Cancer Therapy: Preclinical

PRIMA-1^Met/APR-246 Induces Apoptosis and Tumor Growth Delay in Small Cell Lung Cancer Expressing Mutant p53

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Abstract

Purpose: Small cell lung cancer (SCLC) is a highly malignant disease with poor prognosis, necessitating the need to develop new and efficient treatment modalities. PRIMA-1^Met (p53-dependent reactivation of massive apoptosis), also known as APR-246, is a small molecule, which restores tumor suppressor function to mutant p53 and induces cancer cell death in various cancer types. Since p53 is mutated in more than 90% of SCLC, we investigated the ability of PRIMA-1^Met to induce apoptosis and inhibit tumor growth in SCLC with different p53 mutations.

Experimental Design: The therapeutic effect of PRIMA-1^Met/APR-246 was studied in SCLC cells in vitro using cell viability assay, fluorescence-activated cell-sorting analysis, p53 knockdown studies, and Western blot analyses. The antitumor potential of PRIMA-1^Met/APR-246 was further evaluated in two different SCLC xenograft models.

Results: PRIMA-1^Met/APR-246 efficiently inhibited the growth of the SCLC cell lines expressing mutant p53 in vitro and induced apoptosis, associated with increased fraction of cells with fragmented DNA, caspase-3 activation, PARP cleavage, Bax and Noxa upregulation and Bcl-2 downregulation in the cells. The growth suppressive effect of PRIMA-1^Met/APR-246 was markedly reduced in SCLC cell lines transfected with p53 siRNA, supporting the role of mutant p53 in PRIMA-1^Met/APR-246-induced cell death. Moreover, in vivo studies showed significant antitumor effects of PRIMA-1^Met after i.v. injection in SCLC mouse models with no apparent toxicity.

Conclusion: This study is the first to show the potential use of p53-reactivating molecules such as PRIMA-1^Met/APR-246 for the treatment of SCLC.

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Introduction

Lung cancer is one of the most frequent causes of cancer deaths in the world. Small cell lung cancer (SCLC) accounts for 15% to 20% of all lung cancers diagnosed, and is the most aggressive of all lung cancer subtypes. SCLC is characterized by early dissemination, fast growth, and in most cases development of therapy resistance. Current treatments are radiotherapy and chemotherapy, but despite high rate of initial response, relapse is seen in almost all patients (1–3).

Radiotherapy and most chemotherapeutic agents used in SCLC treatment directly cause DNA damage within cancer cells and they are thought to eliminate tumor cells through activation of p53 and its signaling pathways (3, 4). When activated, p53 specifically binds to DNA and induces transactivation or transrepression of a number of target genes involved in cell cycle arrest and/or apoptosis (5). Apoptosis can also be induced by p53 in a transcription-independent way by interacting with Bcl-2 family members (6). Once activated, p53 induces upregulation of its negative regulator MDM2, thus forming a negative feedback loop that maintains low levels of p53 in the cells in the absence of cellular stress. This negative feedback loop is however, abrogated in cancer cells with p53 mutations (5). The majority of the mutations found in the p53 gene are missense mutations that cluster in the specific DNA binding core domain, disrupting specific DNA binding and transactivation of target genes (7). Several studies indicate tumors carrying mutant p53 are associated with therapy resistance and poor diagnosis compared with wild type (wt) p53-carrying tumors (8–10).

Mutational alterations in the p53 gene occur in more than 90% of all SCLCs (1). In SCLC cells lacking p53 protein, ectopic expression of p53 induces cancer cell death (11), suggesting p53 gene replacement by gene therapy...
could constitute an effective strategy for SCLC. However, the efficacy of p53 gene therapy depends on the p53 status in the cells. In clinical trials, p53 gene therapy has been shown to be effective only in patients expressing wt p53, but not in patients with high levels of mutant p53, likely due to dominant negative effects of the mutant protein (12). Since mutant p53 tends to accumulate in SCLC, p53 gene therapy is likely to have limited success in this cancer type. Therefore, to restore p53 function in SCLC cells, in which mutant p53 has been accumulated, a more efficient strategy is needed.

