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Mcl-1 targeting strategies unlock the proapoptotic potential of TRAIL in melanoma cells

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Abstract

TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis selectively in cancer cells. For melanoma, the targeting of TRAIL signaling appears highly attractive, due to pronounced TRAIL receptor expression in tumor tissue. However, mechanisms of TRAIL resistance observed in melanoma cells may limit its clinical use. The Bcl-2 family members are critical regulators of cell-intrinsic apoptotic pathways. Thus, the antiapoptotic Bcl-2 protein myeloid cell leukemia 1 (Mcl-1) is overexpressed in many tumor types and was linked to chemotherapy resistance in melanoma. In this study, we evaluated the involvement of antiapoptotic Bcl-2 proteins (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, Bcl-A1, and Bcl-B) in TRAIL resistance. They were targeted by small interfering RNA-mediated silencing in TRAIL-sensitive (A-375, Mel-HO) and in TRAIL-resistant melanoma cell lines (Mel-2a, MeWo). This highlighted Mcl-1 as the most efficient target to overcome TRAIL resistance. In this context, we investigated the effects of Mcl-1-targeting microRNAs as well as the Mcl-1-selective inhibitor S63845. Both miR-193b and S63845 resulted in significant enhancement of TRAIL-induced apoptosis, associated with decreased cell viability. Apoptosis induction was mediated by caspase-3 processing as well as by Bax and Bak activation, indicating the critical involvement of intrinsic apoptosis pathways. These data may indicate a high relevance of Mcl-1 targeting also in melanoma therapy. Furthermore, the data may suggest to consider the use of the tumor suppressor miR-193b as a strategy for countering TRAIL resistance in melanoma.

KEYWORDS
Mcl-1, melanoma, miRNA, siRNA, TRAIL

1 | INTRODUCTION

Selective inhibitors for the mitogen-activated protein (MAP) kinases BRAF and MAP kinase-ERK kinase, as well as immune checkpoint modulators, have significantly improved melanoma therapy in recent years, demonstrating the high potential of targeted strategies in cancer.[1-3] Nevertheless, melanoma remains the major cause of skin cancer-related deaths.[3] Thus, new ideas and strategies for further improvement of melanoma therapy are still urgently needed. Most anticancer therapies focus on two key hallmarks of cancer, either

Abbreviations: BH3, Bcl-2 homology domain 3; MAP kinase, mitogen-activated protein kinase; Mcl-1, myeloid cell leukemia; MEK, MAP kinase-ERK kinase; miRNA, microRNA; siRNA, small interfering RNA; Smac, second mitochondrial activator of caspases; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; XIAP, chromosome X-linked inhibitor of apoptosis protein.

targeting excessive tumor cell proliferation or targeting induction of apoptosis. Even immune-modulating therapies finally aim at the elimination of cancer cells by the induction of apoptosis.[4,5] Two major paths have been described for apoptosis induction, namely (a) extrinsic pathways through death ligands such as TNF-related apoptosis-inducing ligand (TRAIL) and (b) intrinsic, mitochondrial pathways in course of cellular stress situations, for example, DNA damage.[6,7]

TRAIL triggers apoptosis via the two agonistic death receptors TRAIL-R1/DR4 and TRAIL-R2/DR5.[8,9] Upon receptor binding, a membrane-bound, death-inducing signaling complex is formed, where initiator caspase-8 and -10 are activated.[10] In a caspase cascade, effector caspases as caspase-3 are further activated, which cleave a large number of death substrates to finally execute apoptosis' programs.[11] Caspase-3 is negatively regulated through the binding of XIAP (chromosome X-linked inhibitor of apoptosis protein).[12]

A particular advantage of TRAIL is selective apoptosis induction in cancer cells, while normal tissue cells are largely spared.[13,14] Although melanoma cells have been shown to constitutively express DR5, this does not guarantee TRAIL sensitivity, as roughly half of melanoma cell lines reveal an intrinsic TRAIL resistance.[15,16] To address this limitation, different combination strategies have been identified, which can sensitize melanoma cells for TRAIL-induced apoptosis. In this context, the control of mitochondrial apoptosis pathways came into particular focus for explaining TRAIL resistance in melanoma.[17,18]

Mitochondrial apoptosis pathways are decisively controlled by the family of Bcl-2 proteins, which share one to four distinct Bcl-2 homology (BH) domains. Bcl-2 proteins interact and control each other by heterodimerization.[19] In present models, proapoptotic, multidomain proteins with three BH domains (Bax and Bak) are bound and are antagonized by antiapoptotic Bcl-2 proteins with four BH domains (Bcl-2, Bcl-x_L, myeloid cell leukemia 1 [Mcl-1], Bcl-w, Bcl-A1, and Bcl-B). On top of this, proapoptotic BH3-only proteins (Bid, Bim, Bad, and several others), characterized by just the BH3 domain, act as triggers in apoptosis through either binding and antagonizing the antiapoptotic Bcl-2 proteins or through directly activating Bax. BH3-only proteins themselves may be activated by several stimuli, as in particular by cellular stress situations.[20]

Activation of Bax and Bak is associated with permeabilization of the outer mitochondrial membrane and the release of mitochondrial proteins, such as cytochrome c and Smac (second mitochondrial activator of caspases). While cytochrome c promotes initiator caspase-9 activation via formation of the apoptosome, Smac inhibits the caspase-3 antagonist XIAP.[21-23]

Expression of antiapoptotic Bcl-2 proteins provides a basic mechanism to allow survival of normal cells and on the other hand, may prevent apoptosis induction in malignant cells.[24]

Mcl-1 appears of particular importance for the survival of different cell lineages during embryonic development, for example, hematopoietic and neuronal cells.[25,26] Its decisive role is also underlined by embryonic lethality of Mcl-1 knockout mice.[27] In recent years, the oncogenic activity of Mcl-1 received particular attention,[28] which is indicated by the frequent genomic amplification of Mcl-1 in about 40% of tumors of different origin.[29] Increased Mcl-1 protein levels were correlated to chemotherapy resistance of leukemia as well as solid tumors as of the stomach, pancreas, and bile duct.[30,31]

Besides transcription factors, gene expression is critically regulated by small noncoding microRNAs (miRNAs) by the targeting of complementary sequences on messenger RNA (mRNA). Some miRNAs function as tumor suppressors through the downregulation of antiapoptotic or oncogenic proteins, while others may be oncogenic themselves through the downregulation of tumor suppressors. In the context of Mcl-1 regulation, miR-339-3p, and miR-193b were described as tumor suppressors in melanoma.[32,33] On the contrary, upregulated Mcl-1 was associated with downregulation of miRNA-32 in melanoma.[34]

In the present study, we investigated the effects of knockdown of antiapoptotic Bcl-2 proteins with regard to the sensitization of melanoma cells for TRAIL-induced apoptosis. This highlighted the particular role of Mcl-1. Thus, we further investigated and compared different Mcl-1 targeting strategies as the selective inhibitor S63845, miR-193b, and miR-339-3p to overcome TRAIL resistance in melanoma cells.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Origin of human melanoma cell lines used in this study (A-375, SK-Mel-13, SK-Mel-19, SK-Mel-23, Mel-HO, JPC-298, Mel-2a, Mel-JuSo, and MeWo) has been described previously.[35] Cells were cultivated at 37°C, 5% CO_2 in dulbecco's modified eagle's medium (DMEM) (4.5 g/L glucose; Gibco, Invitrogen, Karlsruhe, Germany), supplemented with 10% fetal calf serum (FCS) and antibiotics (Biochrom, Berlin, Germany).

For the different assays, melanoma cells were seeded in flat-bottom 24-well plates at a density of 75.000 to 120.000 cells/well, according to the growth performance of the cell lines. Single treatments started at 48 hours after seeding (cell confluence at 50%-70%). Treatments of 100 ng/mL KillerTRAIL (AG-40T; Adipogen, San Diego) or 5 μM of the selective Mcl-1 inhibitor S63845 (CAS# 1799633-27-4; Abmole Bioscience Inc, Houston, TX) were usually applied for 24 hours, when not stated differently.

Combination treatments with small interfering RNA (siRNA) and miRNA, respectively, started early after seeding. Thus, 10 pmol siRNA/miRNA and 1.5 μL Lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA) were diluted in 100 μL Opti-MEM medium (Invitrogen, Carlsbad, CA), which was given to freshly seeded cells. After 24 hours, transfection medium was replaced by a fresh growth medium (DMEM with 5% FCS, w/o antibiotics) and incubated for an additional 24 hours. After this procedure (48 hours), cells received additional treatments, for example, TRAIL and S63845 (usually for another 24 hours).

