PRIMA-1\textsuperscript{Met}/APR-246 Displays High Antitumor Activity in Multiple Myeloma By Induction of p73 and Noxa

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Abstract

Targeting p53 by the small-molecule PRIMA-1\textsuperscript{Met}/APR-246 has shown promising preclinical activity in various cancer types. However, the mechanism of PRIMA-1\textsuperscript{Met}-induced apoptosis is not completely understood and its effect on multiple myeloma cells is unknown. In this study, we evaluated antitumor effect of PRIMA-1\textsuperscript{Met} alone or its combination with current antimyeloma agents in multiple myeloma cell lines, patient samples, and a mouse xenograft model. Results of our study showed that PRIMA-1\textsuperscript{Met} decreased the viability of multiple myeloma cells irrespective of p53 status, with limited cytotoxicity toward normal hematopoietic cells. Treatment of multiple myeloma cells with PRIMA-1\textsuperscript{Met} resulted in induction of apoptosis, inhibition of colony formation, and migration. PRIMA-1\textsuperscript{Met} restored wild-type conformation of mutant p53 and induced activation of p73 upregulating Noxa and downregulating Mcl-1 without significant modulation of p53 level. siRNA-mediated silencing of p53 showed a little effect on apoptotic response of PRIMA-1\textsuperscript{Met}, whereas knockdown of p73 led to substantial attenuation of apoptotic activity in multiple myeloma cells, indicating that PRIMA-1\textsuperscript{Met}-induced apoptosis is, at least in part, p73–dependent. Importantly, PRIMA-1\textsuperscript{Met} delayed tumor growth and prolonged survival of mice bearing multiple myeloma tumor. Furthermore, combined treatment of PRIMA-1\textsuperscript{Met} with dexamethasone or doxorubicin displayed synergistic effects in both multiple myeloma cell lines and primary multiple myeloma samples. Consistent with our \textit{in vitro} observations, cotreatment with PRIMA-1\textsuperscript{Met} and dexamethasone resulted in enhanced antitumor activity \textit{in vivo}. Our study for the first time shows antmyeloma activity of PRIMA-1\textsuperscript{Met} and provides the rationale for its clinical evaluation in patients with multiple myeloma, including the high-risk group with p53 mutation/deletion. Mol Cancer Ther; 12(11); 2331–41. ©2013 AACC.

Introduction

Multiple myeloma is a neoplasm of terminally differentiated B cells, characterized by aberrant expansion of plasma cells within the bone marrow as well as into extramedullary sites (1). Modern chemotherapy schedules have improved the outcome of patients with multiple myeloma except the p53-mutated/deleted high-risk group (2, 3). At diagnosis, the incidence of p53 gene mutations/deletions in multiple myeloma seems to be low (~10%), however, it increases as the disease progresses (4–6). Importantly, p53 mutations/deletions are considered an adverse risk factor in multiple myeloma as patients with p53 mutation/deletion are resistant to current therapies including stem cell transplantations (4–8). Despite increased response rates in the p53-aberrant patients with clinically approved drugs, more than half display no response to therapy and relapse early, stressing an urgent need for alternative treatment options for this patient subgroup (9–12).

We have previously evaluated two small molecules, nutlin and RITA, which can restore function of wild-type p53 in multiple myeloma (13–16). However, these molecules have been shown to be inactive in multiple myeloma cells with p53 mutation/deletion. PRIMA-1/PRIMA-1\textsuperscript{Met} has been identified as a nongenotoxic agent that can target mutant/deleted p53 (17–20). PRIMA-1, a nongenotoxic candidate drug for mutant p53 reactivation, shows a significant preference for growth inhibition in several human tumor cell lines containing mutant p53, rather than those containing wild-type p53 (17–29). Because of its positive effect on animal models and efficient killing of tumor cells in \textit{ex vivo} experiments, PRIMA-1\textsuperscript{Met} (or APR-246), a more efficient analog of PRIMA-1 (Fig. 1A), is being tested in phase 1/II clinical trials (29).
The mutant p53-dependent antitumor activity of PRIMA-1Met has been shown in vitro and in vivo using human solid tumor xenografts (17, 19–23). In hematologic malignancies, cytotoxic activities of PRIMA-1 have been shown in the samples of patients with chronic lymphocytic leukemia (CLL) and acute myelogenous leukemia (AML). However, none of these studies on PRIMA-1 addressed the underlying mechanisms of its antileukemic effect (18, 24, 25). Interestingly, a recent study showed that PRIMA-1Met could also activate wild-type p53 and trigger a wild-type p53-dependent apoptosis in malignant melanoma cells in three-dimensional culture and in melanoma xenografts in vivo (26). Moreover, PRIMA-1Met has been shown to induce apoptosis in a human prostate cancer and a mouse leukemia cell line lacking p53 expression (27, 28). Induction of apoptosis by PRIMA-1Met in both mutant and wild-type p53 or even in the absence of p53 indicates that multiple signaling pathways related or unrelated to restoration of p53 activity may be involved in this process.

