J. Biochem. **134**, 521–528 (2003)
DOI: 10.1093/jb/mvg169

Triphenyl Tin Benzimidazolethiol, a Novel Antitumor Agent, Induces Mitochondrial-Mediated Apoptosis in Human Cervical Cancer Cells *via* Suppression of HPV-18 Encoded E6

Naseruddin Höti, Jun Ma, Sartaj Tabassum*, Yi Wang and Mian Wu†

Department of Molecular and Cell Biology, Key Laboratory of Structural Biology, School of Life Sciences, University of Science and Technology of China, Hefei, Anhui, 230027, People's Republic of China

Received March 1, 2003; accepted July 14, 2003

Here we report the effect of TPT-benzimidazolethiol, a novel anti-tumor agent developed by our group, on the apoptotic pathway of human cervical carcinoma cells. Treatment of HeLa cells with TPT-benzimidazolethiol arrests the cell cycle at G0/G1 phase and transcriptionally downregulates HPV-encoded E6, restoring p53 expression from E6 suppression. Increased p53 accumulation up-regulates p21/waf and ultimately induces apoptosis. The effect of TPT-benzimidazolethiol is far more potent in inducing apoptosis than cisplatin. Treatment with TPT-benzimidazolethiol in HeLa cells is accompanied by the up-regulation of Bak at the transcriptional level, resulting in the release of cytochrome c and Smac/DIABLO from mitochondria to cytosol and, subsequently, the activation of procaspase-9, -3 and PARP, suggesting that TPT-benzimidazolethiol induced-apoptosis signaling is by an intrinsic mitochondrial pathway. Taken together, we propose that TPT-benzimidazolethiol could has the potential to be developed into a new therapeutic agent for treating HPV-associated cervical neoplasia.

Key words: apoptosis, HeLa cells, HPV/E6, mitochondria, p53, TPT-benzimidazolethiol.

Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DNA, deoxyribonucleic acid; PBS, phosphate buffered saline; K, kilodalton; PI, propidium iodide; DDP, *cis*-diamminedichloroplatinum; bak, Bcl-2 homologous antagonist/killer; Cyto c, cytochrome *c*; Smac, second mitochondria-derived activator of caspases.

Studying cancer treatments at the molecular basis holds promise for developing more effective cancer therapy strategies. It is generally assumed that because cancers are derived from numerous tissues with multiple etiologies and endless combination of genetic and/or epigenetic alterations, therapies for cancers must be as diverse as the disease itself. Chemotherapy is widely used for treating cancers. To be useful in cancer therapy, chemotherapeutic drugs must act broadly across different tumor types. Despite their great efficacy in treating cancers, some types of chemotherapeutic drugs currently used in clinical practice have major problems, such as severe health side effects and the acquisition of drug resistance by tumor. Thus, there is always an exigency for the development of new drugs with minimal side effects and maximal curative potential. The majority of chemotherapeutic drugs are DNA targeted (*1*) and anti-mitotic (*2*). Increasing numbers of biologists have shown more interest in the chemistry of alkyl tin compounds and their interactions with nucleotides (*3*, *4*). Transition element containing-drugs, such as cisplatin or its analogues, intercalate into adjacent bases of the DNA double helix, whereas alkyl tin compounds, such as TPT-benzimidazolethiol developed by our group, interact with phosphate moieties of the DNA backbone (see below). Such molecular pharmacology studies will advance our understanding of the molecular basis of the actions drugs used to treat tumors, and eventually will help in the design of novel anti-tumor drugs. Alkyl tin compounds are reported to be effective against some types of cancers, such as P-338 leukemia (*5*). Although the details of the mechanism by which apoptosis is triggered in response to DNA-targeted drugs have not yet been clearly defined, it is widely accepted that cellular stress can induce the activation and stabilization of the tumor suppressor p53. Loss of p53 function occurs in the early stages of oncogenesis, whereas the restoration of p53 in tumor cells often results in their apoptosis (*6*).

Cervical carcinoma is the third most common malignant disease in women worldwide. The absolute frequency of adenocarcinoma of the cervix uteri has increased dramatically during the last decade and accounts for about 15–20% of invasive cervical cancers (*7*, *8*). The HeLa cell line is a choice model in which to study the effects of drugs on human cervical tumors. However in most cervical carcinomas and cancer derived cell lines, the p53 tumor suppressor pathway has been disrupted by the HPV E6 protein, which binds to p53 and targets it for accelerated ubiquitin-mediated degradation (*9*). The aim of the present study was to investigate whether TPT-ben-

*Dr Sartaj Tabassum is a visiting scientist in the School of Life Sciences at the University of Science and Technology of China.
†To whom correspondence should be addressed: Tel: +86-551-3606264, Fax: +86-551-3606264, E-mail: wumian@ustc.edu.cn

zimidazolethiol is capable of killing human cervical cancer cells through the induction of apoptosis. We demonstrate that TPT-benzimidazolethiol is able to induce apoptosis in HeLa cells via the down regulation of the viral oncogene E6, resulting in the accumulation of p53 promoting the resumption of the p53 dependent apoptotic signaling pathway. Activated p53 upregulates both p21/waf (*10*) and Bak (*11*, *12*), causing cell cycle arrest and subsequently the release of both cytochrome c and Smac/DIABLO from mitochondria. Released cytochrome c activates pro-caspase-9, -3 and PARP in a cascade fashion and ultimately induces apoptosis of HeLa cells. However, the mode of apoptosis induction by TPT-benzimidazolethiol appears to be cell type- dependent; we found that although A549 cells show significant cytotoxicity towards TPT-benzimidazolethiol, the apoptotic signaling pathway is quite distinctive from what has been observed in HeLa cells. TPT-benzimidazolethiol induced-apoptosis in A549 cells does not result in the mitochondrial release of either cytochrome c or Smac/DIABLO.

MATERIALS AND METHODS

Reagents and Antibodies—The following antibodies were used in this study: polyclonal antibody Ab-actin and monoclonal antibody mAb-p53 were purchased from Santa Crutz Biotechnology (USA), monoclonal antibody mAb-caspase-9 from Immunotech (France), monoclonal antibody mAb-caspase-3 and mAb-p21 from BD Biosciences (USA), mAb-cytochrome ***c*** from R&D systems (USA), mAb-PARP from Upstate Biotechonology, Lake Placid, NY (USA), mAb-prohibitin antibody was from Neo Markers (USA) and mAb-Smac/DIABLO from Calbiochem (USA). All chemicals and reagents used in this study were ordered from Sigma unless otherwise specified.

