Preclinical Development

Contrasting Effects of Nutlin-3 on TRAIL- and Docetaxel-Induced Apoptosis Due to Upregulation of TRAIL-R2 and Mcl-1 in Human Melanoma Cells

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Abstract

Wild-type p53 is commonly expressed in melanoma but does not appear to be effective in the induction of apoptosis. One explanation is that p53 is targeted for degradation by the E3 ligase MDM2. However, we found in this study that blockade of the interaction of p53 and MDM2 by the MDM2 antagonist nutlin-3 in melanoma cells did not induce apoptosis, even though it upregulated p53 and its proapoptotic targets. Nevertheless, nutlin-3 enhanced TRAIL-induced apoptosis as a result of p53-mediated upregulation of TRAIL-R2. Unexpectedly, nutlin-3 upregulated Mcl-1, which attenuated apoptotic signaling triggered by TRAIL, and inhibited apoptosis induced by the microtubule-targeting drug docetaxel. The increase in Mcl-1 was related to a p53-independent transcriptional mechanism, but stabilization of the Mcl-1 protein played a dominant role, as nutlin-3 upregulated the Mcl-1 protein to a much greater extent than the Mcl-1 mRNA, and this was associated with prolonged half-life time and reduced ubiquitination of the protein. Knockdown of p53 blocked the upregulation of the Mcl-1 protein, indicating that p53 plays a critical role in the stabilization of Mcl-1. The contrasting effects of nutlin-3 on TRAIL- and docetaxel-induced apoptosis were confirmed in fresh melanoma isolates. Collectively, these results show that nutlin-3 may be a useful agent in combination with TRAIL and, importantly, uncover a novel regulatory effect of p53 on the expression of Mcl-1 in melanoma cells on treatment with nutlin-3, which may antagonize the therapeutic efficacy of other chemotherapeutic drugs in addition to docetaxel in melanoma. Mol Cancer Ther; 9(12); 3363–74. ©2010 AACR.

Introduction

The tumor suppressor p53 is a transcriptional factor that is activated in response to various cellular stresses leading to cell-cycle arrest or apoptosis, thus protecting cells from malignant transformation (1–3). Inactivating mutations in the gene-encoding p53 occur in about 50% of human cancers, which play an important role in cancer development, progression, and resistance to treatment (2, 3). In addition, other mechanisms, such as overexpression of murine double minute 2 (MDM2), an E3 ubiquitin ligase that targets p53 for proteasomal degradation (4), are also involved in the suppression of wild-type p53. There has been considerable interest in the development of small-molecule antagonists of MDM2 to restore p53 function in the treatment of cancer. Nutlin-3, a cis-imidazoline derivative, was identified as a compound that binds MDM2 in the p53-binding pocket and interferes with MDM2-mediated p53 degradation, thus leading to accumulation of p53 (5). Nutlin-3 has been shown to induce p53-mediated cell-cycle arrest and apoptosis in various types of cancer cells (6–8).

Unlike many other cancers, melanoma commonly harbors wild-type p53 (9, 10). However, as judged from its malignant nature and resistance to treatment, p53 does not seem to function effectively as a tumor suppressor in melanoma. Although the mechanism(s) remains to be elucidated, some downstream targets of p53 have been shown to be dysregulated (11, 12). This puts forward a question about whether the application of agents that stabilize p53 such as nutlin-3 may induce apoptosis in melanoma cells. Moreover, it is unknown whether nutlin-3 affects sensitivity of melanoma cells to other apoptosis-inducing agents.

TNF-related apoptosis-inducing ligand (TRAIL) is a promising candidate for cancer therapeutics (13, 14). It induces apoptosis by interaction with 2 death domain-containing receptors, TRAIL-R1 and TRAIL-R2 (13, 14). Previous studies have shown that sensitivity of melanoma
cells to TRAIL-induced apoptosis is in general correlated with the levels of the cell surface expression of these receptors, in particular TRAIL-R2 (13, 15). However, melanoma cells in vivo express low levels of TRAIL death receptors (16, 17), suggesting that melanoma may not respond to treatment with TRAIL unless given with agents that increase the cell surface expression of TRAIL death receptors (18, 19).

