# Cisplatin-induced apoptosis in Hep3B cells: Mitochondria-dependent and -independent pathways

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# Cisplatin-induced apoptosis in Hep3B cells: Mitochondria-dependent and -independent pathways

Directed by Professor Jeon Han Park

The Doctoral Dissertation submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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June 2004

This certifies that the Doctoral Dissertation of Kim, Ji Su is approved

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Thesis Committee Member #4

The Graduate School

Yonsei University

June 2004

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마지막으로, 항상 저를 아끼고 사랑해주시는 가족들께 감사 드립니다.

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# ABSTRACT


## Cisplatin-induced apoptosis in Hep3B cells: Mitochondria-dependent and -independent pathways

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(Directed by Professor Jen Han Park)

An anti-cancer drug, cisplatin (CP) has been known to exert the induction of apoptosis in many cancer cells. Human hepatoma cell lines undergo apoptosis after treatment with CP, by mechanisms that are not fully understood, although our previous study demonstrated that Fas-dependent or -independent pathways are involved.

In the present study, to elucidate the mechanisms of CP-induced apoptosis in Hep3B cells which are Fas and p53 negative, we investigated mitochondria-dependent pathways and -independent pathways. In the mitochondria-dependent apoptotic pathways, the loss of mitochondrial membrane potential ($\Delta\varphi m$), the release of pro-apoptotic molecules from mitochondria into cytosol, and caspase activation were examined. NF-κB and p73 activation were included in the mitochondria-independent pathways. Phase-contrast microscopy and flow cytometry showed that CP induced apoptosis in a time-dependent fashion. Western blot and flow cytometry assay revealed that the translocation of Bax, resulted in the loss of mitochondrial

membrane potential (Δφm) and the efflux of cytochrome c and Smac (second mitochondria-derived activator of caspase)/DIABLO from mitochondria into the cytosol. Caspase-3, -8 and -9 were activated by CP treatment, however, CP-induced apoptosis was not completely blocked by pretreating with the pan-caspase inhibitor, z-VAD-fmk, indicating that caspase-independent apoptotic pathways might also be involved. RNase protection assay confirmed that NF-κB downregulation leading to the suppression of its target genes, such as *XIAP* and *TRAF2*, and p73 accumulation were also observed in CP-treated Hep3B cells. CP-induced apoptosis was inhibited to some extent by transiently overexpressed p73 dominant negative (DN) and XIAP, but not by p73DN or XIAP alone. p73 target genes related with apoptosis, *gadd45*, *pig-7* and *-8* were slightly increased by CP. Transcripts of death receptor-associated and Bcl-2 family genes were not significantly changed.

In conclusion, although we could not completely rule out the involvement of death receptor-mediated apoptosis, in this thesis, apoptosis induced by CP in Hep3B cells was caused by the synergistic effect of both mitochondria-dependent pathways and mitochondria-independent pathways including NF-κB downregulation and p73 activation.

Key words : Cisplatin, Apoptosis, Mitochondria, NF-κB, p73, Hep3B

## ABBREVIATION

CP, cisplatin

PT, permeability transition

Smac, the second mitochondria-derived activator of caspase

$\Delta\varphi_{m,}$ mitochondrial membrane potential

PI, propidium iodide

z-VAD-fmk, benzyloxycarbonyl-valinyl-alaninyl-aspartyl-
(*0*-methyl)-fluoromethylketone

RPA, RNase Protection assay

p73DN, p73 dominant negative

IAP, inhibitor of apoptosis protein

RT-PCR, perverse transcription polymerase chain reaction

SDS-PAGE, sodiumdodecyl sulfate polyacrylamide gel electrophoresis

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# I. INTRODUCTION

Apoptosis is obligatory for normal development of multicellular organism. Apoptotic cell death can be induced by various apoptotic stimuli via numerous mechanisms and it shows cell-type dependency in some cases. Among various apoptotic pathways, it has been reported that there are two cell types, each using almost exclusively one of two different Fas signaling pathways [1]. Figure 1 shows Fas triggering leads to strong caspase-8 activation at the DISC (death inducing signaling complex) which bypass mitochondria directly leading to activation of other caspases and subsequently to apoptosis in type I cells. Unlike type I cells, a weak DISC is formed leading to the activation of mitochondria, resulting in activating caspase-8 and -3 downstream of mitochondria in type II cells.

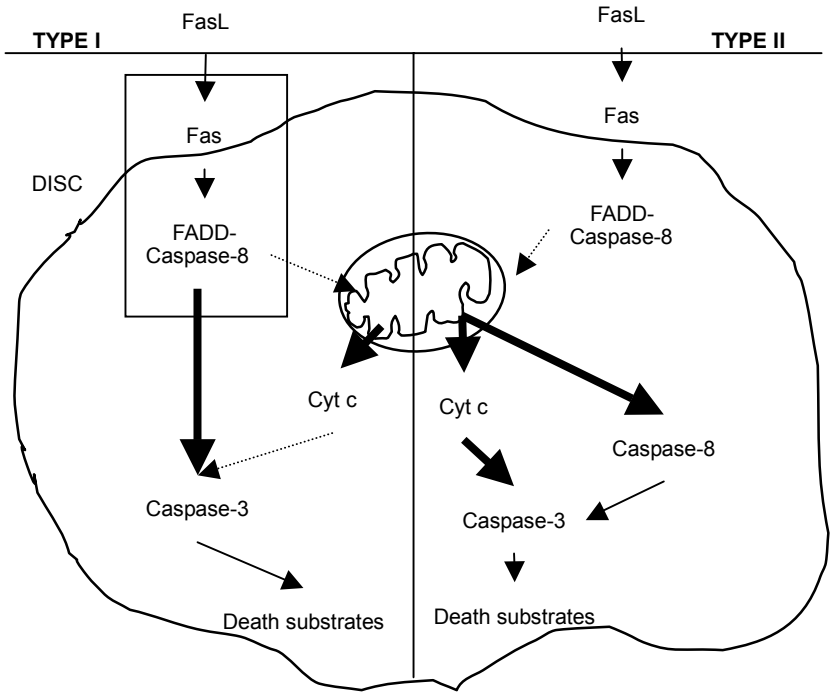



**Figure 1. Two models of Fas signaling pathways.**

Recently, mitochondria has been thought to play an important role in apoptosis [2]. Numerous apoptotic stimuli work through the mitochondria leading to activation of caspases and cell death associated with proapoptotic Bcl-2 family members (Fig. 2).

Cisplatin (CP) is known to induce cell death by generating DNA adducts in many cells [3]. Although CP has been used as a chemotherapeutic agent in many types of cancer and known to regulate death pathways (Fig. 3) [4], uncovered molecular mechanisms of its anti-cancer activity still remain in many types of cancer. Recently, it was reported that CP induces the mitochondria-mediated apoptotic pathway [5]. The major regulatory steps of

apoptosis associated with mitochondria are the disruption of electron transport and energy metabolism and the opening of a large conductance channel, the PT (permeability transition) pore [6]. The opening of PT pore liberates apoptogenic proteins, such as cytochrome c from mitochondria into the cytosol and gives rise to apoptosis [7]. This regulatory step induces the direct activation of caspases by cytochrome c [8]. In some cells, the p53-dependent membrane translocation of Bax, which is known as a mitochondrial PT inducer, is triggered by various DNA damaging agents [8].

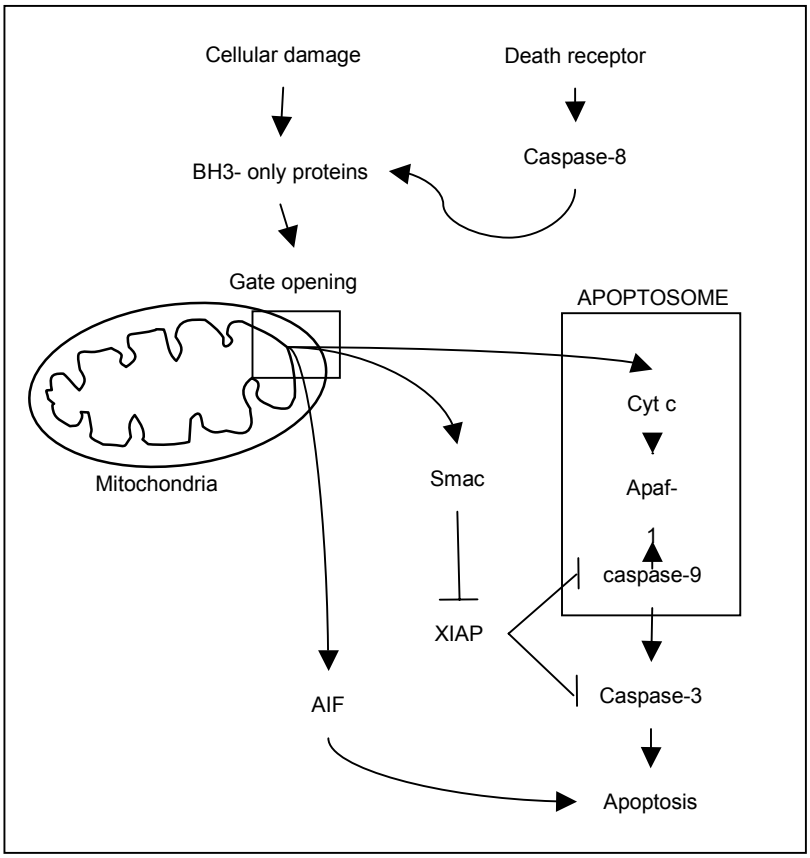



**Figure 2. Mitochondria is a center stage in apoptosis.**

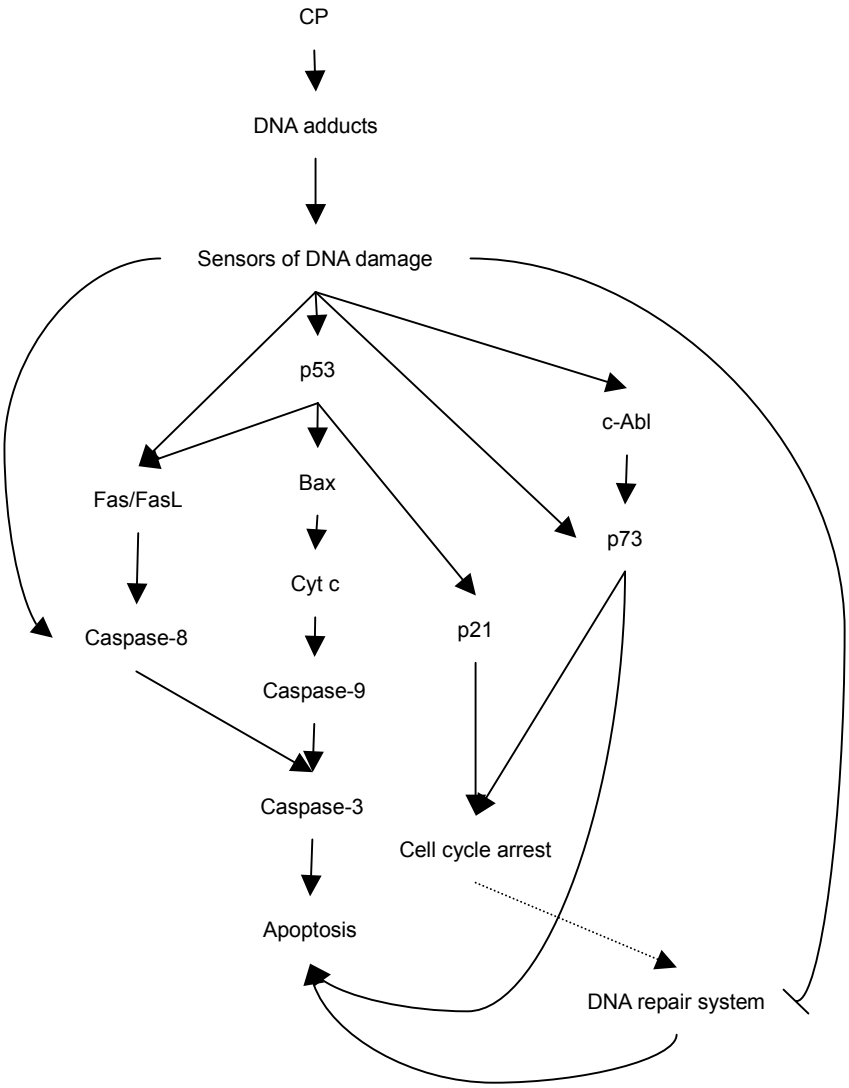



**Figure 3. An overview of pathways involved in mediating cisplatin-induced cellular effects.**

Transiently overexpressed Bax was found to localize to mitochondria and induce apoptosis [9], and inhibitor of apoptosis protein (IAP) blocked the activations of caspase-3, -6 and -7 by inhibiting the cytochrome c-induced activation of caspase-9 [10]. Recently, Smac was identified as a mitochondrial protein that is released with cytochrome c from mitochondria into the cytosol by apoptotic stimuli, and which leads to the promotion of caspase activation by binding and neutralizing IAPs [11, 12].

