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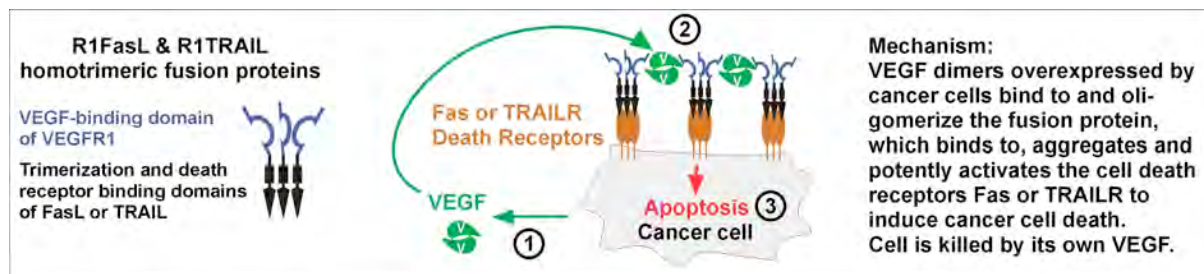
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14. ABSTRACT Two current strategies for targeted cancer drug development are inhibition of vascular endothelial growth factor (VEGF) and activation of pro-apoptotic death receptors such as Fas or TRAILR. The VEGF inhibitor bevacizumab has been shown to prolong progression-free survival in cancer patients, including ovarian cancer patients, but it yields little or no improvement in overall survival. Agent such as soluble TRAIL that target cell death receptors are in early clinical trials but to date have limited efficacy, in part because they do not efficiently aggregate and activate death receptors. We have investigated a novel approach to targeted cancer therapy that combines VEGF inhibition and cell death receptor activation. We generated two recombinant fusion proteins, designated R1FasL and R1TRAIL, that combine the VEGF-binding domain of VEGF receptor-1 with the receptor-binding domain of Fas ligand or TRAIL. The homotrimeric fusion proteins bind VEGF and form oligomeric complexes that efficiently bind to, aggregate and activate Fas or TRAIL cell death receptors. We developed reagents and methods to purify the fusion proteins and demonstrated that they induce cell death only in the presence of VEGF, effectively converting VEGF to act as a cell death factor. When tested on human ovarian cancer cell lines in vitro, R1TRAIL induced cell death when VEGF was present. Chemotherapy agents increased the activity of R1TRAIL. Our results indicate that R1TRAIL may provide a new strategy for combined VEGF inhibition and death receptor activation to more effectively kill cancer cells.				
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Introduction

Two current strategies for targeted cancer drug development are vascular endothelial growth factor (VEGF) inhibition and activation of pro-apoptotic death receptors such as Fas or TRAILR (receptor for TNF-related apoptosis-inducing ligand). VEGF is a well-validated target, and the VEGF inhibitor bevacizumab/Avastin (neutralizing VEGF antibody) is approved for treatment of advanced colon, breast, lung, renal and glioblastoma cancers (1). Recently bevacizumab was reported to prolong progression-free survival in patients with ovarian cancer (2). However, the clinical benefit of bevacizumab has proven to be modest: while progression-free survival is extended by 1 to 5 months, there is little or no increase in overall survival (3). More effective agents that target overexpressed VEGF are needed. Selective activation of death receptors such as TRAILR in tumor cells is also well-validated by preclinical studies (4, 5). Recombinant trimeric soluble TRAIL (the ligand for TRAILR) is in early clinical trials for several cancers, but to date has shown limited efficacy (6-9). In part, its low activity derives from the fact that soluble TRAIL does not efficiently aggregate its receptors. Death receptor aggregation initiates apoptosis (5). Several years ago we conceived a new strategy for a targeted cancer therapy that combines VEGF inhibition and death receptor activation. Our idea was to redirect VEGF to activate cell death receptors within the tumor microenvironment. We created two homotrimeric recombinant fusion proteins that bind to VEGF and thereby become potent activators of apoptosis, designated R1FasL and R1TRAIL. The purpose of this project was to produce purified R1FasL and R1TRAIL fusion proteins, determine if they induce cell death in human ovarian cancer cell lines, and determine if their activity can be potentiated by combining with chemotherapy agents.



Body

Task 1. Generate purified R1FasL and R1TRAIL recombinant fusion proteins

Task 1a. Collect secreted R1FasL and R1TRAIL fusion proteins from the conditioned medium of transfected Cos or CHO cell lines and purify using FLAG antibody affinity chromatography.

We used two approaches to generate the R1FasL and R1TRAIL recombinant fusion proteins: production and secretion by transiently transfected Cos cells or by stably transfected CHO cells. Each approach has advantages and potential disadvantages. Transiently transfected Cos cells can produce high levels of protein for a short period,

but require repeated batch preparations with potential variability. Stably transfected CHO (hamster ovary cells) are widely used by biotech and pharmaceutical companies to produce recombinant proteins, but produce less protein and require generation of producer cell lines. The cDNAs encoding R1FasL or R1TRAIL were constructed with: (1) a signal sequence to allow secretion into the medium (preprotrypsinogen sequence); (2) an N-terminal FLAG epitope tag to allow purification; (3) domain 2 of human VEGFR-1, the principal VEGF-binding domain, including amino acids 129-230; and (4) the trimerization and receptor binding domains of human FasL (amino acids 139-281) or human TRAIL (amino acids 114-281). The cDNAs were subcloned into expression vectors with CMV promoters to allow production and secretion by mammalian cells.

