL ADR. (c) Cell number was determined by Trypan blue dye exclusion assay. (d) Cell viability was assessed by NucleoCounter. (e) ACHN cells were treated with LEXA (1–100 ng/mL) in combination with ADR (0.1–10 μg/mL) for 3, 6, or 12 h. The cells were then washed twice with medium, and cultured for 21, 18, or 12 h, respectively. Cytotoxicity was measured by MTT assay and synergy was assessed by isobolographic analysis. (f) The cytotoxicity of LEXA (1–100 ng/mL) and ADR (0.1–10 μg/mL) used in combination for primary RCC cells from eight patients was measured by MTT assay. Synergy was assessed by isobolographic analysis. Data represent means from three independent experiments.

It is a fully human mAb (IgG_1) isolated using phage-display technology in collaboration with Cambridge Antibody Technology.[9] Enzyme-linked immunosorbent assays (ELISA) and Biacore analyses determined that lexatumumab is highly specific for binding to TRAIL-R2. ADR, Z-LETD-FMK, Z-LEHD-FMK, Z-IETD-FMK, and Z-DQMD-FMK were purchased from Sigma (St Louis, MO, USA). Z-VAD-FMK and human recombinant DR5:Fc chimeric protein were purchased from ICN Pharmaceuticals (Aurora, OH, USA) and Alexis (San Diego, CA, USA), respectively.

Cell lines and primary RCC cells. ACHN, Caki-1, and Caki-2 human RCC cell lines, PC3 and DU145 prostate cancer cell lines, T24 and J82 bladder cancer cell lines, and A549 lung cancer cell line were purchased from the American Type Culture Collection (Rockville, MD, USA). ACHN, Caki-1, Caki-2, PC3, DU145, T24, and J82 cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum, 25 mM HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. A549 cells were maintained in Dulbecco's modified Eagle medium supplemented with serum and antibiotics as described above.

Primary RCC cells were separated from the surgical specimens of eight patients with untreated RCC as described previously.[16] All patients were diagnosed with RCC of the alveolar type and clear-cell subtype by histological examination. Pathological stage and grade were consistent with 2000 World Health Organization criteria as follows: (1) patient 1, $T_2N_4M_0$, grade 2; (2) patient 2, $T_2N_0M_0$, grade 2; (3) patient 3, $T_2N_0M_0$, grade 1; (4) patient 4, $T_2N_0M_0$, grade 1; (5) patient 5, $T_3N_1M_0$, grade 2; (6) patient 6, $T_3N_0M_1$, grade 2; (7) patient 7, $T_2N_0M_0$, grade 2; and (8) patient 8, $T_1N_0M_0$, grade 2.

Cytotoxicity assays. A 100-μL suspension of 1×10^4 cells was seeded into a 96-well flat-bottom microtiter plate. After incubation for 24 h, 100 μL of drug solution or medium (control) was added to the plates in triplicate, and each plate was incubated for an additional 24 h. In sequential experiments cells were preincubated for 8 h with medium only, lexatumumab, or ADR, washed twice with medium, and exposed to lexatumumab and ADR for 16 h. Cytotoxicity was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously.[16]

Cell number and viability was determined using the Trypan blue dye exclusion assay and NucleoCounter (Chemometec, Denmark), respectively. Cells were seeded in six-well plates at 3×10^5 cells per well, treated in duplicate on the second day

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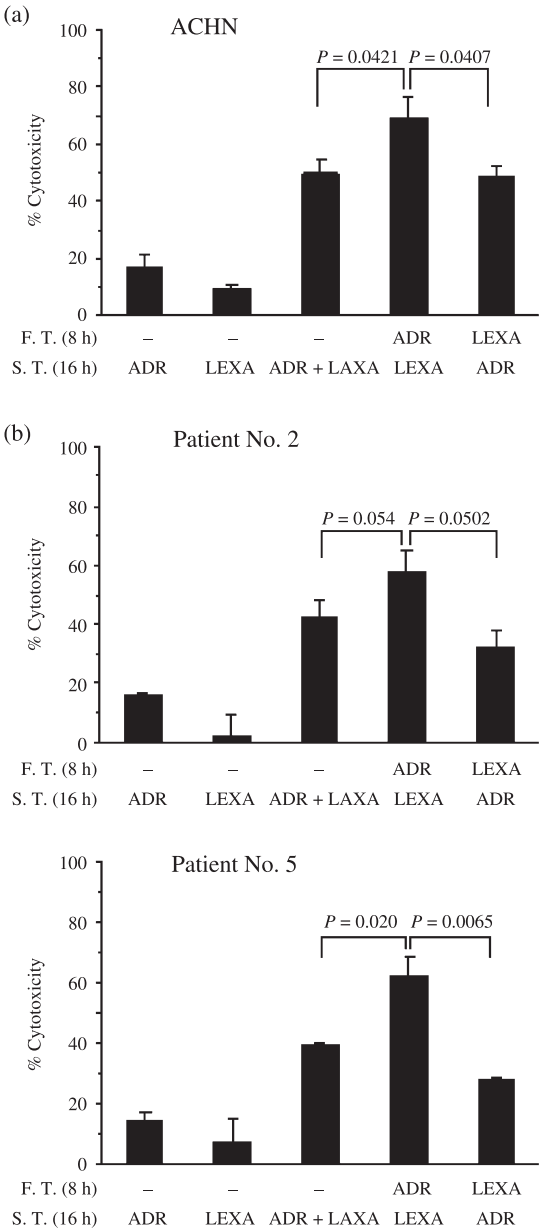

