1,25-Dihydroxyvitamin D₃ Enhances the Apoptotic Activity of MDM2 Antagonist Nutlin-3a in Acute Myeloid Leukemia Cells Expressing Wild-type p53

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Abstract
The tumor suppressor p53 is often referred to as “the guardian of the genome” because of its central role in the cellular response to oncogenic stress and prevention of tumor development. Mutations of p53 in acute myeloid leukemia (AML) are rare but resistance to chemotherapy has been reported because of the deregulation of the p53 signaling and differentiation pathways. It is known that the interaction of the vitamin D metabolite 1,25-dihydroxyvitamin D₃ (1,25D) with its functional vitamin D receptor leads to differentiation, G₁ arrest, and increased cell survival in p53-null AML cells. However, there are no reports on the effect of 1,25D in leukemia cells expressing wild-type p53. Here, we examine vitamin D signaling in AML cells and its effect on p53-null AML cells. We find that 1,25D alone induces monocytic differentiation in these cell lines similar to that seen in p53-null AML cells, suggesting that the presence of wild-type p53 is compatible with activation of vitamin D signaling. Combination of nutlin-3a with 1,25D accelerated programmed cell death, likely because of enhanced nutlin-induced upregulation of the proapoptotic PIG-6 protein and downregulation of antiapoptotic BCL-2, MDMX, human kinase suppressor of Ras 2, and phosphorylated extracellular signal-regulated kinase 2.

Introduction
Acute myeloid leukemia (AML) is a hematologic disease characterized by the failure of hematopoietic stem cells to differentiate (1). As a result, there is accumulation of immature blasts incapable of doing their physiologic roles. A large number of genetic aberrations have been linked to AML, such as altered transcription factors that regulate myeloid maturation and mutation of receptor tyrosine kinases (2), constitutively active antiapoptotic proteins belonging to the BCL-2 and IAP families (3), and Rb protein (4). For example, a prognostically important form of mutation is the in-frame internal tandem duplication of the FLT3 receptor tyrosine kinase (5). These and other mutations ultimately lead to deregulation of the cell cycle and/or the apoptotic machinery.

The tumor suppressor p53 is a key regulator of the G₁/S and G₂/M cell cycle checkpoints, and it is frequently disabled in neoplastic cells through mutation or deletion (6). In proliferating cells, p53 is negatively regulated by MDM2, a specific E3 ubiquitin ligase, which targets p53 for ubiquitin-dependent degradation. MDM2 binds to the amino terminus of p53 and inhibits its transcriptional activity. MDM2 is a transcription target of p53, and thus, p53 and MDM2 form an autoregulatory feedback loop regulating the cellular levels of both proteins (7). MDM2 also functions as an E3 ubiquitin ligase for MDMX, a close analog of MDM2 that binds to the same region in the N-terminus of p53 but inhibits its transcriptional activity. MDM2 is a transcription target of p53, and thus, p53 and MDM2 form an autoregulatory feedback loop regulating the cellular levels of both proteins (7). MDM2 also functions as an E3 ubiquitin ligase for MDMX, a close analog of MDM2 that binds to the same region in the N-terminus of p53, but MDMX does not function as a ubiquitin ligase for p53 (8). Therefore, MDM2 not only regulates p53 activity but also controls the stability of MDMX.

Nutlin-3a, a small-molecule inhibitor of the p53-MDM2 interaction, developed at Roche, binds to the p53 pocket of MDM2 with high selectivity, effectively stabilizes p53, and induces cell cycle arrest and apoptosis in cancer cells with wild-type p53 (9, 10). Nutlin-induced p53 elevates the cellular level of its transcriptional target MDM2. It has been reported that stress-induced phosphorylation of p53 prevents the binding of MDM2 to p53. We have shown that, despite the lack of phosphorylation on key N-terminal serines of p53, nutlin-3a effectively induces cell cycle arrest and apoptosis in cancer cells (11).
In addition to its transcriptional activity, p53 can translocate to mitochondria and modulate the activity of BH3-containing proapoptotic proteins such as Bax by transcriptionally independent mechanisms (12).

1,25-Dihydroxyvitamin D$_3$ (1,25D), the biologically active form of vitamin D$_3$, has profound effects on calcium homeostasis, bone metabolism (13), monocytic differentiation (14, 15), and cell cycle traverse in several human leukemic cell lines (16, 17). When 1,25D enters a cell, it binds to and activates its high-affinity vitamin D receptor. This leads to heterodimerization with the retinoid X receptors (α, β, and γ), and the complexes then bind to vitamin D response elements located in the promoters of vitamin D-responsive genes (18). It has been observed that 1,25D-induced monocytic differentiation in HL60 cells occurs in two overlapping phases. In the first 24 to 48 hours (early differentiation), the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway is activated for expression of genes involved in cell proliferation. Beyond 48 hours (late differentiation), the MAPK/ERK pathway activity is low (because of the increase in p35/NCK5α expression), but kinase suppressor of Ras (KSR)/Raf-1/p90RSK signaling is increased (19–21). KSR1, a protein scaffold that augments interaction of Ras with Raf, mitogen-activated protein/ERK kinase (MEK), and ERK, may amplify this pathway and phosphorylate downstream targets such as CAAT/enhancer binding protein β (22–24). Human KSR2, an evolutionary conserved member of the KSR family is also upregulated by 1,25D and promotes cell survival and optimal monocytic differentiation (25).

