**In vitro and in vivo cytotoxic effects of PRIMA-1 on hepatocellular carcinoma cells expressing mutant p53ser249**

Hong Shi1, Jeremy M.R.Lambert1,2, Agnes Hautefeuille1, Vladimir J.N.Bykov2, Klas G.Wiman2, Pierre Hainaut1,4, and Claude Caron de Fremontel3

1International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France; 2Department of Oncology-Pathology, Cancer Center Karolinska, Karolinska Institutet, Karolinska University Hospital, 171 77 Stockholm, Sweden; and 3Unité Institut National de la Santé et de la Recherche Médicale U590, Université Lyon-1, Centre Léon Bérard, 28 rue Laennec, 69373 Lyon Cedex 08, France

Email: hainaut@iarc.fr

**Hepatocellular carcinoma** (HCC) is highly lethal due to limited curative options. In high-incidence regions, such as parts of Africa and Southeastern Asia, >50% of cases carry an AGG to AGT mutation at codon 249 of the **TP53** gene, considered as a ‘signature’ of mutagenesis by aflatoxins. The protein product, p53ser249, may represent a therapeutic target for HCC. The small molecule p53 reactivation and induction of massive apoptosis (PRIMA)-1 has been shown to induce apoptosis in tumour cells by reactivating the transactivation capacity of some p53 mutants. In this study, we have investigated the cytotoxic effects of PRIMA-1 on HCC cells expressing p53ser249. In p53-null Hep3B cells, over-expression of p53ser249 or p53gln248 by stable transfection increases the cytotoxicity of PRIMA-1 at 50 μM. Furthermore, PRIMA-1 treatment delayed the growth of p53ser249-expressing Hep3B cells xenografted in severe combined immunodeficiency mice. However, PRIMA-1 did not restore wild-type DNA binding and transactivation activities to p53ser249 or to p53gln248 in Hep3B cells. Moreover, in PLC/PRF/5, a HCC cell line constitutively expressing p53ser249, small interfering RNA (siRNA) silencing of the mutant increased the cytotoxic effect of PRIMA-1. These apparently contradictory effects can be reconciled by proposing that p53ser249 exerts a gain-of-function effect, which favours the survival of HCC cells. Thus, both inhibition of this effect by PRIMA-1 and removal of the mutant by siRNA can lead to the decrease of survival capacity of HCC cells.

**Introduction**

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world. Its incidence varies greatly in different geographical areas, with >30% of new cases occurring in less-developed regions, including eastern and Southeastern Asia as well as sub-Saharan Africa (1). Infection by hepatitis B virus (HBV) and dietary contamination by the mycotoxin aflatoxin B1 (AFB1) are two major risk factors in these high-incidence areas (2). The fatality of HCC is notoriously high with its mortality rate being almost equal to its incidence (3). Effective therapies, including surgical resection, liver transplantation and percutaneous ablation, are only feasible at an early stage of the tumour (3). However, most cases are diagnosed at a late stage because of the asymptomatic nature of early HCC and of the poor access to health care in high-incidence areas. Transarterial embolization can serve as a palliative therapy for patients unsuitable for the above treatments, but it is only applicable when the patients have a good liver function and a patent portal vein (4), which is not the case in most patients since HCC often develops from livers with other pathological changes such as cirrhosis or chronic hepatitis. Furthermore, HCC is highly resistant to systemic chemotherapy (5), and whole-liver radiotherapy frequently induces serious liver injury (6), making these two conventional therapies ineffective or unfeasible in the case of HCC. Therefore, most HCC patients cannot benefit from the effective therapeutic methods currently available and there is an urgent need to develop and test new approaches to treat this cancer.

A better understanding of the patterns of genetic alterations in hepatocarcinogenesis may open paths for developing novel-targeted therapies. A promising target in this respect is p53ser249. The latter is the protein product of an AGG (encoding an arginine) to AGT (encoding a serine) transversion at codon 249 of the **TP53** tumour suppressor gene or the R249S mutation. This mutation is present in >50% of subjects with HCC in a context of high exposure to AFB1 and chronic HBV carriage and accounts for 90% of total **TP53** mutations in high-incidence areas (7–9). Moreover, R249S mutation has been shown to be induced by the binding of AFB1 metabolites to the third base of codon 249, resulting in a G to T transversion (10,11). Targeting p53ser249 may thus represent a sensitive and specific approach to induce cytotoxic effects in HCC cells harbouring the R249S mutation.