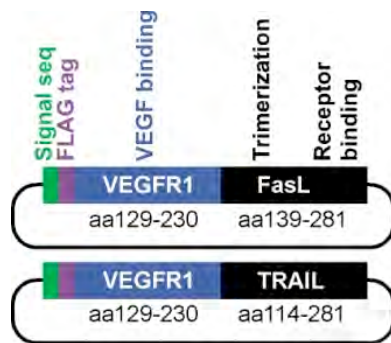


Figure 1. cDNAs encoding R1FasL or R1TRAIL recombinant fusion proteins.

For transient Cos cell production of fusion proteins, cells were transfected using the standard DEAE-dextran method. Briefly, cell were treated with plasmid encoding the fusion protein plus DEAE-dextran for 6 hours, washed and new medium added, and conditioned medium containing fusion protein was collected 48 hours later. For stable production by CHO cells, CHO cells were co-transfected using the calcium phosphate precipitation method with the expression plasmid encoding the fusion protein plus a plasmid encoding resistance to G418. Cells were cultured in G418 (250 µg/mL) for ~2 weeks to allow growth of G418-resistant clones. Stable clones were picked and screened for secretion of the fusion protein by immunoblot of the conditioned medium using anti-FLAG antibody. Positive clones were subcloned two to four times to ensure a stable clonal population. Three stable CHO cell lines expressing either R1FasL or R1TRAIL fusion protein were generated.

To purify the fusion proteins from conditioned medium, we used FLAG antibody affinity chromatography (SIGMA kit). Briefly, conditioned medium from Cos cells or CHO cell lines expressing FLAG-tagged R1FasL or FLAG-tagged R1TRAIL was filtered, loaded onto a FLAG antibody column, washed to remove serum and other medium components, and the fusion proteins were eluted from the column by treatment with excess FLAG peptide. To remove the excess FLAG peptide (molecular weight 2800) from the purified fusion proteins (molecular weight ~160,000), the eluates were spun through centrifugal concentrating filters with 10 kD molecular weight cut-offs.

Task 1b. Quantify R1FasL and R1TRAIL fusion proteins by Bradford protein assay or TRAIL ELISA

The purified R1FasL and R1TRAIL fusion proteins were quantified by Bradford protein assay using BSA as the protein standard. Both proteins were also quantified using ELISA assays (R&D Systems kit) for human FasL or human TRAIL. In addition, both fusion proteins were quantified spectrophotometrically at A280 nM using the calculated molar extinction coefficients for each protein. Comparison of the three methods showed that the Bradford assay and ELISA assays underestimated the concentration of fusion proteins several fold. The likely reason is the use of BSA as the protein standard in the Bradford assay, and the inability of the ELISA assays to accurately quantify the fusion proteins because the presence of the VEGFR-1 domain interfered with binding of the polyclonal ELISA antibodies to the FasL or TRAIL domains. Spectrophotometric quantification by A280 is more accurate and also allows confirmation of protein quality by comparing Abs280 nM to A260 nM. The Abs260/Abs280 ratios of the purified fusion proteins were consistently ~0.55 to 0.6, confirming that the proteins were highly purified. We used the spectrophotometric data to calculate the concentrations of purified R1FasL and R1TRAIL for subsequent experiments.

Task 1c. Characterize molecular weight and purity of R1FasL and R1TRAIL by immunoblot

We used immunoblot with anti-FLAG antibody to characterize the purified fusion proteins. As seen in Figure 2, purified fusion proteins produced from either transiently transfected Cos cells or stably transfected CHO clones migrated at the predicted molecular weights (these are monomers on reduced and denatured PAGE gels).

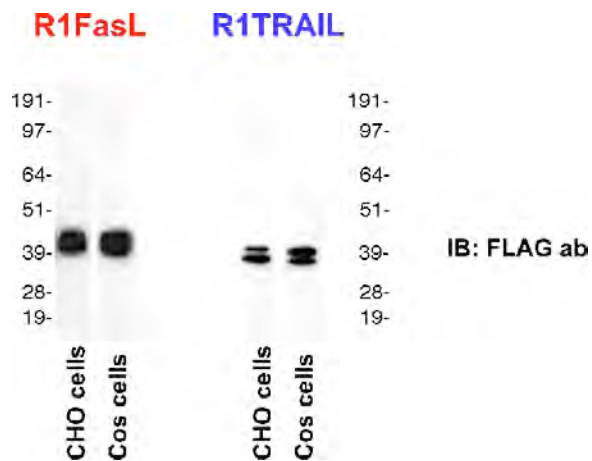
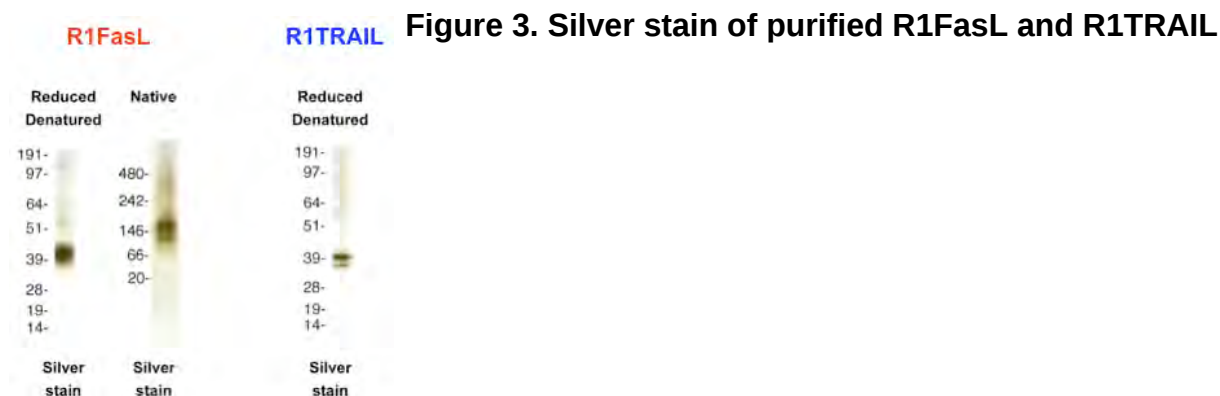


Figure 2. SDS-PAGE of purified fusion proteins

To assess the purity of the fusion proteins we used silver staining (ProteoSilver kit, SIGMA). As seen in Figure 3, both purified fusion proteins were highly pure (left, R1FasL and right, R1TRAIL).



