PRIMA-1^{MET} induces apoptosis through accumulation of intracellular reactive oxygen species irrespective of p53 status and chemo-sensitivity in epithelial ovarian cancer cells

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Abstract. There is an intensive need for the development of novel drugs for the treatment of epithelial ovarian cancer (EOC), the most lethal gynecologic malignancy due to the high recurrence rate. TP53 mutation is a common event in EOC, particularly in high-grade serous ovarian cancer, where it occurs in more than 90% of cases. Recently, PRIMA-1 and PRIMA-1^{MET} (p53 reactivation and induction of massive apoptosis and its methylated form) were shown to have an antitumor effect on several types of cancer. Despite that PRIMA-1^{MET} is the first compound evaluated in clinical trials, the antitumor effects of PRIMA-1^{MET} on EOC remain unclear. In this study, we investigated the therapeutic potential of PRIMA-1^{MET} for the treatment of EOC cells. PRIMA-1^{MET} treatment of EOC cell lines (n=13) resulted in rapid apoptosis at various concentrations (24 h IC_{50} 2.6-20.1 μM). The apoptotic response was independent of the p53 status and chemo-sensitivity. PRIMA-1^{MET} treatment increased intracellular reactive oxygen species (ROS), and PRIMA-1^{MET}-induced apoptosis was rescued by an ROS scavenger. Furthermore, RNA expression analysis revealed that the mechanism of action of PRIMA-1^{MET} may be due to inhibition of antioxidant enzymes, such as Prx3 and GPx-1. In conclusion, our results suggest that PRIMA-1^{MET} represents a novel therapeutic strategy for the treatment of ovarian cancer irrespective of p53 status and chemo-sensitivity.

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Introduction

Each year, more than 100,000 women die of ovarian cancer worldwide (1). Epithelial ovarian cancer (EOC) accounts for the majority of all ovarian malignancies and is one of the most lethal among gynecologic malignancies among women. In many cases, the diagnosis is delayed due to its asymptomatic nature, and, as a consequence, approximately two-thirds of patients with EOC have already developed peritoneal carcinomatosis (2,3). The prognosis of EOC patients is closely related to the stage at diagnosis (4,5).

Most ovarian cancer patients are managed with surgical resection, followed by systemic chemotherapies. Despite recent advances in therapeutic agents, such as platinum-taxane combination chemotherapy, the 5-year survival rate is still less than 40% (6). EOC shows an unfavorable oncologic outcome, based on its asymptomatic features at an earlier clinical stage, and numerous intraperitoneal and/or distant metastases. Despite the relatively high susceptibility of EOC to paclitaxel plus platinum compounds, which are first-line chemotherapeutic agents against EOC, the intrinsic or acquired resistance of tumor cells to these chemotherapies makes the treatment of EOC difficult. In order to overcome chemo-resistance, various additional molecular-targeting therapies combined with conventional anti-neoplastic agents have been developed. High-grade serous ovarian cancer (HGSOC), which is observed much more frequently at an advanced stage, comprises approximately 60% of all histological subtypes of EOC. Recent studies have revealed that most cases of HGSOC carry TP53 mutations, in contrast to other types of EOC, which have a much lower incidence of TP53 mutations (7-9). A recent study using high-throughput sequencing technology revealed that TP53 mutations occurred in 96% of 316 HGSOC samples (10). This suggests that somatic mutation of TP53 is a nearly universal event in HGSOC.

TP53 is located on chromosome 17p and encodes the p53 protein. Wild-type p53 functions predominately as a transcriptional factor, with a potent tumor-suppressive function via its multiple activities, including induction of cell cycle arrest, apoptosis, differentiation and senescence (11). Recent studies

have shown that missense TP53 mutations not only eliminate their own tumor-suppressive function, but also gain oncogenic properties that promote tumor growth, termed gain-of-function (GOF) (12-14). Furthermore, TP53 mutations may be associated with poor prognosis and malignant phenotypes in several types of cancers, including EOC (15-19). Considering the universality of the TP53 mutation in EOC, several novel drugs restoring the p53 pathway have been widely investigated to be utilized in cancer therapy.

PRIMA-1 (p53 reactivation and induction of massive apoptosis) and its methylated form PRIMA-1[MET] are small molecules that can convert mutant p53 to an active conformation, which restores wild-type functions of p53 in several types of cancers, such as breast, neck and thyroid cancer and melanoma (20-23). PRIMA-1[MET] is one of the most promising drugs for clinical use to restore wild-type functions to mutant p53 (24). Although PRIMA-1[MET] is the first compound of such drugs evaluated in clinical trials, the antitumor effects of PRIMA-1[MET] on EOC remain unclear.

In our present study, we investigated whether PRIMA-1[MET] induces growth suppression and apoptosis in EOC cells. Using EOC cells with either wild-type or mutant p53, and with either chemo-sensitivity or chemo-resistance, we demonstrated that PRIMA-1[MET] was able to effectively induce cell death. Therefore, PRIMA-1[MET] can be a promising therapeutic strategy to induce cytotoxic effects and reactivate the p53 pathway in EOC, particularly in HGSOC.

Materials and methods

Cell culture. Human EOC lines, A2780, OVCAR-3, ES-2, SKOV-3, CaOV-3, TOV21G and OV-90, were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). NOS2 and NOS3 cell lines derived from serous EOC were previously established in our institute (25). The NOS2CR and NOS2TR cells with chronic resistance to cisplatin and paclitaxel were previously established from the parental NOS2 cells in our institute (26). Furthermore, we recently established another two chronic cisplatin/paclitaxel-resistant cell lines from the parental NOS3 cells: NOS3CR (cisplatin) and NOS3TR (paclitaxel). All EOC cell lines were maintained at 37˚C with 5% CO_2 in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/ml), and penicillin (100 U/ml). PRIMA-1[MET] was purchased from Santa Cruz Biotechnology, Inc. PRIMA-1[MET] was diluted in dimethyl sulfoxide (DMSO) to create a 50-mmol/l stock solution and stored at -20˚C. Antibodies to p53 (610184) were purchased from BD Pharmingen. Antibodies to cleaved-PARP, PARP and β-actin were purchased from Cell Signaling Technology.

