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PRIMA-1^{MET} inhibits growth of mouse tumors carrying mutant p53

Nicole Zache [a], Jeremy M.R. Lambert [a,b], Klas G. Wiman [a,*] and Vladimir J.N. Bykov [a]
[a] *Department of Oncology–Pathology, Karolinska Institutet, Cancer Centrum Karolinska (CCK), Karolinska University Hospital, Stockholm, Sweden*
[b] *International Agency for Research on Cancer (IARC), World Health Organization, Lyon, France*

Abstract. Reactivation of the tumor suppressor activity to mutant p53 should trigger massive apoptosis and eliminate tumors. The low molecular weight compounds PRIMA-1 and the structural analog PRIMA-1^{MET} reactivate human mutant p53 *in vitro* and suppress growth of human tumor xenografts in SCID mice. However, little is known about their effect on mouse mutant p53 in mouse tumor cells. We have examined the effect of PRIMA-1^{MET} on mouse sarcomas, mammary carcinomas and chemically induced fibrosarcomas. PRIMA-1^{MET} showed potent growth suppression in mutant p53-carrying mouse tumors *in vitro* and a significant anti-tumor effect in syngeneic mice *in vivo*. These results demonstrate that PRIMA-1^{MET} targets mouse tumors carrying mutant p53 and provide strong support for the anti-tumor efficiency of PRIMA-1^{MET} *in vivo*.

Keywords: Mutant p53, PRIMA-1^{MET}, mouse tumors, syngeneic mouse model, cancer therapy

1. Introduction

The p53 tumor suppressor is a potent inducer of cell cycle arrest and apoptosis in response to various forms of cellular stress. p53 is mutated in a wide variety of tumors [9,15,19], indicating a strong selection for inactivation of the p53 tumor suppressor pathway during tumor development. Mutations in p53 promote tumor cell survival and progression through evasion of apoptosis, and often render tumors resistant to chemotherapy and radiation therapy [16,25]. Such protective effect of the mutant p53 protein is attributed not only to the loss of tumor suppressor function due to mutation but also to the acquisition of novel oncogenic properties (gain-of-function) [12,22,23]. Thus, restoration of tumor suppressor activity to mutant p53 is likely to trigger robust apoptosis in tumor cells and benefit treatment of tumors that are resistant to current therapy [4].

Over the past years, several small molecules that restore wild type tumor suppressor activity to mutant p53 have been identified [5]. PRIMA-1 has been shown to rescue both transcription-dependent and transcription-independent p53-mediated apoptosis in human tumor cells [6,7,10,17,26]. PRIMA-1^{MET} is a methylated form of PRIMA-1 that is even more potent in inducing mutant p53-dependent apoptosis than PRIMA-1 itself [8]. Both PRIMA-1 and PRIMA-1^{MET} inhibit human tumor xenograft growth in SCID mice upon systemic administration [7,8].

It has remained unclear if PRIMA-1 and PRIMA-1^{MET} have any effect on mouse mutant p53 in mouse tumors. Therefore, we examined the effect of PRIMA-1^{MET} on a panel of mouse tumors of various origins. Moreover, to avoid the SCID xenograft tumor model, which has several limitations including the species difference between tumor xenograft and host, we determined whether PRIMA-1^{MET} can inhibit growth of mouse tumors *in vivo* in a syngeneic host.

2. Materials and methods

2.1. Cells and cell culture

Our panel of tumors and tumor lines includes the mouse sarcoma MC1M that has wild type p53 in its original solid form but carries V213M mutant p53 when converted to its ascites form [18]. We also used three different mouse mammary carcinomas, including TA3-Stockholm that harbors wild

*Corresponding author: Klas G. Wiman, Department of Oncology–Pathology, Karolinska Institutet, Cancer Center Karolinska (CCK), SE-171 76 Stockholm, Sweden. Tel.: +46 8 5177 9342; Fax: +46 8 32 10 47; E-mail: klas.wiman@ki.se.

type p53 and Ehrlich/ELD that expresses no p53 protein due to a splice acceptor site mutation [18]. The TA3-Hauschka carcinoma carries two p53 mutations, F110L and N236S, but it is unclear if they occur on the same or different alleles [18]. MC1M, TA3-Hauschka, TA3-Stockholm and Ehrlich/ELD are all ascites tumors that grow under highly anaerobic conditions. The MCO1 and MCO4 cell lines are derived from 3-methylcholanthrene-induced mouse fibrosarcomas [13]. MCO1 cells do not express any detectable p53 protein due to an in-frame translational termination codon [13]. MCO4 cells express two mutant p53 species, one carrying the G65R and R277I mutations and one carrying the R246L mutation [13]. MCO1 and MCO4 were grown at 37°C under 5% CO_2 in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 2.5 μM plasmocin. For all other cells Dulbecco's Modified Eagle's Medium was replaced by RPMI medium.

