Piperlongumine exerts cytotoxic effects against cancer cells with mutant p53 proteins at least in part by restoring the biological functions of the tumor suppressor

DEBASISH BASAK, SURENDRA R. PUNGANURU and KALKUNTE S. SRIVENUGOPAL

Department of Biomedical Sciences and Cancer Biology Center, School of Pharmacy, Texas Tech University Health Sciences Center, Amarillo, TX 79106, USA

Received November 23, 2015; Accepted December 29, 2015

DOI: 10.3892/ijo.2016.3372

Abstract. Piperlongumine (PL), a small molecule alkaloid present in black pepper (*Piper longum*), has been reported to kill tumor cells irrespective of their p53 gene status, however, the mechanisms involved are unknown. Since p53 is a redox-sensitive protein, we hypothesized that the redox imbalance induced by PL may affect the structure and/or function of the mutant p53 protein and promote cell death. We used two human colon cancer cell lines, the HT29 and SW620 which harbor the R273H DNA contact abrogatory mutation in p53. PL treatment induced significant ROS production and protein glutathionylation with a concomitant increase in Nrf-2 expression in both cell lines. Surprisingly, immunoprecipitation with wt-p53 specific antibodies (PAb1620) or direct western blotting showed a progressive generation of wild-type-like p53 protein along with a loss of its mutant counterpart in PL-treated HT29 and SW620 cells. Moreover, the EMSA and DNA-affinity blotting revealed a time-dependent restoration of DNA-binding for the mutant p53, which was accompanied by the induction of p53 target genes, MDM2 and Bax. PL, while cytotoxic by itself, also increased the cell killing by many anticancer drugs. In nude mice bearing the HT29 tumors, PL alone (7.5 mg/kg daily) produced a 40% decrease in tumor volume, which was accompanied by diminished intratumoral mutant p53 protein levels. The antitumor efficacy of BCNU or doxorubicin in HT29 xenografts was highly potentiated by PL, followed by expression of apoptotic proteins. These clinically-relevant findings suggest that PL-induced oxidative milieu facilitates a weak functional restoration of mutant p53 through protein glutathionylation and contributes to the increased drug sensitivity.

Correspondence to: Dr Kalkunte S. Srivenugopal, Department of Biomedical Sciences, School of Pharmacy, Texas Tech University Health Sciences Center, 1406 S. Coulter Drive, Amarillo, TX 79106, USA
E-mail: kalkunte.srivenugopal@ttuihsc.edu

Abbreviations: PL, piperlongumine; PMSF, phenylmethylsulfonyl-fluoride; DTT, dithiothreitol; BCNU,1,3 bis (2-chloroethyl)-1-nitro-sourea; DAI, DNA-affinity immunoblotting; Dox, doxorubicin; TMZ, temozolomide; EMSA, electrophoretic mobility shift assay; ROS, reactive oxygen species; DCF-DA, 2, 7'-dichlorofluorescein diacetate, wt, wild-type; Mut, mutant; WB, western blotting

Key words: reactivation of mutant p53, oxidative stress, cancer chemotherapy, protein glutathionylation, drug resistance

Introduction

Human cancers harbor elevated levels of reactive oxygen species and possess increased oxidative stress due to enhanced metabolism, peroxisomal and inflammatory activities (1). Such a heightened metabolic stress has been strongly linked to carcinogenesis via the oxidative and nitrosative damage to DNA, protein and lipids. On the contrary, it has become increasingly apparent that the redox-stress and redox regulatory mechanisms prevalent in cancers may have significant therapeutic implications (2). A huge endeavor is being undertaken to exploit the redox imbalance in cancers for developing novel therapeutic strategies and preferentially eliminating the tumor cells (1,2). Evidence indicates that human malignancies respond to even slight oscillations in their redox milieu with an array of adaptive protein modifications, signaling and gene expression changes, which can render them vulnerable to drugs and natural compounds (1). Piperlongumine (PL) obtained from the fruits and roots of the long pepper plant is a pyridine alkaloid that has been reported to selectively exert cytotoxicity in a wide variety of tumor cell types both *in vitro* and *in vivo*, while sparing non-cancerous normal cell types (3). Although the exact mechanism has not been elucidated, the anticancer effects of PL have been attributed to its pharmacophore containing two active double bonds (C2-C3 and C7-C8 olefins) that act as Michael acceptors and increase the levels of reactive oxygen species (4). PL directly binds to and inhibits the antioxidant enzyme glutathione S-transferase π (GSTP1) resulting in a decrease in glutathione levels and subsequent promotion of cancer-selective cell death by increasing the ROS levels (3). In addition, PL was found to suppress NF-κB transcription factor by inhibiting cys179 indicating that thiol manipulation might play a crucial role in its anticancer effects (5).

p53, also known as the guardian of the genome is found to be mutated in >50% of all human cancers and these mutations drive the emergence of oncogenic genomes and aggressive malignancies (6,7). p53 mutations are highly diverse in their type, position, sequence context and structural impact and can be broadly classified into groups, namely, the DNA contact mutants (p53R273H) where p53 contact with its recognition sequence is disrupted, and the conformational mutants (p53R175H) where structural alterations in the protein mediate the loss of binding to DNA (8). Most p53 mutations confer drug resistance to cancer cells through the impairment of apoptosis by inducing the expression of anti-apoptotic proteins like Bcl-2 and reducing the expression of proapoptotic proteins like Bax and PUMA (9).

A number of reports have been published addressing the reactivation of mutant p53 into its functional form for improved and efficient anticancer therapies (10). Among them, the gene therapy to express the wild-type tumor suppressor and oncolytic adenovirus Onyx 015 has been successful to some extent but are still under investigation due to lack of suitable delivery systems (11). Inhibitors of MDM2-p53 interaction showed promising effects and one of them, nutlin-3 is undergoing clinical trial (12). Several small-molecule screening studies have led to the identification of compounds such as PRIMA-1 (13), MIRA-1 (14), CP-31398 (15), STIMA-1 (16), SCH529074 (17), and NSC319726 (18) that demonstrated the ability to reactivate the mutant p53 protein and confer biological functions such as the activation of the target gene expression. Many of these compounds share a unique feature of possessing chemically active, highly electrophilic double bonds that participate in Michael addition reactions with the nucleophilic thiols in p53. Thus, they are potent electrophiles acting as Michael acceptors that readily react with nucleophilic thiols. Such an interaction also supports the notion that intramolecular or intermolecular disulfide bond formation might be inhibited by thiol modification that could result in the proper folding of the protein core (19).

PL appears to be cytotoxic against tumor cell lines irrespective of their p53 status. Further, PL has demonstrated marked tumor regression in a number of murine cancer models without tumor recurrence incidents or specific toxicities (3). Previously, we reported that human p53 was a substrate for glutathionylation which interferes with the tumor suppressor protein binding with DNA (20,21). Putting these observations together, we hypothesized that PL may promote the conversion of mutant p53 protein into its DNA-bindable and functional form. This study shows indeed that PL possesses the ability to reactivate the R273H mutant form of p53, albeit with a lower efficiency.

Materials and methods

Cell lines, chemicals and antibodies. Human colon carcinoma cell lines HT29, SW620, and HCT116 were obtained from the American Type Culture Collection and were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. Cell cultures were maintained at 37˚C in 5% CO_2 and 95% air. Monoclonal antibodies to actin, GSH, and p53 were purchased from Millipore Corp. 1620 (wt-p53) and 240 (mt-p53) were purchased from Calbiochem. Piperlongumine was purchased from Cayman Chemical (CAS registry no. 20069-09-4, ≥98%, Ann Arbor, MI, USA). Stock solutions of PL were prepared in DMSO.

Western blotting. After trypsinization, the cell pellets were washed with cold phosphate-buffered saline (PBS) and subjected to sonication in 40 mM Tris-HCl (pH 8.0) containing 1% glycerol, 1 mM EDTA, 0.5 mM DTT, 1 mM PMSF and 1 mM benzamidine and centrifuged. Equal protein amounts from different treatments were electrophoresed on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto PVDF membranes (Millipore). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (pH 8.0) containing 0.1% Tween-20 for 3 h, and subsequently incubated with appropriate primary antibodies. Antigen-antibody complexes were visualized by enhanced chemiluminescence.