2.2 | Cell transfection with siRNA or miRNA

Transient reverse cell transfection with siRNA or miRNA was performed with Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturer´s protocol. An amount of 10 pmol siRNA/miRNA and 1.5 µL Lipofectamine was given to 500 µL cell suspension seeded in 24-wells. The following siRNAs from Santa Cruz Biotech (Dallas, TX): were applied: Bcl-2 (sc-61899), Bcl-x_L (sc-43630), Bcl-w (sc-37293), Mcl-1 (sc-35877), Bcl-A1 (sc-37285), and Bcl-B (sc-90043) as well as a scrambled control (sc-37007). The following miRNAs were applied: miR-339-3p (hsa-miR-339-3p: HMI0497, Merck, Darmstadt, Germany) and miR-193b (hsa-miR-193: AM17100, Thermo Fisher Scientific, Life Technologies, Darmstadt, Germany).

2.3 | Determination of apoptosis

For quantification of apoptosis, cell cycle analyses were performed.[36] Cells harvested by trypsinisation were lysed in hypotonic buffer, and isolated nuclei were stained for 1 hour with 40 µg/mL propidium iodide (Sigma-Aldrich, Taufkirchen, Germany). Cells in G1, G2, and S-phase, as well as sub-G1 cells, were quantified by flow cytometry (FL3A) with a FACS Calibur (BD Bioscience, Bedford, MA). Due to the washing out of small DNA fragments, nuclei with less DNA than G1 (sub-G1) correspond to apoptotic cells with fragmented DNA.

2.4 | Determination of cell viability and mitochondrial membrane potential

Cell viability was determined by staining cells with calcein-AM (PromoCell, Heidelberg, Germany), which is converted in viable cells to green-fluorescent calcein by intracellular esterases. Cells, grown and treated in 24-well plates, were harvested by trypsinization and stained with 0.5 µM calcein-AM at 37°C for 1 hour. Labeled cells were washed with phosphate-buffered saline (PBS) and measured by flow cytometry (FL2H).

Mitochondrial membrane potential (MMP) ($\Delta\psi m$) was determined by staining cells with the fluorescent dye $TMRM^+$ (Tetramethylrhodamine methyl ester perchlorate; Sigma-Aldrich). Cells grown in 24-well plates and harvested by trypsinization were stained for 20 minutes at 37°C with 1 µM $TMRM^+$. After two-times washing with PBS, cells were measured by flow cytometry (FL2H).

2.5 | Bax and Bak activation assays

For analysis of Bax/Bak conformational changes related to protein activation, cells were stained with primary antibodies specific for the Bax N-terminal domain (Bax-NT; rabbit; #06-499; Merck Millipore, Darmstadt, Germany) and for the Bak N-terminal domain (Bak-NT; rabbit; #06-536; Merck Millipore, Darmstadt, Germany), respectively. Cells were harvested by trypsinisation and fixed for 30 minutes at 4°C with 0.5% paraformaldehyde in PBS. Then, they were incubated for 1 hour at 4°C in PBS/1% FCS containing Bax/Bak-NT antibodies (1:100) and 0.1% saponine for cell permeabilization. Staining was proceeded by incubation for 1 hour at 4°C in the dark using a goat anti-rabbit secondary antibody (immunoglobulin G [H + L]-FITC; Jackson Immuno Research, West Grove, PA). After washing and resuspension in PBS, cells were measured by flow cytometry (FL1H).

2.6 | Western blotting

For preparing total protein extracts, cells were trypsinized, washed with PBS and lysed in 150 mM NaCl, 1% NP-40 and 50 mM Tris (pH 8.0). To guaranty equal loading of samples in protein gels, concentrations of protein extracts were conscientiously determined in triplicate determinations by Pierce Bicinchoninic acid protein assay (Thermo Fisher Scientific). Western blotting on nitrocellulose membranes was performed as described previously.[35] Primary antibodies were purchased from Cell Signaling (Danvers, MA): Cleaved caspase-3 (rabbit, 1:1000; #9661); caspase-8 (mouse, 1:1000; #9746); cleaved caspase-9 (rabbit, 1:1000; #2002); Bcl-x_L (rabbit, 1:1000; #2762); Mcl-1 (rabbit, 1:1000; #4572); Bcl-2 (rabbit, 1:1000; #2872); Bcl-w (rabbit, 1:1000; #2724) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (rabbit, 1:1000; #2118). Cells were finally stained with secondary antibodies for 1 hour at room temperature, using peroxidase-labeled goat anti-rabbit or goat anti-mouse (dilution of 1:5000; DakoPerkin Elmer, Waltham, MA). Proteins were detected using Immobilon Western Chemiluminiscent HRP Substrate (Sigma-Aldrich, St Louis, MO) and visualized by Fusion FX Image analyzer.

2.7 | Real-time polymerase chain reaction

To quantify the expression levels of Bcl-A1 and Bcl-B mRNAs, a quantitative real-time polymerase chain reaction (PCR) was used (qTower; Analytik Jena, Jena, Germany). Total cellular RNA was isolated with TRIzol lysis reagent (Thermo Fischer Scientific). For reverse transcription, the First Strand complementary DNA Synthesis Kit of Thermo Fisher Scientific was applied. Real-time PCR amplification for Bcl-A1 and Bcl-B was performed with the primers given below using 2× PCR MasterMix (Thermo Fisher Scientific). For normalization, a standard curve for GAPDH was used. Primers: Bcl-A1(FW), 5´-GATAAGGCAAAACGGAGGCTGG-3´; Bcl-A1(Rev), 5´-CTCTTCTTGTGGGCCACTGAC-3´; Bcl-B(FW): 5´-GGACACCGGGACACGG-3´; Bcl-B(Rev): 5´-GGGGGTCCTGAAGAAGTGAC-3´.

2.8 | Statistical analyses

Assays were done in triplicate determinations, and at least two independent experiments were performed. For apoptosis and cell

viability assays, depicted mean values and standard deviations were calculated by enclosing all individual values of the independent experiments. For MMP, Bax, and Bak assays, the mean values of representative experiments are shown. Statistical significance was proven by the ANOVA test (two-way, multiple comparisons), using generally all obtained values of the independent experiments. *P* values of less than .05 were considered statistically significant. Depicted Western blot data were verified by at least two independent series of cellular extracts.

3 | RESULTS

3.1 | Efficient knockdown of antiapoptotic Bcl-2 proteins in melanoma cell lines by siRNA

To address the significance of antiapoptotic Bcl-2 proteins in melanoma, expression of Bcl-2, Bcl-x_L, Mcl-1, and Bcl-w was analyzed by immunoblotting in a panel of nine representative melanoma cell lines (A-375, JPC-298, Mel-2a, Mel-HO, MeWo, Mel-JuSo, SK-Mel-13, SK-Mel-19, and SK-Mel-23) (Figure 1A). Protein expression of antiapoptotic Bcl-2 proteins Bcl-A1 and Bcl-B could not be determined by commercial antibodies, due to low sensitivity (data not shown), as also reported elsewhere.[37,38] Consistently high expression was found for Bcl-x_L, Bcl-w, and Mcl-1, whereas Bcl-2 showed stronger variation, with only weak expression in A-375 and Mel-JuSo (Figure 1A).

For investigating the role of antiapoptotic Bcl-2 proteins in TRAIL sensitivity of melanoma cells, protocols were established for efficient knockdown by RNA interference. The established protocol for siRNA transfection resulted in high knockdown efficiency at the protein level, as demonstrated for Bcl-x_L, Mcl-1, and Bcl-w in Mel-HO and A-375 as well as for Bcl-2 in Mel-HO (Figure 1B). Nonspecific off-target effects of siRNA transfection on Bcl-2 protein expression were largely excluded by transfection with a nonspecific siRNA (OffT). To largely exclude also nonspecific effects of a given siRNA on other Bcl-2 proteins, the expression levels of Bcl-x_L, Mcl-1, Bcl-2, and Bcl-w were determined after all siRNA treatments. Nontarget effects on other Bcl-2 proteins were less pronounced and mostly within the limits of variation (Figure 1B).

As no suitable antibodies could be found for Bcl-A1 and Bcl-B, we demonstrated the efficiency of their siRNA silencing in cell line A-375 at the mRNA level, using real-time PCR. Thus, specific knockdown by siRNAs resulted in a reduction of Bcl-A1 mRNA levels to 16% and 17%, respectively, as shown in two independent experiments. Similarly, Bcl-B mRNA was downregulated to 33% and 27%, respectively (Figure 1C).

3.2 | Sensitization of melanoma cells for TRAIL by Mcl-1 knockdown

To evaluate the significance of antiapoptotic Bcl-2 proteins for cell survival and TRAIL sensitivity of melanoma cells, Bcl-2 proteins were downregulated by six different siRNAs in TRAIL-sensitive (A-375 and Mel-HO) and in TRAIL-resistant melanoma cell lines (MeWo and Mel-2a). Cells received TRAIL at 48 hours after the starting of siRNA treatment, and effects on apoptosis (Figure 2) and on cell viability (Figure 3) were analyzed after another 24 hours. TRAIL-sensitive cell lines responded to TRAIL with 21% (A-375) and 11% apoptosis (Mel-HO), respectively. This effect was strongly enhanced by siMcl-1, resulting in 42% (A-375) and 31% apoptosis (Mel-HO). Resistant cell lines (MeWo and Mel-2a) showed no apoptotic response to TRAIL (<6%). However, TRAIL-induced apoptosis was significantly enhanced by Mcl-1 knockdown resulting in apoptosis values of 22% (MeWo) and 20% (Mel-2a), respectively. In contrast, combinations of TRAIL with the other siRNAs were generally less effective for the induction of apoptosis, and single treatments with siRNAs remained below 12% (Figure 2).