In order for the fusion proteins to function as designed they must be produced as homotrimers, as shown in the figure in the Introduction section. Therefore we performed additional characterization to confirm the presence of homotrimers. As seen in Figure 3 (center), R1FasL run under native gel conditions migrated at ~160 kD, indicated it exists as a homotrimer. We also used size-exclusion chromatography under native conditions to assess the molecular weight of purified R1FasL. As seen in Figure 4, R1FasL migrated as a single peak at ~160 kD, confirming its presence as a homotrimer. Immunoblot of the indicated chromatography fractions confirmed the presence of R1FasL in the A280 peak (reduced and denatured conditions).

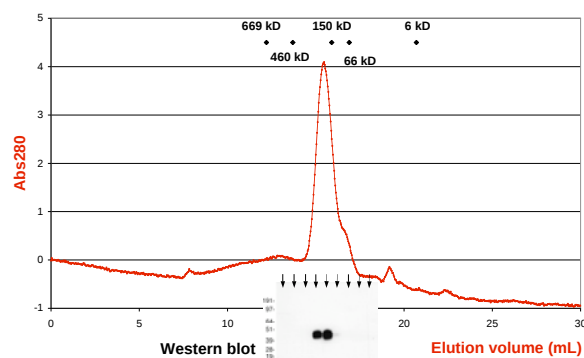


Figure 4. Size-exclusion chromatography R1FasL was run on a Superose-20 column. The indicated column fractions were immunoblotted under reduced and denatured conditions using anti-FLAG antibody, demonstrating the presence of R1FasL monomers.

To confirm the proposed mechanism of action of the fusion proteins – that VEGF binds to the fusion protein homotrimers to induce formation of higher-order oligomeric complexes – we mixed R1FasL with VEGF and ran the sample on size-exclusion chromatography. As seen in Figure 5 (black chromatogram superimposed over the red chromatogram from Figure 4), high molecular weight complexes of ~600 kD were detected (fraction C). Immunoblot analysis confirmed that the high molecular weight complexes were composed of both R1FasL and VEGF (inset). This experiment confirms that VEGF binds to R1FasL to generate multimers of R1FasL homotrimers.

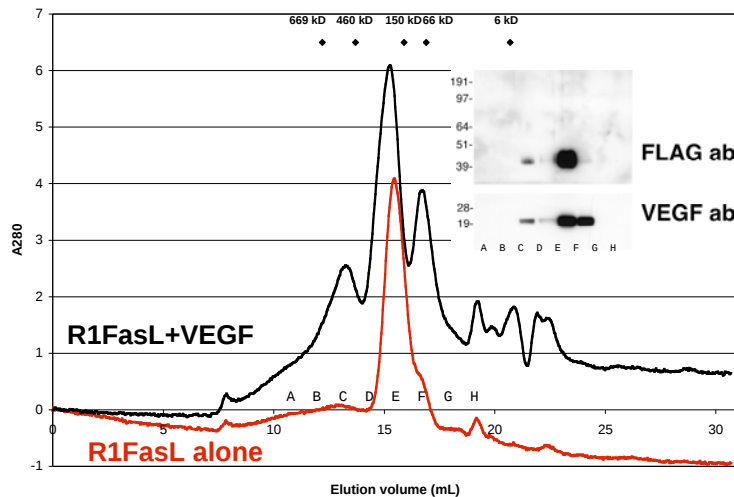


Figure 5. Size-exclusion chromatography of R1FasL plus VEGF complexes

R1FasL was combined with VEGF and run on a Superose-20 column. The indicated column fractions were immunoblotted under reduced and denatured conditions using anti-FLAG or anti-VEGF antibody, demonstrating the presence of oligomeric ~600 kD complexes of R1FasL plus VEGF.

We also confirmed that VEGF forms complexes with R1FasL using PAGE gel electrophoresis under native conditions. As seen in Figure 6, in the presence of VEGF, R1FasL shifted to form higher molecular weight oligomeric complexes.

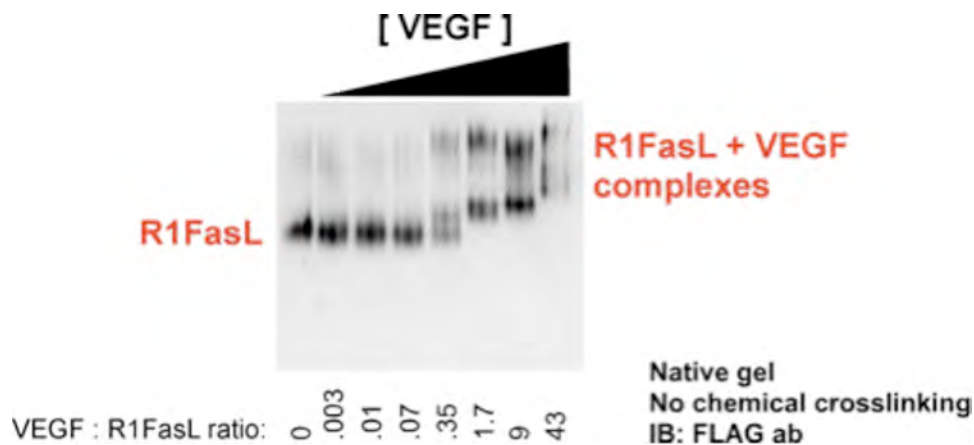


Figure 6. Native gel electrophoresis of R1FasL + VEGF complexes

R1FasL was mixed with increasing concentrations of VEGF and the samples run on native PAGE. Immunoblot using anti-FLAG antibody demonstrates formation of high molecular weight oligomeric complexes.