Animal studies. Female Nu/Nu mice were obtained from Charles River Laboratories (Wilmington, MA, USA) and fed *ad libitum*. Female mice are traditionally used for developing xenografts because the males often tend to be aggressive and bite their littermates. They may also destroy the tumors present on their fellow cage mates or kill them. The animals were allowed to acclimatize to a 12 h light/12 h dark cycle, and all procedures were performed under the guidelines of the institutional animal care and use committee (IACUC). HT29 cells ($5x10^6$) were injected subcutaneously into the right and left flanks. Tumor volume was calculated every 2-3 days with a caliper using the following formula: Volume = [length x $(width)^2$]/2. The mice weighing 25 g were randomized into three groups and were administered i.p. injections of 5 and 10 mg/kg/day of PL dissolved in 1X PBS saline (6 animals per groups), when the agent was used alone. In the second study, the mice were randomized into six groups and they received 7.5 mg/kg/day PL, 1 mg/kg/day cisplatin, 0.75 mg/kg/day doxorubicin and the combinations of PL with cisplatin or doxorubicin. In both cases control mice received only PBS. The animals were sacrificed on day 21 after drug administration. The tumors were excised, washed and homogenized using a polytron in 40 mM Tris-HCl buffer (pH 8.0) containing 5% glycerol, 1 mM EDTA, 20 μM spermidine, 0.5 mM DTT, 1 mM PMSF, 1 mM benzamidine, 0.5% Triton X-100, and 1 mM sodium vanadate. All samples were centrifuged, and the resulting extracts were used for western blot analyses.

Electrophorectic mobility shift assay. Nuclear extracts were prepared using the nuclear extract lysis buffer containing 25 mM Tris-HCl (pH 8.0), 5% glycerol, 1 mM EDTA, 0.5 mM DTT, 1 mM PMSF and 1 mM benzamidine and centrifuged. Five milligrams of each nuclear extract was used in a binding assay with 20 ng/μl of a biotin-labeled p53 probe (5' Biotin AGACATGCCTAGACATGCCT-3') (Signosis, Inc.). The nuclear extract was incubated with p53 probe at room temperature (20-23˚C) for 30 min and the protein/DNA complexes were separated on a non-denaturing 6.5% polyacrylamide gel. The gel was transferred to a nylon membrane, and the biotin-labeled oligonucleotides were detected using streptavidin-HRP and a chemiluminescent substrate according to the manufacturer's protocol.

Binding of p53 to DNA: quantitation by DNA-affinity immunoblotting (DAI). Binding of p53 proteins to their respective consensus recognition sequences was determined by the biotin-labeled oligonucleotide pull-down assays followed by western blotting in a procedure called DNA affinity immunoblotting (22). Nuclear extracts were prepared, and DNA-binding reactions were performed according to our published procedure (20). The following double-stranded consensus recognition sequences were prepared by annealing a strand labeled with biotin at 5' end with an unlabeled complementary sequence; p53 used were 5'-TACAGAACATGTCTAAG CATGCTGGGGACT-3'. The duplex oligonucleotides (50 nM) were bound to streptavidin magnetic beads in TE buffer (pH 8.0) containing 100 mM NaCl. The beads were washed with TE buffer containing 1 M NaCl and then without salt. The oligo-bound beads were subsequently suspended in a binding buffer [20 mM Tris-HCl (pH 8.0), 75 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, 20 mM KCl, 1 μg poly(deoxyinosinic-deoxycytidylic) acid and 5% glycerol] and incubated with nuclear extracts at room temperature for 30 min. The beads were then washed with 300 mM NaCl and the DNA-bound protein was eluted by boiling the beads in SDS sample buffer, electrophoresed on 10% SDS-polyacrylamide gel and western blotting using appropriate antibodies.

Immunoprecipitation. Equal protein amounts in lysates from different treatments were precleaned with 5 μl protein-A agarose beads, and immunoprecipitated using 240 p53 mutant (PAb240) and 1620 p53 wt (PAb1620) antibodies. The immunocomplexes were solubilized in non-reducing SDS-sample buffers, subjected to electrophoresis on 12% gels followed by western blotting with D-1 antibodies for p53 which recognizes both the wt and mutant proteins.

Detection of ROS generation. Intracellular ROS production was determined by 2',7'-dichlorofluorescin diacetate (DCFDA) staining followed by fluorescence detection using a Biotek plate reader (Model-Synergy 2SL) with excitation and emission wavelengths set at 485 and 535 nm, respectively. Briefly, cells were incubated with 10 μM DCF-DA solution at 37˚C for 0.5 h, washed with PBS twice, treated with different concentrations of PL for 1, 3 and 6 h followed by fluorescence measurement.

Cell viability assays. For cell viability assays, the yellow tetrazolium dye [(3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyl tetrazolium bromide) (MTT)] was used. HT29 cells were seeded at a density of 7,000 cells per well in 96-well plates and were treated post-24 h with PL at concentrations specified. In some cases, cells were treated with PL for 24 h. They were treated with PL alone or with PL followed by exposure to BCNU or temozolomide or doxorubicin. The cells were washed, and MTT (10 μl of 5 mg/ml) in 1X PBS was added. The plates were incubated for 4 h followed by the addition of DMSO, stored in the dark for 2 h before reading the absorbance at 570 nm.

Statistical analysis. All experiments were performed three times independent of each other. Results were assessed by Student's t-test. Significance was defined as $P<0.05$. Power analysis was used to calculate the number of animals required to achieve a statistical power of >80%.

Results and Discussion

Chemistry of piperlongumine. PL, an electrophilic small molecule identified in cell-based, high-throughput screening assays was shown to selectively kill cancer cells without harming the normal epithelial cells (3). Structurally, PL has two active double bonds (Fig. 1A) and can conjugate with small-molecule thiols. Adams *et al* synthesized 80 structural analogs of PL to investigate some lead compounds from which they concluded that C2-C3 olefin is critical for the electrophilicity of the molecule. They observed that elimination of C2-C3 olefin did not result in ROS elevation or decreased viability of cancer cells while removal of the C7-C8 olefin was associated with decreased toxicity without affecting the generation of ROS. Several analogs of piperlongumine such as piperine and piperlonguminine also have active double bonds that are located in proximity to the carbonyl group but they lack the crucial C2-C3 olefin in their structures. As a result, although these analogs still possess the ability to elevate cellular ROS, they exhibit reduced toxicities (4). Therefore, the therapeutic properties of PL can be attributed to both the olefins present in the molecule. These structural features of PL also appear to underlie the feeble p53 reactivating properties described in this report.

Protein glutathionylation induced by PL. Glutathionylation is a posttranslational modification where low molecular weight thiols such as the glutathione (GSH or GSSG) form mixed disulfides with protein-bound reactive (anionic) cysteines, apparently, as a defensive/protective strategy during oxidative stress (23). This modification is reversible through an increase in GSH/GSSG ratio or enzymatic reactions involving glutaredoxin, thioredoxin or sulfiredoxins which restore the protein sulfhydryl groups back to their reduced state (24). Similar to phosphorylation, glutathionylation is known to inhibit enzyme activities and transcription protein functions and alter protein-protein interactions (25). Our previous study demonstrated that human p53 is a substrate for glutathionylation *in vitro* and in cells, and p53 function may be modulated by glutathionylation (20,21). Another study suggested that the thiolation induced by PL, and its analogs might play an important role in inducing cancer cell death (4). Because PL induces a redox imbalance, we examined the bulk glutathionylation of proteins as a biochemical marker of oxidative stress. Conjugation of glutathione or glutathione disulfide with bulk proteins was determined by western blotting using a monoclonal antibody against glutathione. In HT29 and SW620 cells, we observed a large increase in protein glutathionylation after PL treatment where a *de novo* or increased glutathionylation of numerous proteins was observed as reflected by enhanced band densities when compared with the untreated cells (Fig. 1B).