Cell Culture—The HeLa cell line was kindly provided by Dr Yao Xuebiao (UC Berkeley, USA). A549 and H1299 human lung cancer cell lines were kind gifts from Dr Zhu Weiguo (Ohio State University, USA). Wild type p53 was generously provided by Dr.Takashi Tokino. (Sapporo Medical University). Cells were grown and maintained according to the instructions provided by the suppliers. Culture media were supplemented with 10% heat inactivated fetal bovine serum (Life Technologies, USA) plus streptomycin (100 μg/ml) and penicillin (100 IU/ml) (Life Technologies).

MTT Assay—Drug-induced cytotoxicity was assessed by (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay carried out in 96-well microtiter plates. The cells were treated with different concentrations of TPT-benzimidazolethiol and untreated cells were used as a control. MTT was added to each well to a final concentration of 1 mg/ml and the plates were 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 DMSO. The plates were read at 562 nm with an EL_x800 Universal Microplate Reader (BIO-TEK Instrument, USA). Assays were performed in triplicate for each of three samples and the means were determined using Excel Software.

Hoechst 33342 Staining—HeLa cells were treated with TPT-benzimidazolethiol at a concentration of 2 μg/ml for 12 h, washed with PBS, fixed with 3.7% Paraformaldehyde at room temperature, and stained with Hoechst 33342 dissolved in 1× PBS at a final concentration of 8 ug/ml. Preparations were then washed 4 times with 1× PBS, and the stained nuclei were visualized under a fluorescence microscope (Olympus Optical). Apoptotic cells were morphologically defined as cells showing aberrant Hoechst 33342 stained nuclei and chromosomal fragmentation.

Oligonucleotides—The sequences of the oligonucleotides used in this study are as follows (all primers are read from 5′ to 3′), Primers P1 and P2 were used in RT-PCR for the amplification of bak, whereas P3 and P4 were used for HPV-18 E6.

P1: GCG AAT TCC ATG GCT TCG GGG CAA GGC
P2: CGC TCG AGT CAT GAT TTG AAG AAT CTT
P3: GGG AAT TCC ATG GCG CGC TTT GAG GAT C
P4: CGC TCG AGT TAT ACT TGT GTT TCT CT

RT-PCR—Total cellular RNA was extracted from confluent HeLa cell cultures (approximately 1×10^7 cells) using an RNA extraction kit (Promega USA). RT-PCR was performed according to the protocol described previously (*13*). RT-PCR products were electrophoretically separated in 1.0% agarose gels (Bio Rad, USA) in the presence of ethidium bromide (0.5 μg/ml) and visualized under UV light.

Preparation of Whole Cell Lysate and Western Analysis—Total cell lysates were prepared and Western blot analysis was performed according to the methods described earlier (*14*).

Cell Cycle Analysis—DNA contents and cell cycle distribution for HeLa cells were determined by flow cytometry. The cells were grown in 6-well plates at a density of 5×10^4 cells/well, and harvested after 2, 6 and 12 h. Harvested cells were washed with ice cold PBS and fixed with 70% ethanol. One hundred microliters of RNase (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. Analyses were performed on a Becton Dickinson (Mountain View, CA) FACScan Flow Cytometer using the Cellquest program (Becton Dickinson). Ten thousand events were analyzed for each analysis.

FACS Analysis of Annexin-V Binding to H1299 Cells Transfected with or without p53 in the Presence of TPT-Benzimidazolethiol—H1299 cells were transfected with or without wild type p53 in the presence of 2 μg/ml of TPT-benzimidazolethiol for 24 h and then trypsinized. Harvested cells were washed twice with ice cold PBS and re-suspended in binding buffer (BAOSAI China) at 1×10^7 cells/200 μl. Ten microliters of Annexin V-FITC and 5 μl of propidium iodide (PI) were added, and the mixtures were incubated for 15 min at room temperature in the dark. Just before analysis by flow cytometry, 300 μl of 1× binding buffer was added to each tube. Ten thousand events were analyzed using a Becton Dickinson (Mountain View, CA) FACScan flow cytometer.

Preparation of Mitochondrial Fraction—Digitonin fractionation of cells into membrane and cytosollic fractions used for the detection of cytochrome c and Smac/DIABLO was performed according to the methods described previously (*15*).

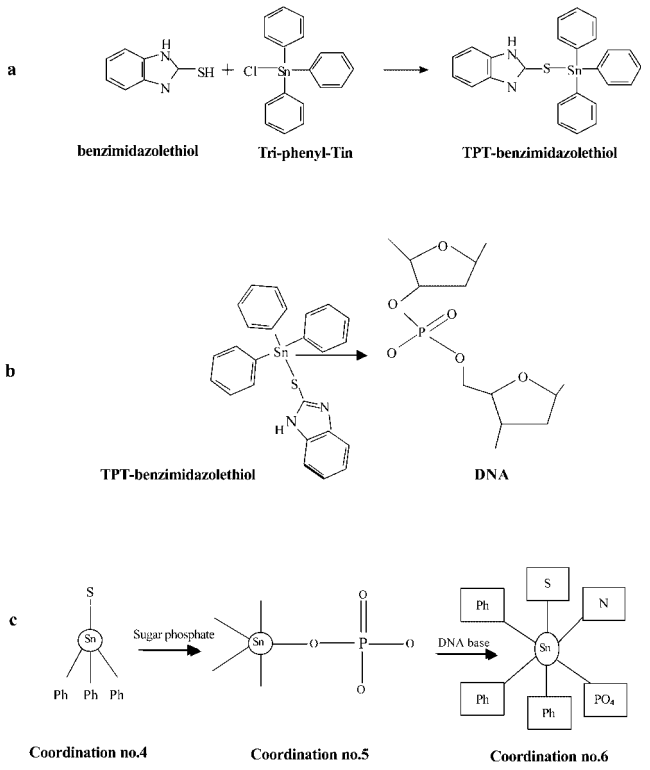

Fig. 1. **Preparation of TPT-benzimidazolethiol and a proposed mechanism for its reactivity towards DNA.** Reaction scheme of TPT-benzimidazolethiol synthesis from benzimidazolethiol and triphenyltin (a). Proposed mechanism of TPT-benzimidazolethiol reactivity towards phosphate sugar moieties on DNA (b). TPT-benzimidazolethiol contains a Sn metal ion with a four-coordinate distorted tetrahedral geometry. The Sn atom forms five coordination bonds by accepting a pair of electrons from an oxygen atom on the sugar phosphate of DNA. The Sn atom further interacts with DNA bases and finally expands its coordination number to six (c).

