Piperlongumine Induces Reactive Oxygen Species (ROS)-Dependent Downregulation of Specificity Protein Transcription Factors
Keshav Karki, Erik Hedrick, Ravi Kasiappan, Un-Ho Jin, and Stephen Safe

Abstract
Piperlongumine is a natural product found in the plant species *Piper longum*, and this compound exhibits potent anticancer activity in multiple tumor types and has been characterized as an inducer of reactive oxygen species (ROS). Treatment of Panc1 and L3.6pl pancreatic, A549 lung, 786-O kidney, and SKBR3 breast cancer cell lines with 5 to 15 μM piperlongumine inhibited cell proliferation and induced apoptosis and ROS, and these responses were attenuated after cotreatment with the antioxidant glutathione. Piperlongumine also downregulated expression of Sp1, Sp3, Sp4, and several pro-oncogenic Sp-regulated genes, including cyclin D1, survivin, cMyc, EGFR and hepatocyte growth factor receptor (cMet), and these responses were also attenuated after cotreatment with glutathione. Mechanistic studies in Panc1 cells showed that piperlongumine-induced ROS decreased expression of cMyc via an epigenetic pathway, and this resulted in downregulation of cMyc-regulated miRNAs miR-27a, miR-20a, and miR-17 and induction of the transcriptional repressors ZBTB10 and ZBTB4. These repressors target GC-rich Sp-binding sites to decrease transactivation. This pathway observed for piperlongumine in Panc1 cells has previously been reported for other ROS-inducing anticancer agents and shows that an important underlying mechanism of action of piperlongumine is due to downregulation of Sp1, Sp3, Sp4, and pro-oncogenic Sp-regulated genes. Cancer Prev Res; 10(8): 467-77. ©2017 AACR.

Introduction
Piperlongumine is an alkaloid natural product found in the plant species *Piper longum* Linn that exhibits a broad spectrum of biological effects (1–5), including antitumorigenic activities in cancer cell lines and animal models (6–19). Raj and colleagues identified piperlongumine in a high-throughput screening assay and demonstrated the highly selective killing of cancer cell lines compared with normal untransformed cells. Their studies also demonstrated in vivo antitumor activity in both mouse and rat models, and they also reported that piperlongumine inhibited ROS in several cancer cell lines (6). It was concluded that piperlongumine was a potent inducer of oxidative stress–dependent cell killing, and this was due, in part, to depletion of glutathione and other thiol-containing proteins involved in maintaining cellular redox homeostasis (6, 10). Several subsequent studies have confirmed the anticancer activities of piperlongumine, and these include pathways/genes that are ROS-dependent (6–13) and other pathways in which the role of ROS was not determined (14–19).

Studies in this laboratory have investigated the anticancer activities and mechanism of action of several ROS-inducing anticancer agents, including curcumin, a nitro-aspirin derivative, betulinic acid, methyl 2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me), histone deacetylase (HDAC) inhibitors, phenethylisothiocyanate (PEITC), celastrol, penfluridol, and benzylisothiocyanate (BITC; refs. 20–28). For some of these drugs such as curcumin and betulinic acid, their induction of ROS was cell context dependent; however, the induction of ROS by these compounds was functionally important as compound-dependent inhibition of cancer cell proliferation and survival were reversed after cotreatment with antioxidants. Drug-induced ROS via alkylation of GSH and redox genes or by direct effects on mitochondria also leads to oxidative stress-induced endoplasmic reticulum (ER) stress and increased apoptosis (29). Studies in this laboratory have demonstrated that ROS inducers (20–28) and also hydrogen peroxide and t-butyl hydroperoxide (30, 31) decrease expression of specificity protein 1 (Sp1), Sp3, and Sp4 transcription factors (TF) and also several pro-oncogenic Sp-regulated genes and noncoding RNAs (32). The mechanism of ROS-dependent downregulation of Sp TFs involves initial ROS-induced repression of cMyc, decreased expression of cMyc-regulated miRNAs, miR-27a and miR-20a/miR-17-5p, which results in the induction of miR-regulated ZBTB10 (ZBTB34) and ZBTB4 (25, 27, 28, 32). ZBTBs are transcriptional repressors that competitively bind GC-rich cis-elements and displace Sp TFs resulting in decreased Sp-regulated gene expression (33, 34).

