Effect of a novel fully human monovalent antigen-binding fragment on the survival of cancer cell lines

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Received January 12, 2007; Accepted April 11, 2007

Abstract. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a cytokine having potent cytotoxic activity specifically to tumor cells. Agonistic antibodies against TRAIL receptors are currently being explored as anti-cancer therapeutics. Here, we report studies on JKTR-18, a monovalent human monoclonal antibody Fab selected against human recombinant TRAIL receptor 2 (DR5) by phage display technology. It induced cell death in Jurkat and HL60 leukemia cell lines without the need for secondary cross-linkers *in vitro*. It did not compete with soluble TRAIL (sTRAIL) for binding to DR5, and its combination with sTRAIL resulted in greater cell death than either agent alone. The cell death induced by JKTR-18 included a caspase-independent mechanism. This is the first report of a monovalent antibody fragment against TRAIL receptor that can induce tumor cell death in the absence of a crosslinker.

Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) exists as either a membrane-bound form or a soluble one. Soluble TRAIL (sTRAIL) can interact with two distinct receptors, TRAIL receptor 1 (DR4) and TRAIL receptor 2 (DR5), leading to apoptosis specific for cancer cells (1). Both DR4 and DR5 contain death domains required for sTRAIL-induced cell death. The binding of sTRAIL to DR4 and/or DR5 can lead to the trimerization of the receptors and the formation of death-inducing signaling complex with adapter proteins, followed by the intracellular signal activation, resulting in cell death (1). The potential use of sTRAIL as an anti-cancer therapeutic agent has been demonstrated (2).

Monoclonal antibodies (mAbs) against DR4 or DR5 with tumoricidal activity have been developed and also demonstrated as potential candidates for cancer therapy (3). Several human or mouse mAbs that activate the receptors and induce cell death have been reported (3-12). Some of these mAbs required a cross-linking reagent (anti-immunoglobulin Ab, protein A, or chemical reagent) to effectively induce tumor cell death (3,7,10,11), but others were able to kill tumor cells without cross-linking (5,6,8,9,12) *in vitro*. The tumoricidal activity of the mAbs without cross-linkers is expected to be advantageous for clinical use in some individuals because of immunosuppressive cancer therapies, or polymorphism of FcR (8). Some anti-TRAIL receptor Abs showed tumoricidal activity by a caspase-dependent mechanism mimicking sTRAIL (12). However, in 2005 Guo *et al* reported that a mouse anti-human DR5 mAb induced both caspase-dependent and caspase-independent cell death in Jurkat cells (8). Therefore, different mAbs to TRAIL receptors can exert their cytotoxic activity via various mechanisms.

In this article, we report studies on JKTR-18 (also named DR5-18 in the GenBank database), a monovalent human monoclonal antibody Fab fragment selected against human recombinant DR5 by phage display technology. It induces cell death in Jurkat and HL60 leukemia cell lines via a caspase-independent mechanism without the need for secondary cross-linkers *in vitro*.

Materials and methods

Reagents. Mouse anti-human IgG (Fab-specific) antibody and 3-(4,5-dimethythiazol-2-yl)-2,5-(3-diphenyl tetrazolium bromide) (MTT) were purchased from Sigma. The zVAD was obtained from Santa Cruz Biotechnology and dissolved in dimethyl sulfoxide (Sigma). Recombinant sTRAIL (amino acids 114-281) and DR5 (amino acids 1-130) were generated as described previously (13). Alternatively, sTRAIL and

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Abbreviations: Ab, antibody; Fab, antigen-binding fragment; PBS, phosphate-buffered saline; DR, death receptor; ELISA, enzyme-linked immunosorbent assay

Key words: tumor necrosis factor-related apoptosis-inducing ligand receptor, human antigen-binding fragment, agonistic antibody, tumor cells, cross-linker

A.

FR1	CDR1	FR2	CDR2
ESGGDVVQPGRSLRLSCAASGFTFT	**NYGMH**	WVRQAPGRGLEWLAV	**IWYDGSKKSYADSVKG**

FR3	CDR3	FR4
RFTISRDNSKNTLYLQMNSLRAEDTAFYYCAR	**PINTFGGVISPFDY**	WGQGTLVTVSS

B.

