Genomically informed small-molecule drugs overcome resistance to a sustained-release formulation of an engineered death receptor agonist in patient-derived tumor models

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Extrinsic pathway agonists have failed repeatedly in the clinic for three core reasons: Inefficient ligand-induced receptor multimerization, poor pharmacokinetic properties, and tumor intrinsic resistance. Here, we address these factors by (i) using a highly potent death receptor agonist (DRA), (ii) developing an injectable depot for sustained DRA delivery, and (iii) leveraging a CRISPR-Cas9 knockout screen in DRA-resistant colorectal cancer (CRC) cells to identify functional drivers of resistance. Pharmacological blockade of XIAP and BCL-XL by targeted small-molecule drugs strongly enhanced the antitumor activity of DRA in CRC cell lines. Recombinant fusion of the DRA to a thermally responsive elastin-like polypeptide (ELP) creates a gel-like depot upon subcutaneous injection that abolishes tumors in DRA-sensitive Colo205 mouse xenografts. Combination of ELPdepot-DRA with BCL-XL and/or XIAP inhibitors led to tumor growth inhibition and extended survival in DRA-resistant patient-derived xenografts. This strategy provides a precision medicine approach to overcome similar challenges with other protein-based cancer therapies.

INTRODUCTION

Over 20 years ago, it was found that TNF (tumor necrosis factor)–related apoptosis-inducing ligand (TRAIL; also Apo2L) kills many cancer cells in vitro and in vivo while remaining innocuous to normal cells (1). Upon binding to death receptor 4 (DR4) or DR5, TRAIL activates the extrinsic death pathway and induces programmed cell death (apoptosis) in a range of human cancer cell lines, including breast, lung, colon, melanoma, lymphoma, pancreatic, and prostate (2). Over the past two decades, TRAIL and other promising death receptor agonists (DRAs) such as monoclonal antibodies (mAbs) have been developed and tested in the clinic, but none of these drugs showed robust therapeutic efficacy in patients (2, 3).

From the substantial efforts expended to understand this outcome, a number of important interrelated conclusions have emerged. Clinical translation of TRAIL, the natural ligand for DR5, was confounded by its short half-life, inadequate delivery to cancer cells, and the presence of TRAIL-resistant cancer cell subpopulations (3). TRAIL forms a homotrimer, and while trimerization of DR5 by TRAIL is adequate for signaling induction, higher-order cross-linking of DR5 is required to trigger optimal signaling (4). Efforts to address TRAIL’s pharmacokinetic and receptor specificity issues focused on the development of high-affinity mAbs. Despite the pharmacokinetic advantages of DR5-specific antibodies, it is thought that the bivalency of these drugs resulted in subpar potency, likely due to their inability to trimerize the death receptors for initiation of apoptotic signaling (5). The DR5-specific antibodies have little to no proapoptotic activity unless they are cross-linked by their Fc domains upon binding to FcRns (6). This process is inefficient, and in the context of DRAs, the bivalent structure of antibodies hinders maximization of proapoptotic activity, as death receptor activation requires, at a minimum, trimerization of the receptor. It has been suggested by Ashkenazi that the failure of these agonists may be overcome through the development of multimeric DR5 agonists with augmented innate potency (7). The recent development of highly potent, multivalent DR5 agonists has addressed this issue, but these agonists still suffer from short half-life, inadequate delivery methods, and intrinsic DRA resistance (4). The mechanistic basis for intrinsic resistance to TRAIL and small-molecule TRAIL inducers have been examined, with studies attributing resistance to diverse signaling mechanisms and identifying combination therapies with the potential to overcome resistance, including those that target nodes within the intrinsic apoptotic pathway (7–9). Despite these studies, no viable therapies that combine TRAIL or related agonists with other drugs to overcome intrinsic resistance have emerged.

This study is motivated by the recognition that DR agonists are attractive as anticancer drugs if their three core limitations—inefficient ligand-induced receptor multimerization, poor pharmacokinetic properties, and tumor intrinsic resistance—can be simultaneously addressed. Here, we systematically address these challenges by engineering a sustained-release formulation of a highly potent, hexavalent DR5 agonist (DRA) and administering the agonist as a sustained-release depot, in combination with targeted drugs, rationally nominated by a CRISPR-Cas9 loss-of-function (LOF) genetic knockout screen, that overcome intrinsic resistance to DR agonists. By combining long-term sustained release of a subcutaneously injected DRA with targeted inhibition of the prosurvival proteins BCL-XL and X-linked inhibitor of apoptosis protein (XIAP), we demonstrate the ability to overcome...
intrinsic resistance to extrinsic pathway activation, suppressing tumor growth and extending survival in patient-derived colorectal cancer (CRC) models.

**RESULTS**

**DRAs induce apoptosis in human CRC cell lines**

We selected a hexavalent DRA protein to maximize receptor engagement and potency. The DRA is a hexameric protein composed of oligomers of the 3rd fibronectin type III domain of tenascin (Tn3) that is engineered to bind DR5, linked by flexible glycine-serine linkers (G₄S)₃ and expressed recombinantly in *Escherichia coli* (10). The DRA induces apoptosis in tumor cells upon binding to its target, DR5. Monomer and dimer oligomers of the Tn3 domain do not induce apoptosis, but the hexavalent DRA construct is extremely cytotoxic in sensitive cell lines, likely due to the enhanced signaling strength owed to higher-order receptor cross-linking (Fig. 1A) (10). A clinically relevant target receptor on the surface of cancer cells, DR5, is highly up-regulated in stage II and III CRC, but without prognostic implications (11, 12). Although human CRC cells express detectable levels of DR5, expression levels do not correlate with sensitivity to DR5 agonists (12, 13).

After recombinant expression of the DRA in *E. coli*, we purified the DRA and characterized its purity by SDS–polyacrylamide gel electrophoresis (PAGE). Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) confirmed that its size is within 0.08% of its theoretical mass (fig. S1). Activity of TRAIL produced in-house was confirmed prior to use for comparative studies (fig. S2A). We next evaluated its potency by cytotoxicity assays in a panel of human CRC cell lines with a range of TRAIL sensitivities (Fig. 1, B to E, and fig. S2, B to J) and a panel of low-passage-number patient-derived cell lines (fig. S2, K to M) (13). To confirm that the DRA induces TRAIL receptor-mediated apoptosis, we showed that pharmacological inhibition of caspase activity with the pan-caspase inhibitor Q-VD-OPh prevents DRA activity in DRA-sensitive human cancer cells (fig. S2N). The DRA is highly potent, as it exhibits EC₅₀ (half-maximal effective concentration) values in the picomolar range in all tested TRAIL-sensitive cell lines (fig. S2) and, in the best case, is over 1000 times more potent than TRAIL (Fig. 1C). The DRA also exhibits cytotoxicity in partially (fig. S2H) or completely TRAIL-resistant cell lines (Fig. 1D), suggesting that in some instances, TRAIL resistance is driven by suboptimal potency that is likely related to inefficient receptor clustering. However, despite the drastically improved potency offered by the DRA, certain cell lines, such as RKO, remained resistant to the DRA (EC₅₀ >100 nM) (13), suggesting that additional intrinsic resistance mechanisms are operative in these DRA-resistant tumor cell lines (Fig. 1E).

