Combining radiotherapy with death ligands in cancer treatment: feasibility and molecular mechanisms

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Combining radiotherapy with APO010 in cancer treatment

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Key words: Fas/CD95, death receptor, radiotherapy, apoptosis, xenograft

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Purpose: Various proapoptotic agents are currently being explored to improve the outcome of radiotherapy. We have evaluated whether APO010 - a novel recombinant ligand of the Fas/CD95 death receptor - enhanced the cytotoxic effect of radiation on lymphoid and solid tumor cell types. Experimental Design: A Bcl-2-overexpressing T-leukemic cell line (Jurkat), a colon carcinoma cell line (HCT116), and a mesothelioma cell line were used as model systems in vitro and in a subcutaneous transplant setting in immunodeficient mice. Sensitivity to single and combined treatment was read out by apoptosis hallmarks and clonogenic survival in vitro, and by tumor growth delay using bioluminescence and palpation in vivo. Results: Whereas the three cell lines resisted apoptosis induction by irradiation and APO010 alone, combined treatment greatly enhanced their apoptotic response. In clonogenic survival assays, APO010 reduced the outgrowth of Jurkat-Bcl-2 and HCT116 cells and sensitized the mesothelioma cell line to radiation. In vivo, systemic treatment with APO010 alone caused tumor growth delay in Jurkat-Bcl-2 and HCT116 cells. However, APO010 did not improve the efficacy of radiotherapy in any of the model systems at the selected single dose, which had moderate and reversible systemic toxicity. Conclusions: Although APO010 and radiation had a clear combined cytotoxic effect on tumor cells in vitro, a combined therapeutic effect was not achieved on the same cells subcutaneously grafted in mice, at APO010 doses approximating the maximally tolerable level. These findings suggest that it will be difficult to identify a therapeutic window for this combined modality approach in a clinical setting.

Introduction

The success of radiotherapy in the treatment of cancer is limited by the small window of differential sensitivity between normal tissue and tumor cells and the radioresistance of tumor tissue. Combination of radiotherapy with conventional chemotherapeutics, in particular, cisplatin [1], has improved efficacy, but treatment results are still suboptimal. Current knowledge on the molecular mechanism of apoptosis can now be applied to rationally design new therapeutics that achieve higher cytotoxic efficacy, bypassing death resistance in tumor cells while leaving normal tissues unaffected. These drugs should preferably induce cell death by molecular mechanisms distinct from those activated by conventional chemotherapy/radiotherapy because this would enable additive or synergistic interactions and reduce the risk of therapy resistance. Ionizing radiation, cisplatin, and many other conventional chemotherapeutics induce DNA damage, which is translated into a variety of cellular responses by partially defined signaling events. In response to DNA damage, cells arrest in cycle, in a p53-dependent or p53-independent manner to enable DNA repair. Failing this, apoptotic cell death, irreversible cell cycle arrest, or cell death due to mitotic catastrophe may ensue, all resulting in lack of clonogenicity [2]. Apoptosis induction by DNA-damaging regimens—which can occur in both p53-dependent and p53-independent ways—can be blocked by Bcl-2, indicating that it is reliant on the mitochondrial apoptosis signaling route [3]. To what extent apoptosis contributes to the antitumor effect of (fractionated) radiotherapy, remains a matter of debate and depends on many factors, including cell type. It should be noted, however, that modulation of apoptosis-regulating genes, including Bcl-2, might have an affect on clonogenicity and tumor response after radiation in vitro and in vivo [4].

Death receptor ligands, in particular, Fas (APO1/CD95) ligand (L) and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) are potentially interesting for combination therapy because of their mechanism of apoptosis induction. They operate irrespective of the p53 status of cells and can activate effector caspases independently of the mitochondria, that is, via a pathway other than that used by DNA-damaging regimens [5]. In this scenario, with ionizing radiation hampering clonogenicity (by cell cycle arrest, mitotic catastrophe, and mitochondrial-dependent apoptosis) and death receptors inducing apoptosis (in part via a mitochondrial-independent pathway), it can be envisioned how combined treatment can increment the antitumor response and improve therapeutic outcome in radiotherapy. Preclinical studies indicated a number of years ago that soluble recombinant TRAIL and agonistic antibodies directed against TRAIL receptor-1 and -2 are promising anticancer therapeutics. TRAIL induced apoptosis in a large proportion of long-term established tumor cell lines, but was not toxic to normal tissue [6, 7]. Presently, several phase I and II clinical trials with TRAIL receptor agonists have been completed and have corroborated low toxicity [8]. Preclinical studies indicate, however, that for optimal efficacy, these reagents must be
combined with conventional or novel therapeutics. These studies have also revealed that radiation and conventional anticancer drugs can sensitize tumor cells to TRAIL-induced apoptosis by a variety of mechanisms (ref. [9] and references therein). Clinical trials will have to point out whether these encouraging experimental observations are predictive of the patient response to combined modality treatment.