PRIMA-1 (p53-dependent reactivation of massive apoptosis) and its methylated and more potent form, PRIMA-1\textsuperscript{Met}/APR-246, which we henceforth will refer to only as PRIMA-1\textsuperscript{Met}, are low molecular weight compounds that selectively inhibit the growth of a number of cancer cells expressing mutant p53 (13–15). They are capable of restoring the sequence-specific DNA-binding and transcriptional transactivation by mutant p53 in tumor cells and inhibiting the growth of human tumor xenografts in mice (13, 15, 16). PRIMA-1\textsuperscript{Met} is currently being tested in a clinical phase I/II trial (17). However, the mechanisms by which PRIMA-1 and PRIMA-1\textsuperscript{Met} restore the transcriptional transactivation function to mutant p53 and induce cancer cell death are not completely clear. PRIMA-1 has been suggested to induce cell death in a mutant p53–dependent manner by either (a) directly binding to the mutant protein leading to protein refolding or (b) inducing mutant p53 refolding via chaperones, or (c) mediating the release of p73 and p63 from mutant p53, which then results in p73- or p63-induced growth suppression (18).

The cytotoxic effect of PRIMA-1 and PRIMA-1\textsuperscript{Met} in various cancer types prompted us to study the effect of small p53 reactivating molecules in SCLC cells. We reasoned, due to the high prevalence of p53 mutations in SCLC cells and lack of effective treatment modalities for this cancer, reactivation of mutant p53 with PRIMA-1 or PRIMA-1\textsuperscript{Met} might provide a novel and specific approach for the treatment of SCLC patients.

In this study, we show that PRIMA-1\textsuperscript{Met} can induce apoptosis in SCLC cell lines with p53 mutation in a mutant p53–dependent manner. We also show a significant tumor growth delay in human SCLC mouse models after PRIMA-1\textsuperscript{Met} treatment without any apparent toxicity. These results suggest that PRIMA-1\textsuperscript{Met} has antitumor effects in SCLC, and could thus serve as prototype for the development of new p53-targeting agents in future treatment of SCLC patients.

**Materials and Methods**

**Cell lines**

The origin and propagation of the SCLC cell lines used in this study has been previously described in detail (19). The human non-immortalized lung fibroblast cell line, CCD32Lu, and the breast carcinoma cell line, MDA-MB-231, were obtained from American Type Culture Collection (ATCC) and the non–small cell lung carcinoma cell line, H1299, was provided by Dr. R.J. Christiano (Houston, TX). Propagation of CCD32Lu, MDA-MB-231, and H1299 cell lines is described elsewhere (20, 21). The cell lines were grown in monolayer, suspension or semiadherent/suspension cultures (Table 1). All cell lines were tested for mycoplasma upon arrival in the laboratory. Authentication of the SCLC cell lines was performed years ago prior to arrival in the laboratory (22–27). The p53 mutation status in the above-mentioned SCLC lines is listed in Table 1 and has previously been evaluated by us (28) and others (29–31). p53 mutation status in CCD32Lu, MDA-MB-231, and H1299 cell lines has also been previously reported (32–34) and is included in Table 1.

**Reagent**

PRIMA-1\textsuperscript{Met} [APR-246; 2-hydroxy-methyl-2-methoxy-methyl-aza-bicyclo[2.2.2]octan-3-one] was kindly provided by Aprea AB. Stocks (100 mmol/L) were prepared in dimethyl sulfoxide (DMSO) and stored at −20°C. Dilutions were made in PBS.

**Semiquantitative RT-PCR**

Total cell RNA was isolated from cell lines using the RNAeasy kit according to the manufacturer (Qiagen). cDNA was synthesized with Superscript RT II Reverse Transcriptase (Invitrogen) and amplified using Platinum TaqPolymerase (Invitrogen) with 25 cycles of amplification. The primers used were purchased from DNA Technology.

**Primers**

p53 (PCR product from 616 to 1,164; annealing temperature 57°C); sense, 5'-GGAGGACCCGATCATGATG-3'; antisense, TCTGACCAGGCTAGTGGT. Actin (PCR product from 768 to 1,124; annealing temperature 57°C); sense, 5'-GGAAGGGCGGATGCGATG-3'; antisense, TCTGACCAGGCTAGTGGT. Actin
product from 101 to 619, annealing temperature 57°C): sense, 5'-GTCGACACGGCTCGGAGATTG; anti-sense, 5'-GCCAGCGGTCCAGACGGAGATG.