Direct sequencing of TP53 mutations. Genomic DNA was extracted from the NOS2 and NOS3 cells using the Genomic DNA purification kit (Promega, Madison, WI, USA). The exons and flanking introns of TP53 were amplified by polymerase chain reaction (PCR). The primers used are shown in Table I. The resulting PCR products were sequenced and the mutation status was confirmed.

RNA extraction. RNA extraction from the cells was undertaken using the Qiagen RNeasy Mini kit according to the

Table I. The specific primers used for direct sequencing of the TP53 gene.

Primer	Sequence	Length (bp)
p53		
Exon 2-4	F: GTGTCTCATGCTGGATCCCCACT	23
	R: GGATACGGCCAGGCATTGAAGT	22
Exon 5-6	F: TGCAGGAGGTGCTTACGCATGT	22
	R: CCTTAACCCCTCCTCCCAGAGAC	23
Exon 7-9	F: ACAGGTCTCCCCAAGGCGCACT	22
	R: TTGAGGCATCACTGCCCCCTGAT	23
Exon 10	F: GTCAGCTGTATAGGTACTTGAAGTGCAG	28
	R: GCTCTGGGCTGGGAGTTGCG	20
Exon 11	F: CCTTAGGCCCTTCAAAGCATTGGTCA	26
	R: GTGCTTCTGACGCACACCTATTGCAAG	27

F, forward; R, reverse.

manufacturer's protocols. The cells were lysed in 250 μl of buffer RLT and filtered through the filtration spin column. The samples were applied to the RNeasy Mini spin column. Total RNA bound to the membrane and contaminants were removed by washing consecutively with buffers RW1 and RPE. RNA was eluted in RNase-free water. Extracted RNA was immediately stored at -80˚C. The RNA concentration was determined with the NanoDrop 1000 spectrophotometer.

Reverse transcription. To obtain complementary DNA (cDNA), 1 μg of RNA and 0.2 μg of random primers (Promega) were used. After incubation at 72˚C for 4 min, the mixture of RNA and random primers were placed on ice for 4 min. M-MLV RT 1X reaction buffer, M-MLV Reverse Transcriptase RNase Minus, and 10 mM dNTP (Promega) were added to the mixture and then incubated at 42˚C for 90 min, followed by 70˚C for 15 min. cDNA was stored at -20˚C.

Quantitative real-time PCR (qRT-PCR). Quantitative RT-PCR (qRT-PCR) was performed on the Takara PCR thermal cycler using the SYBR Green detection system (Takara, Tokyo, Japan). Cycling conditions consisted of a 3-min hot start at 95˚C, followed by 40 cycles of denaturation at 95˚C for 10 sec, annealing at 58-60˚C for 10 sec, extension at 72˚C for 10 sec, and then a final inactivation at 95˚C for 10 sec. Dissociation curve analyses were carried out at the end of the cycling to confirm that one specific product was measured in each reaction. Relative quantification was performed using the ΔΔCT method (27). Expression normalization was conducted by the expression of GAPDH, a housekeeping gene shown to have stable expression in cancer cell lines (28). The specific primers for each gene are shown in Table II. All experiments were performed in triplicate.

Protein extraction and western blot analysis. Cultured ovarian cancer cells were washed with PBS and lysed in RIPA buffer (Millipore). The cells were scrapped into lysis buffer,

Table II. The specific primers used for quantitative real-time RT-PCR.

Primer	Sequence	Length (bp)
PRX3	F: GACGCTCAAATGCTTGATGA	20
	R: GATTTCCCGAGACTACGGTG	20
GPX1	F: AAGAGCATGAAGTTGGGCTC	20
	R: CAACCAGTTTGGGCATCAG	19
GAPDH	F: TTGGTATCGTGGAAGGACTCA	22
	R: TGTCATCATATTTGGCAGGTT	21

F, forward; R, reverse.

Table III. TP53 status of the EOC cell lines.

Cancer cell lines	p53 status
ES-2	S241F
OV-90	S215R
OVCAR-3	R248Q
TOV21G	Wild-type
A2780	Wild-type
CaOV-3	Q136Term
SKOV-3	Null
NOS2	Wild-type
NOS3	L257P

centrifuged at 12,000 x g at 4˚C for 15 min, and then diluted in 2X sample buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, 0.01% bromophenol blue, and 10% 2-mercaptoethanol]. Equal amounts of protein ($10 \mu g$) were mixed with the 2X sample buffer and were boiled at 95˚C for 5 min. The samples were loaded and separated by 7.5-15% SDS-polyacrylamide gel electrophoresis (PAGE) with running buffer. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 1% skim milk, incubated with each primary antibody overnight at 4˚C, washed with TBS-T buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Tween-20) and incubated with the secondary antibodies. The proteins were visualized using enhanced chemiluminescence (GE Healthcare Bio-Sciences, Uppsala, Sweden).

Cell viability assay. The effect of PRIMA-1[MET] on the viability of human EOC cells was evaluated with the CellTiter-Glo Luminescent Cell Viability Assay (Promega), which quantifies living cells by ATP signal intensity. The luminescent signal was determined with a luminometer. Cells were seeded in triplicate in 96-well plates at a density of 2,000 cells/well. After a 24-h culture, the cells were treated with various concentrations of PRIMA-1[MET], and then incubated for 24-72 h. Control cells were treated with the same concentration of DMSO as that of the PRIMA-1[MET]-treated cells.
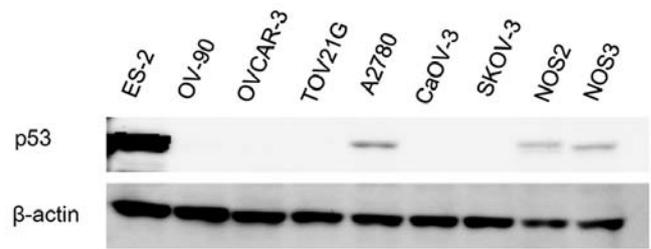

Figure 1. p53 protein expression in the EOC cells. EOC cells were culture in 6-well plates, and then lysed with lysis buffer. Immunoblot analysis revealed the p53 protein expression of EOC cells. The samples were loaded and separated by 10% SDS-PAGE with running buffer. The separated proteins were transferred to a PVDF membrane. After blocking with 1% skim milk, the membrane was incubated with anti-p53 or anti-β-actin at 4˚C for 1 day. The proteins were visualized using enhanced chemiluminescence. β-actin was used as a loading control.