Wild-type **TP53** exerts its antitumour function through its protein product, p53, that can be stabilized and can be activated by diverse stress stimuli including DNA damage, oncogene activation and hypoxia. Activated p53 can induce cell-cycle arrest, apoptosis or senescence by transactivating a large panel of target genes involved in these cellular responses (reviewed in ref. 12). Mutations in **TP53** are found in 5–70% of human cancers, depending on site, histology and stage (IARC TP53 Mutation Database [R1]), http://www-p53.iarc.fr, (13)). Many of these mutations are missense and lead to the synthesis of structurally altered proteins that have lost their transactivation activity due to single amino acid changes, resulting in impaired tumour-suppressing function (14). Recently, the small molecule, p53 reactivation and induction of massive apoptosis (PRIMA)-1 has been identified as suppressing function (14). The anti-proliferative effect of PRIMA-1 exerted its antitumour function through its protein product, p53, that can be stabilized and can be activated by diverse stress stimuli including DNA damage, oncogene activation and hypoxia. Activated p53 can induce cell-cycle arrest, apoptosis or senescence by transactivating a large panel of target genes involved in these cellular responses (reviewed in ref. 12). Mutations in **TP53** are found in 5–70% of human cancers, depending on site, histology and stage (IARC TP53 Mutation Database [R1]), http://www-p53.iarc.fr, (13)). Many of these mutations are missense and lead to the synthesis of structurally altered proteins that have lost their transactivation activity due to single amino acid changes, resulting in impaired tumour-suppressing function (14). Recently, the small molecule, p53 reactivation and induction of massive apoptosis (PRIMA)-1 has been identified as preferentially inhibiting the proliferation of tumour cells expressing a mutant p53, as compared with those deficient for **TP53** or with those expressing wild-type p53 (15,16). The anti-proliferative effect of PRIMA-1 is attributed to its capacity to induce apoptosis in a mutant p53-dependent manner (16). There is some evidence that PRIMA-1 may restore wild-type transcriptional transactivation function to mutant p53 proteins, notably to some of those commonly found in human cancers, and to induce the expression of p53-target genes such as wild-type p53-activated fragment 1 (WAF1), Bcl2-associated X protein and p53-up-regulated modulator of apoptosis (PUMA) (16,17). Moreover, in vivo tests using severe combined immunodeficiency (SCID) mice carrying human tumour xenografts have provided support for a mutant p53-specific antitumour effect of PRIMA-1 (16).

In the present study, we have analysed the capacity of PRIMA-1 to exert cytotoxic effects on HCC cell lines expressing p53ser249. We reasoned that, because of the high prevalence of **TP53** codon 249 mutation in HCC in low-resource countries, reactivating the wild-type antitumour function of p53ser249 with PRIMA-1 may provide a novel, affordable and specific approach to treat or to limit the progression of HCC in a considerable percentage of patients who cannot benefit from currently available treatments. However, the effect of PRIMA-1 on the p53ser249 mutant and on HCC cells carrying this mutant has so far not been tested. Therefore, we evaluated the mutant p53-dependent and -independent cytotoxicity of PRIMA-1 on p53ser249-expressing HCC cells. We have used a previously developed cellular model based on the stable transfection of the p53-null HCC cell line Hep3B with either p53ser249 or p58gln248 mutant H.Shi, K.Szymanska, O.Galy, J.Lambert, V.J.Bykov, L.Egevad,

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C. Caron de Fromentel, P. Hainaut, submitted for publication) as well as PLC/PRF/5, a cell line that constitutively expresses p53ser249. Our results show that PRIMA-1 exerts p53-dependent and -independent cytotoxic effects, and that expression of p53ser249 confers an increased survival capacity that is inhibited by treatment with PRIMA-1. These observations are compatible with the notion that p53ser249 may have a gain-of-function effect on HCC cells.