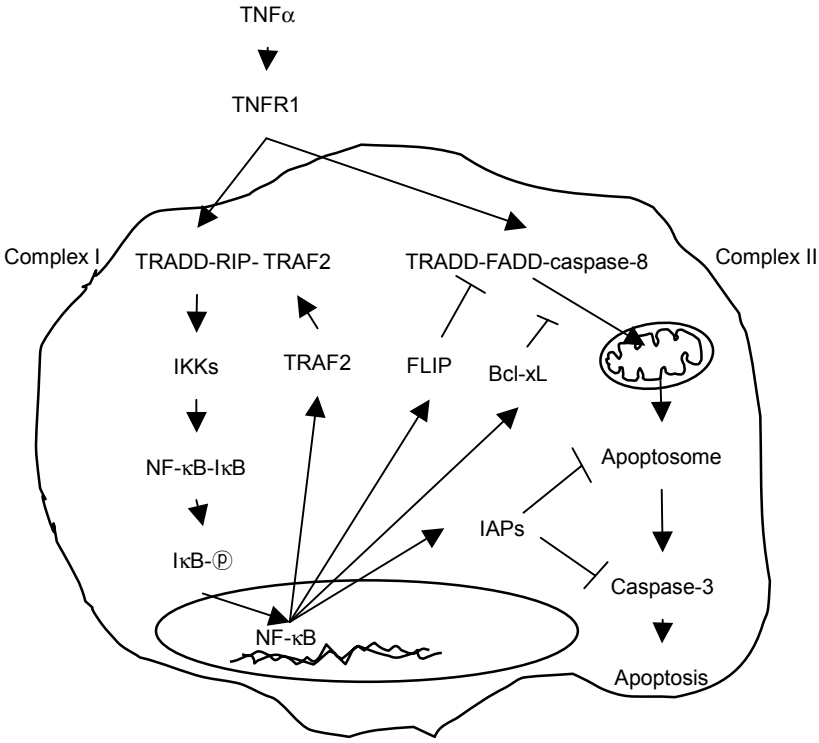



**Figure 4. TNF-R1 activation upon biding of cytokine TNFα leads to the formation of two sequential complexes.**

NF-κB regulates cell proliferation and inhibits apoptosis by

transactivating cell survival factors [13], and NF-κB activation allows some cells to resist chemotherapy [14]. The anti-apoptotic function of NF-κB has been reported to act via the induction of TRAF1 and TRAF2. NF-κB also induces c-IAP1, c-IAP2 and XIAP blocking caspases [15, 16], and FLIP inhibiting caspase-8. Figure 4 shows that complex I is comprised of TRADD, RIP1 and TRAF2, and is responsible for activation of NF-κB signaling, which upregulates anti-apoptotic genes. After modification such as ubiquitination and endocytosis, the protein complex recruits FADD, procaspase-8 and -10, leading to produce complex II, which initiates apoptotic pathways. The fate of cells is determined by the balance between NF-κB-dependent antiapoptotic genes and proapoptotic factors. Binding of FLIP competes with procaspase-8 in complex II, while the antiapoptotic Bcl-2 family members Bfl-1/A1, Bcl-xL, NR13 and Bcl-2 block the release of cytochrome c from mitochondria. IAPs, including XIAP, c-IAP1 and c-IAP2, can bind and silence effector caspases [17]. In addition, the association XIAP and PI3K/Akt pathway causes chemoresistance in many cancers (Fig. 5) [18].

p73 has been identified as another member of the p53 family and may function in the regulation of stress response and development [19]. Although the ectopic expression of p73 can transactivate p53-responsive genes such as *p21, mdm2, gadd45, bax, btg2, 14-3-3σ, p85, KILLER/death receptor 5 (DR5), p53-induced gene (pig)-2, -3, -6, -7, -8,* and *-11* [19], and induce cell cycle arrest or apoptosis, in p53-like manner, the role of p73 in apoptosis and tumorigenesis remains unclear [20]. In recent report, p73 elicits apoptosis via the mitochondrial pathway using PUMA (p53 up-regulated modulator of apoptosis) and Bax as mediators [21] (Fig. 6).

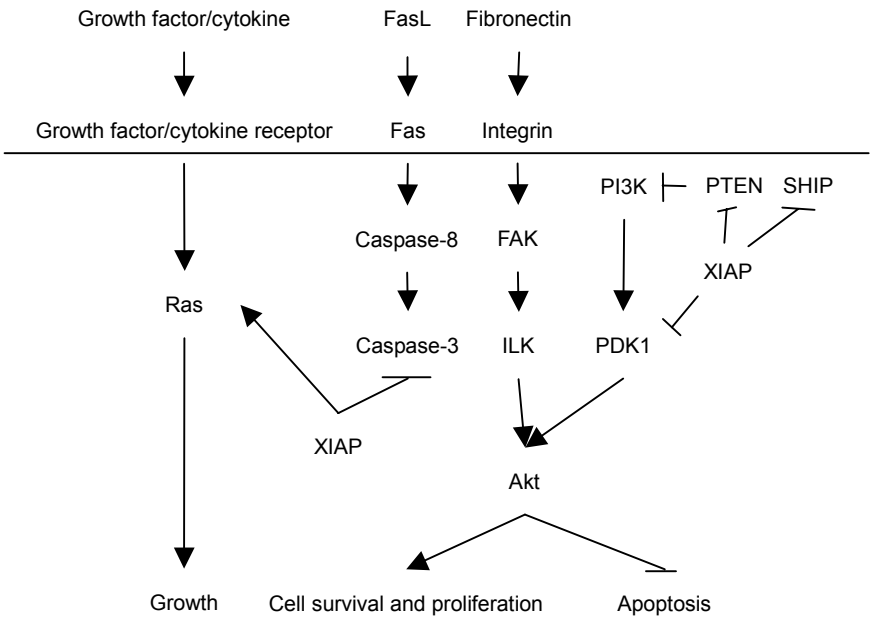



**Figure 5. The proposed pathways regulated by XIAP.**

Previously, we reported that CP induced apoptosis in the human hepatoma cell lines, HepG2 and Hep3B, via Fas-dependent and -independent pathway, respectively [22]. Although our previous study showed that the Fas-independent pathway was involved in the apoptosis of CP treated Hep3B cells, the mechanisms of this process are not fully understood. Therefore, in the present study, to elucidate the mechanism of CP-induced apoptosis in Hep3B cells, we investigated mitochondria-associated apoptotic events, NF-κB transcriptional activity, p73 activation, and their target genes. Our results suggested that CP-induced apoptosis in Hep3B cells is engaged in both mitochondrial dysregulation (mitichondria-dependent pathway) and the

synergistic effect of both NF-κB downregulation and p73 activation (mitochondria-independent pathway).

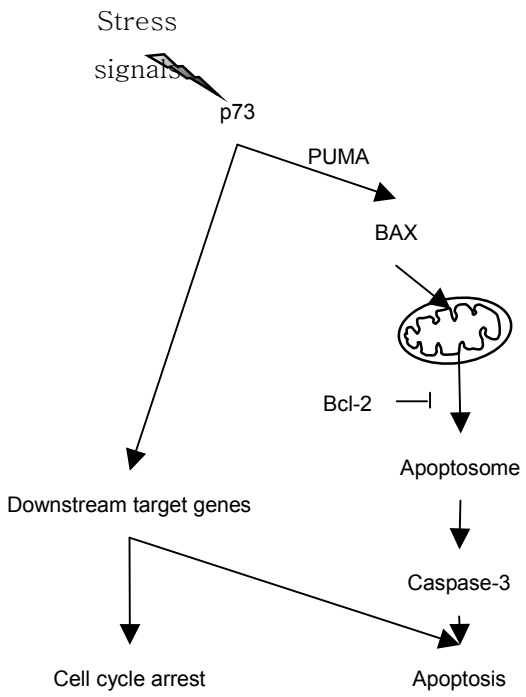



**Figure 6. The possible p73 pathways leading either to apoptosis or to cell cycle arrest.**

# II. MATERIALS AND METHODS

### 1. Cell culture

The human hepatocellular carcinoma cell line, Hep3B (ATCC HB 8064), was grown in minimum essential medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GIBCO BRL), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

### 2. Reagents and plasmids

Cisplatin (CP) was purchased from Sigma Chemical Co (St. Louis, MO, USA). Rabbit anti-Smac antibody was kindly provided from Dr. X. Wang (Howard Hughes Medical Institute and Department of Biochemistry, University of Texas Southwestern Medical Center at Dallas, USA). Rabbit anti-p50 and anti-p65, goat anti-14-3-3-σ, and mouse anti-HSP60 antibodies were purchased from Santa Cruz Bio Technology (Santa Cruz, CA, USA). Mouse anti-cytochrome c, rabbit anti-Bax, mouse anti-abl, and anti-caspase-3, -8, and -9 antibodies were purchased from PharMingen (San Diego, CA, USA). Mouse anti-α tubulin and mouse anti-MLH1 antibodies were purchased from Oncogene Research Products (Boston, MA, USA). Mouse anti-p73 antibody was purchased from NeoMarkers (Fremont, CA, USA). FITC-conjugated mouse anti-CD8 antibody was purchased from Becton-Dickinson (Dickinson, Sunnyvale, CA). Donkey anti-rabbit IgG conjugated with rhodamine and goat anti-mouse IgG. conjugated with FITC were purchased from Jackson ImmunoResearch (Philadelphia, PA, USA) and Zymed Laboratories (San

Francisco, CA, USA), respectively. The caspase inhibitor, z-VAD-fmk (R&D System, Minneapolis, MN, USA) was added to the culture to a final concentration of 100 μM - 300 μM and incubated for 1 h before CP treatment. p73-α and -β constructs were kindly donated by Dr. J. Y. J. Wang (Department of Biology and the Cancer Center, University of California, San Diego, La Jolla, CA, USA). p73DN was a gift from Dr. W. G. Kaelin Jr. (Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA, USA).