RESULTS

Properties of TPT-Benzimidazolethiol—$C_{25}H_{20}ON_2SSn$ was synthesized from benzimidazolethiol and triphenyltin chloride. Briefly, 1mmol of benzimidazolethiol was dissolved in 50 ml of methanol and 1mmol of triphenyl tin chloride was added to this solution in a 1:1 molar ratio (Fig. 1a). The reaction mixture was boiled under reflux for *ca*. 80 h and then allowed to stand overnight in a refrigerator. A shinning, creamy colored complex was isolated, washed with ether and dried *in vacuo*. NMR analysis of the synthesized compound mixed with calf thymus DNA showed a 6 nm red shift, showing a strong tendency for TPT-benzimidazolethiol to interact with DNA molecules (data not shown). This interaction might be due to a change in the coordination environment of the tin atom from tetrahedral to trigonal bi-pyramidal when the compound binds to the phosphate moiety of the DNA backbone as schematized in Fig. 1b. The tin atom in this compound can further expand its coordinating sphere and stabilize into an octahedral geometry by intercalating into DNA bases. The mechanism proposed for DNA binding is shown in Fig. 1c.

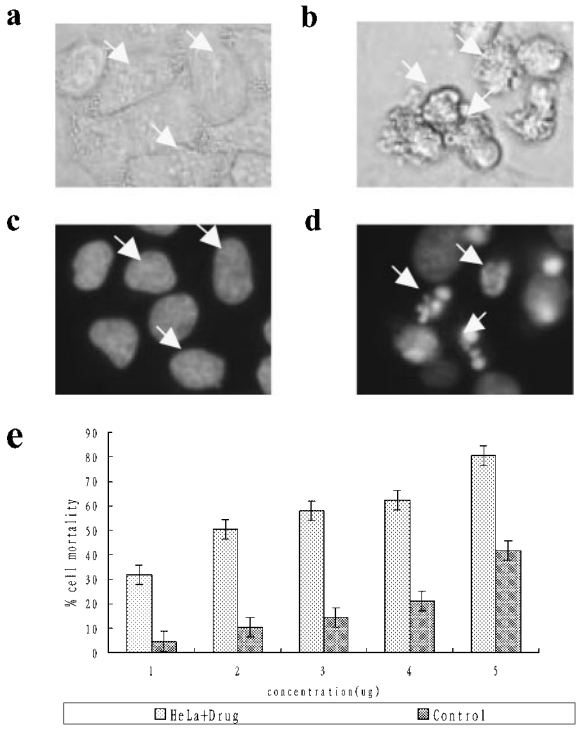

Fig. 2. **Induction of apoptosis by TPT-benzimidazolethiol in HeLa cells.** HeLa cells were treated with 2 μg of TPT-benzimidazolethiol for 12 h. Both the TPT-benzimidazolethiol treated cells and control cells were fixed in 3.7% paraformaldehyde and stained with Hoechst-33258. The morphological changes associated with apoptosis were photographed under an inverted fluorescence microscope. Cells in different stages are indicated by arrows; most dead cells display densely stained and compacted nuclei (b, d). Untreated HeLa cells showing viable and intact nuclei served as controls for the TPT-benzimidazolethiol treated cells (a, c). HeLa cells were plated into a 96-well plate and cell viability was determined by the MTT method. One hundered percent of viable cells was defined as the measurement obtained for untreated cells. Values shown are means ± SD of triplicate samples. Since TPT-benzimidazolethiol is DMSO-soluble, cells treated with DMSO without TPT-benzimidazolethiol were used as controls (e).

TPT-Benzimidazolethiol Causes Cell Cycle Arrest at Go/G1 Phase and Subsequently Induces Apoptosis in HeLa Cells—To determine whether TPT-benzimidazolethiol is able to deliver an apoptotic signal to cervical carcinoma HeLa cells, cells stained with Hoechst 33342 were examined under a fluorescence microscope for morphological changes of apoptosis such as cells shrinkage, nuclear segmentation and chromatin condensation. After treatment with TPT-benzimidazolethiol for 12 h, a majority of cells had undergone apoptosis and showed typical apoptotic characteristics such as densely stained and compacted nuclei as shown in Fig. 2 (b and d). These results were further confirmed by MTT assay, which serves as an index of cell viability by measuring the reduction of tetrazolium salt to a blue formazan product by the intact mitochondrial fraction (*16*). TPT-benzimidazolethiol in the range of 1 μg/ml to 5 μg/ml caused dose-dependent cytotoxicity with approximately 80% of the cells having lost viability after TPT-benzimidazolethiol treatment at a concentration of 5 μg/ml as shown in Fig. 2e. These MTT results are in accordance with those of the

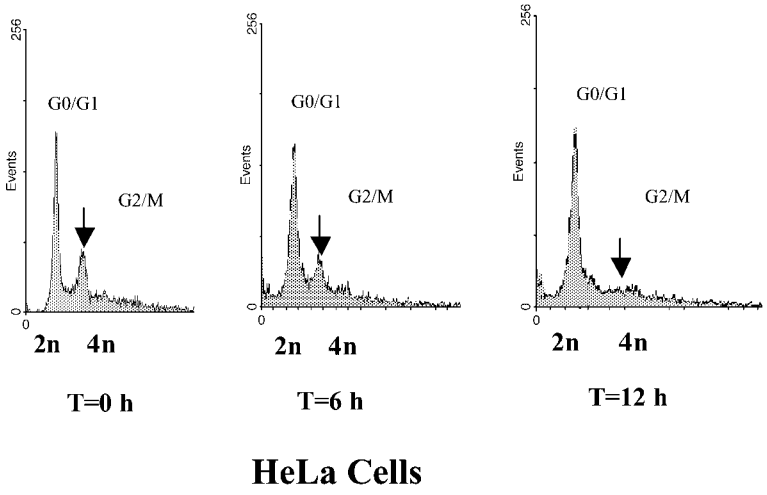

Fig. 3. **FACS analysis of TPT-benzimidazolethiol–induced apoptosis in HeLa cells.** Flow cytometric histograms of HeLa cells prior to TPT-benzimidazolethiol treatment (left) compared with 6 h (center) and 12 h (right) after treatment. Cells were treated with PI and subjected to flow cytometric analysis. The horizontal axis of each histogram represents DNA content; the vertical axis denotes cell number. The arrows indicate cells in G2/M phase of the cell cycle.

Hoechst 33342 nuclear staining assay. FACS analysis of HeLa cells exposed to the low concentration (1 μg/ml) of TPT-benzimidazolethiol showed a marked decrease in the G2 peak after incubation for 12 hours (Fig. 3), indicating a decrease in G2/M cells and an increase in G0/G1 and sub G1 phase cells in the cell population during the 12 h treatment. This response is likely to be attributable to an elevated expression of the cell cycle inhibitor p21/WAF1 in response to an accumulation of p53 induced by either DNA damage and/or E6 suppression upon TPT-benzimidazolethiol treatment.