Materials and methods

Cell lines, transfections and chemicals

Hep3B cells (ATCC HB-8064) do not express p53 protein due to a large deletion of the TP53 gene (18). Both Mahlavu (19) and PLC/PRF/5 (ATCC CRM-8024) (20) express an endogenous p53ser249 mutant. The above cell lines were cultured at 37°C under 5% CO2 in minimum essential medium (Invitrogen, San Diego, CA) supplemented with 10% fetal calf serum (Invitrogen), 100 IU/ml penicillin/100 µg/ml streptomycin/2 mM glutamine (Sigma-Aldrich, Gillingham, Dorset, UK), non-essential amino acids and 1 mM sodium pyruvate (both from Invitrogen). The development of Hep3B stable clones expressing p53ser249 or p53gln248 is described elsewhere (H. Shi, K. Szymanska, O. Galy, J. Lambert, V. J. Bykov, L. Egevad, C. Caron de Fromentel, P. Hainaut, submitted for publication). Briefly, Hep3B cells were first transfected with a control vector pcDNA4/TO (Invitrogen) or with a pcDNA4/TO vector containing a cDNA fragment that encodes either p53ser249 or p53gln248. Individual clones were isolated after selection with 50 µg/ml Zeocin (InvivoGen, San Diego, CA) and were cultivated in the presence of 25 µg/ml Zeocin. Small interfering RNA (siRNA) silencing of p53ser249 in PLC/PRF/5 cells was carried out in six-well plates at 1.5 × 105 cells per well. The next day, cells were transfected using 6 µl HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) with 5 nM p53-specific siRNA duplex (5'-CAA UGG GCC ACC GAA GCC GAT CTT-3' and 5'-GGU CUU CAG UAC ACC AUU CTT-3') or a control siRNA duplex (5'-CAU AGA UGA CCG UAG CTA GAC ATG G-3' and 5'-GUCUCAGGUAUCAUUGT3') (Euorgenet, Seraing, Belgium). Two subsequent transfections at a 1 day interval were performed to obtain maximum p53 inhibition. PRIMA-1 was produced by Syngene (Bangalore, India) and was dissolved in 100% dimethyl sulphoxide (DMSO) for cellular treatments and in phosphate-buffered saline (PBS) for mouse in vivo assays.

Flow cytometry

For Hep3B stable clones, cells were seeded in 60 mm dishes at 3 × 103 cells per dish (~1.5 × 105 cells/cm2) and the next day were treated with PRIMA-1 (25 or 50 µM) or with equivalent volume of DMSO as control for 48 h. For PLC/PRF/5, cells were treated 1 day after two subsequent siRNA transfections with 25 µM PRIMA-1 or with equivalent volume of DMSO for 24 h. After being harvested, both adherent and floating cells were processed using CycleTest™ Plus DNA Reagent Kit (Becton Dickinson, Franklin Lakes, NJ) according to the manufacturer’s instructions. Flow cytometry analyses were performed using a FACSCalibur cytometer (Becton Dickinson). Data were analysed using CellQuest™ software (BD Bioscience, Bedford, MA).

Trypan blue exclusion assay

Hep3B stable clones were seeded in six-well plates in triplicates at three different densities (1, 2 and 3 × 105 cells per well, equivalent to 1, 2 and 3 × 106 cells/cm2) and were treated the next day with 50 µM PRIMA-1. After 0 and 24 h of treatment, cells were stained with 0.2% trypan blue (Invitrogen). Both viable and dead cells were enumerated using light microscopy.

In vivo assay

All experiments with mice were approved by the local animal ethical committee in Stockholm, Sweden, and animal care was in accordance with institutional guidelines. Hep3B stable clones (248/3 or 249/6) were first re-suspended in PBS-containing 50% Matrigel™ basement membrane matrix (BD Bioscience) at a concentration of five million cells/100 µl. Cellular suspensions were then injected subcutaneously into the right flank of 8-week-old SCID mice, with five million cells per mouse. Twelve mice were used for each clone. PRIMA-1 treatment began 10 days after the injection of the cells. Six, mice inoculated with each clone received one dose (100 mg/kg) per day of PRIMA-1 diluted in 100 µl of PBS through intravenous injection for 5 days on two consecutive weeks (total, 10 injections). The other six mice received 100 µl of PBS as controls. During the whole experiment, body weight and tumour size were measured every 2–3 days and tumour volumes were estimated (length × width × height). Mice were killed when tumours reached 1 cm3.

TransAM™ enzyme-linked immunosorbent assay

Hep3B stable clones were seeded at 9 × 105 cells/100 mm dish (~1.5 × 106 cells/cm2) 1 day before treatment and then 25 µM PRIMA-1 was applied. Cells were harvested 0, 16 and 24 h after treatment and lysed in electrophoretic gel mobility shift assay buffer containing 20 µM 2-n-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, pH 7.6, 10 mM NaCl, 1.5 mM MgCl2, 0.2 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 20% glycerol, 0.1% NP40 and protease inhibitors. Nuclei were then isolated by centrifugation and lysed in electrophoretic gel mobility shift assay buffer (the same composition as buffer A except for 500 mM NaCl instead of 10 mM). Nuclear extracts were then clarified by centrifugation. The assays were performed using the TransAM™ p53 Transcription Factor Assay Kit (version C) (Active Motif, Carlsbad, CA) following the manufacturer’s instructions.

