Therapeutic Discovery

Targeting TRAIL Death Receptor 4 with Trivalent DR4 Atrimer Complexes

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Abstract

TRAIL is a trimeric protein that potently induces apoptosis in cancer cells by binding to the trimeric death receptors (DR4 or DR5). Death receptors are attractive therapeutic targets through both the recombinant TRAIL ligand as well as receptor agonist monoclonal antibodies. Although efficacy of the ligand is hampered by its short half-life, agonistic antibodies have a much longer half-life and have shown some clinical efficacy as antitumor agents. However, the efficacy of these antibodies may be limited by their bivalent nature that does not optimally mimic the trimeric ligand. To overcome limitations of currently used death receptor-targeting agents, we engineered trimeric proteins called Atrimer complexes that selectively bind DR4 and potently induce apoptosis in a variety of cancer cells. Atrimer complexes are based on human tetranectin, a trimeric plasma protein of approximately 60 kDa. Loop regions within the tetranectin C-type lectin domains (CTLD) were randomized to create a large phage display library that was used to select DR4-binding complexes. A panel of unique and potent agonist DR4 Atrimer complexes with subnanomolar affinity to DR4 and no detectable binding to DR5 or the decoy receptors was identified. Mechanism of action studies with a selected Atrimer complex, 1G2, showed that Atrimer complexes induce caspase-dependent and DR4-specific apoptosis in cancer cells while sparing normal human fibroblasts and, importantly, hepatocytes. This proof-of-principle study supports the use of alternative proteins engineered to overcome limitations of therapeutically desirable molecules such as TRAIL. Mol Cancer Ther; 11(10); 2087–95. ©2012 AACR.

Introduction

The death receptor pathway is engaged by TNF members to trigger apoptosis through the intrinsic or extrinsic death pathway (1). TRAIL is a member of the TNF family that engages death receptors on the surface of tumor cells to induce apoptosis but generally exerts no appreciable cytotoxicity on normal cells unlike other family members such as FasL. The apparently favorable therapeutic index has led to clinical trials of the recombinant TRAIL protein as an antitumor agent. In most physiologic environments, TRAIL binds to proapoptotic death receptors, DR4 or DR5, or decoy receptors, DcR1 or DcR2. Proapoptotic receptors contain intracellular death domains whereas the decoy receptors have truncated intracellular regions that cannot induce cell death signaling. TRAIL and its bound receptor form a homotrimeric binary complex that results in the colocalization of the receptors’ intracellular death domains. This colocalization recruits pro-caspase-8 and Fas-associated death domain to form the death-inducing signaling complex (DISC). DISC formation results in the autocatalytic cleavage and thereby activation of the initiator caspase-8, which ultimately activates effector caspses directly through the extrinsic death pathway or indirectly through the intrinsic death pathway depending on the cell type.

Clinical trials with recombinant TRAIL as an antitumor agent have shown limited success given its promising preclinical activity (2). Despite a favorable toxicity profile, TRAIL has been reported to induce cell death in human hepatocytes, which has been linked to DR5, though this is dependent on TRAIL preparation (3–6). Another potential limitation of recombinant TRAIL is its short half-life (7) that has led to the development of proapoptotic agonist monoclonal antibodies that target either DR4 or DR5 and have longer half-lives than recombinant TRAIL. These antibodies are being explored in clinical trials but it remains unclear whether bivalent binding may hinder efficacy.

To overcome potential limitations of current TRAIL-based therapeutics, we engineered human tetranectin to specifically target DR4. Tetranectin is an abundant...
[10 mg/L in serum (8)] and highly stable 60 kDa non-covalently linked homo-trimeric protein found in human serum and tissue. Tetranectin belongs to the family of C-type lectins and binds through its loop regions to plasminogen (9) and fibrin (10). Knockout mouse studies have implicated tetranectin in developmental tissue remodeling (11) and wound repair (12). Each 20 KDa subunit of the tetranectin homo-trimer is composed, starting at the N-terminus, of a short lysine rich domain, a coil-coil trimerization domain, a hinge region, and a C-type lectin-binding domain (CTLD). The CTLD contains 5 flexible loops that can tolerate considerable amino acid diversity without disruption of the overall CTLD structure (13). One or more of these loops can be reprogrammed to generate CTLDs that bind strongly and specifically to any protein target of interest, replacing tetranectin’s specificity for its natural ligands. Moreover, trimerization of engineered CTLDs has been shown to boost affinity up to 100-fold (13), presumably through an avidity effect. Combined with its fully human origin and high stability it is highly desirable platform for engineering serum-stable therapeutic proteins, which we term Atrimer complexes. Here, we show that tetranectin is a robust platform for the discovery of a new class of protein therapeutics and provide proof-of-principle evidence in the form of a panel of potent and specific DR4 agonists.