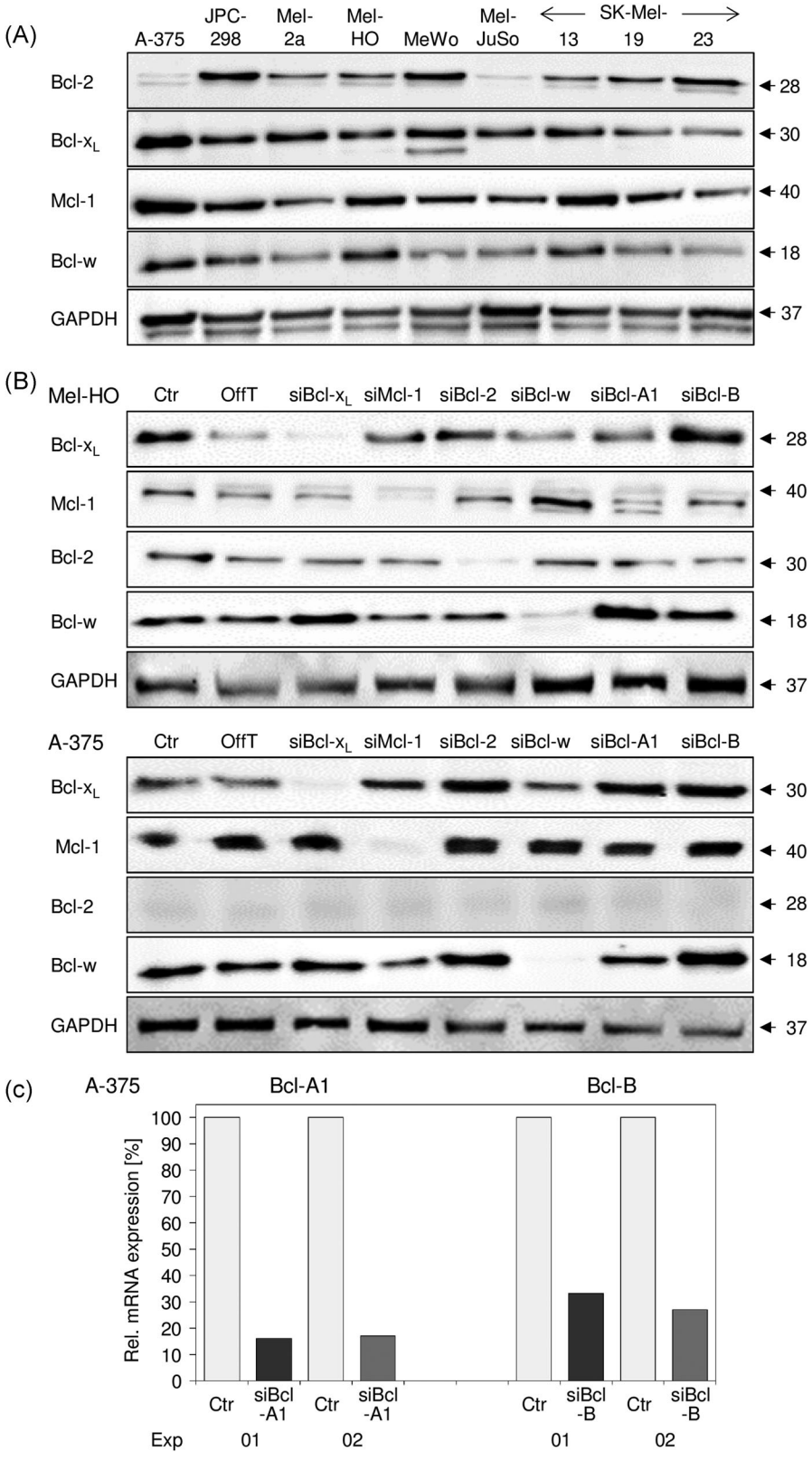
The effects at the level of cell viability were largely in parallel. Thus in A-375 and Mel-HO, TRAIL-induced loss of cell viability was enhanced by Mcl-1 knockdown, leaving 39% and 28% viable cells, respectively. In MeWo and Mel-2a, the week effects of TRAIL on cell viability were strongly enhanced, resulting in remaining viable cells of only 57% (MeWo) and 35% (Mel-2a). Again, other treatments were less effective (Figure 3). Thus in four representative melanoma cell lines, Mcl-1 targeting turned out as the most promising strategy for enhancing TRAIL sensitivity and for overcoming TRAIL resistance.

3.3 | Activation of mitochondrial apoptosis pathways by TRAIL and Mcl-1 knockdown

For better understanding, the pathways mediating enhanced TRAIL sensitivity, activation of extrinsic initiator caspase-8, intrinsic initiator caspase-9, and the predominant effector caspase-3 were investigated by Western blotting in TRAIL-sensitive A-375 and in resistant MeWo and Mel-2a. In A-375, TRAIL alone already resulted in strong activation of the caspase cascade, seen by cleavage products of caspase-9 (35 kDa), caspase-8 (43, 41, and 18 kDa) and caspase-3 (21, 19, and 17 kDa) (Figure 4A). In contrast, almost no caspase processing was seen in resistant MeWo and Mel-2a in response to TRAIL alone. In particular, no mature 17 kDa caspase-3 cleavage product appeared (Figure 4B).

Mcl-1 knockdown alone had some effect on caspase-3 in the cell lines as seen by the slightly enhanced 17 kDa product. This came along with some processing of caspase-9 in A-375 and MeWo (35 kDa) as well as some processing of caspase-8 in Mel-2a (18 kDa). Strong activation of the caspase cascade as indicated by high expression of caspase-3 cleavage products was seen in MeWo and Mel-2a under combination treatment (siMcl-1 + TRAIL). In A-375, the effects of combination treatment were visible by a higher ratio of caspase-3 mature product (17 kDa) vs intermediate cleavage products of 19 and 21 kDa (Figure 4A). The finding of caspase-8 activation in combinations may be at least partly explained by a positive feedback loop leading from activated caspase-3 up to caspase-8, as described earlier.[39,40]

Loss of MMP, as an indicator of mitochondrial pathway activation, was investigated in A-375, MeWo, and Mel-2a at 72 hours post-siRNA transfection (TRAIL treatment for the last 24 hours). Significant loss of MMP in course of TRAIL was seen in A-375 (80%), whereas MeWo and Mel-2a were not responsive. Knockdown of Mcl-1 alone significantly decreased MMP in all three cell lines resulting in 30% to 47% cells with low MMP. This effect was strongly enhanced by combination treatment resulting in 96%, 51%, and 87%



cells with low MMP in A-375, MeWo, and Mel-2a, respectively (Figure 4B).

The proapoptotic effectors Bax and Bak critically control intrinsic apoptosis pathways. Activation of both proteins was determined due to characteristic conformational changes monitored by Bax-NT (N-terminus) and Bak-NT antibodies, respectively. Significant activation of both Bax and Bak was seen in A-375 and MeWo as a result of Mcl-1 knockdown, as compared to Off-target controls. Thus in A-375, Bax and Bak were activated 51% and 38% of cells; in MeWo, 71% and 45% of cells showed activation of Bax and Bak, respectively. Bax and Bak activation were further enhanced in A-375 in the combination with TRAIL (71%/75%), whereas TRAIL remained without additional effect in MeWo (Figure 4C,D). The fact that TRAIL remained without effect on Bax/Bak activation in TRAIL-resistant cell line MeWo may be at least partly explained by previous findings showing a strong relation of TRAIL resistance and lacking Bax activation in melanoma cells.[17,41]

These data demonstrate that although the Mcl-1 knockdown did not efficiently induce apoptosis by itself, it preactivated mitochondrial apoptosis pathways, thus opening a "mitochondrial gate." This was then used by TRAIL for enhanced apoptosis induction.

3.4 | Mcl-1 targeting by the Mcl-1 inhibitor S63845 and by miRNAs

The role of Mcl-1 in TRAIL resistance was further investigated in the TRAIL-resistant cell line MeWo by the novel BH3 mimetic and specific Mcl-1 antagonist S63845. When applied alone, S63845 resulted in 15% apoptosis induction and reduction of viable cells to 60%. The proapoptotic Bcl-2 proteins Bax and Bak were strongly activated by S63845 (44%). In combination with TRAIL, apoptosis was strongly enhanced (42%) and cell viability was further decreased (15% remaining viable cells), while Bax and Bak activation were not further affected in MeWo cells by additional TRAIL (Figure 5A).

