Combining an Aurora Kinase Inhibitor and a Death Receptor Ligand/Agonist Antibody Triggers Apoptosis in Melanoma Cells and Prevents Tumor Growth in Preclinical Mouse Models

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Abstract

Purpose: Preclinical studies show that inhibition of aurora kinases in melanoma tumors induces senescence and reduces tumor growth, but does not cause tumor regression. Additional preclinical models are needed to identify agents that will synergize with aurora kinase inhibitors to induce tumor regression.

Experimental Design: We combined treatment with an aurora kinase inhibitor, MLN8237, with agents that activate death receptors (Apot2L/DR5 agonist death receptor 5) and monitored the ability of this treatment to induce tumor apoptosis and melanoma tumor regression using human cell lines and patient-derived xenograft (PDX) mouse models.

Results: We found that this combined treatment led to apoptosis and markedly reduced cell viability. Mechanistic analysis showed that the induction of tumor cell senescence in response to the AURKA inhibitor resulted in a decreased display of Apo2L/TRAIL decoy receptors and increased display of one Apo2L/TRA3L receptor (death receptor 5), resulting in enhanced response to death receptor ligand/agonists. When death receptors were activated in senescent tumor cells, both intrinsic and extrinsic apoptotic pathways were induced independent of BRAF, NRAS, or p53 mutation status. Senescent tumor cells exhibited Bid-mediated mitochondrial depolarization in response to Apot2L/TRA5 treatment. In addition, senescent tumor cells had a lower apoptotic threshold due to decreased XIAP and survivin expression. Melanoma tumor xenografts of one human cell line and one PDX displayed total blockage of tumor growth when treated with MLN8237 combined with DR5 agonist antibody.

Conclusions: These findings provide a strong rationale for combining senescence-inducing therapeutics with death receptor agonists for improved cancer treatment.

Introduction

Antitumor therapies cause both apoptosis and senescence. We previously reported that an aurora kinase A inhibitor reduces melanoma growth through inducing senescence in vitro and in vivo (1). The role of cellular senescence in restricting tumorigenesis upon oncogenic activation or tumor suppressor loss has been established (2–4). Spontaneous induction of senescence is an initial defense against unrestrained proliferation. Expression of an oncogene in premalignant cells without loss of a tumor suppressor often results in oncogene-induced senescence (OIS). However, through a series of changes, premalignant cells can by-pass senescence, undergo transformation, and become tumor cells (5). When tumor cells are treated with cytostatic or cytotoxic drugs, they can be forced into another state of senescence, therapy-induced senescence (TIS). In vitro and in vivo data suggest that low doses of conventional anticancer compounds or gamma radiation can trigger TIS (6). A mouse lymphoma model demonstrates that TIS can result in improved animal survival (7), which is of clinical interest. More than 20 anticancer reagents have been reported to induce senescence, resulting in stable cell-cycle arrest and inhibition of tumor growth (8).

Alisertib (MLN8237), an aurora kinase A (AURKA) inhibitor, blocks the G2–M cell-cycle progression, induces polyploidy, causes DNA damage, and results in senescence (1, 9–11). MLN8237 is currently in clinical trials in liquid and solid tumors (12–14). We have recently shown in preclinical mouse models that combining MLN8237 with an MDM2 antagonist will activate p53-mediated apoptosis and markedly enhance the therapeutic response of p53 wild-type tumors to MLN8237, which involves enhanced recruitment of immune cells. Previous studies demonstrated that natural killer (NK) cells and macrophages can...
eliminate senescent premalignant cells and tumor cells, and thus participate in immune-editing (15, 18–20).

In an effort to identify a therapeutic regimen of combined therapies that would result in the killing of both p53-mutant and p53 wild-type tumor cells, we sought to evaluate of the efficacy of combining MLN8237 with agents that activate death receptors. Death receptor 5 (DR5) agonist antibodies are currently in clinical trials, but often exhibit a limited therapeutic index alone. Our in vitro studies have shown that combining MLN8237 with Apo2L/TRAIL, or death receptor 5 agonists, we are able to achieve tumor regression. Moreover, the response is independent of p53, BRAF, or NRAS mutation status. Both AURKA inhibitors and DR5 agonist antibodies are currently in clinical trials as single agents, making rapid translation of our results feasible.