Taxanes, such as paclitaxel and docetaxel, represent a class of anticancer agents that target microtubules (20). Past studies have shown that docetaxel induces apoptosis of melanoma cells by the mitochondrial apoptotic pathway (21, 22), which is regulated by pro- and anti-apoptotic Bcl-2 family proteins (21, 22). Proapoptotic Bcl-2 family proteins are divided into 2 subgroups, BH3-only proteins, such as PUMA and Noxa, and multidomain proapoptotic proteins, including Bak and Bax. Activation of BH3-only proteins leads to the activation of Bax and Bak, resulting in the release of mitochondrial apoptogenic proteins (23, 24). Antia apoptotic Bcl-2 family proteins may also constitute more than 1 functional classes that possess different potency in antagonizing apoptotic signaling (25, 26). For example, Mcl-1 is of particular importance in the protection of melanoma cells from apoptosis (27).

We have examined the effect of nutlin-3 on survival and growth of melanoma cells and its potential interactions with TRAIL and docetaxel. We show in this report that nutlin-3 alone does not kill melanoma cells, even though it upregulates p53 and its proapoptotic targets. Nevertheless, nutlin-3 enhances TRAIL-induced apoptosis by p53-mediated upregulation of TRAIL-R2. In contrast, it inhibits apoptosis induced by docetaxel, which is largely due to an increase in Mcl-1 that is primarily mediated by p53-related posttranslational stabilization of the Mcl-1 protein.

Materials and Methods

Cell culture and reagents

Human melanoma cell lines ME4405, IgR3, Mel-FH, Mel-RM, Mel-CV, Mel-JD, Sk-Mel-28, Sk-Mel-110, and MM200 have been described previously (15). No further authentication of the cell lines was done. Nutlin-3 was purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide to make up a stock solution of 12.5 mmol/L. Recombinant human TRAIL, the TRAIL-R2/Fc chimera, and mouse monoclonal antibodies (mAbs) against TRAIL receptors were supplied by Genentech Inc. Docetaxel was provided by Aventis Pharma SA. The cell-permeable pan-caspase inhibitor Z-Val-Ala-Asp(OMe)-CH₂F (z-VAD-FMK) and the caspase-8 specific inhibitor Z-Ile-Glu (OMe)-Thr-Asp(OMe)-CH₂F (z-IETD-FMK) were purchased from Calbiochem. The rabbit polyclonal antibodies (pAbs) against caspase-3 and -9 and the mouse mAbs against caspase-8 were from Stressgen. The mouse mAb against cleaved form of PARP was from BD Pharmingen (Bioclone). The mouse mAbs against Bcl-2, Mcl-1, Apaf-1, and Bax were from Santa Cruz Biotechnology, Inc. The mouse mAb against Noxa was from Imgenex. The mouse mAbs against p21 and p53 were from Upstate (Millipore). The rabbit pAb against PUMA and Bid were purchased from Cell Signaling Technology.

Fresh melanoma isolates

Isolation of melanoma cells from fresh surgical specimens was carried out as described previously (16).

Apoptosis

Quantitation of apoptotic cells was carried out by measurement of sub-G₁ DNA content with propidium iodide (PI) on a flow cytometer as described elsewhere (15).

Flow cytometry

Immunostaining on intact and permeabilized cells was carried out as described previously (15).

Mitochondrial membrane potential

Mitochondrial membrane potential (ΔΨm) was quantitated with JC-1 dye staining in flow cytometry as previously described (28).

Western blot analysis

Western blot analysis was carried out as described previously (27, 29). Labeled bands were detected by Immun-Star HRP Chemiluminescent kit, and images were captured and the intensity of the bands was quantitated with the Bio-Rad VersaDoc image system (BioRad, Regents Park).

Preparation of mitochondrial and cytosolic fractions

Methods used for subcellular fraction were similar to the methods previously described (18).