In this study, we investigated in vitro and in vivo antitumor potential of PRIMA-1Met in multiple myeloma and explored the molecular mechanisms associated with this process.

Materials and Methods

Primary human myeloma samples and cell lines

Freshly isolated multiple myeloma samples were collected from newly diagnosed patients. Multiple myeloma cell lines, MM.1S, NCI-H929 (H929), RPMI-8226 (8226), LP1, and U266 used in this study were obtained from American Type Culture Collection. No authentication was done by the authors. 8226R5 cell line that do not express p53 was kindly provided by Dr. R. Buzzeo (H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL) as described previously by us (15, 16). The cell lines were maintained as described previously (13–16). Peripheral blood mononuclear cells (PBMC) and bone marrow mononuclear cells (BMMNC) were obtained from healthy donors.
volunteers and processed as described previously (15). This study was approved by the research ethics committee of University Health Network, Toronto, Canada, in accordance with the declaration of Helsinki.

Drug treatment
PRIMA-1\textsuperscript{Met} was purchased from Cayman Chemical and dissolved in dimethyl sulfoxide (DMSO) to create a 10 mmol/L stock solution and stored at −20°C. In each experiment, the final DMSO concentration was kept constant and did not exceed 0.05% (v/v). In some experiments, cells were simultaneously exposed to PRIMA-1\textsuperscript{Met} and dexamethasone or doxorubicin. The pan-caspase inhibitor, ZVAD-FMK was purchased from Biovision Inc. After drug treatment, cells were harvested and subjected to further analysis as described later.

Cell viability, apoptosis, colony formation, and migration assay
Cell viability in cell lines and patient samples was assessed by MTT assay as previously described (13–16). To examine apoptotic cell death, multiple myeloma cells were treated with various concentrations of PRIMA-1\textsuperscript{Met} and stained for flow cytometry with Annexin V–FITC (Abcam) and propidium iodide (Sigma-Aldrich). Apoptotic cells were analyzed on a BD FACS Canto II (BD Biosciences) using FACSDiva (BD Biosciences) as described by us previously (16). For colony formation assays, multiple myeloma cells (5 × 10\textsuperscript{4} cells/mL) were plated into 6-well plates in 1 mL RPMI medium (20% FBS) containing 1% methylcellulose and maintained with DMSO control or indicated concentration of PRIMA-1\textsuperscript{Met}. Ten days after plating, the total number of colonies was calculated and enumerated by morphologic assessment, as previously described (30, 31). Migration assays were conducted in triplicate with 24-well Transwell insert chambers (8 μm insert; Costar, Corning Inc.) according to the manufacturer’s instruction. In brief, multiple myeloma cells (5 × 10\textsuperscript{5} cells/mL) were added to the upper chamber in the presence or absence of PRIMA-1\textsuperscript{Met} at the indicated concentrations and allowed to migrate for 4 hours at 37°C. The migration of control DMSO-treated cells on the Transwell was normalized to 100% (32).

Western blot analysis and immunoprecipitation
Western blot analysis was conducted as described elsewhere (13–16). Primary antibodies used were those against p53 (DO-7; Sigma-Aldrich); Noxa (Abcam); Mcl-1, caspase-3, and PARP (Cell Signaling Technology); c-Jun, EGR1, and p73 (Signalway Antibody); p53 (pAb1620) and p53 (pAb240; Calbiochem); and α-tubulin (BioLegend). Goat anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase were purchased from Cell Signaling Technology and Santa Cruz Biotechnology, respectively. Immunoprecipitation using anti-p53 antibody was carried out by using PureProteome Protein A Magnetic Beads (Millipore) as described by the manufacturer.

Gene expression analysis by microarray and quantitative PCR
Protocols for microarray analysis and quantitative PCR (qPCR) are described in the supplementary section of the article (Supplementary Data). All of our microarray data are deposited to the gene expression omnibus repositories (accession no. GSE46609) following the minimum information about a microarray gene experiment guidelines.

Genetic knockdown of selective target genes
MM.1S, U226, or 8226R5 cells were transfected with target-specific siRNAs against p53, p73, and Noxa (Sigma), c-Jun (Qiagen) or EGR1 (Dharmacon), and control scrambled siRNA (Sigma) using the Cell Line Nucleofection Solution Kit V (Amaxa, GmbH) according to the manufacturer’s instruction with the Amaxa Nucleofector II device (Amaxa; ref. 16). siRNAs synthesized for wild-type or mutant p53 were previously shown to differentiate between wild-type and mutant p53 (33). Following transfection, cells were treated with PRIMA-1\textsuperscript{Met} and analyzed for inhibition of expression of p53, p73, Noxa, c-Jun, and EGR1 and apoptotic targets including caspase-3 and PARP. The effect of apoptosis induction by PRIMA-1\textsuperscript{Met} following knockdown of p53, p73, Noxa, EGR1, or c-Jun was analyzed for Annexin V–positive cells by flow cytometry.