Materials and Methods

Cell lines, tissue culture, and chemical reagents

A375, Hs294T, SK-Mel-2, and SK-Mel-28 melanoma cells were purchased from the ATCC and cultured in DMEM/F12 media supplemented with 10% FBS. All the cell lines were meticulously passaged and tested for Mycoplasma monthly. The p53 inhibitor pifithrin-α (PIT-α) was obtained from Tocris Bioscience. The caspase inhibitors were purchased from R&D Systems. MLN8237 (alisertib) was obtained from Millennium Pharmaceuticals, Inc. Recombinant human Apo2L/TRAIL and OPG were from Peprotech. DR5 activator, Bioymi, was from Xcess Biosciences Inc. An AURKA B inhibitor (AZD1152) and a pan-AURKA inhibitor (VX-680) were purchased from Selleckchem. DR5 agonist antibody was from R&D Systems and Genentech.

siRNA transfection

Cells were cultured in serum-reduced media. siRNA and transfection reagent (lipofectamine RNAiMAX; Life Technologies) were each diluted in serum-reduced media. The siRNA solution and RNAiMAX solution were combined and incubated at room temperature for 5 minutes, the siRNA and RNAiMAX complexes were added to the cultured cells. siRNAs targeting DR5 or BID were from Life Technologies.

Senescence assay

Senescence was examined by a senescence-associated β-galactosidase assay kit (Sigma-Aldrich). Briefly, cells were washed with phosphate-buffered saline (PBS) buffer, fixed and stained at 37°C according to supplier protocols. TaqMan pimers for senescence markers were from Life Technologies.

Western blot antibodies

Anti-RIP (#3493), anti-BID (#2002), anti-caspases 3 (#9662), 8 (#9746), 9 (#9502), anti-DR1 (#4756), anti-BIM (#2933), anti-BAX (#2772), anti-Survivin (#2808), anti-p53 (#2524) antibodies were from Cell Signaling Technology. Anti-FLIP (06-697) antibody was from Upstate. Anti-OPG (500-P149) was from Peprotech. Anti-Dr2 (TA305975) was from Origene. Anti-DR5 (NB100-56618) was from Imgenex.

Cell viability assay

Cells were trypsinized and treated with Trypan blue. The viable cells that excluded Trypan blue were counted using a hemocytometer.

Flow cytometry

For DR5 analysis, cells were filtered, washed, and then stained for 30 minutes on ice with anti-DR5 antibody or IgG control. For flow cytometry, anti-DR5 (12-9908-41) was from eBioscience. For apoptosis analysis, cells were stained with propidium iodide and Annexin V. Analysis was performed on a custom 5-laser LSRII (BD Biosciences).

Animal studies

All of the animal experiments were performed in accordance with the Vanderbilt University Institutional Animal Care and Use Committee (IACUC) guidelines and regulations. The experiments were approved under the IACUC protocol number M/10/034. The PDX tumor exhibited a BRAFV600E mutation and was p53 wild-type, NRAS wild-type, GNAQ and GNA11 wild-type, and cKIT wild-type. Balb/c nu/Foxn1 athymic nude mice (female, 6–8 weeks of age and weighing 22–25 g) were purchased from Harlan Sprague Dawley. Briefly, tumor-bearing mice were given vehicle (water), IgG control (10 mg/kg), DR5 antibody (10 mg/kg), MLN8237 (30 mg/kg), or combination, respectively. According to information available from Takeda, this dose of MLN8237 may possibly result in an exposure and PD effect 1.3-fold greater than that achievable in patients at the 50 mg BID d1–7 dose (16, 17). Vehicle control and MLN8237 were delivered once daily five times per week by oral gavage. IgG control and DR5 antibody were delivered twice weekly by intraperitoneal (i.p.) injection. Tumor volume and mouse body weight were measured weekly and the volume was calculated using the formula: V = 1/2 × L × W², where L is the long and W is the short diameter of the tumor. When any tumor reached a size of 1.5 cm diameter, mice were sacrificed and tumors were harvested. DR5 antibody and IgG control were kindly provided by Avi Ashkenazi from Genentech.