Detection of apoptosis by staining with Annexin V-FITC and propidium iodide. Cells ($2x10^5$) were cultured in 6-well plates for 24 h before treatment with DMSO (control) or an appropriate concentration of PRIMA-1[MET] for 24 h. The cells were trypsinized, washed once with PBS, and then stained with Annexin V-FITC and propidium iodide (PI) to determine the early/late apoptotic cell population (MBL, Japan).

Results

Protein expression of p53 and the TP53 status in EOC cells. Firstly, we evaluated the levels of p53 protein expression in the EOC cells by western immunoblot analysis. The mutation status of TP53 in the EOC cells was acquired from previous studies. The mutation status of TP53 of NOS2 and NOS3 cells was evaluated by direct sequencing. The TP53 status of EOC cells is shown in Table III. The protein expression of p53 is shown in Fig. 1. EOC cells with wild-type p53, A2780 and NOS2, displayed a basal expression of p53 to some extent. On the contrary, we could not detect the protein expression of p53 in the EOC cells with mutant p53, except for the ES-2 cells. This result demonstrates that EOC cells bearing mutant p53 do not always express a higher level of p53 than those bearing wild-type p53.

The effect of PRIMA-1[MET] on cell death and apoptotic morphological changes in EOC cells. To assess the effect of PRIMA-1[MET] on EOC cells, the anti-proliferative effects of various concentrations of PRIMA-1[MET] (approximately 0-100 μM) were determined in a total of 9 EOC cell lines: TOV21G, A2780, ES-2, OV-90, OVCAR-3, CaOV-3, SKOV-3, NOS2 and NOS3. Fig. 2a shows the cell viability of wild-type p53 cell lines (TOV21G, A2780, and NOS2) and mutant p53 cell lines (ES-2, OV-90, OVCAR-3, CaOV-3, NOS3 and SKOV-3) treated with PRIMA-1[MET] for 48 h. PRIMA-1[MET] reduced cell viability after 48 h in all EOC cell lines in a dose-dependent manner. The IC_{50} values of PRIMA-1[MET] ranged from 2.6 to 20.1 μM, which were independent of the mutation status of TP53 (Fig. 2b). Furthermore, PRIMA-1[MET] treatment induced a morphological change which was consistent with the apoptotic change within 6-24 h (Fig. 2c). We next investigated whether PRIMA-1[MET] had sufficient effects on cisplatin- and paclitaxel-resistant cell lines, which were previously developed
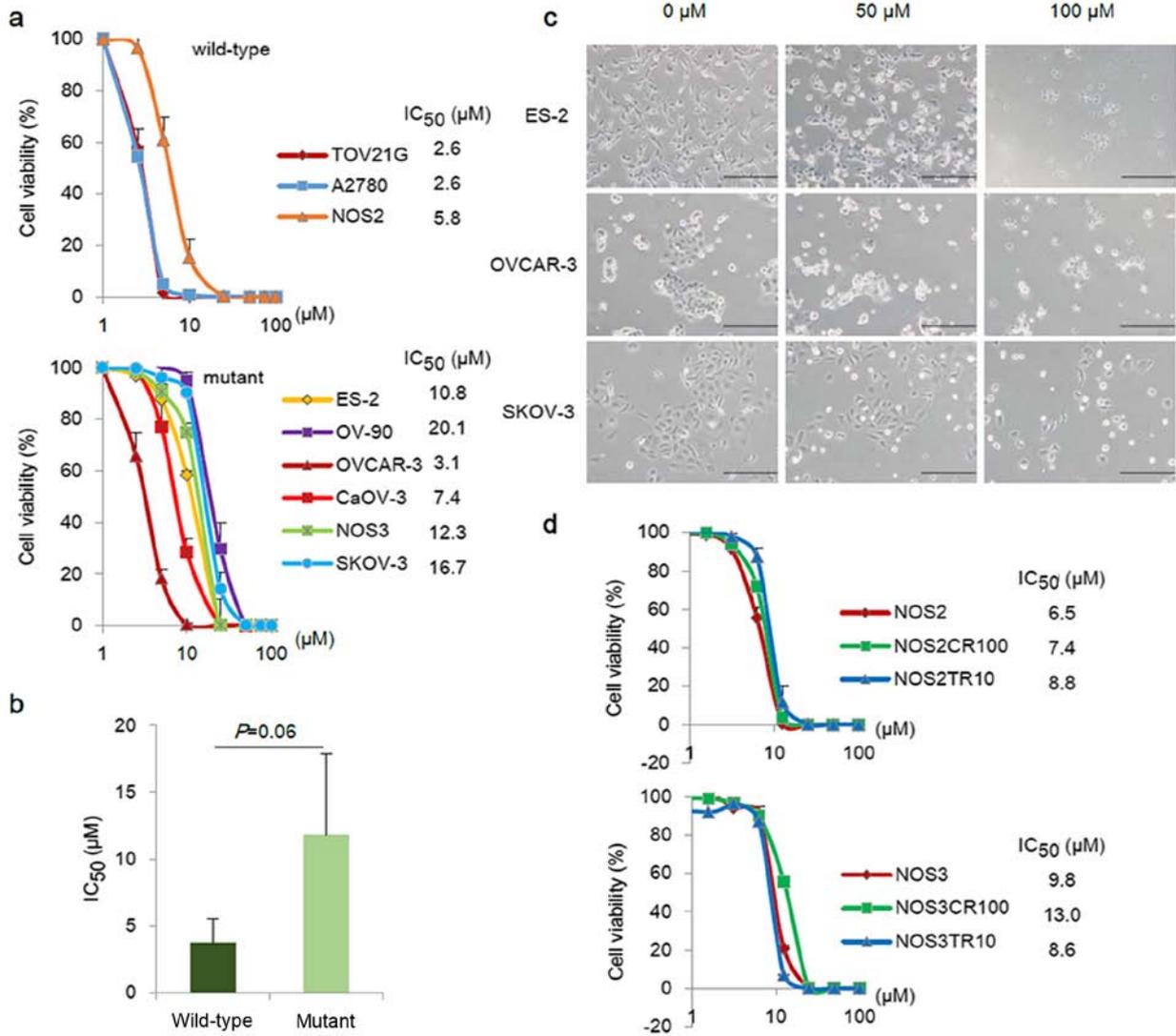
Figure 2. The effects of PRIMA-1[MET] on tumor cell growth in ovarian cancer cells. (a) PRIMA-1[MET]-induced growth suppressive dose response curves of EOC cells following 48 h of PRIMA-1[MET] treatment. The IC$_{50}$ values of PRIMA-1[MET] were determined in a total of 9 EOC cell lines by cell viability assay. Data represent the mean ± standard deviation (SD) from triplicate reactions. (b) Correlation between PRIMA-1[MET] 48 h IC$_{50}$ values and TP53 status. EOC cell lines with wild-type p53 were slightly more sensitive than those with mutant p53 (not significant). (c) PRIMA-1[MET]-induced morphological changes in EOC cells. ES-2, OVCAR-3, and SKOV-3 cells were cultured in a 6-well plate, and then treated with 0, 50, 100 μM PRIMA-1[MET] for 24 h. PRIMA-1[MET]-induced morphological changes were observed using microscopy (scale bar, 200 μm). (d) PRIMA-1[MET]-induced growth suppressive dose response curves of NOS2, NOS3, and their chemo-resistant cells. The IC$_{50}$ values of 48 h PRIMA-1[MET] were determined by cell viability assay. Data displays mean ± standard deviation (SD) from triplicate reactions.

