Agonists of the TRAIL Death Receptor DR5 Sensitize Intestinal Stem Cells to Chemotherapy-Induced Cell Death and Trigger Gastrointestinal Toxicity

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Abstract

The combination of TRAIL death receptor agonists and radiochemotherapy to treat advanced cancers continues to be investigated in clinical trials. We previously showed that normal cells with a functional DNA damage response (DDR) upregulate the expression of death-inducing receptor DR5/TRAIR2/TNFRSF10B in a p53-dependent manner that sensitizes them to treatment with DR5 agonists. However, it is unclear if targeting DR5 selectively sensitizes cancer cells to agonist treatment following exposure to DNA-damaging chemotherapy, and to what extent normal tissues are targeted. Here, we show that the combined administration of the DR5 agonist monoclonal antibody (mAb) and chemotherapy to wild-type mice triggered synergistic gastrointestinal toxicities (GIT) that were associated with the death of Lgr5⁺ crypt base columnar stem cells in a p53⁻ and DR5-dependent manner. Furthermore, we confirmed that normal human epithelial cells treated with the human DR5 agonistic mAb and chemotherapeutic agents were also greatly sensitized to cell death. Interestingly, our data also indicated that genetic or pharmacologic targeting of Chk2 may counteract GIT without negatively affecting the antitumor responses of combined DR5 agonist/chemotherapy treatment, further linking the DDR to TRAIL death receptor signaling in normal cells. In conclusion, the combination of DR5-targeting agonistic mAbs with DNA damaging chemotherapy may pose a risk of developing toxicity-induced conditions, and the effects of mAb-based strategies on the dose-limiting toxicity of chemotherapy must be considered when establishing new combination therapies.

Introduction

Novel targeted therapeutics in oncology hold the promise to selectively kill the tumor cell population with reduced toxicity to normal tissues that hampers current standard radiochemotherapy that often has a narrow therapeutic index. One strategy that has been under development is the targeting of the extrinsic apoptotic signaling pathway for the treatment of advanced systemic malignancies. Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is a member of the tumor necrosis factor superfamily. Endogenous TRAIL and its death-inducing receptors DR4 and DR5 (TNFRSF10B) in humans play a powerful role in tumor immune surveillance, inflammation, and tumor suppression in vivo (1–4). Ligand-dependent clustering of the DR4 and DR5 receptors and activation of downstream caspases triggers fairly rapid and fulminant apoptosis selectively in cancer cells. The specific molecular mechanisms that render cancer cells increasingly susceptible to apoptosis triggered through the TRAIL system remain to be fully understood, although a role for TRAIL decay receptors has been suggested to protect normal cells.

Despite the observations that TRAIL death receptor agonists (TGRA) are generally nontoxic to normal cells and are overall well tolerated, some precautions have been suggested. Certain preparations of recombinant TRAIL have been found to be toxic to human hepatocytes in vitro (5, 6) and some agonistic mAbs targeting DR4 and DR5 can kill normal human hepatocytes in vitro (7). Human hepatocytes isolated from steatotic and hepatitis C–positive livers appear to be sensitive to both untagged and tagged TRAIL (8). In experimental mouse models, high-dose treatment with MD5-1, an agonistic mAb targeting mouse DR5, triggered cholangitis with a histologic appearance reminiscent of human primary sclerosing cholangitis (9). Some early clinical trials reported DLTs of DR5 mAbs that may involve liver toxicity.
High-dose (20 mg/kg) treatment with lexatumumab resulted in asymptomatic and reversible transaminase and amylase elevations in a phase 1 trial of patients with advanced malignancies (10). Similarly, transaminitis was noted in 1 of 37 patients subjected to apomab, an agonistic DR5 mAb (11). It is important to note that it can be difficult to attribute liver toxicity to any particular therapy in patients with metastatic disease to the liver especially when it is progressing. Activation of p53 has been shown to sensitize spermatocyte-like cells to recombinant TRAIL or DR5-targeting mAbs (12).

More recent clinical trials have focused on the integration of DR5-targeting mAbs, which have significantly longer plasma half-life than recombinant TRAIL, with first-line radiochemotherapy that remains the mainstay in oncology in order to help improve response rates. Some data from early-phase clinical trials suggest toxicity when DR5-targeting mAbs are administered in combination with chemotherapy. A clinical phase 1 and II study assessing the DR5-targeting antibody conatumumab in combination with FOLFIRI6 plus bevacizumab for the treatment of metastatic colorectal cancer was unable to document an improved response rate with conatumumab. In general, conatumumab was well tolerated but 5% of the patients receiving 10 mg/kg biweekly of conatumumab experienced grade 4 diarrhea and 15% experienced grade 3 hypokalemia (13). A randomized, placebo-controlled phase II study of conatumumab in combination with FOLFIRI for second-line treatment of mutant KRAS metastatic colorectal cancer was able to document a trend toward improved response in the FOLFIRI/conatumumab arm (14). However, the potential for improved response in the FOLFIRI/conatumumab arm was associated with a trend toward an increased number of adverse events, such as diarrhea, neutropenia, fatigue, anemia, and abdominal pain as compared with the FOLFIRI/placebo arm.