**3. Annexin V-FITC and propidium iodide (PI) staining**

Annexin V and PI staining was performed using an Annexin V-FITC Apoptosis Kit (BioSource, International Inc., Camarillo, CA, USA) to measure apoptosis. Briefly, after treatment with 15 μg/ml CP, cells were washed with PBS twice, collected and resuspended in 100 μl of 1 x Annexin V-FITC binding buffer. Five microliters of Annexin V-FITC conjugate and 10 μl of PI buffer were added, and the cells were then incubated at room temperature for 15 min in the dark. After adding 400 μl of 1 x Annexin V-FITC binding buffer, the cells were analyzed using a FACScan flow cytometer (Becton Dickinson).

**4. Immunofluorescence assay (IFA)**

Cells ($1 \times 10^4$ cells per well) were seeded on chamber slides and treated 15 μg/ml CP. After 24 h, cells were washed with cold PBS containing 0.1% BSA and fixed in 100% methanol for 15 min at -20℃. Fixed cells were washed and primary antibodies were incubated at the following dilutions: anti-Bax antibody 1:100 and anti-HSP60 antibody 1:100. Secondary antibodies were used at the following dilutions: rhodamine-conjugated anti-rabbit IgG, 1:250 and FITC-conjugated anti-mouse IgG, 1:250. Slides were mounted with

Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA) and images were captured using a confocal microscope with COMPIX software.

**5. Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)**

Nuclear extracts of Hep3B cells were prepared and EMSA was carried out as descried by Kim et al [23]. The synthetic oligonucleotides used as a probe DNA in assay consisted of double-stranded NF-κB sequence (Promega, Madison, WI, USA). To assess the composition of NF-κB complex, a supershift assay was carried out with anti-p50 and anti-p65 antibodies.

**6. NF-κB reporter assay**

Cells (2 x $10^6$ cells/well) were seeded onto a 6 well plate, and 0.75 μg p2x NF-κB-Luc and 0.25 μg pCMV-β-gal plasmids were cotransfected into the cells using Lipofectamine (GIBCO-BRL). After 24 h, the cells were treated with CP and harvested at the indicated times. Luciferase activity was determined using a Luciferase Assay System (Promega), and normalized against β-galactosidase activity [24].

**7. RNase protection assay (RPA)**

RPA was performed by using a RiboQuantTM multi-probe RNase protection assay system (PharMingen) following the standard protocol provided by the manufacturer. Briefly, anti-sense probes for hAPO-2b (human apoptosis multi-probe template sets-related with Bcl-2 family), hAPO-3c (-related with death

receptor), hAPO-5 (-related with NF-κB downstream target genes) were synthesized using an in vitro transcription kit (PharMingen) in the presence of 137.5 μM rNTPs, and 100 μCi [$^{32}$P]UTP (3,000 Ci/mmol; NEN, Boston, MA, USA). Total RNA was prepared using an RNeasy kit (Qiagen Inc., Chatsworth, CA, USA) and 10 μg RNA was hybridized with $^{32}$P-labeled anti-sense probes. After hybridization, 20 ng RNase A and 50 U RNase T1 were added to digest the unhybridized RNA. The duplex RNA hybrids were then resolved on 5% denaturing polyacrylamide gels containing 8 M urea and analyzed by autoradiography.

**8. RT-PCR**

Cells were collected and lysed and total RNA was isolated using a RNeasy kit. cDNA was synthesized by reverse transcription with 5 μg total RNA, 0.5 μg of random hexamer (Promega) and 200 unit of Murine Molony Leukemia Virus-Reverse Transcriptase (MMLV-RT) (GIBCO BRL) in 20 μl reaction mixture. The primer sequences used were as described *mlh1*, (sense) 5′-GTGCTGGCAATCAAGGGACCC-3′, (antisense) 5′-CAAGGTTGAGGCAT TGGGTAG-3′ (42); *pig-7*, (sense) 5′-CAG CCAGCGCCCATCCCCAATA AC -3′ (antisense) 5′-GCGTGAAGCTGGATGAGAGGTG GAAAG-3′; *pig-8*, (sense) 5′-TTGGGTCGTGGCTGGGATTCTT-3′(antisense) 5′-TTGCCAGGG GTCTTTGCTTCAT-3′; *β-actin*, (sense) 5′-CGTGGGCCGCCCTAGGCA CCA-3′ (antisense) 5′-TTGGCTTAGGGTTC AGGGGGG-3′; and *gadd45*, (sense) 5′-GAAGACCGAAAGGATGG-3′(antisense) 5′-GGGAGATTAATCA CTGG-3′ (39). Each 25 μl of PCR mixture containing 3 μl of reverse transcripted mixture, 10 pmol of forward and reverse primers and 2 unit of Taq

polymerase (Perkin Elmer, Norwalk, CT, USA) were amplified 25-27 cycles of denaturation at 94℃ for 30s, anealing at 56-59℃ for 30s, and extension at 72℃ for 30s. The PCR products were analyzes by agarose gel electophoresis.

**9. Determination of mitochondrial membrane potential (Δφm)**

To detect Δφm changes, cells (2 x $10^5$ cells/well) treated with CP were harvested at the indicated times and stained with 10 μg/ml JC-1 (Molecular Probes, Inc. Eugene, OR, USA) for 10 min at room temperature in the dark. Cells were then washed twice with cold PBS, and resuspended in 400 μl PBS for flow cytometry.

**10. Separation of the cytosolic and mitochondrial proteins**

Cytosolic and mitochondrial proteins were separated as described by Gao et al., [8] with minor modifications. Briefly, cells treated with CP were collected and suspended in mitochondria isolation buffer (20 mM HEPES-KOH, pH 7.5, 210 mM sucrose, 70 mM mannitol, 1 mM EDTA, 1 mM DTT, 1.5 mM $MgCl_2$, 10 mM KCl) and protease inhibitor cocktail (Boehringer Mannheim, Germany) supplemented with 10 mM digitonin (Sigma Chemical Co.). Suspensions were incubated at 37°C for 10 min and centrifuged at 12,000 g for 15 min. The supernatant (cytosolic fraction) and pellet containing the mitochondria were collected for Western blotting.

**11. Western blot**

After CP treatment, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 1.8 μg/ml aprotinin, 100 mM NaCl, 0.2% NP-40, 2 mM $MgCl_2$, 0.5 mM PMSF), and the lysates were cleared by centrifugation at

15,000 rpm for 15 min. One hundred micrograms of cell lysates were used for 8-15% SDS-PAGE, which was followed by Western blotting using the appropriate antibodies. Proteins were visualized by using Western Blotting Luminol Reagent (Santa Cruz Bio Technology).

**12. Transfection**

Cells (2 x $10^5$ cells/well) seeded onto a 12-well plate, were co-transfected with 0.75 μg p73-α or -β construct and 0.25 μg pCDNA-CD8TN plasmid expressing an extracellular and a transmembrane domain of human CD8 α chain [25] using Lipofectamine (Invitrogen, Carlsbad, CA, USA). After 48 h and 72 h of transfection, cells were stained with FITC-conjugated mouse anti-CD8 antibody and cell death was analyzed by FACS. To determine the effect of p73DN or XIAP on CP-induced apoptosis in Hep3B cells, cells (2 x $10^6$ cells/well) seeded onto a 6-well plate, were transfected with 10 μg p73DN and/or XIAP constructs using Lipofectamine (Invitrogen). After 48 h of transfection, cells were treated with CP for 48 h and stained with Annexin V-FITC conjugate and PI.

# III. RESULTS

## 1. Induction of apoptosis in Hep3B cells by cisplatin

In our previous study, we determined the dose- and time-dependencies of the CP-induced apoptosis of Hep3B cells using various methods, i.e., MTT and TUNEL assays and DNA fragmentation. We also found that 15 μg/ml CP optimally induced apoptosis in Hep3B cells at 48 h [22]. In the present study, we confirmed the effect of this condition on apoptosis, by finding that cells had a shrunken appearance and were detached from the bottom of dish (Fig. 7A). To quantify apoptosis, the cells were stained with Annexin V-FITC and PI, and analyzed by flow cytometry. As illustrated in Fig. 7B, apoptosis was found to occur in a time dependent manner, which is consistent with the results of our previous study [22].

## 2. Translocation of Bax to mitochondria in Hep3B cells treated with cisplatin

Recently, it was reported that CP-induced apoptosis involves Bax translocation to mitochondria in mouse collecting duct cells [3]. To investigate whether CP induces Bax translocation in Hep3B cells undergoing apoptosis, we examined the intracellular localization of Bax.

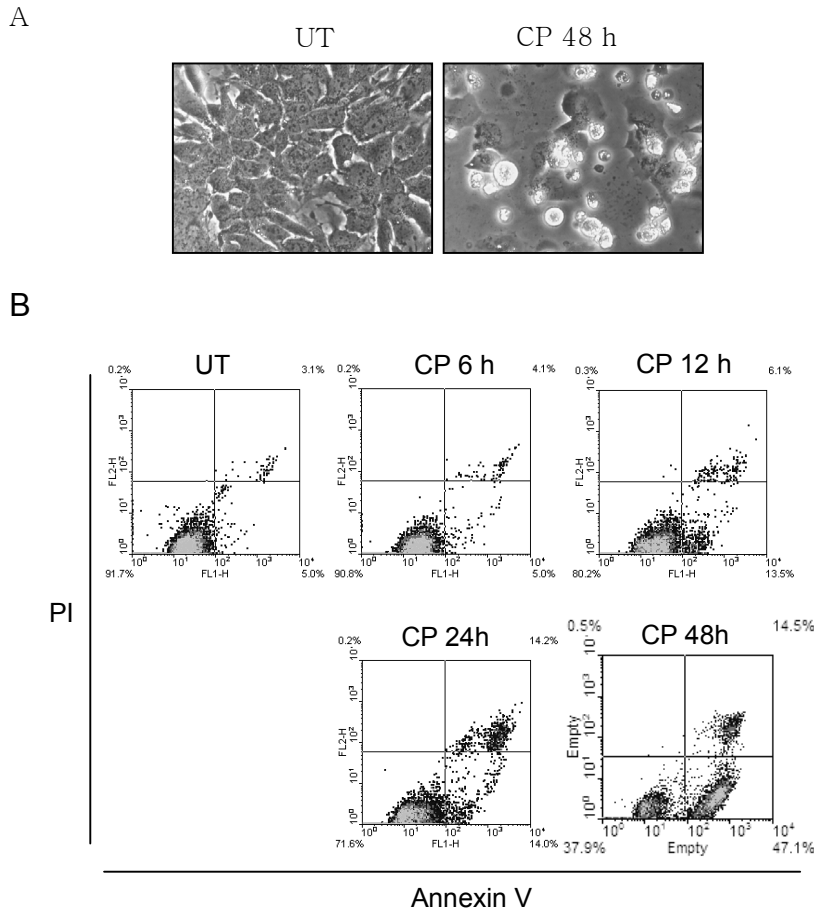



**Figure 7. Cisplatin-induced apoptosis in Hep3B cells.**

(A) After 48 h treatment of Hep3B cells with 15 μg/ml CP, cell morphology was observed under a phase-contrast microscope (× 400). (B) Hep3B cells treated with 15 μg/ml CP were harvested at the indicated times, stained with Annexin V-FITC (X axis) and PI (Y axis), and analyzed by flow cytometry. Data is representative of three independent experiments. UT: untreated, CP: cisplatin treated

Fractionated cytosolic sample and pellet containing mitochondria of Hep3B cells undergoing apoptosis were subjected to Western blotting. To verify

correct fractionation, we detected the expression of HSP60 in each fractionation. Cechetto *et al* examined the location of HSP60 in cellular components and determined that Hsp60 in different rat tissues is primarily localized in the mitochondrial matrix compartment [26]. HSP 60 is only detected in pellet fraction containing mitochondria but not in cytosolic fraction (Fig. 8A). Cytosolic Bax was found to have decreased and mitochondrial Bax accumulated in a time dependent manner (Fig. 8A), indicating that CP induces the translocation of Bax from the cytosol to mitochondria during the apoptosis of Hep3B cells. To confirm the Bax translocation, IFA was performed with anti-Bax and anti-HSP60 antibodies. Punctuated form of HSP60 was detected in mitochondria [26] and Bax was displayed in the diffuse pattern. CP induced cell shrinkage and translocalization of Bax from cytosol to mitocondria and colocalized with HSP60 protein (Fig. 8B). These results suggest that CP promotes translocation of Bax to mitochondria and might lead to the dysregulation of mitochondria and apoptosis in Hep3B cells.