PL treatment upregulates Nrf-2 expression and generates ROS. The Nrf-2 (nuclear factor erythroid 2-related factor) is a cytoprotective transcription factor activated during oxidative stress and functions to restore the redox homeostasis. Nrf2 controls the basal and induced expression of an array

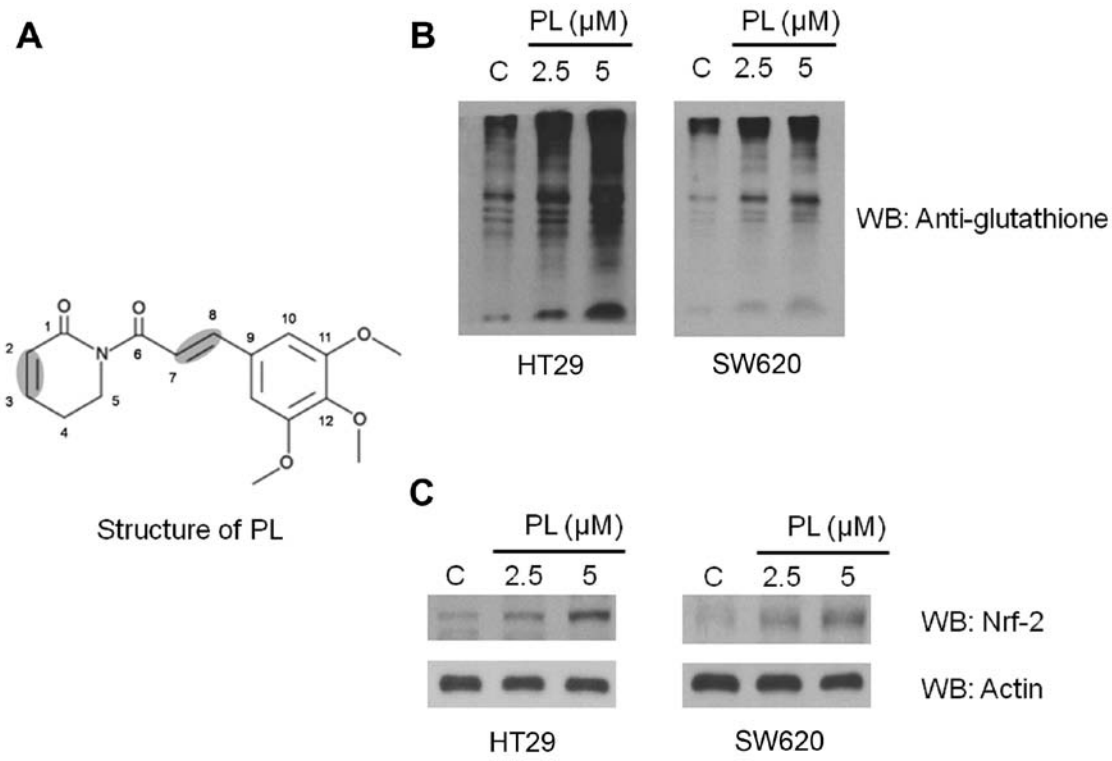

Figure 1. (A) Structure of piperlongumine (PL). The shaded regions show the C2-C3 and C7-C8 olefin reactive double bonds. (B) Immunoblot pattern of protein glutathionylation in HT29 and SW620 cells treated with 2.5 and 5 μM PL for 24 h. (C) Western blot analyses of Nrf-2 expression was performed in the same conditions.

of antioxidant response element-dependent genes such as the γ-glutamylcysteine synthase, glutathione S-transferases, and NAD(P)H oxidoreductase 1 to regulate the physiological and pathophysiological outcomes of oxidant exposure (26). Consistent with the properties known for PL, we observed an increase in the Nrf-2 protein levels in HT29 and SW620 cells (Fig. 1C). Therefore, an increase in Nrf-2 demonstrates the development and maintenance of oxidative stress by PL in cancer cells. The ability of PL to induce oxidative stress in tumor cell lines with mutant p53 was further explored using the redox probe DCF-DA. DCF-DA oxidizes rapidly to a highly fluorescent 2', 7'-dichlorodihydrofluorescein (DCF) in stressed cells and the fluorescence intensity is proportional to the cytosolic ROS levels (27). The increase in ROS levels by PL was confirmed when the HT29 and SW620 cells were incubated with DCF-DA for 30 min, followed by PL treatment for 1, 3 and 6 h (Fig. 2B and C). However, there was no significant difference in ROS levels in MCF10a cells after PL treatment for the same time periods (Fig. 2A) indicating that ROS production is entirely selective for cancer cells, not for normal epithelial cells.

Restoration of functional status to mutant p53 by piperlongumine. p53 is the most commonly mutated gene in human cancers and hence has emerged as a huge target for novel cancer therapies (28). These mutations occurring in the DNA-binding domain abrogate binding of p53 protein to its target sequences, leading to a suppression of target gene products involved in cell cycle arrest, apoptosis and a multitude of oncogenic pathways (29). Additionally, p53 mutations are also associated with the inhibition of two other p53 family members, p63 and p73 through oligomerization, and this leads to the inhibition of apoptosis (30). Reactivation or restoration of functional properties to the mutant p53 proteins is expected to sensitize the human tumors to therapy-induced DNA damage and facilitate a greater antitumor efficacy through a pronounced apoptosis. Most compounds reported to impart at least some wild-type properties to mutant p53 proteins are all electrophilic with great affinity for nucleophilic groups such as the protein bound cysteines or glutathione present in abundance (19). PL is a reactive compound as well (4); therefore, we hypothesized that anticancer effects of PL may stem at least partially from the oxidative stress-induced modification of the p53 protein. Our own findings implicating the reactive cysteines 124, 141 and 182 in the DNA-binding domain of p53 as targets for oxidation and thiolation further supported the above hypothesis (20,21).

We used a number of approaches to validate the functionalization of mutant p53. Chief among these were the conformation specific monoclonal antibodies for p53. pAb 1620 can recognize the wild-type structure, and many cancer mutations perturb this conformation (31). The epitope comprising Arg156, Leu209, Arg 209, and Gln/Asn210 in p53 DNA-binding domain is recognized by pAb1620 (32). On the other hand, a highly conserved, denaturation-resistant epitope on p53 is targeted by the antibody pAb240; it binds to p53 in the mutant conformation and also to denatured p53. Both antibody epitopes are located away from the p53-DNA interface (33).

As a first step to verify the reactivation of mutant p53, the cell lysates from PL-treated and untreated HT29 and