Down Regulation of the Viral E6 Oncogene and Up Regulation of Tumor Suppressor p53 upon TPT-Benzimidazolethiol Treatment—Since the reduced half-life of p53 in HeLa cells is a result of its enhanced ubiquitination mediated by the HPV-encoded E6 protein (*9*), we studied the effect of TPT-benzimidazolethiol on E6 expression. RT-PCR results showed that the cellular level of E6 mRNA was significantly reduced by treatment with TPT-benzimidazolethiol for 6 h as depicted in Fig. 4B. To further confirm that p53 escaped E6 suppression, we performed Western analysis to examine the expression of p53 and the results are shown in Fig. 4A. As expected, the level of p53 was found increase with time in cells treated with TPT-benzimidazolethiol. The effect of TPT-benzimidazolethiol on the nuclear accumulation of p53 appeared to be rapid. An accumulation of p53 was detected within 2 h of the start of treatment and reached a peak in 6–8 h. The activation of p53 was evidenced by the up-regulation of its direct downstream substrate, p21/WAF1, which increased gradually as shown in Fig. 4A. To demonstrate the apoptotic pathway in HeLa cells upon treatment of TPT-benzimidazolethiol, we evaluated another important apoptotic factor downstream of p53, Bak, a bcl-2 family member, and found that in TPT-benzimidazolethiol treated HeLa cells, Bak is transcriptionally up-regulated by p53 (*11*, *12*), indicating the involvement of Bak in TPT-benzimidazolethiol induced apoptosis. To determine whether p53 is truly involved in TPT-benzimidazolethiol-induced apoptosis, we examined the expression of p53 and its direct downstream protein, p21/WAF1, in both A549 (p53 +/+) and H1299 (p53 –/–) human lung cancer cells treated with TPT-benzimidazolethiol. The induction of p53 and p21/WAF1 became evident in A549 cell at 2 μg/ml TPT-benzimidazolethiol, whereas no detectable level of either p53 or p21/WAF1 could be detected in H1299 cells (Fig. 4C). To further confirm that TPT-benzimindazolethiol-induced apoptosis is p53-dependent, we transfected a p53-null mutant cell line, H1299, with wt-p53 or empty vector, and added TPT-benzimindazolethiol 8 h after transfection to the final concentration of 2 μg/ml, and incubated the culture for another 16 h. The resultant cells were analyzed for apoptosis by Flow Cytometry Annexin V-FITC and Hoechst 33342 DNA staining. As shown in Fig. 4F (left), fewer than 13% of the empty vector-transfected H1299 cells treated with 2 ug/ml of TPT- benzimindazolethiol were AnnexinV-positive and PI-negative (early apoptotic cells). In contrast, approximately 54% of the wt p53-transfected H1299 cells were Annexin-V positive and PI negative when treated with the same concentration of TPT-benzimindazolethiol. We noted that the percentage of both AnnexinV and PI-positive cells was roughly the same in both the vector-transfected (36.2%) and wt-p53–transfected (33.8%) H1299 cells in the presence of 2 μg/ml of TPT- benzimindazolethiol. These doubly positive cells represent either late stage apoptotic cells or necrotic cells, which are independent of apoptotic induction by p53. This is in agreement with our result that nuclear condensation in H1299 cells transfected with p53 in the presence of TPT-benzimindazolethiol was much greater than in control cells, which further confirms that TPT-benzimidazolethiol-induced apoptosis acts in a p53-dependent manner (4D).

TPT-Benzimidazolethiol Is More Potent than Cisplatin—To evaluate the cytotoxicity of TPT-benzimidazolethiol, we compared TPT-benzimidazolethiol with a commonly used chemotherapeutic drug cisplatin (DDP) and found TPT-benzimidazolethiol to be more effective against a human cervical cancer cell line. Treating confluent HeLa cells with these two compounds separately for different time intervals resulted in a significant difference in cell viability as shown by MTT assay (Fig. 5A), showing that TPT-benzimidazolethiol is a stronger inducer of apoptosis than cisplatin. Treating HeLa cells with TPT-benzimidazolethiol at a concentration of 2 μg/ml caused a dramatic decrease in cell viability. After 10 h treatment, fewer than 20% of the HeLa cells were viable.

In contrast, treating HeLa cells with cisplatin at the same concentration killed only approximately 10% of cells. TPT-benzimidazolethiol cytotoxicity is not restricted to human cervical cancer cells; when applied to A549 human lung cancer cells, similar results were obtained, as shown in Fig. 5A. To compare the cytotoxic effect of cisplatin and TPT-benzimidazolethiol on HeLa cells further, cell viability was measured after treatment with various molar concentrations of the compounds for 12 h by MTT cell death assay (Fig. 5B). We found that treatment of HeLa cells with 0.2 mM TPT-benzimidazolethiol resulted in significant apoptosis (80%) as compared with the same concentration of cisplatin, which caused approximately 25% cell death (Fig. 5B), indicating that TPT-benzimidazolethiol is a stronger inducer of apoptosis than cisplatin. This may be attributable to the change in the coordination environment of the Tin (Sn) atom (Fig. 1c). We reasoned that because TPT-benzimidazolethiol not only interacts electrostatatically with sugar phosphate moieties in the DNA backbone, it also intercalates into bases within the genome structure, which makes its binding to DNA more stably than that of cisplatin.

TPT-Benzimidazolethiol–Induced Apoptosis Causes a Release of Cytochrome c and Smac/DIABLO in HeLa Cells but Not in A549 Cells—Cytosolic cytochrome *c* forms an essential subcomponent of the apoptotoic death complex, apoptosome, leading to the activation of cellular caspases. To explore the role(s) of mitochondria in the induction of apoptosis by TPT-benzimidazolethiol, we examined the mitochondrial release of cytchrome *c* and Smac/DIABLO following treatment. Figure 6A shows that cytochrome *c* was released when HeLa cells were treated with TPT-benzimidazolethiol for 16 h. In addition to cytochrome *c*, HeLa cells were also found to release another pro-apoptotic factor, Smac/DIABLO, when treated with TPT-benzimidazolethiol. However, the release of cytochrome *c* or Smac/DIABLO from mitochondria to the cytosol appears to be cell type-dependent. Although TPT-benzimidazolethiol is able to induce apoptosis in p53-proficient A549 cells, neither cytochrome c nor Smac/DIABLO release from mitochondria was detected, as shown in Fig. 6B. The mechanism for this effect remains uncharacterized.