To evaluate alternative strategies for Mcl-1 targeting, miR-193b and miR-339-3p, reported to downregulate Mcl-1 expression, were applied in MeWo cells and were compared to siMcl-1. Comparable to the effects of siMcl-1, miR-193b enhanced TRAIL-induced apoptosis by 4-fold (15%, Figure 5B) and further decreased cell viability (53% remaining viable cells, Figure 5C), as compared to the off-target controls. The effects of miR-339-3p showed a similar tendency but were less pronounced. Clearly indicating the activation of mitochondrial apoptosis pathways, miR-193b alone significantly activated Bax (64%, Figure 5D) and Bak (52%, Figure 5E), comparable to siMcl-1 effects. In conclusion, the targeting of Mcl-1 appeared as a highly promising strategy to enhance the antineoplastic effects of TRAIL, which may finally pave the way for therapeutic approaches. The activated pathways were generally based on the activation of Bax and Bak, underlining their particular roles in TRAIL-induced apoptosis in melanoma cells.

4 | DISCUSSION

Despite the development of new therapeutic approaches for metastatic melanoma, survival prognosis is still limited, particularly due to acquired therapy resistance.[3] New ideas and additional strategies may help to finally defeat this deadly disease. Apoptosis resistance represents a critical hallmark in cancer,[4] and the targeting of apoptosis pathways, for example, by the death ligand TRAIL, appears as a promising antitumor strategy.[18] The particular advantage of TRAIL is based on its capability to selectively induce apoptosis in cancer cells, while normal cells are largely spared.[13,14]

TRAIL agonists have proven good tolerability and safety profiles in clinical trials, however, an additional clinical benefit, when TRAIL was applied in combination therapies, so far remained on a low level. For example, in B-cell lymphoma patients treated with recombinant TRAIL (dulanermin) in combination with rituximab (phase I) as well as in nonsmall-cell lung cancer patients treated with dulanermin in combination with paclitaxel, carboplatin, and bevacizumab (phase II), positive combination effects of TRAIL were reported only within the first 6 months.[42,43] Comparable results were obtained in a phase III trial for advanced nonsmall-cell lung cancer patients treated with dulanermin in combinations with vinorelbine and cisplatin. Also here, the early improvement of progression-free survival seen for the combination, vanished after 12 months.[44]

Lack of sustained TRAIL efficacy can be attributed to resistance mechanisms, as reported in different cancer cells, for example, of the

FIGURE 1 Efficient knockdown of antiapoptotic Bcl-2 proteins by siRNA. A, Expression of Bcl-2, Bcl-x_L, Bcl-w, and Mcl-1 was determined by Western blot analysis in nine human melanoma cell lines (A-375, JPC-298, Mel-HO, MeWo, Mel-JuSo, SK-Mel-13, SK-Mel-19, and SK-Mel-23). Equal protein amounts (30 μg) were loaded in each lane, as proven by Ponceau staining and GAPDH, used as a loading control. Molecular weights (in kDa), as determined by a protein standard, are given on the right side. Three independent series of protein extracts revealed comparable results. B, Melanoma cell lines Mel-HO and A-375 and were transfected with each 10 pmol of siRNA against Bcl-x_L, Mcl-1, Bcl-2, Bcl-w, Bcl-A1, and Bcl-B or scrambled control (OffT), as indicated. Proteins were harvested at 48 hours posttransfection for analysis of Bcl-2, Bcl-x_L, Bcl-w, and Mcl-1 expression by Western blotting. Equal protein amounts (30 μg) were loaded in each lane as proven by Ponceau staining and GAPDH, used as a loading control. Some variations seen here for the GAPDH signals may result from technical challenges in Western blotting. But two independent series of protein extracts revealed highly similar results. C, Results of real-time RT-PCR determined in cell line A-375 for Bcl-A1 (left) and Bcl-B mRNA expression (right) are shown. The values of siRNA-treated cells (siBcl-A1, siBcl-B) vs nontreated controls (Ctr, set to 100%) are shown in two independent experiments (Exp 01/02). GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; Mcl, myeloid cell leukemia; mRNA, messenger RNA; siRNA, small interfering RNA

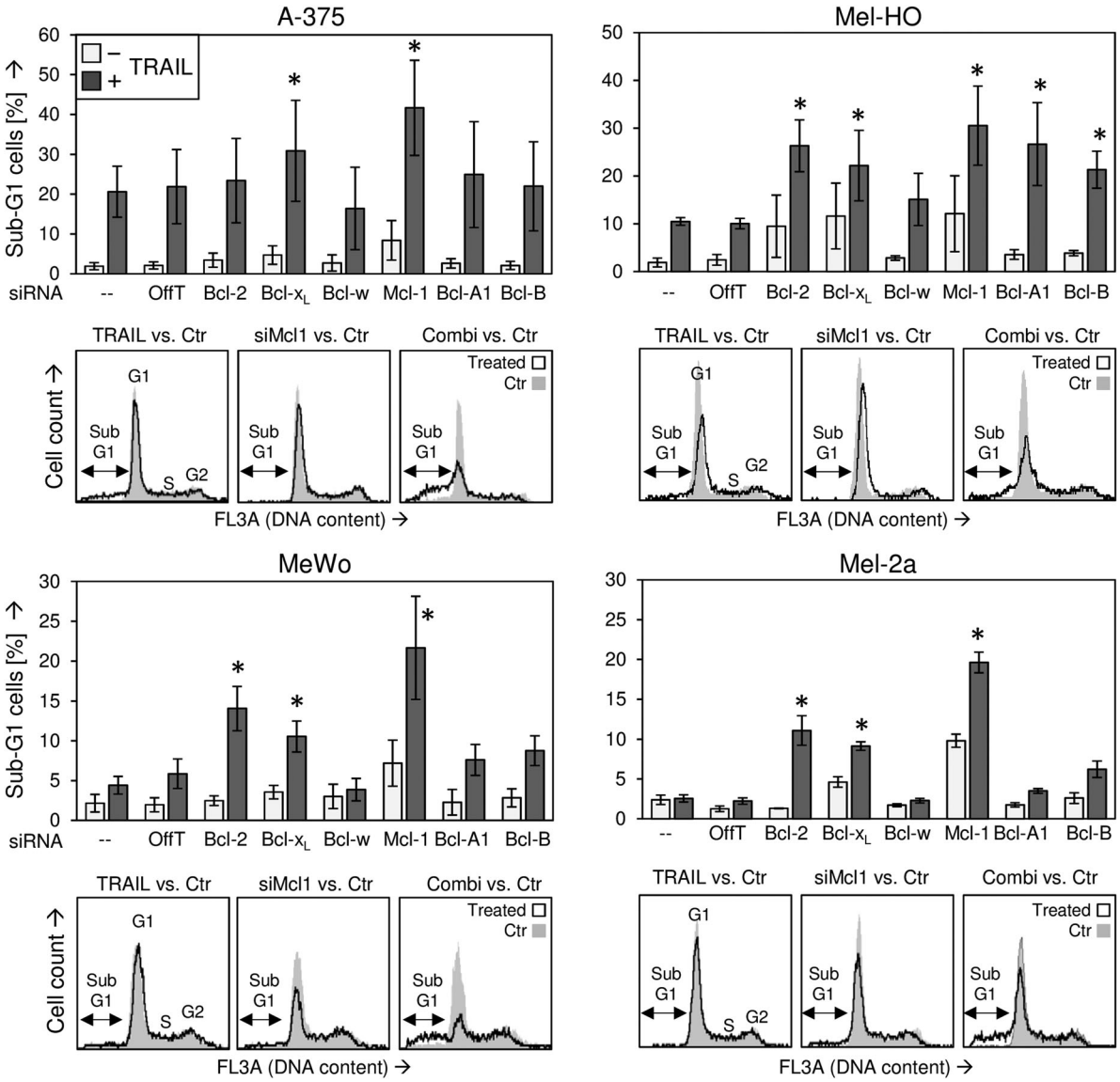


FIGURE 2 Sensitization for TRAIL-induced apoptosis by Bcl-2 protein knockdown. Effects of Bcl-2 protein knockdown by siRNA on TRAIL-induced apoptosis were determined in TRAIL-sensitive cell lines A-375 and Mel-HO as well as in the TRAIL-resistant cell lines MeWo and Mel-2a. Assays were performed at 72 hours after the transfection of indicated siRNAs and at 24 hours after TRAIL treatment (100 ng/mL). Apoptosis was determined by PI staining and flow cytometry (cell cycle analysis). Cell cycle phases (G1, G2, and the S-phase) are indicated in overlays given below; apoptotic cells correspond to weakly PI-stained cells (sub-G1 cells). Indicated mean values and SDs correspond to all individual values of at least two independent experiments (each one with triplicates, at least six independent values). Statistical significance is indicated for the comparison of the combinations vs TRAIL treatment alone (*$P < .05$, ANOVA, two-way, multiple comparisons). ANOVA, analysis of variance; SD, standard deviation; siRNA, small interfering RNA; TRAIL, TNF-related apoptosis-inducing ligand

breast, colon, and ovary[45,46] as well as of melanoma.[47,48] In the present study, consistent TRAIL resistance in melanoma cells is represented by the cell lines MeWo and Mel-2a, while TRAIL-sensitive cell lines (A-375, Mel-HO) may still develop inducible TRAIL resistance.[47] Countering TRAIL resistance mechanisms represents a basic condition for the development of efficient TRAIL-based clinical approaches.