2.2. Determination of IC_{50} values

Cells were grown overnight in 96-well plates (3000 cells per well per 100 μl) at 37°C and treated with 0–100 μM of PRIMA-1^{MET} for 96 h. Ten μl of WST-1 reagent (Roche, Basel, Switzerland) were added to each well, samples were incubated for 1 h at 37°C and absorbance was measured at 490 nm. IC_{50} values (the concentration at which cell proliferation is inhibited by 50%) were determined using the WST-1 proliferation assay.

2.3. Trypan blue staining

Cells were plated (15,000 cells per cm^2) in 12-well plates, grown overnight at 37°C and treated with 0–30 μM of PRIMA-1^{MET} for 48 h. Cells were then harvested by trypsinization, centrifuged and resuspended in medium. Cells were stained with 0.2% trypan blue (Sigma, Sweden). Both dead and viable cells were counted using light microscopy.

2.4. Flow cytometry

Cells were plated (15,000 cells per cm^2) in 12-well plates, grown overnight at 37°C and treated with 0–30 μM of PRIMA-1^{MET} for 48 h. The proportion of cells in sub-G1 was then analyzed by flow cytometry. Briefly cells were harvested and fixed with 70% ethanol, treated with RNase (0.25 mg/ml) and stained with propidium iodide (0.05 mg/ml). Samples were incubated at 37°C for 30 min before analysis on a FACSCalibur flow cytometer (Becton Dickinson, CA, USA) according to standard procedures.

2.5. Western blotting

Cells were plated (15,000 cells per cm^2) in 6-well plates, grown overnight at 37°C and treated with 0–30 μM of PRIMA-1^{MET} for 20 h. After gel electrophoresis, filters were blotted with anti-PUMA rabbit polyclonal (#4976, Cell Signalling, MA, USA) and anti-β-actin mouse monoclonal (AC-15, Sigma, Sweden) antibodies. Anti-rabbit and anti-mouse HRP conjugates (Amersham Biosciences, Buckinghamshire, UK) and SuperSignal West Femto Maximum Sensitivity substrate (Pierce, Rockford, IL, USA) were used for signal development.

2.6. Real time PCR

Cells were plated (15,000 cells per cm^2) in 6-well plates, grown overnight at 37°C and treated with 0–30 μM of PRIMA-1^{MET} for 6 h. RNA was extracted using the RNeasy Mini Kit (Qiagen, Sweden) according to the manufacturer's instructions. cDNA was synthesized by first incubating RNA 5 min at 65°C with 1 μl Oligo dT (Invitrogen, Sweden) and 2 μl of 10 mM dNTP. Samples were then cooled on ice before adding 4 μl 5× first strand buffer, 2 μl of 0.1 M DTT and 1 μl RNase out (Invitrogen, Sweden) and incubating 2 min at 42°C. Finally, 1 μl of Supercript II (Invitrogen, Sweden) was added and samples were incubated 50 min at 42°C. Reactions were inactivated by 15 min incubation at 70°C. Real time PCR reactions were performed on 100 ng RNA. TaqMan Gene Expression Assay Mm00519268_m1 (PUMA) or Mm99999915_g1 (GAPDH) were used (Applied Biosystems, CA, USA). Amplification consisted of a first step with 2 min at 50°C and 10 min at 95°C followed by 40 cycles with 15 s denaturation at 95°C and 1 min annealing/extension at 60°C. Reactions were performed using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, CA, USA). Results were analyzed with the comparative Ct method using GAPDH as the endogenous control.

2.7. Toxicology

NMRI male mice were separated into 2 groups of 5 animals. One group received intraperitoneal (i.p.) injections of 100 mg/kg body weight PRIMA-1^{MET} in PBS at day 1, 3 and 5, while the other group received only PBS. The mg/kg dose was calculated from the average weight of 35 g. Animals were checked daily for signs of changes in food intake or activity, as signs of

general health status. Body weight was determined before the first injection and before the animal was sacrificed. At day 7 all animals were anaesthetized and 200 µl of blood were collected by orbital puncture to investigate hematological parameters. Animals were then sacrificed and an autopsy was performed to inspect the presence of pathological changes in the major organs.

