p53-Dependent Apoptotic Mechanism of a New Designer Bimetallic Compound Tri-phenyl Tin Benzimidazolethiol Copper Chloride (TPT-CuCl₂): In Vivo Studies in Wistar Rats as Well as in Vitro Studies in Human Cervical Cancer Cells

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ABSTRACT

We have studied the effect of tri-phenyl tin benzimidazolethiol-copper chloride (TPT-CuCl₂), a novel bimetallic compound, on the regulation of apoptosis in HeLa cells, MCF-7 cells, and in vivo Wistar rat model. TPT-CuCl₂ induces significant apoptosis in HeLa cell line characterized by DNA fragmentation and chromosome condensation. Comet assay revealed that TPT-CuCl₂ targets and causes severe damage to the DNA. Treatment of HeLa cells with TPT-CuCl₂ rescues the accumulation of p53 from the suppression of human papilloma virus E6, resulting in a dramatic up-regulation of Bax and Bak and down-regulation of the antiapoptotic factor Survivin. Apoptotic induction by TPT-CuCl₂ was shown to mediate in a p53-dependent manner; loss of p53 impairs the release of cytochrome c and Smac/DIABLO from mitochondria to cytosol. Moreover, we have shown that TPT-CuCl₂ induced-apoptosis was through an intrinsic mitochondrial pathway, which was inhibited by viral oncoprotein E1B19K. Caspase-3 was found to be indispensable in TPT-CuCl₂-triggered apoptosis signaling pathway, because caspase-3 deficient cell line MCF-7 was resistant to TPT-CuCl₂. Furthermore, in vivo studies using C6 glioblastoma xenograft rat model revealed that TPT-CuCl₂ exhibits significant antiproliferative activity against tumor development with minimal cytotoxicity toward normal physiological function of the experimental rats. These findings imply the attractiveness of TPT-CuCl₂ as a drug candidate for further development.

Cancer is the result from a multistep process by accumulation of mutations in either tumor-suppressor genes or various dominant oncogenes (Blume-Jensen and Hunter, 2001). Awareness of the molecular basis and evolutionary nature of the cancer can offer a better therapeutic regime, resulting in eradication of the cancer or prolongation the survival rates. Chemotherapy is widely used for treating metastatic cancers either alone or in combination with surgery or radiotherapy (Einhorn et al., 2003; Savarese and Cognetti, 2003). To be useful in cancer therapy, the chemotherapeutic drugs must act broadly across different tumor types. Despite their effective efficacy in treating cancers, most of chemotherapeutic drugs have intrinsic problems such as severe health side effects and the acquisition of drug resistance by tumor cells. To overcome these problems, there is always a need for developing new drugs with minimal side effects and maximal curative potential. The majority of chemotherapeutic drugs are DNA targeted (Eastman, 1990) and antimitotic (Schneider et al., 2003). Although the detailed mechanism by which drugs trigger apoptosis in response to the DNA damage has not been clearly defined, it is widely accepted that cellular

ABBREVIATIONS: HPV, human papilloma virus; mAb, monoclonal antibody; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; TPT-CuCl₂, tri-phenyl tin benzimidazolethiol copper chloride; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; RT-PCR, reverse transcription-polymerase chain reaction; TBS, Tris-buffered saline; PI, propidium iodide; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; Smac/DIABLO, second mitochondria-derived activator of caspases; PARP-1, poly(ADP-ribose) polymerase-1; ALT, alanine aminotransferase; RBC, red blood cell.
stress can induce the activation and stabilization of tumor suppressor p53, resulting in the cell cycle arrest and/or apoptosis. The importance of the p53 is reflected by the fact that it is the most frequently mutated gene in more than one-half of the human malignancies (Hollstein et al., 1991; Bueso-Ramos et al., 1993).

Although mutations in p53 tumor suppressor accounts for the majority of abnormal transformation, there exists additional independent mechanisms, which hinder p53 tumor suppressor function. For example, high-risk human papilloma virus (HPV) serotype 16 or 18, identified as a causative agent of cervical carcinoma (Schwarz et al., 1985; Durst et al., 1987), is responsible for the degradation of p53. The HPV oncogenic protein E6 was reported to directly interact with p53 and through this binding, E6 recruits cellular ubiquitin-protein ligase E6-AP that in turn targets p53 for its accelerated degradation (Munger et al., 1989; Scheffner et al., 1993), thus disrupting the p53-mediated response to DNA damage.

Cervical carcinoma is the third most common malignant disease in women worldwide. The absolute frequency of adenocarcinoma of cervix uteri has increased dramatically during the last decade and accounts for about 15 to 20% of invasive cervical cancers (Andersson et al., 2000).

In the present study, HeLa cell line was used as a model cell line to study the effects of drug candidate on human cervical cancer cells. We demonstrated that a novel bimetallic compound tri-phenyl tin benzimidazolethiol copper chloride (TPT-CuCl2) is capable of restoring p53 and inducing apoptosis in HeLa cells via mitochondrial pathway. Cellular level of wild-type p53 protein increased dramatically after TPT-CuCl2 treatment. The accumulated p53 triggers apoptosis through transactivation of specific target genes, including p21/WAF-1 and Bax, leading to the release of downstream mitochondrial factors cytochrome c and Smac/DIABLO from mitochondria to cytosol. In addition, TPT-CuCl2 was shown to suppress transcription of E6 and Survivin, favoring the apoptosis of target cells to proceed. We further evaluated the efficacy of TPT-CuCl2 in C6 glioblastoma multiform xenograft model in rats and concluded that TPT-CuCl2 was effective in inhibition of tumor formation at a tolerable dose without compromising the normal physiological function of the animal.

