MDM2 Antagonist Nutlin-3 Displays Antiproliferative and Proapoptotic Activity in Mantle Cell Lymphoma

Yoko Tabe,1,2 Denise Sebasigari,1 Linhua Jin,2 Martina Rudelius,1 Theresa Davies-Hill,1 Kazunori Miyake,2 Takashi Miida,2 Stefania Pittaluga,1 and Mark Raffeld1

Abstract Purpose: Mantle cell lymphoma (MCL) has one of the poorest prognoses of the non-Hodgkin's lymphomas, and novel therapeutic approaches are needed. We wished to determine whether Nutlin-3, a novel small-molecule murine double minute 2 (MDM2) antagonist that efficiently activates TP53, might be effective in inducing cell death in MCL.

Experimental Design: MCL cell lines with known TP53 status were treated with Nutlin-3, and biological and biochemical consequences were studied. Synergies with the prototypic genotoxic agent doxorubicin and the novel proteasome inhibitor bortezomib were assessed.

Results: Nutlin-3 resulted in a reduction in cell proliferation/viability (IC50 < 10 μmol/L), an increase in the apoptotic fraction, and cell cycle arrest in wild-type (wt) TP53 Z-138 and Granta 519 cells. These effects were accompanied by TP53 accumulation and induction of TP53-dependent proteins p21, MDM2, Puma, and Noxa. Cell cycle arrest was characterized by suppression of S phase and an increase in the G0-G1 and G2-M fractions and accompanied by suppression of total and phosphorylated retinoblastoma protein and a decrease in G2-M-associated proteins cyclin B and CDC2. The combination of Nutlin-3 with doxorubicin or bortezomib was synergistic in wt-TP53 MCL cells. Nutlin-3 also induced cell cycle arrest and reduced cell viability in the mutant TP53 MINO cells but at a significantly higher IC50 (22.5 μmol/L). These effects were associated with induction of the TP53 homologue p73, slight increases in p21 and Noxa, and caspase activation. Nutlin-3 and bortezomib synergistically inhibited cell growth of MINO.

Conclusion: These findings suggest that the MDM2 antagonist Nutlin-3 may be an effective agent in the treatment of MCL with or without wt-TP53.

The TP53 tumor suppressor gene encodes for a critical cellular protein that monitors the integrity of the cell and, when activated by cellular stress, is capable of inducing apoptotic cell death (1). Thus, it is no wonder that loss of TP53 function occurs in an estimated 50% of all cancer (2). Inactivation of the TP53 pathway may occur by direct mutation of the TP53 gene or through mechanisms that affect TP53 signaling and activation. These include overexpression of murine double minute 2 (MDM2), a TP53-specific E3 ubiquitin ligase that mediates the ubiquitin-dependent degradation of TP53 and inactivation of p19/ARF, a small MDM2-binding protein that controls the activity of MDM2 by displacing TP53 and preventing its degradation, and the inactivation of the ATM kinase, an upstream TP53 regulator whose activity is critical for the activation of TP53 in response to cellular stress.

There has been considerable interest in developing compounds that are capable of restoring the TP53 apoptotic response in cancer. Nutlin-3 is a novel small-molecule antagonist of MDM2 that binds MDM2 in the TP53-binding pocket, thereby interfering with MDM2-directed TP53 degradation (3). This stabilization of TP53 results in its activation, leading to cell cycle arrest, growth inhibition, and apoptosis. The ability of Nutlin-3 to restore the apoptotic response requires that TP53 be wild-type (wt) and capable of trans-activating its target genes. Reactivation of wt-TP53 by Nutlin-3, leading to cell cycle arrest and apoptosis, has been shown in both solid tumors and lymphoid neoplasms such as chronic lymphocytic leukemia, primary effusion lymphomas, and most recently Hodgkin's lymphomas (4–7). Interestingly, Nutlin-3 has also been shown to have a TP53-independent mode of action that may further broaden its potential therapeutic range (8, 9).