Multiple strategies have been tested in melanoma cells for overcoming TRAIL resistance.[17] These have revealed an insufficient caspase cascade via caspase-8/caspase-3 as well as the requirement of the mitochondrial amplification loop. The level of Bcl-2 proteins thus represents a highly critical step in the control of TRAIL sensitivity.[17,41,49,50] Upregulation of antiapoptotic Bcl-2 proteins is a frequent issue in cancer, which was also associated with TRAIL resistance, for example, in pancreatic carcinoma and prostate cancer cells.[51-53] TRAIL-induced apoptosis in melanoma cells was particularly correlated with Bax activation and was abrogated by Bcl-2 overexpression.[17,54] Thus, the targeting of antiapoptotic Bcl-2 proteins represents a promising strategy to sensitize melanoma cells for TRAIL. This approach was further investigated here.

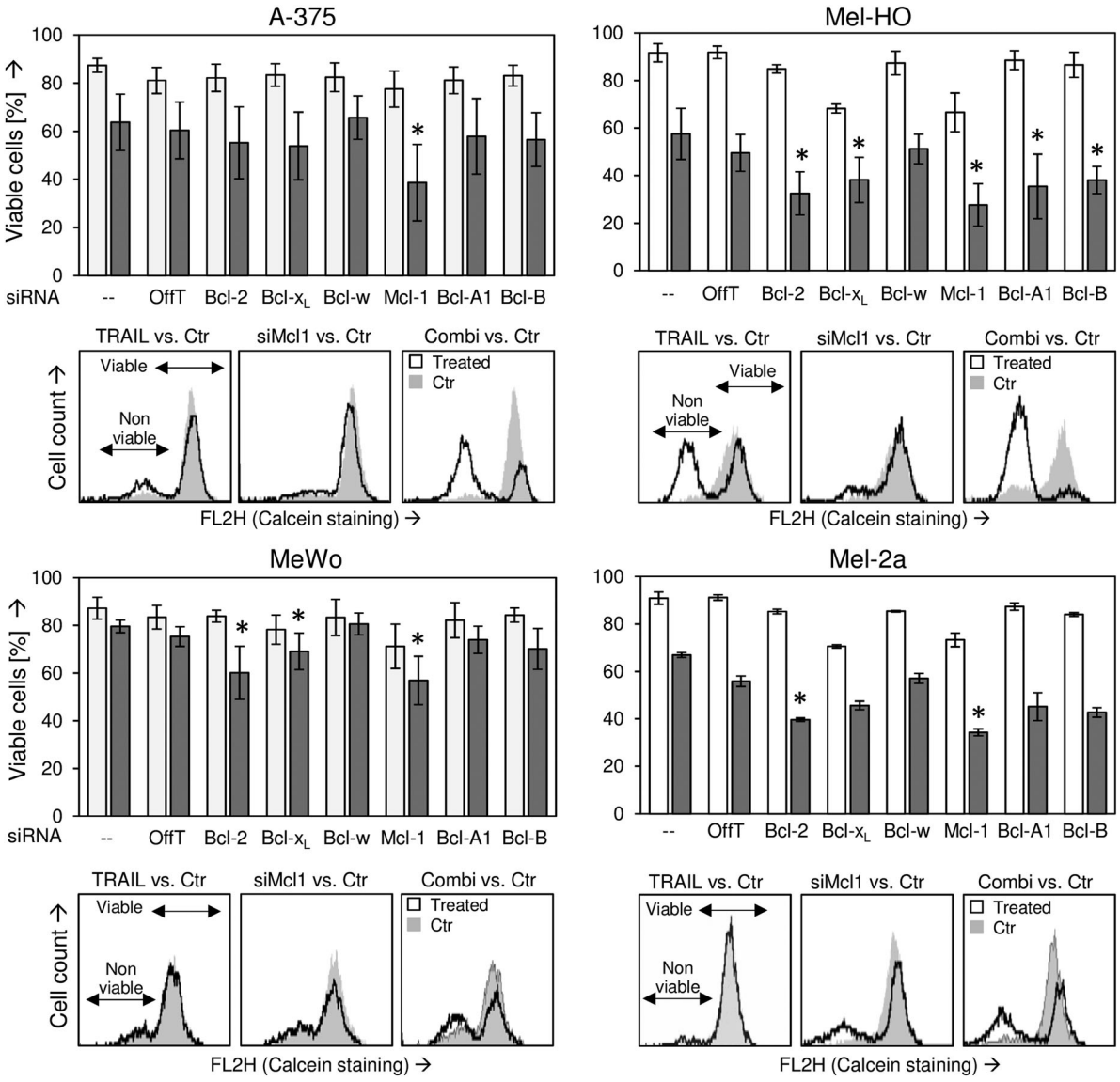


FIGURE 3 Loss of cell viability by combined TRAIL and Bcl-2 protein knockdown. Effects of Bcl-2 protein knockdown by siRNA on TRAIL-induced loss of cell viability were determined in TRAIL-sensitive cell lines A-375 and Mel-HO as well as in the TRAIL-resistant cell lines MeWo and Mel-2a. Assays were performed at 72 hours after the transfection of indicated siRNAs and at 24 hours after TRAIL treatment (100 ng/mL). Cell viability was determined by calcein-AM staining and flow cytometry; viable and nonviable cell populations are indicated in the overlays given below. Indicated mean values and SDs correspond to all individual values of at least two independent experiments (each one with triplicates, at least six independent values). Statistical significance is indicated for the comparison of the combinations vs TRAIL treatment alone (*$P < .05$, ANOVA, two-way, multiple comparisons). ANOVA, analysis of variance; SD, standard deviation; siRNA, small interfering RNA; TRAIL, TNF-related apoptosis-inducing ligand

The particular roles of six antiapoptotic Bcl-2 proteins (Bcl-2, Bcl-x_L, Mcl-1, Bcl-w, Bcl-A1, Bcl-B) have been acknowledged in models explaining the mutual regulation of Bcl-2 proteins.[19] A major goal of the present study was to evaluate their particular contribution to the regulation of TRAIL sensitivity in melanoma cells. The established siRNA strategies mediated high efficiency and largely specific effects, as verified by Western blotting. Some improvement of the TRAIL effects was seen upon the downregulation of several antiapoptotic Bcl-2 proteins in different melanoma cell lines. However, in terms of apoptosis induction and loss of cell viability, knockdown of Mcl-1 turned out as the most efficient strategy, which was effective in all four melanoma cell lines, investigated.

The Mcl-1 gene was reported as amplified in different human cancer types, also in melanoma, and high Mcl-1 expression has often been associated with therapy resistance.[28,29,55] A particular significance of Mcl-1 for melanoma cell survival was also demonstrated when Mcl-1 and Bcl-A1 knockdown were combined with chemotherapy.[38,56]

Addressing the mechanisms, by which Mcl-1 knockdown enhanced TRAIL-induced apoptosis in melanoma cells, activation of the caspase cascade and increased loss of MMP was shown. Of particular