ROS-dependent targeting of Sp TFs represents an important pathway that contributes to the anticancer activity of ROS inducers, as this results in downregulation of pro-oncogenic Sp-regulated genes, including survivin, cyclin D1, VEGF and its receptors, EGFR, and other receptor tyrosine kinases (32, 33). In this
study, we show that piperlongumine induces ROS, inhibits cell growth, and induces apoptosis in several cancer cell lines, and cotreatment with glutathione reverses these responses. Piperlongumine also induces ROS-dependent downregulation of Sp1, Sp3, Sp4, and pro-oncogenic Sp-regulated genes, demonstrating that the anticancer activity of this compound is also due, in part, to targeting of Sp TFs.

Materials and Methods

Cell lines, antibodies, and reagents
Pancreatic cancer cells (Panc1, L3.6pl), kidney (786-O), lung (A549), and breast (SKBR3) cancer cell lines were purchased from ATCC, and the SKBR3, L3.6pl, and Panc1 cells were authenticated in 2016 by Biosynthesis. Cells were grown and maintained at 37°C in the presence of 5% CO2 in DMEM/Ham’s F-12 medium supplemented with 10% FBS or RPMI1640 medium with 10% FBS. DMEM, RPMI1640 medium, FBS, formaldehyde, trypsin, and glutathione (98% pure) were purchased from Sigma-Aldrich. CMyc, survivin, cleaved PARP (cPARP), and cMete antibodies were from Cell Signaling Technology. ZBTB4 and Sp1 antibodies were from Abcam, and ZBTB10 antibody was from Bethyl Laboratories Inc. Chemiluminescence reagents (Immobilon Western) for Western blot imaging were purchased from Millipore, and piperlongumine was purchased from INDOFINE Chemical Company, Inc. The Apoptotic, Necrotic, and Healthy Cells Quantiﬁcation Kit was purchased from Biotium. The ROS Determination Kit was purchased from Invitrogen, Chromatin Immunoprecipitation Kit was purchased from Active Motif, and XTT Cell Viability Kit purchased from Cell Signaling Technology.

Cell viability assay
Cells were plated in 96-well plates at a density of 3,000 per well with DMEM containing 10% charcoal-stripped FBS. After 24 hours, cells were treated with DMSO and different concentrations of piperlongumine with DMEM containing 2.5% charcoal-stripped FBS for 0 to 48 hours. After treatment with piperlongumine, 25 μL (XTT with 1% of electron coupling solution) was added to each well and incubated for 4 hours as described in the manufacturer’s instructions (Cell Signaling Technology). After incubation, absorbance was measured at a wavelength of 450 nm in a 96-well plate reader.

Measurement of ROS
Cell permeable probe CM-H2DCFDA [5-(and 6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester] as described in the manufacturer’s instructions (Life Technologies) was used to measure ROS level in cancer cells. Cells were seeded at a density of 1.5 × 10^5 per mL in 6-well plates, allowed to attach for 24 hours, pretreated with GSH for 30 minutes, and treated with vehicle (DMSO), piperlongumine alone, or with GSH for 30 minutes to 9 hours. ROS levels were measured by flow cytometry as described previously (27, 28).

Measurement of apoptosis (Annexin V staining)
Cells were seeded at a density of 1.5 × 10^5 per mL in 6-well plates and allowed to attach for 24 hours, pretreated with GSH for 30 minutes, and treated with either vehicle or piperlongumine or combination with GSH for 24 hours. Cells were then stained and analyzed by flow cytometry using the Vybrant Apoptosis Assay Kit according to the manufacturer’s protocol (Biotium).