Mantle cell lymphoma (MCL) is a distinctive subtype of B-cell lymphoma believed to originate from follicle mantle cells (10). It represents ~5% to 10% of all non-Hodgkin's lymphomas and has one of the poorest prognoses of the non-Hodgkin's lymphoma. This lymphoma is characterized by the t(11,14)(q13;32) translocation, which juxtaposes the cyclin D1 gene on chromosome 11 to the immunoglobulin heavy chain locus.
Mantle cell lymphoma (MCL) has one of the poorest prognoses of the non-Hodgkin’s lymphomas, and novel therapeutic approaches are needed. Nutlin-3 is a novel small-molecule antagonist of the murine double minute 2 ubiquitin ligase, interfering with murine double minute 2-directed TP53 degradation. This stabilization of wild-type (wt) TP53 results in its activation, leading to cell cycle arrest, growth inhibition, and apoptosis. In this study, we show that Nutlin-3 is highly effective in inducing cell death in MCL with wt-TP53 and also has activity in MCL with mutant TP53. Furthermore, we show that the combination of Nutlin-3 treatment with the proteasome inhibitor bortezomib results in synergistic cytotoxicity in MCL cells harboring both wt-TP53 and mutant TP53. These results provide a basis for the rational use of Nutlin-3 in MCL either alone or in combination with other compounds known to have activity in MCL.

MCL show frequent alterations of genes associated with the TP53 signaling pathway. Mutations of ATM occur in as many as 70% of both typical and blastic MCL (20), whereas deletions of the p19/ARF locus (15, 19), overexpression of the MDM2 protein (21), and inactivating mutations of the TP53 gene itself occur primarily in the more aggressive MCL variants (14, 18, 22). Although abnormalities of the TP53 signaling pathway are common in MCL, mutation of the TP53 gene itself is relatively uncommon, occurring in ~30% of the less common blastic MCL variants or ~6% to 20% of the total number of cases (14, 18, 22, 23). The implicit suppression of TP53 signaling in this lymphoma, combined with the relatively low overall rate of mutational inactivation of TP53 itself, suggests that MCL may be a good candidate for biological therapies that up-regulate TP53 and potentially induce apoptosis.

In this study, we wished to assess whether Nutlin-3 would be effective in reactivating wt-TP53 and inducing cell death in MCL cell lines and whether the mechanism of its activity might be affected by the constitutively high levels of cyclin D1. The latter question was of interest because cyclin D/CDK complexes are capable of sequestering and functionally inactivating p21 (11), a primary TP53 target responsible for inducing G1-S cell cycle arrest, and in light of a recent study in which Nutlin-3 treatment of cyclin D1/CDK2-transfected cells was resistant to inactivation of Rb and G1-S arrest presumably through sequestration of induced p21 (24). In addition, the use of a mutant (mt) TP53 “control” cell line also allowed us to assess the potential presence of TP53-independent mechanisms of actions, as reported by other investigators (8, 9).

We show here that Nutlin-3 stabilizes TP53 and results in the efficient induction of apoptosis, cell cycle arrest, and Rb inactivation in MCL cell lines with wt-TP53 despite the presence of high levels of cyclin D1. In addition, we show that Nutlin-3 and a genotoxic chemotherapeutic agent, doxorubicin, or a selective proteasome inhibitor, bortezomib, display synergistic cytotoxic activity in the MCL cell lines with wt-TP53. Of further interest, Nutlin-3 also displayed antiproliferative and proapoptotic activity toward the mt-TP53 MINO cell line but at a significantly higher IC50 (22.5 μmol/L). These effects were accompanied by an increase in protein levels of the TP53 homologue p73 (25), which has been implicated in a TP53-independent antitumor effect of Nutlin-3 (8, 9). Notably, Nutlin-3 and bortezomib acted synergistically to inhibit cell growth in MINO.

These findings establish a basis for the rational use of MDM2 inhibitors alone or in combination with traditional chemotherapeutic agents or the novel proteasome inhibitor bortezomib as a new therapeutic strategy for the treatment of MCL.

Materials and Methods

Cell lines and culture conditions. Five MCL cell lines [Z-138 (26), Granta 519 (27), JVM-2 (28), Rec-1 (29), and MINO (30)] and two non-MCL cell lines [one B-lymphoblastoid cell line GM18154 and one primary effusion lymphoma cell line BC-1 (31)] were used in this study. Z-138, Granta 519, JVM-2, and Rec-1 have wt-TP53, and MINO possesses a mutation in codon 147 (valine-to-glycine; refs. 30, 32, 33). Z-138, JVM-2, GM11854, and BC-1 were cultured in RPMI 1640 containing 15% fetal bovine serum and 1% penicillin/streptomycin. Granta 519 was grown in DMEM supplemented with 15% fetal bovine serum and 1% penicillin/streptomycin. For cell viability assays, Western blot, and cell cycle analysis, cells were first acclimated in DMEM containing 5% fetal bovine serum for 24 h before exposure to Nutlin-3 (Calbiochem), doxorubicin (Calbiochem), or bortezomib (Millennium). Control cells were treated with an equivalent amount of DMSO under the same growth conditions.