Before proceeding to test the fusion proteins on ovarian cancer cell lines we needed to confirm that they were active. For these experiments we used the Jurkat human T cell line. These cells express Fas and TRAILR death receptors but not VEGF, and so can be used as a testbed to demonstrate that the fusion proteins function as designed: they induce apoptosis only in the presence of VEGF. As seen in Figure 7, neither fusion protein induced Jurkat cell death in the absence of VEGF, but both fusion proteins induced substantial cell death when VEGF was added. This demonstrates that the fusion proteins effectively convert VEGF from a growth factor into a cell death factor.

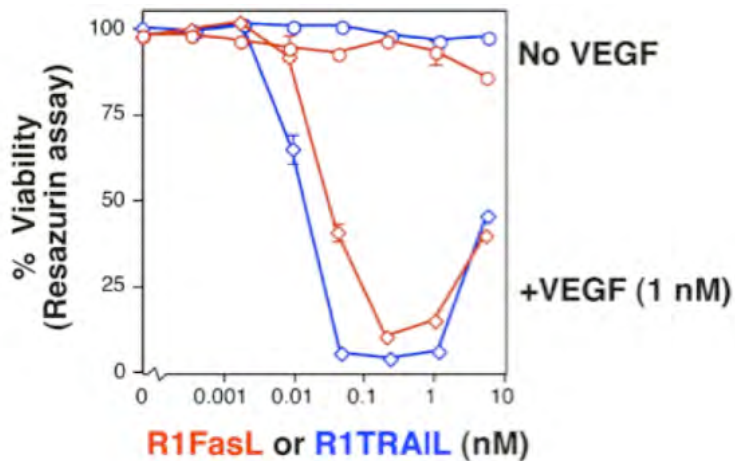


Figure 7. Fusion proteins kill Jurkat cells only when VEGF is present

Jurkat human T cells were plated in 96-well plates and treated with the indicated concentrations of R1FasL or R1TRAIL fusion proteins $\pm$ human VEGF (1nM). Cell viability was assayed 24 hours later using the resazurin/AlamarBlue assay.

Activation of Fas or TRAILR cell death receptors stimulates intracellular apoptosis signals that begin with caspase-8 (5). To demonstrate that the fusion proteins activate caspase-8, we compared the activity of the fusion proteins on Jurkat cells versus caspase-8-deficient Jurkat cells. As seen in Figure 8, caspase-8-deficient Jurkat cells were not killed by the fusion proteins plus VEGF, confirming as predicted that the fusion proteins require caspase-8 to induce apoptosis.

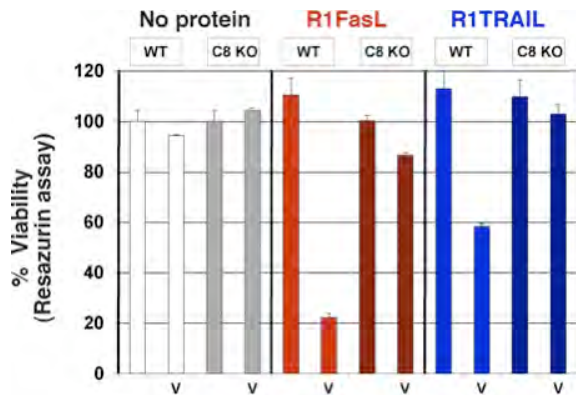


Figure 8. R1FasL and R1TRAIL require caspase-8 to induce apoptosis

Jurkat cells and caspase-8-deficient Jurkat cells were treated with R1FasL or R1TRAIL (1 nM) $\pm$ VEGF (1 nM). At 24 hours viability was assayed using the resazurin/AlamarBlue assay.

In other experiments we confirmed that the activity of the fusion proteins was blocked as predicted by neutralizing antibodies against the FasL or TRAIL domains, and by addition of the caspase inhibitor ZVAD.

Together these experiments complete Task 1. They demonstrate production, purification, quantification and characterization of the R1FasL and R1TRAIL fusion proteins.

The data shown above are the culmination of a process that required very extensive troubleshooting and optimization. The principal challenge was producing and purifying the fusion proteins as homotrimers without aggregation (i.e., without higher order aggregates of the fusion protein homotrimers). Aggregation of recombinant proteins

during production, purification or storage is a well-recognized problem (10). For almost all recombinant proteins, a small degree of aggregation is not a significant problem because the aggregated proteins are inactive (10). In contrast, the R1FasL and R1TRAIL fusion proteins are activated by aggregation, whether by VEGF (as designed) or as a consequence of production or purification. There would be no value in producing aggregated fusion proteins because they would induce apoptosis in many cells and could never be useful as a therapy. For example, FasL that was made in an aggregated form induced fulminant liver necrosis and death in mice, and aggregated TRAIL induced liver cell apoptosis in vitro (11, 12). Our goal was to produce homotrimeric fusion proteins that are specifically and only activated by VEGF.

Some of the problems we encountered and our solutions:

Cos cell production of fusion proteins: Cos cells produce high levels of recombinant protein over several days. We found that after 72 hours the recombinant fusion proteins often had small but unacceptable levels of aggregates that were constitutively active (i.e., they killed Jurkat cells in the absence of VEGF). Solution: use low levels of FBS (1-5%), decrease the amount of plasmid, and collect conditioned medium at 48 hours.