Materials and Methods

Phage library

A library with modifications in loops 1 and 4 of human tetranectin was first used to select DR4-specific clones. For the construction of the Loop 1 to 4 library (human 1–4 library), the coding sequences for Loop 1 in human tetranectin were modified so that the 7 amino acids DMAAEGT were substituted with 7 randomized amino acids. The coding sequences for Loop 4 were modified and extended so that the 2 amino acids KT of Loop 4 were replaced with 5 random amino acids. The human 1 to 4 library was cloned into a phage display vector that expresses the modified CTLDs on the phage surface as a phage gene III fusion protein. A library size of 2 × 10^9 was obtained. Phages generated from the human 1 to 4 library were selected for specific binding on recombinant DR4-Fc (R&D Systems) by solid phase panning according to standard protocols (14).

Affinity maturation

Libraries were constructed from 2 DR4 agonist clones from primary selections through the randomization of amino acids in Loop 3 of the CTLD. For the construction of these libraries, 6 of the amino acid positions in Loop 3 (ETEITA) were replaced with random amino acids. A library size of 2.65 × 10^9 was obtained for the 1B3 affinity maturation library. Affinity maturation was done on biotinylated DR4-Fc bound to streptavidin beads. Five rounds of selection were conducted in which the amount of target was successively decreased.

DR4 Atrimer complex expression and purification

DR4 Atrimer complexes were either expressed in E. coli or 293 cells. For E. coli, a pBAD-derived vector with a Strept tag was used. DR4 Atrimer complexes were extracted from periplasm by gentle sonication, and then purified by Strept-Tactin affinity beads (Qiagen) following the manufacturer’s instructions. DR4 Atrimer complexes were produced in suspension 293 cells by transient transfection with 293Fectin (Invitrogen). Supernatant was harvested at 6 to 7 days after transfection, and DR4 Atrimer complexes were purified with an affinity column using an anti-human tetranectin trimer domain monoclonal antibody.

Purified DR4 Atrimer complexes were dialyzed into TBSC buffer (150 mmol/L NaCl, 100 mmol/L Tris, 2 mmol/L CaCl_2, pH 8.5). Protein concentrations were calculated by OD280 absorbance and extinction coefficient. Endotoxin levels were less than 5 EU/mg protein as determined by the Limulus amebocyte lysate method. The relative affinities of the DR4 Atrimer complexes were determined on a Biacore 3000 (GE Healthcare) using recombinant DR4F/Fc (R&D Systems) captured by anti-human IgG (GE Healthcare) on a CMS chip. Mouse anti-human IgG was immobilized onto CMS sensor chips using standard amine coupling chemistry and for each binding cycle, receptor/Fc fusion protein was captured to one flow cell at a low density while retaining an anti-human IgG flow cell blank. Each Atrimer complex sample was injected at 30 μL/min flow rate over the DR4/Fc surface in a concentration series ranging from 1 to 250 nmol/L. The association was measured for 3 minutes, followed by dissociation in buffer for 5 minutes. Sosorgrams obtained were double referenced and analyzed with Biareview software using a 1:1 Langmuir-binding model.

Cell culture and reagents

All cell lines were obtained from American Type Culture Collection (ATCC) except for HCT116 Bax/- and HCT116 p53/- cells, which were a kind gift of Dr. B. Vogelstein (Howard Hughes Medical Institute, Chevy Chase, MD). Cell lines used in this study were not authenticated. Hepatocytes were obtained from Triangle Research Laboratories. Cells were cultured in ATCC-recommended media and maintained in a humidified incubator at 5% CO_2 and 37°C. Z-DEVD, Z-IETD, AEVD-FMK, and ZVAD-FMK were obtained from R&D Systems. For all sub-G1 DNA content analysis and cell viability assays except with hepatocytes, recombinant his-tagged TRAIL was produced as previously described (15). For the cell viability assay with hepatocytes, recombinant TRAIL was obtained from R&D Systems.