Immunohistochemistry. Untreated cells (Z-138 and MINO) exposed to Nutlin-3 for 8 h were fixed overnight in buffered formalin and embedded in paraffin. Sections (5 μm) were deparaffinized through xylene and graded alcohols. Immunohistochemical stains for TP53 (DO-7; DAKO), p21Cip1/WAF1 (BD-Bioscience), and MDM2 (DAKO) were done after antigen retrieval using Target Retrieval solution, low pH (DAKO). Slides were incubated in Tris goat (3%) for 15 min and then incubated for 1 to 2 h at room temperature with primary antibodies (TP53 dilution 1:2000, p21 dilution 1:200, and MDM2 1:200) (DO-7; DAKO), p21Cip1/WAF1 (BD-Bioscience), and MDM2 (DAKO) were done after antigen retrieval using Target Retrieval solution, low pH (DAKO). Slides were incubated in Tris goat (3%) for 15 min and then incubated for 1 to 2 h at room temperature with primary antibodies (TP53 dilution 1:2000, p21 dilution 1:200, and MDM-2 dilution 1:200). Detection was carried out on an automated system (AutoStainer; DAKO) using a horseradish peroxidase/3,3’-diaminobenzidine polymer-based detection system (Envision; DAKO) according to the manufacturer’s recommendations.

Images were taken using an Olympus BX41 microscope, objective UPlanF 40/0.75 ×/0.17, with an adaptor U-TV0.5/C2 measuring 40 ×/0.75 ×/0.17, with an adaptor U-TV0.5/C2 using a digital camera Q-imaging Micropublisher 5.0RTV. The images were captured using “Q-Capture Version 3.1” and imported into Adobe Photoshop 7.0.

Western blot analysis. Cells treated with Nutlin-3 for various amounts of time were solubilized in lysis buffer [PBS, 1% cell lysis buffer (Cell Signaling), 1 × protease inhibitor (Roche), 1 × phosphatase inhibitor cocktail I and II (Calbiochem)] and incubated for 30 min on ice. Subsequently, the lysates were centrifuged for 15 min at 13,000 rpm...
Nutlin-3 Induces Apoptosis in Mantle Cell Lymphoma

Nutlin-3-mediated effects on TP53 and TP53 target proteins. To assess the effects of Nutlin-3 treatment on TP53 activation in MCL cells, we examined the expression levels of TP53 and TP53 target proteins in Z-138, Granta 519, JVM-2, and Rec-1 cells harboring wt-TP53 and Mino cells with mt-TP53. Treatment with Nutlin-3 resulted in the rapid accumulation of TP53 protein in all wt-TP53 cell lines as assessed by both Western blot analysis (Fig. 1A) and immunohistochemistry (Fig. 1B). TP53 accumulation was followed by a marked increase in the classic TP53 targets, p21 and MDM2. In addition, we noted a modest increase in Ser15 phosphorylation of TP53, a site generally associated with activation by genotoxic agents (36), and at Ser166 of MDM2, a site associated with MDM2 feedback induction and activation (37). In mt-TP53 Mino cells, no change in TP53 was detected following Nutlin-3 treatment. However, a small but consistent increase in p21 protein was evident in Western blot analysis (Fig. 1A), although this minimal change was not apparent in the immunohistochemical analysis possibly due to the different sensitivities of the techniques. These studies confirmed that Nutlin-3 was capable of reactivating TP53 in MCL cells with wt-TP53 and suggested the possibility of a TP53-independent mechanism of action as well.

Nutlin-3 inhibits cell growth and activates apoptosis in both wt-TP53 and mt-TP53 MCL cells. The effect of Nutlin-3 on proliferation and apoptosis induction was assessed in the same 5 MCL cell lines (Z-138, Granta 519, Rec-1 JVM-2, and Mino).