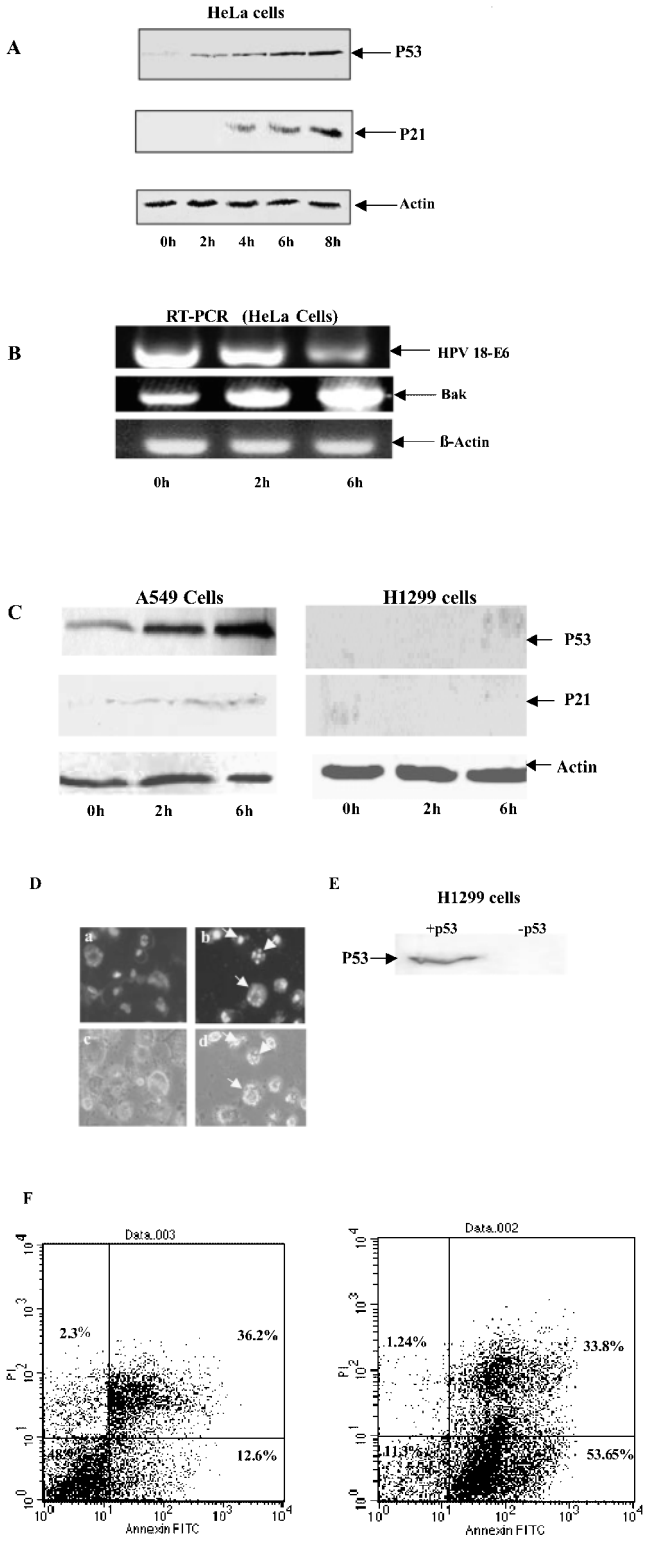

Fig. 4. **Down-regulation of E6 and up-regulation of p53 and Bak.** The Immunoblot analysis of p53 and p21 in total cell lysates of HeLa cells. Immunoblot analysis for actin was also performed as an internal control to ensure the equal loading of total cellular proteins (A). RT-PCR analyses for HPV-18 E6 and Bak were performed using total RNA extracted from HeLa cells exposed to TPT-benzimidazolethiol for 0 h, 2 h, and 6 h. Beta actin was used as an internal control to ensure equal amount of templates used in RT-PCR (B). Human lung cancer cell lines, A549 cells, which express functional p53, and H1299 cells, which are devoid of p53 expression, were treated with 2 μg/ml of TPT-benzimidazolethiol. Total cellular proteins were extracted at the indicated times and analyzed by Western blotting for detection of p53 and p21 (C). Morphological changes associated with apoptosis of H1299 cells. H1299 cells transfected with or without wt-p53 in the presence of 2 μg/ml TPT-benzimidazolethiol were photographed under an inverted fluorescence microscope (D). Arrows indicate cells in different stages of apoptosis; most dead cells display densely stained and compacted nuclei (b, d). A majority of H1299 cells transfected with vector were viable and displayed intact nuclei in the presence of 2 μg/ml TPT-benzimidazolethiol (a, c). Western blot analysis of H1299 cells transfected with p53 and empty vector showing the presence and absence of the p53 protein. (E). FACS analysis of wt-p53 transfected and vector transfected H1299 cells in the presence of 2 μg/ml TPT-benzimidazolethiol. H1299 cells without p53 (left) and with p53 (right) were stained with fluorescent isothiocyanate (FITC) conjugated with Annexin-V and with the fluorescent dye propidium iodide (PI). Ten thousand events were analyzed. The percentages of cells are indicated in each box (F).

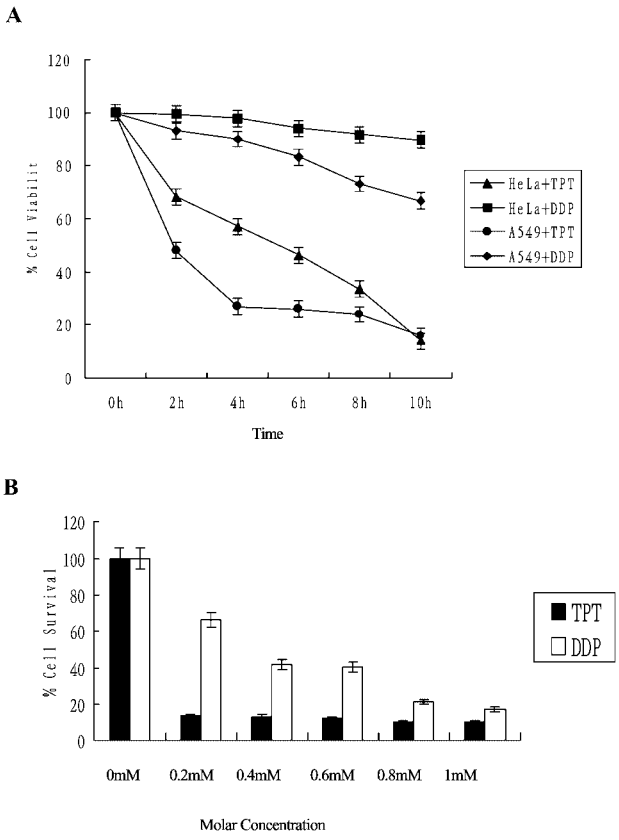

Fig. 5. **MTT assay of TPT-benzimidazolethiol and cisplatin cytotoxicity.** Cell viability was measured by MTT following treatment of HeLa and A549 cells with TPT-benzimidazolethiol (2 μg/ml) or cisplatin (2 μg/ml). Viable cells were counted at the indicated times from 0 h to 10 h and a cell survival curve was drawn (A). The dose-dependent response of HeLa cells toward different molar concentration of TPT-benzimidazolethiol or cisplatin after 24 h of treatment were measured by MTT assay. Cell viability is shown as the means ± SD of triplicate samples (B).