The p53 and vitamin D pathways share some common molecular mediators and biological functions that may interact when activated. Maruyama et al. (26) have found p53 binding sites in the promoter of vitamin D receptor, suggesting that p53 may affect the vitamin D pathway. Because activation of p53 and vitamin D pathways lead to growth arrest and affect cell survival of AML cells, we hypothesized that agents activating both signaling pathways may synergize in their activity. This prompted us to investigate the combination effect of an MDM2 antagonist and 1,25D. Here, we show that 1,25D accelerates the onset of p53-dependent apoptosis induced by nutlin-3a in MOLM-13 and OCI-AML3 cells. Our results suggest that the modulation of some p53 targets, such as enhanced upregulation of PIG-6 and downregulation of BCL-2, MDMX, hKSR2, and p-ERK2 proteins, is the likely contributor to the enhanced apoptotic effect of the MDM2 antagonist.

Materials and Methods

**Chemicals and antibodies.** Nutlin-3a and its inactive enantiomer, nutlin-3b, were synthesized at Hoffmann-La Roche. Compounds were dissolved in Dimethyl sulfoxide (DMSO) and kept as frozen aliquots. Anti-CD14 My4-RD-1–phycoerythrin (PE) and anti-CD11b MO1–fluorescein isothiocyanate (FITC) antibodies were obtained from Coulter Corp. Antibodies used for Western blotting were as follows: β-actin (A5316), MDMX (A300–287A), PIG-6 (ab48001), and KSR1 (ab52196) antibodies were purchased from Sigma, Bethyl Laboratories, and Abcam, respectively. Phospho-MEK1/2 (Ser217/221; 9121), phospho-MEK1/2 (Thr202; 9127), MEK1/2 (9122), and phospho-ERK1/2 (Thr202/Tyr204; 4377) were purchased from Cell Signaling, Inc. BCL-2 (sc-7382), ERK1/2 (sc-94), MDM2 (sc-965), p53 (sc-263), poly (ADP-ribose) polymerase (PARP; sc-7150), and vitamin D receptor (C-20; sc-1008) were purchased from Santa Cruz Biotechnology, Inc. hKSR2 (H00283455-A01) antibody was purchased from Abnova Corporation/Novus Biologicals. Secondary antibodies, anti-goat IgG–horse-radish peroxidase (HRP; sc-2020), anti-rabbit IgG–HRP (sc-2301), and anti-mouse IgG–HRP (sc-2302) were also purchased from Santa Cruz Biotechnology, Inc.

**Cell culture and treatments.** MOLM-13 cell line was purchased from DSMZ, and the OCI-AML3 cell line was a kind gift from Dr. Mark Minden, Princess Margaret Hospital (Ontario, Canada). Both cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). MOLM-13 cells harbor internal tandem duplication mutations in FLT-3 tyrosine kinase receptors and OCI-AML3 cells carry gene mutation in nucleophosmin (DSMZ cell database). HL60 and THP-1 cells, purchased from the American Type Culture Collection were cultured in Iscove’s and RPMI-1640 medium supplemented with 20% and 10% heat-inactivated FBS, respectively. Cells were cultured in fresh tissue culture media at a cell density of $1 \times 10^6$ cells per 10 mL medium in 25-cm$^2$ flasks and treated immediately with compounds or the equivalent volume of ethanol or DMSO as vehicle control. Cell lines were mycoplasma-free and cultured in a humidified environment with 5% carbon dioxide. For combination experiments, cells were exposed to 1,25D for a total of 48 hours, and nutlin-3a was added during the last 2 to 36 hours.