Western blot analysis
Panc1, L3.6pl, SKBR3, 786-O, and A549 cells were seeded at a density of 1.5 × 10^5 per mL in 6-well plates and allowed to attach for 24 hours. Cells were treated with various concentrations of piperlongumine alone or in combination with GSH, and whole-cell proteins were extracted using RIPA lysis buffer containing 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100 (w/v), 0.5% sodium deoxycholate, and 0.1% SDS with protease and phosphatase inhibitor cocktail. Protein concentrations were measured using Lowry method, and equal amounts of protein were separated in 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were incubated with primary antibodies overnight at 4°C and incubated with corresponding HRP-conjugated secondary IgG antibodies, and immunoreacted proteins were detected with chemiluminescence reagent.

Chromatin immunoprecipitation assay
Panc1 cells were seeded at a density of 5 × 10^4 and allowed to attach for 24 hours. Cells were treated with piperlongumine for 3 hours and subjected to chromatin immunoprecipitation (ChIP) analysis using the ChIP-IT Express Magnetic Chromatin Immunoprecipitation Kit (Active Motif) according to the manufacturer’s protocol using 1% formaldehyde for crosslinking. The sonicated chromatin was immunoprecipitated with normal IgG (Santa Cruz Biotechnology), and antibodies for RNA polymerase II (pol II; GeneTex), H3K27me3 (Abcam), H3K4me3 (Abcam), H4K16Ac (Active Motif), Sp1 (Abcam), Sp3, and Sp4 (SantaCruz) were incubated with protein A–conjugated magnetic beads at 4°C for overnight. Magnetic beads were extensively washed, and cross-linked protein-DNA was reversed and eluted. DNA was extracted from the immunoprecipitates, and PCR was performed using following primers. The primers for detection of the c-Myc promoter region were 5'-GCC CIT CTC CCA GCC TTA GC-3' (sense) and 5'-AAC CGC ATC TCT CTT TGA GTA-3' (antisense); the primers for the detection of the β-actin (ACTB) promoter region were 5'-CTC CCT CTT CCT TCT CCT CA-3' (sense) and 5'-TCC ATG CAT AAA AGG CAA CTT-3' (antisense); the primers for detection of the Sp1 promoter region were 5'-CTA ACT CCA ATC AAT AGC TTC C-3' (sense) and 5'-GAG ATG ATT GCC TGG TGG-3' (antisense). PCR products were resolved on a 2% agarose gel in the presence of ethidium bromide (Denville Scientific Inc.).

Real-time PCR
Expression of miR-17, miR-20a, and miR-27a after treatment with piperlongumine alone or in combination with GSH was measured using RT-PCR. Panc1 cells were plated at a density of 4 × 10^4 in 60-mm dish and were allowed to attach for 24 hours. Cells were treated with piperlongumine alone or in combination with GSH for 0 to 24 hours. Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer’s instruction. TaqMan microRNA assays (Life Technologies) were used to quantify expression of miR-17, miR-20a, and miR-27a, and RNU6 was used as a control to determine relative miRNA expression.

Xenograft study
Female athymic nu/nu mice (4–6 weeks old) were purchased from Harlan Laboratories. L3.6pl cells (1 × 10^6) were harvested in 100 μL of DMEM and suspended in ice-cold Matrigel (1:1 ratio) and subcutaneously injected to either side of the flank area of the
mice. After 1 week of tumor cell inoculation, mice were divided into two groups of 5 animals each. The first group received 100 μL of vehicle (corn oil), and second group of animals received an injection of 30 mg/kg/day of piperlongumine in 100 μL volume of corn oil intraperitoneally for 3 weeks. All mice were weighed once a week over the course of treatment to monitor changes in body weight. Tumor volumes could not be determined over the period of treatment because xenografted tumors were relatively deep. After 3 weeks of treatment, mice were sacrificed and tumor weights were determined. All animal studies were carried out according to the procedures approved by the Texas A&M University Institutional Animal Care and Use Committee.