**MTT assay**

All SCLC cells, except DMS273, were seeded in 96-well plates at a density of 3 × 10^4 cells/well to achieve exponential growth. DMS273, H1299, and CCD32Lu cells were seeded at a density of 1 × 10^4 cells/well. The cells were incubated overnight and treated with 0, 25, 50, and 100 μmol/L PRIMA-1Met for 72 hours before addition of MTT (Sigma-Aldrich). Solubilization buffer (10% SDS; 0.01 mol/L HCl) was added after 4 hours and absorbance was measured the day after using a microplate reader.

**Flow cytometry**

All SCLC cells, except DMS273, were seeded in 6-well plates at a density of 8 × 10^5 cells/well. DMS273, H1299, and CCD32Lu cells were seeded at a density of 2.7 × 10^5 cells/well. The following day, cells were treated with 0, 50, or 100 μmol/L PRIMA-1^Met for 72 hours after which they were harvested. The cells were fixed in 70% ethanol, centrifuged, and resuspended in a propidium iodide solution (50 μg/mL propidium iodide, 10 mmol/L Tris, 5 mmol/L MgCl2, and 10 μg/mL ribonuclease A and 1 μL/mL of NP-40; propidium iodide and ribonuclease A were from Sigma-Aldrich). Samples were analyzed using flow cytometry (FACScan; BD Biosciences).

**siRNA transfection studies**

DMS273 and DMS53 cells were seeded in 6-well plates 1 day prior to transfection at a density of 3 × 10^5 and 5 × 10^5 cells/well, respectively. The suspension cell line, GLC16, was seeded in 6-well plates on the day of transfection at a density of 7 × 10^5 cells/well. The cells were transfected with 50 nmol/L p53 siRNA or scrambled siRNA

### Table 1. p53 mutation status in SCLC and non-SCLC cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Morphology</th>
<th>Mutation</th>
<th>Amino acid change</th>
<th>Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMS456</td>
<td>A/S</td>
<td>G469T</td>
<td>V157F</td>
<td>C</td>
</tr>
<tr>
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<td>A/S</td>
<td>A536T</td>
<td>H179L</td>
<td>C</td>
</tr>
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<td>A</td>
<td>G733T</td>
<td>G245C</td>
<td>C</td>
</tr>
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<td>A/S</td>
<td>A463C</td>
<td>T155P</td>
<td>C</td>
</tr>
<tr>
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<td>A</td>
<td>C637T</td>
<td>R213UGA (STOP)</td>
<td>C-E</td>
</tr>
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<td>M237I</td>
<td>C</td>
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<td>G279GFrameshift</td>
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<tr>
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<td>S241F</td>
<td>C</td>
</tr>
<tr>
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<td>G511T</td>
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<td>C-E</td>
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<td>delta 96–114</td>
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<td>C</td>
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<tr>
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<td>G159A</td>
<td>W53UGA (STOP)</td>
<td>A-E</td>
</tr>
<tr>
<td>GLC2</td>
<td>A/S</td>
<td>Delta 780–803</td>
<td>Delta 261–268</td>
<td>C</td>
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</table>

Breast carcinoma

| MDA-MB-231 | A    | G839A    | R280K             | C      |

Normal lung fibroblast

| CCD32Lu | A | None (wt) |

NSCLC

| H1299   | A | Null    |

NOTE: The p53 protein is 393 amino acids (aa) long and consists of 5 domains referred to as domain A–E. Domain A (aa 1–62): transactivation domain; B (aa 63–94): proline-rich domain; C (aa 95–292): DNA-binding domain; D (aa 325–356): tetramerization domain; E (aa 357–393): negative auto-regulatory domain. The morphology of the cell lines indicates whether the cells were grown in suspension (S), adherent (A), or semiadherent/suspension (A/S) cultures.
(both from Santa Cruz) using Lipofectamine 2000 (Invitrogen). p53 downregulation was investigated 24, 48, 72, and 96 hours after transfection by Western blot analysis to determine the optimal conditions for p53 downregulation. Subsequently, cells were transfected with either p53 or scrambled siRNA for 48 hours after which they were treated with 50 or 100 μmol/L PRIMA-1Met for 72 hours. At the end of incubation, the cells were counted after Tryphan blue staining.

siRNA transfection efficiency was assessed by transfection of cells as above using a fluorescein-labeled nonsilencing siRNA.