FR1	CDR1	FR2	CDR2
TQSPGTLSVFPGERATLSC	**RASQSLTDKSLA**	WYQQKPGQAPRLVVY	**GASSRAT**

FR3	CDR3	FR4
GIPDRFSGSGSGTDFSLSISSVEPEDFAVYFC	**HQYDSSPRTF**	GQGTKVEIKR

Figure 1. Amino acid sequences of the V_H (A) and V_L regions (B) of the JKTR-18 (DR5-18) Fab. The GenBank accession nos. are DQ344022 and DQ344023, respectively.

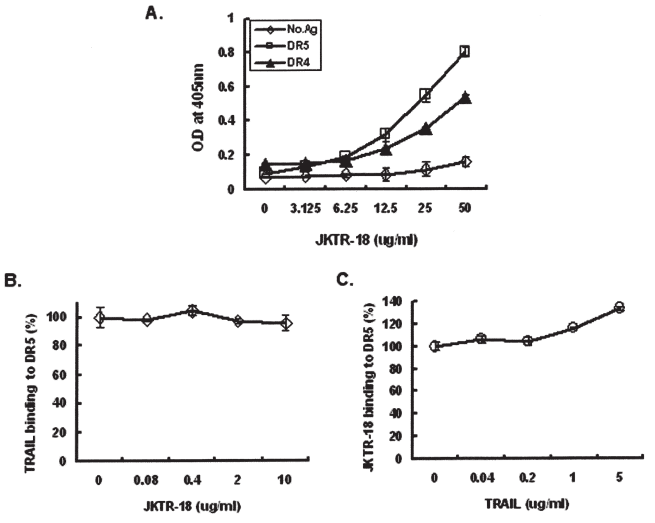

Figure 2. (A) Direct binding ELISA for binding of JKTR-18 to 5 μg/ml DR4, DR5, or PBS. JKTR-18 bound to the coated antigen was incubated with anti-human IgG (Fab-specific) alkaline phosphatase. (B,C) Competitive ELISA for binding to DR5. ELISA plates were coated with 5 μg/ml of DR5, and then incubated with 5 μg/ml TRAIL (B) or 50 μg/ml JKTR-18 (C) and various concentrations of JKTR-18 (B) or TRAIL (C). The results are presented as the percentage of the signal in the absence of added JKTR-18 (B) or TRAIL (C). Results shown are the mean ± standard deviation of triplicate measurements.

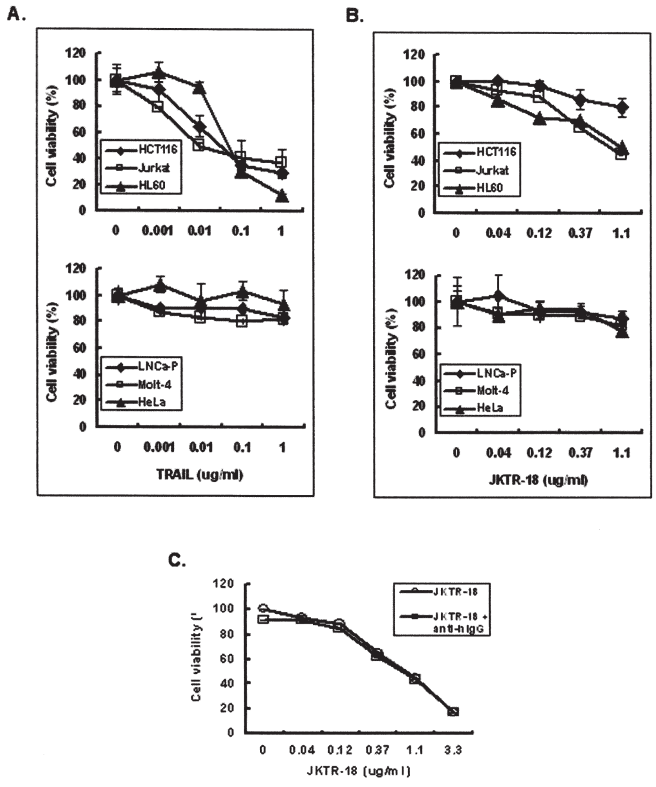

Figure 3. Cytotoxic effects of JKTR-18 on various tumor cell lines. Cells ($4x10^4$) were treated with the indicated concentrations of TRAIL for 24 h (A) or JKTR-18 for 48 h (B). Cell viability was measured by MTT assay. Jurkat cells were treated with JKTR-18 in the presence or absence of mouse anti-human IgG (Fab-specific) antibody (diluted 1:15,000) for 48 h (C). Results shown are the mean ± SD of at least triplicate measurements.