CHO cell production of fusion proteins: Fusion protein aggregates or fragments were present in conditioned medium. After ruling out that the problem was due to particular clones, we tried a variety of modifications to cell culture: low FBS, add ZnSO₄, add CuSO₄, add 2-ME, collect conditioned medium at 48 hours, lower cell density, or change basal medium. Solution: 1% FBS, 10 μ M ZnSO₄, 48 hour collection.

Elution of fusion proteins from FLAG affinity columns: Elution with glycine-HCl was efficient but inactivated the fusion protein. Solution: elute with excess FLAG peptide.

Removal of FLAG peptide after elution: Repeated centrifugal concentration and buffer replacement induced aggregation or fragmentation of R1TRAIL. An example of the problem is shown in Figure 9. R1TRAIL in the conditioned medium looked fine, but after purification the sample showed high molecular weight aggregates and 20 kD fragments requiring the purified prep to be discarded. Solution: add ZnSO₄ (50 μ M) to all buffers and avoid highly concentrating R1TRAIL.

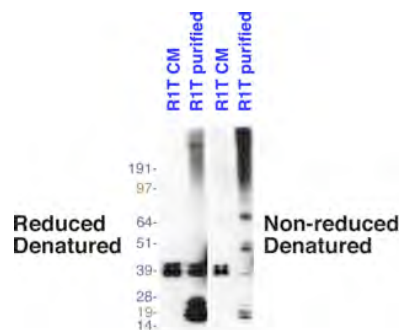
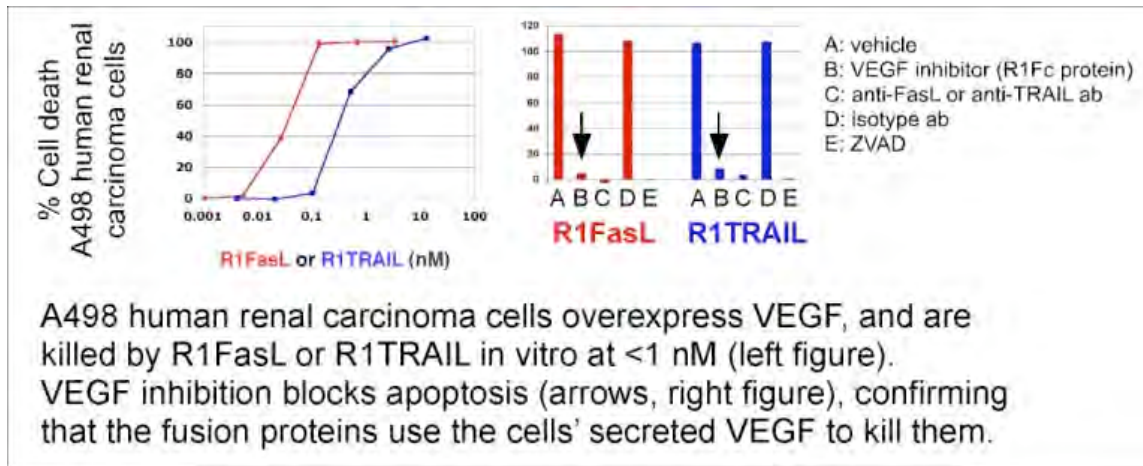


Figure 9. R1TRAIL aggregates and fragments in non-optimal purification

After optimization of multiple variables involved in producing R1FasL and R1TRAIL, the final purified fusion proteins functioned as designed. The fusion proteins were tested on A498 cells, a human renal cell carcinoma cell line that produces VEGF and express Fas and TRAILR. As seen in Figure 10, both R1FasL and R1TRAIL induced cell death in

these cells (left figure). Importantly, cell death was blocked when VEGF produced by the cells was neutralized (arrow in right figure), demonstrating that the fusion proteins are not constitutively active and that they use VEGF secreted by the cancer cell to induce cell death.

Figure 10. R1FasL and R1TRAIL induce apoptosis in renal cancer cells that is blocked when VEGF is neutralized.

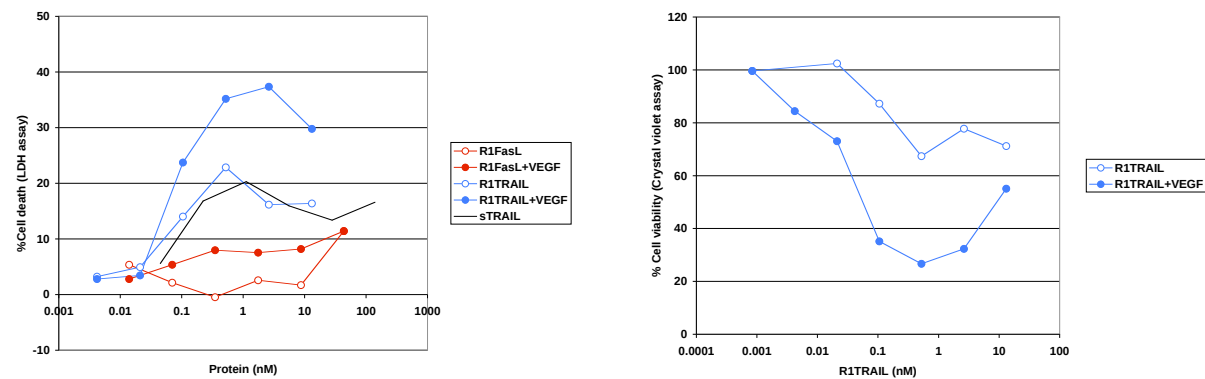


Task 2. Determine if R1FasL or R1TRAIL induce apoptosis in ovarian cancer cell lines in vitro