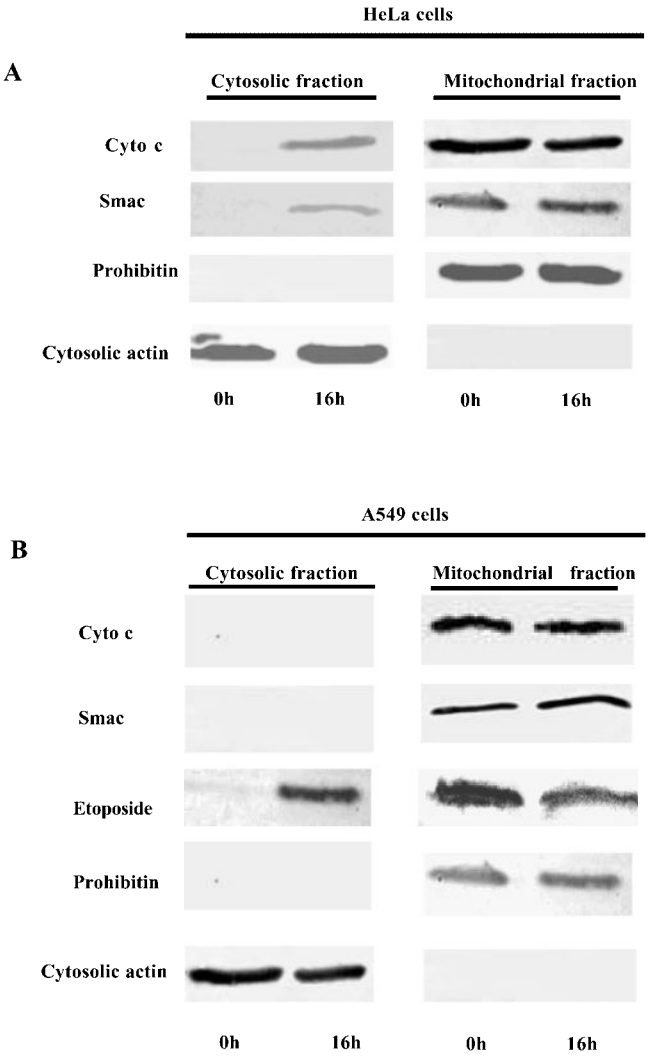

Fig. 6. **Western analysis showing the release of cytochrome c and Smac/DIABLO in HeLa cells but not in A549 cells.** After treatment with TPT-benzimidazolethiol, equal amount of proteins from the cytosolic fractions and mitochondrial pellets were resolved by SDS-PAGE and the gels were incubated with anti-cytochrome *c* and anti-Smac/DIABLO antibodies. Cytosolic fractions and mitochondrial pellets were analyzed for the presence of cytochrome *c* and Smac/DIABLO by Western blotting. The mitochondrial protein prohibitin and cytosolic actin were used as positive controls to correct for sub-cellular compartmental fractionation. Both cytochrome c and Smac/DIABLO were detected in the cytosolic fraction as well as in the mitochondrial fraction in HeLa cells after 16 h treatment with TPT-benzimidazolethiol (A). No release of cytochrome c or Smac/DIABLO into the cytosolic fraction of A549 cells was detected, even after 16 h of treatment with TPT-benzimidazolethiol; all the cytochrome *c* and Smac/DIABLO remained in the mitochondrial fraction (B).

TPT-Benzimidazolethiol–Induced Apoptosis in HeLa Cells Occurs by an Intrinsic Mitochondrial Pathway—Caspases play a vital role in triggering apoptosis. To identify which caspase is involved in the process of apoptosis induced by TPT-benzimidazolethiol, HeLa cells were treated with TPT-benzimidazolethiol for various time intervals, harvested, and lysed for Western analysis. TPT-benzimidazolethiol was found to activate caspases-9 and -3 in HeLa cells as shown in Fig. 7, a and b. After 16 h of treatment with TPT-benzimidazolethiol, procaspase-9 (45 kDa) was cleaved into the p37 and p35 active forms. Meanwhile, the level of uncleaved procaspase-3 (32 kDa) decreased dramatically as shown in Fig. 7b, indicating that procaspase-3 was processed during TPT-benzimidazolethiol treatment. The activation of caspase-3 resulted in the cleavage of PARP-1 (*17*) as shown in Fig. 7c. The level of an 89 kDa PARP-1 fragment generated by activated caspase-3 increased beginning after 8 h of TPT-benzimidazolethiol treatment. Consistent with the observation that there was no release of cytochrome *c* or Smac/DIABLO in A549 cells following treatment with TPT-benzimidazolethiol, it is not surprising that the initiator caspase-9 and effector caspase-3 as well as PARP were not activated by TPT-benzimidazolethiol treatment (data not shown). This can be explained by the fact that various cell types differentially utilize distinct apoptotic pathways. Ofir *et al.* recently reported similar results (*18*): They demonstrated that Taxol induced apoptosis occurs in the absence of the activation of caspase-3 and caspase-9 using caspase-3-deficient-MCF-7 breast carcinoma cells transfected with the caspase-3 gene.

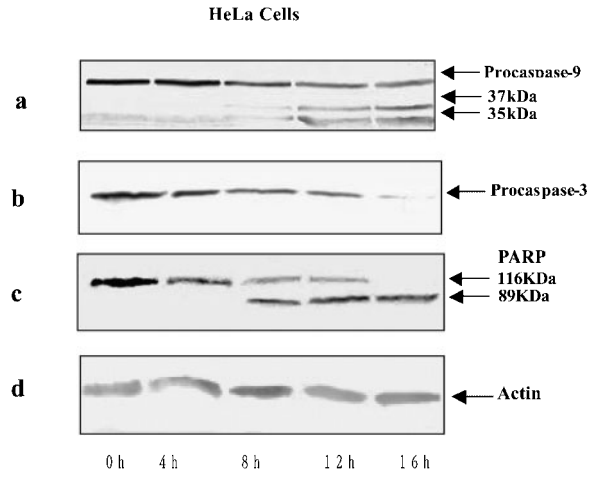

Fig. 7. **Activation of caspases-9, -3 and PARP in HeLa cells.** HeLa cells were treated with TPT-benzimidazolethiol, and then samples of whole cell lysate were subjected to SDS-PAGE followed by Western analysis. As shown in a, procaspase-9 was cleaved into 37 kDa and 35 kDa following 12 h of treatment with TPT-benzimidazolethiol. Similarly, the level of procaspase-3 decreased with incubation time indicating its processing (b). After 8 h of treatment of TPT-benzimidazolethiol, the 89 kDa cleaved PARP-1 fragment began to appear and by 16 h of treatment, almost all the PARP had been processed (c).

