

Multivalent DR5 Peptides Activate the TRAIL Death Pathway and Exert Tumoricidal Activity

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Abstract

Ongoing clinical trials are exploring anticancer approaches based on signaling by TRAIL, a ligand for the cell death receptors DR4 and DR5. In this study, we report on the selective apoptotic effects of multivalent DR5 binding peptides (TRAIL^{mim/DR5}) on cancer cells *in vitro* and *in vivo*. Surface plasmon resonance revealed up to several thousand-fold increased affinities of TRAIL^{mim/DR5}-receptor complexes on generation of divalent and trivalent molecules, the latter of which was achieved with a conformationally restricted adamantane core. Notably, only multivalent molecules triggered a substantial DR5-dependent apoptotic response *in vitro*. In tumor models derived from human embryonic kidney cells or primary foreskin fibroblasts, TRAIL^{mim/DR5} peptides exerted a cancer cell-selective action that could synergize with resveratrol in a manner independent of p53. In a xenograft model of human colon cancer, a divalent TRAIL^{mim/DR5} peptide inhibited tumor growth. Our results offer a proof-of-principle for the development of synthetic small molecules to trigger the TRAIL apoptosis pathway for cancer therapy. *Cancer Res*; 70(3): 1101–10. ©2010 AACR.

Introduction

Tumor necrosis factor- α -related apoptosis-inducing ligand (TRAIL/Apo2L/TNFSF10) is the only known endogenous molecule with the unique characteristic of inducing apoptosis in tumor cells while sparing normal ones (1). TRAIL is a type II trans-membrane protein that, like other tumor necrosis factor (TNF) superfamily members, binds as a homotrimer to its receptors. Four membrane-bound TRAIL specific receptors have been described: two death receptors (DR4/TRAIL-R1/TNFRSF10A and DR5/TRAIL-R2/TNFRSF10B; refs. 2, 3) that mediate the apoptogenic signal and two decoy receptors (DcR1/TRAIL-R3/TNFRSF10C and DcR2/TRAIL-R4/TNFRSF10D). Albeit on binding the ligand, DcR1 and DcR2 do not transduce the apoptogenic signal,

on TRAIL binding, the ligand-receptor complexes oligomerize and transmit the apoptotic signal through DR4 or DR5 intracellular domains, which provide a scaffold for specific adaptor proteins [such as Fas-associated death domain (FADD)] and initiator caspases, thereby forming the death inducing signaling complex (DISC). DISC formation enables autocleavage of the initiator caspases resulting in activation of the death-executing caspase cascade and ultimately cell death (for recent reviews, see refs. 1, 4–6). Despite its regulation by p53 (7), the apoptogenic action of TRAIL or other member of the TNF family (8) is largely independent of p53 integrity (9).

In addition to its role in tumor surveillance (10–16), TRAIL exerts potent tumoricidal activity also in a cell-autonomous fashion. Indeed, several antitumor signaling pathways (e.g., retinoids, rexinoids, and IFNs) and novel drugs (e.g., histone deacetylase inhibitors) converge on TRAIL (17–21). Soluble recombinant TRAIL exhibits antitumor activity on primary tumor cells cultured *in vitro* and human tumor xenografts *in vivo* (22–24). Moreover, agonistic humanized monoclonal antibodies that engage DR4 or DR5 exhibit potent antitumor activity *in vivo* (25–28). Tumor cells frequently display constitutive or acquired resistance to TRAIL, which, however, can be relieved by a poorly understood phenomenon generally referred to as sensitization. Indeed, a plethora of sensitizing agents, including chemotherapeutic drugs and cytokines, or ionizing radiation can revert the TRAIL-resistant phenotype (for recent reviews, see refs. 4, 6).

Currently, two main strategies are used to explore the TRAIL pathway for cancer therapy. On the one hand, recombinant human TRAIL (Apo2L/AMG951) is tested in clinical trials⁶ for combination therapies of colorectal, non-small cell lung cancer, and non-Hodgkin's lymphoma. The second

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therapeutic option is the use of activating humanized antibodies directed against the death receptor DR4 (mapatumumab/HGS-ETR1) or DR5 (Iexatimumab/HGS-ETR2/AMG655). Several clinical trials assessing the therapeutic potential of these drugs in mono- and combined therapies for a diverse set of cancers are ongoing (29). A major issue in the use of therapeutic proteins, such as recombinant human TRAIL (rhTRAIL) and humanized antibodies, is the production of a stable and biologically active product. Because also the storage/transport necessities of such molecules can be expensive, major efforts are ongoing to find new treatment paradigms that display tumor and receptor selectivity and efficacies that are comparable or superior to endogenous TRAIL.