Statistical analysis
Student t test was used to determine the statistical significance between two groups. The experiments for treatment group were performed at least three independent times, and results were expressed as means ± SEM. P values less than 0.05 were considered to be statistically significant.

Results
Piperlongumine induces ROS-dependent growth inhibition and apoptosis
In this study, we initially used 786-O kidney, SKBR3 breast, Panc1 and L3.6P L pancreatic, and A549 lung cancer cell lines to investigate the growth-inhibitory effects of piperlongumine. Treatment of 786-O cells with 5, 10, and 15 μmol/L piperlongumine for 24 and 48 hours significantly decreased cell proliferation at all concentrations (Fig. 1A), and these effects were blocked after cotreatment with 5 mmol/L GSH. Interestingly, we observed that GSH alone enhanced proliferation of these cells at the 24-hour but not the 48-hour time point, suggesting that endogenous ROS may have been decreasing cell proliferation at the former time point. The same experimental protocol was used for SKBR3 (Fig. 1B), Panc1 (Fig. 1C), A549 (Fig. 1D), and L3.6P L (Fig. 1E) cells, and the results confirmed that for this panel of cancer cell lines that piperlongumine-induced ROS was a major factor in the growth-inhibitory effects observed for this compound.

We also investigated induction of ROS by piperlongumine by FACS analysis using the cell-permeable CM-H2DCFDA dye. Piperlongumine induced ROS in 786-O, SKBR3, Panc1, A549, and L3.6P L cells (Fig. 2A–E); this response was attenuated in cells cotreated with GSH, and these results are consistent with previous studies showing that piperlongumine induces ROS (6). Treatment of these cells with piperlongumine also induced Annexin V staining and PARP cleavage, which are markers of apoptosis (Fig. 3A–E), and cotreatment with GSH attenuated these responses, demonstrating that piperlongumine induces ROS, which in turn inhibits cell growth and induces apoptosis. Supplementary Figure S1 illustrates the flow cytometric analysis of the piperlongumine-induced Annexin V staining.

Piperlongumine downregulates Sp1, Sp3, Sp4, and Sp-regulated genes
The effects of piperlongumine on downregulation of Sp1, Sp3, Sp4, and Sp-regulated genes, including cyclin D1, EGFR, hepatocyte growth factor receptor (cMET), and survivin, were also investigated in the five cancer cell lines. Treatment of 786-O cells with 5 or 10 μmol/L piperlongumine decreased expression of Sp1, Sp3, and Sp4, and after cotreatment with GSH, these responses were blocked, and similar results were observed for effects of piperlongumine on Sp-regulated cMyc, EGFR, survivin, and cMET (Fig. 4A). This same approach was used to investigate the effects of piperlongumine alone or in combination with GSH on Sp TFs and Sp-regulated genes in SKBR3 (Fig. 4B), Panc1 (Fig. 4C), A549 (Fig. 4D), and L3.6P L (Fig. 4E) cells. The higher concentration of piperlongumine (15 μmol/L for A549 cells and 10 μmol/L for the other cell lines) decreased expression of Sp1, Sp3, Sp4, and Sp-regulated genes, and this response was attenuated after cotreatment with GSH. We also observed that 5 μmol/L piperlongumine was effective in reducing one or more Sp proteins and Sp-regulated genes in 786-O, SKBR3, Panc1, and L3.6P L cells and 10 μmol/L piperlongumine in A549 cells, which was the most piperlongumine-resistant cell line after treatment for 24 hours.