We show that targeting of DR5 in mice concomitant with treatment with 5-fluorouracil (5-FU) and CPT-11 triggers a moribund state in the animals not present following either treatment alone, and this phenotype is associated with p53-dependent erosion of the gastrointestinal (GI) epithelium. In the case of CPT-11 plus DR5 targeting, this GI toxicities (GIT) is also dependent on the presence of the gene encoding cell-cycle checkpoint kinase 2 (Chk2). GIT is preceded by apoptosis in the stem cell region of the GI tract associated with loss of Lgr5+ cells. Furthermore, toxicity following this treatment is highly dependent on specific components of the apoptotic machinery downstream of the receptors in a chemotherapy-specific manner. Our results point to an unanticipated molecular complexity of the interaction between the extrinsic and intrinsic apoptosis pathways that has relevance to proapoptotic cancer therapy combinations in the clinic. Interestingly, we show that pharmacologic targeting of Chk2 protects the GI epithelium and facilitates dose escalation without negative impact when treating p53-deficient syngeneic colon cancer cells. A detailed understanding of these interactions along with insights into their potential reversal through pharmacologic intervention may improve the therapeutic index during cancer therapy with proapoptotic drugs that target the TRAIL death receptor pathway.

**Material and Methods**

**Mice and treatment**

Mice were purchased from The Jackson Laboratory. Chk2−/− and DR5−/− mice have been described previously (3, 15) p53+/−; mt/mG; lgr5 and DR5−/−; mt/mG; lgr5 mice were generated in house. All animal care and treatment procedures used were approved by an Institutional Animal Care and Use Committee.

**In vivo labeling of apoptotic cells**

B6.129P2-Lgr5tm1(cre/ERT2)Cle/J mice were injected with SR-FLIVO (ImmunoChemistry Technologies LLC) according to the manufacturer’s instructions. Small intestinal crypts were isolated as previously described (16). Labeled cells (GFP+) and/or FLIVO (red fluorescent) were quantitated with an inverted fluorescence microscope (Zeiss, Axiovert100) coupled to a CCD camera.

**Quantitation of histologic and immunohistochemical findings**

All slide sections were coded, analyzed, and quantified blindly by counting 10 randomly selected 60× or 100× image fields of three nonserial sections of the same specimen. The area percentage of the total GI area subjected to histologic evidence of injury was estimated using ImageJ (NIH Image 1.62 software). Also, the number of TUNEL-positive cells (see Histology and immunohistochemistry in Supplementary Material) was assessed on digital images of specimens with the use of ImageJ (NIH Image 1.62 software). Appropriate statistics were applied to the generated data (see Statistical analysis in Supplementary Material).

**Noninvasive high-resolution near-infrared optical imaging**

Noninvasive near-infrared (NIR) imaging was performed as described previously (17).

**Expression and purification of recombinant human TRAIL**

Expression and purification of rhTRAIL was carried out as described previously (18).