Recently, small apoptogenic peptides that bind to specific TRAIL receptor interfaces have been reported (30, 31). It is well established that death receptor oligomerization is important for DISC function/efficacy, and structural studies provide novel insight into this process (32). Indeed, it has been shown that strategies that increase receptor oligomerization amplify TRAIL-induced apoptosis (33, 34). Therefore, we used previously described sequences (30) to systematically study the effect of peptide dimerization and trimerization on DR5 binding and selective DR5-mediated death induction. By using two different stepwise tumorigenesis models (35), we revealed the tumor-selective activity of such TRAIL mimics (TRAIL^{mim}) *in vitro* and determined their capacity to synergize with resveratrol. We show that neither the DR5-selective mimic (TRAIL^{mim/DR5}) nor resveratrol affects the viability of normal cells and that resveratrol sensitization to TRAIL^{mim/DR5} takes place in a *p53*-independent manner. Finally, we show the antitumor activity of divalent TRAIL^{mim/DR5} peptides *in vivo*.

Materials and Methods

Reagents. Recombinant human DR4, DR5, DcR1, and DcR2 were from Alexis Biochemicals. Resveratrol was from Sigma. Sources of antibodies and the cell lines used in this study are described in Supplementary Materials and Methods.

Peptide synthesis. Linear precursors of peptides **1m–3m** were prepared using standard Fmoc chemistry starting from Rink amide resin (36) and with BOP (37) as a coupling agent. The peptides were cleaved from the resin using a trifluoroacetic acid/triisopropylsilane/DTT/H₂O solution, purified by preparative C₁₈ reverse-phase high-performance liquid chromatography (RP-HPLC), and lyophilized. Disulfide bridge formation was done by air oxidation in aqueous medium under slightly alkaline conditions (pH 8.0) at a peptide concentration of 0.8 mg/mL. Crude cyclic peptides were purified by preparative C₁₈ RP-HPLC and lyophilized. Divalent **1d–3d** were prepared as described (30) by reaction of cyclic peptides (2.2 equiv) with the bis-succinimidyl carboxymethoxyacetate (1 equiv) in dimethylformamide in the presence of a tertiary base. The detailed synthesis of triva-

lent **1t–3t** based on adamantane core will be reported elsewhere. Briefly, triamine adamantane (38) was reacted with succinic anhydride. The resulting triacid was converted to the corresponding tris-succinimidyl ester by reaction with *N*-hydroxysuccinimide and dicyclohexylcarbodiimide. Trivalent **1t–3t** molecules were obtained by reacting the corresponding cyclic peptides **1m–3m** (3.3 equiv) with the adamantane tris-succinimidyl ester in dimethylformamide in the presence of a tertiary base. Divalent and trivalent molecules were purified by C₁₈ RP-HPLC and their identity was confirmed by mass spectrometry. The fraction of active molecules was determined by surface plasmon resonance (SPR) under conditions of mass transport limitation; for details, see Supplementary Data.

Surface plasmon resonance. All experiments were done with a BIAcore 3000 (BIAcore) instrument. To determine the kinetic parameters of binding of the various peptides on DR5, peptides were injected on a CM5 sensor chip (Research Grade, Biacore AB) previously immobilized with the TRAIL receptors (DR5, DR4, DcR1, and DcR2) and three other receptors of the TNF superfamily (RANK, CD95, and TNFR1). The sensorgrams were analyzed with BIAevaluation version 4.1 using the simple 1:1 Langmuir binding model. To assess the ability of the various peptides to inhibit the binding of rhTRAIL to DR5, the DR5 protein was preincubated with different concentrations of the peptides and then injected on the sensor chip containing His-TRAIL. The sensorgrams were analyzed by global fitting using the Biaeval 1.4 program and the IC₅₀ was calculated from a logit plot (Sigmaplot).

Apoptosis measurement. Apoptosis was determined either by detection of phosphatidylserine externalization after co-labeling with Annexin V-FITC/propidium iodide or by APO 2.7 immunostaining, according to the manufacturer's instructions. Apoptosis is displayed as the percentage of cells presenting a positive staining on rhTRAIL or synthetic peptide treatment compared with nontreated cells as control. Each experiment was carried out independently at least three times.

Western blot assays. BJAB cells were treated during 16 h with 5 μmol/L of peptides or with 0.1 or 10 nmol/L of rhTRAIL as a positive control. Samples were harvested in radioimmunoprecipitation assay buffer, separated by SDS-PAGE, electrotransferred onto nitrocellulose membranes, and processed as described in Supplementary Materials and Methods.

DISC experiments. Cells (10⁸) were stimulated with either 5 or 30 μmol/L of peptides or 5 μg/mL His-TRAIL as a positive control for 1 h at 37°C. Cells were then washed with cold PBS and then lysed in 1 mL of lysis buffer [containing 1% NP40, 20 mmol/L of Tris-HCl (pH 7.5), 150 mmol/L of NaCl, and 10% of glycerol]. Lysates were precleared with Sepharose 6B (Sigma-Aldrich) and the DISC was immunoprecipitated using 5 μg of anti-caspase-8 antibody bound to protein G-Sepharose beads (Amersham Biosciences). Immunoprecipitates were processed for immunoblotting to detect DR5, DR4, FADD, and caspase-8.