from the parental NOS2 and NOS3 cells (NOS2CR, NOS2TR, NOS3CR, and NO3TR). Dose-responsive cell viability assays with PRIMA-1[MET] were performed to evaluate the sensitivities of the chemo-resistant cells. As shown in Fig. 2d, PRIMA-1[MET] displayed anti-proliferative effects on both the parental and chemo-resistant cells. The IC$_{50}$ values of the NOS2, NOS2CR, and NOS2TR cells were 6.5, 7.4, and 8.8 μM, respectively. The IC$_{50}$ value of the NOS3CR cells was slightly higher than the values of the NOS3 and NOS3TR cells (not significant). PRIMA-1[MET] had sufficient growth-suppressing activity regardless of the mutation status of the TP53 and the chemo-sensitivity in the EOC cells.

PRIMA-1[MET] induces apoptosis in a dose-dependent manner in the EOC cells. We next performed an Annexin V-FITC/PI staining assay to investigate whether PRIMA-1[MET] actu-

ally induced apoptosis in the EOC cells. Treatment with PRIMA-1[MET] for 16 h against EOC cells, TOV21G and A2780, increased the fraction of early and late apoptotic cells (Fig. 3a). In the TOV21G cells, the fractions of early and late apoptotic cells were significantly increased from 1.1 and 4.3% following control vehicle treatment to 3.3 and 54.5% following 20 μM of PRIMA-1[MET] treatment, respectively (Fig. 3b). In the A2780 cells, the proportion of late apoptotic cells was significantly elevated from 5.3% following control vehicle treatment to 17.6% following 20 μM treatment (Fig. 3b). To determine whether PRIMA-1[MET] also induced apoptosis in chemo-resistant EOC cells, we evaluated cell apoptosis in another manner using fluorescence microscopy. The cells after a 24-h treatment with PRIMA-1[MET] were fixed with 4% paraformaldehyde, stained with Hoechst 33342, and then we identified apoptosis with fluorescence microscopy. The cells which had fragmented
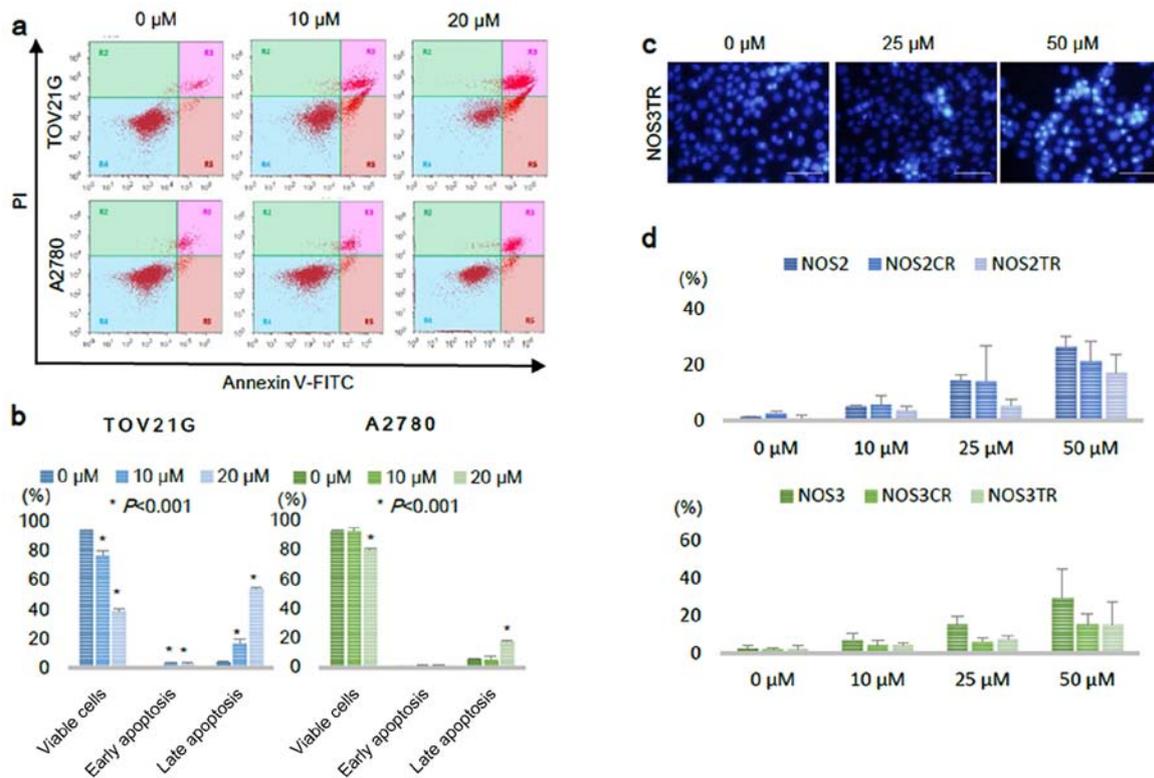
Figure 3. PRIMA-1[MET] induces apoptosis in a dose-dependent manner in EOC cell lines. (a) TOV21G and A2780 cells were treated with 0, 10, 20 μM of PRIMA-1[MET] for 16 h, and then stained with Annexin V-FITC and PI. The Annexin V-positive and PI-negative cells were defined as early apoptotic, and the Annexin V-positive and PI-positive cells were defined as late apoptotic (dead cells). (b) Viable, early apoptotic, and late apoptotic cells were quantified from triplicate samples. Asterisk indicates statistical significance (P<0.001). Error bars represent standard deviations. (c) Representative images of Hoechst 33342 staining of NOS3TR cells after 24 h of PRIMA-1[MET] treatment. Apoptotic cells were defined by their condensed and fragmented nuclei with fluorescence microscopy (scale bar, 200 μm). (d) Apoptosis levels of NOS2 and NOS3 cells, and their chemo-resistant cells after 0, 10, 25, 50 μM 20 h PRIMA-1[MET] treatment. Bars represent the percentages of apoptotic nuclei counted in each treatment group and are expressed as the mean ± SD.