Quantitative reverse transcription and real-time PCR

Quantitative real-time PCR (qPCR) was done using the ABI Prism 7900 sequence detection system (Applied Biosystems, Carlsbad, CA) as described previously (27). Gene-specific primers used were TRAIL-R2 forward, 5'-GCC GAT CCA CAC GGA GTA CT-3'; TRAIL-R2 reverse, 5'-GGG TCT TCA GTG AAC CAT TG-3'; Mcl-1 forward, 5'-GCC TCT TCG CAC TGA CA-3'; p53 forward, 5'-TGG AAA CTA CCT CCT GAA AAC AAC-3'; p53 reverse, 5'-GGG TCT TCA GTG AAC CAT TG-3'; Mcl-1 forward, 5'-GGG TCT TCG CAC TGA CA-3'; p53 forward, 5'-TGG AAA CTA CCT CCT GAA AAC AAC-3'; p53 reverse, 5'-GGG TCT TCA GTG AAC CAT TG-3'; Mcl-1 forward, 5'-GGG TCT TCG CAC TGA CA-3'; p53 forward, 5'-TGG AAA CTA CCT CCT GAA AAC AAC-3'; p53 reverse. 

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Small RNA interference

The small RNA interference (siRNA) constructs used were obtained as the siGENOME SMARTpool reagents (Dharmacon), TRAIL-R2 siGENOME SMARTpool (M-004448-00-0010), p53 siGENOME SMARTpool (M-003329-03-0010), and siGENOME Nontargeting siRNA pool (D-001206-13-20). Transfection of siRNA pools was carried out as described previously (27).

Short hairpin RNA

Sigma MISSION Lentiviral Transduction Particles for short hairpin RNA (shRNA)-mediated knockdown of Mcl-1 were used as described previously (27).

Results

Nutlin-3 does not induce apoptosis, even though it upregulates p53 and its proapoptotic targets in wild-type p53 melanoma cells

Our initial studies in 2 wild-type p53 melanoma cell lines (Mel-JD and MM200) showed that nutlin-3 caused rapid and sustained upregulation of p53 and inhibition of cell growth (Fig. 1A and B) but did not induce significant apoptosis even at 20 μm/L for 48 hours (Fig. 1C). The inability of nutlin-3 to induce apoptosis was confirmed in a panel of melanoma cell lines carrying either wild-type (IgR3, ME1007, Mel-RM, Mel-CV) or mutant (Mel-FH and Sk-Mel-28) p53 and in a p53-null melanoma cell line (ME4405; ref. 30) (data not shown). Nutlin-3 did not inhibit proliferation of mutant p53 and p53-null melanoma cells (Supplementary Fig. 1), suggesting that the inhibition of melanoma cell growth is p53 dependent.

We examined whether the failure of nutlin-3 to induce apoptosis is due to the dysregulation of proapoptotic transcriptional targets of p53. As shown in Fig. 1A, the BH3-only proteins Bid, PUMA, and Noxa, the multidomain proapoptotic Bcl-2 family protein Bax, and the essential component of the apoptosome Apaf-1 were all increased by nutlin-3, with varying kinetics. Figure 1D shows that another transcriptional target of p53, TRAIL-R2 (31), was increased on the cell surface by nutlin-3. As anticipated, p21 was also upregulated, which...
is conceivably involved in the inhibition of melanoma cell growth (Fig. 1A and B).

**Nutlin-3 enhances TRAIL-induced, but protects against docetaxel-triggered apoptosis, in melanoma cells harboring wild-type p53**

Upregulation of TRAIL-R2 is of particular interest as the expression of TRAIL-R2 is a major determinant of sensitivity of melanoma cells to TRAIL-induced apoptosis (13, 15). We therefore examined whether nutlin-3 enhances TRAIL-induced apoptosis. Because nutlin-3 also upregulated multiple proapoptotic proteins that play important roles in apoptosis mediated by the mitochondrial apoptotic pathway, which is essential in docetaxel-induced apoptosis of melanoma cells (21, 22), we also studied whether nutlin-3 affects sensitivity of melanoma cells to apoptosis induced by docetaxel. As shown in Fig. 2A, nutlin-3 enhanced sensitivity of Mel-JD and MM200 cells to TRAIL-induced apoptosis (P < 0.05, 2-tailed Student’s t test), but surprisingly, it inhibited apoptosis induced by docetaxel (P < 0.05, 2-tailed Student’s t test). These contrasting effects were confirmed in Mel-RM and Mel-CV cells that carry wild-type p53 (Supplementary Fig. 2). Notably, nutlin-3 did not cause significant changes in sensitivities of mutant p53 or p53-null melanoma cells to apoptosis induced by TRAIL or docetaxel (Supplementary Fig. 2).