In vivo analysis of antitumor activity of synthetic peptides. Five-week-old athymic (nu/nu) female mice were

⁶ <http://clinicaltrials.gov/>

used for all *in vivo* experiments. Injections of 5×10^6 HCT116 cells were done in both flanks. Xenografts were allowed to develop for 4 to 5 d after grafting, and tumor volume was determined by caliper measurement. Animals were randomly sorted into treatment groups presenting a mean tumor volume of 20 mm^3 . The initial value for each group was arbitrarily established as 100 and all subsequent changes in tumor volume for each group were expressed as percent change in comparison with the starting tumor mass [(tumor volume day n) \times 100 / (tumor volume at day 1)] and is referred to as normalized tumor volume. Doses of 4 milligram per kilogram (mpk) **1d**, 8 mpk **1d**, and 8 mpk **2d** (in 0.9% NaCl), or vehicle (0.9% NaCl), were daily administered by i.p. injection. Growth curves were obtained by externally measuring tumors everyday during 10 or 14 d. Percentage of growth inhibition was calculated as follows: $100\% - [\text{normalized tumor volume at last day of treatment in treated group} \times 100\% / \text{normalized tumor volume at last day of treatment in control group}]$. The remaining animals (at least six per group) were released from treatment and the tumors were measured everyday during 5 d. Mice body weight was determined at the beginning and at the end of the treatment.

Results

TRAIL-mimicking peptides selectively bind to DR5.

Monovalent TRAIL^{mim/DR5} peptides (**1m**, **2m**, and **3m**; Fig. 1A) were synthesized using standard Fmoc chemistry and cyclized as described in Materials and Methods. Divalent TRAIL^{mim/DR5} peptides **1d**, **2d**, and **3d** were prepared by coupling with bis-succinimidyl carboxymethoxyacetate. As adamantane derivatives are mechanically rigid and conformationally well defined, they represent a useful scaffold for trivalent display (38). Therefore, an adamantyl core was chosen to generate the trivalent TRAIL^{mim/DR5} peptides **1t**, **2t**, and **3t** (Fig. 1A).

The selectivity of the peptides for the four TRAIL receptors was validated by SPR. For this, recombinant DR4, DR5, DcR1, or DcR2 proteins were covalently immobilized on a sensor chip and TRAIL^{mim/DR5} or rhTRAIL was injected. As previously reported, rhTRAIL binds to all its receptors, whereas the synthetic monovalent peptides exclusively recognize DR5 (Fig. 1B; Supplementary Fig. S1). Similar results were obtained for divalent molecules (Supplementary Fig. S1). Moreover, TRAIL^{mim/DR5} neither binds to other death domain-containing receptors such as TNFR1 and CD95 (Supplementary Fig. S1) nor recognizes the mouse homologue of DR5 (data not shown). Taken together, these results reveal the stringent selectivity of these synthetic molecules for human DR5.

Multivalent TRAIL^{mim/DR5} peptides display increased affinity to DR5. Ligands of the TNF superfamily are generally organized as trimers, and it is postulated that trimerization is requisite for inducing death receptor clustering and the induction of a substantial apoptogenic response (33, 39). Indeed, SPR experiments in which recombinant human DR5 (rhDR5) was covalently immobilized on a sensor chip and flushed with monovalent, divalent, or trivalent

TRAIL^{mim/DR5} peptides revealed that all monovalent peptides bound to rhDR5 with affinity in the micromolar range. Divalent peptides substantially improved the binding affinity to rhDR5 between 100- and 6,000-fold, with the strongest effect observed for **2** (Table 1; Supplementary Fig. S2). Moreover, trivalent **3t** resulted in a 45-fold increased affinity to rhDR5 relative to **3d**. That this effect was less evident for **1** and **2** is most likely due to the different lysine anchoring of the peptide to the adamantyl core, which may not be compatible with optimal cooperative binding to the receptor. Note that a low solubility observed for **1t** may also be a contributing factor.

Analysis of the association rate of the peptides to DR5 (k_{on}) revealed that the kinetics of TRAIL^{mim/DR5} peptide association was increased for divalent molecules (Table 1). This effect was more evident for **2d** (800-fold) than for **3d** (50-fold) or **1d** (8-fold). Interestingly, trivalent peptides did not substantially increase the association rates relative to the divalent ones.

As is apparent from the corresponding dissociation kinetics (k_{off}), divalent **1** and **2** stabilized the peptide-receptor complex by 1 order of magnitude, whereas trivalent molecules for each of these peptides (**1t** and **2t**) did not further improve complex stability (Table 1; Supplementary Fig. S2). In contrast, generation of a trivalent **3t** stabilized the complex by 65-fold compared with its divalent molecule. Together, the above data reveal that, for optimally designed TRAIL^{mim/DR5} peptides, generation of multivalent molecules can result in a several thousand-fold increased affinity, which originates from the effects on both the association and dissociation kinetics and is sensitive to subtle changes of the sequence (R to K) and/or the anchoring point, as well as the nature of the core used.