**Materials and Methods**

**Reagents and Antibodies.** The following antibodies were used in this study: monoclonal antibody mAb-p53 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibody mAb-caspase-9 was from Immunotech (Marseille, France), monoclonal antibodies mAb-PARP-1 and mAb-p21 were from BD Biosciences (San Jose, CA); mAb-cytochrome c was from R&D Systems (Minneapolis, MN), and mAb-Smac/DIABLO was from Calbiochem (San Diego, CA). Caspase inhibitor Z-LDH-FMK was obtained from BD Biosciences Pharmingen (San Diego, CA), and Z-VAD-FMK was from Sigma-Aldrich (St. Louis, MO). Most of the chemicals and reagents used in this study were ordered from Sigma-Aldrich unless otherwise specified.

**Rats.** Young adult Wistar rats weighing approximately 70 g were obtained from the Animal Laboratory Unit, University of Science and Technology of China. Rats were quarantined for a minimum of 5 days in the animal house. Littermates were used in all experiments under a 12-h light/dark cycle at 20 to 22°C with a relative humidity of 30 to 50%. Institutional guidelines were followed in handling the animals. Tumors were established by s.c injection of 2 × 10^6 glioblastoma multiform C6 cells in both flanks of the animals.

**Cell Culture.** H1299 human lung cancer cell line was a kind gift from Dr. Zhu Wei-Guo (Peking University, People’s Republic of China). HeLa, MCF-7, and C6 cell lines were purchased from Shanghai Institute of Life Sciences (Chinese Academy of Sciences, People’s Republic of China). Cell lines were grown and maintained according to the instructions provided by the suppliers.

**MTT Assay.** Drug-induced cytotoxicity was assessed by MTT assay carried out in 96-well microtiter plates. The cells were treated with TPT-CuCl2 in different concentrations, and untreated cells were used as control. MTT was added to each well to a final concentration of 1 mg/ml and incubated at 37°C for 3 h. The reaction was terminated by removing the medium and the MTT dye was dissolved by adding 100 μl of dimethyl sulfoxide. The plates were read at 570 nm on ELx800 universal microplate reader (Bio-Tek Instruments, Winooski, VT). The assay was performed in triplicate, and the means were determined by using Excel Software.

**Hoechst 33342 DNA Staining.** DNA staining with Hoechst 33342 was performed as described previously (Galli and Fratelli, 1993). Briefly, cells were fixed by Carnoy solution [methanol/acetic acid, 3:1 (v/v)], stained with Hoechst 33342 (0.1 mg/ml in PBS for 10 min at 37°C), washed with water for 5 min, air-dried, and mounted with 50% glycerol in PBS. Cells were observed under fluorescence microscope.

**Trypan Blue Exclusion Assay.** After collecting floating cells, attached cells were exposed to 0.05% trypsin, 0.02% EDTA. All the attached and detached cell populations were combined to determine the proportion of dead cells. Trypan blue (Invitrogen, Carlsbad, CA) was mixed with cells (1:1), and trypan blue exclusion by living cells was scored using phase contrast microscopy.

**DNA Fragmentation Assay.** HeLa cells were grown in the six-well tissue culture plates. After TPT-CuCl2 treatment, cells were harvested at different time points, washed once with ice-cold PBS, and resuspended in 0.5 ml of lysis buffer [10 mM Tris- HCl, pH 7.6, 20 mM EDTA, pH 8.0, and 0.5% (w/v) Triton X-100]. After centrifugation at 14,000 rpm for 5 min, the supernatant was extracted once with phenol/chloroform (1:1) and once with chloroform/isoamyl alcohol (24:1). DNA was precipitated with sodium acetate (pH 5.2) at −20°C overnight. The DNA was then pelleted and subsequently digested with Dnase-free RNase A (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) at 37°C for 20 min. The extracted genomic DNA fragments were fractionated by 1.5% agarose gel and were further visualized by ethidium bromide under UV light.

**Comet Assay for Detecting DNA Strand Breaks.** The comet assay, also called the single-cell gel electrophoresis, was performed as described previously (Anderson et al., 1994). Briefly, precold microscopic slides were overlaid with 110 μl of 0.5% normal melting agarose at 60°C. The slides were immediately covered with a coverslip and then kept at 4°C for 15 min to allow the agarose to solidify. About 10^3 cells of TPT-CuCl2-treated or untreated cells in 40 μl of PBS were mixed with an equal amount (40 μl) of 1% lower melting agarose to form a cell suspension. After gently removing the coverslip, the cell suspension was pipetted onto the first agarose layer and maintained at 4°C for 15 min to solidify. After removal of the coverslip, the slides were immersed in fresh prepared cold lysing solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, pH 10.0, and 1% sodium sarcosinate) with 1% Triton X-100 for 40 min at 4°C. The slides were then placed in a horizontal gel electrophoresis tank filled with fresh electrophoresis solution (1 mM Na3EDTA and 300 mM NaOH, pH 13) for 10 min. The slides were then placed in Tris-buffer (0.4 M Tris, pH 7.5) for 15 min to neutralize the excess alkali. After electrophoresis at 4°C, the slides were stained with 75 μl of ethidium bromide (5 μg/ml) for 30 min and were examined under fluorescent microscope (400 × magnification).

**Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) Assay.** Formalin-fixed sections (5 μm) from the
liver and kidney of trailied or controlled rats were deparaffinized in xylene and were hydrated in graded ethanol. The TUNEL-positive cells were identified using the in situ cell apoptosis detection kit (Huangyong Biological Products Co., Ltd., People’s Republic of China). Liver and kidney sections were examined by higher-powered field microscope (400× magnification).

**Oligonucleotides.** The sequences of the oligonucleotides used in this study are as follows (all primers are read from 5′ to 3′): P1, GGG CAT GCC TTT AGT ATG CAT; P2, GCC TCG ATG TAT ACT TGT CTT CT; P3, GCA GTA CTA GGG GTG CCC CGA CTT TG; P4, GCC TCG ATG CAA TCC ATG GCA GCC AG; and P5, GAC CTG CAT GAC TAG CTC ATG AAC ATG; and P6, GTC ACA CTT CAT ATG GAG TTT TTG AAG G. Primer pair P1/P2 was used to amplify GAC CTG ACT GAC TAC CTC ATG AAG AT; and P6, GTC ACA CTT CAT ATG GAG TTT TTG AAG G. Primer pairs P3/P4 and P5/P6 were used for amplification of Survivn and Actin, respectively.

**RT-PCR.** Total cellular RNA was extracted from confluent HeLa cell cultures (approximately 1 × 10⁷ cells) using an RNA extraction kit (Promega, Madison, WI). RT-PCR was performed according to the protocol described previously (Wu et al., 2000). RT-PCR products were electrophoretically separated by 1.0% agarose gel (Bio-Rad, Hercules, CA) in the presence of ethidium bromide (0.5 μg/ml) and visualized under UV light.

**Preparation of Whole Cell Lysates.** To prepare the whole cell lysates, cells were first grown in 5-cm dishes (Greiner Bio-One GmbH, Frickenhausen, Germany), and the medium was then removed. Cells were washed twice with ice-cold Tris-buffered saline (TBS) (150 mM NaCl and 10 mM Tris, pH 7.6) and lysed with 0.5 ml of lysis buffer (10 mM Tris, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 25 mM-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 mg/ml apotinin, 10 mg/ml leupeptin, and 1 mM sodium orthovanadate) for 15 min. The lysed cells were transferred to 1.5-ml Eppendorf tubes, boiled in SDS sample buffer, and analyzed by Western blot using the indicated antibodies.

**Western Blot Analysis.** Cultured cells were lysed and extraction of cellular proteins was performed using standard procedures. The samples were separated by 12% SDS polyacrylamide gel and electrophoretically transferred onto polyvinylidene difluoride membrane (Amersham Biosciences UK, Ltd.). The blot was blocked with 5% skim milk, 0.1% Tween PBS solution followed by incubation with the relevant antibodies. After washing, the membrane was incubated with activator protein-conjugated secondary antibody for 1 h, washed again in TBS/Tween 20, and developed by using the Western Blue stabilized substrate for alkaline phosphatase (Promega). The blot was blocked with 5% skim milk, 0.1% Tween PBS solution followed by incubation with the relevant antibodies. After washing, the membrane was incubated with activator protein-conjugated secondary antibody for 1 h, washed again in TBS/Tween 20, and developed by using the Western Blue stabilized substrate for alkaline phosphatase (Promega).

**Cell Cycle Analysis.** DNA contents and cell cycle distribution for HeLa cells were determined by flow cytometry. The cells were grown in six-well plates at a density of 5 × 10⁴/well and were then harvested at 0, 2, 6, and 12 h. After washing with ice-cold PBS and fixing with 70% ethanol, 100 μl of RNase A (1 mg/ml) and 400 μl of propidium iodide (50 μg/ml) were added to the cell pellet, which was resuspended and incubated at 37°C for 30 min. Analysis was performed on FACScan flow cytometer (BD Biosciences) using the Cellquest program (BD Biosciences). Ten thousand events were analyzed for each analysis.

**Preparation of Mitochondrial Fraction.** Digitonin fractionation of cells into membrane and cytosolic fractions used for detection of cytochrome c and Smac/DIABLO was performed according to the methods described by Ekert et al. (2001).

**Results**

**Synthesis of Heterobimetallic Complex C₂₅H₂₀N₄S₂Sn₂CuCl₂.** C₂₅H₂₀N₄S₂Sn₂CuCl₂ (0.499 g; 1 mmol) was dissolved in ethanol (100 ml) and CuCl₂ (0.085 g; 0.5 mmol) was added in at 2:1 molar ratio. The reaction mixture was boiled under reflux for ca. 20 h and allowed to cool in a refrigerator. A dark green complex was collected, washed with ether, and dried in vacuo. The formula and structure of this final product are illustrated in Fig. 1A. The complex tri-phenyl tin benzimidazolothiol copper chloride (from Dr. Sarta Tabassum, Department of Chemistry, Aligar University, Aligar, India) was further characterized by various physicochemical methods, and some of its physical properties are listed in Table 1.