**Western blotting.** Cells were harvested and washed twice with ice-cold PBS. Cell pellets were solubilized with radioimmunoprecipitation assay lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L sodium chloride, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% NP40, 1% sodium deoxycholate, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, and 1 μg/mL aprotinin] followed by centrifugation at 16,000 $\times g$ for 20 minutes. The protein concentrations of the extracts were determined using the Bio-Rad protein assay kit and then incubated in (3:1) 4× SDS sample buffer [150 mmol/L Tris-HCl (pH 6.8), 30% glycerol, 3% SDS, 1.5 mmol/L bromophenol blue dye, and 500 mmol/L dichlorodiphenyltrichloroethane]. Proteins (15 or 30 μg per lane) in cell extracts were separated on 4% to 20% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes (Amersham).
Membranes were blocked with 5% milk in TBS/0.1% Tween 20 for 1 hour, incubated overnight with primary antibodies, and then blotted with a HRP-linked secondary antibody for 1 hour. The protein bands were visualized using a chemiluminescence assay system (GE Healthcare), and the absorbance of each band was quantitated using MultiGuage (Fuji).

**Determination of differentiation markers.** Approximately 1 × 10^6 cells were washed twice with cold PBS and incubated with 0.5 μg MO1-FITC and 0.5 μg My4-RD1-PE antibodies at room temperature for 45 minutes to analyze the surface expression of CD11b and CD14, respectively. As an isotype control, cells were incubated with FITC and PE-conjugated mouse IgG1 antibodies. Cells were washed twice with ice-cold PBS. Stained cells were then resuspended in 0.5 mL PBS and analyzed by a Fluorescence Activated Cell Sorting Calibur flow cytometer (Becton Dickinson). Two-parameter analysis was done using CellQuest software. For assessment of monocyte-specific esterase, also known as nonspecific esterase, smears were made by resuspending cells in 100 μL PBS and spread onto slides. The air-dried smears were fixed in formalin-acetone mixture buffer for 30 seconds, then washed with distilled water, and stained for 45 minutes at room temperature with the following solution: 8.9 mL of 67 mmol/L phosphate buffer (pH 7.6), 0.6 mL of hexazotized pararosaniline, 1 mg/mL α-naphthyl acetate, and 0.5 mL ethylene glycol monomethyl ether. The percentage of monocyte-specific esterase–stain cells was determined by counting 100 cells in triplicate.

**Cell cycle analysis.** For analysis of cell cycle distribution, treated cells were washed twice with cold PBS, fixed with 70% cold ethanol, and stored at −20°C until analysis. After thawing, cells were washed twice with cold PBS, resuspended in 0.5 mL propidium iodide/RNase staining buffer (BD Biosciences), and incubated for 15 minutes at 37°C. Stained cells were filtered and analyzed using a Fluorescence Activated Cell Sorting Calibur flow cytometer (Becton Dickinson). Analysis was done using CellQuest software. For bromodeoxyuridine (BrdUrd) incorporation analysis, 20 μmol/L BrdUrd (Sigma) was added 1 hour before harvesting treated cells. Cells were fixed as described above, pelleted, and washed with cold PBS containing 1% FBS. Samples were permeabilized with 2N HCl and 0.5% Triton X-100 for 30 minutes, and neutralized with 0.1 mol/L sodium tetraborate (pH 8.5) at room temperature. Cells were then labeled with anti-BrdUrd FITC conjugated monoclonal antibody (BD Biosciences) for 1 hour in the dark. Stained cells were washed once with PBS containing 0.5% Tween 20 and resuspended in 0.5 mL propidium iodide/RNase staining buffer (BD Biosciences). Stained cells were filtered and analyzed for BrdUrd incorporation using dual-color flow cytometric DNA techniques.

**Cell viability and apoptosis assays.** MOLM-13 and OCI-AML3 cells (1 × 10^5 per well) were seeded in six-well tissue culture plates and treated immediately with drugs. For quantification of apoptosis, cells were collected by centrifugation at 1,500 rpm for 10 minutes at 4°C and stained with the Guava Nexin Assay kit as recommended by the manufacturer. Apoptotic indices were determined using the Guava Personal Cell Analyzer (Guava Technologies). Three cell populations were distinguished in this assay: nonapoptotic cells (Annexin V negative and 7-Amino Actinomycin D (AAD) negative), early apoptotic cells (Annexin positive and 7-AAD negative), and late apoptotic cells (annexin V positive and 7-AAD positive).
and late-stage apoptotic cells (Annexin positive and 7-AAD positive). Another aliquot of cells was used for assessment of cell viability by Trypan blue exclusion. For the Caspase-Glo 3/7 Assay, MOLM-13 cells (1 × 10⁴) were seeded per well in clear-bottom 96-well tissue culture plates and treated immediately with drugs at the indicated time points. Cells were lysed and processed according to the manufacturer’s protocol (Promega; G8091). For the fluorimetric terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay, cells (2 × 10⁶) were seeded in 75-cm² flasks and treated immediately with drugs at the indicated time points. The manufacturer’s protocol (Promega; G3250) was used for determination of TUNEL-stained cells by flow cytometry.