The DR5 binding surfaces of TRAIL^{mim/DR5} peptides were explored by competitive SPR inhibition assays. For this, rhTRAIL was immobilized on a sensor chip, and solutions consisting of a constant concentration of rhDR5 and stepwise 2-fold increasing concentrations of peptides were successively injected. All peptides competed with rhTRAIL for binding to rhDR5 in a concentration-dependent manner, and divalent molecules of all three peptides (**1**, **2**, and **3**) resulted in largely increased competition at equimolar concentration, as revealed by the left shift of the dose-response curve (Fig. 1C). Whereas solubility issues precluded conclusion about the ability of **1t** to inhibit TRAIL binding to DR5, **2t** showed a similar inhibitory capacity as **2m**. In the case of **3**, the inhibitory capacity gradually increased from monovalent to divalent to trivalent molecules (Fig. 1C; k_i). These competition experiments confirm that TRAIL and TRAIL^{mim/DR5} peptides target, directly or indirectly, common surface(s) at the cognate DR5 receptor.

TRAIL^{mim/DR5} activates DR5-mediated apoptosis in vitro. The efficiency and selectivity of peptides to trigger DR5-mediated apoptosis *in vitro* were analyzed using the BJAB (Burkitt lymphoma) model, which consists of cells that express endogenous levels of DR4 and either lack (DR5-DEF) or express (DR5-DEF+DR5) the DR5 receptor (40). BJAB cells

either 15 or 30 $\mu\text{mol/L}$ of these peptides was negligible compared with their multivalent homologues (Supplementary Fig. S4), emphasizing that increasing the valency enhances the apoptogenic potential of TRAIL^{mim/DR5} peptides.

Western blot analysis confirmed that TRAIL^{mim/DR5} induces cell death in a caspase-dependent manner involving initiator (caspase-8 and caspase-9) and executor (caspase-3) caspases and resulting in substrate (PARP) cleavage in DR5-DEF+DR5, but not in DR5-DEF, cells (Fig. 2C). Albeit **2d** and **3t** can reach similar apoptogenic activity on prolonged treatment (Fig. 2A, 16 hours), time-course experiments revealed that whereas **3t** reaches its maximal effect after 4.5 hours, **2d** only does it after 7.5 hours (Fig. 2D). This indicates that increasing the valency dictates not only the extent of the apoptotic response observed but also the death kinetics. These observations confirm the specificity of TRAIL^{mim/DR5} to trigger a DR5-dependent apoptosis and indicate that divalent molecules (or higher order valency) are required to trigger substantial cell death.

Resveratrol sensitization to TRAIL^{mim/DR5} induces extensive tumor-selective apoptosis without affecting normal cells. To determine whether active TRAIL^{mim/DR5} triggers apoptosis only in tumor cells, we used a stepwise tumorigenesis model in which primary normal human embryonic kidney (HEK) cells have been transformed into tumorigenic cells (HA1ER) by overexpressing telomerase, the SV40 early region, and activated RAS_{G12V} (35). Normal and tumor cells were exposed to 5 $\mu\text{mol/L}$ TRAIL^{mim/DR5} or 10 nmol/L rhTRAIL as a positive control. Importantly, rhTRAIL induced a significant apoptotic response in tumor cells without affecting the viability of the parental normal counterpart (Fig. 3A). However, compared with DR5-DEF+DR5, HA1ER cells displayed only a weak apoptotic response toward **1d** under identical conditions.

Because low sensitivity of solid cancers to TRAIL^{mim/DR5} would represent an important limitation, we explored if sensitization, which is well described for rhTRAIL (1), would also enhance TRAIL^{mim/DR5} activity. Among the various sensitizing agents, resveratrol has been described as having only negligible effect on normal tissues (42, 43). Notably, resveratrol dramatically enhanced the sensitivity of HA1ER to **1d** by

10-fold, and apoptosis induced by rhTRAIL was increased 6-fold (Fig. 3B). To assess the tumor selectivity of this sensitization to TRAIL^{mim/DR5}, we used the above-described stepwise model and the corresponding model derived from human foreskin fibroblasts composed of normal BJ and fully transformed BJELR cells (35). Whereas normal HEK and BJ cells were entirely resistant to the combination of resveratrol (100 $\mu\text{mol/L}$) and either **1d** (5 $\mu\text{mol/L}$) or rhTRAIL (0.1 nmol/L), more than 30% of HA1ER and BJELR cells were killed by TRAIL^{mim/DR5} at doses at which none of the compounds efficiently triggered apoptosis as a single agent (Fig. 3C). Similar results were obtained for rhTRAIL. These results show that resveratrol dramatically sensitizes tumor cells to TRAIL^{mim/DR5} without affecting tumor selectivity.

Resveratrol sensitization to TRAIL^{mim/DR5} is largely independent of p53. The p53 protein, which mediates critical cell functions including the response to genotoxic stress, is one of the most commonly altered proteins in human cancer, and its loss usually results in resistance to cell death stimuli. Therefore, abnormal p53 function could block the effects of sensitizers acting in a p53-dependent manner. However, HCT116 wild-type and p53^{-/-} colon carcinoma cells responded similarly (Fig. 3D), indicating that resveratrol sensitization to TRAIL^{mim/DR5} is largely independent of the p53 status of the cell.