DR4/DR5 were purchased from Koma Biotechnology (Korea) and Strathmann Biotechnology, respectively.

Phage display and purification of soluble JKTR-18. Detailed methods for phage display and purification of soluble Fab were described previously (14). For panning, the wells of an ELISA plate were coated with 7 μg/ml DR5 in PBS (pH 7.4).

Cell culture. Jurkat human T cell leukemia, HL60 human myeloid leukemia, and LNCa-P human prostate cancer cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, and 100 μg/ml penicillin/streptomycin. For Molt-4 human acute lymphoblastic leukemia cells, RPMI-1640 with 20% FBS and the supplements were used. HCT116 human colorectal carcinoma and HeLa human cervix adenocarcinoma cells were cultured in Dulbecco's modified Eagle's medium containing the same supplements.

Direct binding and competitive ELISA. Enzyme-linked immunosorbent assay (ELISA) was performed as previously described (15). The bound Fabs were detected with biotin-conjugated anti-human IgG (Fab-specific) antibody.

Cytotoxicity test. Cytotoxicity was measured by the mitochondrial conversion of MTT as described previously (1).

FACS analysis for apoptosis. Cells after the treatment were stained using the annexin-V-FITC/PI kit according to the manufacturer's instructions (Pharmingen). After the staining, samples were analyzed with a flow cytometer (FACSVantage, BD Biosciences). Cells (10,000) were counted per sample.

DNA laddering assay. Cells ($1x10^6$) were treated as presented. DNA were collected using the ApopLadder Ex kit according to the manufacturer's instructions (Takara Bio) and analyzed by electrophoresis on a 1% agarose gel.

Measurement of activity of caspase-3 and -7. Activation of caspase-3 and -7 was measured using an Apo-ONE Homogeneous Caspase-3/7 assay (Promega) according to the manufacturer's instructions. Caspase activity was measured by reading the plates at the wavelengths of $485_{Excitation}/530_{Emission}$ using a model LS55 luminescence spectrometer (Perkin Elmer).

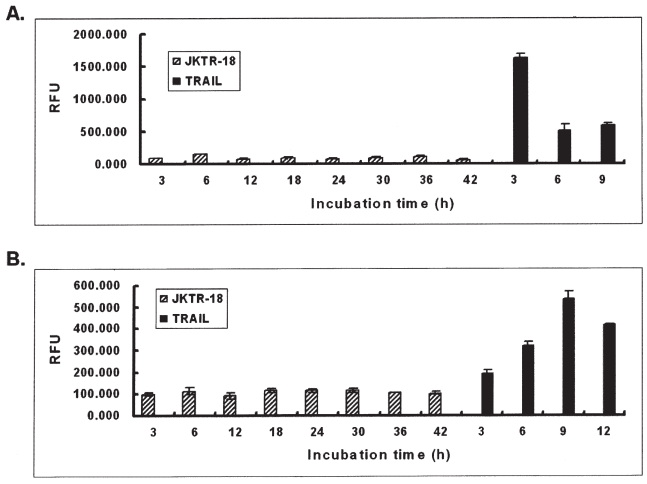

Figure 4. Determination of levels of caspase-3/7 activation in cells treated with JKTR-18. Jurkat (A) or HL60 (B) cells ($2x10^4$/well) were incubated with 0.1 μg/ml TRAIL or 1 μg/ml JKTR-18 for various times. Results shown are the mean ± SD of triplicate measurements. RFLU, relative fluorescent units.

Results

Isolation of a Fab clone, JKTR-18. In the current studies, we isolated a Fab clone against recombinant DR5 by panning of a phage-displayed human Fab library. The amino acid sequences of the V_H and V_L regions of the isolated Fab, JKTR-18, are shown in Fig. 1.