Fig. 2. Effect of the sequence of treatment with lexatumumab and doxorubicin (ADR) on cytotoxicity in renal cell carcinoma (RCC) cells. (a) ACHN and (b) primary RCC cells were preincubated for 8 h with medium only, 100 ng/mL lexatumumab, or 1 μg/mL ADR, washed twice with medium, and exposed to 100 ng/mL lexatumumab and/or 1 μg/mL ADR for 16 h. Cytotoxicity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data represent means of three independent experiments. F.T., first treatment; S.T., second treatment.

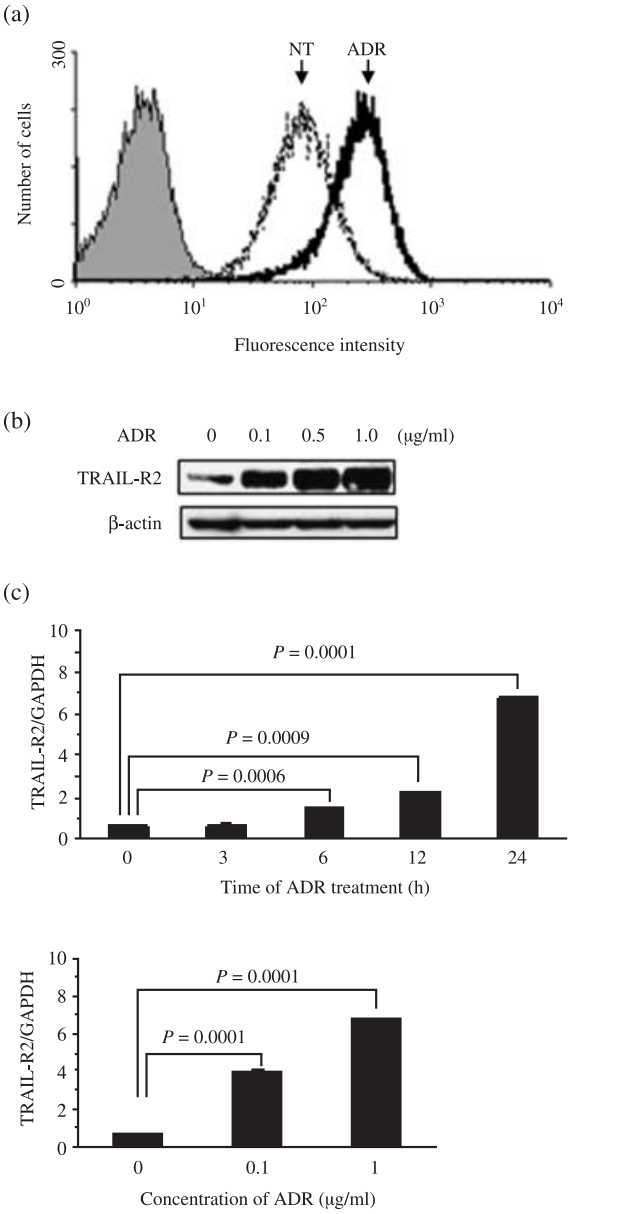

Fig. 3. Effect of doxorubicin (ADR) on the protein and mRNA levels of tumor necrosis factor-related apoptosis-inducing ligand-receptor 2 (TRAIL-R2) in renal cell carcinoma cells. (a) ACHN cells were treated for 24 h with medium only or 1 μg/mL ADR. The cells were harvested, incubated with phycoerythrin-conjugated anti-TRAIL-R2 monoclonal antibody for 30 min at 4°C, and analyzed by flow cytometry. Gray areas represent IgG1 isotype staining. The thin histogram indicates TRAIL-R2 staining, whereas the thick histogram indicates TRAIL-R2 staining after ADR treatment. (b) TRAIL-R2 expression in ACHN cells was monitored by western blot analysis after ADR treatment at different concentrations for 24 h. β-Actin was used as a loading control. (c) ACHN cells were treated with 1 μg/mL ADR for variable times or at different concentrations for 24 h. Total RNA was extracted with TRIzol reagent and used for cDNA synthesis. The mRNA levels of TRAIL-R2 were determined by real-time reverse transcription–polymerase chain reaction. Data represent the means of three independent experiments.

with lexatumumab and ADR, and viable cells were then counted with 0.5% Trypan blue dye (Sigma). The viable cells were also counted using NucleoCounter, as described previously.[17]

Flow cytometry. Cell-surface expression of TRAIL-R2 was determined by flow cytometry with an EPICS XL (Beckman Coulter, Miami, FL, USA). Briefly, cells were seeded in 60-mm dishes at 5×10^5 cells per dish and treated on the second day with ADR. After treatment, the cells were harvested from the substrate and washed twice in phosphate-buffered saline containing 0.2% fetal bovine serum and 0.01% NaN_3. The cells were incubated with phycoerythrin-conjugated anti-TRAIL-R2 mAb (Genzyme Techne, Minneapolis, MN, USA) at 4°C for 30 min, washed, and analyzed.