2.8. In vivo assays

C3H/Hen mice were divided into 3 groups of 5 animals each and inoculated i.p. with 10^5 MC1M tumor cells. Seven days after inoculation the mice were treated with either PBS or 100 mg/kg of PRIMA-1^{MET} intravenously (i.v.) or i.p. once a day during 10 days. The mice perimeter (at midpoint of the body) and weight were monitored during the observation period, starting from the time of tumor inoculation. When the size of the largest tumor reached the permitted upper limit according to local ethical guidelines, all mice were sacrificed and ascites fluid was collected from each animal separately. Viable cells were counted by trypan blue staining as previously described.

Three groups of 5 Balb/c mice were inoculated subcutaneously with 5×10^5 MCO4 cells. Seven days after the inoculation, the mice were treated by i.v. administration in the tail vein with PBS or 25 mg/kg or 100 mg/kg PRIMA-1^{MET} once a day during 10 days. Weights and tumor sizes were monitored during the observation period, starting from the time of inoculation. Tumors were excised and immediately fixed in 4% formaldehyde for 12 h and kept at +4°C in 70% ethanol before being embedded into paraffin. Hematoxylin and eosin staining was performed according to a standard protocol. *In situ* detection of apoptotic cells by TUNEL was performed according to the manufacturer's instructions (Roche Applied Science, Germany).

2.9. Statistical analysis

All data were analyzed using the Microcal Origin statistical software (OriginLab, MA, USA). Results are shown as mean ± s.e.

3. Results

We treated the mouse tumors and tumor lines (Table 1) with PRIMA-1^{MET} and determined IC_{50} values (concentration that causes 50% growth inhibition) using the WST-1 proliferation assay (Fig. 1(a) and (b)). The mutant p53-carrying MC1M sarcoma was most sensitive to PRIMA-1^{MET} treatment, whereas TA3-Stockholm, TA3-Hauschka and Ehrlich/ELD were less sensitive (Fig. 1(a)). We observed similar IC_{50} values for TA3-Hauschka that carries mutant p53 and the wild type p53-carrying TA3-Stockholm. The reason could be that the particular mutant or mutants carried by TA3-Hauschka, F110L and/or N236S, are not amenable to reactivation by PRIMA-1^{MET}. Figure 1(b) shows that the mutant p53-carrying MCO4 cell line has a higher sensitivity to PRIMA-1^{MET} treatment than the p53 null MCO1 cell line. After 48 h incubation with 30 µM of PRIMA-1^{MET} 45.8% of the MCO4 cells were dead according to trypan blue staining whereas only 11.6% of the MCO1 were dead under the same

Table 1
Mouse tumors and tumor lines with different p53 status

Name	P53 status	Mouse strain	Type	Codon	Amino acid	Tumor
MC1M	mt	C3H/Hen	Ascites	213	Val-Met	Sarcoma
TA3-Hauschka	wt/mt	A/Snell	Ascites	110	Phe-Leu	Mammary
				236	Asn-Ser	carcinoma
TA3-Stockholm	wt	A/Snell	Ascites	–	–	Mammary
						carcinoma
Ehrlich/ELD	null	Non-inbred	Ascites	Deletion*	Splice acceptor	Mammary
					site mutation	carcinoma
MCO1	null	Balb/c	Solid	–	–	Fibrosarcoma
MCO4	mt	Balb/c	Solid	277	Arg-Ile	Fibrosarcoma
				65	Gly-Arg	
				246	Arg-Leu	

*A to C base substitution in highly conserved dinucleotide AG, the splice acceptor site of exon 9.

Abbreviations: mt, mutant; wt, wild type.