### 3. Reduction of mitochondrial membrane potential ($\Delta\varphi_m$) in Hep3B cells treated with cisplatin

Since the loss of $\Delta\varphi_m$ is one of the early apoptotic changes, which is induced by Bax through an interaction with the PT pores [27, 28], we investigated whether $\Delta\varphi_m$ is reduced in Hep3B cells showing CP-induced Bax translocation. Mitochondrial membrane potential was measured using

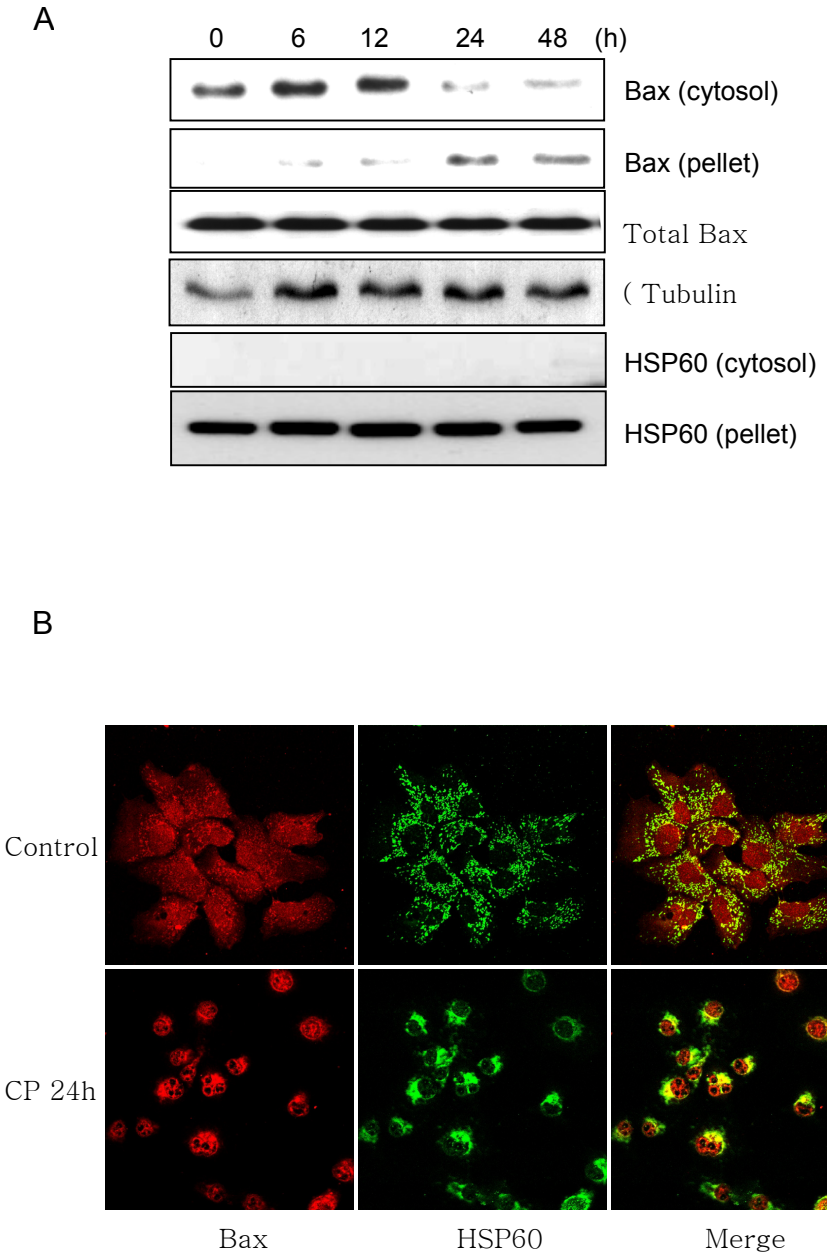



**Figure 8. Translocation of Bax to mitochondria by cisplatin.**

(A) To detect Bax translocation from the cytosol to mitochondria, samples were fractionated

from Hep3B cells treated with 15 μg/ml CP, and blotted with anti-Bax antibody. To determine the total level of Bax, total cell extracts from Hep3B cells were subjected to Western blot. The membrane was stripped and reprobed with anti-α tubulin antibody as a loading control. To confirm fractionation, each fraction was blotted with anti-HSP60 antibody. (B) Translocation of Bax to mitochondria in CP-treated Hep3B cells. At 24 h CP treatment, the cells were fixed and labeled with anti-Bax and anti-HSP60 antibodies followed by the rhodamine-conjugated anti-rabbit and FITC-conjugated anti-mouse immunoglobulins, respectively. The labeled cells were visualized by confocal micorscopy. In the merged confocal images, Bax (red) and HSP60 (green) were colocalized (yellow).

the lipophilic cation dye, JC-1, which produces a color change from red to green as the mitochondrial membrane becomes depolarized [28]. JC-1 exists in monomeric form emitting at 527 nm after excitation at 490 nm in depolarized membrane. However when mitochondrial membrane becomes more polarized, JC-1 is able to form J-aggregates that are associated with large shift in emission (590 nm). Fig. 9 shows that cells with depolarized mitochondria gradually increased according to CP treatment time. In contrast, CP-untreated Hep3B cells still have polarized mitochondria until 48 h. It is implied that translocation of Bax induced mitochondrial dyslagulation including the loss of mitochondrial membrane potential in CP-treated Hep3B cells.

## 4. Release of cytochrome c and Smac from mitochondria into the cytosol by cisplatin

Since it has been reported that cytochrome c is released from mitochondria

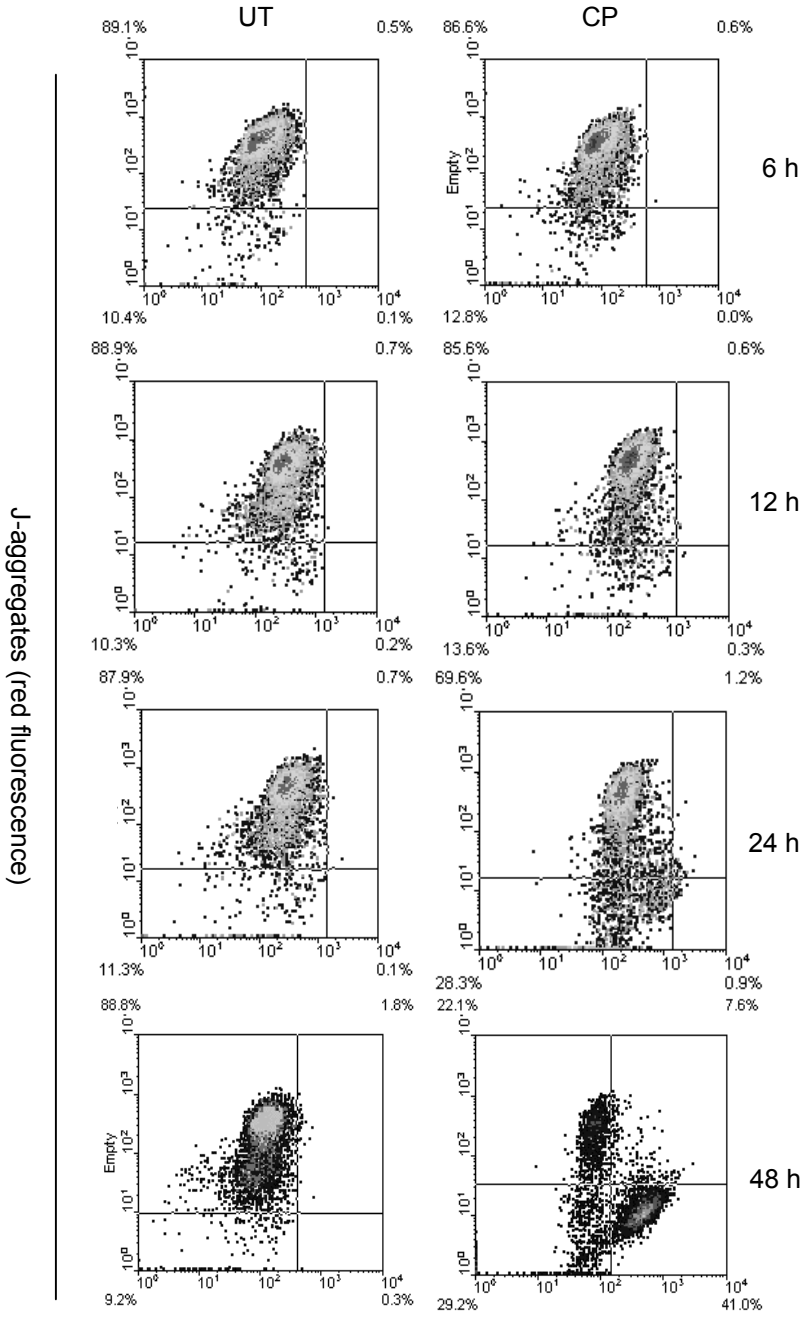

UT
CP
6 h
12 h
24 h
48 h
Empty
89.1%
0.5%
10.4%
0.1%
86.6%
0.6%
12.8%
0.0%
88.9%
0.7%
10.3%
0.2%
85.6%
0.6%
13.6%
0.3%
87.9%
0.7%
11.3%
0.1%
69.6%
1.2%
28.3%
0.9%
88.8%
1.8%
9.2%
0.3%
22.1%
7.6%
29.2%
41.0%
J-aggregates (red fluorescence)
JC-1 monomer (green fluorescence)

**Figure 9. The loss of mitochondrial membrane potential (Δφm) by cisplatin.**

For the cytofluorometric analysis of φΔm, Hep3B cells treated with (CP) or without (UT) 15 μg/ml CP were stained with JC-1. Green emission was measured in fluorescence channel 1 (X axis) and red emission in channel 2 (Y axis). The data shown is the representative of two independent experiments.

into the cytosol during Bax translocation-mediated apoptosis and anticancer drugs-induced apoptosis [9, 28], we expected that cytochrome c might be released from mitochondria into the cytosol in accord with the mitochondrial translocation of Bax and the reduction of $\Delta\varphi_m$. To observe the efflux of cytochrome c from mitochondria, cytosolic and pellet fractions isolated at the various times were subjected to Western blotting. It was found that CP induced the release of cytochrome c from mitochondria into the cytosol in a time dependent manner, however total cytochrome c expression did not affected by CP treatment (Fig. 10A). Recent studies associated with cytochrome c have shown that another pro-apoptotic mitochondrial protein, Smac, is released along with cytochrome c during apoptosis, and that this then neutralizes IAPs [11, 12]. To investigate the release of Smac in Hep3B cells undergoing CP-induced apoptosis, we determined cytosolic and mitochondrial Smac by immunoblotting fractionated cell lysates. It appears that Smac is released with cytochrome c from mitochondria into the cytosol during CP-induced apoptosis in Hep3B cells in a time dependent manner (Fig. 10B). Like cytochrome c, total Smac expression did not change in CP-treated Hep3B cells.