Although TRAIL receptors and Fas induce apoptosis by highly similar mechanisms [5], Fas agonists have not been pursued for cancer therapy. Such application was discouraged by the early observation that systemic treatment of mice with anti-Fas antibody resulted in acute, lethal hepatotoxicity [10]. Recently, however, a novel form of soluble recombinant FasL has been generated. This so-called MegaFas Ligand—currently named APO010—is composed of a fusion protein of the extracellular domain of FasL and the collagen domain of ACRP30. Whereas FasL naturally forms a trimer, the ACRP30 domain imposes a hexameric configuration of the molecule, i.e., a dimer of FasL trimers. The dimeric state allows APO010 to crosslink two adjacent Fas receptor trimers, which renders it highly agonistic as compared with other available forms of soluble recombinant FasL or anti-Fas antibodies [11-13]. We reasoned that, in radiotherapy, a local combined therapeutic effect might be achieved at APO010 concentrations that have acceptable systemic toxicity or none at all. Therefore, this potential application was evaluated. We have tested—both in short-term apoptosis assays as well as in clonogenic survival assays—the in vitro responsiveness of lymphoid and solid tumor types to combined treatment with ionizing radiation and APO010. We next grafted these tumor cells into nude mice and monitored tumor regression as well as normal tissue toxicity upon single and combined modality treatment.

**Results**

**Apoptic responses to ionizing radiation and APO010 in vitro**

The apoptotic response of the model cell lines was tested in short-term assays. Whereas wild-type T-leukemic J16 cells are sensitive to radiation-induced apoptosis, Bcl-2 overexpression is known to render them resistant [9, 14], as also observed here (Fig. 1A). J16-Bcl-2 cells underwent apoptosis upon treatment with APO010 alone at doses higher than 0.1 ng/mL, but their sensitivity was significantly increased when APO010 was combined with radiation (Fig. 1A).

HCT116 colon carcinoma cells (Fig. 1B) and 03JJ0215 mesothelioma cells (Fig. 1C) underwent low-level apoptosis upon treatment with radiation alone and were hardly sensitive to APO010 alone at doses up to 10 ng/mL, but displayed significantly increased apoptosis.

**Translational Relevance**

APO010, a novel recombinant form of Fas/CD95 ligand, is currently in a phase I dose-escalation trial in patients with solid tumors. We have investigated the feasibility of combination therapy with APO010 and radiotherapy in leukemic and solid tumor cell types. This approach is attractive because a potential combined treatment effect would be local and limit normal tissue toxicity. By analogy to what has been documented for tumor necrosis factor-related apoptosis-inducing ligand and radiotherapy, we found a significant combined effect of both treatment regimens on cell death induction, as read out by apoptosis and clonogenic survival in vitro. In vivo, however, the combination of systemically administered APO010 and radiotherapy did not significantly enhance tumor growth delay, as compared with the single modality treatments. Toxicity data indicated that further dose-escalation of APO010 was not achievable. We suggest therefore that future studies should address the efficacy of locally applied APO010 as a radiation-enhancing agent.

![Fig. 1](image-url)
Antitumor effects of radiotherapy and Fas/CD95 stimulation

We found that radiation could sensitize all three tumor cell lines for APO010-induced apoptosis in a dose-dependent fashion (data not shown). A single dose of 10 Gy was found to produce optimal sensitizing conditions as compared with the lower doses of irradiation. We conclude that for all three cell lines, combined treatment with radiation and APO010 strongly enhanced the apoptotic response.