Real-time reverse transcription–polymerase reaction. Total RNA was extracted from RCC cells using TRIzol Reagent (Life

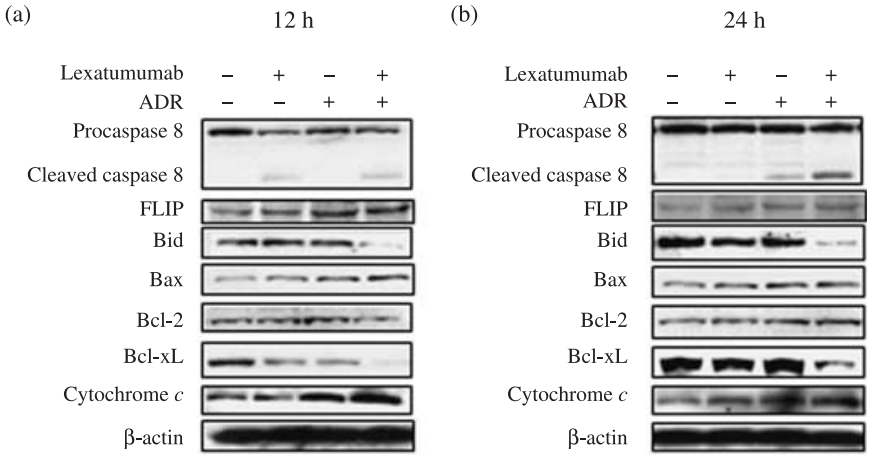

Fig. 4. Effect of lexatumumab and doxorubicin (ADR) on the expression of caspase 8, FLIP, Bid, Bax, Bcl-2, Bcl-xL, and cytochrome *c* in ACHN cells. Cells were treated with 100 ng/mL lexatumumab and/or 1 μg/mL ADR for (a) 12 h or (b) 24 h. The expression of caspase-8, FLIP, Bid, Bax, Bcl-2, Bcl-xL, and cytochrome *c* was monitored by western blot analysis. β-Actin was used as a loading control.

Technologies, Grand Island, NY, USA) and cDNA was synthesized with the First-Strand cDNA Synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The primer set for TRAIL-R2 was as follows: forward 5′-GGGAGCCGCTCATGAGGAAGTTGG-3′ and reverse 5′-GGCAAGTCTCTCTCCCAGCGTCTC-3′.[18] Real-time reverse transcription (RT)–polymerase chain reaction (PCR) was done using LightCycler Fast-Start DNA Master SYBR Green 1 (Roche Diagnostics, Mannheim, Germany). The protocol applied for TRAIL-R2 was 40 cycles at 95°C for 10 s, 63°C for 1 s, and 72°C for 4 s. Quantitative analysis of the data was carried out using LightCycler software, version 3.5 (Roche Diagnostics). Standard curves for templates of TRAIL-R2 and glyceraldehyde-3-phosphate dehydrogenase were generated by serial dilution of the PCR products.

Western blotting. Western bolt analysis was carried out as described previously.[13] Forty micrograms of protein was loaded in each lane. Rabbit anti-TRAIL-R2 polyclonal antibody (Millennium Biotechnology, Ramona, CA, USA), goat anti-Bid polyclonal antibody, rabbit anti-Fas-associated polypeptide with death domain-like interleukin-1-converting enzyme-inhibitor protein (FLIP) antibody, mouse anticaspase-8 mAb (MBL, Nagoya, Japan), Bcl-2, Bcl-xL, Bax, cytochrome *c* mAb (Santa Cruz Biotechnology, Santa, CA, USA), and mouse anti-β-actin mAb (Abcom, Cambridge, UK) were used as primary antibodies. Signals were detected using an ECL kit (Amersham Pharmacia Biotech).

Caspase activity and caspase inhibition assays. Caspase 3, 6, 8, and 9 activities were measured using a quantitative colorimetric assay with Caspase 3, 6, 8, and 9 Colorimetric Protease Assay Kits (MBL), as described previously.[19] The caspase inhibition assay was carried out using specific caspase 3, 6, 8, and 9 inhibitors, and a general caspase inhibitor. The cells were pretreated with these caspase inhibitors for 1 h, then exposed to 1 μg/mL ADR and 100 ng/mL lexatumumab for 23 h. Cell viability was assessed by MTT assay.

Apoptosis assays. Apoptosis was determined in two ways. Following incubation with lexatumumab or ADR for 12 or 24 h, floating and adherent cells were harvested. DNA was extracted from the prepared cells using an Apoptosis Ladder Detection Kit (MBL), as described previously.[20] Extracted DNA samples were resolved by electrophoresis on 2% agarose gels and stained with ethidium bromide. DNA fragmentation was evaluated quantitatively using an ELISA kit (Roche Diagnostics) according to the manufacturer's instructions.