![Fig. 1. Nutlin-3 effects on TP53 and TP53 target proteins. A, Z-138, Granta 519, and Mino cells were treated with Nutlin-3 (Granta 519 and Mino, 10 μM/L; Z-138, 5 μM/L). At the indicated time points, cells were lysed and analyzed by Western blot. Carrier (DMSO) alone did not result in any increase in pTP53 or pTP53 target proteins. The protein levels of TP53 and its traditional targets p21 and MDM2 were increased by Nutlin-3 treatment along with a modest increase in p-TP53 (Ser15) and marked increase in p-MDM2 (Ser166) in Z-138 and Granta 519 cells. A modest increase in p21 is observed in the mt-TP53-bearing cell line Mino. B, immunohistochemistry for TP53, p21, and MDM2 done on paraaffin-embedded sections of Z-138 and Mino prepared following treatment with Nutlin-3 (Z-138, 5 μM/L; Mino, 10 μM/L) for 8 h. The wt-TP53-bearing Z-138 cell line shows a marked increase in both nuclear TP53 and target proteins p21 and MDM2, no increase in TP53 or in p21 and MDM2 can be discerned by immunohistochemistry in Mino. The high constitutive level of TP53 in Mino is a result of its mutant status.](www.aacrjournals.org)
As shown in Fig. 2A, Nutlin-3 resulted in a dose-dependent reduction in cell viability in the wt-TP53 cell lines as assessed by the MTS assay (IC_{50} at 48 h: 1.0 μmol/L for Z-138, 7.5 μmol/L for Granta 519, 9.2 μmol/L for Rec-1, and 5.7 μmol/L for JVM-2). These findings were accompanied by a significant increase of trypan blue-positive dead cells at 48 h (Fig. 2B). Nutlin-3 treatment also induced dose-dependent growth inhibition in the mt-TP53 MINO cells. However, this occurred at significantly higher dose levels (IC_{50} = 22.5 μmol/L) than with the wt-TP53 MCL cells.

To characterize the mechanism of Nutlin-3-induced cell death in wt-TP53 MCL cells, we analyzed the expression of several apoptosis-related targets of TP53. The TP53 transcriptional target BH3-only proteins, Puma and Noxa, were induced in both Z-138 and Granta 519 following TP53 stabilization by Nutlin-3. Peak induction of Puma was observed at 8 h following Nutlin-3 treatment in both cell lines, whereas Noxa peaked at 8 h in Granta 519 and at 24 h in Z-138. These changes were accompanied by the cleavage of caspase-3 and -9 in both cell lines, suggesting activation of the intrinsic apoptotic pathway (Fig. 2C). Another TP53 target protein, the proapoptotic multidomain Bcl-2 family member Bax, showed no increase in response to Nutlin-3-induced TP53. Bcl-2 was expressed in all tested cell lines, and its expression levels were not affected by Nutlin-3. Interestingly, Nutlin-3 also induced a small but reproducible increases in Noxa and cleaved caspase-3 and -9 in the mt-TP53 MINO cell line, whereas other TP53 apoptotic target proteins, including Puma and Bax, were not increased (Fig. 2C). These results confirm that Nutlin-3 has antiproliferative and proapoptotic effects in both wt-TP53- and mt-TP53-bearing MCL cell lines.

Nutlin-3-related effects on cell cycle and related proteins in wt-TP53 and mt-TP53 MCL cells. As shown in Fig. 3A and B, Nutlin-3 impeded cell cycle progression, resulting in a depression of the S-phase fraction in all tested MCL cell lines. The reduction in the S-phase fraction was associated with accumulation of cells in the G_{0}/G_{1}-G_{2}-M phases, suggesting that Nutlin-3 may impede cell cycle progression at both the G_{1}-S and G_{2}-M checkpoints in wt-TP53 Z-138 and Granta 519. In the mt-TP53 MINO cell line, the cell cycle arrest observed was predominantly in G_{2}-S. The histograms also revealed an increase in the sub-G_{1} fraction in all cell lines; however, in MINO, this increase was less than in the wt-TP53 cell lines. The increase in the sub-G_{1} fraction was temporally correlated with
the accumulation of cleaved caspase-3 and -9 products as discussed previously.

Activated TP53 results in inhibition of Rb phosphorylation and cell cycle arrest through the up-regulation of the CDK inhibitor p21 (TP53-p21-Rb signaling; ref. 38). However, the high levels of constitutively expressed cyclin D1 in MCL could potentially sequester and functionally inactivate Nutlin-3 induced p21, thereby preventing its ability to inhibit active CDK complexes, inactivate Rb, and lead to G0-G1 arrest. Therefore, we wished to know whether Nutlin-3 treatment could successfully repress Rb phosphorylation in the presence of constitutive cyclin D1 expression in wt-TP53 MCL cells. As shown in Fig. 3C, Nutlin-3 treatment resulted in a significant decrease in the Rb phosphorylation at Ser780, Ser795, and Ser807/Ser811 in wt-TP53 Z-138 and Granta 519 but not in mt-TP53 MINO cells.