**Extrinsic pathway gene expression and apoptotic competency fail to explain intrinsic resistance**

In a panel of CRC cell lines (Fig. 1, B to E, and fig. S2, B to J), DRA sensitivity and resistance could not be trivially explained by differential expression of individual genes in the extrinsic and intrinsic apoptotic pathways, as mRNA expression data from the Cancer Cell Line Encyclopedia (CCLE) revealed no notable differences in the expression of these genes (Fig. 2A and fig. S3). Further, hierarchical clustering of the cell lines did not lead to stratification of resistant and sensitive cell lines into separate clusters, implying that the expression pattern of these genes is not a strong discriminator between the two groups. These results are consistent with a large body of literature, suggesting that cancer cell resistance to TRAIL and other proapoptotic drugs cannot be easily explained by simple analyses of differential gene expression (1). mRNA expression is not always indicative of protein levels or posttranslational modification states, which, themselves, may be predictive of sensitivity and resistance. In addition, differential DRA responses were unrelated to apoptotic competence, as DRA-resistant cell lines were capable of undergoing apoptosis by treatment with the topoisomerase inhibitor etoposide for 48 hours,

![Fig. 1. Multivalent proapoptotic DRAs can induce cell death in TRAIL-sensitive and TRAIL-resistant human CRC cell lines.](image-url)

(A) The DRA is composed of oligomers of the third type III fibronectin domain of tenascin engineered to bind DR5 with a *Kₐ* (dissociation constant) = 43 nM for the DRA monomer, linked by flexible glycine-serine linkers (G₄S)₃ and expressed recombinantly in *E. coli* (10). Monomers and dimers do not induce cell death (top), whereas the hexameric DRA increases apoptotic signaling in sensitive cell lines (bottom). (B and C) Human CRC cell lines’ responses to DRA treatment are superior to TRAIL in TRAIL-sensitive cell lines HCT116 and Colo205. (D) DRAs can induce cell death in TRAIL-resistant cell line HT29 cells. (E) RKO cells are resistant to both TRAIL and DRA treatment.
as indicated by annexin V binding assays in two intrinsically resistant cultures: the established cell line, RKO, and a low-passage patient-derived line, CRC247 (Fig. 2, B and C). Together, these data motivated the use of a systematic CRISPR-Cas9 knockout screen to identify functional drivers of DRA resistance.

CRISPR-Cas9 knockout screen reveals functional drivers of resistance to DRA

First, we used a CRISPR-Cas9 LOF screen to map the genetic landscape of resistance to the DRA (Fig. 3A) (14). We previously demonstrated that by coupling the results of resistance pathway screening with newly developed, long-term culture methods, it is possible to discover and creditential combination therapies that delay resistance evolution (15). Specifically, this screening approach identifies genes that, when knocked out, confer sensitivity to the DRA in resistant CRC cells. The screen used a recently created lentiviral short guide RNA (sgRNA)/Cas9 library targeting key nodes in major oncogenic growth, survival, and DNA damage response pathways, as well as many other potential drug targets and sensitivity modifiers (e.g., kinases, histone deacetylases, metabolic enzymes, BCL-2 family proteins) (15).

Briefly, after exposure of library-transduced cells to TRAIL or DRA for 2 weeks, cell samples were isolated, DNA was extracted, and polymerase chain reaction (PCR) was used to amplify and index barcode sgRNA amplicons.

The sgRNA depletion metric was defined as the normalized relative abundance of each construct in the presence of TRAIL or DRA to the same quantity in the presence of vehicle. sgRNA–level depletion metrics were converted to gene-level scores using the “3-score,” which represents the average of the three most depleted sgRNAs for a particular gene and is used to rank genes that, when knocked out, sensitize cells to drug treatment. Genes that drive resistance to TRAIL or DRA exhibit low 3-scores, as knockout of the gene leads to cell death in the presence of TRAIL or DRA, thus depleting cells expressing associated sgRNAs. Close correspondence between the results of two technical replicates is indicated in replicate plots; these plots demonstrate the reproducibility of the screen, as matching replicate values for each gene result in a clustering of the data around the diagonal (Fig. 3, B and C). The sgRNA depletion data are provided in table S1. All genes with depletion 3-score below 0.8 for both replicates were extracted for follow-up investigation; this threshold ensures that knockout of the gene results in at least 20% loss in relative cell abundance upon drug treatment. These genes were considered “hits” and examined to identify possible small–molecule inhibitors that target their associated proteins. Examples of putative hits and their corresponding 3-scores for each replicate are shown in table S2, alongside candidate small-molecule drugs that target their encoded protein products. The strongest hit in both TRAIL and DRA resistance screens was the gene for XIAP, a result that corroborates recent findings reporting XIAP’s involvement in TRAIL resistance (16–18). Other hits, albeit with lower 3-scores, included antiapoptotic proteins such as BCL-XL and the kinase CDK6.

BCL-XL, CDK4/6, and XIAP inhibitors potentely sensitize cancer cells to DRA

After hits were ranked based on their 3-score of depletion in each replicate, we performed drug sensitization studies by evaluating the in vitro cytotoxicity—in the RKO cell line—of the DRA in combination with commercially available small-molecule drugs that target the proteins encoded by these hits. The cells were treated with a “background dose” of the small-molecule drug and increasing doses of DRA. The selected background doses were chosen to be high enough to engage the target yet lower than or equal to the EC25 of the sensitizer drug in the cell line; thus, cytotoxicity observed in cells treated with the sensitizer and DRA would reflect DRA-induced cytotoxicity. Cellular viability measurements associated with each combination treatment were then normalized to the viability measurement associated with the background dose alone, and as such, all leftward shifts in the viability plots in Fig. 3D represent sensitization and not additive toxicity. As DRA monotherapy resulted in no cytotoxicity regardless of dose, the results of certain combination treatments were marked. Guidelines were set to identify the most effective sensitizers; cytotoxicity of at least 50% at 100 nM (1.3) and an ED50 of less than 1 nM were required.

The most efficacious drug combinations were not necessarily those targeting the hits with the best scores. This is likely due to limitations of the small-molecule drugs and genetic pleiotropy. Instead, the robustness of the screen lies in its ability to provide a pool of potential sensitizer drugs for combination with the drug of interest. Drugs resulting in the lowest cell viability (described as the maximum effect or
Fig. 3. CRISPR-Cas9 knockout screen reveals the genetic drivers of resistance to DRA, confirmed by in vitro cytotoxicity testing of screen-informed drug combinations. (A) Overview of LOF CRISPR screen experimental setup. First, sgRNA and Cas9 expression, packaging, and envelope plasmids were transfected into 293T cells, and lentiviral particles were harvested. The RKO cell line was then infected with the pooled lentiviral library, and puromycin selection was completed. The transfected cell line was subcultured for each desired condition (in duplicate), and treatment continued for 2 to 3 weeks, after which DNA was extracted from all samples. Constructs were barcoded by polymerase chain reaction (PCR) and sent for Illumina sequencing. (B) Results of TRAIL LOF CRISPR screen in RKO cells. (C) Results of DRA LOF CRISPR screen in RKO cells. Gray box indicates genes with depletion metric scores <0.8. Each dot represents a gene and is plotted on the depletion metric of each of its two replicates (replicate 1 on the x-axis and replicate 2 on the y-axis). Red dots indicate common hits between TRAIL and DRA screens. Blue dots indicate hits uniquely generated in the DRA screen. (D) Cell viability assay results of combination treatment with the CDK4/6 inhibitor Palbociclib, XIAP inhibitor BV6, BCL-X\textsubscript{L} inhibitor WEHI-539, and DRA in RKO cells and three human patient-derived cell lines (DRA concentration on the x-axis and cell viability on the y-axis). (E) Flow cytometry data show increased cytotoxicity (positive annexin V staining) for combination treatment conditions in RKO cells. A-1155463 (A-11) is a BCL-X\textsubscript{L} inhibitor (23). One-way ANOVA followed by Bonferroni multiple comparisons test was used to establish significance between A-11/BV6 (dark gray) and A-11/BV6/DRA for both DRA concentrations (red). (F) Clonogenic 2D growth assay experiments of >7 days in CRC247 cells indicate markedly slower cell growth upon treatment with DRA in combination with increasing doses of A-1155463 and BV6 each at 0, 50, and 150 nM (left to right) and 0, 3.7, or 7.5 pM DRA (top to bottom). Percent colony area has been graphed for visual purposes below the primary result. For all panels, error bars show SEM. ****P < 0.0001.
$E_{\text{max}}$ were identified as the XIAP inhibitor BV6, the CDK4/6 inhibitor Palbociclib, and the BCL-X$_L$ inhibitor WEHI-539 (Fig. 3D) (9, 19–21). The most effective sensitizers were then paired to evaluate the most potent three-drug combinations composed of DRA and two small-molecule inhibitors. As seen in Fig. 3D, Palbociclib/WEHI-539/DRA, and BV6/WEHI-539/DRA are the most efficacious drug combinations in RKO cells, resulting in picomolar DRA EC$_{50}$ concentrations.