**Western blot analysis**

Cells were lysed and analyzed according to standard procedures. Bands were detected using Supersignal West Dura Extended Duration Substrate (Pierce-Thermo Scientific) in an Autochemi system (UVP).

The antibodies used were as follows: anti-PARP (Cell Signaling), anti-Tubulin (Cell Signaling), anti-p21 (BD Biosciences), anti-p53 (N-term; Epitomics), anti-caspase-3 (Santa Cruz), anti-Bax (Cell Signaling), anti-Bcl-2 (Merck), anti-MDM2 (Calbiochem), anti-ubiquitin (Santa Cruz), and anti-Noxa (Calbiochem).

**Immunoprecipitation**

Cell lysates were precleared with protein G agarose beads (Upstate) and equal amounts of protein (1500 μg) were immunoprecipitated with 10 μL anti-p53 antibody (N-term; Epitomics) followed by incubation with protein G agarose beads (Upstate). Antibody control was prepared by incubating 1 mL of precleared radioimmunoprecipitation assay buffer (RIPA) lysisbuffer with 10 μL anti-p53 antibody (Epitomics) followed by incubation with protein G agarose beads. Immunoprecipitated and coimmunoprecipitated proteins were analyzed by Western blotting.

**Animal studies**

The SCLC mouse models were established by inoculating 1 x 10^6 GLC16 or DMS53 cells s.c. per flank on each flank into 6- to 8-week-old nude male NMRI mice (Taconic). Xenografts were allowed to grow for up to 6 weeks before initiation of treatment; weight and tumor sizes were measured up to and including the treatment period. Tumor sizes were measured using a digital caliper. Tumors were between 30 and 200 mm^3 when treatment was initiated. The mice were randomized into 2 groups. One group received intravenous (i.v.) injections of 100 μL PBS (control) while the other received i.v. injections of 100 mg/kg PRIMA-1Met. Injections were performed once daily for 5 days on 2 consecutive weeks.

All animal experiments were done in accordance with ethical guidelines under valid license from the Danish Animal Experimentation Board, and the mice were sacrificed once the tumors reached the maximum allowed volume of 1000 mm^3.

**Statistical analysis**

One-way ANOVA with Dunnet’s post hoc test or Student’s t-test were used to compare the values of the test and control samples in the cell viability and flow cytometric studies.

The effect of PRIMA-1Met on the tumor growth rate as compared with PBS control was analyzed using tumor doubling times (TDT) defined as the time elapsed when each tumor had doubled in size from treatment start time. For tumors that did not reach double size during the first 32 days after treatment start, TDT was censored at 32 days. Cox regression analysis was performed to test the effect of PRIMA-1Met on TDT. Robust standard errors were used to obtain P-values because several mice with 2 tumors were used in the analysis. The choice of using TDT as the end-point for the statistical analysis is based on the fact that some animals were sacrificed during the course of the experiment due to tumors reaching maximum allowed size. Therefore, the amount of data collected for each animal during the entire experiment varied depending on their lifespan making direct analysis of mean tumor volumes of control and treated animals difficult to conduct and subject to bias. In addition, complete eradication of some of the tumors during and after treatment leads to negative gradients of the growth curves and makes it difficult to analyze commonly used parameters of growth curves such as the gradients on a log-scale. TDT time is a parameter of the growth curves, which circumvents these difficulties.

P < 0.05 was considered statistically significant.

**Results**

**p53 protein and mRNA levels in SCLC cell lines**

Total levels of p53 in a large panel of SCLC cell lines expressing mutant p53 (Table 1) were evaluated by Western blot analysis to choose those with high p53 levels, as reactivation of abundant mutant p53 would be expected to trigger a massive apoptotic response. The cell lines, CCD32Lu (wt p53), MDA-MB-231 (mutant p53), and H1299 (p53-null) were included as controls. As shown in Figure 1A, the level of mutant p53 in the SCLC cell lines varied. However, in the majority of the SCLC cell lines carrying mutant p53, the level of the protein was higher than in the CCD32Lu cell line (Fig. 1A). To determine if the low or negligible levels of mutant p53 observed in some of the SCLC cell lines were due to high MDM2 protein levels or markedly decreased mRNA expression levels, Western blot and semiquantitative reverse transcriptase (RT)–PCR analyses were performed, respectively. As seen in Figure 1A, low levels of mutant p53 were not associated with high levels of MDM2 compared with the high p53 expressing cell lines. Nor was it due to lack of p53 mRNA as evident by the presence of p53 transcript (Fig. 1B). Although p53 mRNA could not be detected after 25 cycles of amplification in the NCIH69 cell line (Fig. 1D), it was detectable after 30 cycles (data not shown), suggesting that NCIH69 expresses low levels of mutant p53.
PRIMA-1<sup>Met</sup> suppresses the growth of SCLC cell lines with high levels of mutant p53 by activating the apoptotic pathway