Clonogenic survival of irradiated cells cultured in the absence or presence of APO010

To investigate the combined effects of radiation and APO010 on cell survival, we used long-term clonogenic assays that incorporate effects on cell cycle activity and apoptotic as well as nonapoptotic modes of cell death [2]. A direct comparison of clonogenicity after treatment with radiation alone revealed that J16-Bcl-2 and HCT116 cells had a similar degree of radiosensitivity, whereas 03JJO215 cells were more radioresistant (Supplemental Fig. S1). It should be noted that at low doses of ionizing radiation, the survival curve of HCT116 and 03JJO215 cells showed a small shoulder region, indicative of sublethal DNA damage repair. In agreement with the sensitivity to APO010-induced apoptosis, the number of surviving colonies of J16-Bcl-2 cells was clearly reduced after treatment with APO010 alone (Fig. 2A). This was observed to a lesser degree for HCT116 cells and was not noticeable for 03JJO215 cells (Fig. 2B and C). The clonogenicity of untreated cells or cells treated with APO010 alone at 0 Gy was normalized to 100% to yield the curves depicted in Fig. 3. In case of J16-Bcl-2 and HCT116, the curves indicating clonogenic survival after treatment with different doses of radiation alone (control) and combined treatment with APO010 were overlapping (Fig. 3A and B). This is in agreement with the similar slopes of the curves depicted in Fig. 2 and implies that APO010 had no significant effect on the intrinsic radiosensitivity of these cells. In case of 03JJO215, however, the slope of the survival curve was steeper after combined treatment with radiation and APO010 than after treatment with radiation alone (Fig. 3C). This data set indicates that APO010 reduced the initial clonogenicity of J16-Bcl-2 and HCT116 cells by increasing the apoptotic response, but had no effect on radiosensitivity, whereas it enhanced the intrinsic radiosensitivity of 03JJO215 cells.

Effects of radiotherapy and APO010 on tumor growth in vivo

The effects of combined treatment in vitro prompted us to explore the in vivo potential of this treatment in a xenograft setting, using immunodeficient mice as recipients. Tumor cell inoculation protocols were explored to find the optimal setting for subcutaneous establishment of the different tumor cell types and dose finding for local irradiation was done to arrive at a noncurative treatment dose of 10 Gy. APO010 delivery (intraperitoneal) and dosage (3 x 15 µg/kg) were based on previous experience [13]. Caliper measurements showed that J16-Bcl-2 tumors in control mice grew steadily and reached the DEP at ≈6.2 (±1.4) weeks (Fig. 4A). Tumors of mice that were treated with APO010 alone showed a slight growth delay compared with mock-treated animals (control) and reached the DEP...
after 9.2 ± 2.6 weeks (P = 0.05). Tumors of mice that were treated with irradiation alone or in combination with APO010 regressed initially, but grew out again. Compared with mock treatment, irradiation caused tumor growth delay (P < 0.01), but the addition of APO010 did not further promote this. The evaluation of tumor growth with bioluminescence imaging corroborated the slight growth delay of J16-Bcl-2 tumors after treatment with APO010 alone and clearly revealed tumor regression in mice treated with radiation alone, as well as tumor regrowth in subsequent weeks. In agreement with caliper measurements, bioluminescence imaging did not show an additional effect of combined treatment with APO010 and ionizing radiation on J16-Bcl-2 tumor growth (Supplemental Fig. S2A). Survival curves were constructed based on the time required to reach the DEP (Fig. 5). These indicated that treatment with APO010 alone caused a slightly improved survival of J16-Bcl-2 tumor-bearing animals as compared with control (Fig. 5A). Radiation alone clearly enhanced overall survival of the mice as compared with control, but no additional effect was observed of combined treatment with radiation and APO010. HCT116 colon tumors reached the DEP at 8.3 ± 1.5 days and showed slight growth delay when mice were treated with APO010 alone, reaching the DEP at 11.5 ± 2.1 days (P = 0.02; Fig. 4B). Radiation caused a significant delay in the growth of HCT116 tumors, which reached the DEP at 21 ± 4 days (P < 0.01), however, combined treatment with APO010 and radiation did not further delay tumor growth (DEP, 18.4 ± 2.9 days; Fig. 4B). Bioluminescence imaging corroborated the effects of radiation and combined treatment (Supplemental Fig. S2B). In concordance, overall survival of APO010-treated animals was slightly better than in the control situation. Radiation significantly enhanced survival, but APO010 did not promote the therapeutic effect of radiation (Fig. 5B).

