February, 2014

Thesis for Master Degree

Platinum nanoparticles induce apoptosis in Raw 264.7 cells through p53-mitochondria pathway

Graduate School of Chosun University

College of Dentistry

TA THI LOAN

플라티늄 나노입자에 의한 p53-미토콘드리아 의존성 세포사멸기전 연구

2014 년 2 월 25 일

Graduate School of Chosun University

College of Dentistry

TA THI LOAN

Platinum nanoparticles induce apoptosis in Raw 264.7 cells through p53-mitochondria pathway

지도교수 유훈

이 논문을 이학석사 학위신청 논문으로 제출함

2013년 10월

Graduate School of Chosun University

College of Dentistry

TA THI LOAN

This thesis is examined and approved for TA THI LOAN's master degree

Approved as to style and content by:

위원장	조선대학교	교수	**김명수**	(인)
위 원	조선대학교	교수	**방일수**	(인)
위 원	조선대학교	교수	**유훈**	(인)

2013년 11월

Graduate School of Chosun University

CONTENTS

LIST OF FIGURES ii

Abstract iii

초 록 v

I. INTRODUCTION 1

II. MATERIALS AND METHODS 9

2.1 Reagents 9
2.2. Cell line and cell culture 9
2.3. Cytotoxicity assay 9
2.4. Western Blot analysis 10
2.5. FACS analysis of apoptosis using Annexin V 10
2.6. Nuclear staining with DAPI 11
2.7. DNA fragmentation assay 11
2.8. Cell cycle analysis by Flow cytometry 11
2.9. Determination of cytochrome c release from Mitochondria 12

III. RESULTS 13

3.1. Cytotoxicity of PNPs on Raw 264.7 cells 13
3.2. Morphology of PNP-treated Raw 264.7 cells …… 15
3.3. Nucleus fragmentation by PNPs 17
3.4. PNPs induce an increase in the Annexin V binding on Raw 264.7 cells 19
3.5. PNPs induce DNA fragmentation 21
3.6. Induction of caspase activation by PNPs 23
3.7. Effect of PNP on the cell cycle regulation 25
3.7. PNP induces p53, p21 expression 27
3.8. Effects of PNP on cell cycle proteins 29
3.9. PNP induces cytochrome c release, activates caspase-9 , down-regulates Bcl2 and up-regulates Bax protein 31

IV. DISCUSSION AND CONCLUSION 36

REFERENCES 40

ACKNOWLEDGEMENT 45

LIST OF FIGURES

Fig 1. Schematic representation of the main molecular pathways leading to apoptosis..3

Fig 2. Apoptosome structure..... 4

Fig.3. p53-mediated apoptotic signaling..... 5

Fig. 4. The cell cycle and its regulation by cyclins, CDKs, and CDKIs 7

Fig. 5. Cytoxicity of PNPs on Raw 264.7 cell 14

Fig. 6. Effects of PNPs on morphology of Raw 264.7 cell..... 16

Fig.7 . Visualization of nuclei after exposing Raw 264.7 cells to PNPs 18

Fig.8 . PNPs induced an increase in the Annexin V-binding Raw 264.7 cells. 20

Fig.9 . PNPs cause DNA laddering on Raw 264.7 cells 22

Fig.10 . PNPs induced the activation of caspase-3, -7 on Raw 264.7 cells 24

Fig. 11. Effect of PNP on cell cycle regulation 26

Fig. 12. PNP induces p53, p21 activation..... 28

Fig. 13. Effect of PNP on cell cycle proteins..... ……..30

Fig. 14. Effect of PNP on cytochrome c release 32

Fig.15. Effect of PNP on pro-Caspase-9 expression ..... ……33

Fig.16. Effect of PNP on Bcl2/Bax expression ..... ……35

Fig. 17. Scheme of PNP-induced apoptotic pathways 39

ABSTRACT

Platinum nanoparticles induce apoptosis in Raw 264.7 cells through p53-mitochondria pathway