In association with sensitization of melanoma cells to TRAIL, nutlin-3 enhanced TRAIL-induced activation of caspase-8, reduction in ΔΨm, mitochondrial release of cytochrome c, activation of caspase-9 and -3, and cleavage of PARP (Fig. 2B and Supplementary Fig. 3). Inhibition of TRAIL-R2 by siRNA markedly blocked TRAIL-induced apoptosis in the presence of nutlin-3 (P < 0.05, 2-tailed Student’s t test; Fig. 2C). Similarly, the sensitization was also blocked by either the general caspase inhibitor z-VAD-fmk or the caspase-8 specific inhibitor z-IETD-fmk (Supplementary Fig. 4). Along with inhibition of docetaxel-induced apoptosis, nutlin-3 inhibited docetaxel-induced activation of caspase-9 and -3 and cleavage of PARP (Fig. 2D). Similarly, it blocked reduction in ΔΨm and mitochondrial release of cytochrome c induced by docetaxel (Supplementary Fig. 5), suggesting that nutlin-3 interferes with docetaxel-induced apoptotic signaling upstream of mitochondria.

**Upregulation of TRAIL-R2 by nutlin-3 is mediated by p53**

As shown in Fig. 3A and B, nutlin-3 increased the total protein levels of TRAIL-R2, as measured in permeabilized Mel-JD and MM200 cells, and upregulated the levels of TRAIL-R2 mRNA. The increase in TRAIL-R2 mRNA levels could be inhibited by actinomycin D (Fig. 3C), suggesting that this was due to a transcriptional increase rather than a change in the mRNA stability. Nutlin-3 did not alter the levels of TRAIL-R2 mRNA in a p53-null (ME4405) and 2 mutant p53 melanoma cell lines (Mel-FH and Sk-Mel-28; Supplementary Fig. 6), suggesting that wild-type p53 is required for upregulation of TRAIL-R2 by nutlin-3. This was further demonstrated in Mel-JD and MM200 cells with p53 knocked down by siRNA (Fig. 3D).

**Nutlin-3-mediated protection of melanoma cells from docetaxel-induced apoptosis involves upregulation of Mcl-1**

We also studied the mechanism(s) by which nutlin-3 protects melanoma cells from docetaxel-induced apoptosis. Because nutlin-3 appeared to act upstream of mitochondria (Fig. 2D and Supplementary Fig. 5), we tested whether nutlin-3 impinges on the expression of antiapoptotic Bcl-2 family proteins. Fig. 4A shows that treatment with nutlin-3 did not alter the expression levels of Bcl-2 but resulted in upregulation of Mcl-1 with a ~4.3-fold increase in Mel-JD and a ~3.7-fold increase in MM200 cells (Fig. 4A).

As shown in Fig. 4B, inhibition of Mcl-1 by shRNA reversed nutlin-3–mediated protection against docetaxel-induced apoptosis. Similarly, it enhanced docetaxel-induced activation of caspase-9, and -3 and cleavage of PARP in the presence of nutlin-3 (Fig. 4C). Fig. 4D shows that deficiency in Mcl-1 resulted in a further increase in sensitivities of Mel-RM and MM200 cells to TRAIL-induced apoptosis in the presence of nutlin-3. Notably, nutlin-3 alone induced moderate levels of apoptosis in melanoma cells when Mcl-1 was knocked down (Fig. 4C and D), indicating that the failure of nutlin-3 to induce apoptosis is partially due to upregulation of Mcl-1.

**Upregulation of Mcl-1 by nutlin-3 in melanoma cells involves both transcriptional and posttranslational mechanisms**

We studied the mechanism of upregulation of Mcl-1 by nutlin-3. As shown in Fig. 5A, nutlin-3 caused a ~1.5-fold increase in Mcl-1 in both Mel-JD and MM200 cells, which was efficiently blocked by actinomycin D, indicating that it is a consequence of enhanced Mcl-1 transcription (Fig. 5A). However, the increase in Mcl-1 mRNA was not adequate to account for the greater increase in its protein levels (Figs. 4 and 5A). Because Mcl-1 is a short-lived protein (32), we sought to determine whether the increase in the protein levels involves changes in its stability. Figure 5B shows that exposure to nutlin-3 prolonged the half-life time of Mcl-1. This was associated with reduced ubiquitination of Mcl-1 (Fig. 5C).