Statistical analysis. All determinations were done at least three times and the results are presented as the mean and SD of three experiments. Significance was analyzed by Student's *t*-test, with $P \leq 0.05$ considered significant. Synergistic cytotoxicity was assessed by isobolographic analysis, as described previously.[21]

Table 1. Effect of CAT002 on the sensitivity of ACHN cells to doxorubicin (ADR)

CAT002 (ng/mL)	ADR (μg/mL)			
	0	0.1	1	10
0	0	9.80 ± 0.8	23.54 ± 4.7	30.62 ± 6.5
1	3.88 ± 2.9	9.94 ± 7.5	21.62 ± 10.6	25.79 ± 10.2
10	6.02 ± 3.2	13.5 ± 5.2	19.81 ± 4.0	24.25 ± 7.1
100	6.21 ± 1.3	13.0 ± 0.4	23.2 ± 6.9	25.80 ± 6.3

The cytotoxic effect of CAT002 and ADR on the ACHN cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The results are expressed as the mean cytotoxicity (%) ± SD of three different experiments.

Results

Synergistic cytotoxicity of lexatumumab and ADR against RCC cells. We examined the susceptibility of the ACHN human RCC cell line to lexatumumab in the absence or presence of five commonly used chemotherapeutic agents: ADR, 5-fluorouracil (5-FU), vinblastine (VBL), paclitaxel, and docetaxel. In the absence of chemotherapeutic agent, ACHN cells showed limited sensitivity to lexatumumab, with cytotoxicity reaching a maximum of 9.4% at the highest dose (100 ng/mL). The cells were also insensitive to these chemotherapeutic agents. However, among these chemotherapeutic agents, ADR, but not 5-FU, VBL, paclitaxel, or docetaxel (data not shown), significantly enhanced cell death induced by lexatumumab and had a synergistic cytotoxic effect with lexatumumab (Fig. 1a,b).

The synergistic cytotoxicity of lexatumumab and ADR was confirmed by both the Trypan blue dye exclusion assay (Fig. 1c) and NucleoCounter (Fig. 1d). The synergistic effect was also achieved even when the treatment with lexatumumab and ADR was shortened from 24 h to 12 or 6 h, whereas it was weak when the treatment was shortened to 3 h (Fig. 1e). This synergy was specific for lexatumumab, because ADR in combination with isotype control antibody (CAT002; Human Genome Sciences) had no synergistic effect (Table 1). Furthermore, the synergy obtained with the ACHN cell lines was not selective for this cell line, as two other RCC cell lines, Caki-1 and Caki-2, were also sensitized to lexatumumab-mediated cytotoxicity in the presence of ADR (data not shown).

To determine whether the synergy was a reflection of the properties of the established cancer cell lines, we tested for synergy in eight primary RCC cell cultures. In all cases, significant

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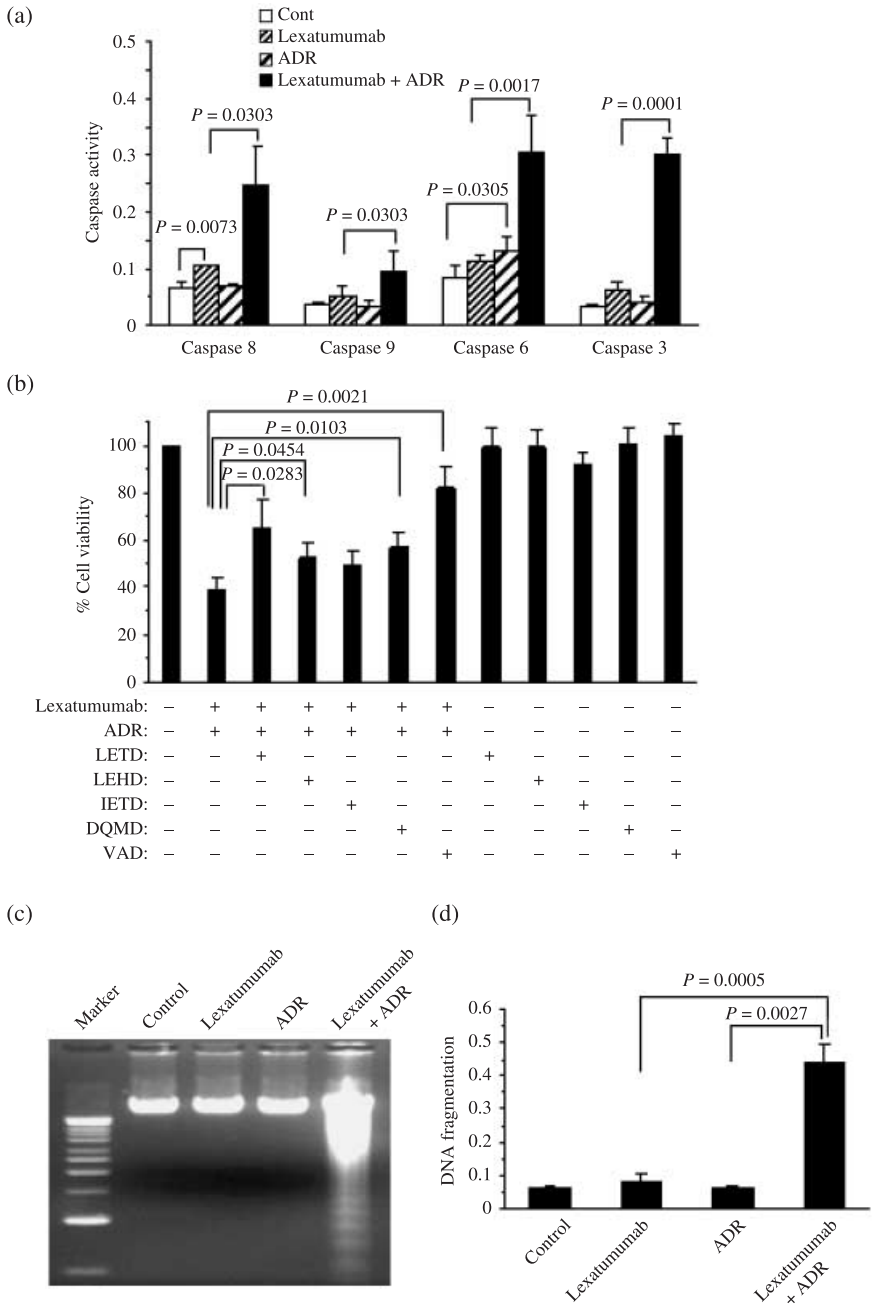