These drugs were then tested in three human patient-derived CRC cell lines with a range of baseline sensitivities to DRA. In each case, the Palbociclib/WEHI-539/DRA and BV6/WEHI-539/DRA triple combinations were extremely potent, sensitizing cells to picomolar concentrations (EC$_{50}$ values) of DRA and corroborating the results obtained in RKO cells (Fig. 3D). We also conducted a series of complementary experiments to assess the relative toxicity of these treatments in normal, nontumorigenic human cells. Specifically, we assessed the toxicity of DRA alone, or DRA in the presence of background doses of the IAP inhibitor BV6 and the BCL-X$_L$ inhibitor WEHI-539, in human embryonic kidney (HEK293T), smooth muscle (P70), and primary patient-derived platelet cells. Platelets are of particular interest, as the dose-limiting toxicities (DLTs) associated with BCL-X$_L$ inhibition in patients are driven by thrombocytopenia (22). We observed that the EC$_{50}$ for DRA, both alone and in combination with BV6 and WEHI-539, in these cells (fig. S4, A and B) was, in all cases, at least 10-fold—and in most cases, up to 100-fold—greater than tumor cells. Together, these results indicate that treatment with DRA and its combination with BV6 and WEHI-539 is selective for tumor cells relative to normal, nontumorigenic cells.

In short-term in vitro studies, WEHI-539 was used to inhibit BCL-X$_L$, but this drug could only be used as an in vitro tool due to the presence of a labile and potentially toxic hydrazine moiety and poor physicochemical properties. Fortuitously, as our long-term in vitro studies commenced, A-1155463, a potent BCL-X$_L$ inhibitor with in vivo activity, became commercially available, and we used this inhibitor for subsequent studies (23). Further confirmation of cellular apoptosis was provided by quantification of annexin V–positive cells using flow cytometry; RKO cells incubated with triple drug combinations of DRA, the BCL-X$_L$ inhibitor A-1155463, and BV6 resulted in increased apoptosis of cells compared with single drug—or double drug–treated cells (Fig. 3E and table S3). Immunoblotting from extracts derived from RKO cells treated with DMSO control, A-1155463, BV6, DRA, and dual/triple combinations reveals that proapoptotic extrinsic and intrinsic pathway caspases 3, 8, and 9, and BID are activated by the drug combinations (fig. S4, A and D).

Before testing the effective drug combinations in animals, we evaluated their potency and robustness in longer-term two-dimensional (2D) growth assays. The 2D assays consist of a drug dosing matrix in which DRA-resistant CRC247 and RKO cells are treated with increasing doses of the sensitizing drugs and DRA. The DRA drug concentrations were chosen as the minimum concentration required for complete growth inhibition (MinC) and two times MinC, as identified from cell viability assays. Percent colony area was quantified for each treatment condition using the ImageJ ColonyArea plugin, which determines the percent area of the well covered by crystal violet and simultaneously accounts for intensity and, thus, cell density (Fig. 3F and fig. S4E) (24). Images of the 2D growth assays and quantified percent colony area both demonstrate the efficacy of DRA when combined with small-molecule sensitizers. The triple drug combination of A-1155463/BV6/DRA is the most effective inhibitor of cell growth, as seen in the bottom right of the 2D growth assay matrices for human CRC RKO and CRC247 cells [Fig. 3F (top) and fig. S4E]. As shown in the bar graphs, the maximum concentration of each triple drug combination results in <5% colony growth area in both cell lines and triple drug combinations [Fig. 3F (bottom) and fig. S4F].

ELP$_{\text{depot}}$-DRA fusions form gel-like depots at body temperature and abolish tumors in vivo
As suggested by the discussion of the pitfalls of TRAIL in the clinic, the rational development of broadly efficacious drug combinations can enhance a targeted protein’s anticancer activity, but if unaddressed, inadequate drug delivery can still prevent clinical translation. In the case of the DRA used in our studies, the short half-life (approximately 36 min) required at least daily injections for in vivo efficacy (10). Since proteins require systemic administration, the need for chronic outpatient therapy on a daily basis is impractical (25).

The most common approach for improving pharmacokinetic properties of protein drugs is polymer conjugation, which increases the overall size of the molecule and can reduce the rate of renal filtration and proteolytic degradation (26). Our biopolymer fusion approach integrates the advantages of polymer conjugation—longer plasma half-life than the native drug—with the unique phase behavior properties of protein–based thermally responsive polymers to generate “gel-like” coacervate depots for slow release of the DRA (27, 28). In previously published work, we demonstrated that recombinant fusion of protein drugs to elastin-like polypeptides (ELPs) not only increases molecular weight, which decreases renal clearance (29), but also endows the fusion protein with the thermally triggered phase behavior of the ELP (30). ELPs are repetitive, artificial, genetically encoded biopolymers in which the repeat unit is the pentapeptide (VPGXG), where X can be any amino acid except proline. ELPs exhibit lower critical solution temperature (LCST) phase behavior; they are soluble below their cloud point temperature, also commonly referred to as the inverse transition temperature ($T_t$) and undergo a phase transition, leading to the formation of an ELP-rich insoluble coacervate phase and an ELP-depleted aqueous phase. The identity of the guest residue (X) and the number of pentapeptide repeats are two orthogonal variables that control the $T_t$ of an ELP. The phase behavior of ELPs is retained upon fusion to soluble peptides and proteins and produces an injectable slow-release protein drug depot, prolonging the availability of the protein therapeutic (28, 30, 31).