**Results**

**DR5 targeting sensitizes to the GI toxicity inflicted by 5-FU and CPT-11**

In order to assess the toxicity and dose–response characteristics of two commonly used chemotherapeutics fluorouracil (5-FU) and irinotecan (CPT-11) in the absence and presence of DR5-targeting mAbs, we treated mice with an isotype-specific control antibody (IgG) or MD5-1. MD5-1 triggers apoptosis in malignant cells that is dependent on FLIP and completely inhibited by the pan-caspase inhibitor z-VAD-fmk (19). To our surprise, the supplementation of MD5-1 to either 5-FU or CPT-11 caused anorexia and increased lethality at lower than expected doses of the chemotherapeutic agents (Fig. 1A and B and Supplementary Fig. S1A and S1B). For example, all the mice in the 250 mg/kg group of 5-FU (Fig. 1A, right) succumbed when challenged with MD5-1, whereas 60% of the mice were alive when IgG was administered in place of MD5-1. Administering MD5-1 alone at this dose level, without the presence of either chemotherapeutic, was not toxic to mice for an observational period of up to 90 days (Supplementary Fig. S1C and data not shown). From the two parameters (weight loss and moribund state) that were being assessed, weight loss was the more sensitive endpoint (with an approximately 2-fold lower ED50 data not shown). Subsequently, we used weight loss area under the curve (AUC) values to generate dose–response relationships for CPT-11 and 5-FU in the presence or absence of the DR5-targeting mAb MD5-1 (Supplementary Fig. S1A and S1B; Fig. 1C and D). From the dose–response curves
it is clear that the presence of the DR5-targeting antibody MD5-1 sensitized the mice to weight loss and anorexia. The ED$_{50}$ values for CPT-11 was 416.6 mg/kg (95% CI, 383.9–452.0 mg/kg) in the presence of control IgG and 175.7 mg/kg (95% CI, 143.5–215.2) in the presence of MD5-1 (Fig. 1B, Table). The corresponding values for 5-FU were 375.7 mg/kg biweekly (95% CI, 260.6–541.6) and 106.1 mg/kg (95% CI, 97.4–115.6) in the absence (IgG) and presence of MD5-1, respectively (Fig. 1B, Table). Thus, based on weight loss as a toxicity endpoint, mice were able to withstand 2.4- and 3.6-fold less CPT-11 and 5-FU, respectively, following targeting of DR5 with agonistic Abs in vivo. Given that CPT-11 and 5-FU are commonly administered to patients in combination for the treatment of metastatic colorectal cancer as part of the FOLFOX regimen, we used allometric dose-conversion factors from human to mouse for doses of 5-FU and CPT-11 used in clinical protocols. Following treatment with 5-FU/CT-11/MD5-1, we found that wild-type mice developed anorexia and succumbed to treatment when the DR5-targeting antibody MD5-1 was included in the treatment regimen (Fig. 1E and F and data not shown). Interestingly, mice lacking one or both alleles of DR5 (DR5$^{-/}$ and DR5$^{-/-}$) were resistant to anorexia (P < 0.001, Student t test) and protected from the lethality (P < 0.001, log-rank Mantel–Cox test) triggered by the CPT-11/5-FU/MD5-1 combinatorial treatment (Fig. 1E and F). In contrast to our findings with MD5-1, when recombinant mouse TRAIL (rmTRAIL) or rhTRAIL was combined with CPT-11, no sustained anorexia or moribund state was encountered (Supplementary Fig. S1D). This could potentially implicate a protective role for DcRs that may be abundantly expressed on normal cells. Collectively, our data suggest that DR5-agonistic antibodies may exacerbate toxicity when combined with chemother-apy commonly used for the treatment of colorectal cancer. 

**DR5-dependent liver toxicity occurs following high-dose treatment with MD5-1**

Previously reported data have shown that liver toxicity may arise in mice following higher doses of MD5-1 than applied here (9). Mice that became anorexic in our model did not show visible signs of liver cholestatic disease nor where there histologic signs of injury in other organs than the GI tract (Supplementary Fig. S2A). We found that MD5-1 alone was well tolerated without any obvious signs of toxicity up to a dose level of 20 mg/kg (Supplementary Fig. S2B and S2C). Consistent with the previous report, necropsy indicated that weight loss was well correlated with yellow skin discoloration, enlarged gall bladders, and histologic signs of periporal loss of cholangiocytes and hepatocyte atrophy (Supplementary Fig. S2D). Interestingly, the addition of a single dose of CPT-11 (40 mg/kg i.v.) caused sensitization of mice to MD5-1 with respect to cholestatic disease at a dose of 10 mg/kg of MD5-1 (Supplementary Fig. S2C). Furthermore, we assessed the impact of mouse strain on the observed GIT inflicted by combined DR5 targeting and chemotherapy because a strict strain dependency has been described for the manifestation of liver toxicity following MD5-1-exposure (9). In contrast to what has been reported, we found that the combination of 5-FU/MD5-1 triggered anorexia and a moribund state in previously reported “resistant” strains with a slightly delayed response compared with that of C57BL6 (Supplementary Fig. S2E and S2F). One exception to this was the CB17 SCID mouse strain that was found to be highly sensitive to the 5-FU/MD5-1 combination. This may indicate that chemotherapy sensitizes to DR5 target-
injected mice with MD5-1 (Supplementary Fig. S3C). Wild-type mice subjected to WBR/MD5-1 and lacking AS showed a trend to increased weight loss as compared with wild-type mice that had their mid-section shielded and received WBR/MD5-1 (Supplementary Fig. S3D and S3E). Furthermore, we subjected mice to lethal doses of WBR and performed BM transplantation (BMT) with BM lacking DR5 (DR5−/− BM) (Supplementary Fig. S3F). Female recipient mice received male DR5−/− BM, and 40 weeks
after BMT we were able to confirm complete engraftment of the DR5−/− BM in the mice (Supplementary Fig. S3G). However, mice receiving DR5−/− BM showed equal sensitivity toward 5-FU and CPT-11, suggesting that loss of DR5 specifically in the BM did not rescue mice from the toxicity of DR5 targeting in combination with chemotherapy (Supplementary Fig. S3H and S3I). Taken together, our data indicate that the GI tract and not the BM is the bona fide site for our observed acute organ toxicity following targeting of DR5 in combination with 5-FU and CPT-11.