or condensed nuclei were defined as undergoing apoptosis and counted manually with fluorescence microscopy (29). The representative images of condensed nuclei are shown in Fig. 3c. PRIMA-1[MET] treatment significantly increased the fractions of apoptotic cells with fragmented or condensed nuclei in both parental cells and their chemo-resistant cells in a dose-dependent manner (Fig. 3d). These results indicate that PRIMA-1[MET] induces apoptotic cell death in both chemo-sensitive and chemo-resistant EOC cells.

PRIMA-1[MET] activates PARP cleavage. In order to confirm whether PRIMA-1[MET]-induced cell death is apoptotic, we evaluated the apoptosis-related protein levels after treatment with PRIMA-1[MET] in EOC cells by western blot analysis. Immunoblot analysis elucidated that PRIMA-1[MET] induced dose-dependent PARP cleavage in the NOS2 and NOS3 cells and in their chemo-resistant cells (Fig. 4). This result showed that PRIMA-1[MET] induces apoptosis in EOC cells through PARP cleavage.

PRIMA-1[MET] increases intracellular ROS in EOC cells. As it was reported that PRIMA-1[MET] induces intracellular ROS accumulation, we investigated intracellular ROS accumulation using 5-6-chloromethyl-2'7'-dichlorodihydroflorescein diacetate, acetyl ester (CM-H$_2$DCFDA; Molecular Probes Invitrogen, Carlsbad, CA, USA) (20,30). PRIMA-1[MET] treatment for 24 h promoted intracellular ROS accumulation in the

NOS2 and NOS3 cells, and their chemo-resistant cells (Fig. 5a). To quantify the proportion of fluorescence-positive cells in the TOV21G cells after a 24-h treatment with PRIMA-1[MET], fluorescence activated cell sorting (FACS) was performed. The proportion of fluorescence-positive cells was increased in the cells treated with PRIMA-1[MET] in a dose-dependent manner, and the increase was significant (Fig. 5b and c). These results demonstrate that PRIMA-1[MET] promotes intracellular ROS accumulation in EOC cells.