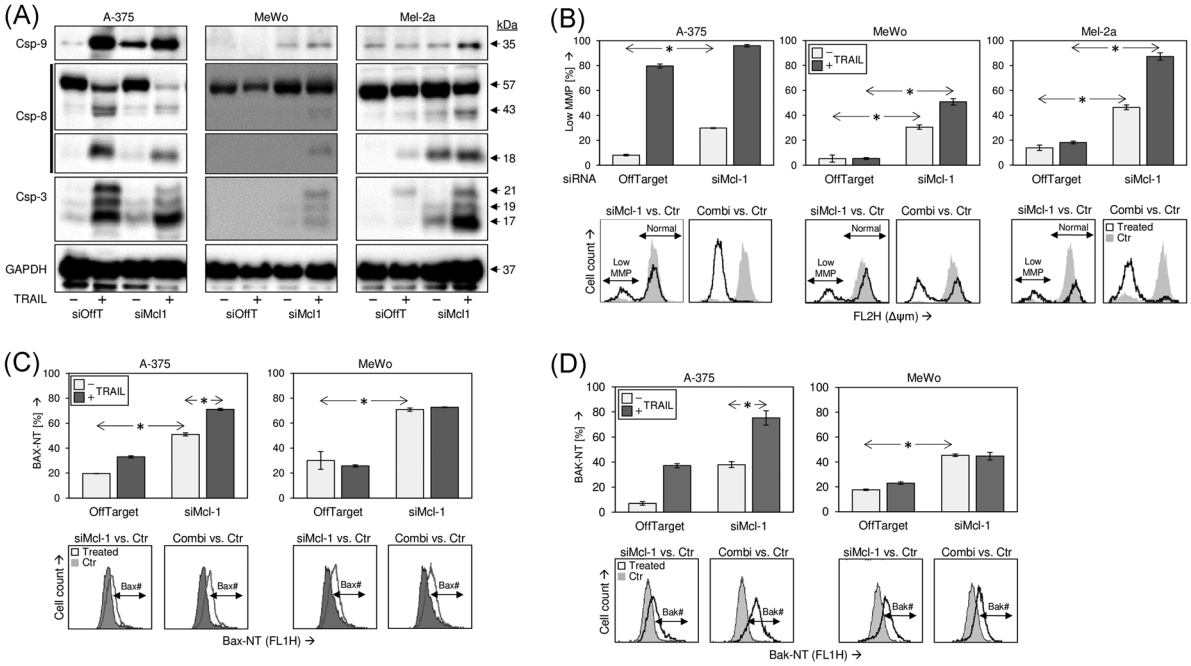


FIGURE 4 Apoptosis mechanisms in response to Mcl-1 knockdown. A, Processing of caspases-8, -9, and -3 was monitored in A-375, MeWo, and Mel-2a by Western blotting at 72 hours after starting siMcl-1 or scrambled control siRNA (OffT) transfection and at 24 hours of TRAIL treatment. Equal protein amounts (30 μg) were loaded as proven by Ponceau staining and GAPDH, used as a loading control. Molecular weights (in kDa) determined by a protein standard, are given on the right side. Some variations seen here for the GAPDH signals may result from technical challenges in Western blotting. But Western blots of two independent series of protein extracts revealed highly comparable results. The caspase-8 blot was cut to show the different protein fragments. B, Mitochondrial membrane potential (MMP) in response to Mcl-1 knockdown and TRAIL treatment was determined by $TMRM^+$ staining in A-375, MeWo, and Mel-2a at 72 hours after transfection and at 24 hours after TRAIL treatment. C and D, Bax and Bak activation was determined by staining with Bax-NT (N-terminus) and Bak-NT antibodies, at 60 hours after transfection and at 12 hours after starting TRAIL treatment. B-D, At least two independent experiments, each one with triplicates, revealed highly comparable results; mean values and SDs of a representative experiment are shown. Statistical significance was determined by ANOVA test (two-way, multiple comparisons) using all individual values (at least six); it is indicated for siMcl-1 treatment vs controls or for combination treatment vs siMcl-1 alone (*$P < .05$). Examples of treated cells vs controls are given below the bar charts (overlays), and cell populations with low MMP or activated Bax/Bak (#) are indicated. ANOVA, analysis of variance; Mcl, myeloid cell leukemia; SD, standard deviation; siRNA, small interfering RNA; TRAIL, TNF-related apoptosis-inducing ligand

note, the proapoptotic Bcl-2 proteins Bax and Bak were consistently activated in course of Mcl-1 knockdown, indicating the dominant role of Mcl-1 in suppressing the activity of these important proapoptotic agonists. We already showed that Bax serves as a master regulator of TRAIL-induced apoptosis in melanoma cells.[17,18] On the other hand, apoptosis in melanoma cells can also be mediated by Bak pathways, as shown for Bcl-x_S-induced apoptosis.[57] Bak has been described as a particular target of Mcl-1.[58,59]

Antiapoptotic Bcl-2 proteins may be antagonized by BH3 mimetics, which mimic the activity of proapoptotic BH3-only proteins. For preparing clinical applications, several BH3 mimetics have been developed, for example, ABT-199 (venetoclax) directed against Bcl-2; ABT-263 (navitoclax) directed against Bcl-2 and Bcl-x_L as well as ABT-737 directed against Bcl-2, Bcl-x_L, and Bcl-w. Monotherapy with ABT-199 resulted in improved overall survival of multiple myeloma patients.[60,61] However, patients with advanced solid tumors frequently did not profit, for example, as reported for the combination of ABT-263 and the EGFR inhibitor erlotinib.[60,61] In preclinical studies, BH3 mimetics, in general, have shown less efficiency in melanoma as compared to hematological cancers.[62]

Several previous BH3 mimetics did not affect Mcl-1, which may at least partly explain their insufficiency in melanoma. In the meantime, also Mcl-1-specific BH3 mimetics have been established (S63845, AMG-176, AMG-397, and AZD-599), which are planned for clinical trials in patients with hematological malignancies.[63] For S63845, antitumor effects and low toxicity have been reported in preclinical studies of multiple myeloma and leukemia. Most melanoma cells, however, showed pronounced resistance to S63845 when it was applied alone.[64] Also melanoma cell line MeWo, as shown here, was almost not responsive to S63845 in terms of apoptosis induction and loss of cell viability. However, MeWo cells showed strong activation of Bax and Bak in response to S63845, thus suggesting that a proapoptotic gate was opened. This resulted in sensitization for TRAIL-induced apoptosis. S63845 has shown also positive effects in melanoma cells when combined with other BH3 mimetics or the proteasome inhibitor bortezomib.[62]