**RNA interference experiments.** Approximately 3 × 10⁶ OCI-AML3 cells were suspended in 100 μL of solution L (Amaxa) and nucleofected with 10 nmol/L nontargeting negative-control human small interference RNA (siRNA; Dharmacon; D-001810-01-20), 10 nmol/L human BCL-2 siRNA (Dharmacon; D-003307-04), or 10 nmol/L human PIG-6 siRNA (Ambion silencer select; s11219) with an Amaxa nucleofector device. The target sequence of human BCL-2–specific siRNA were 5′-AGAUAGUGAUGAAGGCUACAU. The sequences of the human PIG-6–specific siRNA were 5′-GGACAGGCGCAUUCACATT and 3′-UGUUGUAUAGCCUCUGUCTTA. Cells were then transferred to a 12-well plate containing 2 mL of pre-warmed media for 24 hours before exposure to drugs.

**Quantitative real-time PCR.** Total cellular RNA was extracted from cells using RNeasy Mini kit (Qiagen) and treated with RNase-free DNase following the manufacturer’s protocol. Total RNA (2 μg) was incubated with a reverse transcription mix (Applied Biosystems) containing 25 mmol/L MgCl₂, PCR buffer II, deoxyribonucleotide triphosphate mixture (2.5 mmol/L each), 50 μmol/L random hexamers, RNase inhibitor, and 50 U/μL Multi-Scribe reverse transcriptase. The parameters for reverse transcription were as follows: 40 cycles of 25°C for 10 minutes followed by 42°C for 30 minutes and 98°C for 5 minutes. cDNA (200–400 ng) was used for quantitative PCR with the Taqman 2× PCR Master Mix on an Applied Biosystems 7900 Real-Time PCR System following the manufacturer’s instructions. Triplicate reverse transcriptase experiments were done for each gene, and the relative amount of transcript was normalized to 18S rRNA. Specific primer/probe sets for BCL-2 and PIG-6 in 20× expression assays (Applied Biosystems; Hs00608023_m1 and Hs00275205_m1, respectively) were used for quantitative reverse transcriptase-PCR. The parameters for PCR were as follows: 40 cycles of 50°C for minutes, 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 1 hour. The C<sub>t</sub> value, the cycle number at which signal fluorescence surpassed fluorescence background noise, was recorded, and relative fold values were calculated based on the equation 2<sup>ΔΔC<sub>t</sub></sup>, in which ΔΔC<sub>t</sub> = ΔC<sub>t-treated</sub> − ΔC<sub>t-calibrator</sub> (vehicle or drug-treated samples). To determine p53 target expression level, cDNAs were subjected to quantitative real-time PCR with Taqman low-density array microfluidic cards. These cards contain Taqman gene expression assays for 90 targets and three internal controls (Supplementary Table S1).
Statistical analysis. Each data point represents the mean and SD of at least three independent experiments. The significance of differences between indicated data groups was assessed by the Student’s t test.

Results

1,25D induces differentiation of human AML cells expressing wild-type p53. Numerous studies have shown that 1,25D can induce differentiation of leukemia cells with p53-mutant or p53-null status (14, 15). However, there are no published reports of vitamin D effects in cancer cells expressing wild-type p53, raising the possibility that wild-type p53 status is not compatible with vitamin D-induced differentiation. Therefore, we investigated the effect of 1,25D on AML cell lines expressing wild-type p53. For these experiments, we chose human AML cell lines MOLM-13 (AML M5 subtype) and OCI-AML3 (AML M4 subtype) expressing wild-type p53, and for comparison, HL60 (AML M2 subtype) and THP-1 (AML M5 subtype), p53-null cell lines. Experiments with HL60 and THP-1 cells indicated that vitamin D-induced expression of cell surface differentiation markers in these cell lines were similar. However, HL60 cells were chosen for comparative in-depth studies with p53-expressing AML cells because our laboratories have documented that vitamin D derivatives have most striking effects on this cell line. In initial experiments, the cells were incubated with 1, 10, or 100 nmol/L 1,25D for 48 hours, and the expression of CD11b and CD14 myeloid differentiation markers was analyzed by flow cytometry. 1,25D induced the surface expression of CD11b and CD14 in a dose- and time-dependent manner on all three cell lines (Fig. 1A and B). The monocyte-specific esterase was expressed on MOLM-13 and OCI-AML3 cells not exposed to 1,25D, whereas the expression of this cytoplasmic differentiation marker was minimal in HL60 cells. Nonetheless, monocyte-specific esterase expression increased in a dose-dependent manner in all cell lines studied (Fig. 1A). Exposure of HL60 cells to 10 nmol/L 1,25D for 48 hours caused ~50% of the cell population to flatten and adhere to culture vessels, and the nonadherent cells remained as single-cell suspension or aggregates of variable sizes. In contrast, only 10% to
20% of the total population of MOLM-13 and OCI-AML3 cells remained attached to flasks, with most cells remaining in single-cell suspension or aggregates. The time-dependent increase in expression of CD11b and CD14 on HL60 and OCI-AML3 cells displayed a similar pattern in that ∼80% of the cells were CD14 positive within 24 hours, which was sustained at 48 and 72 hours. However, the population of HL60 cells expressing CD11b was significantly (P < 0.01) greater and continued to increase with time when compared with MOLM-13 and OCI-AML3 cells (Fig. 1B). In contrast, ∼60% of MOLM-13 cells were positively stained for CD14 at 24 and 48 hours, which was slightly reduced to 50% at 72 hours. Trypan blue exclusion test indicated that cells were viable throughout the duration of the treatment (Supplementary Fig. S1). Because MOLM-13 and OCI-AML3 cells were responsive to 1,25D, this indicated that expression of wild-type p53 is compatible with 1,25D-induced differentiation. Therefore, we used these cell lines to study the interaction between 1,25D and nutlin-3a because previous studies have shown that MDM2 antagonists require wild-type p53 for activity (27).