Immunofluorescence staining

Immunofluorescence staining was performed to analyze Ki67 and cleaved caspase-3. Methods have been previously described (1). Anti-Ki67 (M7240) antibody was from Dako. Anti–cleaved-caspase-3 (#9644) antibody was from Cell Signaling Technology. JC-1 was from Selleckchem. Anti-GLB1 (ab139288) was from Abcam.
Statistical analysis
For multiple-group comparison, one-way ANOVA was used; for two-group comparison, the Student two-sided t test was used.

Results
Apo2L/TRAIL triggers caspase-dependent apoptosis in therapy-induced senescent tumor cells independent of p53 status

We have previously reported that MLN8237 induces senescence in melanoma cells based upon beta-galactosidase staining (1). Here, we performed quantitative real-time PCR to determine the mRNA expression levels of markers for senescence after treatment with 1 μmol/L MLN8237, which is comparable with the plasma concentration of MLN8237 in mice (15, 21). We evaluated DEC1 (deleted in esophageal cancer 1), GLB1 (β-galactosidase), CCNA2 (cyclin A2), CDKN1A (p21), CDKN2A (p16), IL8, IL6, and CXCL1 in two p53WT (A375, Hs294T) and two p53mut (SKMEL28, SKMEL2) melanoma cell lines in response to MLN8237 treatment. DEC1 was not detectable in all four melanoma cell lines; GLB1 was upregulated in A375 cells; CCNA2 was downregulated in all four cell lines; CDKN1A was upregulated in p53WT cells (A375 and Hs294T); CDKN2A was upregulated in A375 cells; IL8, IL6, and CXCL1 were elevated in p53WT melanoma cell lines, but not in the p53mut cell lines compared with vehicle-treated control cells (Supplementary Fig. S1A).

To determine whether cytokines, such as death receptor ligands, secreted by innate immune cells in the tumor can contribute to the killing of senescent tumor cells, we treated melanoma cells with MLN8237 and a death receptor ligand, Apo2L/TRAIL (25 ng/mL) simultaneously for 5 days. We observed that MLN8237-induced senescence (Fig. 1A) and the combination treatment significantly reduced tumor cell viability compared with single treatment.

Figure 1.
Apo2L/TRAIL triggers cell death in drug-induced senescent tumor cells independent of p53. A, Hs294T, SK-Mel-28, A375, and SK-Mel-2 melanoma cells were treated with MLN8237 (1 μmol/L) for 5 days, followed by SA-β-Gal staining. B, cells were treated with vehicle (V), MLN8237 (M; 1 μmol/L), Apo2L/TRAIL (T; 25 ng/mL), or a combined treatment as indicated for 5 days, followed by crystal violet staining. C, cells were treated with vehicle, Apo2L/TRAIL (25 ng/mL), MLN8237 (1 μmol/L), or combination for 5 days. Then cells were lysed and levels of p53 were analyzed by Western blot analysis. D, Hs294T and A375 cells were treated with MLN8237 or the p53 inhibitor, PFT-α (10 μmol/L) for 5 days, followed by SA-β-Gal staining. Quantification is shown by β-Gal-positive cell counting. n = 5 independent experiments. E, Hs294T and A375 cells were treated with Apo2L/TRAIL (25 ng/mL) and MLN8237 (1 μmol/L) in the absence or presence of PFT-α for 5 days. Quantification was performed by cell counting. Data are shown as mean ± SD from three independent experiments.
In addition, other aurora kinase inhibitors, such as aurora kinase B inhibitor, AZD1152, and pan-aurora kinase inhibitor, VX-680, also induced senescence in melanoma cells (Supplementary Fig. S1B) and reduced cell viability when combined with Apo2L/TRAIL (Supplementary Fig. S1C).

To determine whether p53 activation is required for this effect, we analyzed p53 after combined MLN8237 and Apo2L/TRAIL treatment in melanoma cells with different p53 status. As shown in Fig. 1C, p53 was not increased in p53-mutant SK-Mel-2 or SK-Mel-28 cells in response to treatment, even though these treatments induced senescence and apoptosis. To further investigate whether p53 is necessary for Apo2L/TRAIL–induced cell death, we treated cells with a p53 inhibitor, Pifithrin-α (PFT-α). When nonsenescent melanoma cells with p53WT (Hs294T and A375 cells) were treated with PFT-α (10 μmol/L) for 5 days, cell viability was not affected (Supplementary Fig. S1D). When senescent Hs294T or A375 melanoma cells were treated with Apo2L/TRAIL in the presence of PFT-α (10 μmol/L), no significant change in either senescence (Fig. 1D) or cell viability (Fig. 1E) occurred. These results indicate that the therapeutic response of senescent tumor cells to Apo2L/TRAIL does not require p53 activation.