03JJ0215 mesothelioma tumors reached the DEP in absence of treatment at 16.2 ± 3.1 days. Tumors in mice receiving APO010 reached the DEP after 18.2 ± 5.3 days (Fig. 4C). Radiation alone caused a clear reduction in tumor growth, resulting in a DEP of 27.2 ± 3.6 days (P < 0.01). However, as for the other tumors, combined treatment with radiation and APO010 did not further reduce tumor growth (DEP at 23.5 ± 3.3 days). Bioluminescence imaging (Supplemental Fig. S2C) and survival curves (Fig. 5C) reflected similar effects of radiation and combined treatment with radiation and
APO010 on tumor growth. In addition, we orthotopically transplanted 03JJ0215 cells in the intrathoracic cavity of the mice, where tumor cells grow in their normal biological environment. Upon the appearance of a bioluminescent signal, mice were divided into treatment groups as described above. 03JJ0215 mesothelioma tumors reached the DEP in the absence of treatment at 7.1 ± 2.2 days (Supplemental Fig. S3). Tumors in mice receiving APO010 reached the DEP after 7.8 ± 0.8 days. Radiation alone caused a clear reduction in tumor growth, resulting in a DEP of 11.3 ± 2.5 days (P < 0.0001). However, combined treatment with radiation and APO010 did not further reduce tumor growth (DEP at 9.6 ± 3.2 days). We conclude that APO010 did not improve the therapeutic effect of radiation on the three tumor types in the regimen employed here.

**Normal tissue toxicity in vivo**

Changes in body weight were used as an index of general toxicity of the treatments (Fig. 6A). Radiation alone had no effect. In contrast, the body weight of mice receiving APO010 alone or in combination with radiation decreased rapidly after the start of treatment to ~85% of their initial value. After treatment, body weight returned to the pretreatment level within approximately 2 weeks. To examine potential liver toxicity, serum AST and ALT levels were determined on days 3 and 7 after the start of treatment (Fig. 6B). Elevated AST and ALT levels were found on day 3 (16 h after the second i.p. injection with APO010) in mice treated with APO010 alone or in combination with radiation. On day 7 (3 days after the third i.p. injection with APO010), the AST and ALT levels of these mice had returned to levels found in mock-treated animals. These data are in good agreement with previous findings using APO010 in vivo [13]. Livers excised from mice treated with APO010 on day 3 showed a minor vacuolization of hepatocytes in zone I of the portal area of the liver. On day 18, the normal morphology of hepatocytes in APO010-treated animals was restored (Supplemental Fig. S4). We conclude that APO010 induces moderate and reversible systemic and liver toxicity, indicating that the dose used is close to the maximally tolerable level.