Cell viability assays

Cell killing was measured on ST486 (Burkitt’s lymphoma), Colo205 (colon carcinoma) cell lines using the ViaLight Plus kit (Lanza). Briefly 1–5 × 10^4 cells per well were incubated with the purified DR4 Atrimer complexes or TRAIL-his (R&D Systems). After 24 hours, cell viability was determined by measuring ATP levels and detected on...
a Glomax luminometer (Promega). Data were expressed as percent cell death relative to the respective buffer control. EC_{50} values were calculated using nonlinear regression curve fitting (GraphPad Prism). For DLD-1, HCT116, HT-29, and HCT15 cell lines cells were seeded into 96-well black-walled plates (Nunc) at a concentration of 1 × 10^5 cells per mL in fresh media and a volume of 100 μL per well. Cells were allowed to adhere for a minimum of 12 hours and then treated with indicated concentrations of Atrimer complexes. CellTiter-Glo (Promega) assays were carried out according to the manufacturer’s protocol 48 hours posttreatment and bioluminescent imaging was recorded on an IVIS imaging system (Xenogen).

**Flow cytometry**

For sub-G, DNA content analysis adherent and floating cells were harvested after trypsinization at indicated time points and fixed in 80% ethanol at 4°C. Cells were then stained with propidium iodide in the presence of RNase and analyzed on an Epics Elite flow cytometer (Beckman Coulter). For death receptor expression, cells were harvested by brief trypsinization and fixed in 4% paraformaldehyde to avoid receptor internalization. Cells were then incubated with primary antibodies at 1:250 (DR5, Imgenex Img-120A; DR4, Imgenex Img-141) for 1 hour, washed in PBS, incubated with Alexafluor 488 as a secondary antibody, and resuspended in PBS for analysis.

**Western blot analysis**

Cells were grown in 6-well plates and treated in log-phase as indicated. At each endpoint, cells were harvested by cell scraping, centrifugation, and lysed on ice for 2 hours with cell lysis buffer. The supernatant was collected following centrifugation and its protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories). Samples were electrophoresed under reducing conditions on NuPAGE 4% to 12% Bis-Tris gels (Invitrogen), transferred to polyvinylidene difluoride membranes, and blocked in 10% nonfat milk in TBST for 1 hour. Membranes were then incubated with incubated with primary antibodies obtained from Cell Signaling (DR4, Santa Cruz sc-7863; DR5, Cell Signaling 3969; DcR1, BD 550622; DcR2, Imgenex IMG-121-1) at 1:1,000 in TBST overnight at 4°C. Membranes were washed in TBST, incubated with the appropriate HRP-conjugated secondary antibody (Thermo-Scientific) for 1 hour, washed in TBST, and visualized using ECL-Plus (Amersham) and X-Ray film (Thermo-Scientific).

**Results**

**Generation of DR4-agonist Atrimer complexes**

To obtain agonistic Atrimer complexes against DR4, we built a phage display library in which 12 amino acids within the loop regions of the tetranectin CTLD were randomized. As depicted in Fig. 1, we altered, by overlap PCR, 7 positions in Loop-1 and 5 positions in Loop-4 (inserting 3 extra residues) and obtained a library of more than 10^9 clones. We then used standard phage display techniques to select clones from this primary library with the ability to specifically bind DR4. Late rounds of selection were screened by phage ELISA and 16 unique DR4-binding CTLD sequences were identified (Supplementary Table S1). These CTLDs were then reformatted into full-length trimeric Atrimer complexes, purified and tested for affinity matured DR4 agonists are highly specific for human DR4. Binding of purified DR4 Atrimer complexes (10 ng/well) is shown to recombinant human DR4-Fc, IgG-Fc, DR5-Fc, DcR1-Fc, DcR2-Fc, murine DR-Fc, and mock-coated (PBS) wells.
agonist activity against a panel of cell lines. 13 of the Atrimer complexes showed varying degrees of agonist activity against DR4 bearing cells, but not against DR4 negative cell lines (data not shown). We selected the most potent of these, clone 1B3, to carry into an affinity maturation selection with the intent of improving both affinity and activity.