Nutlin-3 also increased the Mcl-1 mRNA levels in mutant p53 (Mel-FH) and p53-null (ME4405) melanoma cells to a similar extent, as it did in wild-type p53 melanoma cells (Fig. 5A and D), indicating that transcriptional upregulation of Mcl-1 by nutlin-3 in melanoma cells is independent of wild-type p53. This was also shown by siRNA knockdown of p53 in wild-type p53 melanoma cells (Supplementary Fig. 7). Strikingly, knockdown of p53 inhibited the upregulation of Mcl-1 at the protein level in wild-type p53 melanoma cells (Fig. 5D), suggesting that wild-type p53 is involved in nutlin-3-mediated stabilization of the Mcl-1 protein in melanoma cells.
Figure 2. Nutlin-3 enhances TRAIL-induced apoptosis, but protects against docetaxel-induced apoptosis in wild-type p53 melanoma cells. A, Mel-JD and MM200 cells were treated with nutlin-3 (10 μmol/L), TRAIL (200 ng/mL), or the combination of nutlin-3 and TRAIL for 24 hours (left panel), or nutlin-3 (10 μmol/L), docetaxel (20 nmol/L), or the combination of nutlin-3 and docetaxel for 48 hours (right panel). Apoptosis was quantitated by the PI method. B, upper panel, whole-cell lysates from Mel-JD and MM200 cells treated with nutlin-3 (10 μmol/L), TRAIL (200 ng/mL), or the combination of both for 16 hours were subjected to Western blot analysis. Low panel, mitochondrial and cytosolic fractions of Mel-JD and MM200 cells before and after treatment with nutlin-3 (10 μmol/L), TRAIL (200 ng/mL), or the combination of both for 16 hours were subjected to Western blot analysis. Western blot analysis of COX IV and β-actin was included as controls for the relative purity of mitochondrial and cytosolic fractions, respectively. C, upper panel, Mel-JD and MM200 cells transfected with the control or TRAIL-R2 siRNA. Twenty-four hours later, total RNA was extracted and subjected to real-time qPCR analysis of TRAIL-R2 and TRAIL-R1 mRNA expression. Lower panel, Mel-JD and MM200 cells transfected with the control or TRAIL-R2 siRNA. Twenty-four hours later, cells were treated with nutlin-3 (10 μmol/L), TRAIL (200 ng/mL), or the combination of both for a further 24 hours before apoptosis was measured by the PI method. D, whole-cell lysates from Mel-JD and MM200 cells treated with nutlin-3 (10 μmol/L), docetaxel (20 nmol/L), or the combination of both for 48 hours were subjected to Western blot analysis. Bars, SE (n = 3).
Nutlin-3 differentially regulates sensitivities of fresh melanoma isolates to apoptosis induced by TRAIL and docetaxel

As shown in Fig. 6A, nutlin-3 increased TRAIL-R2 on the cell surface to varying degrees in a panel of 5 fresh melanoma isolates harboring wild-type p53. This was associated with increases in TRAIL-R2 mRNA, as shown for Mel-MC and Mel-JC cells (Fig. 6A). Figure 6B shows that cotreatment with nutlin-3 and TRAIL enhanced apoptosis in fresh melanoma isolates, which was inhibited by a recombinant TRAIL-R2/Fc chimera.