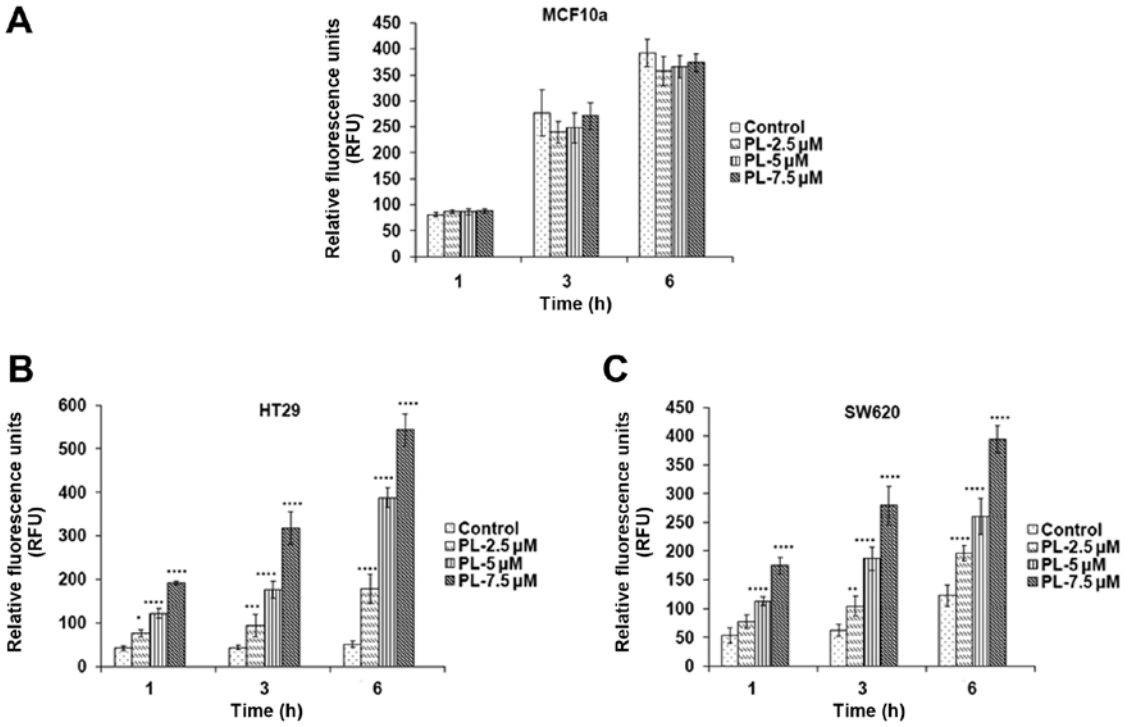

Figure 2. PL induces ROS elevation in: (A) MCF10a normal breast epithelial cells, (B) HT29, and (C) SW620 cells was measured by fluorescence enhancement associated with the DCF-DA in an oxidative milieu. Tumor cells were treated with 2.5, 5 and 7.5 μM PL for 1, 3, and 6 h after the addition of DCF-DA. ROS levels were then measured using a plate reader as described in Materials and methods. The values shown are mean ± SD. *Statistically significant difference as compared with the control. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

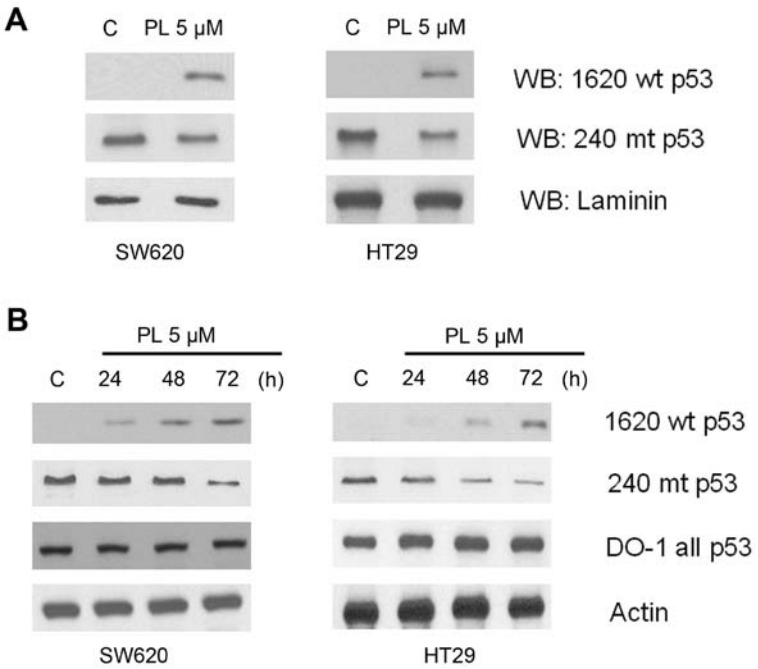

Figure 3. (A) Immunoprecipitation and western blotting evidence for the appearance of wild-type like p53 protein and reduction of the mutant protein. Lysates from both HT29 and SW620 cells treated with 5 μM PL for 24 h were immunoprecipitated using monoclonal antibodies pAb1620 or pAb240, respectively. The resulting immunoblots were probed with p53 DO-1 antibody, which recognizes both the mutant and wt-p53 proteins. An increase in wt-protein and a decrease in mutant protein are evident. (B) Immunoblot results of mutant p53 conversion into its wild-type like form in HT29 and SW620 cells. The cells were treated with 5 μM PL for 24, 48 and 72 h. 1620 Ab detects a wild-type, 240 detect a mutant type and pan DO-1 Ab detects the total p53 levels, respectively. The results show the upregulation of wt-like protein and downregulation of mutant form.

SW620 cells were immunoprecipitated with pAb1620 or pAb240 followed by immunoblotting with the DO1 anti-p53 antibodies. The western blots in Fig. 3A show that in both cell lines, the bands corresponding to the wt-like p53, which were

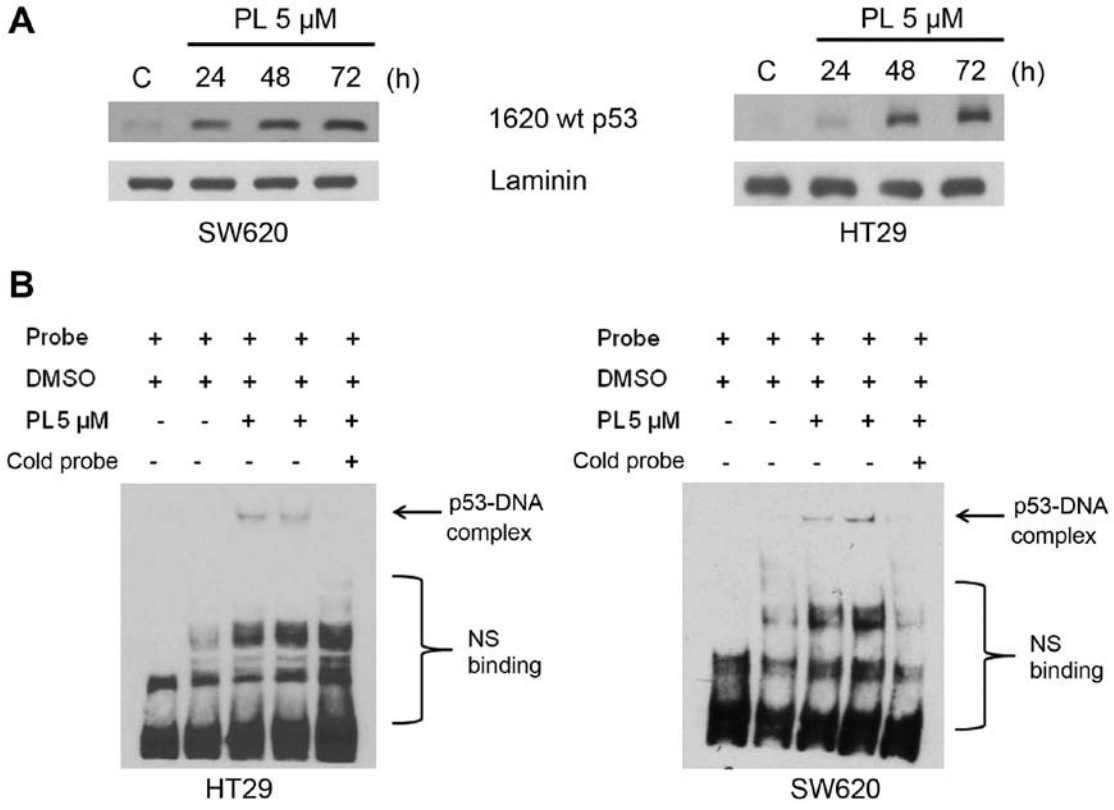

Figure 4. (A) PL weakly reactivates and promotes the binding of mutant p53 present in HT29 and SW620 cells with DNA. Cells were treated with 5 μM PL for 24, 48 and 72 h. p53 protein present in the nuclear extracts was subjected to DAI assays as described in Materials and methods. (B) Nuclear extracts (NE) from 5 μM PL-treated or untreated HT29 and SW620 cells were processed for EMSA as described in Materials and methods. Lane 1, probe alone; lane 2, NE from untreated cells; lane 3, NE from 5 μM PL (24 h) treated cells; lane 4, NE from 5 μM PL (48 h); lane 5, cold probe - NE was mixed with 1X non-biotinylated probe in DNA binding assays.

absent in control cells appeared after PL-treatment. Further, a concomitant decrease of the mutant p53 protein was evident in extracts immunoprecipitated with pAb240. Next, the above results were verified by direct western blotting using the p53 conformation specific antibodies. The untreated HT29 and SW620 cells which harbor a mutant p53 exhibited a very weak immunoreactivity for pAb 1620, which, however, increased significantly in a time-dependent manner after PL treatment. On the contrary, the intrinsic mutant p53 in these cells decreased gradually as evident from the diminished pAb240 immunoreactivity (Fig. 3B).