Multiple myeloma mouse xenograft model
Animal studies were carried out according to the guidelines of the Institutional Review Board. A total of 3 × 10\textsuperscript{7} 8226 cells mixed with Matrigel (BD Biosciences) were subcutaneously injected into the right flank of 6-week-old severe combined immunodeficient mice (SCID) mice. In first experiment, mice were randomized into two groups when tumors are palpable, 5 mice for treatment with 100 mg/kg PRIMA-1\textsuperscript{Met} and 5 mice used as control groups. In second experiment, mice were divided into four groups. Each group contained 5 mice, with 50 mg/kg of PRIMA-1\textsuperscript{Met} or 1.0 mg/kg dexamethasone or with their combination. Control group was treated with PBS. Mice were monitored for body weight and tumor volume by caliper measurements every alternate day. Tumor volumes were calculated with the formula $V = 0.5ab^2$, where $a$ is the long and $b$ is the short diameter of the tumor. Mice were euthanized in the event of tumor size of more than 1.5 cm or major compromise in their quality of life. Tumors were harvested for further analysis and tumor weight was recorded. For Western blot analysis and immunohistochemical analysis, tumors (two from each group, after three injections) were snap frozen or fixed in 10% formalin solution and processed routinely. TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling; Promega) and Ki-67 (Novus Biologicals) staining of the tumor sections were conducted as described by the manufacturer. A group of mice was followed up until the mice in the treated or control groups die or event occurs (tumor exceeds the allowable size).
Survival of mice between the two treatment groups was determined by the Kaplan–Meier test using the log-rank analyses to compare the mean overall mouse survival with 95% confidence intervals.

**Statistical analysis**

The synergistic effect [combination index (CI) <1.0] of the combination of PRIMA-1Met and dexamethasone or doxorubicin was analyzed using CalcuSyn (Biosoft), a software program based on the Chou–Talalay method, as described by us previously (13–16). Statistical significance levels were determined by two-tailed Student t test analysis. P values of more than 0.05 were considered significant.

**Results**

**PRIMA-1Met induces apoptosis in multiple myeloma cells but has minimal cytotoxicity on normal hematopoietic cells**

We first studied in vitro activity of PRIMA-1Met against different multiple myeloma cell lines harboring wild-type (MM.1S, H929), mutant (LP1, U266, and 8266), or null p53 (8266R5). The effect of PRIMA-1Met on viability of multiple myeloma cells was determined by MTT assays. Multiple myeloma cell lines of different p53 status were cultured in the presence of increasing doses of PRIMA-1Met (0–40 μmol/L) for 48 hours. As shown in Fig. 1, PRIMA-1Met treatment resulted in dose-dependent decrease in the viability of multiple myeloma cells. The IC50 of PRIMA-1Met varied among the cell lines tested (Fig. 1B and C). By interphase FISH analysis we detected hemizygous p53 deletions in U266 (~90%) and LP1 (~20%) cells (data not shown). To evaluate the cytotoxic effect of PRIMA-1Met in patient with multiple myeloma cells, we isolated CD138+ tumor cells from 9 newly diagnosed patients with multiple myeloma, three (sample #2, #5, and #9) of the nine samples carried hemizygous p53 deletions (data not shown). PRIMA-1Met treatment resulted in a dose-dependent cytotoxicity of patient-derived multiple myeloma cells (Fig. 1D). However, PRIMA-1Met showed only a limited cytotoxicity in normal PBMCs and BMMNCs obtained from healthy donors (Fig. 1E). These results indicate that PRIMA-1Met preferentially kills malignant myeloma cells. We next examined whether the declining of the viability of multiple myeloma cells upon PRIMA-1Met stimulation was due to apoptosis induction in these cells. Results showed that PRIMA-1Met induced apoptosis in multiple myeloma cells as evidenced by enhanced Annexin V binding in cells treated with PRIMA-1Met. The percentage of apoptotic MM.1S, H929, U226, and 8226R5 cells increased in a dose-dependent manner (Fig. 1F). The sensitivity of wild-type, mutant, or p53-null cells to PRIMA-1Met suggest that it can induce apoptosis irrespective of p53 functional status in multiple myeloma cells.