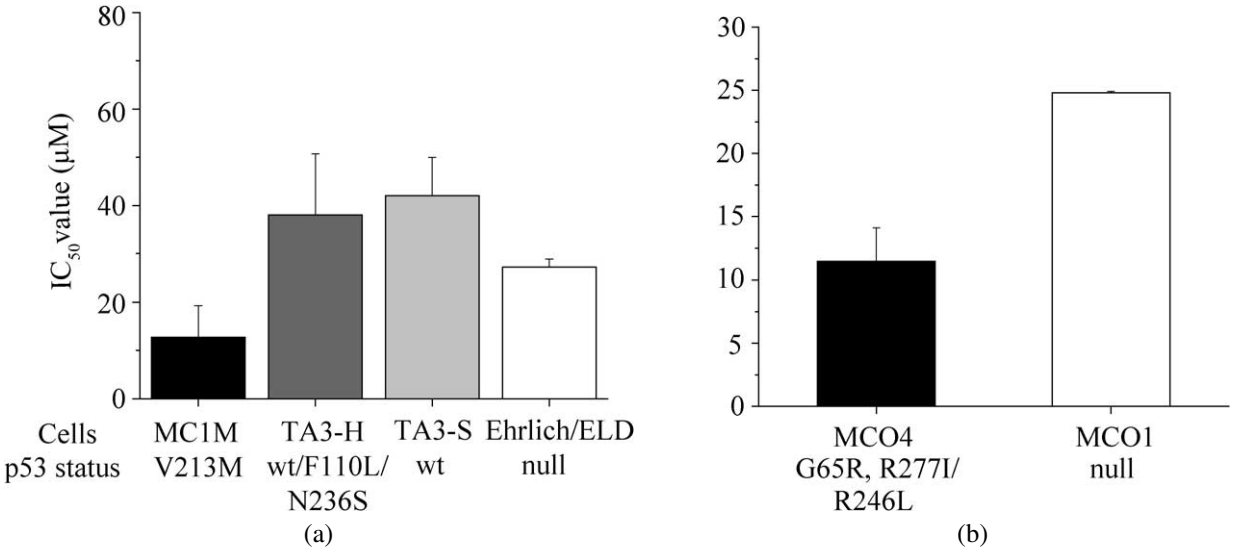

Fig. 1. PRIMA-1^{MET} inhibits growth of different mouse tumors and tumor cell lines. (a) The MC1M sarcoma carrying mutant p53 is most sensitive to PRIMA-1^{MET} ($IC_{50} = 12 \pm 6.6$ μM), whereas the wild type p53-carrying tumor TA3-Stockholm (TA3-S) is least sensitive (42 ± 8.0 μM) among all tested tumors. Neither TA3-Hauschka (TA3-H) with an IC_{50} value of 38 ± 12.7 μM nor Ehrlich/ELD with a value of 27.3 ± 1.6 μM are as sensitive as MC1M. (b) MCO1 cells containing no p53 protein are less sensitive to PRIMA-1^{MET} (24.8 ± 2.7 μM) than the mutant p53-harbouring MCO4 cell line (11.4 ± 0.1 μM).

conditions (Fig. 2(a)). Real time PCR showed a mutant p53 specific upregulation of the proapoptotic p53 target gene PUMA. We observed a ∼1.3 fold upregulation of PUMA mRNA levels in MCO4 cells after 6 h incubation with 10 μM PRIMA-1^{MET}, but not in MCO1 cells. Western blotting showed PUMA upregulation at the protein level in a mutant p53 dependent manner in full agreement with the real time PCR data (Fig. 2(c)). DNA fragmentation assessed by FACS analysis and PUMA upregulation assessed by Western blotting and by real time PCR suggest that the PRIMA-1^{MET}-treated cells die by apoptosis. The fact that only a fraction of the dead cells have a sub-G1 DNA content could indicate cell death in the absence of substantial DNA fragmentation, as previously reported [14,21]. Overall these results demonstrate that PRIMA-1^{MET} is not only effective against human tumor cells carrying mutant p53, but also against several mutant p53-expressing mouse tumors and cell lines.

Next we investigated whether PRIMA-1^{MET} can suppress growth of MC1M and MCO4 cells *in vivo* in syngeneic mice. MC1M cells were grown as ascites tumors in C3H/Hen mice. We treated the mice daily with 100 mg/kg PRIMA-1^{MET} intravenously (i.v.) or intraperitoneally (i.p.) for 10 days. Tumor growth was assessed by measuring the abdominal perimeter of the mice. As shown in Fig. 3(a), PRIMA-1^{MET} resulted in a significant inhibition of tumor growth whereas PBS treatment had no effect. To obtain a more exact measurement of tumor growth, we counted viable ascites cells from each mouse by trypan blue staining. The mean number of viable tumor cells in the PBS-treated control mice was $(9.8 \pm 1.9) \times 10^7$, whereas the mean number of tumor cells in PRIMA-1^{MET}-treated mice was $(4.9 \pm 4.6) \times 10^6$ (i.v.) and $(1.1 \pm 1.1) \times 10^7$ (i.p.) (Fig. 3(b)). Thus, PRIMA-1^{MET} treatment i.v. or i.p. caused a marked inhibition of tumor growth in this ascites tumor model. The effect was statistically significant at $p < 0.05$, according to the independent t-test. Treatment with PRIMA-1^{MET} did not result in any weight loss.