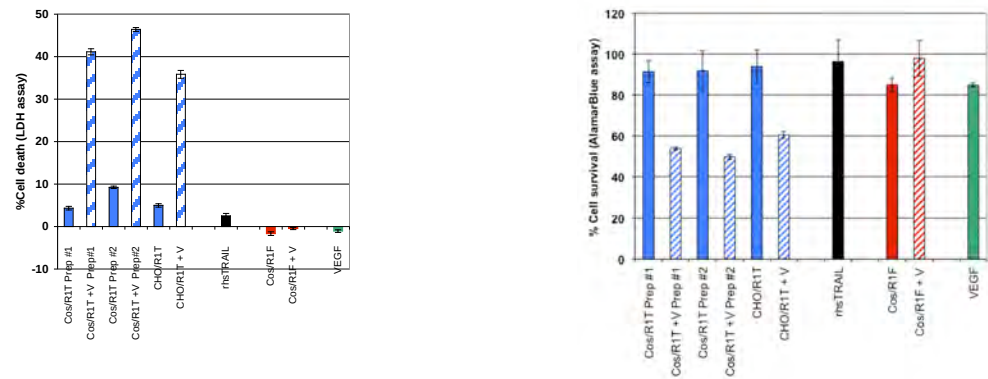
The R1FasL and R1TRAIL fusion proteins were tested on human ovarian cancer cell lines (SKOV-3, Caov-3, OVCAR-3, and OV-1063) to determine if they induce cell death. Methods: cells were plated in 96-well plates (~20,000 cells/well) in medium with 10% FBS and allowed to grow for 72-96 hours. During that time secreted VEGF was allowed to accumulate in the medium. At 72-96 hours purified R1FasL or R1TRAIL (0.01 to 10 nM final concentration) was added to the unchanged medium without VEGF or with human VEGF (1 nM) added. The rationale for adding exogenous VEGF is that in vitro culture conditions (4 mm column of medium above a monolayer of cells) dilute any VEGF secreted by the ovarian cancer cells. After 24 – 48 hours cell death was assayed using the LDH release assay and cell viability was assayed using the resazurin/AlamarBlue assay. Both are plate-read spectrophotometric assays.

SKOV-3 cells (Figures 11-14): R1FasL did not induce cell death with or without added VEGF. R1TRAIL alone weakly induced cell death, but strongly induced cell death when VEGF was added (40-50% cell death). Recombinant soluble TRAIL was equivalent to R1TRAIL, and less active than R1TRAIL+VEGF (Figure 11). R1TRAIL produced from Cos cells or CHO cells performed similarly.

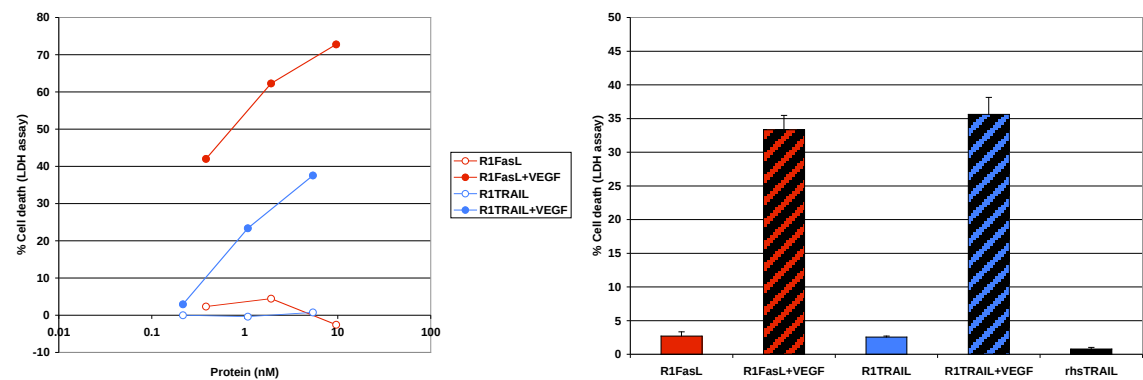
Figures 11-12:



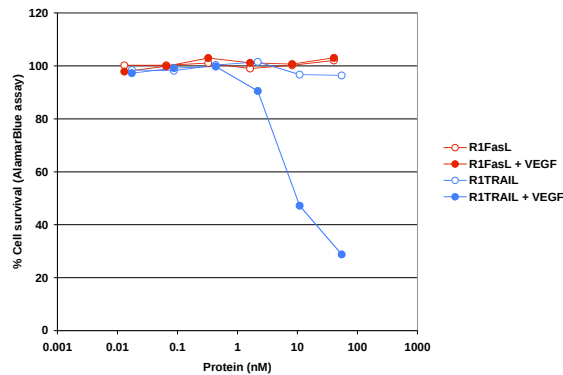
Figures 13-14:



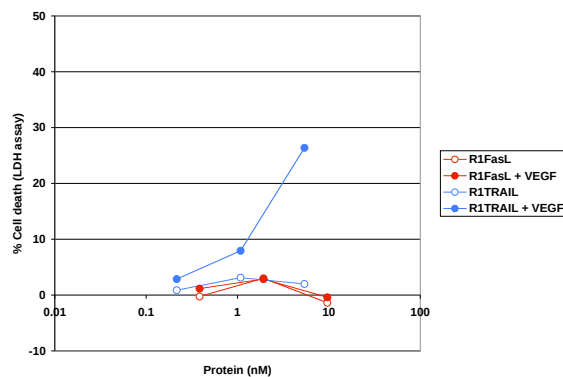
Caov-3 cells (Figures 15-16): R1FasL alone or R1TRAIL alone did not stimulate cell death, nor did recombinant TRAIL. However, cells were efficiently killed when VEGF was added.



OVCAR-3 cells (Figure 17): R1FasL did not stimulate cell death alone or with VEGF added. R1TRAIL strongly stimulated cell death only when VEGF was added. The Y-axis plots cell survival.