DISCUSSION

Adenocarcinoma of the cervix uteri remains the third most common malignant disease among women worldwide (*8*, *9*). Surgery combined with radiotherapy and chemotherapy help in its treatment to some extent, but due to the ability of the tumor to acquire high resistance, the prognosis remains dismal. Chemotherapy provides a promising and affordable approach to cancer treatment. The newly characterized TPT-benzimidazolethiol is a promising drug candidate for cancer chemotherapy for several reasons. First, unlike cisplatin and its analogues, which have large dose requirements and resistance build-up problems, TPT-benzimidazolethiol can efficiently kill tumor cells at very low doses, thus minimizing the toxic side effects. Our dose-dependent experiment confirms that a low dose of TPT- benzimidazolethiol (2 μg/ml) is able to kill HeLa cells significantly. In contrast, to achieve significant cell death in tumor cells, high dosages of cisplatin, not less than 16 μg/ml (*19*), are required. As discussed in the result section, TPT–benzimidazolethiol at a concentration of 2 μg/ml induced apoptosis in HeLa cells at a rate of almost 90%, whereas the same concentration of cisplatin killed fewer than 10% of HeLa cells. Secondly, TPT-benzimidazolethiol interacts with DNA more tightly than cisplatin, as detailed in the result section. Our research group was the first to synthesize TPT-benzimidazolethiol, a member of the alkyl tin compound family, and its interaction with nucleotides in aqueous media and solid state has been described previously (*3*, *4*). In this study, we reported for the first time that TPT-benzimidazolethiol acts as a potent inducer of apoptosis in HeLa cells. Several lines of evidence suggest that the apoptotic effects of TPT-benzimidazolethiol on tumor cells are mediated through p53. Firstly, upon treatment of HeLa cells with TPT-benzimidazolethiol, p53 is induced when the suppression of HPV-18 E6 is lifted; secondly, the downstream substrates p21 (*10*) and Bak (*11*, *12*) are up-regulated by p53. Our preliminary NMR results showed that the formation of a complex between TPT-benzimidazolethiol and calf thymus DNA resulted in a red shift of 6 nm and produces a large hypochromism (data not shown), indicating that TPT-benzimidazolethiol forms an adduct in DNA. This assault on DNA may activate the genomic guardian p53 (TPT-benzimidazolethiol treatment lifts the suppression of E6 in HeLa cells; therefore p53 should be induced by DNA damage), which in turn initiates the expression of the downstream p21/waf protein and arrests the cells at the G0/G1 phase of the cell cycle. Our finding of TPT-benzimidazolethiol-induced apoptosis in HeLa cells is similar to the results of Reed (*20*).

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 release of cytochrome c and/or Smac/DIALBO from the mitochondrial intermembrane space into the cytosol. The released cytochrome *c* activates Apaf-1 and procaspase-9, and the latter in turn activates caspase-3 and other effector caspases (*21*). Similar to many other apoptotic stimuli, such as gamma irradiation, anticancer drugs and staurosporine, TPT-benzimidazolethiol presumably induces apoptosis by activating the intrinsic apoptotic pathway (*22*, *23*). A number of studies have implied a central role for the p53 tumor suppressor in the integration of the death signal to the intrinsic apoptotic pathway (*24–26*). Soengas and coworkers (*27*) demonstrated that the induction of apoptosis in oncogenic transformed murine embryonic fibroblasts requires wild type p53 as well as Apaf-1 and caspase-9. Thus, p53 appears to transduce a signal to the apoptotic machinery downstream of mitochondria. This conclusion is reinforced by our results obtained in the present study using H1299 cells (p53–/–). The rate of apoptotic induction in empty vector–transfected H1299 cells treated with TPT-benzimidazolethiol was much less than that in wt-p53 transfected H1299 cells. We propose the following pathway for the induction of apoptosis by TPT-benzimidazolethiol. TPT-benzimidazolethiol treatment dramatically stabilizes nuclear p53 by down regulating the HPV E6 viral oncoprotein, which in turn up-regulates the downstream p21/waf protein and arrests tumor cells at G0/G1 phase of the cell cycle. p53 might induce the release of mitochondrial cytochrome c and Smac/DIABLO by a pathway involving Bak, which is transcriptionally elevated upon TPT-benzimidazolethiol treatment. The released cytochrome *c* is known to oligomerize with both Apaf-1 and procaspase-9, and the latter is then activated by cytochrome *c*. Processed caspase-9 cleaves procaspase-3 directly, which in turn cleaves its downstream substrate, PARP, and ultimately causes tumor cell death. Whether p53 induces cytochrome *c* release by an enhanced transcriptional level of Bak or by other Bcl-2 family member(s) is not yet clear. Similarly the link between p53 activation and E6 suppression upon treat-

ment with TPT-benzimidazolethiol remains to be elucidated in HeLa cells.

In conclusion, we propose that TPT-benzimidazolethiol is able to induce apoptosis effectively in tumor cells and has potential for being developed as a better and safer therapeutic agent for the treatment of human cervical carcinomas.

We express our gratitude to USTC for providing the facilities in which to carry out our research work. We are also thankful to Matthew Christensen for careful reading of the manuscript and Dr Takashi Tokino (Department of Molecular Biology-Cancer Research Institute, Sapporo Medical University) for providing the wild type p53 gene. This research was supported by the Key Project Fund (KSCX2-2-01-004) from the Chinese Academy of Sciences, grants from the National Natural Science Foundation of China (30121001 and 90208027) and a 973 grant (2002CB713702) from the Ministry of Science and Technology of China.