**PRIMA-1Met inhibits the clonogenic and migratory properties of multiple myeloma cells**

To further explore the antitymoma effects induced by PRIMA-1Met, we carried out a clonogenic assay to study the colony formation activity of multiple myeloma cells treated with PRIMA-1Met. We found a 30% and 33% reduced MM.1S and 8226 colony formation, respectively (Fig. 2A). As migration plays an important role in the progression of multiple myeloma, the effect of PRIMA-1Met on cell migration was then examined using Transwell inserts system. As shown in Fig. 2B, treatment of either

![Figure 2. PRIMA-1Met inhibits colony formation and cell migration of multiple myeloma cells. A, colony formation assay using multiple myeloma cells. RPMI-8226 and MM.1S cells (5 × 10^4 cells/mL) were seeded into 12-well plate in methylcellulose-based medium. After 10 days, colony formation capacity was evaluated by counting colonies including more than 50 cells. B, MM.1S and 8226 cells treated with DMSO control or PRIMA-1Met were grown in 24-well Transwell migration plates and the migratory potential of the cells were determined as described in Materials and Methods. Data are expressed as mean ± SD of triplicate cultures. * P < 0.05; ** P < 0.01.](image-url)
Western blot analysis revealed that PRIMA-1 Met triggered multiple myeloma cells using immunoblotting analysis. Apoptosis, we investigated the effect of PRIMA-1 Met on p53 status. Indications that PRIMA-1 Met inhibits clonogenic and migratory ability of multiple myeloma cells irrespective of p53 status.

**PRIMA-1 Met treatment results in caspase-dependent apoptosis of multiple myeloma cells**

To understand the mechanism of PRIMA-1 Met–induced apoptosis, we investigated the effect of PRIMA-1 Met on multiple myeloma cells using immunoblotting analysis. Western blot analysis revealed that PRIMA-1 Met triggered time- (Fig. 3A) and dose-dependent (Fig. 3B) activation of p73 and HSP70 followed by caspase-3 and PARP cleavage in MM.1S, U266, and 8226R5 cells. Moreover, activation of caspase was associated with upregulation of a proapoptotic marker, Noxa and downregulation of an antiapoptotic marker, Mcl-1 in a time- and dose-dependent manner (Fig. 3A and B). Furthermore, to determine PRIMA-1 Met–induced cell death is dependent on caspase activity, we treated U266 and MM.1S cells with the pan-caspase inhibitor ZVAD-FMK (25 μmol/L) for 2 hours before treatment with PRIMA-1 Met for additional 12 or 48 hours to analyze by Western blot analysis and MTT assay, respectively. As shown in Fig. 3C and D, PRIMA-1 Met–induced apoptosis was inhibited by ZVAD-FMK pretreatment, evidenced by inhibition of cleavage of caspase-3 and PARP induced by PRIMA-1 Met in U266 cells (Fig. 3C) and confirmed by increase in survival by MTT assay in MM.1S and U266 cells (Fig. 3D; P < 0.05). These results suggest that apoptosis induced by PRIMA-1 Met in multiple myeloma cells is caspase-dependent.

**Apoptosis induction of multiple myeloma cells by PRIMA-1 Met is not totally dependent on p53 activation**

Having shown that PRIMA-1 Met induces apoptosis in multiple myeloma cells harboring either wild-type or mutant p53 or even in p53 null cells, we further examined whether selective knockdown of p53 expression by siRNA has any effect on the induction of apoptosis in multiple myeloma cells. MM.1S and U266 cell lines were transfected with small interfering RNA targeting p53 and were treated with PRIMA-1 Met, and apoptosis was evaluated by Western blot analysis and MTT assay, respectively. As shown in Fig. 3C and D, PRIMA-1 Met–induced apoptosis was inhibited by ZVAD-FMK pretreatment, evidenced by inhibition of cleavage of caspase-3 and PARP induced by PRIMA-1 Met in U266 cells (Fig. 3C) and confirmed by increase in survival by MTT assay in MM.1S and U266 cells (Fig. 3D; P < 0.05). These results suggest that apoptosis induced by PRIMA-1 Met in multiple myeloma cells is caspase-dependent.

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**Figure 3.** PRIMA-1 Met induces time- and dose-dependent apoptosis in multiple myeloma cells. PRIMA-1 Met treatment results in upregulation of p73, HSP70, and Noxa and downregulation of Mcl-1 in a time- and dose-dependent manner with associated activation of caspase-3 and/or PARP. A, total levels of the indicated proteins were evaluated by Western blot analysis in the indicated cell lines after treatment with 25 μmol/L MM.1S or 50 μmol/L U266 and 8226R5 cells for up to 12 hours. B, multiple myeloma cells (MM.1S, U266, and 8226R5) were treated with indicated concentrations of PRIMA-1 Met for 12 hours. Total cell lysates were prepared and analyzed by Western blot analysis for the expression of the indicated protein. Cl, cleaved. C, U266 cells were treated with the pan-caspase inhibitor ZVAD-FMK (20 μmol/L) before treatment with 50 μmol/L PRIMA-1 Met for additional 12 hours. Cells were lysed and analyzed by Western blot analysis for the expression of caspase-3 and PARP. D, MM.1S and U266 cells were treated with the pan-caspase inhibitor ZVAD-FMK (20 μmol/L) before treatment with PRIMA-1 Met for additional 48 hours and analyzed for cell survival by MTT assay (P < 0.05).
with siRNAs specific for wild-type or mutant p53, respectively, treated with PRIMA-1\textsuperscript{Met} (10 and 20 \textmu mol/L, respectively), and examined for its apoptotic activity by Western blot and flow cytometry analysis. The results showed that knockdown of p53 in either MM.1S or U266 cell lines had little effect on the cleavage of PARP induced by PRIMA-1\textsuperscript{Met} (Fig. 4A) as well as on the binding of Annexin V (Fig. 4B), indicating that PRIMA-1\textsuperscript{Met}-induced apoptosis in multiple myeloma cells is not totally dependent on p53.