Our toxicology study in NMRI mice did not reveal any weight loss during the course of treatment with PRIMA-1^{MET} treatment. None of the animals treated with 100 mg/kg of PRIMA-1^{MET} showed any signs of discomfort or changes in behavior during the study. No significant differences in hematological parameters we observed between this group and the control group treated with PBS (Table 2). Finally no pathological changes in liver, kidneys, heart, stomach, spleen and lungs could be detected at autopsy.

We also inoculated mutant p53-harboring MCO4 cells in Balb/c mice and treated daily with PBS or 25 mg/kg or 100 mg/kg PRIMA-1^{MET} i.v. for 10 days. Treatment with 25 mg/kg PRIMA-1^{MET} resulted in a noticeable but not statistically significant inhibition of

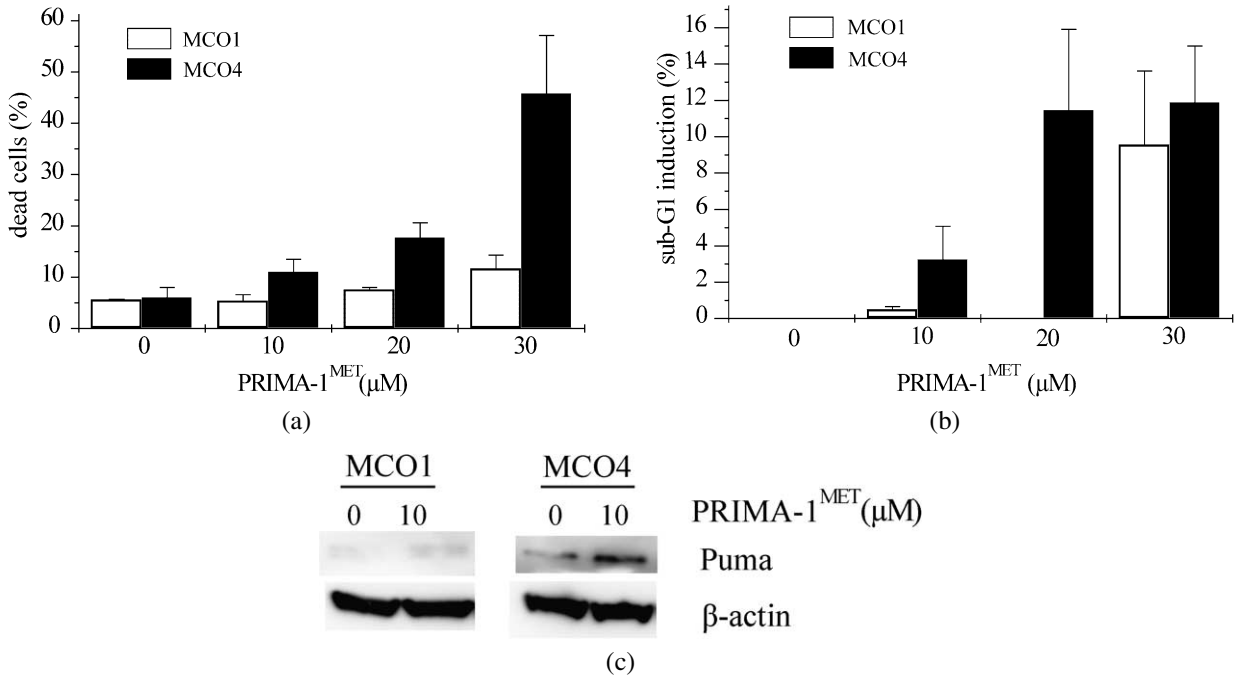

Fig. 2. PRIMA-1^{MET} induces cell death in MCO4 cells. The proportion of dead cells after 48 h of treatment was analysed (a) by trypan blue staining to detect all dead cells and (b) by flow cytometry to assess the sub-G1 population. (c) Western blot analysis of PUMA revealed upregulation in MCO4 but not in MCO1 cells upon PRIMA-1^{MET} treatment.