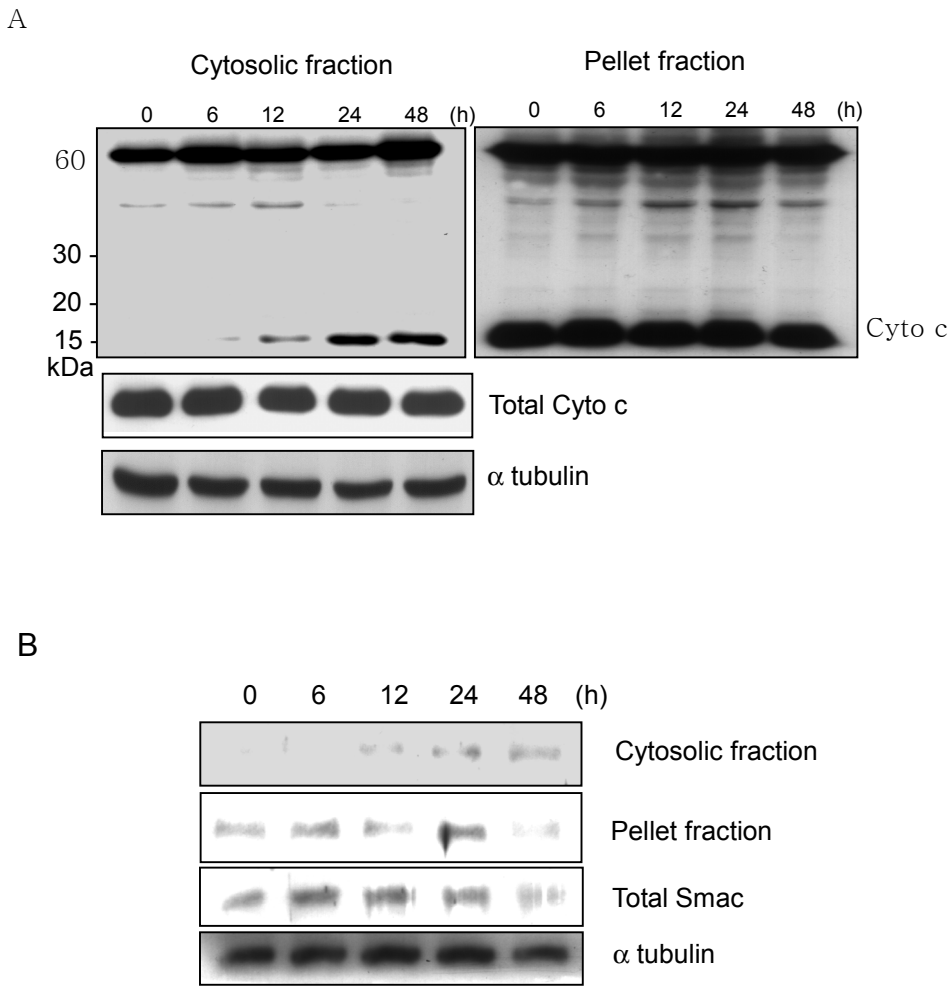



**Figure 10. Release of cytochrome c and Smac from mitochondria into the cytosol by cisplatin.**

CP treated Hep3B cells were harvested at various time intervals and cell lysates were fractionated into cytosolic and pellet fractions. (A) Immunoblot was performed upon each fraction with anti-cytochrome c antibody. (B) Cytosolic and mitochondrial Smac were immunoblotted in cytosolic and pellet fractions using anti-Smac antibody. Total extracts were analyzed to examine the effect of CP on the basal expression level of cytochrome c and Smac. The membrane was stripped and reprobed with anti-α tubulin antibody as a loading control.

Cyto c; cytochrome c

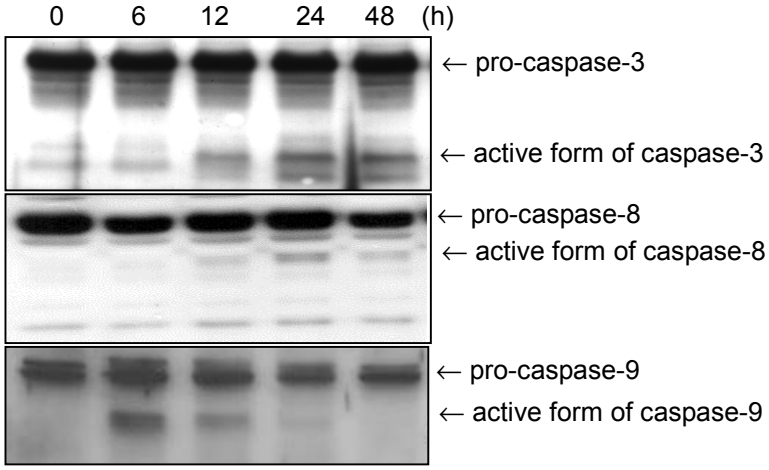
## 5. Partial inhibition of cispaltin-induced apoptosis by a pan-caspase inhibitor

Cytochrome c and Smac released into cytosol are known to promote caspase activation [29]. To confirm caspase activation, Western blotting was performed for caspase-3, -8, and -9. CP led to the cleavage of procaspase-3, -8 and procaspase-9, and their cleaved forms were detected until 48 h (caspase-3, -8) and 12 h (caspase-9), respectively (Fig. 11A). To examine whether the pan-caspase inhibitor, z-VAD-fmk, inhibits caspase-dependent apoptosis by CP, for cells were pretreated with various concentration of z-VAD-fmk for 1 h before adding CP, and cytotoxicity was measured by Annexin V and PI staining. CP-induced apoptosis was found to be decreased in Hep3B cells by z-VAD-fmk in a dose-dependent manner (Fig. 11B). However, this apoptosis was not completely inhibited by z-VAD-fmk, we speculated that other apoptotic (mitochondria-independent) pathways might be also involved, and thus, we searched for NF-κB and p73 related events as mitochondria-independent pathways.

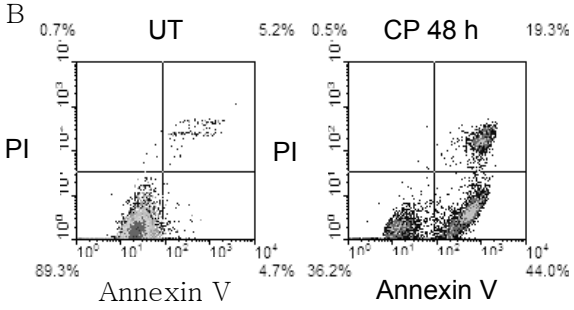
## 6. Downregulation of NF-κB transcriptional activity by cisplatin

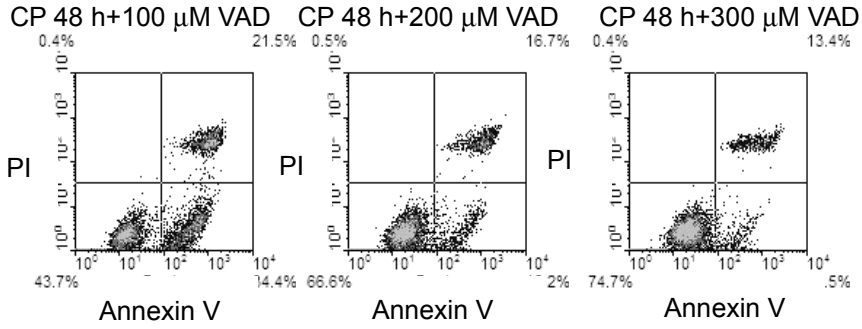
To investigate the effect of CP on NF-κB binding activity in Hep3B cells, we carried out EMSA. After 6 h treatment of CP, a significant decrease of NF-κB binding activity in Hep3B cells was observed. To assess the composition of NF-κB complex in Hep3B cells, supershift assay was performed with anti-p50 and anti-p65 antibodies. Our result showed that CP led to reduction of NF-κB binding activity comprised of the p50 and p65 heterodimer in Hep3B cells (Fig. 12A). We also examined the transcriptional activity and expression level of NF-κB. Consistent with Mandonado's report [30], the expression level of p65 was unchanged by CP treatment. However, CP steadily downregulated NF-κB transcriptional activity in Hep3B cells in a time dependent manner (Fig. 12B). In terms of the anti-apoptotic role of NF-κB, it has been reported that its target genes related with cell survival, such as *c-IAP1, c-IAP2, TRAF1, TRAF2* [15], *XIAP, bcl-2* and *bcl-$_{XL}$* [16]. To determine the effect of CP on the transcription of NF-κB target genes, RPA was performed with APO-5 probe set for NF-κB downstream target gene, such as *TRAF* and *c-IAP* family members. We found that *XIAP* and *TRAF2* mRNA transcript levels were dramatically reduced in Hep3B cells treated with CP, in a time dependent manner (Fig. 12C). These results imply that the CP-induced downregulation of *XIAP* and *TRAF2* transcription might play a role in enhancing the apoptotic signal by affecting cell survival.

A


0
6
12
24
48
(h)
← pro-caspase-3
← active form of caspase-3
← pro-caspase-8
← active form of caspase-8
← pro-caspase-9
← active form of caspase-9

B


UT
0.7%
5.2%
89.3%
4.7%
PI
Annexin V
CP 48 h
0.5%
19.3%
36.2%
44.0%
PI
Annexin V


CP 48 h+100 μM VAD
0.4%
21.5%
43.7%
PI
Annexin V
CP 48 h+200 μM VAD
0.5%
16.7%
66.6%
PI
Annexin V
CP 48 h+300 μM VAD
0.4%
13.4%
74.7%
PI
Annexin V

| | UT | CP 48 h | CP 48 h | | |
|---|---|---|---|---|---|
| | | | 100μM z-VAD | 200μM z-VAD | 300μM z-VAD |
| Annexin $V^-/PI^-$ | 89.3 % | 36.2 % | 43.7 % | 66.6 % | 74.7 % |
| Annexin $V^+$ | 4.7 % | 44.0 % | 34.4 % | 6.2 % | 11.5 % |
| Annexin $V^+/PI^+$ | 5.2 % | 19.3 % | 21.5 % | 16.7 % | 13.4 % |

**Figure 11. Activation of caspases during cisplatin-induced apoptosis.**

(A) Total cell lysates from Hep3B cells treated with 15 μg/ml CP at the various times were immunoblotted with anti-caspase-3, -8 and -9 antibodies, respectively. (B) To inhibit the action of caspase, 100 μM - 300 μM z-VAD-fmk was pretreated 1 h before adding of CP. Cells were then harvested at 48 h and stained with Annexin V-FITC and PI, and analyzed by flow cytometry. Data is representative of three independent experiments.