**Nutlin-3a activates p53 in MOLM-13 and OCI-AML3 cells leading to cell cycle arrest and apoptosis.** Nutlin-3a is a small molecule that binds to MDM2, thereby preventing the ubiquitin-dependent degradation of p53. Accumulation of p53 enables the transcriptional activation of genes that induce cell cycle arrest and/or apoptosis. For these experiments, exponentially growing cells were exposed to 10 μmol/L nutlin-3a for 2 to 24 hours, and whole cell lysates were analyzed for protein levels of p53 and its transcriptional targets, p21 and MDM2. Exposure of MOLM-13 and OCI-AML3 cells to nutlin-3a led to stabilization and accumulation of p53 in a time-dependent manner. The protein level of the p53 targets, MDM2 and p21, increased during treatment (Fig. 2A). In MOLM-13 cells, the drop of MDM2 and p21 protein levels beyond 8 hours and that of p53 beyond 12 hours is a likely consequence of degradation as cells undergo apoptosis. This was shown by the activation of caspase-3, with the concomitant cleavage of PARP evident beyond 8 hours in MOLM-13 cells. Assays for early apoptosis (caspase-3/caspase-7 activation) and late-stage apoptosis (TUNEL assay) confirmed that nutlin-3a induces apoptosis in MOLM-13 cells (Supplementary Fig. S1B). Incorporation of BrdUrd revealed that nutlin-3a induced G1 and G2/M cell cycle arrest in OCI-AML3 cells (Fig. 2B). We then tested MOLM-13 and OCI-AML3 cells for apoptosis by the Annexin V assay. Exposure of exponentially proliferating cells to 10 μmol/L nutlin-3a, but not the inactive enantiomer nutlin-3b, for 5 to 48 hours, led to an increase in Annexin V-positive cell fraction (Fig. 2C and D). However, a marked difference in response to nutlin was observed. MOLM-13 cells were more sensitive with 80% of the cell population undergoing apoptosis at 24 hours. OCI-AML3 cells were relatively slow to respond to nutlin-3a, with only 18% of the cell population undergoing apoptosis at 24 hours but increased to 80% after 48 hours. Nutlin-3b, the inactive form of nutlin-3a (10), did not cause cell death in both cell lines (Fig. 2C and D).

1,25D accelerates the onset of nutlin-induced apoptosis in AML cells. The OCI-AML3 cells, showing lower sensitivity to nutlin-3a than MOLM-13 cells, were used to determine the optimal conditions for combination studies. In comparison with nutlin-3a (5 and 10 μmol/L) or 1,25D (0.1, 1, and 10 nmol/L) alone, the combination of both agents markedly enhanced apoptotic cell death in OCI-AML3 cells (Fig. 3A). Most prominent was the combination of 10 μmol/L nutlin-3a with all three concentrations of 1,25D (Fig. 3A, groups 3, 6, 9, and 12) when
compared with nutlin-3a or 1,25D alone (Fig. 3A, groups 3, 4, 7, and 10). Western blot analysis revealed that both concentrations of nutlin-3a upregulate p53, whereas 1,25D has no effect on p53 protein levels (Fig. 3B). Most notable was the high level of PARP cleavage in the combination of 10 nmol/L 1,25D and 10 μmol/L nutlin-3a (Fig. 3B). For further studies, OCI-AML3 cells were exposed to 10 nmol/L 1,25D for 48 hours, and 10 μmol/L nutlin-3a was added during the last 2 to 36 hours. The apoptotic response to nutlin-3a alone was minimal at 24 hours, but the addition of 1,25D increased the apoptotic fraction to ~40% (Figs. 3C and 4A). The most prominent morphologic change observed was membrane blebbing, which is associated with cell injury and death (data not shown). Similarly, 1,25D enhanced the apoptotic effect of nutlin-3a in MOLM-13 cells. At 12 hours, 1,25D increased the percentage of apoptotic cells from ~50% to 70% (Fig. 3D) and, at 24 hours, from 76% to 96% (Fig. 4B).