**Effective knockdown of p73 and Noxa attenuates the apoptotic activity of PRIMA-1\textsuperscript{Met} in multiple myeloma cells**

Because treatment of multiple myeloma cells with PRIMA-1\textsuperscript{Met} resulted in increased p73 protein levels in multiple myeloma cells carrying wild-type, mutant, or null p53 we next sought to determine whether PRIMA-1\textsuperscript{Met}-induced apoptosis is specifically dependent on activation of p73. To this aim, p73 expression was silenced by siRNA. p73 knockdown using siRNA specific to p73 resulted in more than 90\% inhibition of p73 expression, as measured by densitometry. Having shown upregulation of the proapoptotic protein Noxa in multiple myeloma cells irrespective of p53 status, we also silenced the expression of Noxa. Knockdown of p73 diminished expression of Noxa, however, silencing Noxa did not significantly modulate the expression of p73, indicating that Noxa is the downstream target of p73 (Fig. 4C). Functionally, knockdown of either p73 or Noxa resulted in attenuation of PRIMA-1\textsuperscript{Met}-induced apoptosis as shown by decrease in percentage of Annexin V-positive cells (Fig. 4D). Taken together, our data suggest that induction of p73 is required for PRIMA-1\textsuperscript{Met}-mediated apoptosis in multiple myeloma cells.

**PRIMA-1\textsuperscript{Met} treatment retards growth of multiple myeloma tumors harboring mutant p53 in vivo**

Next, we sought to establish whether our in vitro observations would translate to antimyeloma activity in vivo by studying the antitumorogenic potential of PRIMA-1\textsuperscript{Met} in multiple myeloma xenograft SCID mouse models. Of note, 8266 cells, which are capable of forming tumors, were inoculated into SCID mice and...
the animals received intraperitoneal injections of either 100 μL PBS (control) or 100 mg/kg PRIMA-1<sup>Met</sup> once daily for 15 days after tumor formation was evident. Administration of PRIMA-1<sup>Met</sup> resulted in significant inhibition of multiple myeloma tumor growth (Fig. 5A; \( P < 0.05 \)) and increased the survival (Fig. 5B; \( P = 0.007 \)). There were no obvious toxic effects of the treatments as evaluated by mouse body weight data (Fig. 5C).

To assess in vivo activation of p73 signaling, we examined whether PRIMA-1<sup>Met</sup> could induce apoptosis through activation of p73 in human tumor xenografts. Treatment of 8226 tumor xenografts with PRIMA-1<sup>Met</sup> resulted in induction of p73 and cleavage of caspase-3 (Fig. 5D) in accordance with our in vitro results (Fig. 3). Given the therapeutic efficacy of PRIMA-1<sup>Met</sup> in multiple myeloma mouse xenograft model, we next examined the associated histologic events. As seen in Fig. 5E, treatment of PRIMA-1<sup>Met</sup> resulted in decrease of the Ki-67–positive and increase of TUNEL–positive cells in mice tumor samples. These findings therefore support our in vitro data showing induction of apoptosis in multiple myeloma cells by PRIMA-1<sup>Met</sup>.

**PRIMA-1<sup>Met</sup> has synergistic or additive antimyeloma activity when combined with current therapeutics**

Tumors with alterations of p53 or its family usually do not respond adequately to genotoxic chemotherapeutic agents including glucocorticoids (e.g., dexamethasone) or topoisomerase inhibitors (e.g., doxorubicin) that act through the p53 pathway. We, therefore, examined whether combination of PRIMA-1<sup>Met</sup> with dexamethasone or doxorubicin can enhance their cytotoxic response in multiple myeloma cells. MM.1S cells were treated with 5 μmol/L PRIMA-1<sup>Met</sup>, dexamethasone (0.25 μmol/L), or doxorubicin (0.25 μmol/L) alone or in combination of these doses of PRIMA-1<sup>Met</sup> plus dexamethasone or PRIMA-1<sup>Met</sup> plus doxorubicin. The cytotoxicity of the cells was assayed by MTT at 48 hours. A significant decrease in cell viability was observed in response to treatment with combined low doses of PRIMA-1<sup>Met</sup> and dexamethasone or PRIMA-1<sup>Met</sup> and doxorubicin than with either agent alone (\( P < 0.05 \)) in MM.1S cell line (Fig. 6A). Synergism was noted at these combinations of the drugs tested, evidenced by CI of less than 1. Combination of PRIMA-1<sup>Met</sup> and dexamethasone in two primary