Piperlongumine modulates the expression of or inhibits redox enzymes, and the conjugated en-one structure alkylates thiol-containing molecules (7, 15), and we therefore further investigate the effects of the non–thiol-containing redundant Tiron on piperlongumine-dependent Sp downregulation (Supplementary Fig. S2). The results were similar to that observed after treatment with piperlongumine ± GSH (Fig. 4); piperlongumine decreased Sp1, Sp3, Sp4, and Sp-regulated genes in 786-O, SKBR3, Panc1, A549, and L3.6P L cells and cotreatment with 5 μmol/L Tiron blocked the effects of piperlongumine in all but SKBR3 cells, where some responses were decreased by 10 μmol/L Tiron (Supplementary Fig. S2). These data confirm the piperlongumine-induced ROS results in downregulation of Sp TFs and pro-oncogenic Sp-regulated genes, and this was similar to the effects observed for other ROS-inducing anticaner agents (20–28).

Mechanism of piperlongumine-induced downregulation of Sp TFs and in vivo studies
Figure 5A outlines the mechanism of ROS-induced downregulation of Sp TFs by initially targeting cMyc, which results in downregulation of cMyc-regulated miRNAs and induction of miRNA-suppressed ZBTB transcriptional repressors (32). Using Panc1 cells as a model, Fig. 5B shows that 5 μmol/L piperlongumine decreases cMyc expression within 3 hours after treatment, and similar results were observed for Sp1, Sp3, and Sp4. Piperlongumine-dependent downregulation of cMyc was blocked after cotreatment with GSH (Fig. 5C), and piperlongumine-induced downregulation of miR-27a and miR-17/20 (Fig. 5D) was also inhibited by cotreatment with GSH (Fig. 5E), and at longer time points (12 and 24 hours), GSH enhanced miR expression. We also observed that 5 μmol/L piperlongumine induced expression of ZBTB10 and ZBTB4 proteins (Fig. 5E), and cotreatment with GSH attenuated this response (Fig. 5F), and these effects are consistent with the pathway illustrated in Fig. 5A.

ROS induces rapid shifts of chromatin-modifying complexes from non–GC-rich to GC-rich sequences (36), and ChIP analysis of the cMyc promoter showed that piperlongumine increased the gene inactivation mark H3K27 and slightly decreased the activation marks H3K4me3 and H4K16Ac and pol II (Fig. 6A). GSH reversed the piperlongumine-induced interactions with the cMyc promoter, and GSH alone enhanced H4K16Ac. In contrast, the major piperlongumine-dependent changes on the GC-rich region of the Sp1 promoter were decreased in the H3K4me3 and H4K16Ac histone marks.
Figure 1.
Piperlongumine inhibits cancer cell proliferation. A–E, 786-O (A), SKBR3 (B), Panc1 (C), A549 (D), and L3.6pL (E) cancer cell lines were treated with different concentrations of piperlongumine or 5 mmol/L GSH alone or in combination for 24 and 48 hours, and cell numbers were determined as outlined in the Materials and Methods. Results are means ± SEM for at least three replicate determinations, and significant (P < 0.05) growth inhibition by piperlongumine (*), growth induction or reversal of piperlongumine-dependent growth inhibition by GSH (**) are indicated.
Piperlongumine induces ROS in cancer cell lines. A–E, 786-O (A), SKBR3 (B), Panc1 (C), A549 (D), and L3.6pL (E) cells were treated with piperlongumine or 5 mmol/L GSH alone or in combination, and ROS was determined by FACS analysis of the cell permeant dye CM-H2DCFDA as outlined in the Materials and Methods. Results are expressed as means ± SEM for at least three replicate determinations, and significant (P < 0.05) induction of ROS by piperlongumine (*) and inhibition by GSH (**) are indicated.
(Fig. 6B), which is consistent with the decreased expression of cMyc (Fig. 5B). We further confirmed the critical role of cMyc in ROS-dependent downregulation of Sp TFs by showing that piperlongumine-induced decreases in Sp1, Sp3, Sp4, and Sp-regulated gene products were rescued by overexpression of cMyc (Fig. 6C). We also observed that piperlongumine (30 mg/kg/day) decreased tumor weight but not body weight in athymic nude mice bearing L3.6pL cells as a xenograft (Fig. 6D), and this was accompanied by significant downregulation of Sp1, Sp3, Sp4, and pro-oncogenic Sp-regulated gene products and induction of PARP cleavage in tumors from piperlongumine-treated mice compared with the vehicle controls.