To further establish the conformational transition and acquisition of DNA binding of the R273H mutant p53 present in HT29 an SW620 cells, we performed DNA-affinity immunoblotting (DAI) and EMSA, both of which detect the protein specifically associated with DNA. The representative results from these experiments are shown in Fig. 4. The nuclear extracts (NE) from control cells failed to bind with DNA, whereas the NE from PL-treated cells showed a time-dependent increase in DNA binding in the DAI assays (Fig. 4A). Similar data showing the binding of p53 with DNA in both HT29 and SW620 cells were obtained in EMSAs; the bands corresponding to DNA-bound proteins were diminished by the addition of the non-biotinylated cold probe, demonstrating the specificity of interaction (Fig. 4B). Consistent with the mutant p53 conversion to its functional form, we observed upregulation of the target genes, MDM2 and Bax in both cell lines after PL treatment (Fig. 5). A significant increase in the levels of apoptotic markers, cleaved caspase-3 and cleaved PARP was also observed (Fig. 5), suggesting that the oxidative milieu may prime and usher the tumor cells along the cell death pathways.

Piperlongumine exerts marked cytotoxicity by itself and enhances cell killing by anticancer drugs. To investigate the selective anticancer effects of PL in the context of the DNA contact mutants of p53 and our observations that PL can impart wt-like the protein conformation to the mutant proteins (Figs. 3-5), we performed cell survival assays with PL alone or PL in combination with some clinically used drugs against cancers. PL by itself, over a concentration range of 2.5-15 μM for 24 h, showed significant anti-proliferative effects against cell lines of various cancer types. These included the breast cancer MCF-7, colon cancer HT29, HCT116, SW620, and the Mia Paca of pancreatic origin (Fig. 6). However, consistent with earlier results (3) PL was 10-12-fold less toxic to MCF10a normal breast epithelial cells as also shown in Fig. 6. Based on these observations, we chose 7.5 μM PL, which caused 35-55% cell-kill for 24 h as the preincubation period for testing the potentiation of cytotoxicities of temozolomide (TMZ), 1,3 bis(2-chloroethyl)-1-nitrosourea (BCNU) and doxorubicin (Dox). Three cell lines, the HT29, HCT116 and SW620 were tested to determine the drug potentiation by PL. In this setting, the TMZ (250-1,000 μM range) + PL combination showed 2- to 3-fold increased cytotoxicity compared with TMZ alone

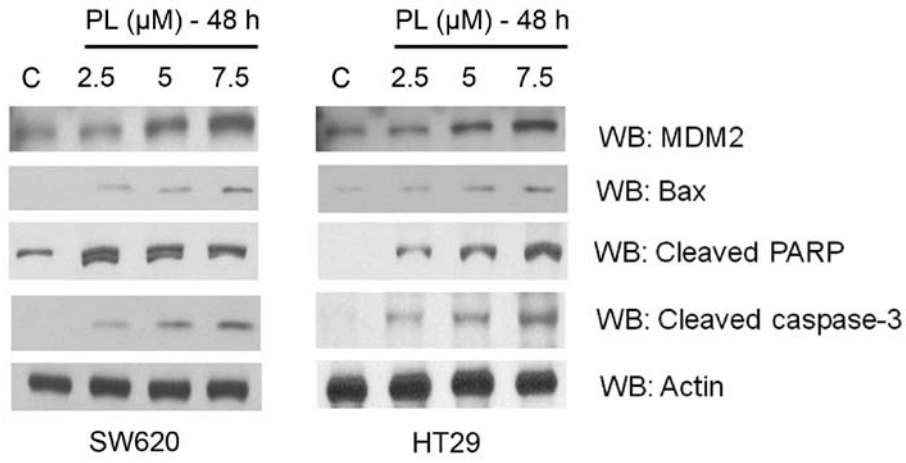

Figure 5. Restoration of p53 transcriptional function. Western blots showing the induction of p53 target genes MDM2, Bax and apoptosis markers cleaved caspase-3 and cleaved PARP in HT29 and SW620 cells treated with 2.5, 5 and 7.5 μM PL for 48 h are shown.

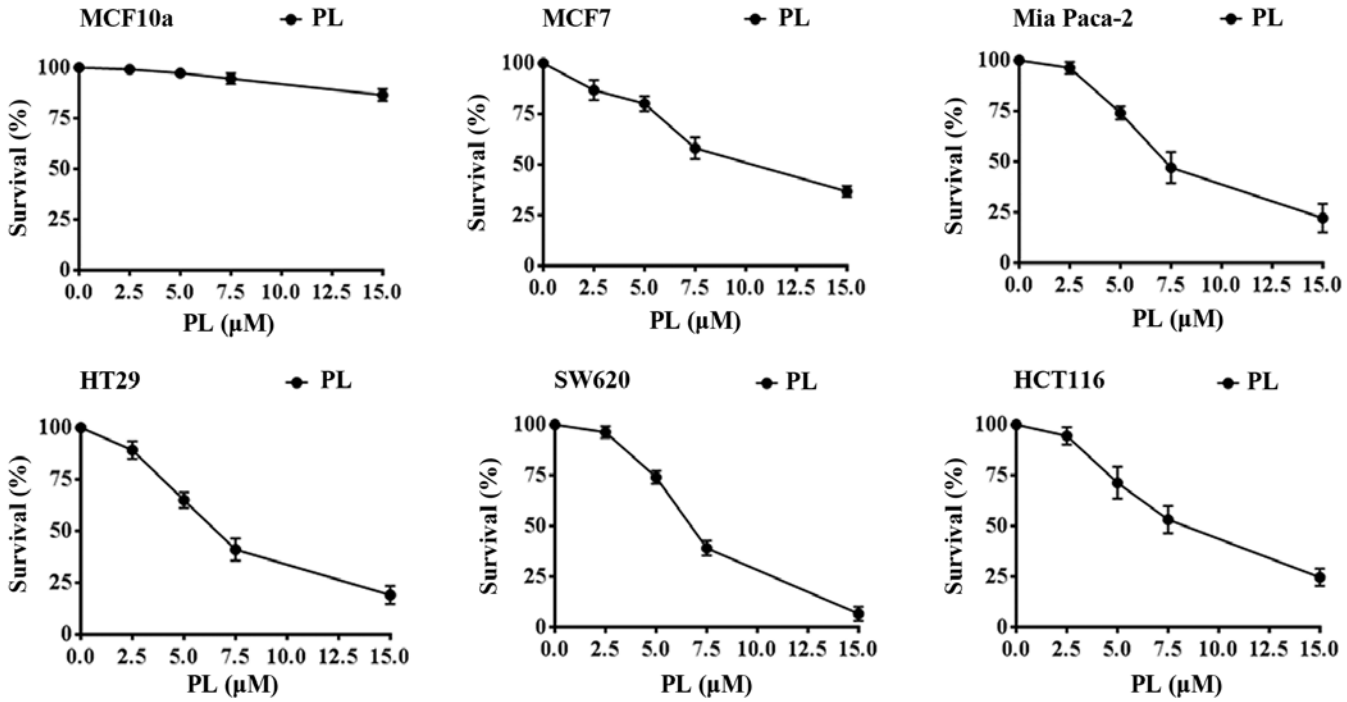

Figure 6. Cytotoxicity of PL against MCF10a (normal cells), MCF7 (p53-wt), Mia Paca-2 (R248W), HT29 (R273H), SW620 (R273H), and HCT116 (p53-wt) cancer cell lines. Cell growth inhibition was analyzed using the MTT assay. Cells were treated with different concentrations of PL (2.5, 5, 7.5 and 15 μM) for 48 h. The data represent the results of three independent experiments performed in triplicate. Values are mean ± SD.

(Fig. 7). For BCNU and doxorubicin, the potentiation was ~2-fold in different cell lines. Our findings suggest that addition of PL to the anticancer regimens will be beneficial and result in increased tumor cell killing. The enhanced anticancer effects are likely to occur at least in part through the functional restoration of the p53 tumor suppressor observed in this study.