Constitutive expression of cyclin D1 remains unaffected. Cyclin B1 and CDC2 levels are down-regulated between 8 and 24 h following Nutlin-3 treatment in only wt-TP53 cells. D, p73 expression is assessed in Nutlin-3-treated MINO cells by Western blot analysis; p73 level is increased by Nutlin-3 treatment.

Fig. 4. Nutlin-3 treatment does not result in induction of novel TP53 targets PTEN, TSC2, and AMPKα1. Z-138, Granta 519, and MINO cells were treated with Nutlin-3 (Z-138, 5 μmol/L; Granta 519 and MINO, 10 μmol/L) for the indicated amount of time, and protein expression was analyzed by Western blot. None of the novel TP53 transcriptional targets TSC2, PTEN, or AMPKα1 showed a significant increase in expression following Nutlin-3 treatment.
capable of activating TP53-p21-Rb even in the presence of high levels of cyclin D1 in wt-TP53 MCL cells. In contrast, and consistent with the minimal G1-S cell cycle arrest, no effect on p-Rb or total Rb was detected in the mt-TP53 Mino cell line, suggesting that the lower levels of p21 induced are insufficient to affect Rb phosphorylation and protein level.

Recently, Kan et al. (24) showed that Nutlin-3-activated TP53 could also induce a G2-M cell cycle arrest through a pathway leading to the suppression of both CDC2 and cyclin B in cells overexpressing a cyclin D1/CDK2 artificial construct. For this reason, we also assessed the protein levels of these two cell cycle regulators following Nutlin-3 treatment. Consistent with this previous report, both CDC2 and cyclin B levels declined at 24 h following exposure to Nutlin-3 in wt-TP53 MCL cell lines. Nutlin-3-treated Mino cells, however, showed no consistent change in total or p-Rb levels or in CDC2 or cyclin B levels (Fig. 3C), suggesting that the mechanism of cell cycle arrest in Mino may be different.

Recently, several groups (8, 9) have shown that Nutlin-3 is capable of inducing cytotoxicity in TP53-null or mt-TP53 cell lines through a mechanism that involves the TP53 homologue p73. The investigators speculated that Nutlin-3 might be capable of activating p73 by competitively displacing it from MDM2 complexes, in a manner similar to that invoked for activation of wt-TP53. In both studies cited above, Nutlin-3 treatment resulted in an increase in p73 protein levels and p73 target gene expression. We therefore investigated p73 expression levels in Mino cells following Nutlin-3 treatment and observed a significant increase in p73 levels at 8 h (Fig. 3D). These data suggest that the TP53-independent cytotoxicity observed following Nutlin treatment in wt-TP53 Mino might be mediated by p73.

**Lack of effect on the AKT and mTOR regulators PTEN, TSC2, and AMPKβ1.** Recently, attention has been focused on novel TP53 target genes that function to inhibit cell growth and proliferation rather than by inducing apoptosis (41). These include the AKT and mTOR regulators PTEN, TSC2, and AMPKβ1, which have been shown to be inducible in a tissue-specific and stress-specific fashion. We therefore investigated whether Nutlin-3-induced TP53 activates these novel targets. Western blot results showed no significant increase in these novel TP53 targets in any of the cell lines analyzed (Fig. 4), suggesting that Nutlin-3-stabilized TP53 activates only a subset of its possible targets.

**MDMX is not responsible for the differential sensitivity to Nutlin-3.** MDMX is a MDM2-related protein that is similarly capable of binding wt-TP53 and blocking its transcriptional activity, although it has minimal E3 ubiquitin ligase activity. Unlike MDM2, its interaction with TP53 is not affected by Nutlin-3. Recently, several groups have reported that TP53 activation by Nutlin-3 is inversely proportional to the levels of MDMX in the cancer cells (42, 43). Because Z-138 was much more sensitive to Nutlin-3 than other MCL cell lines with wt-TP53, we wished to know whether this difference in cytotoxicity was correlated with the expression levels of MDMX. Western blot analysis showed no significant difference in the levels of MDMX between Z-138 and other MCL cell lines with wt-TP53 (Fig. 5), indicating that the sensitivity to Nutlin-3 was independent of the MDMX expression status in the tested MCL cell lines.