Reverse transcription and quantitative polymerase chain reaction

Hep3B stable clones were seeded in 100 mm dishes at 1.2 × 106 cells per dish (~2 × 107 cells/cm2). The next day they were treated with 50 µM PRIMA-1 and were harvested at 0, 12 and 24 h after treatment. Total cellular RNA was extracted using Nucleospin® RNA II Total RNA Isolation Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. Reverse transcription was performed using SuperScript™ II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA). For WAF1 and cyclooxygenase-2 (COX-2), quantitative polymerase chain reaction (QPCR) was performed using TaqMan® Gene expression assays from Applied Biosystems (Foster City, CA), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control (assay ID: Hs00355782_m1 for GAPDH). For WAF1, Hs99999905_m1 for COX-2 and Hs0099999051_m1 for GAPDH. The reactions were carried out in Brilliant® QPCR Master Mix (Stratagene, Cedar Creek, TX). For PUMA, QPCR was carried out using Quantitect™ SYBR® Green PCR kit (Qiagen) with the following primers: PUMA F, 5'-ATG CCG GAC GAC CTC AAC-3'; PUMA R, 5'-AGG GCC CCT AGG AGA AGA TCG TAC ATG AC-3' (21); GAPDH (endogenous control): F, 5'-GGG GGA AAC TGT GGC GTG ATG G-3' and R, 5'-AGG TGG AGG AGT GGT TCG GCC TGT T-3'. All QPCRs were performed using MX3000P™ Multiplex Quantitative PCR system (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. For each gene tested, samples from three independent experiments were analysed in duplicates. Relative quantifications were performed using the Comparative Quantitation module in MxPro™ QPCR Software from Stratagene.

Western blotting

For Mahlavu, cells in experimental phase were treated with 25 or 50 µM PRIMA-1 before being harvested at 0, 8 and 24 h after treatment. For PLC/PRF/5, cells were harvested 24 h after two subsequent siRNA transfections. Total cellular protein extracts were prepared using RIPA-like buffer (50 mM Tris–HCl, pH 7.4, 250 mM NaCl, 2 mM dithiothreitol, 0.1% sodium dodecyl sulphate and 0.5% NP40 and protease inhibitors). For the detection of p53, PUMA and actin, western blotting was performed according to the standard procedures. For that of Noxa, Tricine–sodium dodecyl sulphate–polyacrylamide gel electrophoresis (22) was performed. The antibodies used were rabbit polyclonal antibody PUMA (Abcam, Cambridge, UK, 1:1000), mouse monoclonal anti-Noxa (Calbiochem, San Diego, CA, 1:250), mouse monoclonal anti-p53 (DO-7, Dako, Glostrup, Denmark, 1:1000) and goat polyclonal anti-actin (1:19) (Santa Cruz Biotechnology, Santa Cruz, CA, 1:500).

Immunoﬂuorescence

Cells were plated in eight-well slides (Lab-Tek® II Chamber Slide™ System, Nalg Nunc International, Rochester, NY) at 1.5 × 105 cells per well (~2 × 106 cells/cm2). The next day, they were treated with 25 µM PRIMA-1 or with equivalent volume of DMSO for 16 h. Cells were then fixed in 4% formaldehyde/PBS, p53 expression was detected using a rabbit polyclonal antip53 antibody (CM-1, Novocastra, Newcastle, UK, 1:100) and Rhodamine (tetramethyl) rhodamine isothiocyanate-conjugated AffiniPure F(ab')2 Fragment Donkey anti-rabbit IgG (7.5 µg/ml) (Jackson ImmunoResearch, West Grove, PA). The nuclei were stained with 4',6-diamidino-2-phenylindole (0.1 µg/ml). Labelled samples were analysed using an epifluorescence microscope (Axioskop 2, Zeiss, Jena, Germany) with a video camera (Quantix™, Photometrics, Tucson, AZ). The entire surface of each well was examined and the proportion of cells with mutant p53 nuclear localization was estimated with reference to the number of seeded cells.

Results

Mutant p53-dependent and -independent cytotoxicity of PRIMA-1 in HCC cells

To evaluate the impact of mutant p53 on the effect of PRIMA-1 in HCC cells, we have first assessed and compared the effect of different p53 status on PRIMA-1-induced cell death in cell lines clonally derived from stably transfected Hep3B cells. The expression of either p53ser249 or p53gln248 in these cells was shown to be on high levels
and homogenous, with >90% of cells of each clone showing strongly positive nuclear staining for p53 (H.Shi, K.Szymanska, O.Galy, J.Lambert, V.J.Bykov, L.Egevad, K.G.Wiman, C.Caron de Fromentel, P.Hainaut, submitted for publication).