Piperlongumine induces tumor growth delay and loss of p53 mutant protein in HT29 xenografts. To probe whether PL induces a conversion of mutant p53 protein to its wt-like counterpart *in vivo*, we developed subcutaneous xenografts by injecting the HT29 cells in nude mice. PL (5-10 mg/kg) was administered every day to the tumor bearing mice for 20 days. A significant and PL dose-dependent delay of tumor growth was observed (Fig. 8A). No changes were discernible in the body weight of animals indicating a lack of toxicity (Fig. 8B). Further, when the lysates from the excised tumors were immunoblotted, there was an apparent decrease in the R273H mutant p53 protein levels (Fig. 8C). This decrease was accompanied by enhanced levels of both the cleaved PARP and cleaved caspase-3 (Fig. 8C), verifying that PL can trigger apoptotic initiation in tumor tissues as well.

Effect of PL on the anticancer effects of cisplatin and doxorubicin in HT29 xenografts. To explore the possibility of combining PL with current chemotherapy drugs, we evaluated

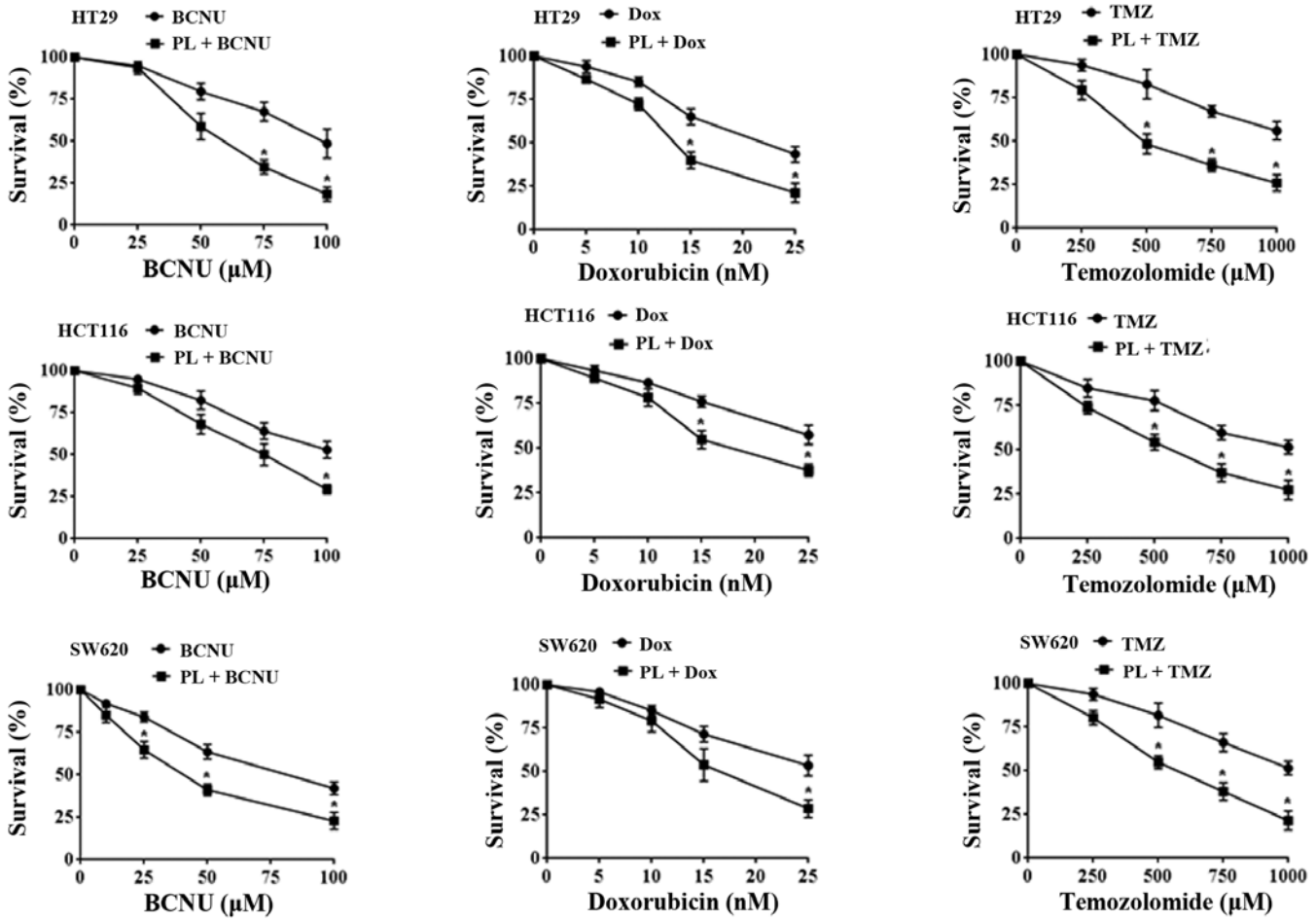

Figure 7. BCNU, doxorubicin, and TMZ mediated cell killing with or without PL pre-exposure in HT29, SW620 and HCT116 cancer cells. Cell growth inhibition was analyzed using the MTT assay. Cells were exposed to the drugs with or without pre-exposure to 7.5 μM PL for 24 h. The data represent the results of three independent experiments performed in triplicate. Values are mean ± SD. The results presented are significant at P<0.05. *Statistically significant difference as compared with the controls.

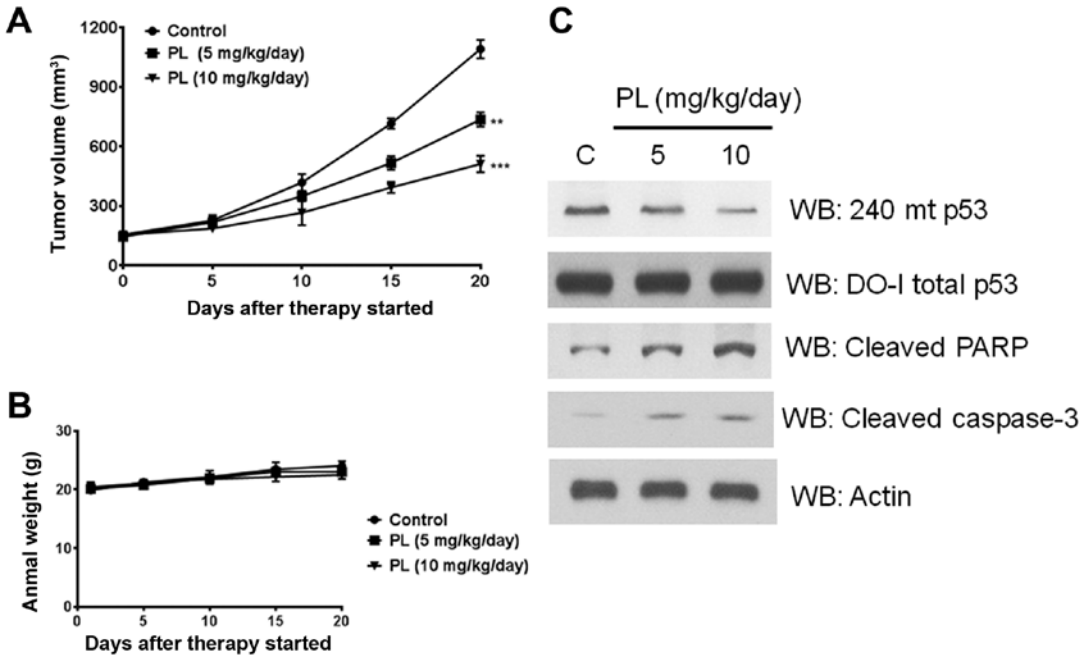

Figure 8. (A) Significant tumor growth delay is observed in HT29 xenografts after PL 5 and 10 mg/kg/day administration. Tumor volumes shown are mean ± SD. *Significant difference as compared with the control. **P<0.01, ***P<0.001. (B) After 20 days of treatment, there were no significant differences in the body weights between the vehicle and treatment groups. (C) Western blot analyses of the mutant p53, total p53, cleaved PARP and cleaved caspase-3 proteins in HT29 tumor tissue lysates from the xenografts with or without PL administration.