On the basis of this concept, we designed a DRA-hydrophobic ELP fusion (ELP$_{\text{depot}}$-DRA) for sustained release of DRA that is soluble at room temperature, and hence, easily injected subcutaneously, and which undergoes an LCST phase transition that is triggered upon warming to body temperature, leading to the formation of a subcutaneous depot of the ELP$_{\text{depot}}$-DRA (Fig. 4A). The design of the ELP$_{\text{depot}}$ is informed by our previous studies of injectable ELP depots for delivery of glucagon-like polypeptide-1 and fibroblast growth factor 21 for treatment of type 2 diabetes (27, 32, 33). We selected the sequence (VPGVG)$_{120}$, with a predicted $T_t$ of $\sim$25°C at an injection concentration of 100 nM in phosphate-buffered saline (PBS), as the depot-forming ELP, based on previous studies that have quantitatively mapped the effect of ELP composition and molecular weight on their $T_t$ as a function of their concentration under physiological solution conditions (34, 35). As a molecular weight–matched ELP control to examine the impact of depot formation on DRA release and therapeutic efficacy, we fused the DRA to a more hydrophilic...
ELP—which where X in the VPGXG repeat alternates between alanine and glycine—that does not undergo its phase transition upon subcutaneous injection; this fusion is named ELP\textsubscript{soluble}-DRA. This ELP is predicted to have a \( T_\text{g} \) of >50°C in the concentration range of 25 to 200 \( \mu \text{M} \), which is the concentration range typically injected for the formation of a subcutaneous depot (27, 33).

Optical turbidity measurements demonstrate a sharp temperature transition of the depot-forming ELP\textsubscript{depot}-DRA formulation at 27.9°C (Fig. 4B), while the soluble ELP\textsubscript{soluble}-DRA fusion remains soluble up to ~60°C. In vitro cytotoxicity results show that the ELP\textsubscript{soluble}-DRA has a similar potency as the DRA in the DRA-sensitive Colo205 cell line, suggesting that appending an ELP to the DRA does not affect the activity of the DRA (Fig. 4C). The DRA is specific for human DRS, as previous studies show no interaction of the DRA with the mouse death receptor (10). Thus, toxicity data were obtained in vitro by assessing cell viability of a variety of normal human cells upon treatment with the DRA (fig. S4A). In vivo results demonstrate that a single intratumoral injection of ELP\textsubscript{depot}-DRA (30 mg/kg; molar equivalent of DRA) on day 0 causes growth inhibition of Colo205 subcutaneous xenografts and improves survival compared with intratumoral treatment with the other drugs: TRAIL, ELP\textsubscript{soluble}-DRA, and soluble DRA—all at a DRA molar equivalent dose (30 mg/kg) (Fig. 4D to F). A follow-up study was conducted to determine the efficacy of subcutaneous injection of ELP\textsubscript{depot}-DRA on the contralateral flank of nude mice xenografted with the Colo205 cell line, as this mode of administration is more clinically relevant for the treatment of CRC. Intratumoral injection was administered at the same dose as the subcutaneous administration at an ELP\textsubscript{depot}-DRA dose of the DRA equivalent (30 mg/kg). Both subcutaneous and intratumoral administration were equally effective, and no difference was observed between the two modes of administration (fig. S5). All subsequent in vivo depot treatments of the ELP\textsubscript{depot}-DRA were, hence, administered subcutaneously.

**Rationally designed drug combinations overcome ELP\textsubscript{depot}-DRA resistance in patient-derived xenografts**

Prior to in vivo efficacy studies, a series of cell viability assays were conducted to confirm activity of the intended drug combinations (Fig. 5A). An improved BCL-X\textsubscript{L} inhibitor, A-1331852, had recently become available; this drug is orally bioavailable and exhibits 10 times the potency of A-1155463 (36). A small pilot toxicity study was conducted, in which A-1331852 was administered in combination with BV6 or Palbociclib to nude mice. Unfortunately, Palbociclib was too toxic in combination with A-1331852 (fig. S6), so we chose to focus the remainder of our studies on the combination of BV6 and A-1331852 with the DRA. First, testing of A-1331852 in combination with DRA and BV6 was necessary to ensure in vitro efficacy in DRA-resistant CRC247 patient-derived cells before proceeding with tumor growth inhibition studies. A background dose of 1 \( \mu \text{M} \) BV6 and 2 \( \mu \text{M} \) A-1331852 (A-13) exhibits no cytotoxicity in CRC247 cells (fig. S7, A and B); any effect seen in combination treatments with DRA is due to the sensitization effect of these drugs to DRA treatment. The DRA has poor efficacy in the patient-derived CRC247 cells across an eight-log range of concentration up to a maximum concentration of 1 \( \mu \text{M} \),

![Fig. 4. ELP\textsubscript{depot}-DRA fusions form gel-like depots at body temperature and abolish tumors in vivo. (A) A hydrophobic ELP (35) was fused to DRA (blue) for a depot-forming formulation (ELP\textsubscript{depot}-DRA), and a hydrophilic ELP (purple) was fused to DRA as a soluble, non–depot-forming molecular weight–matched control (ELP\textsubscript{soluble}-DRA). (B) Optical turbidity, measured at 350 nm (OD\textsubscript{350}), demonstrates phase transition of ELP\textsubscript{depot}-DRA at 27.9°C, while ELP\textsubscript{soluble}-DRA remains soluble up to ~60°C. (C) Cell viability assay data for DRA-sensitive Colo205 cells show that ELP\textsubscript{soluble}-DRA, ELP\textsubscript{depot}-DRA, and DRA lead to similar in vitro cytotoxicity. (D to F) Colo205 subcutaneous xenografts were injected once on day 0 with ELP\textsubscript{depot}-DRA, ELP\textsubscript{soluble}-DRA, soluble DRA, TRAIL, or vehicle (\( n = 8 \) per group). All drugs were injected intratumorally. (D) Tumor growth data, shown as tumor volume versus time. Data were analyzed using two-way ANOVA of matched values, followed by Fisher’s least significant difference (LSD) multiple comparisons test to establish significance (\( P < 0.05 \)) of the difference between groups at each day of treatment. Results indicate statistically significant differences in tumor volumes between and including days 9 and 18 for depot-forming ELP\textsubscript{depot}-DRA compared with other groups, including soluble ELP\textsubscript{soluble}-DRA. (F) Kaplan-Meier survival results demonstrate prolonged survival for mice treated with depot-forming ELP\textsubscript{depot}-DRA formulation. Evaluation of survival data with log-rank test suggests significant differences (\( * P < 0.05 \)) between ELP\textsubscript{depot}-DRA and other treatment groups, with approximately 16 days increased median survival for the slow-release formulation compared with the soluble version.
and addition of the XIAP inhibitor BV6 only has a modest effect on cytotoxicity. In contrast, combination treatment of the BCL-XL inhibitor A-13 with DRA yielded a marked effect on cytotoxicity, as seen by the cell killing at low picomolar concentrations of the DRA (Fig. 5A). Treatment with all three drugs, A-13/BV6/DRA (A + B + DRA), is even more potent than A-13/DRA (A + DRA) treatment, as it results in subpicomolar EC₅₀ for the DRA and >96% cell kill in a 3-day in vitro assay (Fig. 5A). Combination index (CI) calculations confirm synergism of the triple drug combination in CRC247s (fig. S7C).

To assess in vivo efficacy of the triple treatment compared with single drugs or double combinations, we next performed a tumor growth study in highly DRA-resistant CRC247 patient-derived subcutaneous xenografts. Having established the superior efficacy of the ELP depot for sustained DRA delivery compared with DRA by itself, we tested the small-molecule inhibitors of BCL-XL and XIAP—A-13 and BV6—in combination with the ELPdepot-DRA formulation in vivo. The doses for each drug were chosen based on information available in the literature, observations from a pilot toxicity study, and the DRA doses known to be efficacious in DRA-sensitive cell lines (10, 17, 36). The triple drug combination effectively resulted in tumor regression for 1 week and delayed tumor growth between days 5 and 13 compared with all other groups (Fig. 5, B and C). Treatment with single drugs—A-13 [25 mg/kg, per os (po), daily], BV6 [5 mg/kg intraperitoneally (ip), every 4 days (q4d)], or ELPdepot-DRA [30 mg/kg subcutaneously (sc), weekly]—resulted in slowed tumor growth compared with mice in the vehicle control group, but the A + B + ELPdepot-DRA triple drug treatment was much more efficacious at suppressing tumor growth (Fig. 5B). Treatment with the double drug combinations A-13 + BV6, BV6 + ELPdepot-DRA, and A + ELPdepot-DRA was well tolerated (fig. S8) and slowed tumor growth, but addition of the third drug in the A-13 + BV6 + ELPdepot-DRA treatment group resulted in more profound tumor growth inhibition over time (Fig. 5C). Survival data corroborate the advantage of having all three drugs in combination, as mice in this group outlived those in all other groups (Fig. 5D). These in vivo results qualitatively recapitulate those seen in vitro, providing affirmative evidence for the use of this triple drug combination for treatment of a DRA-resistant PDX in mice.