In the first experiment, a mock-transfected clone (TO1) and two p53ser249-expressing clones (249/6 and 249/7) were treated for 48 h with 25 or 50 μM PRIMA-1 or with an equivalent volume of DMSO as control. Cell-cycle analysis by flow cytometry (Figure 1A, upper panel) showed that in DMSO-treated control cells, the sub-G1 proportion that represents cells undergoing DNA fragmentation was similar in the mock-transfected clone (~3%) and in both p53ser249-expressing clones (~4%). When treated with 25 μM PRIMA-1, both p53ser249-expressing clones showed a slightly increased proportion of sub-G1 cells (~10%), as compared with the mock-transfected clone (<5%). At 50 μM, PRIMA-1 significantly increased the sub-G1 proportion to ~30% in p53ser249-expressing clones, but only to ~13% in TO1 clone. Essentially similar results were obtained in another experiment using two mock-transfected clones (TO1 and TO5) and two stable clones expressing p53gln248 (248/3 and 248/4) (Figure 1A, lower panel). With 25 μM PRIMA-1, only a modest proportion (~15%) of sub-G1 cells was observed in mock-transfected clones and in p53gln248-expressing ones; whereas at 50 μM, PRIMA-1 treatment dramatically increased the sub-G1 fraction in the two p53gln248-expressing clones (to ~75 and 43% in 248/3 and 248/4, respectively), but not in the mock-transfected ones. These results suggest that expression of mutant p53, in this case, p53ser249 or p53gln248, can increase the cytotoxic effect of PRIMA-1 on Hep3B cells, as measured by the proportion of sub-G1 cells containing fragmented DNA. Furthermore, upon PRIMA-1 treatment at 50 μM, the proportion of cells in sub-G1 was 10–100% higher in p53gln248-expressing cells than in p53ser249-expressing ones, suggesting that the latter mutant confers on cells a greater sensitivity to PRIMA-1 treatment than the former in vitro.

In the course of these experiments, it became apparent that the cytotoxic effect induced by PRIMA-1 was strongly dependent on cellular confluence in Hep3B cells, regardless of their p53 status. Therefore, we have plated different Hep3B clones at three different densities (1, 2 and 3 × 10^5 cells/cm^2) 1 day before PRIMA-1 treatment and investigated by trypan blue exclusion assay the association between PRIMA-1-induced cytotoxic effect and the number of cells at the beginning of treatment. Figure 1B shows that as cell density prior to treatment increased, PRIMA-1-induced cell death significantly decreased with a similar tendency in clones TO1, 249/6 and 249/7, regardless of their p53 status. However, expression of either p53ser249 or p53gln248 clearly enhanced the toxic effects of PRIMA-1 at lower cell densities. Therefore, in subsequent experiments, we plated Hep3B clones at densities between 1.5 and 2 × 10^4 cells/cm^2 (see Materials and Methods) before PRIMA-1 treatment. These plating densities correspond to those yielding differential sensitivity to PRIMA-1-induced cytotoxicity in a mutant p53-dependent manner.

**In vivo antitumour effect of PRIMA-1 on Hep3B stable clones xenografts**

We next sought to determine whether PRIMA-1 could effectively suppress or limit the growth of xenografts of Hep3B cells expressing mutant p53 in SCID mice. In mice inoculated with 249/6 clone (expressing p53ser249), intravenous injections of PRIMA-1 significantly slowed down tumour growth in three of six mice, as compared with control, PBS-injected ones (Figure 2C). This observation indicates that PRIMA-1 exerts an inhibitory effect on the growth of HCC cells expressing p53ser249. However, in mice inoculated with the 248/3 clone (expressing p53gln248), five of six mice treated with PRIMA-1 appeared to develop tumours earlier and faster than the control ones (Figure 2A). Throughout the experiments, the body weight of treated mice did not show significant variations, indicating an overall good tolerance to PRIMA-1 treatment at 100 mg/kg (Figure 2B and D).

**PRIMA-1 does not restore wild-type DNA binding and transactivation capacities to p53ser249 and p53gln248 mutants expressed in Hep3B cells**

To determine whether PRIMA-1 may act by reactivating mutant p53 in Hep3B cells, we analysed whether PRIMA-1 enhanced the capacity of mutant p53 to bind to a p53 consensus DNA sequence in vitro using the TransAM™ DNA-binding assay. DNA-binding properties of p53ser249 have been described elsewhere (H.Shi, K.Szymanska, O.Galy, J.Lambert, V.J.Bykov, L.Egevad, K.G.Wiman, C.Caron de Fromentel, P.Hainaut, submitted for publication). Results (Figure 3A) indicate that PRIMA-1 did not activate the DNA binding of either p53gln248 or p53ser249. With the latter, a higher constitutive level of binding was observed than with p53gln248. However, this binding decreased upon treatment with PRIMA-1, and the residual binding activity could not be competed out by either wild-type or mutant oligonucleotides corresponding to the binding sequence used in the