Fig. 5. Caspase activation and apoptosis induction by lexatumumab and doxorubicin (ADR) in ACHN cells. (a,c,d) Cells were treated for 12 h with 100 ng/mL lexatumumab and/or 1 μg/mL ADR. Caspase 8, 9, 6, and 3 activities were measured using a quantitative colorimetric assay. (b) Cells were treated for 24 h with 100 ng/mL lexatumumab plus 1 μg/mL ADR in the absence or presence of 50 μM LETD, LEHD, IETD, DMQD, or VAD. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. (c) DNA were extracted and separated by electrophoresis in 2% agarose gels and stained with ethidium bromide. (d) Quantification of DNA fragmentation was assessed using an apoptosis-specific enzyme-linked immunosorbent assay.

synergy was achieved irrespective of the sensitivity of RCC cells to ADR or lexatumumab when each was used alone (Fig. 1f).

Together, these findings clearly demonstrate that treatment of RCC cell lines or primary RCC cells with a combination of lexatumumab and ADR results in the potentiation of cytotoxicity. In all cases synergy was achieved with low concentrations of ADR.

Sensitization of RCC cells to TRAIL-R2-mediated cytotoxicity by ADR. The findings above demonstrate that simultaneous treatment of RCC cells with lexatumumab and ADR results in synergy. To explore the underlying mechanisms of this synergistic cytotoxicity with lexatumumab and ADR, the effect of sequential treatment with these agents was examined. Pretreatment of ACHN and primary RCC cells with 1 μg/mL ADR for 8 h followed by treatment with 100 ng/mL lexatumumab for 16 h induced significantly more cytotoxicity than reverse treatment or simultaneous treatment using these two agents (Fig. 2). This sequential effect was also observed with different concentrations of ADR (data not shown). These findings indicate that ADR sensitized RCC cells to TRAIL-R2-mediated cytotoxicity.

Upregulation of the protein and mRNA levels of TRAIL-R2 by ADR. Flow cytometric analysis showed that TRAIL-R2 was detected in 94.2% of untreated ACHN cells and was increased remarkably after ADR treatment (Fig. 3a). The upregulation of TRAIL-R2 was also observed in Caki-1 and in two primary RCC cell cultures (data not shown). The dose-dependent upregulation of TRAIL-R2 was confirmed by western blot analysis (Fig. 3b). In contrast, other chemotherapeutic drugs, such as 5-FU and VBL, did not affect TRAIL-R2 expression in ACHN cells (data not shown).

By quantitative real-time RT-PCR, we further examined whether ADR regulates the mRNA levels of TRAIL-R2 in RCC cells. ADR significantly increased the mRNA levels of TRAIL-R2 in ACHN cells in a time- and dose-dependent manner (Fig. 3c).