**DR5 targeting sensitizes to 5-FU- and CPT-11-induced cell death in the GI tract**

Given the propensity of DR5 targeting to trigger apoptosis, we assessed the GI tract for apoptotic cells following 5-FU, CPT-11, and MD5-1. H&E staining of colon isolated at 24 hours following treatment showed epithelial erosion and inflammation in mice when combined with 5-FU and CPT-11 (Fig. 2A). Quantification of the number of crypts/mm in histologic sections of the small intestine (B) and the colon (C) from mice treated with chemotherapy and MD5-1 indicates increased probe retention in wild-type animals.
treatment with 5-FU and MD5-1 suggested that the presence of apoptotic cells by morphology and TUNEL staining following both treatments (Fig. 3A–D). Loss of DR5 completely protected the crypts from cell death induced by MD5-1 (Fig. 3D and Supplementary Fig. S4A and S4B). Flow-cytometric sub-G1 analysis and in vivo FLIVO labeling of isolated intestinal epithelial cells confirmed the increased presence of apoptotic cells when DR5-targeting MD5-1 antibodies was combined with CPT-11 (Supplementary Fig. S4C and S4D). We used the untransformed human fetal GI epithelial cell line FHs74Int to assess the impact of DR5-targeting following CPT-11 and 5-FU. Consistent with our mouse in vivo findings, the FHs74Int cells were sensitized to apoptosis when the human DR5-agonistic monoclonal antibody mAb631 was combined with either CPT-11 or 5-FU (Supplementary Fig. S4C and S4D). Furthermore, caspase-3/7 was increasingly activated as compared with control following mAb631 and CPT-11 (Fig. 3E). Indeed, a substantial increase in caspase-3/7 activation over that of either compound alone was observed following the combination of CPT-11/mAb631. The combination treatment significantly inhibited growth of the FHs74Int cells over 72 hours in comparison to either compound alone (Fig. 3F). Interestingly, caspase-3/7 activation following mAb631 alone was poorly correlated with long-term cell survival of mAb631-treated FHs74Int cells. Furthermore, CPT-11 dose-dependent activation of caspase-3/7 was observed only in the presence of mAb631 and subsequently correlated with growth inhibition (Fig. 3G and H and Supplementary Fig. S4G). Thus, untransformed human GI epithelium might be increasingly sensitized to undergo apoptosis following DR5 targeting in the presence of high doses of CPT-11. Our findings are consistent with and appear to validate our earlier mouse model observations on apoptosis induction.

Combined selective DR5 targeting with CPT-11 and 5-FU sensitizes Lgr5+ stem cells to cell death

It was somewhat surprising that MD5-1 triggered cell death in the GI tract of mice without cotreatment with chemotherapy (Fig. 3A–D and Supplementary Fig. S5A–S5C). However, the magnitude of the cell death and its crypt spatial involvement was altered following the addition of either 5-FU or CPT-11. Scoring the crypt positions most frequently affected following CPT-11/MD5-1 and 5-FU/MD5-1 treatments revealed that apoptosis (as determined by TUNEL staining) was more frequently affecting the crypt bottom and crypt base columnar (CBC) cells (Fig. 4A–C and Supplementary Fig. S4A–S4C). In order to verify that CBC cells were increasingly targeted to death following the inclusion of DR5-targeting mAbs with chemotherapy, we performed IHC for Lysozyme C (green fluorescence), a marker for paneth cells in the crypt bases.

Figure 3. Apoptosis in the GI tract following targeting of the GI epithelium with chemotherapy and DR5-targeting antibodies. A, H&E staining of the colon from mice treated with isotype control antibodies (IgG), DR5-agonist antibodies (MD5-1), 5-FU, or 5-FU combined with DR5-agonist antibodies (5-FU/MD5-1, F/M). Red arrows, apoptotic cells in colonic crypts. Representative images are shown. B, image analysis of TUNEL-stained histologic sections of colon isolated from mice treated with 5-FU and DR5-agonist MDS-1 antibodies. Error bars, SE from the mean. C and D, TUNEL staining (brown stain) of the colon from treated mice (C) and the graphical representation of image analysis of TUNEL-stained slide sections from treated mice (D). Error bars, SE from the mean. E and F, caspase-3/7 activation (E), and change in cellularity (F) of the normal GI epithelial cell line FHs74Int following treatment with the DR5-receptor agonist antibody mAb631 (M; 1.0 µg/mL), CPT-11 (C; 280 µg/mL) and the combination thereof (C/M). Error bars, SE from the mean. Statistical analysis was performed by a two-way ANOVA test using Bonferroni correction. P < 0.05 was considered statistically significant.
Figure 4.