Figure 3. Upregulation of TRAIL-R2 by nutlin-3 is dependent on p53. A, left panel, Mel-JD and MM200 cells were treated with nutlin-3 (10 μmol/L) for 16 hours. The levels of expression of TRAIL-R2 in permeabilized cells were measured by flow cytometry. Right panel, whole-cell lysates from Mel-JD and MM200 cells treated with nutlin-3 (10 μmol/L) for 16 hours were subjected to Western blot analysis of TRAIL-R2. B, total RNA was extracted from Mel-JD (upper panel) and MM200 (lower panel) cells treated with nutlin-3 (10 μmol/L) for 16 hours and subjected to real-time qPCR analysis of TRAIL-R2 and -R1 mRNA. The relative abundance of mRNA expression before treatment was arbitrarily designated as 1. C, Mel-JD and MM200 cells with or without pretreatment with actinomycin D (Act D; 100 ng/mL) for 1 hour before the addition of nutlin-3 (10 μmol/L) for a further 16 hours. Total RNA was extracted and subjected to real-time qPCR analysis of TRAIL-R2 and TRAIL-R1 mRNA. The relative abundance of TRAIL-R2 mRNA expression before treatment was arbitrarily designated as 1. Bars, SE (n = 3).
As shown in Fig. 6C, docetaxel alone induced moderate levels of apoptosis in the fresh melanoma isolates Mel-MC and Mel-JC, which were reduced by the combination of docetaxel and nutlin-3, indicative of a protective effect of nutlin-3 on fresh melanoma isolates against docetaxel-induced apoptosis. Similar to findings in cultured melanoma cell lines, nutlin-3 upregulated Mcl-1 in Mel-MC and Mel-JC cells (Fig. 6D).
Discussion

Nutlin-3 has been reported to induce apoptosis in a number of human cancer cells, such as lymphoma and neuroblastoma, by activating the p53-mediated apoptotic pathway (6–8). In addition, E2F1 and p73 that can also bind to the nutlin-3–binding region of MDM2 have also been shown to involve in nutlin-3–induced apoptosis (33). Nevertheless, our finding that nutlin-3 does not induce apoptosis of melanoma cells is not surprising, as failure of nutlin-3 to induce apoptosis has also been shown in various other cancer types (34, 35) and it is in line with the perception that wild-type p53 does not act as an effective tumor suppressor in melanoma. Resistance of

Figure 5. Upregulation of Mcl-1 by nutlin-3 involves both transcriptional and posttranslational mechanisms in melanoma cells. A, left panel, total RNA from Mel-JD and MM200 cells treated with nutlin-3 (10 μmol/L) for indicated periods were subjected to real-time qPCR analysis of Mcl-1 mRNA. The relative abundance of mRNA expression before treatment was arbitrarily designated as 1. Right panel, Mel-JD and MM200 cells were treated with actinomycin D (Act D; 100 ng/mL) for 1 hour before the addition of nutlin-3 (10 μmol/L) for a further 16 hours. Total RNA was extracted and subjected to real-time qPCR analysis of Mcl-1 mRNA expression. The relative abundance of Mcl-1 mRNA expression before treatment was arbitrarily designated as 1. B, whole-cell lysates from Mel-JD and MM200 cells with or without treatment with nutlin-3 (10 μmol/L) for 16 hours followed by the addition of cycloheximide (10 μg/mL) for the indicated periods were subjected to Western blot analysis. C, whole-cell lysates from Mel-JD and MM200 cells with or without treatment with nutlin-3 (10 μmol/L) for 6 hours were subjected to immunoprecipitation using an antibody against ubiquitin. D, left panel, total RNA from Mel-FH (mutant p53) and ME4405 (p53-null) cells treated with nutlin-3 (10 μmol/L) for indicated periods were subjected to real-time qPCR analysis of Mcl-1 mRNA expression. The relative abundance of Mcl-1 mRNA expression before treatment was arbitrarily designated as 1. Right panel, Mel-JD and MM200 were transfected with the control or p53 siRNA. Twenty-four hours later, cells were treated with nutlin-3 (10 μmol/L) for a further 16 hours. Whole-cell lysates were subjected to Western blot. Bars, SE (n = 3).
melanoma cells to nutlin-3–induced apoptosis is not due to dysregulation of proapoptotic transcriptional targets of p53, in that PUMA, Noxa, Bid, Bax, and Apaf-1 were all increased by nutlin-3 along with p53. In contrast, upregulation of Mcl-1 appears to play a role, as nutlin-3 induced moderate levels of apoptosis in wild-type p53 melanoma cells deficient in Mcl-1. Upregulation of Notch1 has been shown to be a negative-feedback antiapoptotic mechanism in hematologic cancer cells after treatment with nutlin-3, but the downstream signaling involved is not known (36). A number of survival pathways such as the MEK/ERK pathway are constitutively activated in melanoma cells (37, 38). It is conceivable that they may contribute to protection against apoptosis induced by nutlin-3, as inhibition of the MEK/ERK pathway has been shown to synergize with