**Discussion**

In this study, we have evaluated the therapeutic efficacy and potential toxicity of APO010 in combination with ionizing radiation, using relatively radioresistant lymphoid and solid tumor cell lines as model systems. The three tumor types tested showed an improved apoptotic response upon combined treatment in vitro and—depending on the tumor type—reduced clonogenicity as a result of treatment with APO010 alone or in combination with radiation. However, improved in vitro sensitivity did not correlate with a combined therapeutic effect of APO010 and radiation in a subcutaneous tumor transplant graft setting with local irradiation and intraperitoneal application of APO010. The tumor models employed included J16-Bcl-2 cells, which we have previously tested in the same type of in vitro and in vivo assays for their responsiveness to combined treatment with soluble recombinant isoleucine zippered–TRAIL and radiation [14]. In that case, we found that increased apoptosis sensitivity and reduced clonogenicity in vitro correlated with an improved therapeutic effect upon combined treatment in vivo. Combined effects of APO010 and radiotherapy on the
clonal outgrowth in long-term nonapoptotic forms of cell death [2]. For this reason, incorporate the effects of radiation on cell cycle and it is known that the apoptotic response is not predictive receptors. not involve increased cell surface expression of the induced apoptosis by a common mechanism that does possible that different DNA-damaging regimens can sensitize tumor cells to both Fas and TRAIL receptor- up-regulation (see ref. [9] and refs therein). We have followed up on such a case in J16-Bcl-2 cells and found that radiation improves the efficacy of the TRAIL receptor to assemble the death-inducing signaling complex upon ligand binding [9]. Our preliminary findings indicate that a similar mechanism underlies the combined apoptotic effect of APO010 and radiation in J16-Bcl-2 cells (I. Verbrugge, results not shown). APO010 was previously shown to act synergistically with cisplatin in the induction of apoptosis in different ovarian carcinoma cell lines, also without up-regulating the cell surface expression of Fas [13]. Therefore, it is possible that different DNA-damaging regimens can sensitize tumor cells to both Fas and TRAIL receptor-induced apoptosis by a common mechanism that does not involve increased cell surface expression of the receptors. It is known that the apoptotic response is not predictive for the in vivo response to therapy because it does not incorporate the effects of radiation on cell cycle and nonapoptotic forms of cell death [2]. For this reason, clonal outgrowth in long-term in vitro cultures is generally used to assess a potential treatment effect. This assay gives an indication of the number of cells that are able to generate progeny after treatment, at least under the in vitro conditions provided. In such clonogenic survival assays, APO010 treatment reduced the clonogenic survival of J16-Bcl-2 and HCT116 cells in accordance with its proapoptotic effect on these cells. However, it did not further reduce clonogenic outgrowth after irradiation, indicating that it did not affect the radiosensitivity of J16-Bcl-2 or HCT116 cells. Treatment with APO010 alone did not reduce the plating efficiency of 03JJO215 cells, but enhanced their radiosensitivity. Using the same assay system, we previously found that isoleucine zippered–TRAIL strongly reduced the plating efficiency of J16-Bcl-2 cells, and in addition, improved their intrinsic radiosensitivity [14]. The molecular basis of this radiosensitizing effect of death receptor ligands is currently unclear. Although APO010 did not affect the radiosensitivity of J16-Bcl-2 and HCT116 cells, it reduced clonogenic survival, thus leaving fewer cells to be eliminated by radiotherapy. Therefore, we considered that a window of combined therapeutic potential might be present and tested the effect of radiotherapy and APO010 on all three tumor types in vivo. APO010 was infused i.p. in three doses of 15 µg/kg, a protocol that was based on previous experience in a model of i.p. implantation of ovarian carcinoma cell lines in nude mice [13]. This APO010 dose had systemic effects, in terms of mild and reversible toxicity, and a small, but evident induction of tumor growth delay in the case of J16-Bcl-2 and HCT116 cells. However, APO010 could not augment the therapeutic effect of radiotherapy on any of the three tumor types tested. In addition, in an orthotopic setting of intrathoracic transplantation of 03JJ0215 mesothelioma cells in nude mice—which is more closely related to a clinically relevant setting—APO010 could not enhance the antitumor effect of radiotherapy. How to explain the lack of correlation between the in vitro and in vivo findings upon combined use of APO010 and radiation? One possibility is that we did not reach the maximally effective dose of APO010 at the tumor site. The isoleucine zippered–TRAIL dose that, upon combination with radiation, effectively combated J16-Bcl-2 tumors in vivo gave no discernable normal tissue toxicity. The APO010 dose used here caused a reversible rapid drop in body weight and liver toxicity, indicating that there was little room for escalation of the dose in our experimental setting. In the intraperitoneal ovarian tumor model, APO010 (MegaFas Ligand) had a combined therapeutic effect with cisplatin in vivo, correlating with synergistic apoptosis induction in vitro [13]. In that study, APO010 was injected i.p. at the same location of the tumor, at 20 µg/kg body weight. The half-life of APO010 as detected in plasma was short, but reached a maximum of ~80 ng/mL within 2 hours after i.p. injection [13]. Therefore, it is likely that the effective dose at the site of the tumor in that case was many fold higher than the IC50 for the same cells, as determined in in vitro apoptosis assays (5 ng/mL). In our case, the tumor was implanted s.c., whereas APO010 was applied i.p. Possibly therefore, the APO010 concentrations reached within the tumor were lower than the concentrations that were effective in apoptosis-induction in the combined setting in vitro. Our findings suggest that it will be useful to explore APO010 for combined modality treatment with chemotherapeutics or radiotherapy in settings of local perfusion of tumor tissue, a similar strategy as used in the treatment of soft tissue sarcomas with tumor necrosis factor-α [15]. In the clinical setting, however, it is important to consider that APO010 may have a degree of species specificity that does not allow a full assessment of its potential toxicity in mouse models. For the in vivo studies, we have employed a relatively high single dose of radiation instead of a fractionated regimen for several reasons. First, our in vitro findings showed a radiation dose–dependent sensitization with optimal antitumor responses (either short-term apoptosis or long-term clonogenic survival) at higher doses of radiotherapy (10 Gy versus 2.5 Gy). Therefore, we expected to achieve a therapeutic effect in vivo using higher doses of radiotherapy. Second, if APO010 were
to be combined with a fractionated radiation schedule, this would require repeated (daily) administration of APO010 to achieve a combined effect. Given APO010’s toxicity, however, this approach would not be feasible.

A key parameter that was not incorporated in our toxicity, however, this approach would not be feasible. APO010 to achieve a combined effect. Given APO010’s toxicity, however, this approach would not be feasible.