The structural framework and the behavior of heterobimetallic compound TPT-CuCl₂ is very distinct from monometallic compounds such as cisplatin, because it provides a unique reactivity pattern in biological processes. It has been reported that Cu²⁺ ions specifically bind to N-7 guanine residue of DNA and cause strand breakage (Sagripanti and Kraemer, 1989). The kinetic analysis (Goldstein and Czapski, 1986; Masarwa et al., 1988) of copper*DNA interaction and its site-specific binding to DNA have been well documented (Chevion, 1988; John and Douglas, 1989). Copper binding site on DNA serves as a center for repeated production of reactive oxygen species (ROS). Similarly, tin (Sn⁴⁺) ion prefers to coordinate with the phosphate backbone of the DNA helix, acting together with Cu²⁺ in a fashion to distort the DNA structure. Based on these findings, we designed a bimetallic Cu²⁺ and Sn⁴⁺-containing compound TPT-CuCl₂ in order to target the DNA of the tumor cells.

To investigate whether TPT-CuCl₂ truly caused damages to DNA, a comet assay was performed. HeLa cells were treated with 4 μM TPT-CuCl₂ for 24 h and harvested for comet assay; the results are shown in Fig. 1B. Compared with untreated control (Fig. 1Ba, left), TPT-CuCl₂ was shown to cause severe DNA damage in treated HeLa cells as evidenced by the presence of profound comet-like tails of the ethidium bromide-stained DNA (Fig. 1Bb, right).
TPT-CuCl₂ Induces Apoptosis in HeLa Cells. To analyze the effect of TPT-CuCl₂, cervical carcinoma HeLa cells were treated with TPT-CuCl₂ at a concentration of 4 μM in a six-well tissue culture plate for 12 h. The typical morphological characteristics of apoptotic cells such as cell shrinkage, nuclear fragmentation, and chromatin condensation were clearly observed under fluorescence microscope after Hoechst 33342 staining. As shown in Fig. 2, Ac and d, the majority of cells that underwent apoptosis displayed densely stained fragmented or compacted nuclei, whereas the untreated con-

<table>
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<th>Complex</th>
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<tr>
<td>C₅₀H₄₀N₄S₂Sn₂CuCl₂</td>
<td>Dark green</td>
<td>280 ± 3</td>
<td>53.3 (53.0)</td>
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Fig. 2. Induction of apoptosis by TPT-CuCl₂ in HeLa cells. HeLa cells were treated with 4 μM TPT-CuCl₂ for 12 h. Both treated and untreated cells were fixed in 3.7% paraformaldehyde and stained with Hoechst 33342. The morphological changes associated with apoptosis were photographed under an inverted fluorescence microscope (A). Arrows indicate apoptotic cells displaying fragmented or compacted nuclei (Ac and d). Untreated HeLa cells showing viable and intact nuclei served as controls for TPT-CuCl₂-treated cells (Aa and b). DNA laddering assay shows the typical pattern of oligonucleosomal-sized fragments of about 200-base pair length (B). Trypan blue exclusion assay was performed to measure cell viability of HeLa cells in response to TPT-CuCl₂ and cisplatin treatment. Concentrations (0–10 μM) used are indicated. Open column, represents cisplatin; hatched column, TPT-CuCl₂. Cell viability is shown as the mean ± S.D. of triplicate samples (C). HeLa cells were treated with increasing concentration of TPT-CuCl₂ from 0 to 6 μM; cells were stained with Annexin V-FITC and PI for detection of apoptosis by FACS analysis (D).
trols showed intact and viable nuclei (Fig. 2Aa and b). To further validate the apoptotic nature resulting from treatment by TPT-CuCl₂, DNA fragmentation analysis was performed. A typical laddering pattern, which is believed to occur at the later stage in apoptosis, is shown in Fig. 2B. The trypan blue exclusion assay, which serves as an index of cell viability, was further conducted to confirm the cell death effect caused by TPT-CuCl₂. HeLa cells treated with different concentrations of TPT-CuCl₂ (0–10 μM) showed a dose-dependent cytotoxicity toward this compound. As shown in Fig. 2C, more cell death was observed as concentration was increased. Approximately up to 70% of the cells were shown to lose viability after TPT-CuCl₂ treatment (10 μM) for 16 h. We also compared the cytotoxic effect of TPT-CuCl₂ with that of cisplatin on HeLa cells and found that TPT-CuCl₂-induced apoptosis was much more potent than cisplatin (Fig. 2C). Treating HeLa cells with TPT-CuCl₂ at a concentration of 10 μM caused a dramatic decrease in cell viability. As mentioned above, fewer than 30% of the HeLa cells were viable after 16 h of treatment. In contrast, treating HeLa cells with cisplatin at the same concentration killed only approximately 40% of the cells, implying TPT-CuCl₂ is more effective than cisplatin in inducing apoptosis in HeLa cells. Sublethal concentration of TPT-CuCl₂ was able to arrest cell division cycle at G0/G1 phase. The FACS data from the exposure of HeLa cells to the low concentration (0.2 μM) of TPT-CuCl₂ showed a marked decrease of cell numbers in S and G2/M phases and consequently an increase of cell population in G0/G1 and sub-G1 phases (Fig. 3), indicating this compound may cause cell cycle arrest at G0/G1 phase. Restraint of treated HeLa cells at G0/G1 is likely to be attributed to the elevated expression of the cell cycle kinase inhibitor p21/WAF-1, which was observed to be up-regulated by accumulation of p53 induced by either DNA damage or E6 suppression upon TPT-CuCl₂ treatment (Fig. 4B). To further quantify the apoptotic induction of the TPT-CuCl₂ in HeLa cells, Annexin V and PI staining was performed on both treated and untreated HeLa cells, and the results are shown in Fig. 2D. Compared with untreated (0 μM) cells, which showed totally about 2% of background cell death, cells treated with 2 μM TPT-CuCl₂ for 16 h were shown to be 35.94% Annexin V-FITC positive and PI negative (early apoptosis) and 9.45% of Annexin V-FITC and PI doubly positive. These doubly positive cells represent either the late stage apoptotic or necrotic cells. By 4 μM treatment, cell death was increased to 39.3% of Annexin V-FITC positive and PI-negative plus 16% of doubly positive for Annexin V-FITC and PI. When a higher concentration of TPT-CuCl₂ (6 μM) was used, more than 38.3% of doubly stained and 23.08% of only Annexin V-FITC stained cells were detected (Fig. 2D), demonstrating that TPT-CuCl₂ causes a dose-dependent cytotoxic response in HeLa cells.
Suppression of E6 by TPT-CuCl₂ Leads to Accumulation of p53, Which in Turn Down-Regulates Survivin.