## 7. Accumulation of p73 in Hep3B cells treated with cisplatin

It has been reported that p73 accumulates and is activated by CP in HCT116 containing wild type p53, and that its proapoptotic function is enhanced by nuclear tyrosine kinase, c-Abl under the presence of the intact mismatch repair gene, mlh1[31]. To verify that p73 plays a role in apoptosis in p53-negative Hep3B cells treated with CP, we checked endogenous MLH1 and c-Abl expressions. Both MLH1 and c-Abl were detected in Hep3B cells (Figs. 13A), though c-Abl was time dependently reduced by CP, which is consistent with Gong's report[31]. Since MLH1 and c-Abl were constitutively expressed in Hep3B cells, we examined the expression of p73 in Hep3B cells treated with CP. An accumulation of p73 was detected in a time dependent manner (Fig. 13B).

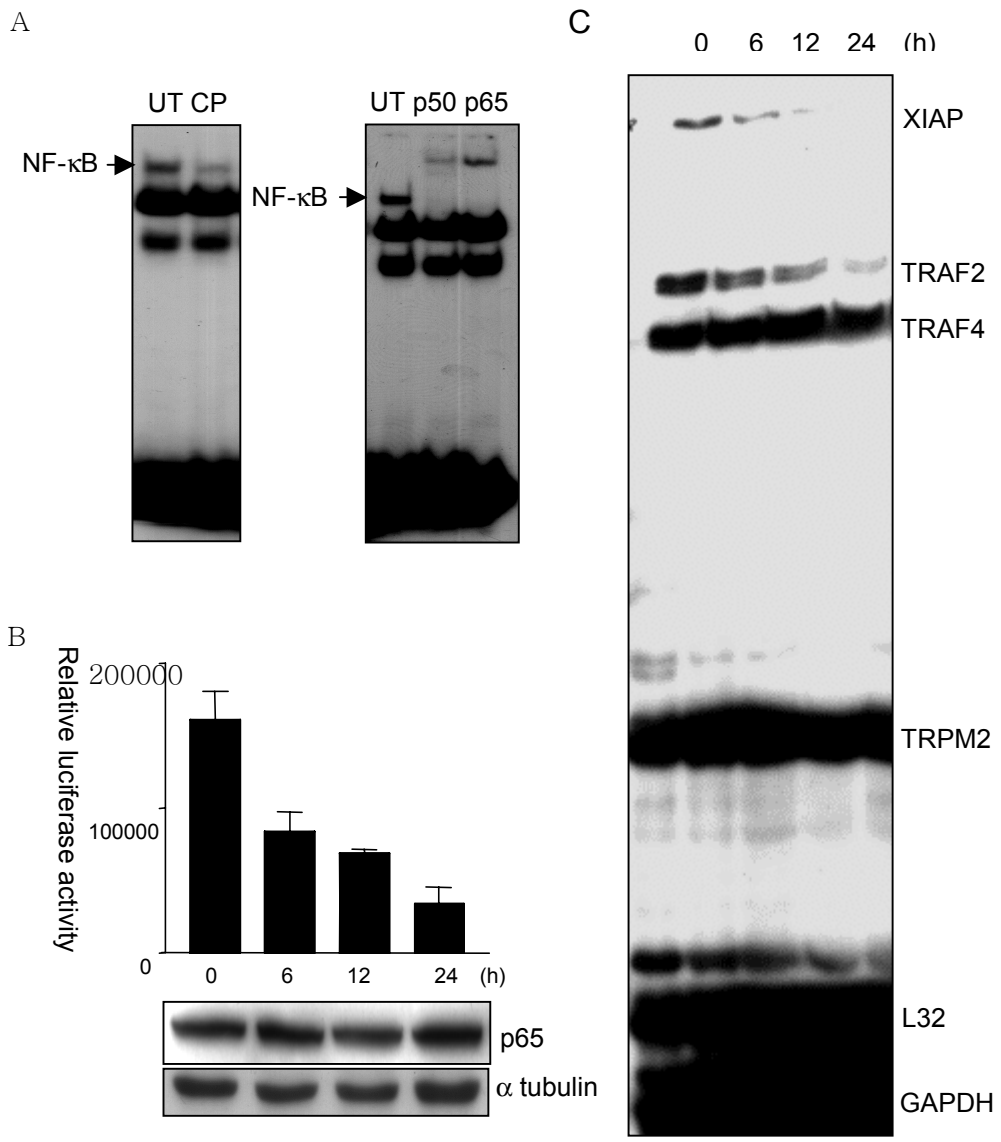



**Figure 12. Downregulation of NF-κB transcriptional activity by cisplatin.**

(A) Nucelar proteins extracted from Hep3B cells treated with or without CP were prepared and EMSA was carried out with double-stranded NF-κB sequence (left panel). Supershift assay was performed with anti-p50 and -65 antibodies (right panel). (B) Cells were transiently

transfected with p2×NF-κB-luc and CMV-β gal constructs. Twenty-four hours post-transfection, the cells were treated with 15 μg/ml CP and harvested at the indicated times. NF-κB transcriptional activity was analyzed by luciferase assay and normalized versus β-galactosidase activity. The data shown is the means and standard deviations of experiments performed in triplicate (upper panel). Total cell extracts from Hep3B cells treated with 15 μg/ml CP, were Western blotted with anti-p65 antibody. The membrane was then stripped and reprobed with anti-α tubulin antibody as a loading control (lower panel). (C) Total RNA extracted from Hep3B cells treated with 15 μg/ml CP was isolated and analyzed using a hAPO-5 template set by RPA at the indicated times. House keeping genes, *L32* and *GAPDH*, were used as internal controls.

Thus, we speculated that accumulated p73 might play a role in CP-induced mitochondria-independent apoptosis in Hep3B cells together with NF-κB downregulation.

To investigate the effect of accumulated p73 on CP-induced apoptosis, we examined p73 target genes which are associated with apoptosis. RT-PCR data showed that CP led to increase slightly the transcripts of *gadd45*, *pig-7* and *-8*, however, 14-3-3-σ protein was not detected in Hep3B cells (Fig. 14). Death receptor-related genes and Bcl-2 family genes were analyzed with PRA. Transcripts of death receptor-related genes (Fig. 15A) and Bcl-2 family genes (Fig. 15B) were not significantly changed by CP in Hep3B cells. Unlike RNA transcripts level, DR5 protein expression was slightly increased in CP treated cells (Fig. 15C). Thus, although CP did not dramatically affect on the transcripts of p73 target genes-related apoptosis, we could not completely exclude the possibility of death receptor-mediated apoptosis by CP in Hep3B cells.

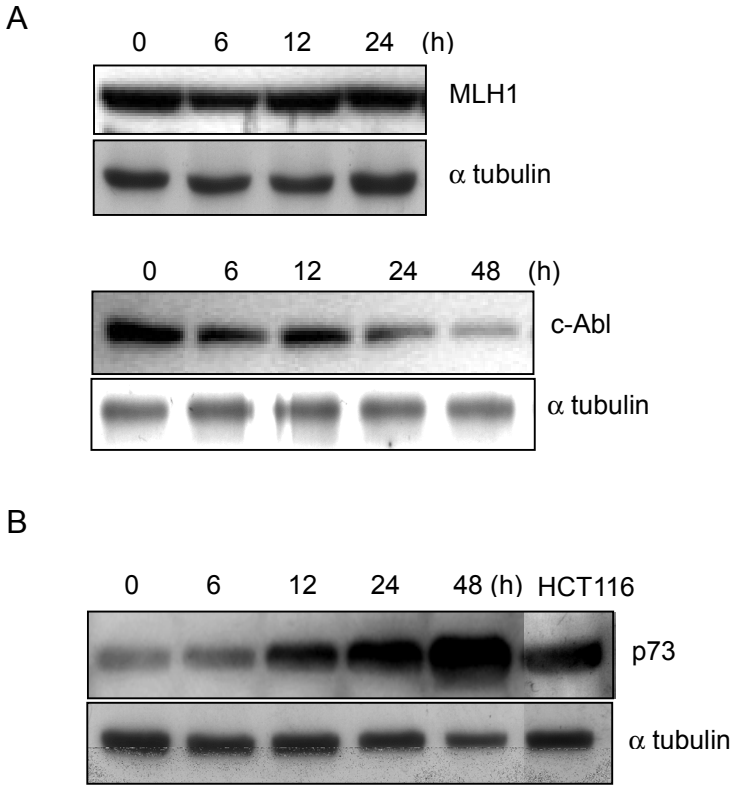



**Figure 13. Accumulation of p73 by cisplatin.**

Total cell extracts from Hep3B cells treated with 15 μg/ml CP were immunoblotted with (A) anti-MLH1(upper panel), anti-c-Abl (lower panel) and (B) anti-p73 antibody. Each membrane was stripped and reprobed with anti-α tubulin antibody as a loading control. HCT116 cells; a positive control of p73.

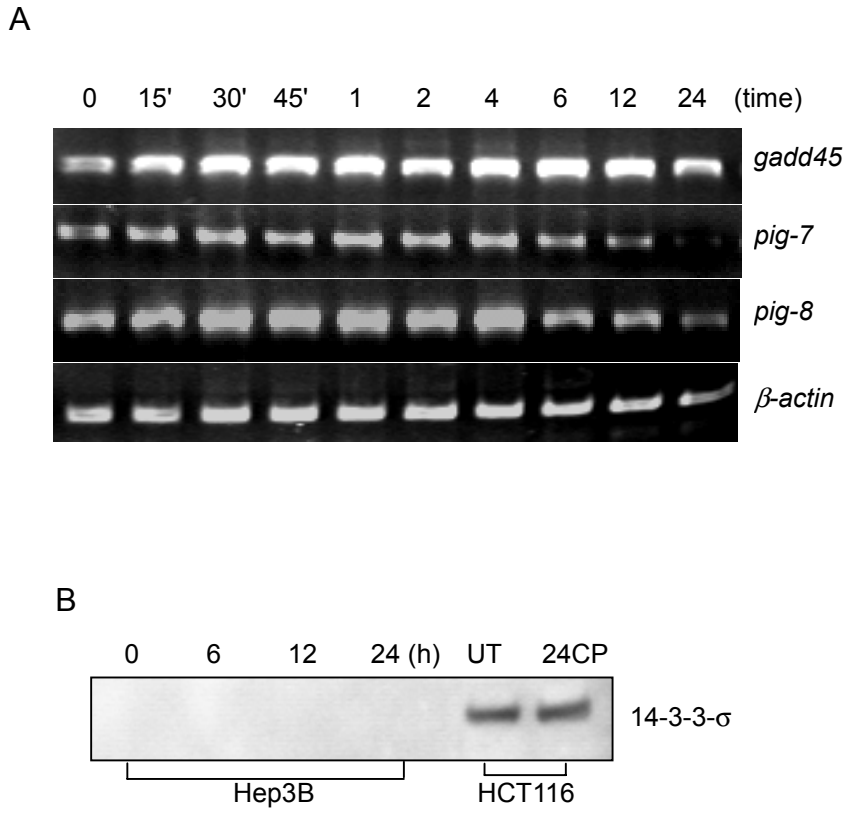



**Figure 14. Regulation of p73 target genes by cisplatin.**

RT-PCR was analyzed with the primer sets of *gadd45*, *pig-7*, and *-8* and *β-actin* at the indicated time periods. *β-actin* transcript amplification is shown as an internal control of RNA amount. (B) Cell lysates from Hep3B cell treated with 15 μg/ml CP for the indicated time periods were prepared and immunoblotted with anti-14-3-3-σ antibody. HCT 116 cell was used as a positive control of 14-3-3-σ.