Thus, like other ROS-inducing anticancer agents, an important underlying mechanism of action is due to targeting of Sp transcription factors (Fig. 5A).

Discussion

Sp1, Sp3, and Sp4 transcription factors are overexpressed in pancreatic cancer lines (20–28, 30–32), and Sp1 is a negative...
Figure 4.
Piperlongumine downregulates Sp1, Sp3, Sp4, and Sp-regulated genes: effects of GSH. A–E, 786-O (A), SKBR3 (B), Panc1 (C), A549 (D), and L3.6pL (E) cells were treated with 5 mmol/L GSH or different concentrations of piperlongumine alone and in combination for 24 hours, and whole-cell lysates were analyzed by Western blot analysis. Effects on Sp proteins and Sp-regulated gene expression and PARP cleavage (Fig. 3) were all obtained in the same experiment and have the same GAPDH loading control. Similar results were observed in duplicate experiments.
Figure 5.
Mechanism of piperlongumine induced Sp downregulation. A, Proposed mechanism of piperlongumine-induced Sp downregulation by initial induction of ROS. B, Panc1 cells were treated with 5 μmol/L piperlongumine for up to 24 hours, and whole-cell lysates were analyzed by Western blots. C, Panc1 cells were treated with 5 μmol/L piperlongumine or 5 mmol/L GSH alone for 4 hours, and whole-cell lysates were analyzed by Western blots. Panc1 cells with 5 μmol/L piperlongumine or 5 mmol/L GSH alone and in combination for up to 24 hours, and the extracted RNA (D) or protein (E) was analyzed by real-time PCR or Western blots, respectively. Results in D are means ± SEM for at least three replicates, and significant (P < 0.05) miRNA downregulation by piperlongumine (*) and reversal by cotreatment with GSH (**) are indicated.
Figure 6. Piperlongumine-dependent Sp downregulation is cMyc dependent and in vivo studies. Panc1 cells were treated with 5 μmol/L piperlongumine or 5 mmol/L GSH alone or in combination for 3 hours, and interactions with the cMyc (A) and Sp1 (B) promoters were determined in ChIP assays. Quantitation of the bands was carried out by quantitative PCR, and results are illustrated in Supplementary Fig. S3. C, Panc1 cells were treated with DMSO or 5 μmol/L piperlongumine alone or after transfection with a cMyc expression plasmid and after 3 hours, whole-cell lysates were analyzed by Western blots. Athymic nude mice bearing L3.6pL cells as xenografts were treated with piperlongumine (30 mg/kg/day), and effects on tumor weights and body weights (D) and expression of various gene products (E) in tumors from control (corn oil) and piperlongumine-treated mice were determined by Western blot analysis of tumor lysates. Expression levels of various proteins in control versus piperlongumine-treated mice were determined (normalized to GAPDH). Significant (P < 0.05) changes in protein levels in tumors from piperlongumine-treated mice compared with controls (*) are indicated.
prognostic factor for patient survival (37, 38), and similar results have been reported for other tumors (32). Results of RNAi studies demonstrate that individual knockdown of Sp1, Sp3, and Sp4 inhibits growth and migration and induces apoptosis in 785-O, SKBR3, Panc1, A549, and L3.6pl cells and other cell lines (24, 35). The responses observed after Sp knockdown are due to the parallel decrease in genes that regulate cancer cell growth, survival, and migration/invasion, and these include multiple receptor tyrosine kinases, angiogenic factors, and prosurvival genes, such as bcl2 and survivin (35). The results suggest that Sp transcription factors are nononcogene addiction genes and are therefore important drug targets for cancer chemotherapy.