ROS scavenger rescues apoptosis induced by PRIMA-1[MET]. To determine whether intracellular ROS accumulation by treatment with PRIMA-1[MET] induces apoptosis, we used a ROS scavenger N-acetyl cysteine (NAC). The compound NAC was added to cultured cells with 20 μM PRIMA-1[MET] medium at a final concentration of 10 mM. Sixteen hours after co-treatment with PRIMA-1[MET] and NAC, apoptotic cells were assessed by Annexin V-FITC and PI staining. The addition of NAC inhibited apoptosis and the growth-suppressing effect induced by PRIMA-1[MET] treatment (Fig. 6a and b). Furthermore, to examine the effect of PRIMA-1[MET] on the expression of antioxidant enzymes including Prx3 and GPx-1, which scavenge intracellular ROS to sustain homeostasis, we treated TOV21G and A2780 cells with PRIMA-1[MET] for 20 h and, thereafter, evaluated the mRNA levels of antioxidant enzymes by real-time RT-PCR. The mRNA levels of Prx3 and GPx-1 were significantly decreased after 20 h of treatment
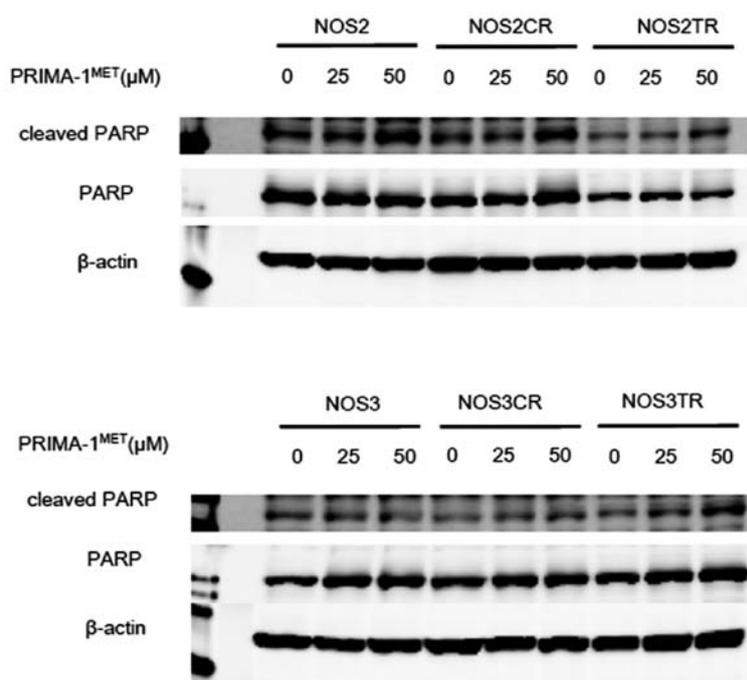
Figure 4. PRIMA-1[MET] induces PARP cleavage in EOC cells. NOS2 and NOS3 cells, and their chemo-resistant cells were treated with 0, 25 and 50 μM PRIMA-1[MET] for 24 h, and then lysed with lysis buffer. Cell lysates were subjected to western blot analysis. β-actin was used as a loading control.
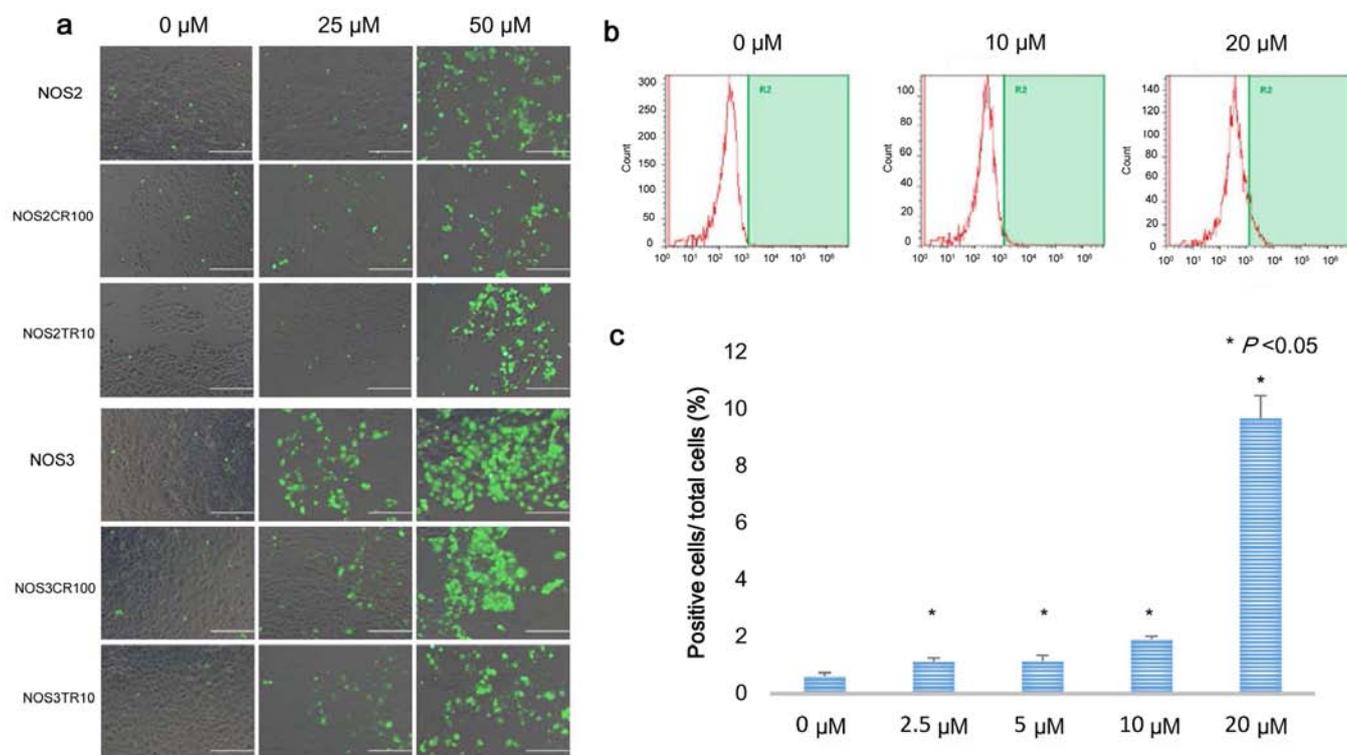

Figure 5. PRIMA-1[MET] induces intracellular ROS accumulation in EOC cells. (a) Intracellular ROS accumulation after 16 h of PRIMA-1[MET] treatment in the NOS2 and NOS3 cells, and their chemo-resistant cells. Intracellular ROS levels were detected by CM-H$_2$DCFDA, resulting in fluorescence positivity under fluorescence microscopy. (b) Dose-dependent intracellular ROS accumulation in TOV21G cells. TOV21G cells were maintained in medium with the indicated concentrations of PRIMA-1[MET], labeled with 5 μM CM-H$_2$DCFDA, and then subjected to flow cytometry. The percentage of cells with fluorescence intensity above the level of 1,000 FL was measured. (c) Bars represent the mean percentage of fluorescence-positive cells. Error bars represent standard deviations.