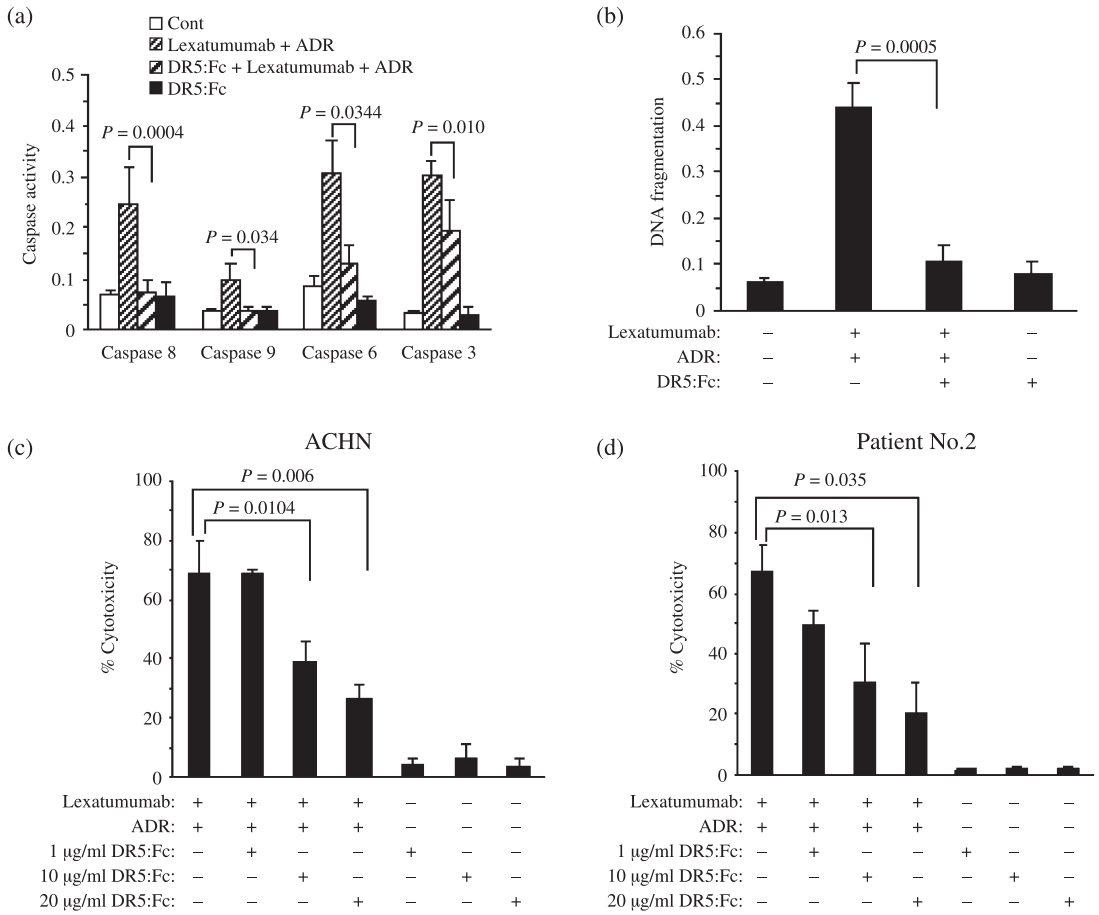

Fig. 6. Suppressive effect of the DR5:Fc chimeric protein on caspases and apoptosis induced by the combination of lexatumumab and doxorubicin (ADR) in renal cell carcinoma (RCC) cells. ACHN cells were treated for 12 h with 100 ng/mL lexatumumab plus 1 μg/mL ADR in the absence or presence of 10 μg/mL DR5:Fc chimeric protein. (a) Caspase 8, 9, 6, and 3 activities were measured using a quantitative colorimetric assay. (b) Quantification of DNA fragmentation was determined by enzyme-linked immunosorbent assay. (c) ACHN and (d) primary RCC cells were treated for 24 h with 100 ng/mL lexatumumab plus 1 μg/mL ADR in the absence or presence of DR5:Fc chimeric protein at the concentrations indicated. Cytotoxicity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Effect of the combination of lexatumumab and ADR on the expression of caspase 8, FLIP, Bid, Bax, Bcl-2, Bcl-xL, and cytochrome c. By western blot analysis, we further analyzed whether the combination of ADR and lexatumumab regulates the expression of caspase 8, FLIP, Bid, Bax, Bcl-2, Bcl-xL, and cytochrome *c* in RCC cells. FLIP, Bcl-2, and Bax expression was not affected when ACHN cells were treated with ADR or lexatumumab for 12 h. However, the combination of ADR and lexatumumab remarkably induced cleavage of caspase 8 into its active fragments, cleavage of Bid, downregulation of Bcl-xL, and mitochondrial release of cytochrome *c* (Fig. 4a). Similar results were achieved when the treatment with ADR and lexatumumab was extended from 12 to 24 h (Fig. 4b).

Activation of the caspase cascade and induction of apoptosis by ADR and lexatumumab. In accordance with the cleavage of procaspase 8, the quantitative colorimetric assays demonstrated that ADR significantly enhanced the ability of lexatumumab to activate caspase 3, 6, 8, and 9 in ACHN cells (Fig. 5a).

To confirm that the synergistic cytotoxicity of lexatumumab and ADR is mediated through the activation of caspase, we further examined the effects of the caspase 8 inhibitor Z-LETD-FMK, caspase 9 inhibitor Z-LEHD-FMK, caspase 6 inhibitor Z-IETD-FMK, caspase 3 inhibitor Z-DQMD-FMK, and general caspase inhibitor Z-VAD-FMK on the death of cells treated with lexatumumab and ADR. The synergistic cytotoxicity of lexatumumab and ADR was partly inhibited by the caspase 8-, 9-, and 3-specific inhibitors, and remarkably inhibited by the general caspase inhibitor (Fig. 5b).

ACHN cells were analyzed to determine whether the synergistic cytotoxicity of lexatumumab and ADR was mediated by apoptosis. When used alone, neither lexatumumab nor ADR caused DNA fragmentation. However, obvious DNA fragmentation was observed when the cells were incubated with the two agents simultaneously (Fig. 5c). The synergistic apoptosis of cells treated with lexatumumab and ADR was confirmed using a quantitative apoptosis-specific ELISA kit (Fig. 5d). These results indicate that the synergistic cytotoxicity of lexatumumab and ADR is realized by inducing apoptosis.