**Combination of Nutlin-3 with bortezomib or doxorubicin has synergistic effects on MCL growth.** Bortezomib, a selective inhibitor of the 26S proteasome, has been reported to induce apoptosis in a caspase-dependent manner in MCL cells (44–46). To determine if inhibition of the TP53-MDM2 interaction by Nutlin-3 in MCL cells might potentiate the effects of bortezomib, we assessed the effect of combining the two drugs on cell viability using the MTS assay at 48 h post-exposure. It has been reported that Nutlin-3 showed significant suppression in growth of human tumor subcutaneous xenografts without any apparent ill effects on the mouse host at steady-state plasma levels of 3.5 μmol/L (3). We used Nutlin-3 near the tolerated level in this combination study.

As shown in Table 1 and Fig. 6A, a synergistic effect between Nutlin-3 and bortezomib was observed in both wt-TP53 and mt-TP53 MCL cells, although the doses needed to obtain this effect differed. The averaged CI values indicated strong and moderate synergism for inhibition of cell viability for Z-138 and Granta 519, respectively. Of note, the Nutlin-3 and bortezomib combination produced very strong synergistic effects on mt-TP53 Mino cells.

**Doxorubicin is a commonly used chemotherapeutic agent whose antineoplastic effects are believed to include genotoxic activation of the TP53 pathway (47).** Similar to the experiments with bortezomib, cells were treated with doxorubicin and Nutlin-3 either as individual agents or in combination, and the

<table>
<thead>
<tr>
<th>Table 1. CI for cell growth inhibiting effects of Nutlin-3/bortezomib and Nutlin-3/doxorubicin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Nutlin-3/bortezomib</td>
</tr>
<tr>
<td>Z-138</td>
</tr>
<tr>
<td>Granta 519</td>
</tr>
<tr>
<td>Mino</td>
</tr>
<tr>
<td>Nutlin-3/doxorubicin</td>
</tr>
<tr>
<td>Z-138</td>
</tr>
<tr>
<td>Granta 519</td>
</tr>
<tr>
<td>Mino</td>
</tr>
</tbody>
</table>

NOTE: CI values < 1.0 indicate synergism.
*Average CI values were calculated from ED50, ED75, and ED90.

CSI plots were generated using CalcuSyn software.

---

Fig. 5. MDMX is not responsible for differential sensitivity to Nutlin-3. Expression level of MDMX was assessed in untreated Z-138, Granta 519, Rec-1, and JVM-2 by Western blot analysis. There was no significant difference of MDMX expression between the highly Nutlin-3-sensitive Z-138 MCL cell line and the other MCL lines.
cell viability was measured 48 h after the drug administration. The results indicated a strong synergistic interaction of doxorubicin and Nutlin-3 on cell viability in Z-138 and Granta 519; however, no synergy was observed for MINO cells (Table 1; Fig. 6B).

**Discussion**

Nutlin-3 is a potent and selective inhibitor of the TP53-MDM2 interaction (48) and has been proposed as a therapeutic agent for tumors maintaining wt-TP53 status. Furthermore, Nutlin-3, a cis-imidizoline analogue, penetrates cellular membranes and can be administered orally (3). Recent studies have shown that the stabilization of wt-TP53 by Nutlin-3 leads to cell death and implicate this agent as a novel therapeutic in cancer. Nutlin-3 has been shown to be effective in both solid tumors and lymphoid malignancies including chronic lymphocytic leukemia (5), Hodgkin’s lymphoma (6, 7), and primary effusion lymphoma (4).

MCL is an aggressive small lymphocytic lymphoma that overexpresses cyclin D1 as a result of a chromosomal translocation that places the cyclin D1 gene under the control of immunoglobulin heavy chain gene control elements. This disease is resistant to standard chemotherapy and novel

---

**Fig. 6.** Synergistic interaction between Nutlin-3 and bortezomib or doxorubicin in MCL cells. Z-138, Granta 519, and MINO cells were cultured in the presence of escalating doses of Nutlin-3 and bortezomib (A) or Nutlin-3 and doxorubicin (B) using fixed ratios. After 48 h, cell growth inhibition was evaluated by MTS assay and displayed as percent absorbance of untreated control cells.
targeted therapies are urgently needed. Multiple studies have shown that TP53 mutations are uncommon in the typical form of MCL that accounts for ~80% of all cases and are present in about one-third of the remaining blastoid variant morphologies (18, 22, 23). This implies that reactivation of TP53 in MCL is potentially achievable in >80% of cases. Therefore, our primary purpose was to assess whether Nutlin-3 might be an effective agent in MCL with wt-TP53 and, if so, to investigate the molecular mechanisms by which this agent might achieve its cytotoxic effect in a lymphoma subtype that is characterized by constitutively overexpressed cyclin D1.