**DISCUSSION**

In these studies, we present solutions to the potency, resistance, and delivery challenges that hinder proapoptotic receptor agonist efficacy. Maximization of potency was achieved through the use of a hexavalent DRA that promoted multimeric DR5 receptor cross-linking and efficient pathway engagement. To address the intrinsic resistance of a subset of CRCs to DRA treatment, an unbiased CRISPR screen in a DRA-resistant CRC was carried out, which identified genes that, when knocked out, overcome intrinsic resistance to the DRA. This unbiased approach does not tailor combination options to conventional knowledge of signaling pathways associated with the drug of interest and, instead, scans a plethora of potential cancer death and survival pathways to evaluate the most important mechanisms of resistance. Among the many cancer pathways interrogated in the screen, most of the primary hits were genes within the extrinsic/intrinsic

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**Fig. 5. Rationally designed drug combinations overcome ELPdepot-DRA resistance in PDX.** (A) Cell viability data for CRC247 show efficacy of triple drug treatment with A-1331852 (A in figure legend), BV6 (B in figure legend), and DRA (A + B + DRA; green) compared with double drug treatments (red). Data were analyzed using two-way ANOVA of matched values to establish significance (*P < 0.05) of the difference between groups and triple drug combination A-1331852 + BV6 + ELPdepot-DRA (*P < 0.05). (B and C) Tumor growth inhibition data; mice (n = 7 per group) were treated with A-1331852 (25 mg/kg, daily po) and/or BV6 (5 mg/kg, q4d) and/or ELPdepot-DRA (30 mg/kg, weekly sc). Data were analyzed using two-way ANOVA of matched values, followed by Fisher's LSD multiple comparisons test to establish significance (*P < 0.05) from days 2 to 13. (D) Kaplan-Meier survival analysis comparing key treatment groups indicates that median survival increases from 29 to 38 days when BV6 is added to the A-1331852 + ELPdepot-DRA combination. A Gehan-Breslow-Wilcoxon test demonstrated statistically significant difference in survival between all single-drug treatment groups and triple drug combination A-1331852 + BV6 + ELPdepot-DRA (*P < 0.05).
pathways, suggesting that failure of DRA monotherapies is driven by an inability to fully engage the cell death machinery. While a number of regulators of the extrinsic apoptotic pathway are conceivably potential drivers of intrinsic DRA resistance, our rational and unbiased genetic knockout screen considerably narrowed down the key players to a shortlist of druggable targets—a task that would be infeasible to conduct in an in vivo model. The screen nominated the gene for XIAP, an antiapoptotic protein, as the greatest driver of resistance to both TRAIL and DRA in the human CRC cell line RKO. The identification of XIAP as a driver is consistent with studies in the literature that have suggested XIAP as a key driver of TRAIL resistance (17, 37, 38). The emergence of the antiapoptotic protein BCL-\(X_L\) as a hit is also consistent with our mechanistic understanding of apoptotic signaling.

The genetic screen then streamlined the testing of potential targeted small molecules that specifically inhibit proteins associated with DRA resistance instead of simply combining DRA with standard-of-care chemotherapeutics. The “druggable” targets were linked to associated, clinically viable small-molecule drugs and tested in combination with DRA to identify the cocktails that most effectively overcome DRA resistance. Most notably, these studies nominated BCL-\(X_L\) inhibition, with or without XIAP inhibition, as a potent strategy for sensitizing tumors to DRA. Although BCL-\(X_L\) and XIAP are well-known inhibitors of TRAIL-mediated apoptosis, previous colon cancer–focused studies have been limited to in vitro studies using RNA interference knockdown of these targets in just a few cell lines (9, 18, 39). Our combination studies included the observation that cancer cells with intrinsic resistance to DRA alone (RKO, CRC247, and CRC119) could be strongly sensitized to death receptor agonism using combined IAP and BCL-\(X_L\) inhibition, whereas normal cells with similar intrinsic insensitivity to DRA could not. This finding is not entirely unexpected, as a growing body of studies suggests that cancer cells are more primed to undergo apoptosis than most normal cell types (40, 41).

Having addressed the in vitro potency and resistance issues of the DRA, we then engineered a DRA fusion with a thermally responsive ELP to create an injectable depot formulation—ELP\(_{\text{depot}}\)-DRA—for sustained delivery of the DRA in vivo. The efficacy of this depot is demonstrated by the substantial antitumor activity of the ELP-DRA gel depot formulation in the DRA-sensitive Colo205 model following only a single injection. Notably, the ELP\(_{\text{depot}}\)-DRA outperformed the DRA, which is soluble upon subcutaneous administration and is rapidly cleared from systemic circulation by renal clearance, highlighting the importance of temporally sustained delivery of drugs such as biologics that are typically systemically injected. Last, we integrated the two strategies to achieve in vivo tumor growth inhibition of DRA-resistant patient-derived xenografts (PDX), demonstrating potent antitumor efficacy when combining BCL-\(X_L\) inhibition with A-1331852, with or without the XIAP inhibitor BV6, with the ELP-DRA conjugate. This work is the first example of the pharmacologic inhibition of BCL-\(X_L\) and XIAP to overcome resistance to extrinsic pathway agonism in vivo in PDX models.

A major potential limitation of most combination antitumor therapies such as A-1331852/BV6/DRA is toxicity. The doses used in this study were based on a small pilot maximum tolerated dose experiment and were not fully optimized, and a rigorous optimization study may enable lowering of drug doses. Even so, we achieved encouraging in vivo results with lower doses of each drug compared with doses used in the literature. For instance, in one study, A-1331852 was given twice daily in combination with venetoclax to achieve effective tumor growth inhibition in vivo (36), while we administered the drug only once a day to reduce potential toxicity. Similarly, in a separate study, BV6 was administered at 10 mg/kg for synergism with another DRA that had not been modified for improved delivery (17). Here, mice were treated with only BV6 (5 mg/kg) at each injection. In addition, instead of daily dosing of the DRA, the slow-release formulation enabled DRA administration only once per week (10). This aspect of our work is encouraging, especially because the major reported DLTs for each class of drug used in this study do not overlap with one another. For instance, DLTs of IAP inhibitors have been associated with cytokine release syndrome in patients, while BCL-\(X_L\) inhibition is limited by thrombocytopenia due to the induction of platelet death (42, 43). DRAs, on the other hand, have historically been associated with potential hepatotoxicity concerns (43). Thus, by taking advantage of their highly synergistic antitumor activities, the therapeutic combinations described here have the potential to be safely administered to patients at active doses. The importance of the current study is not intended to be driven by the biological novelty of the resistance mechanisms, but rather by our modular integration of solutions to the problems of potency, bioavailability, and drug resistance. To extend our modular platform further, next generations of this system will combine the ELP\(_{\text{depot}}\)-DRA with small-molecule encapsulation to promote targeted tumor uptake and minimize toxicities in nontumor tissues.