To study whether Apo2L/TRAIL–induced cell death depends on the senescence state or the continued presence of aurora kinase inhibitors, we treated cells with MLN8237 alone, with Apo2L/TRAIL alone, with Apo2L/TRAIL simultaneously, or with MLN8237 and Apo2L/TRAIL sequentially (MLN8237 for 5 days followed by Apo2L/TRAIL). Melanoma cells (Hs294T and SK-Mel-28) exhibited little sensitivity to Apo2L/TRAIL treatment alone (Supplementary Fig. S2B). However, when one examines the time course of MLN8237–induced senescence (Supplementary Fig. S2A) and the time course of the combined treatment–induced cell death (Supplementary Fig. S2C), it is clear that the induction of cell death by combined treatment was not evident until day 3, the same time required for MLN8237 to induce senescence based on SA-β-Gal staining (Supplementary Fig. S2A). In contrast, if cells

Figure 2. Apo2L/TRAIL induces caspase-dependent apoptosis in senescent tumor cells. A, cells were treated with vehicle, Apo2L/TRAIL, MLN8237 (MLN), or combination of Apo2L/TRAIL and MLN8237, respectively, for 4 days (around 100 hours), stained with propidium and Annexin V, and analyzed by FACS. The results are shown as the percentage of early apoptosis (Annexin V+/PI- ) and apoptosis/necrosis (Annexin V+/PI+ ) over percentage of live cells. B, cells were treated with vehicle, Apo2L/TRAIL, MLN8237, or combination of Apo2L/TRAIL and MLN8237, respectively, for 5 days. The cells were lysed and the levels of the indicated proteins were analyzed by Western blots analyses. C, cells were treated with vehicle (V), Apo2L/TRAIL (T), MLN8237 (M), combination (T+M), or combination with one caspase inhibitor as indicated, respectively, for 5 days, followed by crystal violet staining. D, quantification of results from C is shown by cell counting. Each dataset shows mean ± SD from four independent experiments. ns indicates not significant; **, P <0.01.
were first rendered senescent by pretreatment with MLN8237 and then treated with the Apo2L/TRAIL, a decline in the number of viable cells was evident as early as day 1 of Apo2L/TRAIL treatment (Supplementary Fig. S2D), suggesting that Apo2L/TRAIL–induced cell death was dependent upon MLN8237 induction of senescence.

To determine whether induction of apoptosis would account for the reduction in cell viability after combined MLN8237 and Apo2L/TRAIL treatment, we performed propidium iodide/Annexin V double staining. Results indicated significant apoptosis after treatment of senescent cells with Apo2L/TRAIL (Fig. 2A). Senescent tumor cells exhibited increased cleavage of PARP, caspase-3, -8, and -9 in response to Apo2L/TRAIL treatment (Fig. 2B). It was noted that MLN8237 stabilized the cleaved products of caspase-8 (p43/41 and p18) in both cell lines, which augmented apoptosis stimulation by Apo2L/TRAIL treatment (22). This conclusion is supported by the loss of cell death when a pan-caspase inhibitor (10 μmol/L) or caspase-8 inhibitor (10 μmol/L) was added (Fig. 2C and D). The caspase-9 inhibitor (10 μmol/L) partially rescued Apo2L/TRAIL–induced cell death (Fig. 2C and D), indicating that both intrinsic and extrinsic apoptotic pathways were involved.

Upregulation of DR5 and downregulation of decoy receptors DcR1, DcR2, and OPG sensitize senescent tumor cells to Apo2L/TRAIL