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**Figure 5.** PRIMA-1<sup>Met</sup> inhibits the growth of multiple myeloma tumors with mutant p53 in vivo. SCID mice (\( n = 5/\text{group} \)) were injected subcutaneously with 8226 cells (\( 3 \times 10^7 \)) in RPMI medium along with Matrigel matrix. Following detection of tumor, mice were divided into two groups: the control group that received daily intraperitoneal injection of vehicle (PBS) and the treatment group that received daily intraperitoneal injection of PRIMA-1<sup>Met</sup> (100 mg/kg). A, tumors were measured in two perpendicular dimensions every alternate day and tumor volumes were calculated (\( \cdot P < 0.05 \)). B, survival was evaluated from the first day of treatment until death or an event occurred by using the Kaplan–Meier curve (\( P = 0.007 \)). C, body weight was measured every alternate day till day 15. D, tumors were isolated from PRIMA-1<sup>Met</sup>-treated and control mice and harvested for analysis by Western blot analysis for the expression of p73 and caspase-3. E, immunohistochemical analysis of tumor sections showed that treatment of mice bearing multiple myeloma tumors with PRIMA-1<sup>Met</sup> resulted in decrease in the proliferation index (Ki-67) and increase in the apoptotic index (TUNEL), further verifying the ability of PRIMA-1<sup>Met</sup> to induce apoptosis in multiple myeloma cells in vivo.
multiple myeloma specimens with hemizygous p53 deletions displayed at least additive cytotoxic response (Fig. 6B).

Finally, to test whether enhanced myeloma cell apoptotic response in combination with PRIMA-1<sup>Met</sup> and dexamethasone could be extended in vivo, we used similar subcutaneous 8226 xenograft model of human multiple myeloma in mice as described above. As shown in Fig. 6C, PRIMA-1<sup>Met</sup> or dexamethasone alone showed moderate inhibition of tumor growth. In contrast combination of PRIMA-1<sup>Met</sup> and dexamethasone displayed significant growth inhibition versus control (++, P < 0.01) and versus low-dose PRIMA-1<sup>Met</sup>/dexamethasone alone (on day 15; ++, P < 0.05). Importantly, overall survival of PRIMA-1<sup>Met</sup> and dexamethasone-treated mice was significantly prolonged as compared with mice treated with dexamethasone (P = 0.043) or PRIMA-1<sup>Met</sup> (P = 0.010) alone or control (P = 0.006; Fig. 6D). In addition, treatment with either PRIMA-1<sup>Met</sup> alone or combination with dexamethasone did not affect body weight (Fig. 6E), indicating the doses used for the treatment were tolerable to the mice.

Discussion

In this report, we show that PRIMA-1<sup>Met</sup> exerts a powerful antitumor activity in multiple myeloma cell lines and malignant plasma cells derived from patients as well as in a clinically relevant xenograft model of human multiple myeloma. In <i>vivo</i> results were complemented by <i>in vitro</i> experiments where PRIMA-1<sup>Met</sup> showed significant antiproliferative activity, apoptotic effects, and modulation of gene expression. To our knowledge, this is the first experimental evidence of antitumor activity of PRIMA-1<sup>Met</sup> in preclinical models of multiple myeloma.

Treatment of multiple myeloma cells with PRIMA-1<sup>Met</sup> resulted in significant inhibition of viability, colony