Last, the precision medicine approach described here may serve broadly as a springboard for the design of other protein drug-based combination therapies that combine highly specific activity, favorable delivery properties, and minimal resistance barriers, thereby unlocking the potential of agents that may otherwise fail to exhibit single-agent activity.

**MATERIALS AND METHODS**

**Study design**

This study was designed to improve the delivery and therapeutic efficacy of extrinsic apoptotic pathway agonists in human CRC cells. We addressed this objective by (i) evaluating the potency of a multivalent DRA in a panel of CRC cell lines, (ii) performing a CRISPR-mediated knockout screen in DRA-resistant CRC cells to identify genetic drivers of DRA resistance and overcome resistance by combining DRA with sensitizers informed by the knockout screen, (iii) developing a DRA fusion to ELPs form a subcutaneously injectable gel depot for slow release of DRA into the circulation, and (iv) assessing the in vivo therapeutic efficacy of the ELP\(_{\text{depot}}\)-DRA formulation in combination with the best sensitizers.

**Xenograft tumor studies**

In the xenograft tumor studies, mice were randomized to groups according to tumor volume before treatment. The number of mice per group is specified in the figure legends, tumor sizes were measured by caliper, and the primary endpoint was survival. All procedures were performed as approved by the Institutional Animal Care and Use Committee at Duke University. The investigators were not blinded during the study.

**Statistical analysis**

Results were expressed as means ± SEM and analyzed by one-way analysis of variance (ANOVA) with Tukey’s post hoc test for grouped
analyses. For the depot DRA delivery study, n = 8 per group, and for the drug combination study, n = 7 per group. The exception is in vivo tumor data analyses, in which matched values were analyzed by two-way ANOVA with Fisher’s least significant difference (LSD) multiple comparisons test to establish significance (P < 0.05) of the difference between groups on each day of treatment. Survival times of treatment groups were analyzed and compared using the Mantel-Cox log-rank test. GraphPad Prism 7 Software was used for all statistical analysis and generation of Kaplan–Meier survival plots. Statistical significance of differences was set at *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

**In silico gene expression analysis**

The mRNA gene expression data were obtained from the CCLE (44). Gene-centric robust multiaarray average–normalized expression values of the genes of interest were visualized in a heatmap, where lower expression is represented in blue and higher expression is represented in red. In addition, scatterplots were generated to show the individual expression level of each gene in each cell line more clearly (fig. S3).

**Synthesis and assembly of genes**

The DNA encoding the monomer of the Tn3 in the DRA encoded the following amino acid sequence (GAIEVKDVTDTTALITWAK-
PWVDPPPLWGCETLYIKDVPDRTIDLQKHTAYSIGN-LKPDTETEVLICDPYGRMRSPKAPETFTT) (10). The E. coli codon-optimized gene was purchased as a “G-block” from the Integrated DNA Technologies (IDT). The gene was purchased with a (Gly4Ser)3 linker at the C terminus and designed with restriction sites compatible with recursive directional ligation (RDL) for seamless cloning of oligomeric genes (45). The amplified PCR product was purified using a Qiagen PCR cleanup kit and digested with Bse RI for insertion into a Bse RI/CIP digested pET-24(+) vector modified for RDL. The insert and vector were agarose gel purified and ligated with Quick Ligase to clone the single unit construct. This was followed by digestion of the single unit construct [Tn3 in pET24(+)] with Bse RI/CIP and ligation with Bse RI–digested insert (Tn3 monomer) to clone 2, 4, and 6 Tn3 repeats in the pET24(+) vector (Novagen, Madison, WI). EB50 cells (EdgeBio) were used for cloning steps. All enzymes were purchased from New England Biolabs.

The gene for the depot-forming ELP (ELPdepot) that encodes the amino acid sequence (VPGVG)120 and the soluble ELP (ELPsoluble) that encodes the amino acid sequence (VPGA/GG)120 was recombinantly fused to the hexameric Tn3 fusions using RDL. Both ELP genes were available from previous studies (35). The DNA sequence of the ELP genes can be obtained from previous publications (27, 35). The RDL method for this particular vector called for digestion of the oligomerized Tn3 in modified pET24(+) with Bse RI and Bgl I and digestion of ELP in pET24(+) with Aci I and Bgl I. The digested fragments of DNA were separated using agarose gel electrophoresis, and the DNA bands of the appropriate molecular weights were excised and gel purified using the QIAquick Gel Extraction kit (Qiagen). The purified fragments were ligated using Quick Ligase (New England Biolabs), and successful clones were identified by DNA sequencing analysis.

The hexamer ELPdepot–DRA fusion constructs were expressed in SHuffle T7 Express cells in 2XYT media in 1-liter shake flasks (New England Biolabs). Overnight cultures (50 ml) were used to inoculate 1-liter Erlenmeyer flasks in a shaker incubator (GYROMAX 15,000 rpm at 4°C for 1 to 2 hours to complete protein extraction from periplasmic space. The periplasm extraction samples were then centrifuged at 14,000 rpm for 15 min at 4°C to pellet cell debris. Proteins in solution were precipitated with ammonium sulfate (60%, w/v) and centrifuged for 15 min to pellet the precipitated protein. The protein pellet was then placed on ice and resuspended in ice-cold PBS. Immobilized metal affinity chromatography (IMAC) on a Nickel-NTA agarose resin (Thermo Fisher Scientific) was then used to purify the His-tagged DRA from other periplasmic proteins by following the manufacturer’s protocol. Pure elute then underwent buffer exchange into 20 mM tris, 300 mM arginine (pH 7) using 10-kDa Amicon Ultra centrifugal filters (EMD Millipore).

TRAIL–His6 (TRAIL amino acids 114 to 281) was purchased as a G-block from IDT, cloned into the pET-24(+) plasmid system (Novagen), and grown in BL21(DE3) cells (EdgeBio). Overnight cultures (50 ml) were used to inoculate 1-liter shake flasks, and cells were grown at 37°C for 4 to 5 hours, then induced with 1 mM sterile IPTG, and incubated at 37°C in a shaker incubator (GYROMAX 747 orbital incubator shaker, Amerex Instruments Inc.), and cells were grown for 4 to 5 hours at 37°C and then induced with 1 mM sterile isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated at 180 rpm at 25°C for another 6 to 12 hours. Cells were pelleted, resuspended in 50 mM tris (pH 8), sonicated, and centrifuged at 15,000 rpm at 4°C for 15 min to separate cell debris from the soluble fraction. Proteins were purified from the soluble fraction of the cell lysate using inverse transition cycling (ITC), a method that exploits the LCST phase transition of ELP fusions and involves repeated cycles of protein aggregation and solubilization (46). Specifically, the “hot spin” of ITC was performed by the addition of <2 M ammonium sulfate until the solution became turbid and the salt was fully dissolved and centrifuged at 14,000 rpm at 35°C for 20 min to pellet the protein. The phase-transitioned protein pellet was then resuspended in 20 mM tris, 300 mM arginine at pH 7 and placed in a rotator at 4°C. The “cold spin” of ITC was performed by centrifuging the protein at 4°C, 14,000 rpm and preserving the supernatant, which contained the ELPdepot–DRA fusion. The hot spin/cold spin process was repeated twice before further purification by size exclusion chromatography on a Superdex HiLoad 26 60/200 column in PBS on an ÄKTA chromatography system (GE Healthcare Life Sciences). Pure eluate then underwent buffer exchange into 20 mM tris, 300 mM arginine (pH 7) using 10-kDa Amicon Ultra centrifugal filters (EMD Millipore). All purified proteins were analyzed by SDS-PAGE on Bio-Rad Mini-PROTEAN TGX Tris-HCl Stain-Free gels for correct molecular weight bands.