Figure 6. Nutlin-3 differentially regulates sensitivities of fresh melanoma isolates to apoptosis induced by TRAIL and docetaxel. A, left panel, the expression of TRAIL-R2 on the cell surface was measured by flow cytometry in fresh melanoma isolates with or without treatment with nutlin-3 (10 μmol/L) for 16 hours. Right panel, total RNA from the fresh melanoma isolates Mel-MC and Mel-JC with or without treatment with nutlin-3 (10 μmol/L) for 16 hours was subjected to real-time qPCR analysis of TRAIL-R2 mRNA. The relative abundance of mRNA expression before treatment was arbitrarily designated as 1. B, left panel, fresh melanoma isolates treated with nutlin-3 (10 μmol/L), TRAIL (200 ng/mL), or the combination of both for 24 hours before apoptosis was measured by the PI method in flow cytometry. Right panel, Mel-MC and Mel-JC cells with or without pretreatment with a recombinant TRAIL-R2/Fc chimera (10 μg/mL) for 1 hour were treated with TRAIL (200 ng/mL) or the combination of nutlin-3 (10 μmol/L) and TRAIL for 24 hours before apoptosis was measured by the PI method in flow cytometry. C, Mel-MC and Mel-JC cells were treated with nutlin-3 (10 μmol/L), docetaxel (20 nmol/L), or the combination of both for 48 hours before apoptosis was measured by the PI method in flow cytometry. D, whole-cell lysates from Mel-MC and Mel-JC cells treated with nutlin-3 (10 μmol/L) for 16 hours were subjected to Western blot analysis of Mcl-1. Bars, SE (n = 3).
nutlin-3 in the induction of apoptosis in leukemia cells (39).

Despite the failure of nutlin-3 to induce apoptosis, it enhanced TRAIL-induced apoptotic cell death in melanoma cells harboring wild-type p53. This is of particular interest in that past studies have indicated that melanoma cells in vivo expressed in general low levels of TRAIL death receptors, in particular TRAIL-R2 (16, 17). Sensitization of melanoma cells to TRAIL-induced apoptosis by nutlin-3 was associated with increased activation of caspase-8, consistent with upregulation of TRAIL-2 on the melanoma cell surface. siRNA knockdown of TRAIL-R2 blocked the sensitization, substantiating the essential role of the increased interactions between TRAIL and TRAIL-R2.

Nutlin-3-mediated upregulation of TRAIL-R2 was due to increased TRAIL-R2 gene transcription, which appeared to be mediated by p53. Intriguingly, we found in separate studies that the DNA-damaging drug cisplatin did not upregulate TRAIL-R2 at the protein level, even though it increased TRAIL-R2 mRNA along with p53 (18, 19; data not shown). Together, these findings indicate that the ability of p53 to activate TRAIL-R2 transcription per se is intact in melanoma cells and suggest that p53-mediated upregulation of TRAIL-R2 requires cooperation of posttranscriptional mechanisms that may be impaired in melanoma cells under genotoxic stress.

An unexpected, yet important finding of this study is the inhibition of docetaxel-induced apoptosis by nutlin-3. Blockage of apoptotic signaling induced by docetaxel appeared to occur upstream of mitochondria, as nutlin-3 inhibited reduction in ΔΨm and mitochondrial release of cytochrome c. These observations along with the findings that multiple BH3-only proteins were increased by nutlin-3 suggest that nutlin-3 may also upregulate 1 or more antiapoptotic Bcl-2 family members to neutralize the proapoptotic proteins. Indeed, nutlin-3 markedly upregulated Mcl-1, which is known to be of particular importance in melanoma cells (27, 40). Inhibition of Mcl-1 reversed the inhibitory effect of nutlin-3 on docetaxel-induced apoptosis, indicating that upregulation of Mcl-1 is responsible for the protection of melanoma cells against docetaxel by nutlin-3. Notably, upregulation of Mcl-1 attenuated TRAIL-induced apoptotic signaling, but this inhibitory effect was largely overridden by strong apoptotic signaling resulting from the increased expression of TRAIL-R2 in the presence of nutlin-3.