HeLa cell line is widely assumed to be p53-defective, because the integrated human papilloma viral oncoprotein E6 continually degraded p53 by proteosome-mediated ubiquitination pathway (Goodwin and DiMaio, 2000). Survivin, a novel member of the IAP family, is known to be transcriptionally down-regulated by p53. To evaluate a possible signaling sequence among E6, p53, and Survivin during TPT-CuCl₂-induced cell death, we examined the transcription level of E6 and Survivin in TPT-CuCl₂-treated HeLa cells. Treatment of HeLa cell with TPT-CuCl₂ (4 μM) caused an accumulation of p53 (Fig. 4B) by down-regulating E6 (Fig. 4A). Induction of p53 expression occurred as early as 2 to 4 h after treatment.

Fig. 4. Down-regulation of E6 and Survivin and up-regulation of p53, Bax, and Bak. RT-PCR analyses for HPV-18 E6 and Survivin were performed using total RNA extracted from either HeLa or H1299 cells exposed to TPT-CuCl₂ for 0, 2, 6, and 12 h. β-Actin was used as an internal control to ensure equal amount of templates used in RT-PCR (A). Total cellular proteins were prepared for analysis of p53, p21/WAF-1, Bax, and Bak by Western blotting as described under Materials and Methods. Tubulin was used as an internal control to ensure the equal loading (B). H1299 cells treated with 0.2 μM TPT-CuCl₂ for 12 h were analyzed by FACS analysis for cell cycle distribution as well as for p53 and p21 expression by Western blotting at indicated times (C). HeLa cells stably expressing Survivin and GFP were plated into a 96-well plate, and cell viability was determined by the MTT method. Values shown are means ± S.D. of triplicate samples. Open column, GFP-expressing cells; closed column, Survivin-expressing cells (D).
of TPT-CuCl₂ (Fig. 4B). As expected, level of p21/WAF-1 was concurrently increased as the result of up-regulated p53 expression, resulting in a cell cycle arrest at G0/G1 phase (Fig. 3). To examine whether Survivin is also involved in this TPT-CuCl₂-triggered death signaling pathway, RT-PCR was performed to compare the Survivin expression level before and after the TPT-CuCl₂ treatment. As shown in Fig. 4A, a dramatic down-regulated expression of Survivin was detected in treated HeLa cells, whereas the expression of Survivin in all samples of H1299 (p53 detected in treated HeLa cells, whereas the expression of Survivin remained unchanged) from different time points remained unchanged. These data suggest that down-regulation of Survivin is closely associated with p53. To support the hypothesis that down-regulation of Survivin resulted from TPT-CuCl₂ treatment is due to trans-suppression of p53, a p53 deficient cell line H1299 (p53⁻/⁻) was treated with 4 μM TPT-CuCl₂ for 0, 2, 6, and 12 h. After incubation at the indicated times, treated H1299 cells were harvested, and total cellular proteins were subjected to Western analysis. As shown in Fig. 4C, p53 or p21 expression was undetectable as expected after TPT-CuCl₂ treatment. Our results are in a good accordance with the finding by Zhou et al. (2002). They demonstrated that down-regulation of Survivin in G1- and S-phase-arrested cells is regulated by wt-p53 and is accompanied by cell death. The FACS analysis of the H1299 cells treated with TPT-CuCl₂ (0.2 μM) from 0 to 12 h did not show cell cycle arrest (Fig. 4C), and the transcriptional level of Survivin remained unchanged (Fig. 4A), further confirming the role of p53 in regulating Survivin expression. Reversibly, to demonstrate whether Survivin is able to inhibit p53-mediated TPT-CuCl₂-induced apoptosis, HeLa cells stably expressing GFP-Survivin were selected. We compared the apoptotic effect of TPT-CuCl₂ treatment on HeLa cells either stably expressing GFP-Survivin or GFP alone. As shown in Fig. 4D, the difference in cell viability shown in MTT assay between cells expressing Survivin and GFP was significant. Treatment of the TPT-CuCl₂ with a sublethal concentration of 4 μM at different time points resulted in marked apoptosis in HeLa cells stably expressing mock GFP, whereas in HeLa cells stably expressing Survivin, much less cell death were observed by the same treatment. For example, after 12-h incubation, less than 50% of the treated Survivin-expressing cells underwent apoptosis compared with that of GFP-expressing control cells, in which more than 65% of the cells were killed (Fig. 4D).