Antitumor activity of divalent TRAIL^{mim/DR5} in vivo. To assess if the apoptogenic activity of divalent TRAIL^{mim/DR5} on cultured HCT116 cells is indicative of an antitumoral activity *in vivo*, **1d** and **2d** were tested in the HCT116 tumor xenograft model. For that, nude mice were s.c. injected with 5×10^6 HCT116 cells and a daily i.p. administration of **1d** or **2d** was started 4 days after tumor grafting (mean tumor volume $\sim 20 \text{ mm}^3$). Doses of 4 mpk and 8 mpk of **1d** were administered during 10 days. Interestingly, under these conditions, we observed major dose-dependent antitumor activities with mean growth inhibitions of 30% and 63%, respectively (Fig. 4A). Similar *in vivo* efficiencies were observed for both **1d** and **2d** after 14 days of daily 8-mpk i.p. injections (growth inhibitions of 62% and 51%, respectively; Fig. 4B). That tumor growth increased dramatically when the treatment was discontinued confirms that tumor growth inhibition was

Table 1. Binding constants of TRAIL mimics

Analyte	K_{on} [(mol/L) ⁻¹ s ⁻¹], $\times 10^3$	k_{off} (s ⁻¹), $\times 10^{-5}$	K_{d} (nmol/L)	Affinity (fold increase)
1m	23.90 \pm 0.19	307.00 \pm 1.93	129.00 \pm 3.68	1
1d	196.00 \pm 0.72	24.20 \pm 0.38	1.24 \pm 0.05	104
1t	354.00 \pm 1.00	31.10 \pm 0.41	0.88 \pm 0.03	146
2m	1.67 \pm 0.01	111.00 \pm 0.68	664.00 \pm 18.00	1
2d	1,360.00 \pm 2.79	15.40 \pm 0.30	0.114 \pm 0.005	5,824
2t	1,190.00 \pm 2.50	64.20 \pm 0.29	0.539 \pm 0.007	1,232
3m	28.40 \pm 0.23	642.00 \pm 2.69	226.00 \pm 5.64	1
3d	1,410.00 \pm 12.20	324.00 \pm 1.67	2.29 \pm 0.06	99
3t	2,330.00 \pm 2.84	9.74 \pm 0.26	0.050 \pm 0.003	4,520
TRAIL	61.10 \pm 0.17	1.65 \pm 0.19	0.27 \pm 0.06	