### 1,25D enhances nutlin-induced downregulation of BCL-2 and upregulation of PIG-6 in OCI-AML3 cells.

To examine the underlying mechanisms of the earlier onset of apoptosis in 1,25D and nutlin-treated OCI-AML3 cells, we determined the expression of multiple p53-targeted genes in cells exposed to 1,25D, nutlin-3a, or both. For quantification of differentially expressed genes, we used low-density array PCR cards. These microfluidic cards contained primer/probe sets for 77 genes from the p53 pathway with roles in cell cycle regulation and apoptosis and three endogenous controls (18S rRNA, glyceraldehyde 3-phosphate dehydrogenase, and β-actin). Two independent experiments showed that 3 of 77 genes (survivin, BCL-2, and PIG-6) changed their expression >2-fold in combination-treated cells versus 1,25D or nutlin-3a alone (Supplementary Table S1). The change in the expression of survivin was not studied in depth because it is strictly dependent on cell cycle distribution and changes during cell cycle arrest (28). The other two genes were examined using individual primer/probe sets. Three independent experiments revealed that BCL-2 transcripts, which were downregulated by nutlin-3a alone at 16 hours, were decreased further by 1,25D (Fig. 5A). Statistical analysis confirmed the significant reduction of BCL-2 by 1,25D. 1,25D alone had no effect on the transcriptional expression of BCL-2 in this cell line. Samples were also analyzed for expression of PIG-6 by quantitative reverse transcriptase-PCR. 1,25D alone had no effect on PIG-6 expression, but nutlin-3a treatment produced a time-dependent upregulation of PIG-6. PIG-6 was further enhanced by 1,25D in nutlin-treated OCI-AML3 cells at all indicated time points (Fig. 5B), with a significant enhancement of expression at 16 hours. Further experiments, using a PIG-6 primer/probe sets confirmed this finding. Western blot analysis revealed ~50% reduction in BCL-2 protein in 1,25D- and nutlin-3a–treated samples, but 1,25D or nutlin-3a as single agents had no effect on the protein level of BCL-2 (Fig. 5C; Supplementary Table S2).
Bax protein levels were unchanged in all treated groups. 1,25D alone had no effect on PIG-6 protein level; however, a modest increase of PIG-6 was observed with nutlin-3a alone. The addition of 1,25D increased the protein level ∼2-fold at an early time point of 8 hours, which was not observed at the later time points (Fig. 5C). A possible explanation is that PIG-6 is a transcriptionally and post-transcriptionally regulated gene; therefore, the induction of gene expression presented in Fig. 5B did not correlate with its protein level (Fig. 5C). It is also possible that the commercially available antibody does not detect robust protein changes well as shown in the Supplementary Table S3A.

We further examined the combination of 1,25D and nutlin-3a on their respective signaling pathways (Fig. 5C). Although the protein level of p53 was not increased in the presence of 1,25D, the p53 target MDM2 accumulated in nutlin-treated samples (lanes 3, 5, and 7). The combination of 1,25D with nutlin-3a caused a subtle reduction of MDM2 protein level (lanes 4, 6, and 8). Elevated MDM2 is the likely cause of decrease in MDMX protein through enhanced proteasomal degradation (lanes 3, 5, and 7) as previously shown (29). 1,25D caused further reduction of MDMX protein at all time points (lanes 4, 6, and 8). The mechanism behind the 1,25D-mediated reduction of MDMX will require further studies because there is accumulating evidence of a complex relationship among p53, MDM2, and MDMX proteins. No significant change in the vitamin D receptor protein was observed. There was no change in KSRI protein level, but hKSR2 was reduced by nutlin-3a in a time-dependent manner (lanes 3, 5, and 7). Most notable was the reduction of phosphorylated ERK1/2 by nutlin-3a, which was less reduced in the presence of 1,25D. Calculated optical densities of these key proteins are shown in Supplementary Table S1. The last panel of immunoblots shows marked activation of caspase-3 and PARP cleavage in 1,25D- and nutlin-treated OCI-AML3 cells (lane 8) sensitized to apoptosis by 1,25D at 24 hours.