![Fig. 1](https://academic.oup.com/carcin/article-abstract/29/7/1428/2476436)

**Fig. 1.** Mutant p53-dependent and -independent cytotoxicity of PRIMA-1 in stable clones obtained by transfection of Hep3B cells. The clones used are TO1 and TO5: stable clones with empty vector (mock transfectected); 249/6 and 249/7: clones stably expressing p53ser249 and 248/3 and 248/4: clones stably expressing p53gln248. (A) Levels of PRIMA-1-induced cell death as determined by analysing the sub-G1 proportion of cell-cycle profiles in Hep3B clones treated with PRIMA-1 for 48 h. (B) Confluence-dependent cytotoxicity of PRIMA-1 in Hep3B clones. The percentage of dead cells after 50 μM PRIMA-1 treatment for 24 h was determined by trypan blue exclusion assay. x-axis represents the number of cells per well at the beginning of treatment (0 h). y-axis, the percentage of dead cells after 24 h of treatment minus that observed at 0 h for the same clone. Each point represents the average of a triplicate experiment.
TransAM™ assay. Thus, there is no evidence that PRIMA-1 can reactivate the DNA binding and transcriptional activities of p53ser249 or p53gln248 mutant expressed in Hep3B cells.

To assess whether PRIMA-1 may nevertheless activate the transcription of genes normally transactivated by wild-type p53, we analysed by both real-time reverse transcription–PCR and western blotting the expression levels of several wild-type p53-target genes upon PRIMA-1 treatment in Hep3B clones expressing either p53ser249 (249/6) or p53gln248 (248/3), as well as in a mock-transfected clone (TO5) (Figure 3B). Three p53-target genes involved in cell-cycle control (WAF1), apoptosis (PUMA) or cell survival (COX-2) were studied. Unlike WAF1 and PUMA, which are conventional, direct p53-target genes whose promoters contain a p53-binding consensus element, COX-2 has been shown to be transactivated by wild-type p53 through an indirect mechanism that requires cooperation between p53 and other factors (23). In both 249/6 and 248/3 clones, PRIMA-1 treatment only led to a very modest increase of messenger RNA levels for the three genes tested (1.5– to 2-fold). However, the largest effect (a 4-fold induction at 24 h of treatment) observed by far was with WAF1 in the TO5 clone, which lacks p53 expression. Compatible results were observed at the protein level (data not shown). Moreover, the levels of protein products of p53-target genes such as PUMA or Noxa were not increased by treatment with PRIMA-1 in cell lines that constitutively express p53ser249, such as Mahlavu (Figure 3C) and PLC/PRF/5 (data not shown). Thus, there is no evidence that PRIMA-1 can reactivate the capacity of p53gln248 or p53ser249 to induce the transcription of these p53-dependent genes in HCC cell lines. In contrast, these data suggest that in Hep3B cells, PRIMA-1 may be able to activate WAF1 through a p53-independent mechanism, but that this effect is blocked in cells expressing p53ser249 or p53gln248.

Effects of PRIMA-1 on nucleolar translocation of mutant p53 in Hep3B cells

It was recently shown that PRIMA-1 and its methylated derivative PRIMA-1MET could induce mutant p53his175 expressed in the human lung adenocarcinoma cell line H1299 to relocate in discrete nuclear localization corresponding to nucleolar translocation. This phenomenon was observed in a small percentage (2–3%) of H1299 cells expressing p53his175 treated with 25 μM PRIMA-1, but this proportion increased to >75% in cells treated with an equivalent concentration of PRIMA-1MET. This relocalization is correlated with the activation of apoptosis (24). To determine whether PRIMA-1 could induce similar effects in Hep3B cells expressing p53ser249, we analysed the subcellular localization of p53ser249 in these cells upon treatment with 25 μM PRIMA-1. Nucleolar translocation and accumulation of p53ser249 were detected in a small proportion of cells, estimated to ~1%, whereas no such translocation was observed in control cells treated with DMSO (Figure 4).