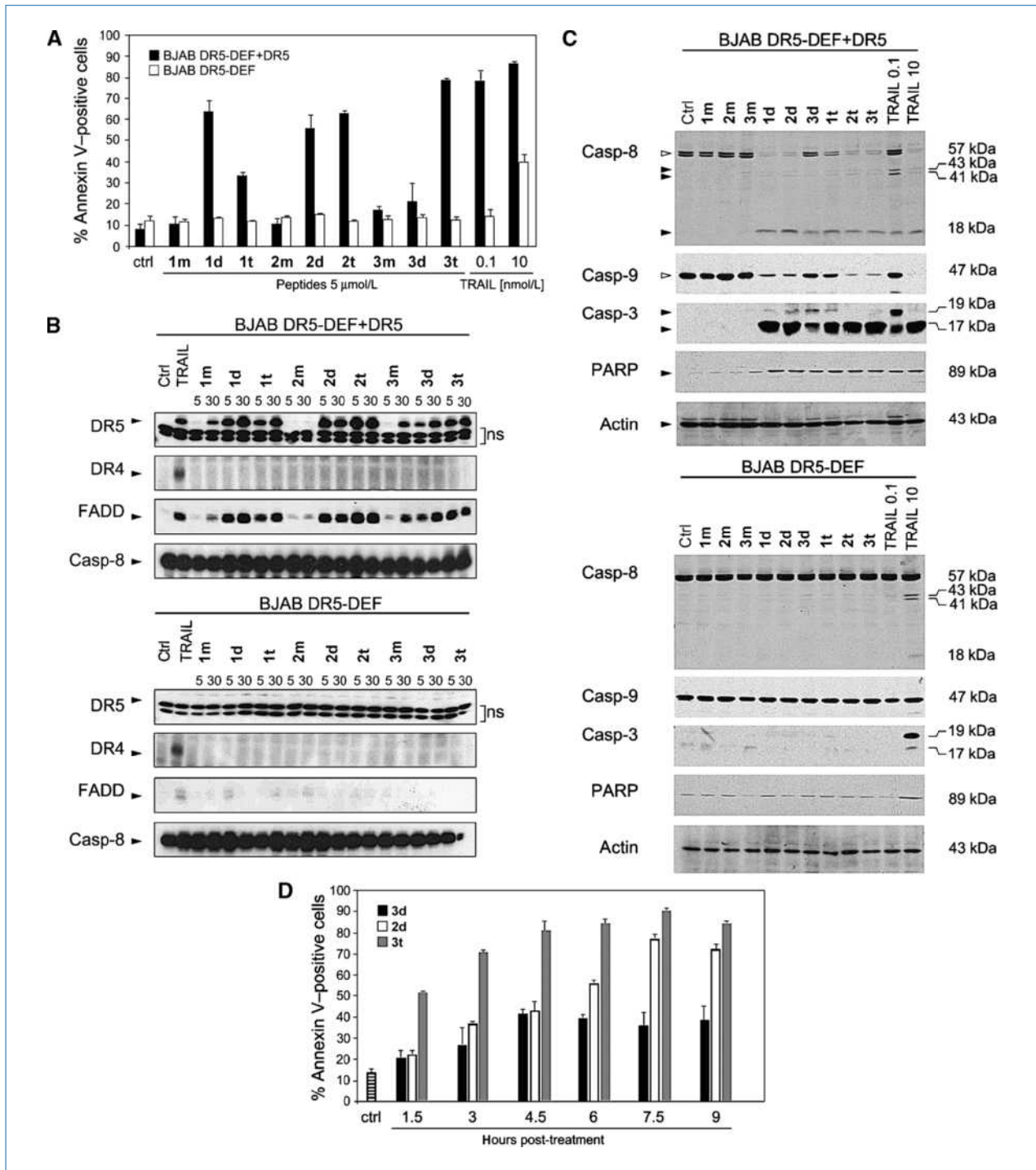


Figure 2. TRAIL^{min} peptides trigger apoptosis in BJAB cells through the DR5 receptor. **A**, DR5-DEF+DR5 or DR5-DEF cells were treated with monovalent (m), divalent (d), and trivalent (t) peptides (5 μmol/L). Apoptosis was determined as the percentage of Annexin V-positive cells by fluorescence-activated cell sorting (FACS) analysis. RhTRAIL (TRAIL) was included as positive control at 0.1 and 10 nmol/L. Columns, mean from three independent biological replicates; bars, SD. **B**, DR5-DEF+DR5 or DR5-DEF cells were treated with different peptides or TRAIL, subjected to immunoprecipitation with antibodies against caspase-8 (Casp-8), and later immunoblotted to analyze DISC components (DR5, DR4, and FADD). ns, nonspecific. **C**, DR5-DEF+DR5 or DR5-DEF cells were treated as in **A**, and total cell extracts were processed for Western blot. Caspase-8 (full-length and cleaved), caspase-9 (full-length), caspase-3 (cleaved), and PARP (cleaved) were detected using specific antibodies. Samples treated with TRAIL (0.1 and 10 nmol/L) were included as a positive control. Full-length blots are presented in Supplementary Figs. S7 to S9. **D**, DR5-DEF+DR5 cells were treated with 5 μmol/L of 2d, 3d, or 3t; samples were collected at the indicated time points after treatment; and apoptosis was determined as the percentage of Annexin V-positive cells. Columns, mean from one representative experiment (three samples per treatment and per time point) of three independent biological replicates; bars, SD.