**TPT-CuCl₂-Induced Apoptosis in HeLa Cells Is by an Intrinsinc Mitochondrial Pathway Involving Bax and Bak.** The most intuitive link between p53-mediated transcription and apoptosis comes from the ability of p53 to control transcription of proapoptotic members of bcl-2 family. These include the Bcl-2 family members Bax (Miyashita et al., 1994), Noxa (Oda et al., 2000), and Bid (Thomas et al., 2001). To support the hypothesis that apoptosis induced by TPT-CuCl₂ is mediated by p53, we examined the level of its two direct downstream factors Bax and Bak. HeLa cells were treated with 4 μM TPT-CuCl₂ harvested at different time points indicated in Fig. 4B and were subjected to Western blot analysis with anti-Bak and anti-Bax antibodies. Expression level of the Bak and Bak in HeLa cells were shown to increase as the incubation time proceeded after the treatment of TPT-CuCl₂. It has been previously shown that viral oncoprotein E6 binds to the Bak protein and stimulates its degradation in vivo (Thomas and Banks, 1998). Treatment of the HeLa cells with TPT-CuCl₂ transcriptionally suppressed E6, which might cause the escape of Bak from HPV E6 mediated protein degradation (Fig. 4B). E1B19K was known to bind to Bax and Bak (Sundararajan and White, 2001) and thus to eliminate the Bak*Bak interaction or oligomerization, resulting in the block of mitochondrial-mediated apoptosis pathway (Mikhailov et al., 2003). To testify whether up-regulation of Bak by p53 is involved in the apoptosis induced by TPT-CuCl₂, HeLa cells stably expressing E1B19K were treated with TPT-CuCl₂ (Fig. 5A), and drug-induced apoptosis was significantly attenuated in HeLa cells. The majority of GFP-E1B19K-expressing cells treated with TPT-CuCl₂ (4 μM) for 12 h displayed intact nuclei similar to that in untreated control cells (Fig. 5A). The results were further verified by MTT assay as shown in Fig. 5C. We concluded that interaction between Bak and Bak is involved in the apoptosis induced by TPT-CuCl₂, because E1B19K, which disconnects Bak and Bak, was able to block TPT-CuCl₂-triggered cell death (Fig. 5C).

**TPT-CuCl₂ Induces Mitochondrial Release of Cytochrome c and Smac/DIABLO in a p53-Dependent Manner.** Cytosolic cytochrome c forms an essential subcomponent of the apoptotic death complex apoptosome, leading to the activation of cellular caspases. To explore the role of mitochondria in the induction of apoptosis by TPT-CuCl₂, we examined the mitochondrial release of cytochrome c and Smac/DIABLO after TPT-CuCl₂ treatment. As shown in Fig. 6A, cytochrome c was released from the mitochondria into cytosol when HeLa cells were treated with TPT-CuCl₂. In addition to cytochrome c, another proapoptotic factor Smac/DIABLO was also found to release from mitochondria to cytosol after treatment with TPT-CuCl₂ in HeLa cells. The level of the cytochrome c and Smac/DIABLO from mitochondria to the cytosol was increased gradually as the incubation time proceeded (Fig. 6A). To further demonstrate that release of cytochrome c and Smac/DIABLO was indeed due to p53, H1299 cells (p53⁻/⁻) were infected with adenovirus expressing either wild-type p53 or a mock GFP. After 12-h infection, cells were further incubated with or without TPT-CuCl₂ for an additional 12 h. Cells were lysed, fractionated, and subjected to immunoblotting with anti-cyt c and anti-Smac/DIABLO antibodies. As shown in the Fig. 6B, both cyt-c and Smac/DIABLO were detected in cytosolic fraction from H1299 cells infected with wt-p53-expressing adenovirus. In contrast, neither of these two mitochondrial proteins was detected in the cytosolic fraction of the cells infected with GFP-expressing mock adenoviral particles. These data suggest that the release of cyt-c and Smac/DIABLO by TPT-CuCl₂ is a p53-dependent event.

**TPT-CuCl₂ Induced Cell Death Requires Caspase-3 Activation.** Caspases have been reported to play vital roles in executing apoptosis. To identify the role of caspase involved in the signaling of apoptosis induced by TPT-CuCl₂, a caspase-3-deficient cell line MCF-7 and caspase-3-competent HeLa cells were used to examine whether caspase-3 is required for the apoptotic induction by TPT-CuCl₂. Both MCF-7 and HeLa cells were treated with TPT-CuCl₂ (4 μM) for 16 h and stained with Hoechst 33342. Unlike that in HeLa cells, the majority of stained nuclei of both treated and untreated MCF-7 (caspase-3⁻/⁻) cells remained intact (Fig. 7A), indicating that MCF-7 cells were highly resistant to apoptosis induced by TPT-CuCl₂. To substantiate the notion...
that caspase-3 is indispensable for TPT-CuCl₂-triggered apoptosis, activation of caspase-9 and its downstream nuclear substrate PARP-1 in both MCF-7 and HeLa cells were compared. As shown in Fig. 7B, both procaspase-9 and PARP-1 in HeLa cells were significantly processed upon TPT-CuCl₂ treatment, whereas in MCF-7 cells, activation of procaspase-9 was greatly hampered and the cleavage of PARP-1 was abolished. These data clearly demonstrate that caspase-3 is crucial for both caspase-9 and PARP-1 activation. Our data are in good accordance with the result reported by Blanc et al. (2002). They demonstrated that caspase-3 is essential for the procaspase-9 processing in cisplatin-induced apoptosis of MCF-7 cells. Treatment with general caspase inhibitor zVAD-FMK was shown to abolish the cleavage of caspase-9 and PARP-1, as shown in Fig. 7C. In the case of caspase-9, zVAD-FMK prevents the appearance of p37 form (which is the caspase-3-cleaved product) but not of the p35 form (which is generated by autocleavage of caspase-9). In contrast, Z-LEHD-FMK, a specific caspase-9 inhibitor, was shown to partially block the PARP-1 activation (Fig. 7C), implying that TPT-CuCl₂-triggered apoptosis pathway was not linear, and there might exist some other caspase-mediated pathway(s) during TPT-CuCl₂-induced apoptosis. This hypothesis, however, remains to be further investigated.