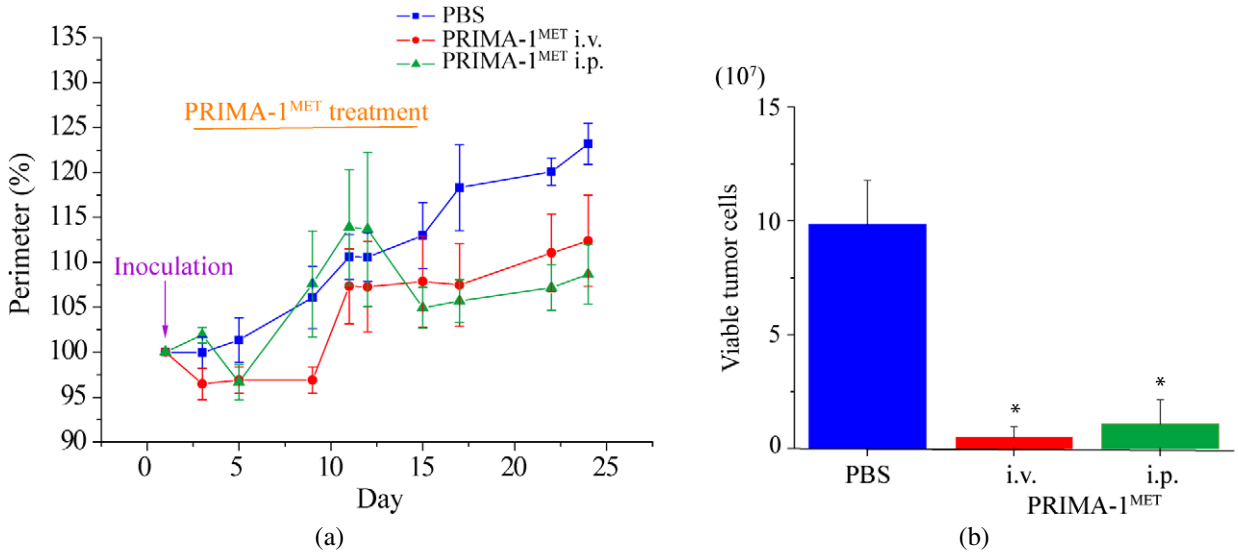

Fig. 3. Treatment with PRIMA-1^{MET} i.v. or i.p. inhibits ascites tumor growth in syngeneic mice. (a) The abdominal perimeter was measured to assess ascites tumor growth throughout the experiment. Increase in the perimeter size was more important in PBS treated cells compared to PRIMA-1^{MET} treated (b) PRIMA-1^{MET} i.v. and i.p. reduced the number of viable tumor cells by ∼20- and ∼9-fold, respectively, as compared to treatment with PBS.

tumor growth as compared to the PBS-treated group. However, treatment with 100 mg/kg of PRIMA-1^{MET} caused a marked suppression of tumor growth compared to the control group that was statistically significant from day 9 ($p < 0.05$, independent t-test) (Fig. 4(a)). Again, PRIMA-1^{MET} treatment did not result in any weight loss or any obvious toxicity. We further investigated morphological changes in the tu-

Table 2
PRIMA-1 ^{MET} toxicology data

	PBS	PRIMA-1^{MET} (100 mg/kg)
Weight, day 1 (g)	35.4 ± 1.0	35.3 ± 1.8
Weight, day 7 (g)	38.3 ± 1.1	38.1 ± 1.9
White blood count (10^9/l)	8.40 ± 0.64	9.52 ± 1.90
Red blood count (10^{12}/l)	9.58 ± 0.17	10.02 ± 0.31
Heamoglobin (g/l)	151.8 ± 3.6	157.4 ± 3.6
Hematocrite	0.488 ± 0.009	0.484 ± 0.015
Tronibocytei (10^9/l)	1174 ± 53	1060 ± 73