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Figure 6. PRIMA-1<sup>Met</sup> in combination with dexamethasone or doxorubicin display synergistic cytotoxic response in a multiple myeloma cell line and two primary multiple myeloma samples. A, MM.1S cells were treated with PRIMA-1<sup>Met</sup> (5 μmol/L) in combination with dexamethasone (0.25 μmol/L) or doxorubicin (0.25 μmol/L). Forty-eight hours after treatment, cells were assessed for survival by MTT viability assay (*, P < 0.05). Close bar (■), PRIMA-1<sup>Met</sup>; open bar (□), dexamethasone or doxorubicin; and open hatched bar (△), combination of PRIMA-1<sup>Met</sup> plus dexamethasone or doxorubicin. B, primary multiple myeloma samples from 2 newly diagnosed patients carrying hemizygous p53 deletion were treated with PRIMA-1<sup>Met</sup> (5 μmol/L) alone or combination of PRIMA-1<sup>Met</sup> plus dexamethasone or doxorubicin. Primary multiple myeloma samples were inoculated subcutaneously with 3 × 10<sup>4</sup> RPMI medium together with Matrigel matrix. Twenty tumor-bearing mice were randomly assigned to four groups and treated for 15 days with control (PBS), PRIMA-1<sup>Met</sup> (50 mg/kg), dexamethasone (1 mg/kg), or the combination once daily. At day 15 the treatment was discontinued and mice were monitored for tumor dynamics and body weight. C, PRIMA-1<sup>Met</sup> and dexamethasone combination therapy markedly prolonged survival compared with mice treated with PRIMA-1<sup>Met</sup> or dexamethasone alone (++, P < 0.05) or control (*, P < 0.01). D, combination therapy markedly prolonged survival compared with mice treated with control (P = 0.006), PRIMA-1<sup>Met</sup> (P = 0.010), or dexamethasone (P < 0.043) alone. E, mice body weight was used to assess toxicity of the treatment. C, control; P, PRIMA-1<sup>Met</sup>; D, dexamethasone; P + D, PRIMA-1<sup>Met</sup> + dexamethasone.
formation and induction of apoptosis. As migration is necessary for the homing of tumor cells to the bone marrow, for expansion within the bone marrow microenvironment, and for egress into the peripheral blood we also examined whether PRIMA-1Met can inhibit the migration of multiple myeloma cells. As expected, PRIMA-1Met significantly inhibits the migratory capacity of multiple myeloma cells. Interestingly, although PRIMA-1Met was originally described as a mutant p53–reactivating drug, antimyeloma activity of PRIMA-1Met was not limited to the cells harboring mutant p53. In fact, PRIMA-1Met displayed its cytotoxic and apoptotic response regardless of p53 status. Despite its relatively high concentrations required for optimum cytotoxic response in multiple myeloma cells, the similar concentrations of PRIMA-1Met has been used to induce apoptosis in other tumor cell types (17–28). Moreover, in agreement with our findings, a number of previous studies provided the evidence that PRIMA-1Met showed its cytotoxic effect in melanoma, prostate, AML, and mouse leukemia cells irrespective of p53 mutational status (26–28). Importantly, cytotoxic response of PRIMA-1Met was observed in three multiple myeloma primary samples with hemizygous p53 deletion. Similar to the previous observations in B-CLL (18), there were no obvious differences in cytotoxicity observed in multiple myeloma samples with or without p53 deletion. In contrast, PRIMA-1 was more cytotoxic in the subgroup of patient’s AML samples with hemizygous p53 deletion (24). As the function of p53 correlates with its wild-type conformation, we tested whether PRIMA-1Met can change a mutant p53 to a wild-type conformation in multiple myeloma, specifically recognized by the antibody pAb1620. The epitope for pAb1620 is near the epitope of the mutant conformation antibody pAb240 (reactive to conformation-sensitive pAb240 antibody for mutant conformation; refs. 34, 35). Immunoprecipitation studies showed an increase in p53 level detected with pAb1620 and decrease in p53 level detected with pAb240 in U266 cells treated with 50 μmol/L PRIMA-1Met. However, only wild-type (pAb1620) but not mutant (pAb240) p53 was detected in MM.1S cells harboring endogenous wild-type p53 (Supplementary Fig. S1). Moreover, our results showed that selective knockdown of either wild-type or mutant p53 resulted in significant inhibition of apoptosis in multiple myeloma, specifically recognized by the antibody pAb1620. The epitope for pAb1620 is near the epitope of the mutant conformation antibody pAb240 (reactive to conformation-sensitive pAb240 antibody for mutant conformation; refs. 34, 35). Immunoprecipitation studies showed an increase in p53 level detected with pAb1620 and decrease in p53 level detected with pAb240 in U266 cells treated with 50 μmol/L PRIMA-1Met. However, only wild-type (pAb1620) but not mutant (pAb240) p53 was detected in MM.1S cells harboring endogenous wild-type p53 (Supplementary Fig. S1). Moreover, our results showed that selective knockdown of either wild-type or mutant p53 had a little effect on the apoptosis induction by PRIMA-1Met. Although we cannot exclude the role of p53 in PRIMA-1Met–induced apoptosis our results suggest that functional restoration of p53 may not have a direct role in PRIMA-1Met–induced apoptosis of multiple myeloma cells.

To identify the potential targets of PRIMA-1Met, we conducted gene expression profiling (GEP) by microarray in three different cell lines–harboring wild-type, mutant, or null p53 and analyzed differential expression of target genes between PRIMA-1Met–treated and untreated samples (Supplementary Table S1–S3). GEP identified a number of genes up- or downregulated in these cell lines including c-Jun and EGR1 (Supplementary Fig. S2A and S2B). These data led us to hypothesize that PRIMA-1Met–induced apoptosis in multiple myeloma cells is mediated by c-Jun/EGR1 signaling. However, genetic inhibition of c-Jun/EGR1 activation did not significantly modulate PRIMA-1Met–induced apoptosis (Supplementary Fig. S2A and S2B). Thus, it is unlikely that c-Jun/EGR1 signaling plays a major role in PRIMA-1Met–induced apoptosis.