To arrive at more potent DR4 agonist Atrimer complexes, we randomized 6 positions of Loop 3 of the 1B3 clone (Supplementary Fig. S1) and generated a large phage display library. This library was panned under stringent conditions and target antigen was limited in a stepwise fashion to select for Atrimer complexes with improved binding characteristics. To identify binders with enhanced cytotoxic activity we built a single point killing assay into the screening strategy. Briefly, late round phage pools were reformatted en masse into full length Atrimer complexes, expressed as individual clones in the 96-well format, and then screened in parallel for DR4 binding and cytotoxic activity. Previously identified agonist Atrimer complexes, of known activity, affinity, and expression level, were included as comparators. In this way, we were able to get a first read on multiple true positive probability parameters.

Clones that performed better than previously isolated candidates in both assays (such as 1C9 in Supplementary Fig. S2) were selected for further characterization. This strategy led to the identification of several unique Atrimer complexes (Supplementary Table S1) that showed significantly improved affinity and cytotoxic activity as compared with the 1B3 parental molecule (Table 1). As measured by Biacore the apparent affinity was improved up to 12-fold in the affinity-matured Atrimer complexes. Cytotoxic activity was improved up to 160-fold against ST486 cells and up to 4,000-fold against the TRAIL-resistant Colo205 cells (Table 1). These improvements brought the efficacy of the DR4 Atrimer complexes into a range comparable with, or better than, recombinant TRAIL as well as such reported values for mapatumumab (Ref. 16–17; Table 1).

Importantly the affinity matured Atrimer complexes were highly specific to DR4 and showed no binding to the homologous DR5, DcR1, DcR2 or the murine death receptor (mDR), as measured by ELISA (Fig. 1).

**DR4 Atrimer complexes are cytotoxic to TRAIL-sensitive cancer cells but not normal human fibroblasts and hepatocytes**

To select the best DR4 Atrimer complexes, we directly compared the cytotoxic activity of the candidate DR4 Atrimer complexes. We found that the candidate DR4 Atrimer complexes led to decreased cell viability of TRAIL-sensitive colorectal cancer cell lines DLD-1, HT-29, HCT116, and HCT15 (Fig. 2) but not TRAIL-resistant cancer cell lines (Supplementary Fig. S3). Although all DR4 Atrimer complexes had cytotoxic activity at the tested concentrations, 1G2 and 1C9 consistently outperformed the other candidate DR4 Atrimer complexes at least in terms of decreasing cell viability at the tested concentrations and time points.

A key motivation for engineering DR4 Atrimer complexes was to generate therapeutic molecules that induce apoptosis in cancer but not normal cells, particularly hepatocytes as has been reported with some versions of TRAIL. Neither recombinant TRAIL nor DR4 Atrimer complexes decreased the cell viability of normal fibroblasts with a 2-day incubation at the same doses where activity was observed against tumor cells (Fig. 3A). Importantly, DR4 Atrimer complexes did not exhibit toxicity

<table>
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<th>Atrimer</th>
<th>Affinity (KD) nmol/L</th>
<th>EC₅₀ Colo205 nmol/L</th>
<th>EC₅₀ ST486 nmol/L</th>
<th>EC₅₀ Colo205/EC₅₀ ST486 ratio</th>
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<td>483</td>
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<td>0.03</td>
<td>6.0</td>
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<td>0.200</td>
<td>0.06</td>
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NOTE: The relative affinities of the DR4 Atrimer complexes were measured by Biacore using recombinant DR4/Fc captured by anti-human IgG on a CM5 chip. Atrimer complex binding was evaluated at 1 to 250 nmol/L. Cell viability was determined by measuring ATP levels after 24 hours and EC₅₀ values were calculated using nonlinear regression curve fitting (GraphPad Prism). Mean of 2 to 4 independent experiments is shown.
with human hepatocytes as opposed to TRAIL (Fig. 3B). The lack of normal cell toxicity associated with Atrimer complexes is likely due to lack of expression of DR4 that has characterized for human hepatocytes (18). Based on this cytotoxicity profile, we selected 1G2 and 1C9 for further characterization.