JKTR-18 binds to both DR5 and DR4. A direct binding ELISA showed that the purified JKTR-18 Fab bound to both DR5 and DR4 which were expressed in *E. coli* BL21(DE3) as previously described (13), although its binding activity was higher to DR5 (Fig. 2A). Since human DR5 (GenBank accession no. BAA33723) and DR4 (no. O00220) share ~ 50% identity in their amino acid sequences, it is possible that an mAb to one antigen could cross-react to another. It is suggested that the anti-DR5, JKTR-18 has cross-reactivity to DR4. The binding of JKTR-18 to the antigens increased with the concentration of JKTR-18, whereas there was no signal

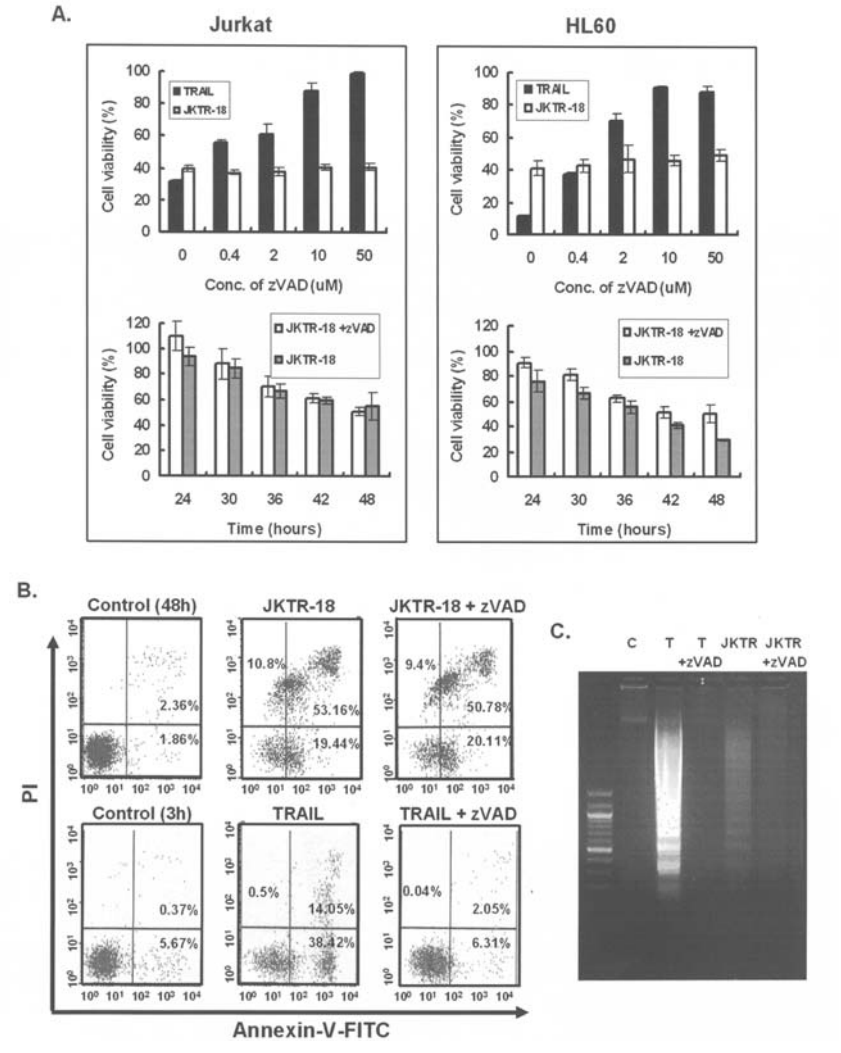

Figure 5. Effects of zVAD in JKTR-18-treated cells. (A) Jurkat or HL60 cells ($4x10^4$) were treated with 1 μg/ml TRAIL for 24 h or JKTR-18 for 48 h in the presence of the indicated concentrations of zVAD (upper panels). JKTR-18 (1 μg/ml) and 50 μM of zVAD were incubated for various times (lower panels). Results shown are the mean ± SD of triplicate measurements. (B) Jurkat cells were treated with 1 μg/ml JKTR-18 for 48 h or 0.5 μg/ml TRAIL for 3 h in the presence or absence of 30 μM zVAD. PS exposure and cell death were analyzed by annexin-V/PI staining on a flow cytometer. Control cells were also assayed after either 48 h or 3 h. The data shown are representative of at least three independent experiments. (C) DNA laddering assay. HL60 cells were treated with 1 μg/ml TRAIL for 6 h or 10 μg/ml JKTR-18 for 52 h in the presence or absence of 30 μM zVAD.