REFERENCES

1. Eastman, A. (1990) Activation of programmed cell death by cancer agents: cisplatin as a model system. *Cancer Cell* **2**, 275–280
2. Gerard, I.E. and Vousden, K. (2001) Proliferation, cell cycle and apoptosis in cancer. *Nature* **411**, 342–347
3. Jancso, A., Nagy, L., Moldrheim, E., and Slelten, E. (1999) Potentiometric and spectroscopic evidence for coordination of dimethyl tin (IV) to phosphate groups of DNA fragment and related ligands. *J. Chem. Soc. Dalton Trans.* 1587–1592
4. Barbieri, R., Silvestri, A., Guilani, A.M., Piro, V., Disimone, F., and Madonia, G. (1992) Organotin compounds and deoxyribonucleic acid. *J. Chem. Soc. Dalton Trans.* 577–585
5. Huber and Barbceri, R. (1986) *Tin as a Vital Nutrient*, p. 175, CRC Press, Boca Raton
6. Chou, R.H. and Huang, H. (2002) Restoration of p53 tumor suppressor pathway in human cervical carcinoma cells by sodium arsenite. *Biochem. Biophys. Res. Commun.* **26**, 298–306
7. Andersson, S., Alemi, M., Rylander, E., Strand, A., Larsson, B., Sallstrom, J., and Wilander, E. (2000) Uneven distribution of HPV 16 E6 prototype and variant (L83V) oncoprotein in cervical neoplastic lesions. *Br. J. Cancer* **83**, 307–310
8. Andersson, S., Rylander, E., Larsson, B., Strand, A., Silfversvard, C., and Wilander, E. (2001) The role of human papillomavirus in cervical adenocarcinoma carcinogenesis. *Eur. J. Cancer* **37**, 246–250
9. Jozefa, W.G., Daniela, S., Vladimir, K., and Marcel, H. (2002) Escape of p53 protein from E-6 mediated degradation in HeLa cells after cisplatin therapy. *Int. J. Cancer* **101**, 128–136
10. Wu, L. and Levine, A.J. (1997) Differential regulation of the p21/WAF-1 and mdm2 genes after high-dose UV irradiation: p53-dependent and p53-independent regulation of the mdm2 gene. *Mol. Med.* **3**, 441–451
11. Pearson, A.S., Francis, R., Spitz, S., Swisher, G., Masafumi, K., Mona, G.S., Meyn, R.E., McDonnell, T.J., Cristiano, R.J., and Roth, J.A. (2000) Up-Regulation of the Proapoptotic Mediators Bax and Bak after Adenovirus-mediated *p53* Gene Transfer in Lung Cancer Cells. *Clin. Cancer Res.* **6**, 887–890
12. Swisher, S.G., Roth, J.A., Komaki, R., Gu, J., Lee, J.J., Hicks, M., Ro, J.Y., Hong, W.K., Merritt, J.A., Ahrar, K., Atkinson, N.E., Correa, A.M., Dolormente, M., Dreiling, L., El-Naggar, A.K., Fossella, F., Francisco, R., Glisson, B., Grammer, S., Herbst, R., Huaringa, A., Kemp, B., Khuri F.R., Kurie, J.M., Liao, Z., McDonnell, T.J, Morice, R., Morello, F., Munden, R., Papadimitrakopoulou, V., Pisters, K.M., Putnam, J.B. Jr., Sarabia, A.J., Shelton, T., Stevens, C., Shin, D.M., Smythe, W.R., Vaporciyan, A.A., Walsh, G.L., and Yin, M. (2003) Induction of p53-regulated genes and tumor regression in lung cancer patients after intratumoral delivery of adenoviral p53 (INGN 201) and radiation therapy. *Clin. Cancer Res.* **9**, 93–101
13. Wu, M., Das, A., Tan, Y., Zhu, C., Cui, T., and Wong, M.C. (2000) Induction of apoptosis in Glioma cell lines by TRAIL/APO-2L. *J. Neurosci. Res.* **61**, 464–470
14. Yang, Y., Ma, J., Song, Z., and Wu, M. (2002) HIV-1 TAT-mediated protein transduction and subcellular localization using novel expression vectors. *FEBS Lett.* **532**, 36–44
15. Ekert, P.G., Silke, J., Hawkins, C.J., Verhagen, A.M., and Vaux, D.L. (2001) DIABLO promotes apoptosis by removing MIHA/XIAP from processed Caspase-9. *J. Cell Biol.* **152**, 483–490
16. Ronca, F., Chan, S.L., and Victor, C.Y. (1997) 1-(5-Isoquinolinesulfonyl)-2- methylpiperazine induce apoptosis in human neuroblastoma cells SH-SY5Y, through a p53 dependent pathway. *J. Biol. Chem.* **272**, 4252–4260
17. Horky, M., Wurzer, G., Kotala, V., Anton, M., Vojtesek, B., Vacha, J., and Wesierska-Gadek, J. (2001) Segregation of nuclear components coincides with caspase-3 activation in cisplatin-treated HeLa cells. *J. Cell Sci.* **114**, 663–670
18. Ofir, R., Seidman, R., Rabinski, T., Krup, M., Yavelsky, V., Weinstein, Y.N., and Wol, S. (2002) Taxol induce apoptosis in human SKOV3 ovarian and MCF-7 breast carcinoma cells is caspase-3 and caspase-9 independent. *Cell Death Differ.* **9**, 636–642
19. Lan, F.Q. and Irene, O.L.N. (2002) Induction of apoptosis by cisplatin and its effect on cell cycle-related proteins and cell cycle changes in hepatoma cells. *Cancer Lett.* **175**, 27–38
20. Reed, J.C. (1999) *Cisplatin in Cancer Chemotherapy and Biological Response Modifiers* (Pinedo, H.M., Longo, D.L., and Chabner, B.A., eds.) pp. 141–151, Elsevier Science BV, Amsterdam
21. Green, D.R. and Reed, J.C. (1998) Mitochondria and apoptosis. *Science* **281**, 1309–1312
22. Fuchs, E.J., McKenna, K.A., and Bedi, A. (1997) p53-dependent DNA damage-induced apoptosis requires Fas/APO-1-independent activation of CPP32beta. *Cancer Res.* **57**, 2550–2554
23. Sun, X.M., MacFarlane, M., Zhuang, J., Wolf, B.B., Green, D.R., and Cohen, G.M. (1999) Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. *J. Biol. Chem.* **274**, 5053–5060
24. Eischen, C.M., Kottke, T.J., Martins, L.M., Basi, G.S., Tung, J.S., Earnshaw, W.C., Leibson, P.J., and Kaufmann, S.H. (1997) Comparison of apoptosis in wild-Type and Fas-resistant cells: chemotherapy-induced apoptosis is not dependent on Fas/Fas ligand. Interactions. *Blood* **90**, 935–943
25. Lowe, S.W., Ruley, H.E., Jacks, T., and Housman, D.E. (1993) a p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* **74**, 957–967
26. Lowe, S.W., Ruley, H.E., Jacks, T., and Housman, D.E. (1993b) p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* **29**, 847–849
27. Soengas, M.S., Alarcón, R.M., Yoshida, H., Giaccia, A.J., Hakem, R., Mak, T.W., and Lowe, S.W. (1999) Apaf-1 and Caspase-9 in p53-dependent apoptosis and tumor inhibition. *Science* **2**, 156–159