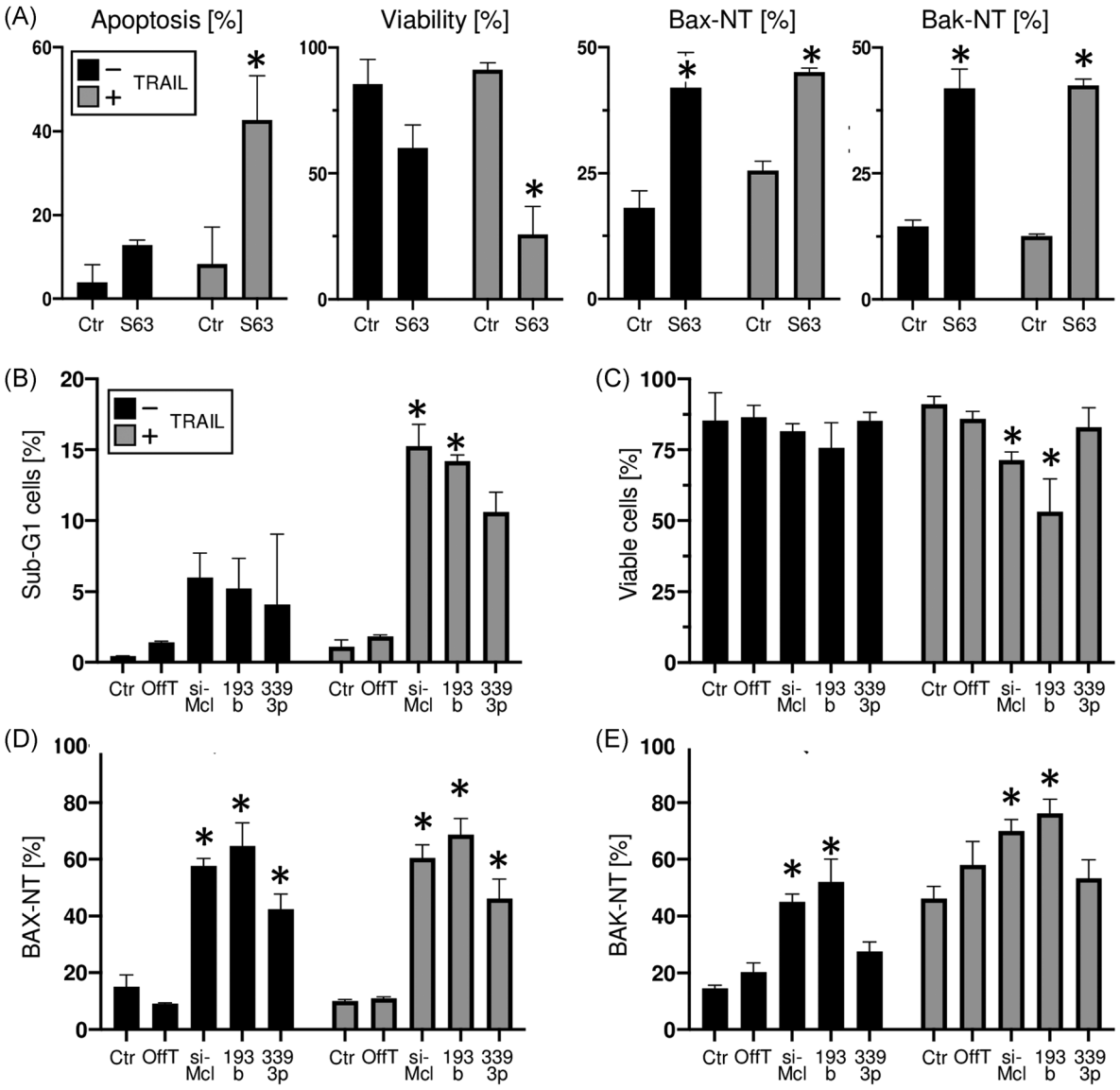


FIGURE 5 Use of miRNAs and S63845 to overcome TRAIL resistance. A, MeWo cells were treated with 5 μM S63845 and TRAIL (100 ng/mL, light grey bars, following at 48 hours by determination of apoptosis (PI assay) and cell survival (calcein assay). Bax and Bak activation was quantified by NT antibodies at 12 hours posttreatment. B-E, MeWo cells were transfected with scrambled siRNA (OffT), siMcl-1, miR-193b, miR-339-3p, or were nontransfected (Ctr). In addition, they were treated with TRAIL at 48 hours posttransfection (100 ng/mL, light grey bars). TRAIL treatment was for 48 hours for determination of apoptosis (B) and cell survival (C), whereas for Bax (D) and Bak activation assays (E), TRAIL treatment was for only 12 hours. A-E, At least two independent experiments were performed, each one with at least duplicates; data are expressed as means ±SDs of all individual values (n = 4). Statistical significance of single-treated cells (w/o TRAIL, dark grey bars) is indicated vs nontreated controls, whereas the statistical significance of combination treatments ($TRAIL^+$, light grey bars) is indicated vs TRAIL treatment alone (*$P < .05$). SD, standard deviation; siRNA, small interfering RNA; TRAIL, TNF-related apoptosis-inducing ligand

Limiting efficiency of BH3 mimetics in melanoma cells has raised the question about their general applicability for melanoma. We thus investigated further strategies for posttranscriptional Mcl-1 control. miRNAs can efficiently regulate gene expression. Due to the small size, they allow an efficient packaging in viral vectors, which may be used in gene-based therapy.[65] Both miR-339-3p and miR-193b have been shown to suppress melanoma cell proliferation and/or invasion, which was associated with Mcl-1 downregulation.[32,33] They were used in the present study to restrict Mcl-1 expression in MeWo. Whereas miR-339-3p was less effective in combination with TRAIL, the combination of miR-193b and TRAIL resulted in significant loss of cell viability and increased apoptosis. In parallel with the siMcl-1 approach, strong Bax and Bak activation was observed, indicating the activation of intrinsic apoptotic pathways.

In conclusion, tumor cells can be targeted by direct apoptosis agonists, for example, by TRAIL, as well as by inhibition of

antiapoptotic mechanisms. The present study demonstrates the only limited value of single treatments, while the combination of TRAIL and Mcl-1 targeting revealed high efficiency in 2D cultures of melanoma cells. The principle strategy of combining apoptosis agonists and the targeting of antiapoptotic pathways appears generally promising in cancer therapy. Also, further combinations with MAPK inhibitors may be considered, in particular also due to crosstalks between MAPKs and Bcl-2 proteins. Thus, MAPK activation results in downregulation of the proapoptotic protein Bim, which serves as an important antagonist of Mcl-1.[66,67]

Of course, 2D cell cultures represent only a highly simplified model of cancer. The situation may improve when more complex models as 3D cultures or animals are used. Indeed, we have shown previously the antitumor effects of TRAIL in melanoma nude mouse models, when TRAIL was expressed by a replication-competent adenoviral vector.[68] As other drugs, BH3 mimetics may reveal varying efficacy in different tumor models as 2D, 3D, and in vivo, as previously shown.[56] Nevertheless, BH3 mimetics are presently promising enough for their clinical testing in patients with different tumors.[60-62] Thus, it appears conceivable that the good combination effects we saw here in a basic melanoma model may be finally translated into a clinical situation. Thus, after possibly further testing the particular effectors identified here in animal models, pharmacological use of TRAIL and S63845 or gene therapeutic use of miR-193b may be considered for melanoma therapy.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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REFERENCES

1. Franklin C, Livingstone E, Roesch A, Schilling B, Schadendorf D. Immunotherapy in melanoma: recent advances and future directions. *Eur J Surg Oncol*. 2017;43(3):604-611.
2. Schadendorf D, van Akkooi ACJ, Berking C, et al. Melanoma. *Lancet*. 2018;392(10151):971-984.
3. Gershenwald JE, Scolyer RA, Hess KR, et al. Melanoma staging: evidence-based changes in the American Joint Committee on Cancer eighth edition cancer staging manual. *CA Cancer J Clin*. 2017;67(6):472-492.
4. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646-674.
5. Eberle J, Kurbanov BM, Hossini AM, Trefzer U, Fecker LF. Overcoming apoptosis deficiency of melanoma-hope for new therapeutic approaches. *Drug Resist Updat*. 2007;10(6):218-234.
6. Colin DJ, Hain KO, Allan LA, Clarke PR. Cellular responses to a prolonged delay in mitosis are determined by a DNA damage response controlled by Bcl-2 family proteins. *Open Biol*. 2015;5(3):140156.
7. Dickens LS, Powley IR, Hughes MA, MacFarlane M. The "complexities" of life and death: death receptor signalling platforms. *Exp Cell Res*. 2012;318(11):1269-1277.
8. Pan G, Ni J, Wei YF, Yu G, Gentz R, Dixit VM. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science*. 1997;277(5327):815-818.
9. Walczak H, Degli-Esposti MA, Johnson RS, et al. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. *EMBO J*. 1997;16(17):5386-5397.
10. Krammer PH, Arnold R, Lavrik IN. Life and death in peripheral T cells. *Nat Rev Immunol*. 2007;7(7):532-542.
11. Dickens LS, Boyd RS, Jukes-Jones R, et al. A death effector domain chain DISC model reveals a crucial role for caspase-8 chain assembly in mediating apoptotic cell death. *Mol Cell*. 2012;47(2):291-305.
12. Deveraux QL, Roy N, Stennicke HR, et al. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J*. 1998;17(8):2215-2223.
13. Walczak H, Miller RE, Ariail K, et al. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med*. 1999;5(2):157-163.
14. Ashkenazi A, Pai RC, Fong S, et al. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest*. 1999;104(2):155-162.
15. Geserick P, Drewniok C, Hupe M, et al. Suppression of cFLIP is sufficient to sensitize human melanoma cells to TRAIL- and CD95L-mediated apoptosis. *Oncogene*. 2008;27(22):3211-3220.
16. Kurbanov BM, Geilen CC, Fecker LF, Orfanos CE, Eberle J. Efficient TRAIL-R1/DR4-mediated apoptosis in melanoma cells by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). *J Invest Dermatol*. 2005;125(5):1010-1019.
17. Quast SA, Berger A, Plotz M, Eberle J. Sensitization of melanoma cells for TRAIL-induced apoptosis by activation of mitochondrial pathways via Bax. *Eur J Cell Biol*. 2014;93(1-2):42-48.
18. Eberle J. Countering TRAIL resistance in melanoma. *Cancers (Basel)*. 2019;11(5):656.
19. Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, Green DR. The BCL-2 family reunion. *Mol Cell*. 2010;37(3):299-310.
20. Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nat Rev Mol Cell Biol*. 2014;15(1):49-63.
21. Goldstein JC, Waterhouse NJ, Juin P, Evan GI, Green DR. The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. *Nat Cell Biol*. 2000;2(3):156-162.
22. Dorstyn L, Akey CW, Kumar S. New insights into apoptosome structure and function. *Cell Death Differ*. 2018;25(7):1194-1208.
23. Bhola PD, Letai A. Mitochondria-judges and executioners of cell death sentences. *Mol Cell*. 2016;61(5):695-704.
24. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science*. 1998;281(5381):1322-1326.
25. Steimer DA, Boyd K, Takeuchi O, Fisher JK, Zambetti GP, Opferman JT. Selective roles for antiapoptotic MCL-1 during granulocyte development and macrophage effector function. *Blood*. 2009;113(12):2805-2815.
26. Arbour N, Vanderluit JL, Le Grand JN, et al. Mcl-1 is a key regulator of apoptosis during CNS development and after DNA damage. *J Neurosci*. 2008;28(24):6068-6078.
27. Rinkenberger JL, Horning S, Klocke B, Roth K, Korsmeyer SJ. Mcl-1 deficiency results in peri-implantation embryonic lethality. *Genes Dev*. 2000;14(1):23-27.