Interestingly, we found that PRIMA-1Met treatment resulted in activation of p73, a p53-related protein, in multiple myeloma cells retaining or not functional p53 status. In this regard, we addressed whether the depletion of endogenous p73 can abolish the apoptotic effects of PRIMA-1Met. Our results showed that efficient knockdown of p73 by siRNA attenuated the apoptotic effect of PRIMA-1Met. Similarly, a previous study showed involvement of p73 in PRIMA-1Met–mediated mutant p53-dependent apoptosis in small-cell lung cancer (23). However, unlike the study in lung cancer (23), the association of p73 is not limited to mutant p53 in our study. Our data showed that silencing the expression of p73 resulted in significant inhibition of apoptosis in multiple myeloma cells with wild-type, mutant, or null p53 providing the evidence for an important role of p73 in PRIMA-1Met–mediated apoptosis of multiple myeloma cells irrespective of p53 status. Our data also indicate that like several other drug candidates such as nutlin, NSC176327, and RETRA, p73 is an important target of PRIMA-1Met. Interestingly, we found that PRIMA-1Met treatment induced an increase of Noxa together with a decrease in Mcl-1 in multiple myeloma cells irrespective of p53 status. We have also shown the role of Noxa as a downstream target of p73 in PRIMA-1Met–induced apoptosis of multiple myeloma cells. A balance between proapoptotic and antiapoptotic protein may control the PRIMA-1Met–mediated apoptotic pathways.

Furthermore, our results identified transcriptional changes of several cancer-related genes and signaling pathways including c-Myc (Supplementary Fig. S3 and Supplementary Table S1–S3). The Myc oncogene is one of the important regulators of cell growth and protein...
synthesis and Myc overexpression has been proposed to be associated with progression of multiple myeloma (1, 2). Recent reports have shown that c-Myc–dependent apoptosis of multiple myeloma cells by small-molecule inhibitors such as pazopanib (41) and 10058-F4 (42) or a growth factor, bone morphogenetic proteins (43). Therefore, in addition to activation of p73 and/or Noxa, downregulation of c-Myc may play a role in PRIMA-1Met–induced apoptosis of multiple myeloma cells. Further studies will be required to understand the significance of PRIMA-1Met–induced downregulation of c-Myc in the apoptosis of multiple myeloma cells.

To study the clinical relevance of PRIMA-1Met in vivo, mice bearing multiple myeloma tumors were treated with PRIMA-1Met or PRIMA-1Met in combination with dexamethasone. In both in vivo studies, PRIMA-1Met induced a significant tumor growth delay and prolonged survival compared with control mice. In keeping with our in vitro observation, the apoptotic effect of PRIMA-1Met was related to p73 activation in mouse xenograft model. From a therapeutic perspective, it is interesting to note that the doses of PRIMA-1Met that induces apoptosis in multiple myeloma cells in vitro are achievable in vivo, as previously shown by their potent antitumor effect in mouse models of human cancer (17, 19, 23). PRIMA-1Met has recently been administered safely to patients with AML and prostate cancer (29). Furthermore, our in vitro and in vivo studies have shown that apoptosis induced by PRIMA-1Met can synergistically be enhanced by combining it with dexamethasone. This suggest that it could be possible to lower the doses of PRIMA-1Met as well as of the chemotherapeutic drugs used in the treatment of multiple myeloma and thus reduce cytotoxicity to normal cells. Importantly, our findings warrant the clinical evaluation of PRIMA-1Met in a broader patient group, as antimyeloma activity of PRIMA-1Met is not restricted to only mutant p53.

On the basis of the data presented here we conclude that treatment of multiple myeloma cells with PRIMA-1Met lead to induction of p73-mediated apoptosis by upregulating Noxa and downregulating Mcl-1 irrespective of p53 status. Our study provides the preclinical framework to initiate clinical trial of PRIMA-1Met either alone or in combination with a conventional antimultiple myeloma agent, dexamethasone to increase response, overcome drug resistance, reduce side effects, and improve patient outcome in multiple myeloma including the high-risk group with p53 mutation/deletion.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: M.N. Saha, H. Chang
Development of methodology: M.N. Saha, H. Jiang, Y. Yang, H. Chang
 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Yang, D. Reece, H. Chang
 Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.N. Saha, Y. Yang, H. Chang
 Writing, review, and/or revision of the manuscript: M.N. Saha, D. Reece, H. Chang
 Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Jiang
 Study supervision: H. Chang

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References
Antimyeloma Activity of PRIMA-1^{MET}


# Molecular Cancer Therapeutics

**PRIMA-1\textsuperscript{Met}/APR-246 Displays High Antitumor Activity in Multiple Myeloma By Induction of p73 and Noxa**

Manujendra N. Saha, Hua Jiang, Yijun Yang, et al.


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