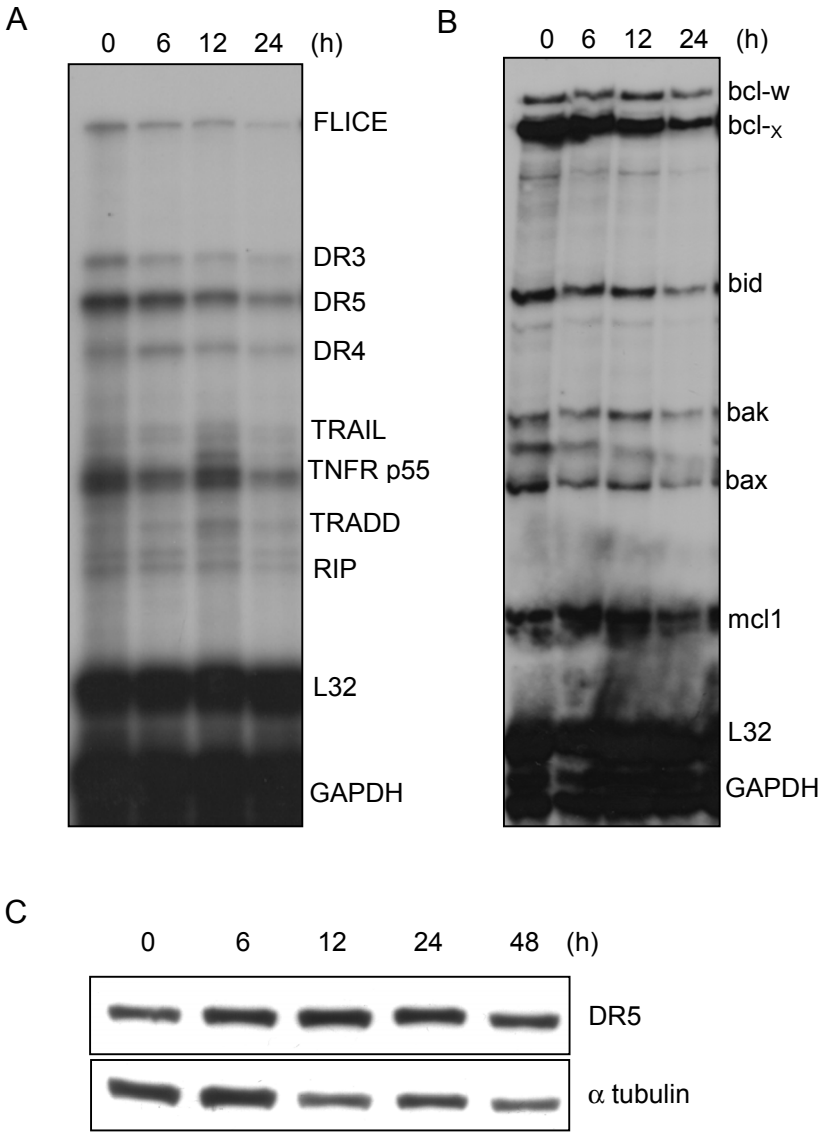



**Figure 15. Effect of cisplatin on death receptor-associated and Bcl-2 family genes.**

Total RNA extracted from Hep3B cells treated with 15 μg/ml CP was isolated and analyzed using a hAPO-3C probe set for death receptor related genes (A) and a hAPO-2b probe set for Bcl-2 family genes (B) template set by RPA at the indicated times. House keeping genes, *L32*

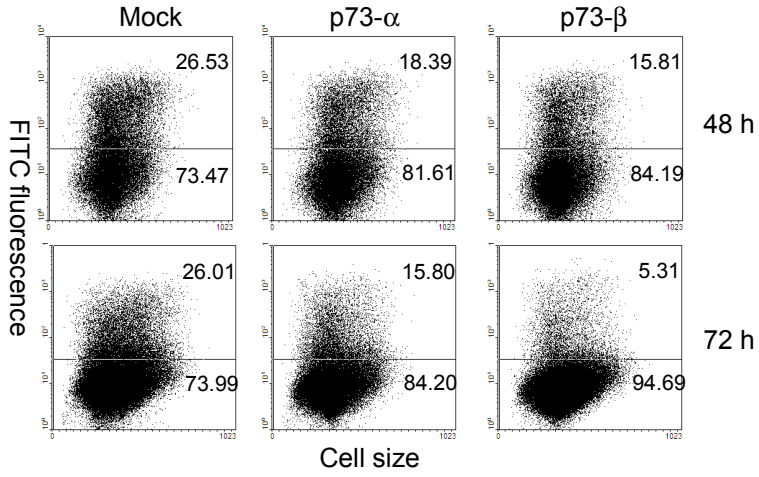
and *GAPDH*, were used as internal controls. (C) Total cell lysates from Hep3B cell treated with 15 μg/ml CP for the indicated time periods were prepared and immunoblotted with anti-DR5 antibody. Membrane was stripped and reprobed with anti-α tubulin antibody as a loading control.

## 8. Effect of p73 and XIAP on Hep3B cells apoptosis induced by cisplatin

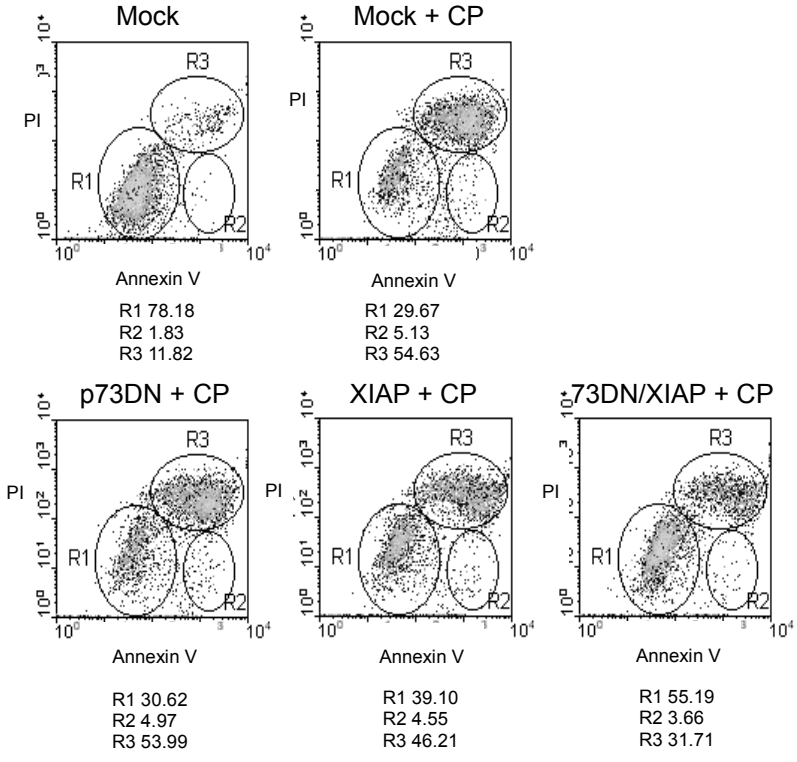
To examine the role of p73 in the apoptosis of Hep3B cells, p73-α or -β construct was co-transfected with pCDNA-CD8TN, and its effect on apoptosis was evaluated by the percentage of CD8+ cell population. CD8+ cell population was decreased by p73-α or -β in a time dependent manner, indicating that the expression of p73 in Hep3B cells led to apoptotic cell death (Fig. 16A). To confirm the role of p73 in CP-induced apoptosis, we transiently transfected p73DN and/or XIAP constructs into Hep3B cells, and then treaed the cells with CP, apoptosis was determined by Annexin V and PI staining. Ectopically expressed p73DN or XIAP alone did not block CP-induced apoptosis. However, the coexpression of p73DN and XIAP did inhibit CP-induced apoptosis to some extent (Fig. 16B). These results indicated that p73DN and XIAP are synergistically involved in the inhibition of CP-induced Hep3B apoptosis.

In conclusion, we could not completely rule out the involvement of death receptor-mediated apoptosis, in this study, CP-induced apoptosis was led to by the synergistic regulations of both mitochondria-dependent pathways containing mitochondrial dysregulations following activation of

A




B




R1 78.18
R2 1.83
R3 11.82

R1 29.67
R2 5.13
R3 54.63

R1 30.62
R2 4.97
R3 53.99

R1 39.10
R2 4.55
R3 46.21

R1 55.19
R2 3.66
R3 31.71

| | Mock | Mock+CP 48 h | CP 48 h | | |
|---|---|---|---|---|---|
| | | | p73DN | XIAP | p73DN+XIAP |
| Annexin $V^-/PI^-$ | 78.18 % | 29.67 % | 30.62 % | 39.1 % | 55.19 % |
| Annexin $V^+$ | 1.83 % | 5.13 % | 4.97 % | 4.55 % | 3.66 % |
| Annexin $V^+/PI^+$ | 11.82 % | 54.63 % | 53.99 % | 46.21 % | 31.71 % |

**Figure 16. The roles of p73 and XIAP in cisplatin-induced apoptosis.**

(A) Hep3B cells were cotransfected with 0.25 μg pCDNA-CD8TN and 0.75 μg p73-α or -β construct, then stained with FITC-conjugated anti-CD8 antibody and analyzed by FACS. The horizontal line represents an arbitrary division between CD8-positive cells (top) and -negative cells (bottom). The data shown is representative of three independent experiments. (B) To examine the effect of p73DN and XIAP on the inhibition of CP-induced apoptosis, p73DN and/or XIAP constructs were transiently transfected in Hep3B cells. Twelve hours post-transfection, 15 μg/ml CP was added for 48 h, and the cells were Annexin V-FITC and PI stained, and assessed by flow cytometry. The data shown is representative of three independent experiments.

caspases, and mitochondria-independent pathways including NF-κB downregulation and p73 activation in Hep3B cells

# IV. DISCUSSION

Previously we demonstrated that cisplatin (CP)-induced apoptosis in the hepatoma cell lines, HepG2 and Hep3B, occurs via Fas-dependent and -independent pathway, respectively [22]. In the present study, to elucidate the possible mechanism of the Fas-independent pathway induced by CP in Fas- and p53-negative Hep3B cells, we investigated mitochondrial involvement, NF-κB activity, p73 function, and their target genes. We found that CP-induced apoptosis in Hep3B cells is associated with mitochondrial dysregulation, NF-κB downregulation, and p73 accumulation.

CP is one of the most potent anti-cancer drugs, showing clinical activity against a various types of tumor. CP interacts with nucleophilic N7-sites of purine bases in DNA to form DNA-protein and DNA-DNA interstrand and intrastrand crosslinks [32]. DNA damage by CP leads to temporally induce a transient S-phase arrest and DNA adducts causes inhibition of G1 phase progression [33]. CP activates various signal transduction pathways, including those containing ATR, p53, p73, and MAPK, and culminates in the activating apoptotic cell death. CP preferencially activates ATR kinase, which phosphrylates and activates p53 following activation of p53 [34, 35]. One of the most common pathways associated with CP is p53-dependent mitochondrial pathway. CP has been shown to induce apoptosis by releasing cytochrome c and activating caspase-3 in breast cancer cells [36] and by triggering Bax translocation in mouse collecting duct cells [3]. In terms of mitochondrial dysregulation, we detected the redistribution of Bax to mitochondria (Fig. 8) and the loss of mitochondrial membrane potential ($\Delta\varphi_m$) (Fig. 9), which

resulted in the efflux of cytochrome c and Smac (Fig. 10), and thereby the activation of caspases (Fig. 11A) during CP-induced apoptosis in Hep3B cells. However, this apoptosis was not completely blocked by various concentrations of z-VAD-fmk (Fig. 11B) and this result is consistent with Gross' report which showed that apoptosis caused by Bax translocation and mitochondrial dysfunction was not blocked with caspase inhibitor [37]. Our results strongly imply that caspase-independent apoptotic pathways might be also involved in CP-induced apoptosis in Hep3B cells.