In vivo, there is intensive crosstalk between tumor cells and the surrounding tissue, by direct cell-to-cell interactions and by secreted factors. Tumor cells are also profoundly influenced by more general conditions, such as vascularization which may dictate drug delivery, the extent of oxygenation and nutrient supply. These parameters may determine the response to APO010 treatment in various ways. In the first place, they may determine whether Fas induces a proapoptotic or an antiapoptotic pathway. Although Fas is viewed primarily as a death-inducing receptor, it can induce a variety of cellular responses via different signaling routes. Fas can activate nuclear factor-kB, all three major mitogen-activated protein kinase pathways [16] and—according to a recent publication—the phosphoinositide-3-kinase pathway as well [17]. Nuclear factor-kB can activate a potent antiapoptotic pathway that involves the new synthesis of inhibitory Bcl-2 family members, c-FLIP, and inhibitor of apoptosis proteins [18]. How the decision between apoptosis induction and nuclear factor-kB activation downstream from Fas is made, is not completely elucidated, but there is evidence that nuclear factor-kB activation requires a lower level engagement of Fas receptors [19]. Therefore, the extent of Fas membrane expression, ligand delivery, or agonistic activity may be decisive for the outcome of treatment with Fas agonists. Moreover, several studies indicate that Fas triggering can promote tissue inflammation [16, 20, 21], which in turn may have an effect on tumor growth [22]. These proinflammatory effects of Fas may proceed via chemotactic factors such as IL-8, which is induced via the c-Jun-NH$_2$-kinase pathway [21]. The nuclear factor-kB signaling pathway is also known to induce proinflammatory mediators [18]. Evidence for a proinflammatory role of recombinant human Super FasL (Alexis Biochemicals) was found in a model of intracranial delivery to a glioblastoma xenograft in nude rats.

In this case, etoposide and Fasl showed a combined proapoptotic effect on the glioblastoma cells in vitro, but promoted inflammation in vivo. Upon concomitant anti-inflammatory dexamethasone treatment, the proapoptotic and therapeutic effect of this combination on the tumor in situ was revealed [23]. Finally, a point of concern is the recent documentation of a stimulatory effect of Fas on tumor cell invasion, mediated via the up-regulation of matrix metalloproteinases [17].

In summary, although in this study, APO010 was proven as a potent proapoptotic agent that could reduce the clonogenicity of tumor cells and showed combined effects with radiation in vitro, an effective therapeutic schedule in vivo was not achieved in the tumor models used here. Future studies will have to point out whether locoregional application of APO010, other combination strategies, or the use of other tumor types may overcome the apparent limitations in obtaining the desired treatment effect.

Materials and Methods

Manufacturing of APO010

An expression construct for ACRP30:FasL was generated according to standard molecular biology protocols, and cloned in a commercial mammalian expression vector. The construct encoded the human ACRP30 (Swiss-Prot P15848, amino acids 16-108) and the human Fasl (Swiss-Prot P48023, amino acids 139-281). The hemagglutinin signal peptide (MAIYLLLFTAVRG) was inserted prior to the predicted cleavage site of the natural human ACRP30 native protein to allow protein secretion. Cell clones expressing APO010 were obtained by transfecting a nonadherent Chinese hamster ovary cell line that was preadapted to grow in the presence of MegaFasL-FLAG, with this construct. Pure APO010 was obtained by growing the Chinese hamster ovary clone F7 for 7 days, up to a viable cell density of 10 million cells/mL. After cell harvest and clarification, contaminants were captured by anion exchange chromatography. The flow-through containing APO010 was diafiltrated and further processed on a cation exchange chromatography resin. After elution by a salt gradient, the APO010-containing fractions were pooled, concentrated by ultrafiltration, and purified by size exclusion chromatography. The final purity of the product, as assessed by Coomassie staining of an SDS-polyacrylamide gel and size exclusion high-performance liquid chromatography was >95%. Using the Edman degradation method, the purified APO010 NH$_2$ terminus was sequenced. Although the NH$_2$-terminal glycine may have been trimmed from the purified protein, no major alterations were found in the primary sequence. The COOH-terminal sequence of the molecule, assessed using an improved procedure based on the method developed by Boyd et al. [24], was found to be complete. For this study, APO010 Lot 340 A12 3.3 F7-0504 was used.