Our study revealed that MCL cell lines with wt-TP53 are highly sensitive to Nutlin-3 treatment, with IC50 values at 48 h ranging from 1.0 to 9.2 μmol/L. In the two wild-type bearing MCL cell lines studied in more detail (Z-138 and Granta 519), cell death was accompanied by a marked decrease in the S-phase fraction in both cell lines and by modest increases in both G0-G1 and G2-M fractions. As expected, Nutlin-3 treatment stabilized wt-TP53 and led to the up-regulation of the classic TP53 targets p21 and MDM2. This was accompanied by the up-regulation of the BH3-only proapoptotic proteins Puma and Noxa and the activation of the intrinsic apoptotic pathway as evidenced by caspase-3 and -9 cleavage products. Although elevations of p53, p21, and MDM2 are universally found in many types of wt-TP53-bearing tumor cell lines following Nutlin-3 treatment, the activation of apoptotic proteins are not as stereotyped even in tumors of the same type. For example, in primary effusion lymphomas, up-regulation of Puma, Noxa, and Bax by Nutlin-3 has been reported (4). In acute myelogenous leukemia, Puma but not Bax was induced. In Hodgkin’s lymphoma, Puma was up-regulated in one reported cell line, whereas in a second cell line Puma was unaffected; instead, Bcl-2 was down-regulated (6). In the two sensitive MCL cell lines studied in the current article, we observed a consistent induction of Puma and Noxa (albeit with different kinetics) with no change in either Bax or Bcl-2.

MCL cells with t(11;14)(q13;q32) translocation constitutively overexpress cyclin D1, which complexes primarily with CDK4 and promotes cell cycle progression by hyperphosphorylating the Rb checkpoint protein. Because cyclin D1/CDK complexes have been reported to be capable of sequestering the Rb checkpoint protein. Because cyclin D1/CDK4 and promotes cell cycle progression by hyperphosphorylating the Rb checkpoint protein. Because cyclin D1/CDK4 complexes have been reported to be capable of sequestering CIP/KIP proteins compared with the naturally occurring cyclin D1/CDK4 complexes found in MCL or possibly to differences in the absolute levels and combinations of these proteins in the two systems.

Although we were initially surprised to find that Nutlin-3 also showed activity in the mt-TP53 cell line MINO, several recent studies have shown a TP53-independent mechanism of action for Nutlin-3 (8, 9). In these studies, higher Nutlin-3 doses were required to induce cytotoxicity in TP53-null or mt-TP53 cell lines, with IC50 values in the 20 to 30 μmol/L range compared with the 1 to 10 μmol/L range for wt-TP53 cell lines (8, 9). This TP53-independent mechanism appears to be mediated, at least in part, through the TP53 homologue p73. Similar to the mechanism of action for TP53, Nutlin-3 interferes with MDM2-P73 binding and is believed to activate a p73-directed transcriptional program that leading to the expression of p21 (9), Puma, and Noxa (8), and the induction of cell cycle arrest and apoptosis. Other TP53-independent mechanisms have also been implicated in Nutlin-3 toxicity including stimulation of apoptotic functions of the E2F1 transcription factor (51, 52).

Consistent with a p73-mediated mechanism of action, Nutlin-3 treatment of MINO cells resulted in dose-dependent toxicity with a significantly higher IC50 (22.5) and a lower apoptosis induction compared with wt-TP53-bearing MCL lines. Toxicity was accompanied by small increases in Noxa, cleaved caspase-3 and -9, and p21. Unlike the wt-TP53 cell lines, there was no change in total Rb, p-Rb, CDC2, and cyclin B levels. There was a consistent decrease in the S-phase fraction and a predominantly G2-M cell cycle arrest. Importantly, p73 levels became elevated following Nutlin-3 treatment, suggesting that at least some of the TP53-independent responses were related to p73 stabilization and activation, as reported for other mt-TP53 tumors (8, 9).