siRNA silencing of p53ser249 sensitized PLC/PRF/5 cells to PRIMA-1 treatment

The results above raise questions about the exact role of p53ser249 in the cytotoxic effects of PRIMA-1, and on whether reactivation of the mutant into wild-type is the main mechanism of PRIMA-1 p53ser249-dependent cytotoxicity observed in Hep3B cells. To address this question, we used RNA interference to transiently knock down the expression of endogenous p53ser249 in PLC/PRF/5 cells, before we exposed these cells to PRIMA-1 treatment. Figure 5A shows the inhibitory efficacy of siRNA transfection on the levels of p53ser249 protein in PLC/PRF/5 cells. Flow cytometry showed that treatment with PRIMA-1 at 25 μM for 24 h significantly increased the percentage of sub-G1 cells in p53 siRNA-transfected cells (~37%), as compared with cells transfected with scramble siRNA or to non-transfected ones (<10%) (Figure 5B and C). These results indicate that inhibition of p53ser249 expression sensitizes PLC/PRF/5 cells to the cytotoxic effects of PRIMA-1. These observations suggest that PRIMA-1 exerts its cytotoxic effects essentially through p53-independent mechanisms. Presence of p53ser249 can at least partially protect the cells against the effects of PRIMA-1, consistent with the notion that the mutant confers a gain-of-function effect which favours cell survival.

Discussion

Small organic molecules targeting mutant p53 have been the subject of great interest as novel mechanisms to restore apoptosis in cancer cells, opening possibilities for the development of more efficient cancer therapies. Assessing and testing such approaches is a very complex task due to the multiple biological mechanisms in which p53 is
involved, as well as to the structural and functional heterogeneity of
mutant p53 proteins. In this respect, HCC in high-incidence areas
represent a unique situation since many patients developing HCC in
a context of high exposure to AFB1 and chronic HBV carriage present
the same mutation at codon 249 in TP53. This particular epidemi-
ological situation opens a clinical opportunity: it may be possible
to develop a standard approach targeting p53ser249 in HCC, which
might be applicable worldwide, especially to the treatment of patients
in all high-incidence areas. This possibility is particularly attractive
since these cancers are currently uniformly lethal, with no access to
manageable curative therapy in the context of low-resource countries.
With these ideas in mind, we have investigated the effects of PRIMA-
1 on HCC cells with different p53 status in culture and as xenografts
in SCID mice. As experimental model, we selected to use Hep3B
cells, which are derived from HCC and carry a large homozygous
deletion in the TP53 gene (18) and to stably transfect these cells with
either p53ser249 or p53gln248 mutants in order to compare the effects
of PRIMA-1 in controlled conditions. We found that clones derived
from stable mutant p53 transfectants had an increased sensitivity to
the cytotoxic effects of PRIMA-1 as compared with clones derived
from mock-transfected Hep3B cells. This effect was not specific for
p53ser249 and was also observed with p53gln248. When cells were
grown as xenografts in SCID mice, we observed that systemic in-
jection of PRIMA-1 delayed the growth of tumours expressing
p53ser249, but not of those expressing p53gln248. These differences
of PRIMA-1 effect between in vitro experiments (which are con-
ducted over up to 48 h) and in vivo experiments (which last up to
40 days) may be due to the fact that the two clones differ by their
intrinsic tumorigenic properties, with p53gln248 tumours developing
~10 days earlier than p53ser249 ones. Despite occurring at adjacent
codons, the two mutations differ by the structural impact on p53
protein. Both mutant proteins have a destabilized structure. Mutation
of p53ser249 removes several intramolecular bridges that stabilize the
folding of loops involved in DNA binding. Mutation of p53gln248 not
only has a destabilizing effect but also modifies a residue that makes
direct contact with target DNA (25,26). It is possible that these

Fig. 3. Expression of p53-target genes upon PRIMA-1 treatment in Hep3B stable clones. (A) Hep3B stable clones were treated with 25 μM PRIMA-1 for 0, 16
and 24 h (0, 16 and 24 h, respectively). Nuclear extracts from these cells were used in TransAM assay to determine p53 DNA-binding activity. Minus represents
negative control without protein extracts and plus represents positive control provided by the TransAM p53 Transcription Factor Assay Kit (version C), which
corresponds to nuclear extract from MCF7 cells treated with H2O2 for 8 h. 'WT' and 'mutant' represent free oligos containing the wild-type and mutated p53-
binding consensus sequence, respectively, which were used in competition assays. (B) Real-time reverse transcription–PCR analysis of expression of WAF1, COX2
and PUMA. Hep3B stable clones were treated with 50 μM PRIMA-1 for 0, 12 and 24 h (0, 12 and 24 h, respectively). Histograms represent the average of three
independent experiments. (C) Protein levels of PUMA and Noxa in Mahlavu cells upon PRIMA-1 treatment. Cells were treated with 25 and 50 μM of PRIMA-1
for 4, 8 and 24 h. Control cells were treated with the equivalent volumes of DMSO. The levels of actin served as loading controls.
other transcription-independent mechanism has been identified by induced by PRIMA-1 or by a degradation product is unknown. An-a role in PRIMA-1-induced apoptosis. Whether this phenomenon is provided evidence that redistribution of mutant p53 to nucleoli plays cytotic leukaemia nuclear body-associated proteins promyelocytic leu-mutant p53 to translocate into the nucleolus together with the promyelo-