GIT following combined DR5 targeting and chemotherapy is associated with depletion of Lgr5\(^+\) GI stem cells. Combinatorial treatment with 5-FU (F)/MD5-1 (M) (A) and CPT-11 (C)/MD5-1 (M) (B) triggered more frequent apoptosis in the crypt bottom (cell position 0–3) as compared with either modality alone. Error bars, SE from the mean. C, IHC for paneth cells (Lysozyme C; Cy2; green fluorescence) and apoptotic cells (TUNEL; Cy3; red fluorescence). Representative pictures from at least 3 mice are shown. D, IHC of transverse SI (ileum) sections showing crypt bottoms positive for EGFP (Lgr5\(^+\) cells) in Lgr5-EGFP-Cre-ERT2\(^+\) and DR5\(^+/−\) transgenic mice following treatment with CPT-11/MD5-1 (C/M). Representative pictures of three mice are shown. E, IHC for GFP on small intestines isolated from Lgr5-EGFP-cre-ERT2 mice subjected to no treatment (V), MD5-1 (M), 5-FU (F), or the combination of 5-FU/MD5-1. F, \textit{in vivo} labeling of apoptotic cells in Lgr5-EGFP-Cre-ERT2 mice using SR-FLIVO and subsequent quantification of crypts containing cells with combined green and red fluorescence following treatment with vehicle, MD5-1, CPT-11, or the combination of CPT-11/MD5-1 (C/M). Error bars, SD from the mean.
and DR5 targeting. However, the combinatorial treatment had no such effect in DR5−/− mice subjected to CPT-11/MD5-1 (Supplementary Fig. S5A–S5C). Considering the deleterious impact the combined DR5 targeting had on the GI cell homeostasis, we sought to address if the inclusion of MD5-1 with chemotherapy increasingly included Lgr5+ CB cells in the cell death response by generating DR5−/−; Lgr5-EGFP-Cre-ERT2 (DR5−/−; Lgr5−/+i) mice (21). These mice express EGFP under the Lgr5 promoter and EGFP-expressing cells can readily be detected by performing IHC for EGFP. Indeed, mice lacking DR5 never displayed loss of EGFP-positive cells following treatment with either CPT-11 or 5-FU combinations as compared with treatment with single modalities of 5-FU, CPT-11, or MD5-1 (Fig. 4D and E). EGFP+ cells with apoptotic morphology were a feature observed almost exclusively in wild-type lgr5-EGFP-CreERT2 (lgr5+) mice treated with the combinations of either CPT-11/MD5-1 or 5-FU/MD5-1 (Fig. 4D). We also used in vivo SR-FIJO labeling to detect apoptotic lgr5+ cells following crypt isolation (Supplementary Fig. SSD and SSE and Fig. 4F). Mice subjected to treatment with CPT-11/MD5-1 showed increased SR-FIJO uptake in GFP+ crypt cells ex vivo compared with crypts isolated from lgr5− mice treated with vehicle, MD5-1, and CPT-11 (Fig. 4F). Moreover, fewer Lgr5+ (EGFP+) cells were obtained following treatment with the combination treatment of CPT-11/MD5-1 compared with treatment with either compound alone (Supplementary Fig. SSE). Taken together, our data indicate that Lgr5+ cells are a de novo target for selective DR5 targeting when combined with chemotherapy.

Both 5-FU and CPT-11 triggered the stabilization of p53 in the colonic epithelium of mice (Fig. 5A). Following 5-FU, induction of p53, p21, and at later time points c-Myc was observed (Fig. 5B). Expression profiling of colonic mRNA at 24 hours following either CPT-11 or 5-FU revealed the induction of several “apoptosis-inducers” as well as “p35-dependent genes” (Supplementary Fig. S6A and S6B). Indeed, mice lacking p53 (p53“null”) in all tissues were completely protected from the morbidity incurred by 5-FU/MD5-1 (Fig. 5C). While these mice were transgenic mice lacking critical molecules for cell-cycle arrest and apoptosis signaling following DNA damage to treatment with 5-FU/MD5-1 and CPT-11/MD5-1 (Supplementary Fig. S7). Predictably, we found that the toxic responses to both treatments in the mice required the presence of DR5 (Supplementary Fig. S7B and S7H); however, surprisingly the reliance on caspase-3, bid, puma, and p21 to trigger GIT was remarkably different for the two drug combinations. Although puma was upregulated in the colonic epithelium following treatment with either 5-FU or CPT-11, puma was only required to trigger toxicity following 5-FU/MD5-1 (Supplementary Fig. S7C and S7I). By contrast, bid appears to be required for toxicity following CPT-11/MD5-1, although this trend did not reach statistical significance (P = 0.1712, log-rank test). Loss of bid appeared to protect the colonic epithelium from apoptosis (as evaluated by TUNEL staining) following CPT-11/MD5-1 but not 5-FU/MD5-1 (data not shown). Interestingly, loss of the cell-cycle regulator p21 also sensitized to CPT-11/MD5-1 (P = 0.0002, log-rank test) (Supplementary Fig. S7D, S7E, S7I, and S7K). Taken together, our data support the requirement of intact p53 signaling to trigger GIT. However, downstream p53 targets show a differential requirement for the triggering of GIT following DR5 targeting in combination with chemotherapy.