with PRIMA-1[MET] in a dose-dependent manner (Fig. 6c). Our results suggest that the antitumor effects of PRIMA-1[MET] may be mediated by intracellular ROS accumulation, and that the intracellular ROS accumulation and the cytotoxic effect induced by PRIMA-1[MET] may be due to downregulation of Prx3 and GPx-1.
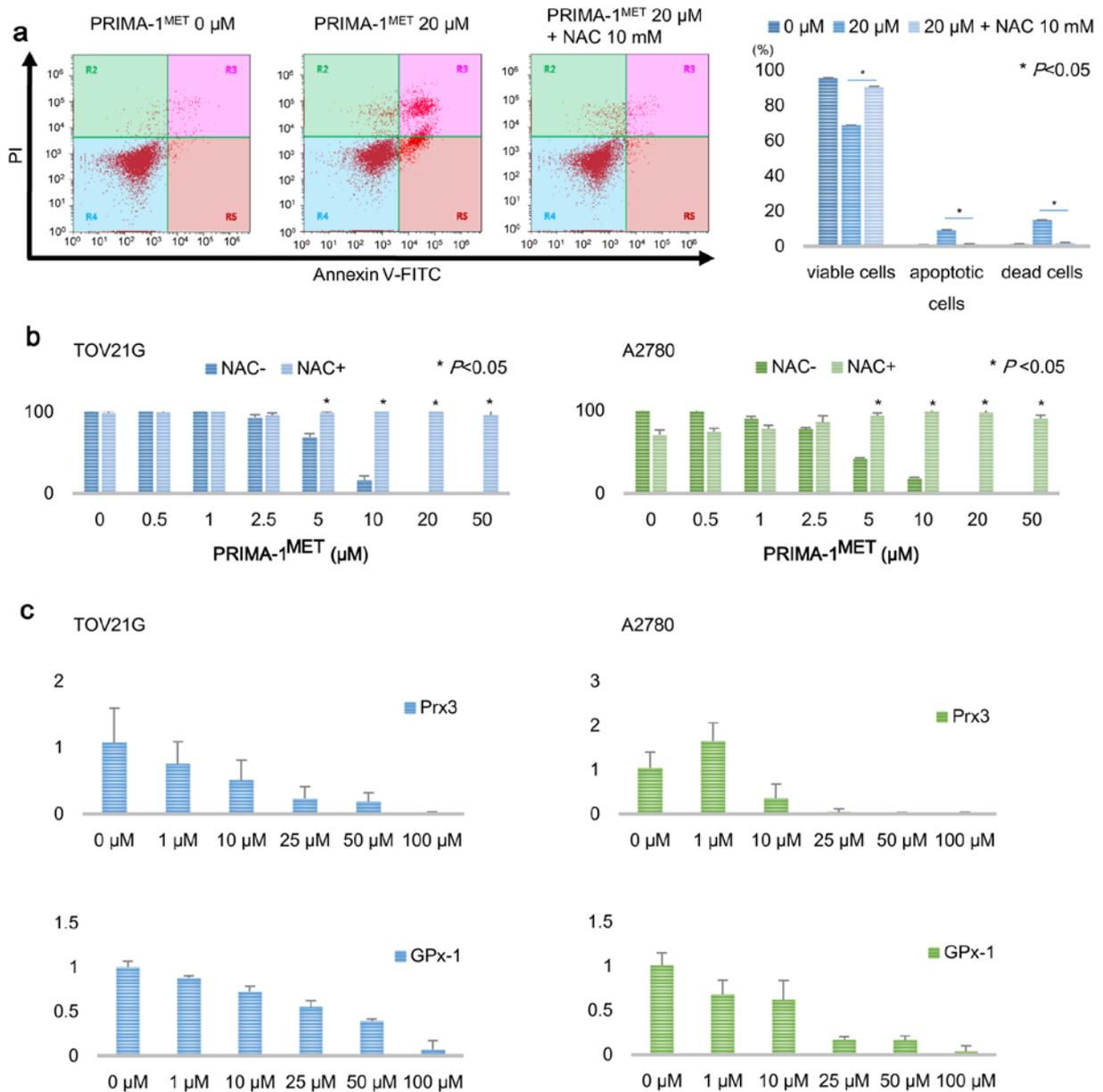
Figure 6. N-acetyl cysteine (NAC) inhibits the biological effect of PRIMA-1[MET]. (a) NAC completely blocked the cytotoxic effect of 16 h of PRIMA-1[MET] treatment in TOV21G cells, as shown by Annexin V-FITC and PI staining analysis. After 20 μM of PRIMA-1[MET] treatment with or without 10 mM NAC for 16 h, the fraction of viable, early apoptotic, and late apoptotic cells were examined. Bars represents the mean percentages. Error bars represent standard deviations. Asterisk indicates statistical significance (P<0.05). (b) Treatment with NAC prevented the PRIMA-1[MET]-induced growth suppressive effect in the TOV21G and A2780 cells according to the cell viability assay. After treatment with several concentrations of PRIMA-1[MET] with or without 10 mM NAC, viable cells were evaluated. Bars represents the mean percentages of viable cells. Error bars represent standard deviations. Asterisk indicates statistical significance (P<0.05). (c) Inhibition of antioxidant enzymes by PRIMA-1[MET]. TOV21G and A2780 cells were treated with PRIMA-1[MET] for 20 h and thereafter the mRNA levels of Prx3 and GPx-1 were evaluated by real-time RT-PCR. Data represent the mean ± SD from triplicate reactions.

Discussion

Most EOC patients experience recurrent disease, despite a high rate of complete clinical remission. Although recurrent EOC patients frequently receive chemotherapy, they are basically incurable due to the acquisition of chemo-resistance. Resistance to cytotoxic agents is a major obstacle to complete cure, and a number of attempts to overcome chemo-resistance have been made in EOC (31,32). While much effort has been made to restore chemo-sensitivity to resistant cells, no promising molecules have been identified. Thus, there is a need to develop novel therapeutics for EOC. Despite the fact that

PRIMA-1[MET] has been confirmed to exhibit tumor-suppressing effects on various types of cancer cells, there have been few reports on the effect of PRIMA-1[MET] on chemo-resistant cells in EOC (33-35). In our present study, we attempted to verify whether PRIMA-1[MET] has antitumor effects on EOC cells.