**DR4 Atrimer complexes induce DR4-mediated caspase-dependent apoptosis**

We previously reported that cancer cells induce apoptosis in response to TRAIL by the extrinsic or intrinsic cell death pathway depending on whether the cells are type-I or -II, respectively (19). We also previously found that HCT116 are type-II cells and that TRAIL-induced apoptosis in such cells relies heavily on Bax, a proapoptotic Bcl-2 family that mediates mitochondrial permeabilization and therefore the intrinsic cell death pathway (20). In accordance with these findings, we found that DR4 Atrimer complexes induce sub-G1 DNA content in HCT116 cells in a Bax-dependent and p53-independent manner (Fig. 3C).

Dose-dependence studies of lead Atrimer complexes and TRAIL found that 1G2 induced higher levels of sub-G1 DNA content relative to equivalent amounts of TRAIL and 1C9 (Fig. 4A). To confirm that cancer cells were undergoing apoptosis in response to the DR4 Atrimer complexes, we explored the involvement of caspases. The selected DR4 Atrimer complexes cause activation of caspase-8 and cleavage of poly ADP-ribose polymerase (PARP) that is a hallmark of apoptosis (Fig. 4B). Furthermore, we found that sub-G1 DNA content induced by DR4 Atrimer complexes could be completely ablated by coincubation with the pan-caspase inhibitor zVAD-fmk (Fig. 4C). We also found that the apoptotic activity of 1G2 was significantly blocked by coincubation with peptides that specifically inhibit the caspase-8 or caspase-3, one of the effector caspases (Fig. 4D). Inhibiting capase-10, which can also play an initiator role in inducing apoptosis, hindered activity but to a lesser extent than observed with inhibitors of capsases-8 and -3.

We found that the selected DR4 Atrimer complexes had similar apoptotic activity to that of TRAIL in HCT15

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![Figure 2](https://example.com/figure2.png)

**Figure 2.** Cytotoxic comparisons of candidate DR4 Atrimer complexes. Cell viability with candidate DR4 Atrimer complex treatment for 48 hours in DLD-1 (A), HCT116 (B), HT-29 (C), and HCT15 (D; n = 2). Exemplary raw data are shown for DLD-1 and HT-29 to the right. Error bars are excluded for visual clarity but are <10% in all cases.
but not SW620 colon cancer cell lines (Fig. 5A and B). Western blot analysis revealed that SW620 had much lower expression of DR4 relative to HCT15 whereas DR5 levels were comparable (Supplementary Fig. S4). In accordance with this observation, we found a significant correlation ($R^2 = 0.91$) between the EC$_{50}$ of 1G2 and surface expression of DR4 (Fig. 5C). To directly test the requirement of DR4 for Atrimer complex-induced apoptosis, we knocked down DR4 in HCT116 cells. Knockdown of DR4 significantly inhibited the ability of DR4 Atrimer complex to induce sub-G$_1$ DNA content in these cells (Fig. 5D; Supplementary Fig. S5). In addition, preincubation of Atrimer complexes with an excess of soluble DR4, but not DR5, significantly blocked the cytotoxic effect of DR4 Atrimer complexes (Supplementary Fig. S6). Combining 1G2 with TRAIL did not increase sub-G$_1$ content in HCT116 cells compared with TRAIL alone (Supplementary Fig. S7). This suggests that TRAIL saturated DR4 binding under these experimental conditions and that perhaps 1G2 and TRAIL compete for similar binding sites on DR4. Together, these results observed in tumor cell culture support the specificity of the lead Atrimer complexes for DR4 to potentiate cell death.