In order to more specifically assess the impact of lost p53 expression in Lgr5+ cells we generated p53fl/fl; lgr5; mTmG mice that would allow deletion of p53 in Lgr5+ cells and subsequent lineage tracking of cell progeny using the Cre-reporter mT/mG transgene (22). Successful deletion of p53 was also assessed by IHC for p53 on serial section of GI tracts isolated from mice subjected to treatment with 5-FU (Fig. 5D). Deletion of the p53 gene in Lgr5+ cells through the use of p53fl/fl;lgr5 mice concomitantly with treatment with CPT-11/MD5-1 partially rescued villous blunting and reduced toxicity (Fig. 5E). Furthermore, lineage tracking of the progeny of Lgr5+ cells was improved when p53 was deleted in Lgr5+ cells through administration of tamoxifen to p53fl/fl; lgr5; mT/mG mice immediately prior to treatment with CPT-11/MD5-1 (Fig. 5F). These data indicate that Lgr5+ cells in the GI tract are increasingly targeted when DR5 agonists are combined with 5-FU and CPT-11.

Targeting of Chk2 prevents GIT following the combination CPT-11/MD5-1

Considering that some key molecules involved in the proapoptotic DNA damage response (DDR) were found to be critical determinants of the toxic response to chemotherapy combined with DR5-targeting mAbs we hypothesized that targeting of the cell-cycle checkpoint kinase 2 (Chk2) could potentially modulate such toxicities. Interestingly, mice lacking the Chk2 gene (Chk2−/−) were susceptible to toxicity following treatment with 5-FU/MD5-1 but not CPT-11/MD5-1 (Fig. 6A). In fact, Chk2−/− mice appeared to be completely resistant to developing a moribund state triggered by the CPT-11/MD5-1 drug combination. These findings translated into less epithelial atrophy and a reduction in crypt apoptosis in the colons of Chk2−/− mice following CPT-11/MD5-1 (Supplementary Fig. SSA and 6C). Using a pharmacologic Chk2 inhibitor (Chk2 inhibitor II [CI-II]; ref. 23) resulted in a decreased moribund state and reduced the P56S0 signal from the GI tract of wild-type mice treated with CPT-11/MD5-1 (Fig. 6D and Supplementary Fig. S8B–S8D). Thus, our data indicate that targeting of Chk2 at the genetic level or the kinase function of Chk2 may prevent GI toxicity when the DR5 receptor is being targeted in combination with CPT-11.

Gene expression analyses indicated that CPT-11 triggered repression of inhibitor-of-apoptosis protein (IAP) survivin (birc5; ref. 6F and data not shown). We targeted expression of survivin using siRNA in human FHS74Int cells and subjected such cells to treatment with CPT-11/mAb631 (Fig. 6F). Indeed, targeting of antiapoptotic survivin resulted in increased activation of caspase-3/7 following CPT-11/mAb631, suggesting that survivin has a functional role in repressing apoptosis following DNA damage and DR5 targeting. Given the in vivo role of Chk2 to protect mice from toxicity following CPT-11/MD5-1 we tested the capacity of three different Chk2 inhibitors in PV1019 (24), CI-II, and CI-III (25) to prevent caspase-3/7 activation in FHS74Int cells following CPT-11/mAb631 (Supplementary Fig. S8E). Indeed, a dose-dependent repression of caspase-3/7 activation was observed following cotreatment with the Chk2 inhibitors, suggesting a functional role of Chk2 in mediating cell death in human cells following DNA damage and DR5 targeting.
To address if the newly found molecular mechanism could be used to reduce GIT and improve treatment efficacy of the combination of CPT-11 and DR5-targeting mAbs we used a reduced dose of CPT-11 (116 mg/kg) to treat syngeneic mouse colon cancer cells (p53dmc/Ras/Myc) overexpressing mutated K-Ras and c-Myc in syngeneic mice in vivo. The combination of CPT-11/MD5-1/CI-II (C/M/CI-II) appeared to improve the response rate of subcutaneous p53dmc/Ras/Myc grafts as compared with that of mice treated with CPT-11/MD5-1/vehicle (C/M/V). Although the observed difference in tumor load was not statistically significantly different between the treatments at the end of the experiment ($P = 0.26$, Mann–Whitney test), the tumor doubling times in days for control (vehicle alone), C/M/V, and C/M/CI-II were 2.4 (95% CI, 2.24–2.54), 4.6 (95% CI, 3.56–6.58), and 6.2 (95% CI, 4.25–11.67) indicated significant differences between the treatment groups (Supplementary Fig. S9A and S9B). Interestingly, mice subjected to the combinatorial treatment of C/M/CI-II lost less body weight throughout the experiment than mice subjected to treatment with C/M/V (Supplementary Fig. S9C and S9D). No sign of increased toxicity was observed in the mice when CI-II was combined with CPT-11 and MD5-1, suggesting that the Chk2 inhibitor was “nontoxic” in this context.
Figure 6.