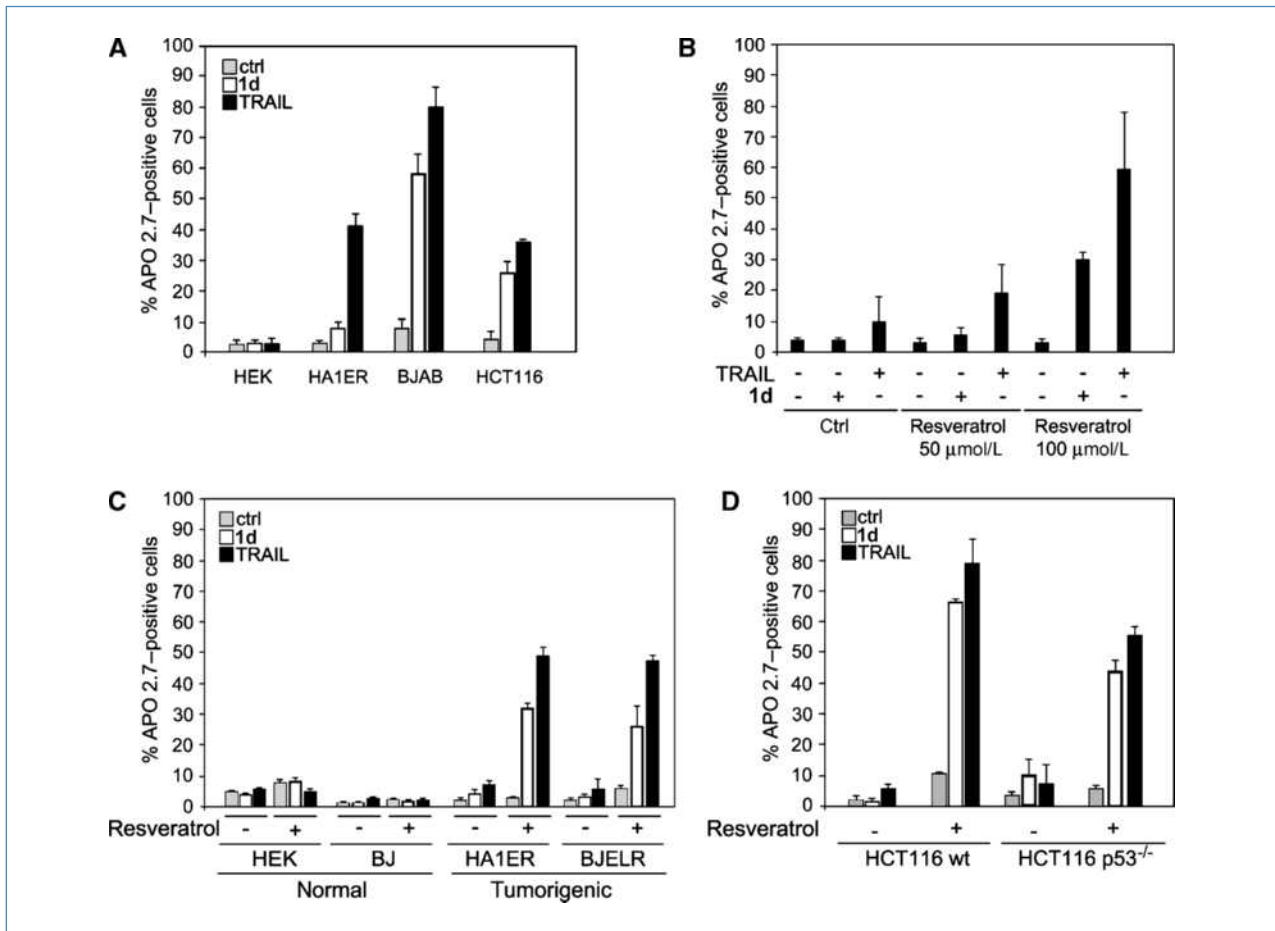


Figure 3. Resveratrol sensitizes tumor cells to TRAIL^{minv/DR5} in a p53-independent manner. A, HEK and HA1ER cells were treated with either **1d** (5 μmol/L) or rhTRAIL (10 nmol/L) and apoptosis was determined as the percentage of APO 2.7-positive cells by FACS analysis. BJAB DR5-DEF+DR5 and HCT116 cells were included in the experimental setup as positive control. B, HA1ER cells were treated with either **1d** (5 μmol/L) or rhTRAIL (0.1 nmol/L) in the presence or absence of resveratrol (50 or 100 μmol/L). Apoptosis was determined as the percentage of APO 2.7-positive cells by FACS. C, normal HEK and BJ cells as well as tumorigenic HA1ER and BJELR cells were treated with either **1d** (5 μmol/L) or TRAIL (0.1 nmol/L) in combination with resveratrol (100 μmol/L) or as a single agent. Apoptosis was determined as the percentage of cells displaying positive labeling for APO 2.7 as determined by FACS analysis. D, HCT116 colon cancer cells, wild-type (*wt*) or p53 knockout (*p53*^{-/-}), were treated with either **1d** peptide (1 μmol/L) or TRAIL (0.1 nmol/L) in combination with resveratrol (100 μmol/L) or as a single agent. Apoptosis was determined as the percentage of cells displaying positive labeling for APO 2.7 as determined by FACS analysis.

directly linked to the activities of **1d** or **2d** (Fig. 4C; Supplementary Fig. S5). Note that the treatments did not cause any changes in body weight or animal behavior in any of the groups (data not shown). However, as the human TRAIL^{mim/DR5} mimics do not bind the mouse TRAIL-R2, no extrapolation to potential toxicity in humans can be made.

Discussion

The majority of current anticancer therapies, in particular chemotherapy and radiotherapy, follow treatment paradigms that attack the general features of cell growth. Consequently, not only the viability of tumor cells is affected but also that of normal ones, thus causing considerable side effects. To overcome this limitation, novel principles that have promise for developing cancer cell-targeted therapies are being intensively explored (44). One such

principle is based on the astounding capacity of the TRAIL signaling pathway to kill tumor but not normal cells (4, 29, 45, 46).

Although recombinant proteins and humanized antibodies without doubt have a major role as therapeutics, they display the inherent disadvantages of proteins associated with production (purity and costs) and functional stability on storage. These aspects provide a strong rationale for the development of more “drug-like” activators of the TRAIL signaling pathway. In addition to the therapeutic aspect, there is a need for tools with which the complexity of the TRAIL signaling pathway, which engages four to five distinct receptors, can be deconvoluted.

For all the above reasons, we set out to characterize and explore the possible therapeutic potential of recently recognized novel synthetic mediators of the TRAIL pathway, the TRAIL-mimicking (TRAIL^{mim}) peptides. Given their synthetic