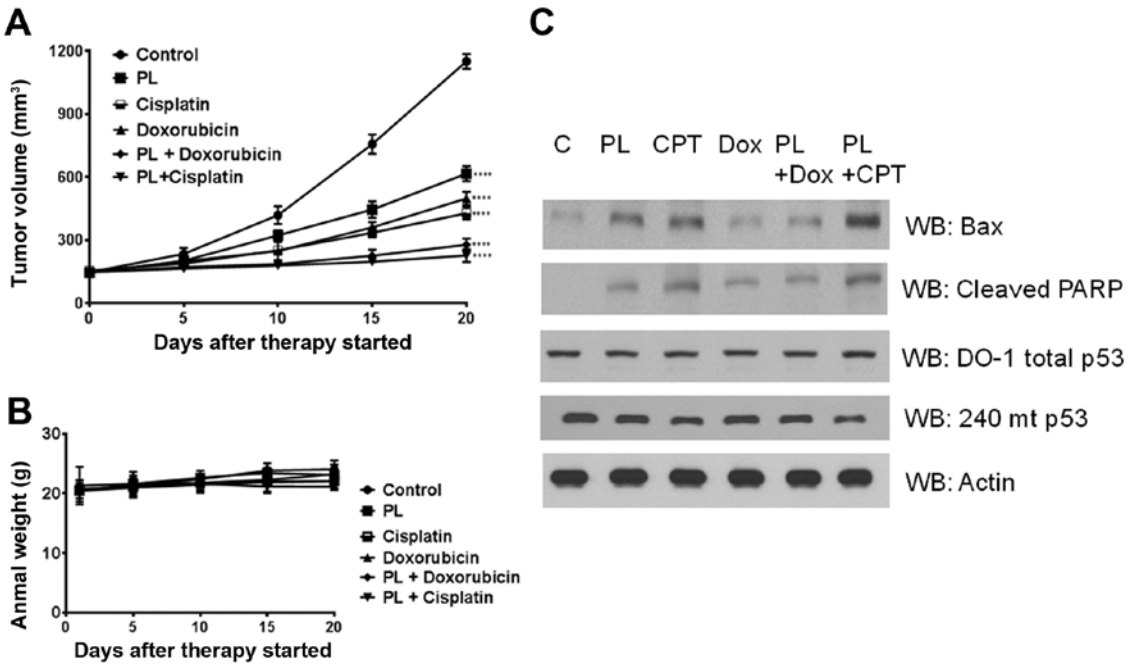

Figure 9. (A) HT29 tumor growth delay in xenografts after PL + cisplatin or PL + doxorubicin treatments. PL was administered at 7.5 mg/kg/day either alone or in combination with cisplatin (1 mg/kg/day) or doxorubicin (0.75 mg/kg/day). A significant increase in tumor regression in the combination groups is evident. Tumor volumes shown are mean ± SD. The results shown are significant at P<0.0001. *Statistically significant differences as compared with controls. (B) The treatment regimens did not produce any statistically significant differences in animal body weights compared with the controls. (C) Western blot analyses in HT29 tumor tissue lysates from animals that received PL alone or PL + chemotherapy drugs.

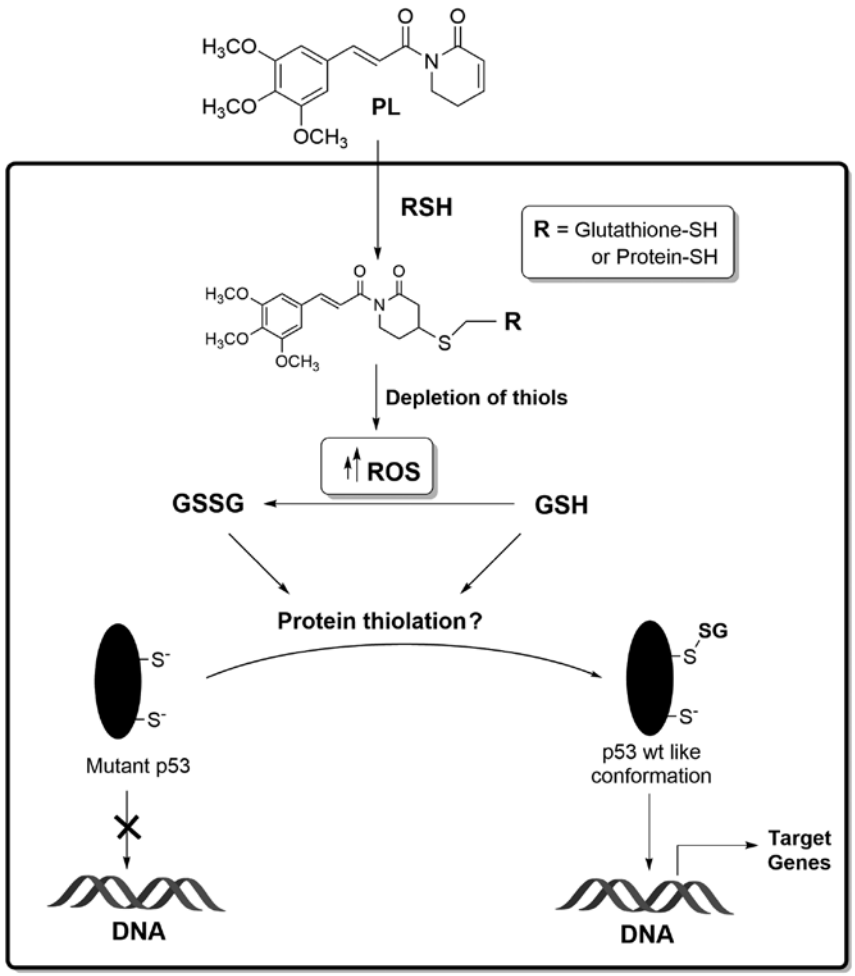

Figure 10. Schematic representation showing PL interaction with the protein-bound or protein-free thiols, consequent oxidative stress and the possible role of protein glutathionylation in mutant p53 reactivation found in this study.

the tumor growth delay in HT29 xenografts given cisplatin or doxorubicin along with PL (7.5 mg/kg/day). As shown in Fig. 9, PL treatment alone produced significant tumor regression. Simultaneous treatment of PL with cisplatin or doxorubicin resulted in a vigorous and significant reduction of tumor volume, compared with either agent alone. Statistical analyses indicated that the antitumor effects were synergistic (Fig. 9A). Changes in body weights were not significantly different between the control and PL-treated groups ($P>0.5$) indicating that no apparent adverse effects were associated with the chemotherapy (Fig. 9B). Western blot analyses again revealed a reduction in mutant p53 levels, unaltered total p53 levels (reactive with the pan DO-1 Ab), along with a marked upregulation of apoptotic proteins such as the Bax and cleaved PARP (Fig. 9C). Taken together, these findings suggest that oxidative stress mediated by PL can potentiate the cytotoxic effects of many clinically used anticancer drugs.

Conclusion. In continuation of our research interests in oxidative stress signaling (34), and regulation of p53 functions by redox imbalance in human cancers (20,21), the present study investigated the biochemical effects of PL on the R273H mutant p53 present in HT29 and SW620 cells and the therapeutic consequences thereof. Fig. 10 summarizes our findings. We propose that PL, as an electrophile, can either react directly with the protein-bound anionic cysteines or conjugate with the SH-group of glutathione (4,5); the glutathione S-transferases may facilitate the latter reaction. The ROS generation by PL and the thiol conjugations are likely to deplete the cellular GSH levels and trigger protein thiolation. We hypothesize that mutant p53 proteins are efficient substrates for glutathionylation, which in turn can induce structural perturbations in the defective DNA-binding domain of the tumor suppressor and restore some functionality (Fig. 10). While the PL-induced p53 reactivation is somewhat feeble, and the mechanism(s) involved are yet to be proven, our recent study with a hybrid C-7 aryl piperlongumine derivative indeed suggests that induction of redox-imbalance may serve as a general platform to rescue the cancer mutations in p53 (35). In conclusion, the findings made here and the evidence that oxidative stress may prevent distant metastasis (36) highlight the therapeutically beneficial and exploitable aspects of tumor redox biology.