TPT-CuCl₂ Has an Antiproliferative and Apoptotic Activity Both in Vitro and in Vivo. We further investigated the effect of TPT-CuCl₂ on the growth of C6 glioblastoma xenografts in Wistar rats. Rats were sedated with ether. Using a 23-gauge needle, C6 cells were injected into the subcutaneous region at a concentration of about 2×10⁶/ml in both flanks of the rat and were then divided into two groups. To verify the injected cells being viable, the remaining cells after injection were cultured again and these recultured C6 cells were found viable and healthy. Two days after C6 cell implantation, TPT-CuCl₂ was delivered intraperitoneally into trial rats at a concentration of 4 mg/kg in 1 ml of PBS every day for 2 weeks. Similarly, 1 ml of sterilized PBS without TPT-CuCl₂ was injected into control rats under
the same conditions. Approximately 5 days postimplantation, tumors began to grow in the rats that received normal saline, and the tiny lumps gradually developed into a solid mass of tumors by the end of day 14. In contrast to the mock trial rats, undeveloped C6 glioblastoma xenografts in TPT-CuCl2-treated rats were confined to the original sites of C6 injection. The development of tumogenesis was completely suppressed after 2 weeks of treatment with TPT-CuCl2 as shown in the Fig. 8A. To verify whether the undeveloped C6 glioblastoma xenografts in TPT-CuCl2-treated rats were due to undergoing apoptosis, TUNEL assay was performed on the paraffin-embedded C6 tissues from both treated and untreated rats to detect apoptosis. Apoptosis was clearly detected in the C6 glioblastoma xenograft tissue but not in the tissues from untreated controls (Fig. 8B). Moreover, TPT-CuCl2 was found to inhibit intraperitoneal tumor growth. In this experiment, exponentially growing C6 cells were injected intraperitoneally in Wistar rats 2 days before TPT-CuCl2 (4 mg/kg) treatment. After 10 days, animals were sacrificed and anatomically opened to observe tumor growth as shown in the Fig. 8Ca and b. Rats treated with TPT-CuCl2 have much smaller tumor mass compared with that of PBS-treated controls in the abdominal region, which were individually confirmed as C6 glioblastoma by H&E staining (Fig. 8Ba and b). We further evaluated whether the compound TPT-CuCl2 elicits any severe side effects on the normal physiology of animal during the course of treatment. Blood tests were carried out to analyze the level of alanine aminotransferase (ALT) in TPT-CuCl2-treated and PBS-treated rats. Red blood cells (RBC) counts and hemoglobin level also were measured. Although levels of ALT in the blood from TPT-CuCl2-treated animals (37 U/l) were marginally elevated compared with that of controls (28 U/l), the RBC counts and hemoglobin level were comparable in both mock and treated groups (data not shown), suggesting TPT-CuCl2 has little, if any, cytotoxicity to normal physiological functions of the experimental rats.
experienced pathologist. As shown in Fig. 8D, a slightly hydrophobic degeneration was observed in the liver cells from TPT-CuCl\(_2\)-treated rats. We further performed In situ cell death assay on the sections of the liver and kidney from the TPT-CuCl\(_2\)-treated and untreated controls by using TUNEL technique. Our TUNEL assay failed to detect any nuclear staining, suggesting that neither liver nor kidney cells underwent apoptosis by TPT-CuCl\(_2\) treatment. The hydrophobic degeneration was more obvious in the liver sections in Fig. 8E, indicated by the arrowheads, which may account for the slightly elevated activity of ALT in the blood of the treated rats. All these data suggest that cytotoxicity of TPT-CuCl\(_2\) is mild, and this compound has a potential to be developed into a novel cancer drug.
Discussion