To determine whether changes in cell membrane–associated Apo2L/TRAIL receptors (death receptors) in senescent cells made them more sensitive to Apo2L/TRAIL, we evaluated the DR5 expression in MLN8237-treated melanoma cells. We observed that induction of senescence increased the levels of membrane presented DR5 in melanoma cells based on FACs analysis (Fig. 3A). In addition, melanoma patient-derived tumor tissues from tumor bearing mice previously treated with MLN8237 (i) also exhibited increased membrane presented DR5 (Fig. 3B). In contrast, senescence reduced decoy receptor 2 (DcR2) and osteoprotegerin (OPG) in Hs294T cells (Fig. 3C). The expression of decoy receptors was cell line specific. Although Hs294T cells did not express decoy receptor 1 (DcR1) and SK-Mel-28 cells did not express DcR2 or OPG (data not shown), the latter cell line had decreased levels of DcR1 when senescence was induced (Fig. 3C). When the levels of DR5 in senescent tumor cells were knocked down by siRNAs (Fig. 3D), the cell death response to Apo2L/TRAIL treatment was partially lost (Fig. 3E). Similarly, when soluble OPG was added to culture media to bind and neutralize Apo2L/TRAIL,
Apo2L/TRAIL–induced cell death was also partially lost (Fig. 3F). These results indicate that the MLN8237-induced changes in surface expression of death receptor and decoy receptors sensitize senescent tumor cells to Apo2L/TRAIL treatment by upregulating the DR5 death receptor and downregulating decoy receptors.

Both intrinsic and extrinsic apoptotic pathways are activated in Apo2L/TRAIL–treated senescent tumor cells
To further investigate the underlying mechanisms of increased sensitivity to Apo2L/TRAIL in senescent tumor cells downstream of death receptors we examined mitochondrial function and the levels of several apoptosis-related proteins. Using JC-1 dye, where red fluorescence indicates intact mitochondrial membrane and green fluorescence indicates loss of the mitochondrial membrane potential, we observed vehicle and Apo2L/TRAIL–treated cells displayed more red fluorescence than green fluorescence (20%); MLN8237–treated cells showed both red fluorescence and green fluorescence (<50%); while MLN8237– and Apo2L/TRAIL–treated cells showed more green fluorescence (80%: Fig. 4A) than untreated cells. These results suggest that Apo2L/TRAIL treatment disrupted the mitochondrial function in senescent tumor cells. Combined treatment of MLN8237 and Apo2L/TRAIL also induced cleavage of BID (tBID; Fig. 4B) and decreased MCL-1 levels in SK-Mel-28 cells but not in Hs294T cells. When the levels of BID were knocked down in senescent tumor cells, tBID was downregulated (Fig. 4C) and cell death was decreased in response to Apo2L/TRAIL treatment (Fig. 4D). These data suggest that activation of both intrinsic and extrinsic apoptotic pathways contributes to the Apo2L/TRAIL–induced apoptosis in senescent tumor cells. DR5 agonist antibody activates apoptosis in TIS tumor cells in vitro and causes tumor regression in preclinical mouse models in vivo
In contrast to Apo2L/TRAIL, which has relatively a short half-life in vivo, DR5 agonist antibody has a half-life of several days in vivo. When Hs294T cells treated with MLN8237 combined with increasing concentrations of DR5 agonist antibody (0–750 ng/mL) there was a dose-dependent loss of cell viability based on cell staining (Fig. 5A). When viable cells were counted after treatment with DR5 antibody alone versus increasing concentrations of DR5 antibody
plus MLN8237, the reduction in cell number was greater in MLN8237-induced senescent cells than in vehicle-treated nonse
necent tumor cell lines (Fig. 5A). The combination of a DR5 agonist antibody with MLN8237 also reduced cell viability in other three melanoma cell lines (Fig. 5B). Similarly, DR5 agonist antibody induced more cleaved caspase-8 and cleaved caspase-3 in the MLN8237-treated senescent melanoma cells than in cells not treated with MLN8237 (Fig. 5C). Pan-caspase inhibitor or caspase-8 inhibitor blocked DR5 agonist antibody-induced cell death (Fig. 5D), suggesting that caspase-dependent apoptosis contributed to the loss of cell viability with treatment. In addition, a DR5 small-molecule activator (23) also produced dose-dependent killing effects (0–15 μmol/L) on MLN8237-treated senescent tumor cells (Fig. 5E and Supplementary Fig. S3A) and only affected the cells not co-treated with MLN8237 at the highest dose (15 μmol/L). These results indicate that DR5 agonist antibody will be highly effective for in vivo studies.