To study the effect of PRIMA-1<sup>Met</sup> in SCLC cell lines, we employed the MTT viability assay. We chose 3 SCLC cell lines with high levels of mutant p53, DMS273, DMS53, and GLC16, carrying missense mutations in the DNA binding domain (Fig. 1A and Table 1). As seen in Figure 2A, PRIMA-1<sup>Met</sup> inhibited the growth of the SCLC cell lines significantly in a dose-dependent manner but caused only a minor reduction in the growth of the CCD32Lu and H1299 control cells. To confirm and extend these results, cell cycle analysis of SCLC and control cells was performed before and after treatment with PRIMA-1<sup>Met</sup> using flow cytometry (Fig. 2B). Quantification of cells with Sub-G0/G1 DNA content showed a significant increase in the SCLC cell lines upon PRIMA-1<sup>Met</sup> treatment compared with the controls (Fig. 2C). To test whether PRIMA-1<sup>Met</sup>-induced cell death was in fact due to reactivation of the p53 transcriptional activity, DMS53, GLC16, and DMS273 cells were treated with PRIMA-1<sup>Met</sup> for up to 72 hours and the level of p53 and its targets were evaluated by Western blot analysis. PRIMA-1<sup>Met</sup> treatment of SCLC cell lines induced the expression of the p53 target genes MDM2, p21, and Bax and resulted in a decrease in total Bcl-2 and procaspase-3 levels and in PARP cleavage (Fig. 2D). A decline in p53 levels was also noted after PRIMA-1<sup>Met</sup> treatment (Fig. 2D). These effects were p53 dependent, since they were not observed in the p53-null H1299 cells. In the CCD32Lu cell line expressing wt p53, transient upregulation of MDM2 and p21 levels were observed upon PRIMA-1<sup>Met</sup> treatment (Fig. 2D). However, in this cell line no Bax upregulation, PARP cleavage, or decrease in procaspase-3 and Bcl-2 levels could be detected.

To investigate whether PRIMA-1<sup>Met</sup>-induced reduction of the level of mutant p53 was due to increased MDM2 binding and hence p53 ubiquitination, p53 was immuno-precipitated from DMS53, GLC16, and DMS273 cells before and after treatment with PRIMA-1<sup>Met</sup>. As seen in Figure 2E and F, PRIMA-1<sup>Met</sup> induced an increase in the binding of MDM2 to p53 and in p53 ubiquitination in the SCLC cell lines GLC16 and DMS273. In DMS53 cells, a decrease in the level of coimmunoprecipitated MDM2 and p53 ubiquitination was detected upon PRIMA-1<sup>Met</sup> treatment, correlating with markedly decreased immunoprecipitated p53 levels (Fig. 2E and F).

Overall, these results suggest that PRIMA-1<sup>Met</sup> induces cell death in SCLC cells with high levels of mutant p53 in a dose-dependent manner by reactivating mutant p53, resulting in the upregulation of proteins involved in cell cycle arrest, apoptosis, and p53 downregulation.

Efficient knockdown of mutant p53 reduces the cytotoxic effects of PRIMA-1<sup>Met</sup> in SCLC cells

To further examine the role of mutant p53 in PRIMA-1<sup>Met</sup>-induced SCLC cell death, the growth suppressive
effect of PRIMA-1\textsuperscript{Met} was tested in SCLC cells in which mutant p53 was downregulated by p53 siRNA. As seen in Figure 3A, the DMS273 and DMS53 cell lines had a higher transfection efficiency compared with GLC16 cells. Mutant p53 was also strongly downregulated after transfection with p53 siRNA in the DMS273 and DMS53 cell lines compared with GLC16 cells (Fig. 3B). Treatment DMS273 and DMS53 cells with PRIMA-1\textsuperscript{Met} after transfection with p53 siRNA resulted in a markedly reduced cytotoxicity compared with scrambled siRNA transfected cells.
In GLC16 cells however, no reduction in PRIMA-1\textsuperscript{Met}-induced cytotoxicity was observed in p53 siRNA transfected cells compared with control cells (Fig. 3C). These results suggest that the growth suppressive effect of PRIMA-1\textsuperscript{Met} in SCLC cells is mutant p53 dependent.