Effect of BCL-2 and PIG-6 modulation by siRNAs on the apoptotic index of OCI-AML3 cells. We have identified several possible contributors to the accelerated nutlin response: downregulation of survival proteins BCL-2, MDMX, hKSR2, and p-ERK1/2 and enhanced upregulation of proapoptotic PIG-6. Because the role of hKSR2 and p-ERK1/2 in 1,25D-induced differentiation was already described (30), we focused on the effect of modulating the protein levels of BCL-2 and PIG-6 during 1,25D and nutlin-induced apoptosis by RNA interference. It has been reported that p53 transcriptionally represses cell survival genes such as BCL-2, leading to induction of apoptosis (31). We knocked down BCL-2 by nucleofecting cells with 10 nmol/L specific BCL-2 siRNAs and examined its effect on the apoptotic index of OCI-AML3. Cells were exposed to 10 nmol/L 1,25D for 48 hours, and 10 μmol/L nutlin-3a was added during the last 24 hours. Samples were analyzed for BCL-2 mRNA expression and...
the percentage of Annexin V-positive cells. Although ~93% reduction of BCL-2 message level was measured by quantitative real-time PCR, the value is likely inaccurate as cell viability was compromised by the BCL-2 knockdown even in control cells (Fig. 6A, group 5). This was not surprising because these cells rely on BCL-2 family members for survival (based on the high basal protein level as well as published work by Kojima et al.; ref. 32). In a reduced BCL-2 background, nutlin increased apoptotic cell death (Fig. 6A, group 7). However, nutlin and 1,25D combination further increased the apoptotic index in the BCL-2 knockdown cells, suggesting that the reduction of BCL-2 protein is a likely contributor to the enhanced apoptosis. For example, at 24 hours, Annexin V positivity was significantly increased from 60% to 80% (Fig. 6A, groups 4 and 8).

For PIG-6 knockdown, OCI-AML3 cells were transfected with 10 nmol/L nontargeting control and PIG-6 siRNAs by nucleofection. We then investigated the effect of siRNA-mediated knockdown of PIG-6 on the apoptotic index and on possible downstream mediators of PIG-6. OCI-AML3 cells were exposed to 10 nmol/L 1,25D for 48 hours, and 10 μmol/L nutlin-3a was added during the last 16 and 24 hours. PIG-6 siRNA was effective at reducing 1,25D and nutlin-induced expression of PIG-6 by 50% (Fig. 6C and Table S3A). The reduction of PIG-6 significantly decreased the apoptotic index of cells treated with combined agents for 24 hours (Fig. 6B, groups 4 and 8), suggesting that enhanced PIG-6 upregulation by 1,25D contributes to the observed apoptotic effects. The apoptotic cell fraction dropped from 60% in combination to 45% in the presence of the PIG-6 siRNA.

It was reported by Liu et al. (33) that upregulation of PIG-6 reduces p-MEK1/2 and p-ERK1/2 in human colon cancer cells. The representative immunoblot and calculated optical densities (Supplementary Tables S2 and S3B and C) revealed a reduction in the phosphorylated forms of MEK1/2 and ERK2 when PIG-6 is upregulated by 1,25D and nutlin-3a. In addition, siRNA-induced downregulation of PIG-6 was sufficient to restore phosphorylated ERK2 to its basal level, but phosphorylated MEK remained downregulated.

Discussion

Because, in many patients, the AML blasts have wild-type p53 (34), any consideration of the use of vitamin D derivatives for treatment of these patients requires a demonstration that such blasts can respond to these compounds. Thus, it is important, as shown here, that AML cells expressing wild-type p53 can be terminally differentiated by exposure to 1,25D as previously shown for cells with mutated or deleted p53 (14, 15, 35). In the presence of 1,25D, MOLM-13 and OCI-AML3 cells expressed the classic differentiation markers similar to that of HL60 cells (14, 15, 20, 36), indicating that p53 status does not interfere with the ability of 1,25D to activate vitamin D signaling and induce differentiation. Our model cell system used AML cells with expression of normal endogenous levels of p53, which is selectively activated by nutlin-3a. In contrast, Chylicki et al. (37) used a model in which a temperature-inducible form of p53 was transduced into U937 cells. Because p53 is normally expressed at very low levels, their system may not accurately reflect the dynamic changes and functions of cellular p53.

MDM2 antagonists, the nutlins, are a novel class of small molecules that selectively activate the p53 pathway to induce apoptosis and may offer a new treatment modality for patients with AML (9). Here, we have studied the effect of combining the proapoptotic MDM2 antagonist nutlin-3a with differentiating agents 1,25D in AML cells expressing wild-type p53, MOLM-13 and OCI-AML3. Addition of 1,25D to nutlin resulted in accelerated apoptotic response in both cell lines (Figs. 3C and D 4A and B). We have identified possible contributors to this enhanced apoptotic effect: the downregulation of antiapoptotic BCL-2, MDMX, hKSR2, and p-ERK1/2 and the enhanced upregulation of PIG-6 by nutlin and 1,25D combination. BCL-2 is overexpressed in OCI-AML3 cells; siRNA-mediated knockdown of BCL-2 was sufficient to induce apoptosis in the absence of 1,25D or nutlin-3a (Fig. 6A). This led to enhanced apoptosis induced by nutlin alone or nutlin in combination with 1,25D (Fig. 6A).