Since PRIMA-1 has been proposed to act by restoring the wild-type activity of mutant p53 (16,17), we next sought to determine whether such an effect and consequent induction of p53-dependent apoptosis accounted for the biological effects observed in Hep3B-derived clones. Our results do not show a reactivation effect by PRIMA-1 on the wild-type activity of the two mutants tested. Upon treatment with PRIMA-1, we were unable to demonstrate an increase in sequence-specific DNA-binding activity or in transcription activation capacity towards three different genes regulated by p53. This is at variance with previous studies on different p53 mutants, which have shown that PRIMA-1 could reactivate wild-type activity of several p53 mutants, with at least partial recovery of wild-type transcriptional capacity (16,17). Interestingly, recent studies have indicated that PRIMA-1 may exert some cytotoxic effects by mechanisms different than renaturation of mutant p53 into wild-type. Rokaes et al. (2007) have shown that PRIMA-1 (and its derivative PRIMA-1MET) induced mutant p53 to translocate into the nucleolus together with the promyelocytic leukaemia nuclear body-associated proteins promyelocytic leukaemia, cAMP response element-binding protein and Hsp70, and provided evidence that redistribution of mutant p53 to nucleoli plays a role in PRIMA-1-induced apoptosis. Whether this phenomenon is induced by PRIMA-1 or by a degradation product is unknown. Another transcription-independent mechanism has been identified by Chipuk et al. (27), who showed that PRIMA-1 modulated mitochondrial status and cytochrome c release in a p53-dependent manner even in the presence of transcription inhibitors. A recent study by V.I.Bykov, J.Lambert, P.Gorzov, D.B.Veprintsev, M.Söderquist, J.Westman, D.Segerbäck, J.Bergman, A.R.Fersht, P.Hainant, K.G. Wiman (submitted for publication) shows that PRIMA-1 may act by targeting thiols in mutant p53, PRIMA-1 may release the mutant protein from interactions with other factors and may facilitate its reactivation and/or its relocalization. The nature, extent and conse-quence of these effects may depend on both the mutant and the cell type considered. There is evidence that different amino acid substitu-tion in p53 may affect the structure or function of the protein in different ways [IARC TP53 Mutation Database (R11), http://www-p53.iarc.fr] (13,25,26). It is possible that the modifications induced by PRIMA-1 may not suffice to reactivate p53ser249, but may nevertheless modify the protein to induce effects that contribute to decrease cell survival.

To assess whether p53ser249 is directly involved in mediating the cytotoxic effects of PRIMA-1 on HCC cells, we used RNA interference to inhibit p53ser249 in HCC cells that constitutively express this mutant. In PLC/PRF/5 cells, knocking down p53ser249 expression significantly enhanced the cytotoxic effect of PRIMA-1, indicating that it is not p53ser249 itself that mediates cell killing by PRIMA-1. Conversely, presence of p53ser249 mutant appears to contribute to protect cells against the cytotoxic effects of PRIMA-1, suggesting that

![Fig. 4. Nucleolar translocation of p53ser249 upon PRIMA-1 treatment in 249/6 clone. Cells were treated for 16 h with 25 μM PRIMA-1 and control cells with an equivalent volume of DMSO. Rhodamine staining represents p53 expression. Nuclei were visualized by 4,6-diamidino-2-phenylindole (DAPI) staining. Immunofluorescence staining was analysed using an epifluorescence microscope.](https://academic.oup.com/carcin/article-abstract/29/7/1428/2476436)

A  

B  

C  

![Fig. 5. Inhibition of p53ser249 expression sensitizes PLC/PRF/5 to PRIMA-1 cytotoxicity. (A) Inhibition of p53ser249 expression in PLC/PRF/5 cells. Total protein extracts were prepared from p53 (sip53) or scramble (sc) siRNA-transfected cells. Inhibition of p53 expression was determined by western blot with actin level as a loading control. N represents non-transfected cells. (B) Flow cytometry cell-cycle profiles of PLC/PRF/5 cells submitted to siRNA silencing of p53ser249 and then to 25 μM PRIMA-1 treatment for 24 h. Controls were treated with an equivalent volume of DMSO. (C) Levels of PRIMA-1-induced cell death determined by analysing the sub-G1 proportion of cell-cycle profiles as represented in (B). Histograms represent averages of three independent experiments. y-axis: percentage of sub-G1 cells in treated cells minus that percentage in non-treated cells transfected with the same siRNA.](https://academic.oup.com/carcin/article-abstract/29/7/1428/2476436)
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References


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