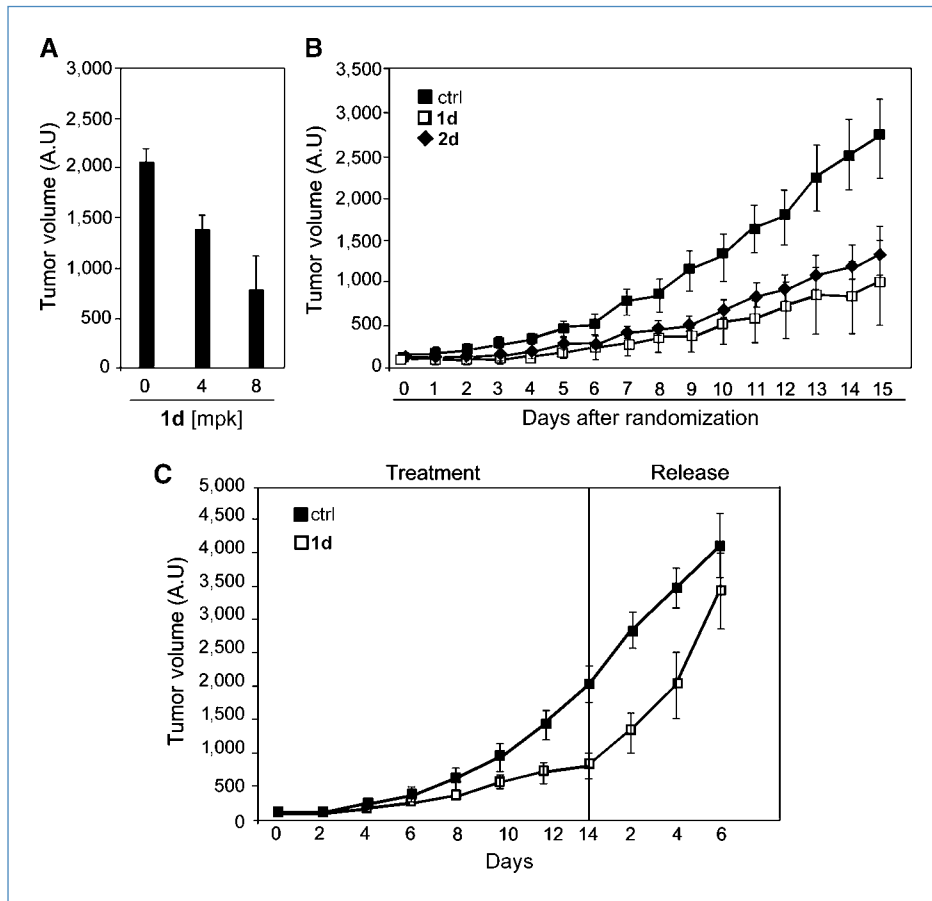


Figure 4. *In vivo* tumoricidal activity of divalent peptides in HCT116 colon cancer xenografts. A, 5-wk-old female nude mice were s.c. injected with 5×10^6 HCT116 cells in both posterior flanks; tumors were then allowed to develop until the mean tumor volume reached 20 mm^3 . Daily i.p. injections of **1d** (4 mpk and 8 mpk) or vehicle (0.9% NaCl) were administered during 10 d. Tumor volume was determined daily by caliper measurement and normalized toward the tumor volume observed at the beginning of the treatment. Columns, mean of normalized tumor volume for each group after 10 d of administration; bars, SEM. $P < 0.05$. B, points, mean normalized tumor volume obtained from groups treated daily with vehicle, **1d**, or **2d** (8 mpk) and followed during 14 d; bars, SEM. C, points, mean normalized tumor volume obtained from control and **1d** (8 mpk) groups treated during 14 d and followed during 6 d after treatment; bars, SEM.

nature, biological contamination and protein stability related with protein production do not have to be considered as potential problems. Moreover, high yield production at a low cost is an additional advantage of these approaches. In the present work, we show that such synthetic peptides recapitulate the action of TRAIL through DR5 by providing evidence that multivalent peptides efficiently trigger the TRAIL apoptotic pathway. It is interesting to note that depending on the peptide, both divalent and trivalent TRAIL^{mim/DR5} can induce DISC formation and apoptosis, supporting previously discussed models for receptor clustering (47).

Moreover, we show that these synthetic peptides retain their tumor-selective apoptogenic activity even when combined with sensitizing compounds and exert a substantial anticancer activity *in vivo* as single agents. Although no "off-target" toxicity was observed in human primary normal fibroblasts and epithelial cells tested, detailed pharmacology and toxicology analyses are required to assess the potential use of TRAIL mimics in human cancer therapy. We observed that increasing TRAIL^{mim/DR5} valency enhances the affinity to DR5 receptor as well as the apoptogenic capacity of these molecules, and determined that the level of multivalency required to achieve a substantial death induction is dictated by the initial peptide sequence and the anchoring point. It is likely that the chemical nature of the scaffold used for polyvalent display may affect the biological prop-

erties (48) and that optimal projection of DR5 binding peptides using more tailored scaffolds may lead to even more potent TRAIL mimics.

Importantly, we have noticed that peptides showing the highest affinity for DR5 and superior association rate/complex stability by SPR were those presenting the strongest apoptogenic activity *in vitro*. This indicates that the kinetic parameters of peptide-receptor interaction could be taken as indicative for the potential apoptogenic capacity of the analyzed molecules. Our experiments show that the peptides efficiently trigger the TRAIL pathway, which is largely independent of the *p53* status of the cell. Notably, the extent of cell death obtained by peptide treatment was enhanced by combined treatment with resveratrol in a *p53*-independent manner without affecting the viability of normal cells. It is worth pointing out that the doses of rhTRAIL used in various xenograft assays to achieve tumor growth inhibition (15–60 mpk; ref. 1) are similar to the doses (4–8 mpk) used in the present study when correcting for the different molecular weights; note in this context that preliminary data reveal that the *ex vivo* half life of **1d** and **2d** in serum is ~4 hours (supplementary information). In view of the observations that other chemo-/radiotherapeutic treatments can enhance TRAIL action, TRAIL mimics like TRAIL^{mim/DR5} and other receptor agonists (29) could pave the way toward a novel type of apoptogenic therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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