For DRA expression without fusion to ELP, the DNA sequence encoding a periplasmic secretion signal, oppA “MTNITKRSLVAA-GVLAALMAGNVALA,” was appended at the 5’ terminus of the hexameric DRA gene by the previously discussed RDL method, and DNA encoding a (His)8 tag was appended at the 3’ end of the DRA gene to create a gene that encodes the following construct—oppA–DRA–His8. This construct was then expressed in BL21(DE3) cells: 50 ml of overnight cultures was used to inoculate 1-liter shake flasks, and cells were grown at 37°C for 4 to 5 hours, then induced with 1 mM sterile IPTG, and incubated at 37°C in a shaker incubator (GYROMAX 747 orbital incubator shaker, Amerex Instruments Inc.) for another 4 to 6 hours. Cells were then pelleted and resuspended in 100 ml of ice-cold 10 mM tris, 1 mM EDTA (pH 8) buffer and placed on a rotator at 4°C for 1 to 2 hours to complete protein extraction from periplasmic space. The periplasm extraction samples were then centrifuged at 14,000 rpm for 15 min at 4°C to pellet cell debris. Proteins in solution were precipitated with ammonium sulfate (60%, w/v) and centrifuged for 15 min to pellet the precipitated protein. The protein pellet was then placed on ice and resuspended in ice-cold PBS. Immobilized metal affinity chromatography (IMAC) on a Nickel-NTA agarose resin (Thermo Fisher Scientific) was then used to purify the His-tagged DRA from other periplasmic proteins by following the manufacturer’s protocol. Pure eluate then underwent buffer exchange into 20 mM tris, 300 mM arginine (pH 7) using 10-kDa Amicon Ultra centrifugal filters (EMD Millipore).
calcium chloride, 10 mM dithiothreitol (DTT) (pH 7.4), sonicated, and centrifuged at 4°C at 15,000 rpm for 15 min to separate cell debris from the soluble fraction. The His-tagged DRA was purified from other periplasmic proteins on a Nickel-NTA agarose resin by IMAC by following the manufacturer’s protocol (Thermo Fisher Scientific). Pure eluate was then underwent buffer exchange into 20 mM tris, 100 μM zinc sulfate, 10 mM calcium chloride, and 10 mM DTT (pH 7.4). All chemicals were purchased from Sigma-Aldrich. The EC50 of the in-house TRAIL was within an order of magnitude of commercial TRAIL.

All proteins used for the in vivo studies were endotoxin purified using Pall Mustang E Membrane sterile/endotoxin filters and tested using the GenScript ToxinSensor Single Test Kit endotoxin test to ensure levels below the U.S. Food and Drug Administration–recommended limit of 0.25 EU/ml (47).

**Optical turbidity**

To determine the T50 of the ELP fusion proteins, the optical turbidity at 350 nm of a 25 μM solution of the ELP fusion proteins was measured at a thermal ramp rate of 1°C/min between 4°C and 60°C on a temperature-controlled ultraviolet-visible (UV-vis) spectrophotometer (Cary 300 UV-Vis, Agilent Technologies). The LCST phase transition is indicated by the sudden increase in optical turbidity, and the inflection point of the OD350 vs. temperature curve was used to calculate the T50.

**MALDI–time-of-flight MS**

MALDI–time-of-flight MS was performed for the DRA-His8 protein using an Applied Biosystems Voyager–DE Pro system with a nitrogen laser, and mass spectra were obtained using α-cyano-4-hydroxycinnamic acid matrix in a 20:1 (v/v) ratio with the analyte.

**Cell lines and reagents**

All cell lines were grown at 37°C in 5% CO2. Colo205 cells were cultured in RPMI 1640 [10% fetal bovine serum (FBS), Heps, pyruvate, 1% penicillin/streptomycin (P/S)]. HCT116 and HT29 were cultured in MEM Earle’s [10% FBS, pyruvate, NEAA (nonessential amino acid), 1% P/S]. LoVo cells were cultured in F-12K (10% FBS, 1% P/S). T84 cells were cultured in 50/50 mix of HAM’s F-12 and MEM Earle’s [10% FBS, pyruvate, NEAA (nonessential amino acid), 1% penicillin/streptomycin (P/S)]. HCT116 and HT29 were cultured in RPMI 1640 [10% fetal bovine serum (FBS), Hepes, pyruvate, 1% penicillin/streptomycin (P/S)]. HCT116 and HT29 were cultured in MEM Earle’s [10% FBS, pyruvate, NEAA (nonessential amino acid), 1% P/S]. LoVo cells were cultured in F-12K (10% FBS, 1% P/S).

**Lentiviral production and titration of CRISPR-Cas9 LOF library**

Lentiviruses were produced from HEK293T cells, which were grown to 50% confluence in 6-cm plates and transfected using FuGENE6 (Promega), 5.6 mg of psPAX2, 0.625 mg of pVSVG, and 6.25 mg of library plasmid. All plasmids were provided by the Wood Lab and had been cloned by previously described methods (49) and sgRNAs (50). After 30 min of incubation at room temperature, the transfection mixture was added to the cells and incubated overnight at 37°C. The next day, harvest medium was added (DMEM, 30% FBS). After the 24- and 48-hour collection points, harvested virus was passed through a 0.45-μm filter (EMD Millipore). Viral titers were calculated using the GenScript ToxinSensor Single Test Kit endotoxin test to ensure levels below the U.S. Food and Drug Administration–recommended limit of 0.25 EU/ml (47).

**CRISPR-Cas9 LOF screen**

To identify genetic drivers of CRC resistance to DRAs, we performed a CRISPR-Cas9–based LOF screen, as previously described (15). A brief overview of the procedure follows. A library of viral vectors encoding LOF sgRNA inserts that targeted a panel of 378 druggable genes and signaling pathways was obtained.
Once the pooled lentiviral library was produced by transfection of 293T cells, it was titrated and used to infect DRA-resistant RKO cancer cells. RKO cells were seeded at 500,000 cells per well in six-well plates, incubated overnight, and transduced at a multiplicity of infection of 0.3 the next day. After puromycin selection, a sample was taken to verify representation of the various knockout genes. The transduced population was maintained under puromycin selection for 1 week, after which the library of cells was then exposed to vehicle, TRAIL, or the DRA (each treatment condition in duplicate) for 2 weeks. Cell samples were obtained, DNA was extracted (DNeasy Blood & Tissue Kit; Qiagen), and sgRNA barcodes were isolated, prepared for sequencing as previously described (49). The samples were sequenced by next-generation Illumina sequencing (HudsonAlpha), and the raw data were processed to identify hits that sensitized RKO cells to each treatment, as evidenced by their depletion in drug versus vehicle treatment conditions. The fractional representation (FR) for a given guide in the final condition after vehicle treatment was compared with its FR final condition after TRAIL or DRA treatment. The depletion level of each sgRNA barcode (drug versus vehicle conditions) was calculated as the FR from treated population normalized to the FR from vehicle control (both at the final time point). Depleted barcodes represent sensitizer genes, as they were specifically depleted in the drug-treated cell populations. Depletion comparisons were used to generate a scoring metric called the "3-score," which represents the average of the three most depleted sgRNAs for a particular gene (52). The depletion median, mean, and 3-scores for both replicates of all genes are provided in table S1. The genes were ranked by their 3-scores; top hits are those that sensitized the cells to DRA treatment when knocked out by the CRISPR-Cas9 machinery. Data are presented as the depletion metric mean of the 3-score per gene in the library. Hits are genes with a low 3-score, and examples of the genes representing top hits are denoted in table S2. All data extractions and calculations were coded and completed using R. The hits were subsequently filtered to retain genes that encoded proteins for which specific inhibitors are commercially available.