DR5:Fc chimeric protein inhibits the activation of caspases and induction of apoptosis. We further analyzed the molecular mechanisms by which ADR sensitizes RCC cells to TRAIL-R2-mediated apoptosis. By using a human recombinant DR5:Fc chimeric protein, which has a dominant-negative function against TRAIL-R2, we observed that it significantly inhibited the activation of caspase 8, 9, 6, and 3, and the apoptosis induced by lexatumumab and ADR in ACHN cells (Fig. 6a,b). Furthermore, the synergistic cytotoxicity of lexatumumab and ADR was also blocked by the DR5:Fc chimeric protein at concentrations of 10 and 20 μg/mL in ACHN and primary RCC cells (Fig. 6c,d).

Sensitization of other human solid cancer cells to TRAIL-R2-mediated cytotoxicity by ADR. We next investigated whether ADR sensitizes other human solid cancer cells to TRAIL-R2-mediated cytotoxicity

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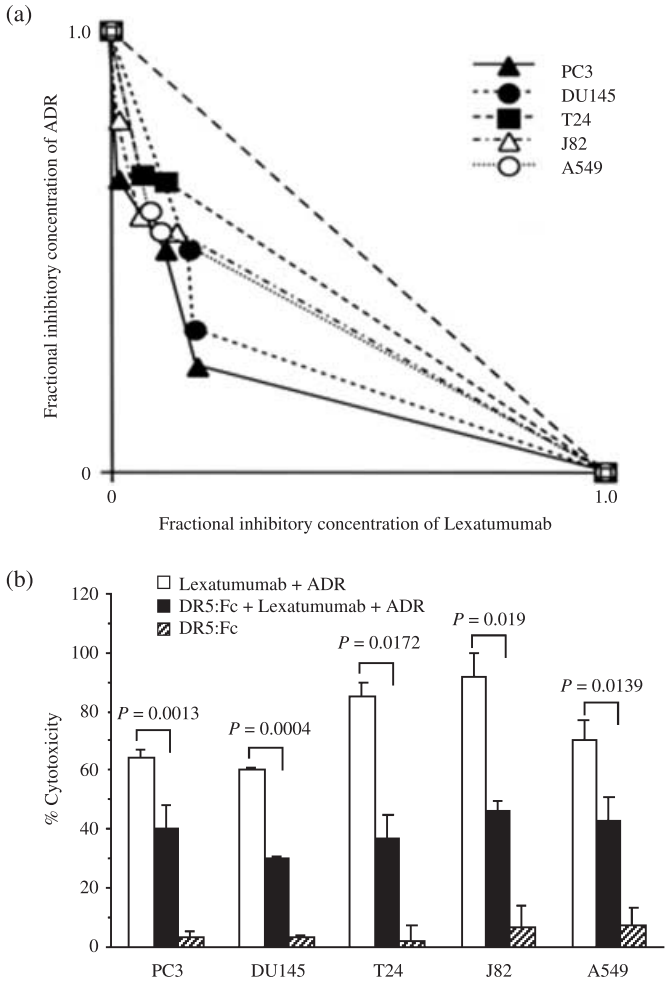

Fig. 7. The DR5:Fc chimeric protein blocks synergistic cytotoxicity of lexatumumab and doxorubicin (ADR) in prostate cancer, bladder cancer, and lung cancer cells. (a) PC3, DU145, T24, J82, or A549 cells were treated for 24 h with lexatumumab (1–100 ng/mL) alone, ADR (1–10 μg/mL) alone, or a combination of the two. Cytotoxicity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and synergy was assessed by isobolographic analysis. (b) Cells were treated for 24 h with 100 ng/mL lexatumumab plus 1 μg/mL ADR (PC3, DU145, T24, and J82 cells) or 10 μg/mL ADR (A549 cells) in the absence or presence of 10 μg/mL DR5:Fc chimeric protein.

using PC3, DU145, T24, J82, and A549 cells. In all cases, significant synergy was observed with lexatumumab in combination with ADR as well as in RCC cells, irrespective of the sensitivity of these cells to either lexatumumab or ADR when each was used alone (Fig. 7a). Furthermore, the synergistic cytotoxicity was also blocked by the DR5:Fc chimeric protein (Fig. 7b).

Effect of other anthracyclines on synergy with lexatumumab. Other anthracyclines, including epirubicin, pirarubicin, and amrubicin, were tested for their cytotoxic effect on ACHN cells when used in combination with lexatumumab. Similar synergistic effects were achieved with these anthracyclines in combination with lexatumumab (Wu *et al.*, unpublished data, 2007).

Discussion

The present study shows that lexatumumab and ADR had a synergistic effect on solid cancer cells, which were resistant to each agent used alone. Synergy was achieved in primary RCC cells as well as in human RCC cell lines. Furthermore, synergy was also observed in human prostate cancer, bladder cancer, and lung cancer cells. It required relatively low concentrations of each agent, thus minimizing drug toxicity and maximizing potential therapeutic applications *in vivo*. These findings strongly suggest that combination treatment using lexatumumab and ADR is promising from a clinical perspective.