Cell culture and stimulation

The J16 clone was derived from the p53 mutant, human T-acute lymphoblastic leukemia cell line Jurkat by limiting dilution and selected for sensitivity to Fas-induced apoptosis [14, 25]. J16 cells were retrovirally transduced as described [14] to stably overexpress Bcl-2 and luciferase to inhibit the mitochondrial apoptosis pathway and for monitoring of tumor growth by bioluminescence, respectively. The resulting J16-Bcl-2 cell line was cultured in Iscove’s modified Dulbecco’s medium with 8% fetal bovine serum and antibiotics. P53 wild-type HCT116 colon carcinoma cells were retrovirally transduced to stably express MegaFasL-FLAG, with this construct. Pure APO010 was obtained by growing the Chinese hamster ovary clone F7 for 7 days, up to a viable cell density of 10 million cells/mL. After cell harvest and clarification, contaminants were captured by anion exchange chromatography. The flow-through containing APO010 was diafiltrated and further processed on a cation exchange chromatography resin. After elution by a salt gradient, the APO010-containing fractions were pooled, concentrated by ultrafiltration, and purified by size exclusion chromatography. The final purity of the product, as assessed by Coomassie staining of an SDS-polyacrylamide gel and size exclusion high-performance liquid chromatography was >95%. Using the Edman degradation method, the purified APO010 NH$_2$ terminus was sequenced. Although the NH$_2$-terminal glycine may have been trimmed from the purified protein, no major alterations were found in the primary sequence. The COOH-terminal sequence of the molecule, assessed using an improved procedure based on the method developed by Boyd et al. [24], was found to be complete. For this study, APO010 Lot 340 A12 3.3 F7-0504 was used.

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and carries a transgenic LucR luciferase reporter allele [27].
O3JJ0215 cells were cultured in DMEM/F12 1:1 + Glutamax
supplemented with insulin-transferrin-selenium (Invitrogen,
Life Technologies), murine epidermal growth factor (Invitrogen),
hydrocortisone (Sigma-Aldrich), 5% fetal bovine serum, and
antibiotics. For apoptosis assays, cells were stimulated with the
indicated doses of APO010 or radiation in their normal culture
medium, after which cells were incubated for the indicated
time periods at 37°C, 5% CO₂. Irradiation of cells was done
using a 137Cs source (415 Ci; Von Gahlen Nederland, B.V.) at an
absorbed dose rate of ~0.6 Gy/min.

Apoptosis assays
Apoptosis was read out by fragmentation of propidium
iodide–stained nuclei for J16-Bcl-2 and HCT116 cells and by
active caspase-3 content for O3JJ0215 cells (because the latter
cell type shows poor nuclear fragmentation). These assays were
carried out as described in refs. [9] and [28], respectively. The percentages of cells with fragmented nuclei or active caspase-3
were determined by flow cytometric analysis. Cellular debris
was excluded from analysis and data were analyzed using FCS
Express software (De Novo Software).

Clonogenic assays
Clonogenic assays for J16-Bcl-2 cells were done as described
[14]. After irradiation, cells were cultured for 11 days in the presence or absence of 0.02 ng/mL of APO010. HCT116 and
O3JJ0215 cells were plated at increasing densities up to 32,000
cells per 10 cm dish. After attachment for 6 h at 37°C, 5% CO₂,
cells were irradiated and the medium was replaced with normal
culture medium containing no APO010 (control), 0.2 ng/mL of
APO010 (HCT116), or 10 ng/mL of APO010 (O3JJ0215). After 9
to 11 days of culture, colonies were counted using an inverted
microscope. Doses of APO010 used in clonogenic assays were
selected for each cell line on the basis of colony outgrowth after
9 to 11 days of culture in the presence of APO010 alone, allowing
for appropriate readout of combined effects with radiotherapy.
Analyses were done using Slide Write Plus software version 5.0
for Windows (Advanced Graphics Software, Inc.).

Mouse model and therapy
Athymic nu/nu mice, backcrossed to a BALB/c background,
were bred at The Netherlands Cancer Institute. Animal
experiments were done in agreement with institutional and
national guidelines and approved by the Experimental Animal
Committee of The Netherlands Cancer Institute. Tumor cell
inoculation was done as described [14] with 2 x 10⁶ J16-Bcl-2
cells or 1 x 10⁶ HCT116 cells resuspended in Matrigel Matrix
Growth Factor Reduced (BD Biosciences), or 25,000 O3JJ0215
cells resuspended in PBS (100 µL). Tumor growth and response
to treatment was monitored by caliper measurement and
by bioluminescence imaging. Tumor volume was calculated
using the formula π/6 x length x width x height (in mm).
Bioluminescence was measured using the IVIS 200 Imaging
System (Xenogen Corporation) as previously described [14].
Signal intensity is reported as a percentage of the last value
before treatment. Treatment of five to six mice per group
started (day 0) after a steady increase in bioluminescent signal
in the case of the fast-growing O3JJ0215 cells and with the
appearance of palpable tumors in the case of J16-Bcl-2 (size,
~50 mm³) and HCT116 (size, ~114 mm³). Treatment consisted
of (a) three i.p. injections every 48 h with 10 µL buffer (PBS +
0.05% human serum albumin) per gram mouse weight (control);
(b) 10 Gy local irradiation followed by three i.p. injections every
48 h with buffer (IR); (c) three i.p. injections every 48 h with 15
µg/kg of APO010 (APO010); (d) 10 Gy local irradiation followed
by three i.p. injections every 48 h with 15 µg/kg of APO010 (IR +
APO010). Injections were started on the same day as irradiation.
Irradiation was done as described [14]. A tumor volume of 200
mm³ for O3JJ0215, 130 mm³ for J16-Bcl-2, and 500 mm³ for
HCT116 were set as the designated end points (DEP). Curves
for treatment groups were terminated when fewer than three
mice remained in that treatment group. The tumor-free survival
curves generated represent the fraction of mice bearing tumors
smaller than their DEP.