PRIMA-1[MET] is a prodrug converted to MQ with potential to bind to cysteine residues and change the conformation of the core domain of mutant p53 (30). PRIMA-1/PRIMA-1[MET] has been reported to synergize with cytotoxic agents to induce apoptotic cell death (33,36,37). Recently, Mohell et al reported that combined treatment with APR-246 and platinum or other drugs could give rise to an improved strategy for

recurrent high-grade serous ovarian cancer (37). In this study, chemo-resistant cells incubated with PRIMA-1^{MET} exhibited apoptosis, which was characterized by morphological features, such as chromosomal DNA condensation and fragmentation. Furthermore, the effect of PRIMA-1^{MET} on cell viability of chemo-resistant cells was similar to that of the parental cells with either wild-type p53 (NOS2) or mutant p53 (NOS3). These findings suggest that PRIMA-1^{MET} may have the possibility to be used for patients with chemo-resistant EOC bearing not only mutant p53 but also wild-type p53.

EOC cells easily spread to the peritoneal cavity and form disseminated metastases with a large amount of ascites. During this metastatic process, tumors may gradually acquire stem-like properties and become chemo-resistant. To our knowledge, there has been no report examining the efficacy of PRIMA-1^{MET} in cancer stem cells (or cancer stem-like cells). On the other hand, a recent study suggested the possibility that PRIMA-1^{MET} may overcome the chemo-resistance of EOC cells (37). Indeed, PRIMA-1^{MET} may be able to target cancer stem cells with chemo-resistant properties although additional studies are warranted.

In the present study, we investigated the efficacy of PRIMA-1^{MET} in the growth suppression and apoptosis induction in ovarian cancer cell lines (n=9) *in vitro*. We demonstrated that PRIMA-1^{MET} suppressed cell viability and induced massive apoptosis, regardless of the TP53 mutational status. Furthermore, ovarian cancer cell lines carrying wild-type p53 were slightly more sensitive than those carrying mutant p53 (not significant). To date, previous reports have shown that PRIMA-1/PRIMA-1^{MET} were more effective on pancreatic and small cell lung cancer cells expressing mutant p53 than on those expressing wild-type p53 or null (38,39). Interestingly, despite the fact that there is evidence that PRIMA-1^{MET} restores the wild-type p53 function to mutant p53, several recent studies have shown that PRIMA-1^{MET} displayed cytotoxic effects on Ewing sarcoma cells, acute myeloid leukemia cells, and human myeloma cells irrespective of the TP53 mutational status (40-42). This controversy is because PRIMA-1^{MET} not only restores wild-type p53 function to mutant p53, but also induces apoptosis in a p53-independent manner through intracellular ROS accumulation and endoplasmic reticulum (ER) stress (40,41). Indeed, in this study, we demonstrated that incubation with PRIMA-1^{MET} resulted in an antitumor effect with intracellular ROS accumulation in ovarian cancer cells, and co-treatment with PRIMA-1^{MET} and an ROS scavenger, NAC, blocked the cytotoxic effects, suggesting that the effects of PRIMA-1^{MET} are due to an intracellular ROS increase in EOC cells. Our results were partly consistent with previous reports, and support the antitumor effects of PRIMA-1^{MET} being universal irrespective of the TP53 mutational status in EOC cells. Unknown diverse mechanisms of PRIMA-1^{MET} may provide a convincing strategy for overcoming chemo-resistance in not only EOC but also other cancers.

ROS can generate oxidative stress in cells inducing DNA damage, protein degradation, peroxidation of lipids, and finally cell death at a high concentration. It is well known that cancer cells are normally more tolerant to high levels of oxidative stress than normal cells (43). One of the underlying mechanisms of cancer cells to survive under high oxidative condition is overexpression of antioxidant enzymes to scavenge

ROS (44). An inhibitor of glutathione synthesis, buthionine sulfoximine (BSO) was used in a clinical situation (45). In the present study, we demonstrated that PRIMA-1^{MET} induced intracellular accumulation and suppressed the expression of antioxidant enzymes, Prx3 and GPx-1, in EOC cells. Prx3 is one of the 2-Cys peroxiredoxin family (PRX 1-4), and operates as a reductase to metabolize ROS (46). Cunniff *et al* reported that knockdown of Prx3 increased oxidative stress and mitochondrial dysfunction in malignant mesothelioma cells, suggesting that Prx3 plays a critical role in cell cycle progression and sustaining the mitochondrial structure (47). Furthermore, a recent report by Song *et al* showed that Prx3 was highly upregulated in colon cancer stem cells, and that knockdown of Prx3 led to decreased cellular viability (48). In addition, several studies have already shown that GPx-1 protects cancer cells upon exposure to severe oxidative stress (49,50). According to our findings, PRIMA-1^{MET} suppressed the expression of both Prx3 and GPx-1, suggesting that it may induce an intracellular ROS increase mediated by downregulation of Prx3 and GPx-1.

To our knowledge, no previous studies have confirmed intracellular ROS accumulation in benign tumor cells or normal epithelial cells. In the present study, we did not evaluate whether PRIMA-1^{MET} induces intracellular ROS accumulation in such non-malignant or normal cells. In the living body, the majority of ROS generated by various stimulation can be degenerated by a higher antioxidant capacity derived from intrinsic ROS scavengers, resulting in weakened efficacy. We believe that the actual effects may depend on the local balance between ROS and such intrinsic scavengers. Certainly, PRIMA-1^{MET} may induce intracellular ROS accumulation in benign or normal cells as well as tumor cells. We speculate that the carcinogenetic effect of PRIMA-1^{MET} in such cells may be minimal. However, we cannot deny that administration of PRIMA-1^{MET} may have some risks. Therefore, further investigation concerning the effect of PRIMA-1^{MET} against benign or normal cells is necessary when used clinically.

In conclusion, we demonstrated that PRIMA-1^{MET} exhibited antitumor effects on chemo-resistant cells through intracellular ROS accumulation and repressed antioxidant enzymes. To utilize PRIMA-1^{MET} for EOC patients including chemo-resistant cases, we need to investigate further how PRIMA-1^{MET} suppresses Prx3 and GPx-1. PRIMA-1^{MET} is a promising compound for further development as a potential cytotoxic agent against EOC.

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