28. Perciavalle RM, Opferman JT. Delving deeper: MCL-1's contributions to normal and cancer biology. *Trends Cell Biol*. 2013;23(1):22-29.
29. Beroukhim R, Mermel CH, Porter D, et al. The landscape of somatic copy-number alteration across human cancers. *Nature*. 2010; 463(7283):899-905.
30. Wertz IE, Kusam S, Lam C, et al. Sensitivity to antitubulin chemotherapeutics is regulated by MCL1 and FBW7. *Nature*. 2011; 471(7336):110-114.
31. Gores GJ, Kaufmann SH. Selectively targeting Mcl-1 for the treatment of acute myelogenous leukemia and solid tumors. *Genes Dev*. 2012; 26(4):305-311.
32. Weber CE, Luo C, Hotz-Wagenblatt A, et al. miR-339-3p is a tumor suppressor in melanoma. *Cancer Res*. 2016;76(12):3562-3571.
33. Chen J, Zhang X, Lentz C, et al. miR-193b regulates Mcl-1 in melanoma. *Am J Pathol*. 2011;179(5):2162-2168.
34. Mishra PJ, Mishra PJ, Merlino G. Integrated genomics identifies miR-32/MCL-1 pathway as a critical driver of melanomagenesis: implications for miR-replacement and combination therapy. *PLoS One*. 2016; 11(11):e0165102.
35. Eberle J, Fecker LF, Hossini AM, et al. CD95/Fas signaling in human melanoma cells: conditional expression of CD95L/FasL overcomes the intrinsic apoptosis resistance of malignant melanoma and inhibits growth and progression of human melanoma xenotransplants. *Oncogene*. 2003;22(57):9131-9141.
36. Riccardi C, Nicoletti I. Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nat Protoc*. 2006;1(3):1458-1461.
37. Vier J, Groth M, Sochalska M, Kirschnek S. The anti-apoptotic Bcl-2 family protein A1/Bfl-1 regulates neutrophil survival and homeostasis and is controlled via PI3K and JAK/STAT signaling. *Cell Death Dis*. 2016;7:e2103.
38. Senft D, Berking C, Graf SA, Kammerbauer C, Ruzicka T, Besch R. Selective induction of cell death in melanoma cell lines through targeting of Mcl-1 and A1. *PLoS One*. 2012;7(1):e30821.
39. Fischer U, Janicke RU, Schulze-Osthoff K. Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ*. 2003;10(1): 76-100.
40. Ferreira KS, Kreutz C, Macnelly S, et al. Caspase-3 feeds back on caspase-8, Bid and XIAP in type I Fas signaling in primary mouse hepatocytes. *Apoptosis*. 2012;17(5):503-515.
41. Berger A, Quast SA, Plotz M, Kammermeier A, Eberle J. Sensitization of melanoma cells for TRAIL-induced apoptosis by BMS-345541 correlates with altered phosphorylation and activation of Bax. *Cell Death Dis*. 2013;4:e477.
42. Soria JC, Márk Z, Zatloukal P, et al. Randomized phase II study of dulanermin in combination with paclitaxel, carboplatin, and bevacizumab in advanced non-small-cell lung cancer. *J Clin Oncol*. 2011; 29(33):4442-4451.
43. Cheah CY, Belada D, Fanale MA, et al. Dulanermin with rituximab in patients with relapsed indolent B-cell lymphoma: an open-label phase 1b/2 randomised study. *Lancet Haematol*. 2015;2(4): e166-e174.
44. Ouyang X, Shi M, Jie F, et al. Phase III study of dulanermin (recombinant human tumor necrosis factor-related apoptosis-inducing ligand/Apo2 ligand) combined with vinorelbine and cisplatin in patients with advanced non-small-cell lung cancer. *Invest New Drugs*. 2018;36(2):315-322.
45. Van Geelen CM, de Vries EG, de Jong S. Lessons from TRAIL-resistance mechanisms in colorectal cancer cells: paving the road to patient-tailored therapy. *Drug Resist Updat*. 2004;7(6):345-358.
46. Li Y, Wang H, Wang Z, et al. Inducible resistance of tumor cells to tumor necrosis factor-related apoptosis-inducing ligand receptor 2-mediated apoptosis by generation of a blockade at the death domain function. *Cancer Res*. 2006;66(17):8520-8528.
47. Kurbanov BM, Fecker LF, Geilen CC, Sterry W, Eberle J. Resistance of melanoma cells to TRAIL does not result from upregulation of antiapoptotic proteins by NF-kappaB but is related to downregulation of initiator caspases and DR4. *Oncogene*. 2007;26(23):3364-3377.
48. Zhang XD, Wu JJ, Gillespie S, Borrow J, Hersey P. Human melanoma cells selected for resistance to apoptosis by prolonged exposure to tumor necrosis factor-related apoptosis-inducing ligand are more vulnerable to necrotic cell death induced by cisplatin. *Clin Cancer Res*. 2006;12(4):1355-1364.
49. Berger A, Quast SA, Plötz M, et al. Sensitization of melanoma cells for death ligand-induced apoptosis by an indirubin derivative—enhancement of both extrinsic and intrinsic apoptosis pathways. *Biochem Pharmacol*. 2011;81(1):71-81.
50. Scaffidi C, Fulda S, Srinivasan A, et al. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J*. 1998;17(6):1675-1687.
51. Hinz S, Trauzold A, Boenicke L, et al. Bcl-XL protects pancreatic adenocarcinoma cells against CD95- and TRAIL-receptor-mediated apoptosis. *Oncogene*. 2000;19(48):5477-5486.
52. Vogler M, Walczak H, Stadel D, et al. Targeting XIAP bypasses Bcl-2-mediated resistance to TRAIL and cooperates with TRAIL to suppress pancreatic cancer growth in vitro and in vivo. *Cancer Res*. 2008;68(19): 7956-7965.
53. Lemke J, von Karstedt S, Zinngrebe J, Walczak H. Getting TRAIL back on track for cancer therapy. *Cell Death Differ*. 2014;21(9): 1350-1364.
54. Quast SA, Steinhorst K, Plotz M, Eberle J. Sensitization of melanoma cells for death ligand TRAIL is based on cell cycle arrest, ROS production, and activation of proapoptotic Bcl-2 Proteins. *J Invest Dermatol*. 2015;135(11):2794-2804.
55. Wei G, Twomey D, Lamb J, et al. Gene expression-based chemical genomics identifies rapamycin as a modulator of MCL1 and glucocorticoid resistance. *Cancer Cell*. 2006;10(4):331-342.
56. Lucas KM, Mohana-Kumaran N, Lau D, et al. Modulation of NOXA and MCL-1 as a strategy for sensitizing melanoma cells to the BH3-mimetic ABT-737. *Clin Cancer Res*. 2012;18(3):783-795.
57. Plotz M, Gillissen B, Hossini AM, Daniel PT, Eberle J. Disruption of the VDAC2-Bak interaction by Bcl-x(S) mediates efficient induction of apoptosis in melanoma cells. *Cell Death Differ*. 2012;19(12): 1928-1938.
58. Willis SN, Chen L, Dewson G, et al. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes & Development*. 2005;19(11):1294-1305.
59. Simmons MJ, Fan G, Zong WX, Degenhardt K, White E, Gelinas C. Bfl-1/A1 functions, similar to Mcl-1, as a selective tBid and Bak antagonist. *Oncogene*. 2008;27(10):1421-1428.
60. Kumar S, Kaufman JL, Gasparetto C, et al. Efficacy of venetoclax as targeted therapy for relapsed/refractory t(11;14) multiple myeloma. *Blood*. 2017;130(22):2401-2409.
61. Tolcher AW, LoRusso P, Arzt J, et al. Safety, efficacy, and pharmacokinetics of navitoclax (ABT-263) in combination with erlotinib in patients with advanced solid tumors. *Cancer Chemother Pharmacol*. 2015;76(5):1025-1032.
62. Lee EF, Harris TJ, Tran S, et al. BCL-XL and MCL-1 are the key BCL-2 family proteins in melanoma cell survival. *Cell Death Dis*. 2019;10(5): 342.
63. Senichkin VV, Streletskaia AY, Gorbunova AS, Zhivotovsky B, Kopeina GS. Saga of Mcl-1: regulation from transcription to degradation. *Cell Death Differ*. 2020;27(2):405-419.
64. Kotschy A, Szlavik Z, Murray J, et al. The MCL1 inhibitor S63845 is tolerable and effective in diverse cancer models. *Nature*. 2016; 538(7626):477-482.
65. Keaveney MK, Tseng HA, Ta TL, Gritton HJ, Man HY, Han X. A microRNA-based gene-targeting tool for virally labeling interneurons in the rodent cortex. *Cell Rep*. 2018;24(2):294-303.
66. Mohana-Kumaran N, Hill DS, Allen JD, Haass NK. Targeting the intrinsic apoptosis pathway as a strategy for melanoma therapy. *Pigment Cell Melanoma Res*. 2014;27(4):525-539.

67. Berger A, Quast SA, Plotz M, Kuhn NF, Trefzer U, Eberle J. RAF inhibition overcomes resistance to TRAIL-induced apoptosis in melanoma cells. *J Invest Dermatol*. 2014;134(2):430-440.
68. Fecker LF, Rückert S, Kurbanov BM, et al. Efficient melanoma cell killing and reduced melanoma growth in mice by a selective replicating adenovirus armed with tumor necrosis factor-related apoptosis-inducing ligand. *Hum Gene Ther*. 2011;22(4):405-417.

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