Chk2 controls GI toxicity following DR5 targeting and CPT-11 treatment. A, body weight loss and moribund state following treatment with CPT-11 plus MD5-1 is controlled by Chk2. Error bars, SE. B, TUNEL staining of colonic crypts at 24 hours following treatment with combinations as indicated and histologic evaluation (right). Representative images are shown. C, weight loss and survival curves generated following cotreatment of mice with CPT-11/MD5-1 (C/M) with Chk2 inhibitor II (CI-II). The data were analyzed for statistically significant differences (P < 0.05) using the two-way ANOVA and the log-rank test. Error bars, SE. D, Western blot of colonic scrapings from wild-type and Chk2−/− mice subjected to treatment with 5-FU and CPT-11 for 24 hours. E, Western blot assessment of siRNA-mediated survivin/birc5 knockdown efficacy in FHs 74Int cells. F, activation of caspase-3/7 in FHs74Int cells subjected to scrambled (CTRL) siRNA, siRNA targeting survivin and following treatment with CPT-11 (280 μg/mL) and mAb631 (1.0 μg/mL) for 18 hours. An unpaired Student t test was used for the statistical analysis. P < 0.05 was considered statistically significant and an N = 3/treatment group was used. The mean with standard error bars are shown.
In summary, our data indicate that the GIT observed in our models differs with respect to the molecular underpinnings in a chemotherapeutic-specific manner despite sharing a similar pathologic manifestation in the GI tract of mice (Fig. 7). Both treatments are dependent on intact DR5 and p53 signaling; however, the relative dependency on canonical downstream molecules of the p53 response is altered depending on the specific chemotherapeutic when DR5-targeting mAbs are included. The combination of CPT-11/MD5-1 relies increasingly on a cell-cycle (p21)–dependent mechanism, bid, and Chk2 to trigger GIT (Fig. 7A), whereas the combination of 5-FU/MD5-1 relies mainly on puma to inflict injury to the GI tract of mice (Fig. 7B). Although we did not address this directly, previous data of others and ours suggest that Myc upregulation may sensitize to DR5 targeting (26). Thus, Myc upregulation and the inherently rapid GI epithelial regeneration following injury might cause Lgr5⁺ GI stem cells to become increasingly sensitized to death receptor agonists.

**Discussion**

Our work addresses the dose-limiting toxicity in the GI tract due to combinations of chemotherapeutic or radiotherapy plus specific targeting of the DR5 death receptor pathway and provides insights that in the future may be pursued to reduce toxicities while maintaining or improving the therapeutic index. In particular, targeting Chk2 may be an attractive strategy to pursue further to reduce dose-limiting toxicity in the GI tract. In order to address the possibility of toxicity when DR5 is targeted in combination with chemotherapy we treated mice with a DR5-agonistic mAb (MD5-1) in combination with 5-FU and CPT-11. Our data indicate that low dose (1.0 mg/kg) supplementation of DR5 targeting mAbs in combination with either 5-FU and CPT-11 is sufficient to trigger a lethal GIT syndrome in mice (Figs. 1 and 2; Supplementary Figs. S1 and S2A–S2F). The GIT observed with combined DR5 targeting plus chemotherapy is completely dependent on the presence of DR5 and the tumor suppressor p53 in mice (Figs. 1E and F and 5C). Furthermore, we found that this toxicity was dose limiting for the treatment of syngeneic colorectal tumor grafts (Supplementary Fig. S9A–S9D) and correlated with increased apoptosis in the TA zone of the gut as the disappearance of Lgr5⁺ CBC cells (Fig. 4A–C and Supplementary Fig. SSD–SSF).

Lgr5⁺ crypt basal columnar cells have been reported to be indispensable for epithelial restitution and as a predictor of lethality in mice from the radiation-induced GI syndrome (27–29). These prior findings are in agreement with findings in our model, where chemotherapy (or DR5 targeting alone) is sufficient to trigger cell death in the TA zone of (ileal) small intestinal crypts. However, cell death in the TA zone per se does not appear to be sufficient to permanently disrupt crypt homeostasis without the addition of a DR5-targeting mAb. Thus, it seems plausible that the result of the perturbed GI epithelial cell homeostasis could result from TA zone injury that sensitized Lgr5⁺ cells to DR5 targeting. Further model development is required to address those observations in depth and elucidate differences in death receptor signaling between normal and stem cells in the GI tract. Our reported data clearly stand in contrast to the majority of preclinical findings that suggest targeting of DR5 is essentially nontoxic to normal cells and tissues in combination with DNA damaging chemotherapy. One potential reason for this is that assessment of toxicity following treatment with TDRA in mice is difficult due to inherent differences between the genome of mice and humans with respect to genes that influence TRAIL signaling. For example, the mouse genome harbors only a single death-inducing TRAIL receptor gene in DR5, in contrast to humans who have two in DR4 and DR5. Subsequently, mouse models would not be useful to address potential toxicity of DR4 targeting that may potentially manifest in a different manner from that of DR5 targeting. Thus, it is important to stress that the mouse model presented here limits itself to assessing toxicity following selective targeting of DR5 due to...
the mentioned differences. It is unclear to what extent any of the clinically relevant DR5 mAbs cross-react with mouse DR5 and toxicity assessment, and the establishment of a LOAEL in rodent models may not be possible for some TDRAs.