Cervical cancer is the second major cause of death in women worldwide (Blume-Jensen and Hunter, 2001). In locally advanced or refractory cervical cancer, chemotherapy still represents the best chance of cure, despite that it usually results in excessive toxicity and short duration of response. In most cases, however, chemotherapy is used as an adjuvant therapy combined with other therapies such as radiotherapy or surgery (Einhorn et al., 2003; Savarese and Cognetti, 2003) to enhance its response. Therefore, chemotherapeutic drug design represents an attractive area for developing novel drugs that possess more tumor suppressive ability while being less toxic to normal physiological functions. The newly developed TPT-CuCl₂ is a bimetallic, DNA-targeted compound that causes DNA damage by binding to the DNA molecule. High-risk human papilloma virus such as HPV-18 plays a central role in the development of essentially all cases of cervical carcinoma. However, carcinoma develops frequently even after infection by HPV, and it typically takes years to decades to occur after the initiation of infection. The E6 and E7 protein of human papilloma virus exerts profound effects on degradation of the tumor suppressors p53 and RB, respectively (Goodwin and DiMaio, 2000). Both p53 and RB play central controls in signaling pathways that regulate the cell cycle and monitor the integrity of the genome. Therefore, the growth regulatory machinery that is active in the normal cells is masked by these viral proteins in cervical cancers. In the present study, we developed a novel DNA-targeted bimetallic compound, TPT-CuCl₂, and used HeLa cells as a model for the study of its apoptotic effects on tumor cells. We demonstrated that TPT-CuCl₂-triggered apoptosis in HeLa cells is a p53-dependent signaling pathway. TPT-CuCl₂ was found to transcriptionally suppress HPV E6 in HeLa cells which in turn to liberate p53 for its accumulation. It has been reported that p53 can act as a trans-activator or repressor for a set of pro- or antiapoptotic genes. Survivin is one of such target genes (Hoffman et al., 2002). We demonstrated that the up-regulated expression of p53 caused a significant down-regulated transcriptionally repression of Survivin in TPT-CuCl₂-treated HeLa cells. To further investigate whether wt-p53 was responsible for the Survivin regulation, we examined the effect of TPT-CuCl₂ on H1299 (p53-/-) cells. We found that the basal level of Survivin transcription in H1299 cells remained unchanged after TPT-CuCl₂ treatment, suggesting a dominant role of the p53 in Survivin regulation. Survivin was shown to protect cells from apoptosis by binding to the activated caspases via BIR (baculovirus IAP repeat) domain (Hinds et al., 1999). Similarly, overexpression of Survivin is reported to block the mitochondrial-induced apoptosis by making complex with upstream mitochondrial initiator caspase-9 (O’Connor et al., 2000). We demonstrated that forced expression of Survivin in HeLa cells greatly inhibits the apoptosis-signaling pathway induced by TPT-CuCl₂. In addition, several lines of evidence suggest that the apoptotic effects of TPT-CuCl₂ on HeLa cells are mediated through p53. First, upon treatment of HeLa cells with TPT-CuCl₂, p53 is induced after the suppression of HPV-18 E6 is lifted. Second, the downstream substrates p21/WAF-1 (Wu and Levine, 1997) and Bak and Bax (Pearson et al., 2000; Swisher et al., 2003) were all up-regulated by p53. Last, results from comet assay clearly show the TPT-CuCl₂ was able to cause DNA damage, and this assault on DNA may activate the genomic guardian p53, which in turn initiates the expression of p21/WAF-1 protein and further arrests the cells at the G0/G1 phase of the cell cycle. This result is in good agreement with that reported by Gatti et al. (2002).

There are two pathways by which death signals are transduced to the cellular apoptotic machinery: an extrinsic pathway via a cell surface receptor and an intrinsic pathway through mitochondria. The mitochondria-mediated apoptotic pathway is activated by a death signal, which leads to the activation of Bax involving release of cytochrome c and/or Smac/DIA BO from the mitochondrial intermembrane space into the cytosol. The released cytochrome c by binding to Apaf-1 activates procaspase-9, and the latter in turn activates caspase-3 and other effector caspases (Green and Reed, 1998). We therefore inquired whether Bax was critical for the p53-induced apoptosis. We introduced E1B19K gene into HeLa cells by selecting cells stably expressing E1B19K protein. It has been previously shown that E1B19K binds to TNFR1 and Bax (Hoffman et al., 2002), blocking the formation of a 500-kDa Bax complex and protects cells from tumor necrosis factor-induced apoptosis. Our experiment showed that E1B19K was able to block apoptosis in HeLa cells treated with TPT-CuCl₂, indicating that Bax is involved in TPT-CuCl₂-induced death signaling. Similarly, another pro-apoptotic factor Bak, which is a cellular target of the HPV-18 E6 (Thomas and Banks, 1998), was also rescued after the E6 suppression by treatment of TPT-CuCl₂. Furthermore, we demonstrated that release of cytochrome c and Smac/DIA BLO from the mitochondria to cytosol was entirely dependent on the expression of p53. It is interesting to note that although the caspase-9 inhibitor Z-LEHD-FMK was unable to abrogate the apoptosis pathway triggered by TPT-CuCl₂, the pan-caspase inhibitor Z-VAD-FMK was found to be able to completely block the downstream PARP-1 processing and apoptosis in HeLa cells after TPT-CuCl₂ treatment. We reasoned that caspase-9 must not be the only pathway involved in TPT-CuCl₂-induced apoptosis, and there must exist additional caspase-involved death signaling pathway that is insensitive to caspase-9 inhibitor.

TPT-CuCl₂ was found to be effective in tumor growth suppression. Studies based on animal model clearly demonstrated that TPT-CuCl₂ was shown to have antiproliferative effect on C6 glioblastoma xenograft rat model and was well tolerated at an effective dose without harmful effects on the organs of the treated animals. Although a slightly elevated response of the ALT in the blood of the treated rats was observed, the RBC counts and hemoglobin level were normal (data not shown). Overall, results from H&E staining and TUNEL assay further confirm that TPT-CuCl₂ does not affect normal physiological functions of organs examined in treated animals.

In summary, we propose that the bimetallic compound TPT-CuCl₂ induces apoptosis in HeLa cells by down-regulating Survivin in an E6-p53-mediated signaling pathways. TPT-CuCl₂-induced apoptosis is both p53- and caspase-3 dependent. TPT-CuCl₂ was found to be antiproliferative on experimental rats with valuable pharmacological potential that could be developed into a better therapeutic drug for treating cervical carcinomas.
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