The effect of PRIMA-1\textsuperscript{Met} on SCLC cell lines with low or undetectable levels of mutant p53

It was previously suggested that the efficacy of PRIMA-1\textsuperscript{Met} depends on mutant p53 levels in the cells (14). Therefore, we sought to determine whether PRIMA-1\textsuperscript{Met} could inhibit the growth of SCLC cell lines with low or negligible levels of mutant p53. Five SCLC cell lines were chosen for this study, DMS153, DMS456, GLC3, GLC28, and NCIH69. The DMS153, DMS456, and GLC28 cells express p53 with a point mutation in the DNA binding domain (Table 1). NCIH69 cells express a p53 protein that lacks a part of the DNA binding domain and the entire C-terminus due to a stop codon at position 171 (Table 1). The GLC3 cell line expresses a heavily truncated p53 protein due to a stop codon at position 53 in the transactivation domain (Table 1). PRIMA-1\textsuperscript{Met} inhibited the growth of the SCLC cell lines DMS153, DMS456, and GLC28 in a dose-dependent manner, whereas in GLC3, H1299, and CCD32Lu cell lines no significant growth suppression was observed even at 100 μmol/L (Fig. 4A). In addition, the growth rate of the NCIH69 cell line was also inhibited despite expressing undetectable levels of a truncated mutant p53 protein (Fig. 4A). To verify these results, cell cycle analysis of the SCLC and control cell lines was performed before and after PRIMA-1\textsuperscript{Met} treatment (Fig. 4B and C). In agreement with the results described above, PRIMA-1\textsuperscript{Met} induced a significant increase in the proportion of Sub-G0/G1 cells in all the SCLC cell lines except GLC3, but not in the control cells (Fig. 4C). These results suggest that PRIMA-1\textsuperscript{Met} is also capable of inducing cancer cell death in SCLC cell lines with low or undetectable levels of mutant p53.

We next investigated whether PRIMA-1\textsuperscript{Met}-induced cancer cell death in SCLC cell lines with low or negligible levels of mutant p53 was due to p53 reactivation and induction of apoptosis. The SCLC cell lines were treated with PRIMA-1\textsuperscript{Met} for up to 72 hours and total levels of mutant p53 and its targets were studied by Western blotting. Bax was upregulated in DMS153, DMS456, and GLC28 cells but not in NCIH69 and GLC3 cells (Fig. 4D and Supplementary Fig. S1). However, no increase in p21 and MDM2 levels could be observed in any of the SCLC cell lines, although a decrease in total levels of mutant p53 was detected in the DMS456, DMS153, and GLC28 cells. To see if PRIMA-1\textsuperscript{Met}
treatment of the SCLC cells results in the upregulation of p53 target genes other than Bax; total levels of the proapoptotic p53 target gene Noxa was evaluated in the SCLC cells before and after treatment with PRIMA-1\textsuperscript{Met} for 72 hours. Noxa was upregulated in the GLC28 and DMS456 cells and to a modest degree in the DMS153 cells (Supplementary Fig. S2). No increase in Noxa protein levels was detected in the GLC3 and NCIH69 cells.
(Supplementary Fig. S2). Furthermore, PRIMA-1^Met induced a decrease in procaspase-3 and Bcl-2 levels as well as PARP cleavage in DMS153, GLC28, and DMS456 but not in GLC3 and NCIH69 cells (Fig. 4D). These results suggest that PRIMA-1^Met induces cancer cell death in DMS153, DMS4856, and GLC28 cell lines by reactivating mutant p53 and hence triggering apoptosis.