In recent report related to NF-κB, some evidences support a protective role for NF-κB in suppressing liver apoptosis [38]. Of this anti-apoptotic role of NF-κB, a link between aberrant NF-κB activity and cancer suggested that its constitutive activity has emerged as a hallmark for many human leukemias, lymphomas, and solid tumors [39]. It was reported that some anti-cancer chemotherapeutic drugs induce the suppression of NF-κB activation, and that this results in the sensitization of cancer cells to apoptosis in cell type specific and signal-dependent manner [40]. This occurs because NF-κB is crucial to cell survival as it regulates the transcription of anti-apoptotic genes, such as *XIAP*. XIAP blocks caspases and neutralizes by interacting with cytosolic Smac [41]. In fact, XIAP plays a important role in cell survival by modulating death signaling pathways and is a determinant of CP resistance in many cancers such as ovarian cancer [18]. Figure 12 shows that the downregulation of NF-κB and the reduction of *XIAP* transcripts in Hep3B cells treated by CP might reduce their chances of survival, and the release of Smac into the cytosol might affect both caspase activation and the enhancement of apoptosis.

It has been reported that the accumulation and ectopic expression of p73 induce apoptosis [31, 42], and it is known that Hep3B cells have nonfunctional

p53 [43]. Therefore, we speculated that p73 accumulation by CP might play a role in mitochondria-independent apoptosis. Transiently overexpressed p73-induced apoptosis in Hep3B cells (Fig. 16A), but p73DN or XIAP alone did not block CP-induced apoptosis, confirming Ferreira's finding that CP-induced apoptosis was not blocked by XIAP alone [44]. However, the coexpression of p73DN and XIAP inhibited CP-induced apoptosis in Hep3B cells to some extent (Fig. 16B). These data support the notion that a CP-induced apoptotic mechanism in Hep3B cells might also be controlled by a p73-associated pathway induced by DNA damage and by the suppression of XIAP , which was cuased by the NF-κB downregulation. However in a recent report, p73 induces apoptosis in p73 inducible system and it is mediated by PUMA induction and translocation of Bax to mitochondria following release of cytochrome c in Saos-2 cells which are p53-null and do not express p73 [21], we cannot not completely exclude the role of p73 in mitochondria-dependent pathway in Hep3B cells. In addition, because of caspase-8 activation, we cannot also completely exclude the possibility of death receptor-mediated caspase activation in Fas-negative Hep3B cells [22]. To investigate this possibility, we examined the transcripts of some death receptors in Hep3B cells treated with CP. Though DR5 protein expression is slightly increased by CP, CP did not show any significant effect on the level of transcripts of death receptor genes such as, *fas*, *DR3*, *DR4*, and *DR5* (Fig. 15). Downregulation of Bcl-2 by p53 has also been reported in HeLa cells treated with CP [30]. However, our RPA result shows that CP did not display any significant effect on the transcription of the *Bcl-2* genes family in Hep3B cells, which might be explained by the lack of functional p53 in Hep3B cells.

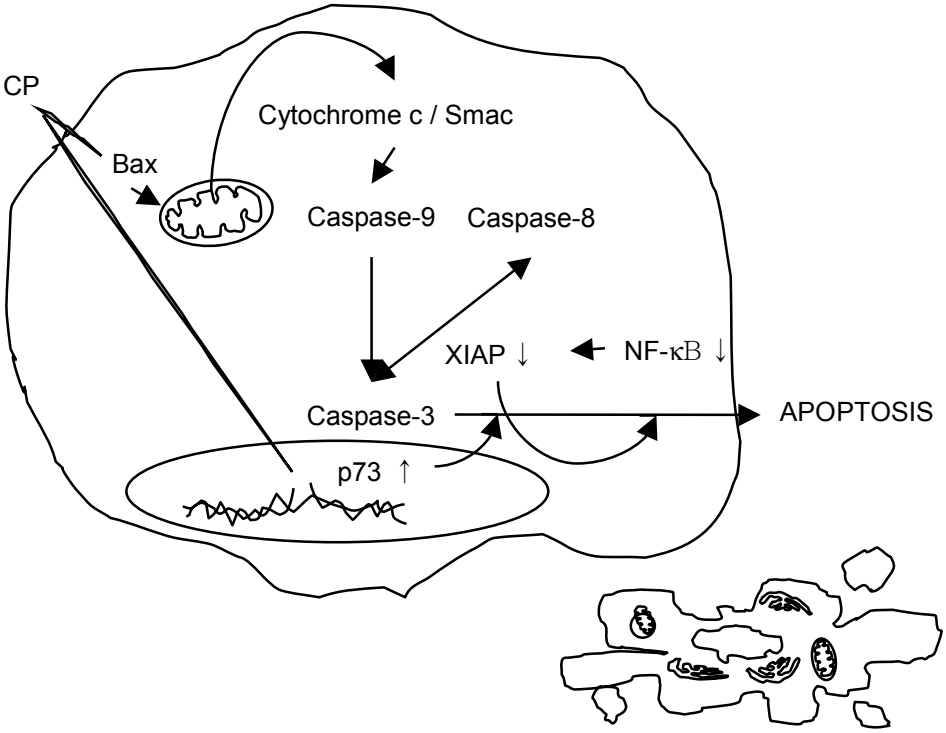



**Figure 17. The possible mechanism of cisplatin-induced apoptosis in Hep3B cells**

In this thesis, the apoptotic mechanism of CP is elucidated in Hep3B cells. The study was investigated mitochondria-associated pathways, the involvement of NF-κB, the activation of p73, and their target genes. Figure 17 shows the possible apoptotic mechanisms induced by CP in Hep3B cells. Although we could not completely rule out the involvement of death receptor-mediated apoptosis, in this study, apoptosis induced by CP in Hep3B cells was caused by the synergistic regulations of both mitochondria-dependent pathways followed by activation of caspases, and mitochondria-independent pathways including NF-κB downregulation, p73 activation.

## V. CONCLUSION

In the present study, to elucidate the mechanisms of CP-induced apoptosis in Hep3B cells which are Fas and p53 negative, we investigated mitochondria-dependent pathways and -independent pathways. In the mitochondria-dependent apoptotic pathways, the loss of mitochondrial membrane potential ($\Delta\varphi m$), the release of pro-apoptotic molecules from mitochondria into cytosol, and caspase activation were examined. NF-κB and p73 activation were included in the mitochondria-independent pathways.

1. Cisplatin (CP) induced a significant apoptotic cell death in a time-dependent way in human hepatoma cell line, Hep3B.

2. Mitochondrial dysregulation caused by CP showed that Bax translocation from mitochondria to cytosol, loss of mitochondrial membrane potential ($\Delta\varphi_m$), the release of cytochrome c and Smac following caspase activation in a time dependent manner.

3. Caspases were activated by CP but pan-caspase inhibitor, z-VAD-fmk, did not completely block CP-induced apoptosis.

4. NF-κB binding activity was reduced and transcriptional activity of NF-κB was downregulated. The expression of its downstream target genes, such as *XIAP* and *TRAF2*, were reduced by CP.

5. CP accumulated p73 in a time dependent manner and its ectopic expression

caused apoptosis in Hep3B cells. Its target genes such as *gadd45, pig-7* and *-8* transcripts were increased but transcripts of death receptor-related and Bcl-2 family genes did not change by CP. Death receptor 5 gene expression was slightly increased.

6. XIAP and p73 DN synergistically affected the inhibition of CP-induced apoptosis in Hep3B cells. Co-transfection of XIAP and p73 DN blocked apoptosis by CP to some extents but each transfection did not effect on CP induced apoptosis in Hep3B cells.

In conclusion, we could not completely rule out the involvement of death receptor-mediated apoptosis, in this thesis, apoptosis induced by CP in Hep3B cells was led to by the synergistic regulations of both mitochondria-dependent pathways containing and mitochondria-independent pathways including NF-κB downregulation and p73 activation.

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## ABSTRACT (in Korean)

### 간암세포주 Hep3B에서 cisplatin에 의해 유도되는 세포고사 기전: 미토콘드리아 의존적 및 비의존적 기전

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김지수

항암제의 하나인 cisplatin (CP)은 많은 암세포에서 세포고사(apoptosis)를 유발한다고 알려져 있다. 사람 간암세포주는 CP 처치 후에 세포고사가 유발된다는 것이 보고되어 있지만, 아직까지 그 기전은 잘 밝혀져 있지 않다. 이전의 보고에서 간암 세포주들 HepG2와 Hep3B에서 CP 처치후에 각각 Fas-의존성 또는 -비의존성 기전에 의해 세포고사가 유발된다는 것이 알려졌었다. 본 연구에서는, Hep3B (Fas 음성 및 p53 음성)에서 CP에 의해 유발되는 세포고사의 기전을 밝히고자 미토콘드리아 연관 기전, NF-κB 및 p73 활성 그리고 이들의 표적 유전자 발현을 조사하였다. CP는 Hep3B에 세포고사를 유도하였으며, 이때 세포질에 존재하던 Bax는 미토콘드리아로 이동하였다. Bax의 미토콘드리아로의 이동이 미토콘드리아막의 막전위 ($\Delta\varphi_m$)에 이상을 초래하여 미토콘드리아로부터 사이토크롬 C 및 Smac (second mitochondria-

derived activator of caspase)/DIABLO를 세포질로 유출시켰다. CP는 caspase-3, -8 and -9를 활성화시켰으며, pan-caspase inhibitor인 z-VAD-fmk 전처리로는 세포고사를 완전히 저해하지 못하였다. 이는 caspase 의존적 세포고사 기전뿐만 아니라 caspase 비의존적 세포고사 기전이 존재함을 시사한다. CP는 NF-κB downregulation을 초래하였으며, RNase protection 분석으로 NF-κB 표적 유전자들로 알려진 *XIAP* and *TRAF2*의 전사체도 감소하였다. p73가 CP 처리된 Hep3B 세포에서 시간별로 축적되었으며, CP에 의해 유도된 세포고사는 p73 dominant negative (DN)와 XIAP를 동시에 과발현시킴으로써 어느 정도 저해되었다. p73 표적 유전자로 세포고사와 관련있다고 알려진 *gadd45*, *pig-7* and *-8*을 조사한 결과, CP는 이들 유전자들의 발현을 다소 증가시켰다. CP는 death receptor 연관 유전자나 Bcl-2 family genes의 전사체에는 큰 영향을 주지 않았다.

결론적으로, CP에 의해 유도되는 Hep3B 세포의 세포고사에서 death receptor가 매개된 기전을 완전히 배제할 수는 없으나, Bax의 미토콘드리아로의 이동, 미토콘드리아 막전위 이상, 사이토크롬 C와 Smac 유출 및 그에 의한 caspase 활성을 통한 미토콘드리아 의존성 세포고사 기전과 NF-κB downregulation 및 p73축적으로 인한 미토콘드리아 비의존성 기전에 의한다는 것을 확인하였다.

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핵심되는 말 : Cisplatin, 세포고사, 미토콘드리아, NF-κB, p73, Hep3B