OV-1063 cells (Figure 18): R1FasL did not stimulate cell death alone or with VEGF added. R1TRAIL stimulated cell death only when VEGF was added.



Summary of cell responses to R1FasL or R1TRAIL

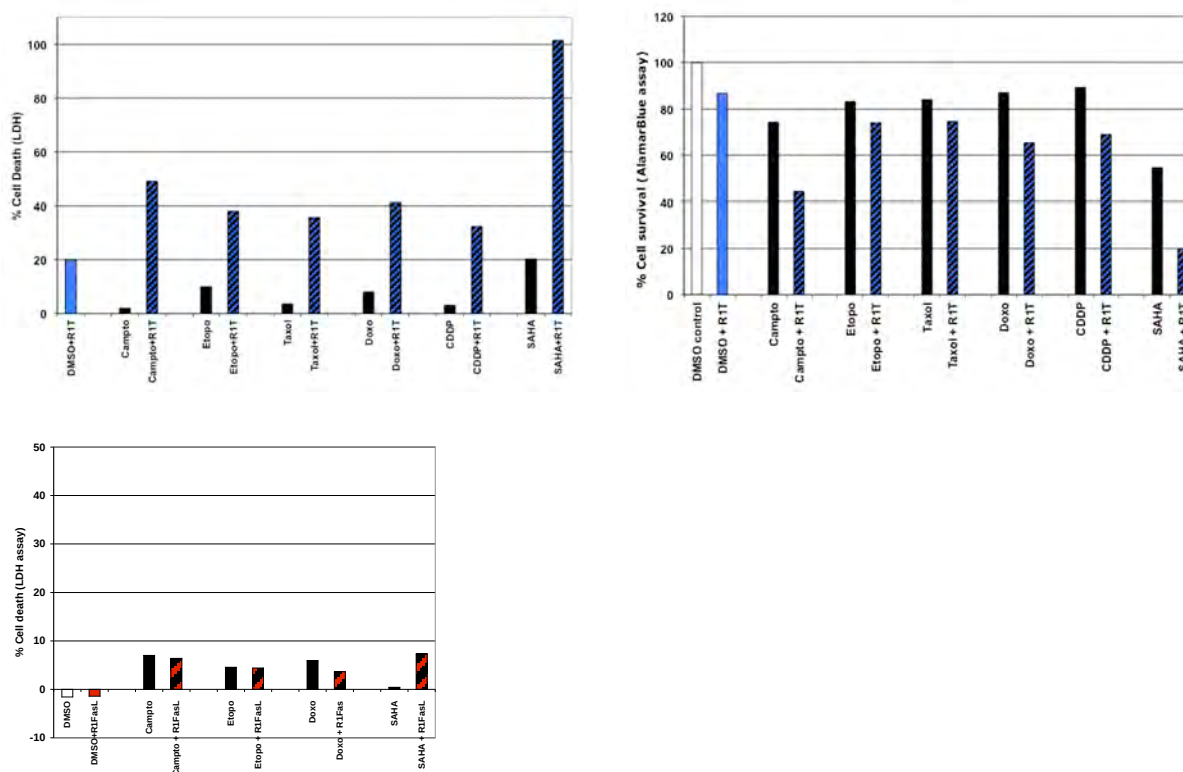
R1FasL alone or R1FasL+VEGF: None of the cell lines were killed by R1FasL treatment alone; one cell line (Caov-3) was significantly killed when exogenous VEGF was added. This indicates that Caov-3 cells have an intact Fas apoptosis signaling system but either do not express significant amounts of VEGF, or the VEGF they do express is diluted in the assay system below the threshold needed to activate R1FasL.

R1TRAIL alone or R1TRAIL+VEGF: SKOV-3 cells were weakly killed by R1TRAIL alone, and the other cells were not. All four cell lines were significantly killed when exogenous VEGF was added, indicating that they have an intact TRAILR apoptosis signaling system but either do not express significant amounts of VEGF, or the VEGF they do express is diluted in the assay system below the threshold needed to activate R1FasL.

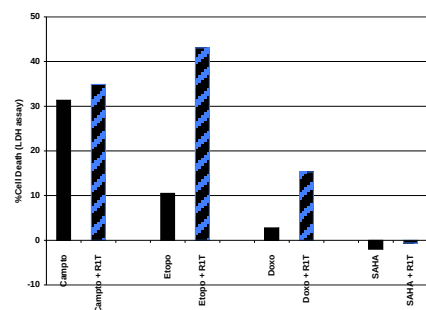
Task 3. Determine if chemotherapeutic agents or signal transduction inhibitors potentiate the apoptotic activity of R1FasL or R1TRAIL

Six chemotherapy agents were examined for evidence that they can potentiate the activity of R1FasL or R1TRAIL: camptothecin, etoposide, taxol, doxorubicin, cisplatin and SAHA. Methods: In preliminary experiments cells were treated with titrated concentrations of the chemotherapy agents alone to identify a concentration that was nontoxic or minimally toxic. For drug+fusion protein combination assays, cells were plated in 96-well plates and allowed to grow for 48 hours, then treated for 24 hours with one agent at the minimally toxic concentration, at which time R1FasL (1 nM) or R1TRAIL (1 nM) was added. After 24 hours cell death was assayed by LDH release or cell viability assayed by resazurin/AlamarBlue assay.