When Balb/C Fox nu/nu mice bearing Hs294T melanoma xenografts were treated with vehicle (water by oral gavage), MLN8237 (30 mg/kg by oral gavage, 5 times per week), vehicle + IgG control (10 mg/kg by i.p. injection, twice per week), vehicle + DR5 antibody (10 μg/kg by i.p. injection, twice per week), IgG + MLN8237 (30 mg/kg) i.p., or DR5 antibody (10 μg/kg) + MLN8237 (30 mg/kg) i.p., respectively, for 3.5 weeks, DR5 antibody treatment resulted in significant killing effects on MLN8237-treated tumors (Fig. 6A). In addition, a PDX from a melanoma patient, who had progressed after BRAF inhibitor treatment, revealed that treatment with DR5 antibody alone did not inhibit...
tumor growth, MLN8237 alone did significantly inhibit tumor growth, but the combined treatment with DR5 antibody and MLN8237 prevented tumor growth (Fig. 6B).

To evaluate the effects of DR5 antibody and MLN8237 treatment in vivo at the cellular level, histologic analysis of paraffin-embedded PDX tumors was performed using special stains. SA-beta-Gal staining showed that MLN8237 strongly induced tumor cell senescence in vivo (Supplementary Fig. S3B). Ki67 staining of tumors showed that MLN8237 treatment alone significantly blocked cell proliferation, while DR5 agonist antibody treatment alone did not significantly reduce cell proliferation. Combined treatment with MLN8237 and DR5 agonist antibody did not further reduce the number of Ki67-positive cells over that of the MLN8237 treatment alone (Fig. 6C). Cleaved caspase-3 staining demonstrated that while MLN8237 treatment alone had little effect on apoptosis, DR5 antibody treatment alone did induce apoptosis and the combined treatment with MLN8237 and DR5 antibody triggered significantly more apoptosis in vivo (Fig. 6D).

Thus, the combination treatment resulted in a significant reduction in Ki67 staining ($P < 0.0001$) and enhanced cleaved caspase-3 ($P < 0.0001$). To determine whether the senescent cells are indeed undergoing apoptosis, we cotained GLB1 with cleaved caspase-3 using tissue slides from the PDX animal study. We observed overexpression of GLB1 in senescent tumor tissues and the GLB1 colocalized with cleaved caspase-3 (Supplementary Fig. S4). Overall, our results show that combined treatment with a DR5 agonist antibody and MLN8237 blocked tumor growth by inducing senescence with a resultant reduction in cell proliferation (Ki67 staining $P < 0.0001$) followed by a triggering apoptosis (cleaved caspase-3 $P = 0.0001$), with the ultimate benefit of total blockage of tumor growth.

**Discussion**

The effects of senescence on tumor growth remain controversial as the senescence-associated secretory phenotype (SASP) is characterized by secretion of many inflammatory cytokines.
and chemokines, which may induce a local or systemic pro- or antitumor reaction (8, 24–27). Some studies have shown senescent cells have cancer-promoting functions, such as in tumors with p53 loss or mutation (27–30), while other studies have demonstrated that SASP is required to maintain senescence and can promote tumor regression (31–33). Therefore, SASP includes both tumor-promoting and inhibitory cytokines and the effect of senescent cells in the tumor microenvironment is highly dependent on cell and tissue context (34).

Accumulating evidence suggests that immune cells, including NK cells, T cells, macrophages, and neutrophils, can clear premalignant cells and senescent tumor cells (18–20), (35, 36). Genetic reconstitution of p53 or stabilization of p53 protein using an Mdm2 inhibitor can promote immune-mediated clearance of OIS and/or TIS tumor cells (19, 15). However, when the antitumor immune response is impaired by the tumor microenvironment or cancer therapies (37–40), the senescent tumor cells may not be eliminated efficiently. Hence, additional treatments that accelerate clearance of therapy-induced senescent tumor cells may be helpful. Considered the frequency of p53 loss or mutation in human tumors, developing therapies that induce tumor cell apoptosis in the absence of active p53 should be highly beneficial.

Apo2L/TRAIL is a potential cancer therapeutic because it induces significant apoptosis in most tumor tissues, but not in normal tissues (41). Preclinical and clinical studies indicate that recombinant human Apo2L/TRAIL is safe for potential therapeutic use (42, 43). However, Apo2L/TRAIL has a very short half-life in vivo (44). To overcome the short half-life of Apo2L/TRAIL, several specific death receptor agonist antibodies that have a long half-life were developed (45). In vitro and in vivo data suggest that administration of DR5 agonist antibody decreases tumor cell proliferation and triggers tumor cell apoptosis in several different types of human cancer (46, 47). Phase I clinical trials demonstrate that DR5 antibody is well tolerated (48). Our in vitro data suggest both a DR5 agonist antibody and a DR5 small-molecule activator can promote cell death in senescent tumor cells.