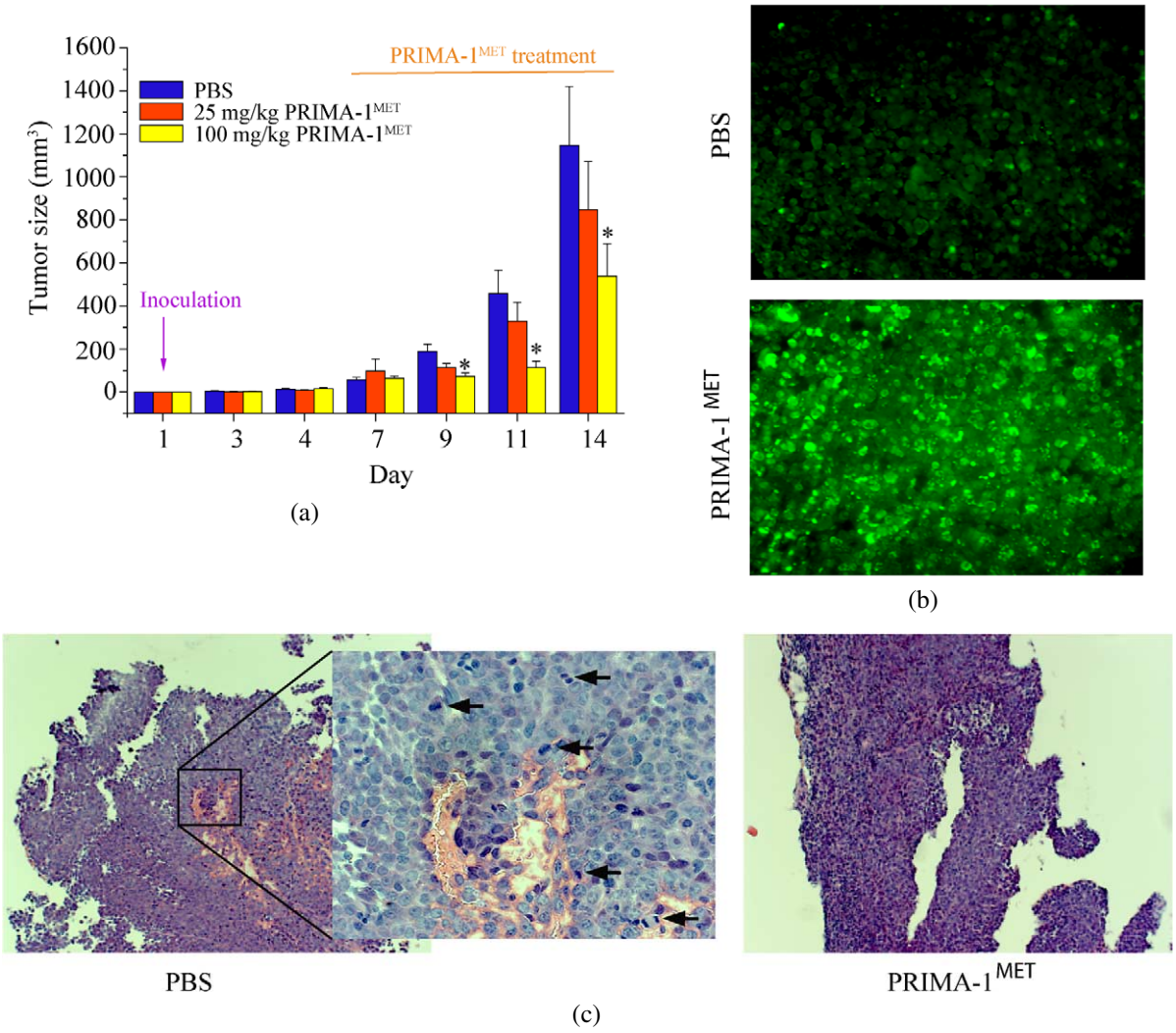

Fig. 4. PRIMA-1^{MET} inhibits MCO4 tumor growth *in vivo* in a dose-dependent manner. (a) Treatment with 25 mg/kg PRIMA-1^{MET} resulted in a small but not statistically significant inhibition of tumor growth, whereas 100 mg/kg PRIMA-1^{MET} caused significant inhibition of tumor growth from day 9 until day 14, as compared to control-treated mice. (b) 100 mg/kg of PRIMA-1^{MET} induced massive apoptosis in MCO4 tumor tissues according to TUNEL staining. In contrast, tumor sections from PBS-treated mice were TUNEL-negative, indicating absence of apoptosis. (c) Tumor sections stained with hematoxylin and eosin. PRIMA-1^{MET}-treated tumors showed significantly fewer mitotic figures and decreased vascularization as compared to the tumors from PBS-treated mice. Black arrows indicate mitotic cells.

mors of control and PRIMA-1^{MET}-treated mice. Interestingly, TUNEL staining revealed a higher fraction of apoptotic cells in the tumors from mice treated with PRIMA-1^{MET} (100 mg/kg) than in the control mice (Fig. 4(b)). Hematoxylin and eosin staining showed less frequent mitotic figures in the tumors of the PRIMA-1^{MET}-treated mice (Fig. 4(c)), indicating that PRIMA-1^{MET} treatment had an anti-proliferative effect on tumors *in vivo*. We also observed fewer blood vessels in the tumors from PRIMA-1^{MET}-treated mice. Thus, PRIMA-1^{MET} showed anti-tumor activity on both MC1M sarcoma and MCO4 fibrosarcoma cells grown in syngeneic mice.