**PRIMA-1^Met has antitumor activity in vivo**

To determine whether the observed inhibitory effects of PRIMA-1^Met in vitro could be shown in vivo, the antitumorogenic potential of PRIMA-1^Met was studied in SCLC xenograft models. DMS53 and GLC16 cells, which are capable of forming tumors, were inoculated into nude mice and the animals received i.v. injections of either 100 μL PBS (control) or 100 mg/kg PRIMA-1^Met once daily for 10 days after tumor formation was evident. Initial tumor volumes did not differ significantly between the treatment groups at treatment start (Supplementary Fig. S3 and Tables S1 and S2). Tumor size was measured several times a week after treatment was initiated and the TDT of each tumor was determined (Fig. 5A and B and Supplementary Tables S1 and S2). Cox regression analysis of the TDT showed a significant growth delay upon PRIMA-1^Met treatment as compared with PBS in both tumor models (P < 0.05). In addition, PRIMA-1^Met treatment reduced the hazard rate of TDT significantly by 68% in the DMS53 study (95% confidence limits [10%;89%]) and by 72% in the GLC16 study (95% confidence limits [43%;87%]). Furthermore, in the DMS53 tumor model, 3 mice in the PBS group were sacrificed 18 days after treatment start, as 5 out of 6 tumors reached the maximum allowed size, whereas in the PRIMA-1^Met group, one mouse was sacrificed with 1 out of 2 tumors reaching the maximum size (Fig. 5A). A complete eradication of both tumors on 2 mice (4 tumors in total) was also seen upon PRIMA-1^Met treatment in the DMS53 study (Fig. 5A). There were no obvious toxic effects of the treatments in any of the experiments as evaluated by histology of resected major mice organs and mouse weight data (data not shown).

**Discussion**

The p53 pathway is disrupted in the majority of the SCLC due to p53 mutations, making functional restoration of this pathway an attractive therapeutic approach (1–3). In this study, we addressed the possibility of using the low molecular weight compound, PRIMA-1^Met, capable of reactivating mutant p53 and inducing apoptosis in various cancer cells, to induce cell death in SCLC cells with different levels of various p53 mutants. To our knowledge, this is the first study to evaluate the effect of such compound in SCLC.

Our results show that PRIMA-1^Met can induce cell death in SCLC cell lines with both high and low (but detectable) levels of mutant p53 by triggering apoptosis, associated with the upregulation of proapoptotic proteins, caspase-3 activation and PARP cleavage. These results are fully consistent with PRIMA-1^Met-induced restoration of wt properties to mutant p53 leading to transactivation of target genes, activation of caspases and hence induction of apoptosis as previously reported (13, 15, 35). The ability of PRIMA-1^Met to restore wt properties to mutant p53 was further supported by the increase in MDM2 and mutant p53 association and in p53 ubiquitination in the GLC16 and DMS273 cells upon PRIMA-1^Met treatment. The fact that ubiquitinated p53 is targeted for degradation (36) could also explain the observed decrease in total p53 levels in the SCLC cell line with both high and low levels of mutant p53 after PRIMA-1^Met treatment. Furthermore, we confirmed that the cytotoxic effect of PRIMA-1^Met in SCLC cells was indeed dependent on mutant p53 as efficient depletion of mutant p53 by siRNA reduced the growth suppressing effect of PRIMA-1^Met.

PRIMA-1^Met had no considerable effect in the SCLC cell line GLC3, expressing undetectable levels of a heavily truncated p53 protein, nor in the 2 control cell lines H1299 (p53-null) and CDD32Lu (wt p53). However, a transient increase in MDM2 and p21 levels was observed in CDD32Lu cells upon PRIMA-1^Met treatment, presumably due to a transient stabilization of wt p53. Stabilization of p53 can be induced by the binding of Hsp90 to p53 and PRIMA-1 has been shown to facilitate this interaction (21, 37, 38). In addition, Lambert and colleagues recently showed that not only can PRIMA-1^Met bind to mutant p53, but it can also bind to unfolded wt p53 (34) and that the degree of binding correlates with the extent of unfolding, suggesting that PRIMA-1^Met can activate unfolded wt p53 in the cells by inducing correct folding (34). Despite the observed effects on MDM2 and p21 levels, no caspase-3 activation, Bax upregulation, Bcl-2 downregulation, or PARP cleavage was detected in the PRIMA-1^Met-treated CDD32Lu cells, which is consistent with the results obtained from the cell viability assay and flow cytometry. The reason could be due to the level of cellular stress, which is likely low in normal cells such as CDD32Lu cells. In the absence of cellular stress, transient apoptotic signals are probably overruled leading to continued cell proliferation. In cancer cells, however, the cellular stress level is high and numerous stress signals converge on p53, which in turn responds by triggering cell cycle arrest or apoptosis unless inactivated by mutation. This could also explain why cancer cells are more sensitive to PRIMA-1^Met than normal cells.