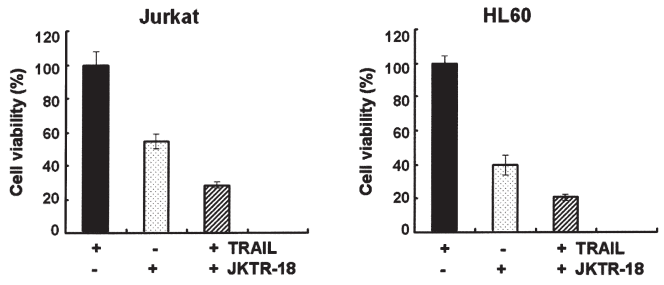

Figure 6. JKTR-18 enhances TRAIL-induced death. Jurkat and HL60 cells were incubated with 1 μg/ml JKTR-18 for 24 h, followed by incubation of 0.1 μg/ml (in Jurkat) or 0.001 μg/ml (in HL60) of TRAIL for an additional 24 h. Cell viability measured by MTT assay is shown as a percentage of the viability in the presence of TRAIL alone (100%).

even at a high concentration of the Fab when the antigen was omitted. Interestingly, a competitive ELISA showed that there was no competition between JKTR-18 and TRAIL for DR5 binding (Fig. 2B and C), suggesting that JKTR-18 and TRAIL bound to different epitopes on DR5.

JKTR-18 induces cell death in Jurkat and HL60 cell lines without cross-linker in vitro. Next, we tested whether, like TRAIL, JKTR-18 can kill tumor cells with both TRAIL-sensitive (HCT116, Jurkat, and HL60) and TRAIL-resistant tumor cell lines (LNCa-P, Molt-4, and HeLa) using MTT assay. The cells were treated with various concentrations of TRAIL for 24 h (Fig. 3A) or the monovalent Fab, JKTR-18 in the absence of crosslinking reagents for 48 h (Fig. 3B). JKTR-18 clearly showed cytotoxicity in Jurkat and HL60 leukemia cell lines, although a higher concentration of JKTR-18 than TRAIL was needed for obtaining the same level of cell death. When Jurkat and HL60 cells were incubated with 1.1 μg/ml JKTR-18 for 48 h, ~ 45-50% of the cells survived. Interestingly, HCT116 cells were not killed efficiently by JKTR-18, whereas the cell line was highly susceptible to TRAIL, which suggested that different mechanisms may be utilized by JKTR-18 and TRAIL. Three TRAIL-resistant cell lines were not susceptible to JKTR-18. Notably, treatment of JKTR-18 induced a similar level of cell death, regardless of the presence of cross-linker (anti-human IgG) in Jurkat (Fig. 3C) and the other five cell lines (data not shown).

JKTR-18 does not activate caspase-3/7. Our results suggested that JKTR-18 and TRAIL might bind to different sites on DR5 and utilize different mechanisms for inducing cell death. Therefore, we compared the abilities of JKTR-18 and TRAIL to activate caspases using a sensitive fluorescent assay for caspase-3/7 activation. In both Jurkat and HL60 cells, JKTR-18 did not efficiently activate caspase-3/7 up to 42 h of treatment, whereas TRAIL activated them at various levels in this time. (Fig. 4A and B)