**Flow cytometry for annexin V apoptosis quantification**

Cells were seeded in six-well plates overnight. The next day, cells were treated with the indicated amount of drug(s) or vehicle control (DMSO). Incubation time was 15 hours for etoposide or 48 hours for DRA alone or DRA with sensitizers. To prepare samples for flow cytometry, cells were drugged at the indicated doses, and drug medium was replaced every 3 to 4 days. Approximately 1 week after treatment, plates were rinsed with PBS and fixed and stained with 0.5% (w/v) crystal violet in 60% (v/v) glutaraldehyde solution (Thermo Fisher Scientific) for 20 min at room temperature. Plates were rinsed in deionized water, dried overnight, and photographed the following day. Percent colony area covered by crystal violet was quantified using the ImageJ Software colony area plugin.

**Immunoblotting**

Cells were seeded at 500,000 cells per 10-cm dish and treated the next day. After treatment for 6 and 16 hours, cells were scraped off the wells in cold PBS, centrifuged at 5000 rpm for 3 min at 4°C, separated from the supernatant, washed with PBS once, frozen at −80°C, and then lysed with cold radioimmunoprecipitation assay buffer (20 mM tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% NP-40, 0.1% SDS, 0.5% nadeoxycholate, 2 mM EDTA (pH 8.0)) supplemented with protease and phosphatase inhibitors (Roche protease inhibitor cocktail; Phosphatase Inhibitor Cocktail I and Phosphatase Inhibitor Cocktail II from Sigma-Aldrich) and centrifuged at 13,300 rpm at 4°C for 10 min. Protein concentration in supernatant lysates was determined using the Bradford assay (Bio-Rad). Proteins from each lysate (10 μg) were resolved on SDS-PAGE (NuPAGE 4 to 12%), transferred to polyvinylidene difluoride membranes, blocked with 5% milk in tris-buffered saline + 0.1% Tween, and probed with primary antibodies in 5% bovine serum albumin overnight at 4°C. Primary antibodies (1:1000 to 1:2000 dilution) recognized BCL-XL (CST#2764); BIM (CST#2933); BID (CST# 2002); caspases 3, 8, and 9; and β-actin.

**Pilot toxicity study of Palbociclib and A-1331852 in nude mice**

A small pilot study was conducted in nude mice to evaluate the toxicity of the double sensitizer A-1331852 (A-13) and Palbociclib combinations at a range of doses. A-13 is an oral drug, while Palbociclib can be administered orally or intraperitoneally. To avoid potential trauma associated with multiple daily dosing of these drugs, we administered Palbociclib intraperitoneally daily and A-13 dosed orally daily. We chose a daily oral dose of 25 mg/kg for A-13, as this dose ensures in vivo activity according to data available in the literature (36). Daily administration of A-13 at this dosage did not affect body weight or cause visible signs of toxicity. Palbociclib dosing was first tested at 50 mg/kg ip in combination with A-13 dosed orally daily at 25 mg/kg. Unfortunately, this combined dose of Palbociclib and A-13 caused visible toxicity in the mice, resulting in lowered body temperature, reduced mobility, closed eyes, and/or sudden death. Thus, lower doses of Palbociclib (25 and 12.5 mg/kg) were tested in combination with A-13 (25 mg/kg) (fig. S6). However, these doses were still too toxic, as mice continued to lose weight each day (fig. S6). Within 1 week, mice lost more than 15% of their body weight; the doses 25 and 12.5 mg/kg were therefore too toxic for drug combination studies. A lower dosage (6.25 mg/kg) of Palbociclib was also tested in combination with A-13 at 25 mg/kg dosed orally daily, and an introductory test in which ELP-DRA (30 mg/kg) was added to this combination treatment caused rapid deterioration in body condition over the course of 2 weeks. Change in dosing frequency was also considered, but the half-life of Palbociclib was only a few hours, making this option infeasible (21).

**Synergy calculations**

Synergy calculations from nonconstant ratio of in vitro CRC247 cell viability study were conducted using the CompuSyn software, which uses the Chou-Talalay method for drug combination based on the median-effect equation (53). The resulting CI-isobologram equation enables quantitative determination of drug interactions of the double...
and triple drug combinations. CI < 1 (logCI < 0) indicates synergism, and logCI = 0 indicates additive effect.

Xenograft tumor studies

All tumor studies were carried out in 5- to 7-week-old, athymic nude/ female mice (the Jackson laboratory), and treatment commenced when tumor size reached 100 to 120 mm³ (as measured by a digital caliper and calculated as $L \times W \times 0.5$). Colo205 engraftment was performed by subcutaneous injection of 1 million cells in the right flank. Colo205 xenografted mice were treated with one single intratumoral or subcutaneous (contralateral flank) injection of the ELPdepot-DRA formulation or ELPsoluble-DRA or DRA or TRAIL on day 0. DRA fusions and TRAIL were administered at the molar equivalent DRA dose of 30 mg/kg. CRC247 patient-derived cells were engrafted subcutaneously at 3 million cells per mouse. On day 0, treatment commenced with daily oral gavage for A-1331852 in previously described vehicle (36), twice weekly intraperitoneal injection of BV6 in sterile saline, and/or weekly subcutaneous injection of ELPdepot-DRA in the contralateral flank. The ELPdepot-DRA formulation for in vivo injection was composed of a 1:1 molar ratio of ELP depot-DRA formulation or ELP soluble-DRA or DRA or TRAIL on one single intratumoral or subcutaneous injection of BV6 in sterile saline, and/or weekly subcutaneous injection of ELPdepot-DRA in the contralateral flank. The ELPdepot-DRA formulation for in vivo injection was composed of a 1:1 molar ratio of ELPdepot-DRA formulation or ELP soluble-DRA or DRA or TRAIL.

SYNTHETICAL MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/9/eaaw9162/DC1

Fig. S1. Protein characterization of DRAs.

Fig. S2. Cytotoxicity dose-response curves for TRAIL and DRA in nine human CRC cell lines and three patient-derived cell lines.

Fig. S3. There is no difference between mRNA expression levels of genes associated with apoptosis in TRAIL-sensitive and TRAIL-resistant cell lines.

Fig. S4. DRA monotherapy and combination of DRA with sensitizer drugs are selectively proapoptotic in human cancer cells, sparing normal cells.

Fig. S5. Subcutaneously injected ELPdepot-DRA formulation is as efficacious as intratumoral injection of BV6 in sterile saline, and/or weekly subcutaneous injection of ELPdepot-DRA in the contralateral flank. The ELPdepot-DRA formulation for in vivo injection was composed of a 1:1 molar ratio of ELPdepot-DRA formulation or ELP soluble-DRA or DRA or TRAIL.

Fig. S6. Palbociclib combination with A-1331852 results in unacceptable toxicity in nude mice.

Table S1. Summary of RKO cell viability results from the combination of DRA with unaffected human cancer cells.
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