In addition, in the NCIH69 cells expressing undetectable levels of a truncated mutant p53 protein, containing only the first 171 amino-terminal residues, PRIMA-1^Met significantly induced cancer death in a dose-dependent manner. PRIMA-1^Met has previously been shown to induce cell death in p53-null cells (13), thus it is possible that the mechanism of PRIMA-1^Met-induced cell death in this line is p53-independent. However, we cannot rule out the possibility that the effect of PRIMA-1^Met in this cell line is mutant p53-dependent, since NCIH69 has low levels of p53 mRNA. PRIMA-1^Met might induce transient...
stabilization of the truncated mutant p53 protein in the NCIH69 cells leading to a transcription- and caspase-independent activation of apoptosis. PRIMA-1 and PRIMA-1Met have been shown to induce mutant p53–dependent but transcription-independent apoptosis in cancer cells (6, 35). PRIMA-1 can also trigger caspase-independent apoptosis by inducing the activation of the c-jun NH, kinase (JNK) signaling pathway in a mutant p53–dependent manner (39) Also, truncated transactivation deficient p53 proteins, encompassing the proline-rich domain but not the DNA binding or carboxy-terminal domain, have been shown to exhibit profound apoptotic activity. While the proline-rich domain is required for p53 tumor suppressing activity, the DNA binding domain seems to be dispensable (6, 40). The fact that NCIH69 cells, unlike GLC3 cells, express a truncated p53 protein with an intact proline-rich domain,

Figure 5. The antitumor activity of PRIMA-1Met in SCLC tumor models. A, mice carrying DMS53 (A) or GLC16 (B) tumor xenografts were treated i.v. with either 100 μL PBS (left graph) or 100 mg/kg PRIMA-1Met (right graph) for 5 days on 2 consecutive weeks. Tumor volume was measured several times a week after treatment start. Graph A depicts the volume of 12 tumors (6 mice) and 11 tumors (7 mice) in the PBS and PRIMA-1Met group, respectively. In the PBS group, each mouse had 2 tumors. In the PRIMA-1Met group, 5 mice carried 2 tumors each, while 2 mice carried only 1 tumor each. In graph B, the volume of 6 tumors (4 mice) in the PBS and PRIMA-1Met group is depicted. In each group, 2 of the mice carried 2 tumors each, while the other 2 had only 1 tumor each. Each mouse is presented by a specific ID number (#) and left and right indicate tumor on the left or right flank. Tumors on the same mice are represented by the same color.
could explain why PRIMA-1\textsuperscript{Met} has an effect in NCI-H69 but not in GLC3 cells. In addition, PRIMA-1\textsuperscript{Met} could exert its effects through the p53 related proteins p73 and p63 (41). PRIMA-1 and PRIMA-1\textsuperscript{Met} have been shown to induce nuclear translocation of not only p53 but also p73, which is suggested to play a role in the induced apoptosis (42, 43). It was also very recently reported that PRIMA-1\textsuperscript{Met} targets mutant forms of p73 and p63 in addition to mutant p53 (44).

To study the clinical relevance of PRIMA-1\textsuperscript{Met} in vivo, mice bearing SCLC tumors were treated systemically with 100 mg/kg PRIMA-1\textsuperscript{Met}. In both in vivo studies performed, PRIMA-1\textsuperscript{Met} induced a significant tumor growth delay compared with control mice. The antitumor activity of PRIMA-1 and PRIMA-1\textsuperscript{Met} in various cancer types with mutant p53 has been shown previously (13, 15, 16). However, to our knowledge, this is the first study to investigate the antitumor activity of PRIMA-1\textsuperscript{Met} in SCLC. The fact that PRIMA-1\textsuperscript{Met} can be administrated systemically is really important since SCLC almost always metastasizes. Therefore, the introduction of PRIMA-1\textsuperscript{Met} and related molecules will be highly relevant for the treatment of patients with SCLC.

Overall, our results show that PRIMA-1\textsuperscript{Met} is capable of reactivating mutant p53 in SCLC cells, which leads to apoptosis and tumor growth delay, providing new perspectives on the future development of novel treatment modalities for SCLC patients.

**Disclosure of Potential Conflicts of Interest**

G. Selivanova is a cofounder and shareholder of Aprea AB.

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