Discussion

We provide the first report of human tetranectin-based Atrimer complexes engineered to specifically targeted DR4, and potently induce cell death in cancer cell lines. Atrimer complexes such as 1G2 induce apoptosis in TRAIL-sensitive human tumor cells that express DR4 and furthermore show an activity profile that correlates with surface DR4 levels. It should be noted that sensitivity of cancer cells to TRAIL-mediated apoptosis is determined by multiple factors and that resistance mechanisms often involve intracellular regulators of apoptosis such as Mcl-1 or cFLIP. Our lab and others have reported on synergistic combinations of TRAIL and chemotherapy or targeted agents to overcome such resistance mechanisms (21–23). Such combinations should be explored with DR4 Atrimer complexes to overcome potential resistance mechanisms and expand their activity profile.
The extent and context of TRAIL-induced hepatotoxicity has been a subject of debate for some time. It has been shown directly that the particular preparation of TRAIL plays a clear role (24). We found that DR4 Atrimer complexes are not hepatotoxic but a commercially available version of recombinant TRAIL seems to be. Although 1G2 seemed to induce higher levels of cell death than TRAIL in some cell lines at equal concentrations this is not necessarily an appropriate comparison. Direct comparisons of DR4 Atrimer complexes and TRAIL or TRAIL-agonist antibodies are limited by several factors. One barrier is that DR4 Atrimer complexes cannot be compared with other TRAIL-agonist antibodies being explored in the clinic due to proprietary restraints. One of the theoretical advantages of DR4 Atrimer complexes over TRAIL and agonistic antibodies is that it does not require trimeric assembly, which is present in the receptor-bound complex. The formation of trimers from monomers is likely to afford an entropic cost, thereby decreasing the affinity of the ligand to complex with its receptor. Although the DR4 Atrimer complexes could alleviate this entropic cost, this is not easily tested, as this requires the ability to measure binding energies between TRAIL-receptor-bound complexes. Combining DR4 Atrimer complexes with TRAIL suggests potentially mutual-binding sites on DR4 that should be examined in future studies.

Ultimately, TRAIL-based therapeutics should be compared based on their biodistribution, affinity, toxicity, and cytotoxic properties as a monoagent and in combination with approved therapies to determine the superior candidates for different clinical situations. Despite practical limitations that hamper such comparisons, DR4 Atrimer complexes could continue to be developed as a first-in-class antitumor agent due to their potent activity and lack of toxicity, at least in cell culture. Exploring the efficacy and toxicity profile of lead DR4 Atrimer complexes in preclinical in vivo models is a clear future direction for the development of these therapeutics. These finding serve as a proof-of-principle to support the use of alternative proteins engineered to overcome limitations of therapeutically desirable molecules such as TRAIL.

Figure 4. DR4 Atrimer complexes induce caspase-dependent apoptosis. A, sub-G₁ DNA content analysis of HCT116 p53⁻/⁻ cells treated with 1G₂, 1C₉, or TRAIL at indicated doses for 48 hours (n = 2). B, Western blot analysis of caspase-8 and PARP processing following a 48 hours incubation of 1G₂, 1C₉, or TRAIL. C, sub-G₁ content induced in HCT116 cells preincubated with or without zVAD-FMK followed by treatment with 1G₂ or 1C₉ for 48 hours (n = 2). D, a similar experiment was carried out but preincubated with zVAD-FMK, Z-DEVD, Z-IETD, or AEVD-FMK (n = 2).
Disclosure of Potential Conflicts of Interest

Authors' Contributions
Conception and design: R. Ferrini, B. Lin, A. Kretz-Rommel, W.S. El-Deiry
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.E. Allen, R. Ferrini, D.T. Dicker, G. Batzer, E. Chen, D.I. Oltean, M.W. Renshaw
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.E. Allen, R. Ferrini, E. Chen, D.I. Oltean, M.W. Renshaw
Writing, review, and/or revision of the manuscript: J.E. Allen, R. Ferrini, D.T. Dicker, D.I. Oltean, B. Lin, M.W. Renshaw, A. Kretz-Rommel, W.S. El-Deiry

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Conflict of Interest Statement

References

Figure 5. DR4 Atrimer complexes induce DR4-mediated apoptosis. Sub-G1 analysis of HCT15 (A) and SW620 (B) cells incubated with 1G2, 1G2, or TRAIL at indicated concentrations for 48 hours (n = 2). C, correlation of EC50 and relative surface DR4 expression for DLD-1, HCT116 WT, HCT116 p53-/-, HCT15, and SW480 determined by flow cytometry. D, sub-G1 content induced by 1G2 at 250 ng/mL in HCT116 knocked down DR4 or a scramble control siRNA following 48 hours incubation (n = 3). Confirmation of knockdown by Western blot analysis is shown on the right.
Molecular Cancer Therapeutics

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