Pan-caspase inhibitor does not recover cell death induced by JKTR-18. Because JKTR-18 did not activate the caspases, we tested further the effect of zVAD, a pan-caspase inhibitor, on JKTR-18-induced cell death by MTT assay and annexin-V/PI staining flow cytometry. As shown in the upper panels of Fig. 5A, zVAD dose-dependently inhibited TRAIL-induced death of both Jurkat and HL60 cells, and from 10 μM, zVAD almost completely suppressed the ability of TRAIL to induce cell death. In contrast, even at 50 μM, zVAD did not protect Jurkat and HL60 cells from JKTR-18-induced cell death. The lower panels in Fig. 5A show that zVAD had no effect on JKTR-18-induced cell death for up to 48 h in both cell lines. As shown in Fig. 5B, annexin-V/PI staining flow cytometry indicated that the presence of 30 μM zVAD had no effect on an annexin-V positive apoptotic level of Jurkat cells treated with 1 μg/ml JKTR-18 (19.44 versus 20.11%), whereas zVAD protected Jurkat cells from 0.5 μg/ml TRAIL-induced apoptosis (38.42 versus 6.31%). Total cell death induced by JKTR-18 alone (80.4%) also was not decreased by co-treatment of zVAD (80.29%), whereas that induced by TRAIL alone (52.97%) was dramatically diminished in the presence of the inhibitor (8.4%). The DNA fragmentation assay showed that HL60 cells treated with TRAIL or JKTR-18 alone formed a DNA ladder, which is one of the typical phenomena of apoptosis (Fig. 5C). No DNA ladder was observed in TRAIL and zVAD co-treated cells. However, in JKTR-18 and zVAD co-treated cells, the laddering did not completely disappear. These results showed that mechanisms of cell death induced by JKTR-18 include at least a caspase-independent pathway.

JKTR-18 enhances the level of cell death induced by TRAIL. Because JKTR-18 does not compete with TRAIL for binding to sDR5, we predicted that JKTR-18 could enhance TRAIL-induced cell death. To examine this possibility, we treated Jurkat and HL60 cells with optimal concentrations of JKTR-18, TRAIL, or both. As expected, the combination of JKTR-18 and TRAIL resulted in greater levels of cell death than either agent alone in both cell lines (Fig. 6).

Discussion

In the current study, we isolated a novel monovalent human monoclonal Fab to human DR5, JKTR-18, which cross-reacts to human DR4. It induced cell death in two leukemia cell lines, Jurkat and HL60 without the need for a secondary cross-linker *in vitro*. A direct agonist of the TRAIL receptor such as JKTR-18 as well as other mAbs (5,6,8,9,12) may provide highly efficient immunotherapy for cancer. However, all other mAbs except JKTR-18 are bivalent whole IgGs. Therefore, we show here for the first time that a monovalent Fab can induce tumor cell death without cross-linking. JKTR-18 caused cell death in the Jurkat cell line, which has been shown to express DR5, but not DR4 (1,16). Therefore, cell death induced by JKTR-18 was probably due to DR5 binding, although JKTR-18 showed binding ability to DR4 in ELISA. HCT116 cells highly sensitive to TRAIL (Fig. 3) (17) are not susceptible to JKTR-18, indicating JKTR-18 and TRAIL utilize different mechanisms for the induction of cell death. The observation that JKTR-18 did not compete with TRAIL for binding to DR5 also indicates that regions other than the TRAIL-binding sites on DR5 may be involved in JKTR-18-induced cell death and mechanisms activated by JKTR-18 and TRAIL are distinct. Therefore, it was strongly suggested that combination treatment of JKTR-

18 and TRAIL could enhance tumor cell killing. The enhanced effect of cell death was actually observed in Jurkat and HL60 cells.

We investigated the differences of the cell signals triggered by TRAIL and JKTR-18 at the level of caspase activation. The important role of DR5 is the cell signaling via activation of the caspase cascade. However, JKTR-18 as well as another previously reported mAb AD5-10 (8), activated a caspase-independent signaling pathway, suggesting DR5 has the ability to trigger a non-classical cell death mechanism by interacting with agonistic Abs. We did not observe any evidence for activation of caspases in Jurkat and HL60 cells treated with JKTR-18. However, we could not confirm whether only the caspase-independent pathway was involved in JKTR-18-induced cell death of these leukemia cells. It is still possible that a caspase-independent mechanism is not sufficient to trigger death of the tumor cells. JKTR-18 requires a higher concentration than TRAIL for achieving the same level of tumor cell death. Converting Fab into the IgG form of JKTR-18 might be helpful for increasing the efficacy of the Ab *in vivo*, although the Fab fragment did not need a cross-linker *in vitro*.

Acknowledgements

This study was supported by the KOSEF grant funded by the Korea governmen (MOST) (No. 2007-01443) and the 'GRRC' project of Gyeonggi Provincial government, Korea. We thank Professor M.H. Kwon and graduate student W.R. Lee (Ajou Univ.) for kindly providing the Molt-4 cell line and technical assistance with purification of sDR5 and sTRAIL, respectively.

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