Our data in melanoma confirm these prior reports that DR5 agonist antibody can inhibit cell growth and trigger apoptosis (43, 44), though the DR5 agonist antibody alone only reduced melanoma tumor viability by approximately 20% to 40%. A potential limitation of Apo2L/TRAIL or DR5 agonist therapy for cancers, including melanoma, is that many types of human cancer have lost sensitivity to Apo2L/TRAIL (49). Although overcoming Apo2L/TRAIL resistance still remains a challenge, our in vitro data suggest that induction of senescence increased the sensitivity to Apo2L/TRAIL. A potential mechanism involves induction of senescence stabilized cleaved caspase-8 products in response to Apo2L/TRAIL treatment. Activated caspase-8 in the cytoplasm may undergo TRAF2-mediated K48-linked polyubiquitination and degradation by proteasome. Stabilization of activated caspase-8 through inhibition of TRAF2 sensitizes cancer cells to DR-mediated apoptosis (22). Our results showed that inhibitors of apoptosis (survivin, XIAP) and DR decoy receptors DR1, DR2, and OPG were downregulated in tumor cells in response to senescence-inducing therapy. Others have reported cleavage of RIP in senescent cells, which provides a potential mechanism for enhanced sensitivity to death receptor activation in that cleaved RIP enhances interaction between the adaptor protein in the death-inducing signaling complex (DISC), FADD, and TRADD, resulting in increased sensitivity to Apo2L/TRAIL (50). Cisplatin and doxorubicin have been shown to increase Apo2L/TRAIL sensitization via upregulation of DR4 and or DR5 in different types of human cancer cells (51, 52). Our data provide new mechanistic insights for enhanced sensitivity of senescent cells to death receptor ligands. We show here that TIS resulted in the downregulation of three decoy receptors, DcR1, DcR2, and OPG, and resulted in enhanced membrane display of DR5. Moreover, knocking down of DR5 or supplement of soluble OPG in the culture media partially rescued the Apo2L/TRAIL-induced cell death in the senescent tumor cells. Thus MLN8237 augments the response to DR5-activating ligands by down-regulating Apo2L/TRAIL decoy receptors and enhancing plasma membrane display of DR5, thus enabling the potent induction of apoptosis in tumor cells. In addition, leukocytes recruited by SASP may also contribute to tumor regression. The downside of this synergy between MLN8237 and DR5 agonist antibody is that if resistance develops to MLN8237, the response to DR5 combinator will likely be lost. Our preclinical studies demonstrate that combining MLN8237 with DR5 activating antibody results in substantial blockade of human melanoma and PDX tumors in mice. In addition, our data showing the effectiveness of combined MLN8237 and DR5 antibody for treatment of vemurafenib-resistant melanoma tumors in a PDX model suggests that senescence-inducing therapy followed by a stabilized Apo2L/TRAIL ligand or DR5 antibody treatment should be considered for treatment of therapy-resistant cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Liu, O.E. Hawkins, A. Richmond
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Liu, O.E. Hawkins, A.E. Vilgelm, J.S. Pawlikowski, J.A. Ecsedy, A. Richmond
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Acknowledgments

The authors thank Avi Ashkenazi and Genentech for DR5 agonist antibody (Drozitumab) and his helpful discussions.

Grant Support

This work was supported by VA Senior Career Scientist Award (to A. Richmond); VA Merit Award 510BX000196 (to A. Richmond), NIH: R01 CA116021 (to A. Richmond), CA-R25-160056 (to A. Richmond and Y. Liu), CA068485 to the Vanderbilt-Ingram Cancer Center; VICTOR funding from Vanderbilt University; VRS353 (to Y. Liu), Y. Liu was also supported by the Vanderbilt MAGEC training program (R25CA160056).

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Received February 4, 2015; revised June 2, 2015; accepted June 30, 2015; published OnlineFirst July 7, 2015.
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Published OnlineFirst July 7, 2015; DOI: 10.1158/1078-0432.CCR-15-0293


Clinical Cancer Research

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Clin Cancer Res Published OnlineFirst July 7, 2015.

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