The inability to detect a decrease in Rb phosphorylation and significant G1 arrest in MINO may be related to the lower levels of p21 achieved by p73 compared with levels achieved by TP53-mediated inhibition. Lack of reduction of cyclin B and CDC2 also may also be related to differences in the p73 and TP53 transcriptional program and also suggests that the G2-M arrest in MINO is likely due to a different mechanism than the G2-M arrest seen in the wt-TP53-bearing MCL cell lines. Although we have not identified the reason for the G2-M arrest in MINO, one plausible explanation could be sequestration of cyclin B/CDC2 complexes in the cytoplasm by 14-3-3 proteins, which are known to be induced by p73 (53, 54). Additional studies will be necessary to investigate this possibility.

Several negative regulators of both AKT and mTOR prosurvival pathways, including AMPKβ1, TSC2, and PTEN, have recently been shown to be novel stress-related transcriptional targets of TP53 (41). The manner in which TP53 selectively targets its many potential targets is poorly understood, and these targets have not been assessed previously following Nutlin-3 treatment. None of these novel TP53 target proteins was reproducibly induced by Nutlin-3 treatment in MCL cell lines. These results suggest that Nutlin-3-stabilized TP53 activates only a subset of its possible targets.
The MDM2 homologue MDMX, a TP53-binding protein, inhibits TP53-dependent transcription (55), and knockdown of MDMX enhances the TP53 response to DNA damage (56). Nutlin-3 is inefficient in disrupting the MDMX-TP53 interaction and, accordingly, has been shown to be ineffective in reactivating wt-TP53 in tumor cells overexpressing MDMX (42, 43, 57). In our study, however, we did not find any correlation between MDMX expression levels and differential sensitivity to Nutlin-3 in wt-TP53 MCL cell lines. These results suggested that the ability of Nutlin-3 to activate TP53 in MCL cell lines is not dependent on the expression level of MDMX.

Phosphorylation of Ser15 is generally believed to be evidence of TP53 activation resulting from genotoxic stress (36). Nutlin-3, a nongenotoxic agent, has initially been reported to activate TP53 signaling without any effect on TP53 phosphorylation status in various tumor cells of nonlymphoid origin (3). However, more recent studies of cancers from different origins, including several studies of lymphoid tumors, have shown modest increases in TP53 phosphorylation at Ser15 following Nutlin-3 treatment (4, 6, 58). Consistent with these latter studies in lymphoid neoplasms, we also observed a modest increase in Ser15 phosphorylation following Nutlin-3 treatment. Further studies will be needed to understand the reasons for the variable presence of Ser15 phosphorylation in some tumor types but not in others.

Because Nutlin-3 was effective in inducing apoptosis and growth inhibition in both wt-TP53 and mt-TP53 MCL cells, we wished to assess whether this MDM2 antagonists could potentiate or synergize with other agents that also up-regulate the apoptotic pathway. In particular, we were interested in the widely used genotoxic chemotherapeutic doxorubicin and the novel proteasome inhibitor bortezomib. Although both drugs have a variety of effects, both are capable of activating the TP53 apoptotic pathway; doxorubicin initiates TP53 activation by DNA damage and bortezomib stabilizes TP53 by inhibiting its degradation through proteasomal blockade (44–47).

We found that the combinations of Nutlin-3 treatment with either doxorubicin or bortezomib resulted in synergistic cytotoxicity in MCL cells harboring wt-TP53. The synergy with doxorubicin, a conventional genotoxic agent to MCL, suggests that addition of Nutlin-3 may allow a reduction in therapeutic dose of this and similar genotoxic drugs, which could potentially reduce some of the unwanted side effects of the genotoxic agents (59).

Bortezomib inhibited tumor cell growth in MCL cells with wt-TP53 and mt-TP53, consistent with both TP53-dependent and TP53-independent effects, as reported previously (42). Importantly, the addition of Nutlin-3 resulted in a significant synergistic cytotoxicity in both wt-TP53 and mt-TP53 MCL cell lines. Because bortezomib alone induces excellent responses in some MCL patients (60, 61), these synergistic effects may allow more flexibility in dosing patients on clinical trials.

In conclusion, our results show that a small-molecule MDM2 antagonist Nutlin-3 is capable of inducing apoptotic cell death and cell cycle arrest in a TP53-dependent and TP53-independent manner in MCL cell lines and provide a basis for the rational use of this agent in MCL.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Drs. M. Andreffe and M. Konopleva for helpful discussions during the course of this study and Tomomi Ikeda for technical assistance.
Cancer Therapy: Preclinical


MDM2 Antagonist Nutlin-3 Displays Antiproliferative and Proapoptotic Activity in Mantle Cell Lymphoma

Yoko Tabe, Denise Sebasigari, Linhua Jin, et al.