BCL-2 proteins localize on mitochondria and smooth endoplasmic reticulum and dimerize with BCL-W, BCL-XL, or MCL-1 to form multidomain complexes with proapoptotic BAX and Bak (38). Therefore, therapeutic intervention that could lower the expression of BCL-2 would be beneficial for the patient by enhancing the action of cell death–inducing agents.

In the presence of 1,25D, the protein levels of p53 regulators MDM2 and MDMX dropped significantly (Fig. 5C). MDMX downregulation could also contribute to the overall apoptotic response. Both MDM2 and MDMX are not known as 1,25D targets, but there is emerging evidence that mitogenic signaling pathways regulate cellular localization of p53. Studies by Kojima et al. (39) showed that an MEK inhibitor and nutlin-3a sensitize AML cells to apoptosis because of the transcriptional activation of p53 target genes in the nucleus.

Another downstream target of p53 is proapoptotic PIG-6, also referred to as proline dehydrogenase and proline oxidase-2, which is localized on the inner mitochondrial membrane. Polyak et al. (40) used serial analysis of gene expression to show that PIG-6 is a p53-induced gene. At low levels, the enzyme couples the oxidation of NADPH to mitochondrial electron transport and thus provides cells with ATP. At high levels, PIG-6 activates oxidative apoptosis by generating reactive oxygen species such as superoxides. These oxygen radicals are known to damage cellular components such as DNA, protein, and lipids. Other groups have shown that overexpression of PIG-6 in a variety of cancer cell types induces apoptosis in a p53-dependent manner (41). Induction of PIG-6 has been shown to reduce phosphorylated levels of MEK1/2 and ERK1/2 during p53-induced
oxidative apoptosis in solid tumor cell lines (33, 41). Therefore, reduced levels of phospho-MEK1/2 and ERK2 in our experiments may be a consequence of PIG-6 induction (Fig. 6C) and activation of p53, but this remains to be further investigated.

Our results indicate that various cellular events may contribute to apoptosis in 1,25D- and nutlin-3a–treated OCI-AML3 cells. It is well documented that the MAPK family proteins are activated during 1,25D-induced differentiation of AML cells with mutant or nonfunctional p53 (14, 15, 36). 1,25D also increases survival in HL-60 cells and other p53-null cells by upregulation of p21, p27, p35, p-ERK1/2, and hKSR2 (16, 17, 30, 42, 43). In addition, the MEK/ERK pathway is known to promote growth and to prevent apoptosis in hematologic malignancies such as AML (44). Western blot analysis depicted the reduction of p-MEK and p-ERK by nutlin-3a alone, whereas 1,25D had no effect on the phosphorylated levels of these proteins under the conditions studied here (data not shown). Upon combination with 1,25D, the level of p-ERK1/2 remained reduced when compared with basal level (Figs. 5C and 6C). One can speculate that sustained repression of p-ERK2 in the presence of 1,25D could be due to induced levels of PIG-6. hKSR2 was also further reduced by 1,25D in nutlin-3a–treated cells during apoptosis. Because hKSR2 seems to function as a survival protein, one could expect a p53 activator such as nutlin-3a to inhibit its expression. Wang et al. (30) showed that siRNA knockdown of hKSR2 sensitized HL-60 cells to undergo apoptosis.

Our data suggest that several factors contribute to the sensitization of OCI-AML3 cells to apoptosis when exposed to 1,25D and nutlin-3a. These are BCL-2, MDMX, phosphorylated ERK2, hKSR2, and PIG-6. One possibility is that 1,25D recruits its coactivators, steroid receptor coactivator-1 and vitamin D receptor interacting protein 205, to response elements in the p53 promoter, thereby enabling p53 to be more effective at inducing cell death.

The use of 1,25D for treatment of AML is limited because of hypercalcemia; hence, deltanoids devoid of this undesirable side effect are being developed. Preclinical work on cultured cells and in animals showed that differentiation induced by 1,25D and its analogs can be markedly increased by combination with plant-derived antioxidants such as carnosic acid extracted from *Rosmarinus officinalis* (45, 46). Ongoing studies also indicate synergistic or additive antiproliferative effect of 1,25D and chemotherapeutic agents in various solid tumors (47–49). Our study suggests that vitamin D may enhance the activity of a novel class of antitumor agents, the MDM2 antagonists.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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1,25-Dihydroxyvitamin D₃ Enhances the Apoptotic Activity of MDM2 Antagonist Nutlin-3a in Acute Myeloid Leukemia Cells Expressing Wild-type p53

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