4. Discussion

While previous studies have demonstrated that PRIMA-1 and PRIMA-1^{MET} can reactivate human mutant p53 and induce apoptosis in human tumor cells, the effect of PRIMA-1 and PRIMA-1^{MET} on mouse mutant p53 in mouse tumor cells has not been addressed. Our results presented here clearly demonstrate that PRIMA-1^{MET} also targets mouse tumor cells carrying endogenous mutant p53. This is consistent with the close structural similarity between human and mouse p53, and suggests that PRIMA-1^{MET} triggers a similar apoptotic program in human and mouse tumor cells.

Both PRIMA-1 and PRIMA-1^{MET} can inhibit human tumor xenograft growth in SCID mice [7,8]. Unfortunately, the SCID model is limited by the lack of an adequate tumor microenvironment [1], and SCID mice are immunodeficient. Furthermore, as result of the tumor-host species barrier, it is possible that an observed inhibition of human xenograft tumor growth in SCID mice in the absence of detectable general toxicity is due to selective effects of the test substance on human cells, rather than specific targeting of the tumor cells. Another flaw of the model is the use of tumor cell lines with an uncertain molecular relationship to the original human tumors from which they were derived. The use of mouse tumors in syngeneic mice overcomes the species barrier problem and allows testing of novel compounds in the presence of a functional immune system and in the context of normal vascularization and extracellular matrix [3,24]. Therefore, our findings described here reinforce and extend our previous observations in SCID mice showing that systemic treatment with PRIMA-1 or PRIMA-1^{MET} can suppress tumor growth *in vivo*.

MC1M and MCO4 carry p53 point mutations that correspond to mutations occurring in human tumors. Mouse V213 corresponds to codon 216 in human p53. V216 is located in the β-sandwich (S7 strand) of the core domain and substitution of this residue will most likely affect DNA binding indirectly by structural destabilization of the core. Codon 216 is mutated at low frequency in human tumors (http://www-p53.iarc.fr/) [19]. Mouse R246 corresponds to human R249 which is situated in the L3 loop in the core domain. This residue is frequently mutated to Ser in hepatocellular cancer (R249S), a classic fingerprint of aflatoxin exposure linked to this disease [20]. Substitution at R249 destabilizes the L3 loop that forms the DNA binding surface in p53 [2]. Codon R249 mutations are found in human tumors at a frequency of around 3%, making this site a hot-spot for mutations. Residue 280 in human p53, corresponding to R277 in mouse p53, is located in the H2 helix domain and makes direct contacts with DNA; substitution of this residue compromises interactions between p53 and DNA [11]. R280 is mutated in about 1% of human tumors, particularly in tumors of the bladder (5%), gallbladder (5%), corpux uteri (3%), prostate (2%) and head and neck (2%) (http://www-p53.iarc.fr/). Thus, our results show that PRIMA-1^{MET} has activity *in vivo* against mouse tumors harboring p53 mutations that are common in human cancer.

According to the IC_{50} values obtained *in vitro*, MC1M and MCO4 cells show similar sensitivity to PRIMA-1^{MET} treatment. However, the MC1M ascites tumor cells appeared to be more sensitive than MCO4 cells *in vivo*. This difference in *in vivo* sensitivity between the two tumors is probably due to better penetration of PRIMA-1^{MET} in MC1M ascites tumor cells than in the MCO4 cells grown subcutaneously.

In conclusion, we have shown that PRIMA-1^{MET} inhibits growth of mutant p53-carrying mouse tumors *in vitro* and *in vivo* in syngeneic mice without any obvious toxicity. This emphasizes the potential of PRIMA-1^{MET}-based compounds for treatment of tumors that express mutant p53. The mouse tumor models described here may be useful for the further development of such compounds towards clinical trials in cancer patients.

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