Although a transcriptional increase is involved in nutlin-3-induced upregulation of Mcl-1, the extent of the increase in Mcl-1 mRNA is not sufficient to account for the greater increase in its protein levels. Treatment with nutlin-3 caused an increase in the half-life time of Mcl-1, which is associated with reduced polyubiquitination of the protein, suggesting that nutlin-3 may affect the mechanism that regulates Mcl-1 ubiquitination and its proteasomal degradation. Interestingly, transcriptional upregulation of Mcl-1 by nutlin-3 appeared independent of p53, but whether other transcription factors such as p73 and E2F1 are involved remains to be studied (33). E2F1 is known to be a transcriptional repressor of Mcl-1 (41). Significantly, the mechanism by which nutlin-3 mediates posttranslational regulation of Mcl-1 appeared dependent on p53. Ubiquitination and proteasomal degradation of Mcl-1 are regulated by the E3 ligase Mule and the deubiquitinase USP9X (32, 42), but whether p53 upregulated by nutlin-3 may impinge on these mechanisms is to be examined. Nevertheless, the present study appears to be the first to reveal the regulatory effect of p53 on the expression of Mcl-1 in melanoma cells after exposure to nutlin-3. It is of note that p53 does not appear to regulate Mcl-1 in every scenario. For example, DNA-damaging agents cause accumulation of p53 but do not increase the levels of Mcl-1 (25, 43).

Besides transcriptional regulation and posttranslational degradation by the ubiquitin–proteasome system (32, 42), Mcl-1 expression can also be regulated both at the translation initiation level and by caspase-mediated degradation during apoptosis (44, 45). We observed that while treatment with TRAIL alone resulted in a moderate decrease in Mcl-1, the combination of nutlin-3 and TRAIL caused further downregulation of the Mcl-1 levels, which could be inhibited by pretreatment with z-VAD-fmk (Supplementary Fig. 8), indicating that increased caspase activation caused by the combinatorial treatment resulted in enhanced caspase-mediated cleavage of Mcl-1 (45). Increased Mcl-1 in some malignancies has been shown to be related to downregulation of microRNA (miR)-29b (46). However, we have found by qPCR-based screening that there was no significant difference in the expression levels of miR-29b in melanoma cells before and after treatment with nutlin-3 (data not shown), suggesting that nutlin-3-mediated upregulation of Mcl-1 may not be associated with downregulation of miR-29b. Similarly, although translation initiation regulators such as elf4E, elf4G, and 4EBP1 have been shown to mediate translational regulation of Mcl-1 (44, 47), they may not play a major role in nutlin-3–triggered upregulation of Mcl-1, as our results clearly indicated that the increase in Mcl-1 induced by nutlin-3 is mediated by transcriptional and posttranslational mechanisms.

Our finding that nutlin-3 could sensitize fresh melanoma isolates to TRAIL-induced apoptosis by upregulation of TRAIL-R2 is of particular importance, as they may reflect more closely the in vivo status of TRAIL death receptor expression in melanoma cells and their susceptibility to TRAIL-induced apoptosis (15, 16). The combination of nutlin-3 and TRAIL may therefore be a useful strategy in improving the therapeutic efficacy of TRAIL in melanoma. However, the finding that nutlin-3 also upregulates Mcl-1 in freshly isolated melanoma cells calls for attention to possibly unexpected adverse effects of the administration of nutlin-3 in vivo. High levels of Mcl-1 may antagonize other chemotherapeutic drugs in addition to docetaxel. The role of p53 in the regulation of Mcl-1 in melanoma cells treated with nutlin-3 may...
have broad implications in understanding melanoma cell survival and resistance to treatment and warrants further investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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Contrasting Effects of Nutlin-3 on TRAIL- and Docetaxel-Induced Apoptosis Due to Upregulation of TRAIL-R2 and Mcl-1 in Human Melanoma Cells

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