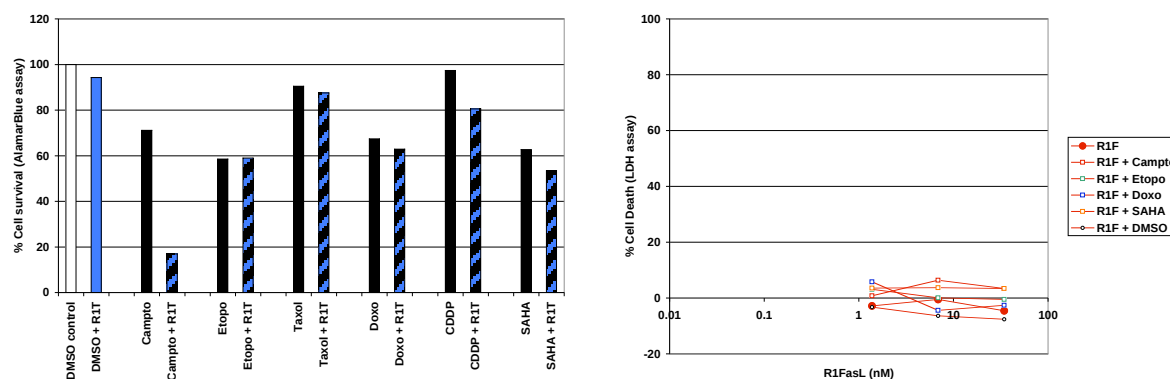
SKOV-3 cells (Figures 19-21): All six agents potentiated the activity of R1TRAIL, whether assayed by cell death (left) or cell survival (right). SAHA (an HDAC inhibitor) was particularly potent. In contrast, no agent potentiated the activity of R1FasL (bottom).



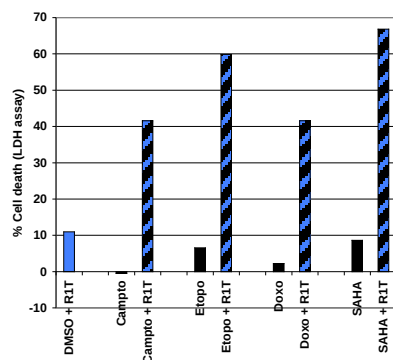
Caov-3 cells (Figure 22): Only etoposide (a topoisomerase inhibitor) potentiated the effect of R1TRAIL.



OVCAR-3 cells (Figures 23-24): Only camptothecin (a topoisomerase inhibitor) potentiated the effect of R1TRAIL (left). No agent potentiated the activity of R1FasL (right).



OV-1063 cells (Figure 25): Camptothecin, etoposide, doxorubicin and SAHA all strongly potentiated the activity of R1TRAIL.



Key research accomplishments

1. Generated purified homotrimeric R1FasL and R1TRAIL fusion proteins using Cos or CHO cell lines and FLAG-affinity chromatography.
2. Demonstrated mechanism of action of R1FasL and R1TRAIL fusion proteins: VEGF induces formation of oligomeric complexes that activate Fas or TRAILR death receptors.
3. Tested R1FasL and R1TRAIL on ovarian cancer cell lines.
4. Ovarian cancer cell lines were not killed by R1FasL and there was no effect of combined chemotherapy treatment.
5. R1TRAIL in the presence of VEGF induced cell death in ovarian cancer cell lines.
6. The activity of R1TRAIL was potentiated by combination with chemotherapy agents including: camptothecin, etoposide, doxorubicin and SAHA.

Reportable outcomes

1. An invited talk presenting this work was given by Dr. Quinn at the Rivkin Ovarian Cancer Research Symposium in Seattle in October 2010. Title: R1FasL and R1TRAIL: Recombinant fusion proteins that redirect VEGF to actively kill cancer cells.
2. The R1FasL and R1TRAIL fusion proteins have been shared with Dr. Mary Nakamura in the Immunology department at the San Francisco VA Medical Center for investigation in mouse models of inflammatory arthritis.
3. CHO cell lines producing R1FasL and R1TRAIL were established.
4. Grants applied for based on work supported by this Concept Award:
NIH R21 Exploratory/Developmental Research Grant 2008
Center for Therapeutic Innovation/UCSF-Pfizer Collaboration 2011

Conclusion

The first goal of this Concept award was to generate purified R1FasL and R1TRAIL fusion proteins. Accomplishing this goal was extremely challenging due to several factors: (1) the homotrimeric form of the fusion protein; (2) the relatively large size of the trimers, 160 kD; and (3) most significantly, the need to avoid any fusion protein aggregation during production and purification because aggregated trimers are constitutively apoptotic in the absence of VEGF. After extensive optimization, experimental conditions were found to produce purified, non-aggregated homotrimeric fusion proteins that induce apoptosis only in the presence of VEGF. The second goal of this project was to test the fusion proteins on human ovarian cancer cell lines. We found that R1TRAIL fusion protein induced apoptosis in ovarian cancer cell lines when VEGF was present, and the activity was potentiated by combining R1TRAIL with several chemotherapy agents. R1FasL did not induce apoptosis even in the presence of exogenous VEGF, demonstrating that these cells have defects in the Fas apoptotic pathway. Since the Fas and TRAILR apoptosis pathways share many intracellular signaling molecules, these results suggest that the defect in Fas signaling lies at the Fas receptor level. Our results suggest that further investigation of combined R1TRAIL treatment + chemotherapy is warranted in ovarian cancer.

Abbreviations

2-ME: 2-mercaptoethanol
BSA: bovine serum albumin
CHO: Chinese hamster ovary cells
CMV: cytomegalovirus
FBS: fetal bovine serum
FLAG: peptide sequence used as epitope tag
HDAC: histone deacetylase
kD: kilodalton
PAGE: polyacrylamide gel electrophoresis
R1FasL: our fusion protein combining VEGFR-1 plus FasL domains
R1TRAIL: our fusion protein combining VEGFR-1 plus TRAIL domains
TRAIL: TNF-related apoptosis-inducing ligand
VEGF: vascular endothelial growth factor
VEGFR-1: VEGF receptor 1
ZVAD: fluormethylketone-Val-Ala-Asp, a pan-caspase inhibitor

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Appendices

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