To the best of our knowledge, MD5-1 is the only reported antibody that targets mouse DR5 in an agonistic manner. A recent study showed that the combination of conatumumab or MD5-1 with recombinant APO2L/TRAIL greatly improved antitumor responses (30). Both conatumumab and MD5-1 require cross-linking to exhibit antitumor activity alone (19, 31), but this requirement is largely abrogated by the addition of APO2L/TRAIL, potentially as a result of higher order of DR5 clustering on the cell surface by the ligand–antibody combination (30). This study also showed that the coadministration of MD5-1 (at a 200 times higher dose than the one used for the majority of the experiments in this study) together with APO2L/TRAIL triggered a gross and histologically similar GIT to what was observed here when MD5-1 was combined with CPT-11 and 5-FU. A proposed reason for the GIT was the abundant expression of DR5 in the GI tract of mice, a characteristic of the mouse alimentary tract that is shared with that of primates and humans as well, indicating a translational nature of this particular finding.

It is unlikely that the severity of toxicity observed in our animal model will translate directly to clinical observation for a number of reasons. For example, the mice in this study were given a single (high-dose) bolus of chemotherapy, whereas 5-FU is administered as a cycled 46-hour intravenous infusion in patients, a schedule that is challenging to mimic in laboratory animal models. Subsequently, no dose adjustment would be possible in our model nor did the mice receive supportive care to the same extent that is typical for cancer patients undergoing chemotherapy treatment. Unfortunately, chemotherapy-induced GIT is a common dose-limiting, costly and quality-of-life prohibitive toxicity, and in contrast to preclinical animal models, accurate quantitative assessment of GIT remains difficult in patients. Estimation of GIT to such treatment is largely based on the presentation of symptoms that may not correlate well with the extent of GI injury or its localization in the GI tract (32). Although MD5-1 can readily generate significant antitumor responses in several preclinical models (Fig. 5F and N; refs. 19, 33), such responses have not been observed in clinical trials where DR5 targeting mAbs are combined with chemotherapy. Therefore, the potential for toxicity would be considered in relation to the limited improvement of the response that may be expected when TDRAs are integrated into current clinical chemotherapy protocols. Furthermore, our data suggest that chemotherapeutics that inflict DNA damage may nonselectively lower the threshold dose to DR5-targeting mAbs in both normal and malignant tissues or perhaps even preferentially do so in normal cells that harbor an intact P53 response.

Based on our gained knowledge of the DDR pathways involved in mediating toxicity following targeting of TRAIL-death receptors in combination of chemotherapy, we aimed to assess if targeting molecules within the DDR pathway could modulate GIT. Molecular targeting of Chk2 has previously been proposed as a method to improve therapeutic indices for DNA-damaging modalities such as radiotherapy (34). To our surprise, we found that mice lacking the Chk2 gene were highly resistant to toxicity inflicted by CPT-11/MD5-1 but not 5-FU/MD5-1 (Fig. 6A and B). Furthermore, pharmacologic targeting of Chk2 kinase function using a 2-arylbenzimidazole Chk2 inhibitor (CI-II; ref. 23) reduced toxicity following CPT-11/MD5-1 (Fig. 6D). Although mice devoid of Chk2 were resistant to lethal GIT inflicted by CPT-11/MD5-1, they were not protected from lethal GIT inflicted by CPT-11 alone following dose escalation (data to be published elsewhere). This finding indicates that the combined agonist targeting of the death receptor DR5 in the face of DNA damage inflicted by the chemotherapeutic CPT-11 triggers GIT through a molecularly distinct mechanism from that observed following DNA damage alone.

Expression data indicate that in contrast to 5-FU, CPT-11 downregulates the expression of Survivin in a chk2-dependent manner (Fig. 6E). Survivin is an established negative regulator of death receptor stimuli (35) suggesting that selective upregulation of Survivin following Chk2 targeting can prevent GIT following CPT-11/MD5-1. Subsequently, we addressed the possibility that targeting of Chk2 could help improve therapeutic indices of CPT-11/MD5-1 when treating colorectal cancer. Indeed, in syngeneic grafts of the mouse colorectal cancer cell line p53dmc/Ras/Myc we were able to show that combining CI-II with CPT-11/MD5-1 resulted in reduced GIT and at the same time improved the tumor response rate (Supplementary Fig. S9A and S9B). Our data indicate that this type of strategy may not only improve the tumor response rate to treatment but also indirectly translate to increased efficacy as a DLT is prevented that otherwise may result in the discontinuation of treatment.

Chemotherapy currently remains the mainstay in oncology, and additional knowledge of how such modalities influence the response to DR5 targeting could help shape strategies that minimize toxicity to normal tissues. The potential of toxicity should also be considered when new combination therapies, including DR5 agonists, are designed because our data indicate that DR5 targeting can augment dose-limiting toxicities of conventional chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Agonists of the TRAIL Death Receptor DR5 Sensitize Intestinal Stem Cells to Chemotherapy-Induced Cell Death and Trigger Gastrointestinal Toxicity

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