Normal tissue toxicity
To investigate normal tissue toxicity, mice were weighed at
least twice per week. In addition, blood was sampled from the
tail vein and levels of aspartate aminotransferase (AST) and
alanine aminotransferase (ALT) were determined in serum 15
h after the second i.p. injection with buffer or APO010 (day 3)
and 3 days after the last i.p. injection (day 7). AST and ALT levels
were determined as described [14]. In a parallel experiment,
animals were sacrificed 15 h after the second i.p. injection
(day 3) or 2 weeks after the last i.p. injection (day 18). Their
livers were fixed in ethanol (50%), acetic acid (5%), formalin
(3.7%), embedded in paraffin, sectioned, stained with H&E, and
analyzed microscopically.

Statistics
Differences between treatment groups were analyzed with
unpaired Student’s t tests and were considered significant at
P < 0.05. Analysis was done using GraphPad Prism version 5
for Windows (GraphPad Software). Statistics described in the
Results indicate mean ± SD, statistics and data points in the
figures are clarified in the legends.

References
1. Bartelink, H., J.H. Schellens, and M. Verheij. The combined use of
radiotherapy and chemotherapy in the treatment of solid tumours. Eur
3. Strasser, A, et al., DNA damage can induce apoptosis in proliferating
lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2.
4. Rupnow, B.A, et al., Direct evidence that apoptosis enhances
58(9): p. 1779-84.
5. Peter, M.E. and P.H. Krammer, The
6. Ashkenazi, A, et al., Safety and
7. Walczak, H, et al., Tumoricidal activity of tumor necrosis factor-
8. Ashkenazi, A, and R.S. Herbst, To
kill a tumor cell: the potential of proapoptotic receptor agonists. J
9. Verbruggue, I, et al., Ionizing radiation
modulates the TRAIL death-inducing


Supplementary Information

Supplementary Figure 1. Direct comparison of clonogenic survival of J16-Bcl-2 cells, HCT116 cells and 03JJ0215 cells after treatment with the indicated doses of ionizing radiation (IR).

Supplementary Figure 2. Luciferin bioluminescence imaging (BLI) of tumor growth. The same mice as analyzed for tumor growth by palpation in Figure 4, implanted with J16-Bcl-2 cells (A), HCT116 cells (B) or 03JJ0215 cells (C) were monitored for tumor response by BLI after treatment with radiotherapy and/or APO010.
Supplementary Figure 3. Tumor response in vivo of orthotopically transplanted 03JJ0215 cells after treatment with radiotherapy and/or APO010. Mice were anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) i.p. and subsequently injected intrathoracically with 25,000 03JJ0215 tumor cells expressing a luciferase reporter gene in 100 μl PBS. Upon appearance of a bioluminescent signal, mice were treated with 10 Gy local irradiation (IR) (▲), three i.p. doses of 15 μg/kg APO010 (▼), or 10 Gy IR + three i.p. doses of 15 μg/kg APO010 (◆). Control mice (●) were injected with buffer. Mice were monitored for tumor response by luciferin bioluminescence imaging (BLI) after treatment with radiotherapy and/or APO010. (A) intrapolated BLI data (at least three data points per week per mouse). Data shown are means (± SD) of pooled data from three independent experiments with 5-18 mice per group in total. (B) BLI of tumor growth from one representative mouse from each treatment group, where D0 (Day 0) indicates start of treatment. (C) Overall survival of mice, which were sacrificed upon obtaining breathing difficulties.

Supplementary Figure 4. Reversible liver toxicity after treatment with APO010. H&E staining of liver sections from a control mouse (left panel), mice treated with APO010, sacrificed on day 3 (middle panel) or day 18 (right panel). P: Portal vein, C: Central vein, B: Bile duct; scale bar indicates 100 μM.