A synergistic effect is achieved by the reciprocal interaction of two agents. Our previous studies using anti-Fas mAb demonstrated that combined treatment with ADR and anti-Fas mAb resulted in a synergistic cytotoxicity against RCC cells.[16,22] In that case anti-Fas mAb and ADR had reciprocal interactions with each other. For example, ADR enhanced the expression of Fas and anti-Fas mAb increased the cellular concentration of ADR. We also reported that pretreatment with ADR followed by TRAIL could induce similar cytotoxicity to that of simultaneous treatment, but the reverse sequence of treatment induced significantly less cytotoxicity.[20] Interestingly, the present study shows that pretreatment of RCC cells with ADR followed by lexatumumab could induce more significant cytotoxicity than reverse treatment or simultaneous treatment using these two agents. These findings suggest that lexatumumab, TRAIL, and anti-Fas mAb have a synergistic cytotoxic effect on RCC cells in a different manner. Additionally, these different sequential effects might provide a foundation to optimize the administration of these drugs for application in the clinical setting.

Cell-surface expression of TRAIL-R1 or TRAIL-R2 is essential for TRAIL-induced apoptosis, although tumor cells expressing these death receptors are not always sensitive to TRAIL due to intracellular mechanisms.[23] It was reported that the efficacy of TRAIL correlates with cell-surface expression of TRAIL-R1 and TRAIL-R2 in leukemia cells.[24] In a previous study, we observed that the cell-surface levels of TRAIL-R1 and TRAIL-R2 mainly qualify the susceptibility of human RCC cells to the effects of agonistic TRAIL-R1 and TRAIL-R2 mAb.[10] The present study shows that low concentrations of ADR (0.1 and 1 μg/mL) significantly upregulated TRAIL-R2 expression in RCC cells at both the mRNA and protein levels in a dose- and time-dependent manner. These findings indicate that the upregulation of TRAIL-R2 by ADR was initiated at the transcriptional level, and TRAIL-R2 has a critical role in the enhancement of TRAIL-R2-mediated apoptosis by ADR.

Previous reports have implicated p53 and nuclear factor κB (NF-κB) as the transactivating factors in TRAIL-R2 and TRAIL-R1 upregulation by DNA-damaging drugs such as etoposide and ADR.[25,26] A recent study further demonstrated that NF-κB differentially regulates etoposide-induced TRAIL-R2 expression.[27] Therefore, we speculate that the molecular mechanism of induction of TRAIL-R2 expression in RCC cells by low concentrations of ADR may be due to the regulation of p53 and NF-κB, although further studies are needed to clarify how ADR upregulates TRAIL-R2 expression.

Caspases are critical protease mediators of apoptosis trigged by TRAIL. However, it is difficult to examine isolated TRAIL-mediated signal transduction because various receptors complicate signal transduction. Using the specific agonistic TRAIL-R2 mAb lexatumumab, we evaluated the involvement of caspases, specifically in TRAIL-R2-mediated apoptosis. We found that the combination of lexatumumab and ADR significantly activated initiator caspases, such as caspase 8 and 9, and effector caspases, including caspase 6 and 3, in RCC cells. Furthermore, the synergistic cytotoxicity of lexatumumab and ADR was significantly inhibited by the caspase 8-, 9-, and 3-specific inhibitors and the general caspase inhibitor. The activities of caspase 6 and 3 were also significantly inhibited by the DR5:Fc chimeric protein, which has a dominant-negative function against TRAIL-R2.[28] Interestingly, the synergistic apoptosis was completely blocked by DR5:Fc. These findings indicate that these caspases might be commonly involved in the synergistic cytotoxicity of lexatumumab

and ADR, and the activation of caspases was initiated via induction of TRAIL-R2.

The present study shows that the combination of lexatumumab and ADR significantly increased the cleavage of Bid, which is a BH3 domain-containing pro-apoptotic member of the Bcl-2 family of proteins that is activated by caspase 8. Bid can bridge these two pathways by mediating death receptor-trigged cyctochrome *c* release from mitochondria.[29–31] In accordance with previous reports,[32,33] we show here that similar events are involved in TRAIL-R2-mediated apoptosis in RCC cells. The lexatumumab and ADR combination further downregulated Bcl-xL and induced release of cytochrome *c* from mitochondria in RCC cells. These results indicate that the combination of lexatumumab and ADR induces synergistic apoptosis and cytotoxicity mainly through the intrinsic apoptotic pathway in RCC cells.

In addition, the synergistic effect of lexatumumab and ADR was achieved in other human solid cancer cells, including bladder, prostate, and lung cancer cells. Furthermore, the synergistic cytotoxicity was also blocked by the DR5:Fc chimeric protein. These findings suggest that the enhancement of TRAIL-R2-mediated apoptosis by ADR through inducing TRAIL-R2 expression is not specific to RCC cells and might be a general mechanism in human solid cancer cells.

In summary, the present study clearly demonstrates that lexatumumab and ADR had synergistic cytotoxicity in human RCC lines, primary RCC cells, and other solid cancer cells. The synergistic cytotoxicity of lexatumumab and ADR was realized by inducing apoptosis through induction of TRAIL-R2 and the intrinsic apoptosis pathway. The concentrations of ADR used to achieve the synergistic effect were quite low compared to the regular dose.[34] These findings suggest that treatment of malignant neoplasms, including RCC, and human prostate, bladder, and lung cancers, using lexatumumab combined with ADR is promising as a potential clinical application.

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