Studies in this laboratory have focused on identifying anticancer agents that target Sp proteins, and these include several ROS-inducing agents, such as BITC, PEITC, curcumin, betulinic acid, and HDAC inhibitors (20–28). Initial studies on the broad-spectrum anticancer activity of piperlongumine showed that this compound also induced ROS (6), and this was confirmed in the five cancer cell lines used in this study (Fig. 2). Like other ROS-inducing agents, we also observed that piperlongumine decreased Sp1, Sp3, Sp4, and pro-oncogenic Sp-regulated genes in vitro and in vivo (Figs. 4, 5, and Supplementary Fig. S2). As GSH also reversed the growth-inhibitory and proapoptotic effects of piperlongumine, we conclude that an important underlying mechanism of action of piperlongumine as an anticancer agent is due to ROS-dependent Sp downregulation; however, we did not further investigate the specific ROS species induced by piperlongumine. Several reports show that piperlongumine also induces many other effects in cancer cell lines (1–19); however, some of the specific piperlongumine-induced downregulated gene products in these studies include NFkB, bcl-2, cMyc, cyclin D1, VEGF, and survivin (7, 14, 19), which are also Sp-regulated genes (32).

It was initially reported by O’Hagan and colleagues that hydrogen peroxide induced genome-wide shifts of chromatin-modifying complexes from non-GC-rich to GC-rich promoters, and this resulted in decreased expression of cMyc (36). This represents a novel epigenetic pathway for ROS-mediated gene repression; moreover, studies in this laboratory have also observed these effects in cancer cells treated with other ROS inducers, including PEITC, celestrol, HDAC inhibitors, BITC, and penfluoridol (20–28). Induction of ROS by these agents was accompanied by decreased expression of cMyc-regulated miRNAs (27a, 17, and 20), resulting in the induction of miRNA-repressed ZBTB10 and ZBTB4 as illustrated in Fig. 5A. Piperlongumine also rapidly decreased cMyc expression in Panc1 cells, and this was accompanied by ROS-dependent downregulation of miR-27a and miR-17/20a (part of the miR-17–92 cluster) and induction of ZBTB10 and ZBTB4 (Fig. 5C–F). Piperlongumine also decreased interactions of pol II, slightly increased H3K27me3, and decreased H3K4me3/H4K16Ac interactions with the GC-rich cMyc promoter (Fig. 6A), and these results were similar to those observed for other ROS inducers (25, 27, 28). Examination of the GC-rich region of the Sp1 promoter in a ChIP assay also showed decreased interactions with pol II and the H3K4me3 and H4K16Ac activation markers (Fig. 6B), consistent with the rapid downregulation of Sp protein (Fig. 5B).

In summary, results of this study demonstrate that the important underlying mechanism of action of piperlongumine is due to the ROS-dependent downregulation of cMyc and a cMyc-regulated pathway (Fig. 5A), resulting in downregulation of Sp1, Sp3, Sp4, and pro-oncogenic Sp-regulated genes. This observation is consistent with previous studies on ROS-inducing anticancer agents, including CDDO-Me, celestrol, PEITC, BITC, HDAC inhibitors, and penfluoridol (20–28). Many ROS-inducing anticancer agents induce important ROS-independent and dependent responses that contribute to the overall compound efficacy. Recognition of the ROS–Sp downregulation pathway could be important for designing drug–drug and drug–radiation combination therapies, as many treatment-related drug resistance genes (e.g., survivin) are Sp regulated.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: E. Hedrick, S. Safe
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Hedrick
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Hedrick, R. Kasiappan
Writing, review, and/or revision of the manuscript: E. Hedrick, S. Safe
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Hedrick, U.-H. Jin, S. Safe
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References
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