Acknowledgements

This study was supported by grants from the Cancer Prevention and Research Institute of Texas (RP130266), the Carson-Leslie Foundation and the Association for Research of Childhood Cancer, all to K.S.S.

References

1. Trachootham D, Alexandre J and Huang P: Targeting cancer cells by ROS-mediated mechanisms: A radical therapeutic approach? Nat Rev Drug Discov 8: 579-591, 2009.
2. Sosa V, Moliné T, Somoza R, Paciucci R, Kondoh H and LLeonart ME: Oxidative stress and cancer: An overview. Ageing Res Rev 12: 376-390, 2013.
3. Raj L, Ide T, Gurkar AU, Foley M, Schenone M, Li X, Tolliday NJ, Golub TR, Carr SA, Shamji AF, *et al*: Selective killing of cancer cells by a small molecule targeting the stress response to ROS. Nature 475: 231-234, 2011.
4. Adams DJ, Dai M, Pellegrino G, Wagner BK, Stern AM, Shamji AF and Schreiber SL: Synthesis, cellular evaluation, and mechanism of action of piperlongumine analogs. Proc Natl Acad Sci USA 109: 15115-15120, 2012.
5. Han JG, Gupta SC, Prasad S and Aggarwal BB: Piperlongumine chemosensitizes tumor cells through interaction with cysteine 179 of IκBα kinase, leading to suppression of NF-κB-regulated gene products. Mol Cancer Ther 13: 2422-2435, 2014.
6. Polyak K, Xia Y, Zweier JL, Kinzler KW and Vogelstein B: A model for p53-induced apoptosis. Nature 389: 300-305, 1997.
7. Petitjean A, Mathe E, Kato S, Ishioka C, Tavtigian SV, Hainaut P and Olivier M: Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: Lessons from recent developments in the IARC TP53 database. Hum Mutat 28: 622-629, 2007.
8. Olivier M, Hollstein M and Hainaut P: Hollstein M and Hainaut P: TP53 mutations in human cancers: Origins, consequences, and clinical Use. Cold Spring Harb Perspect Biol 2: 1-17, 2010.
9. Basu A and Haldar S: The relationship between BcI2, Bax and p53: Consequences for cell cycle progression and cell death. Mol Hum Reprod 4: 1099-1109, 1998.
10. Bykov VJN and Wiman KG: Mutant p53 reactivation by small molecules makes its way to the clinic. FEBS Lett 588: 2622-2627, 2014.
11. Wiman KG: Strategies for therapeutic targeting of the p53 pathway in cancer. Cell Death Differ 13: 921-926, 2006.
12. Saha MN, Qiu L and Chang H: Targeting p53 by small molecules in hematological malignancies. J Hematol Oncol 6: 23, 2013.
13. Bykov VJN, Issaeva N, Shilov A, Hultcrantz M, Pugacheva E, Chumakov P, Bergman J, Wiman KG and Selivanova G: Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. Nat Med 8: 282-288, 2002.
14. Bykov VJN, Issaeva N, Zache N, Shilov A, Hultcrantz M, Bergman J, Selivanova G and Wiman KG: Reactivation of mutant p53 and induction of apoptosis in human tumor cells by maleimide analogs. J Biol Chem 280: 30384-30391, 2005.
15. Tang X, Zhu Y, Han L, Kim AL, Kopelovich L, Bickers DR and Athar M: CP-31398 restores mutant p53 tumor suppressor function and inhibits UVB-induced skin carcinogenesis in mice. J Clin Invest 117: 3753-3764, 2007.
16. Zache N, Lambert JMR, Rökaeus N, Shen J, Hainaut P, Bergman J, Wiman KG and Bykov VJN: Mutant p53 targeting by the low molecular weight compound STIMA-1. Mol Oncol 2: 70-80, 2008.
17. Demma M, Maxwell E, Ramos R, Liang L, Li C, Hesk D, Rossman R, Mallams A, Doll R, Liu M, *et al*: SCH529074, a small molecule activator of mutant p53, which binds p53 DNA binding domain (DBD), restores growth-suppressive function to mutant p53 and interrupts HDM2-mediated ubiquitination of wild-type p53. J Biol Chem 285: 10198-10212, 2010.
18. Yu X, Vazquez A, Levine AJ and Carpizo DR: Allele-specific p53 mutant reactivation. Cancer Cell 21: 614-625, 2012.
19. Bykov VJN, Lambert JMR, Hainaut P and Wiman KG: Mutant p53 rescue and modulation of p53 redox state. Cell Cycle 8: 2509-2517, 2009.
20. Velu CS, Niture SK, Doneanu CE, Pattabiraman N and Srivenugopal KS: Human p53 is inhibited by glutathionylation of cysteines present in the proximal DNA-binding domain during oxidative stress. Biochemistry 46: 7765-7780, 2007.
21. Yusuf MA, Chuang T, Bhat GJ and Srivenugopal KS: Cys-141 glutathionylation of human p53: Studies using specific polyclonal antibodies in cancer samples and cell lines. Free Radic Biol Med 49: 908-917, 2010.
22. Liu Y, Asch H and Kulesz-Martin MF: Functional quantification of DNA-binding proteins p53 and estrogen receptor in cells and tumor tissues by DNA affinity immunoblotting. Cancer Res 61: 5402-5406, 2001.
23. Gallogly MM and Mieyal JJ: Mechanisms of reversible protein glutathionylation in redox signaling and oxidative stress. Curr Opin Pharmacol 7: 381-391, 2007.
24. Ghezzi P, Bonetto V and Fratelli M: Thiol-disulfide balance: From the concept of oxidative stress to that of redox regulation. Antioxid Redox Signal 7: 964-972, 2005.
25. Klatt P and Lamas S: Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress. Eur J Biochem 267: 4928-4944, 2000.
26. Al-Sawaf O, Clarner T, Fragoulis A, Kan YW, Pufe T, Streetz K and Wruck CJ: Nrf2 in health and disease: Current and future clinical implications. Clin Sci (Lond) 129: 989-999, 2015.

27. Brandt R and Keston AS: Synthesis of diacetyldichlorofluorescin: A stable reagent for fluorometric analysis. Anal Biochem 11: 6-9, 1965.
28. Wang Z and Sun Y: Targeting p53 for novel anticancer therapy. Transl Oncol 3: 1-12, 2010.
29. Rainwater R, Parks D, Anderson ME, Tegtmeyer P and Mann K: Role of cysteine residues in regulation of p53 function. Mol Cell Biol 15: 3892-3903, 1995.
30. Li Y and Prives C: Are interactions with p63 and p73 involved in mutant p53 gain of oncogenic function? Oncogene 26: 2220-2225, 2007.
31. Bonsing BA, Corver WE, Gorsira MCB, van Vliet M, Oud PS, Cornelisse CJ and Fleuren GJ: Specificity of seven monoclonal antibodies against p53 evaluated with Western blotting, immunohistochemistry, confocal laser scanning microscopy, and flow cytometry. Cytometry 28: 11-24, 1997.
32. Wang PL, Sait F and Winter G: The 'wildtype' conformation of p53: Epitope mapping using hybrid proteins. Oncogene 20: 2318-2324, 2001.
33. Gannon JV, Greaves R, Iggo R and Lane DP: Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. EMBO J 9: 1595-1602, 1990.
34. Niture SK, Velu CS, Bailey NI and Srivenugopal KS: S-thiolation mimicry: Quantitative and kinetic analysis of redox status of protein cysteines by glutathione-affinity chromatography. Arch Biochem Biophys 444: 174-184, 2005.
35. Punganuru SR, Madala HR, Venugopal SN, Samala R, Mikelis C and Srivenugopal KS: Design and synthesis of a C7-aryl piperlongumine derivative with potent antimicrotubule and mutant p53-reactivating properties. Eur J Med Chem 107: 233-244, 2016.
36. Piskounova E, Agathocleous M, Murphy MM, Hu Z, Huddlestun SE, Zhao Z, Leitch AM, Johnson TM, DeBerardinis RJ and Morrison SJ: Oxidative stress inhibits distant metastasis by human melanoma cells. Nature 527: 186-191, 2015.