TA THI LOAN

Advisor: Prof. Yoo Hoon

Dept. of Bio-dental engineering,

Graduate school of Chosun University

Platinum nanoparticles (PNPs) have been recently reported to have anti-oxidant and anti-inflammatory effects. In this study, we investigated the effects of PNPs on Macrophage cell line Raw 264.7 cells by exposing PNP05 (5 nm in size) or PNP30 (30 nm in size). The PNPs showed severe toxicity on Raw 264.7 cell in a MTT assay and the degree of toxicity was nano-particle size dependent. The growth and survival of Raw 264.7 cells were also dose-dependent. PNP-induced apoptotic evidence was observed in FACS analysis with the increased cell population binding to Annexin-V or PI. PNP-exposed cells exhibited DNA fragmentation with DNA laddering features, and apoptotic protein markers such as cleaved caspase-3/-7 were detected dose-dependently in Western Blotting. Western blotting of cell cycle related proteins revealed that the expression of cell

cycle proteins were slightly decreased (cyclin D, cyclin E1, cyclin B1, Cdc2) or almost unchanged (cyclin A, cdk2, cdk4) at 2 μg/ml of PNP but further decreased at 6 μg/ml of PNP after 24 hours treatment. Furthermore, the cell cycle analysis by flow cytometry revealed that cell cycle was arrested at G2/M phase at low concentration of PNP (2 μg/ml). However, the arrest at G2/M phase was shifted into subG1 phase at high concentration of PNP (6 μg/ml). The expression of p53 and p21 which are necessary for maintaining a cell cycle arrest at G2/M phase were dramatically increased at apoptosis state. In addition, the expression of Bcl-2 protein was decreased and Bax was increased in a dose-dependent manner. Cytochrome c release from mitochondria to cytosol, after treating with PNPs, triggered the activation of caspase-9/-3 and -7. Overall our results suggest that PNP-induced apoptotic signaling may be involved in p53-mitochondrial pathway.

초 록

플라티늄 나노입자에 의한 p53-미토콘드리아 의존성 세포사멸기전 연구

따티로안

지도교수: 유훈

치과대학

조선대학교 대학원

최근 백금나노입자(PNP)가 항산화 및 항염증 효과가 있는 것으로 보고된 바 있다. 본 연구에서는 PNP05 (5 nm 크기) 또는 PNP30 (30 nm 크기)가 대식세포인 Raw 264.7 세포에 미치는 영향을 조사하였다. MTT 분석에 의하면 PNP 는 Raw 264.7 세포에 강한 유독성을 보였고 입자 크기 의존적인 독성을 나타내었다. Annexin-V 및 PI 를 이용한 FACS 분석에 의하면 PNP 는 Raw 264.7 세포의 증식을 억제하면서 세포사멸을 유도함을 확인할 수 있었다. 세포 증식억제에 대한 증거로 세포주기에 관련된 프로틴 (cyclin E1, cyclin D1, cyclin B1, Cdc2, cyclin A, cdk2, cdk4) 들의 발현감소, 그리고 세포사멸에 대한 증거들로 DNA 분절, western blotting 에 의한 단절된 caspase-3/-7 의 농도의존적 검출 등을 확인하였다. 흥미롭게도 2 μg/ml 농도의 PNP 로 Raw 264.7 세포에 24 시간

처리한 경우에 cyclin E1, cyclin D1, cyclin B1, Cdc2 의 발현이 농도의존적으로 감소한 반면 cyclin A, cdk2, cdk4 들은 발현에는 큰 변화가 없었다. 그러나 6 μg/ml 의 PNP 농도로 24 시간 처리한 경우에는 cyclin A, cdk2, cdk4 의 발현도 역시 농도의존적으로 현저히 감소하였다. 또한 이들 단백들의 신호 조절 단백들인 p53 및 p21 발현은 세포주기 억제 조건인 2 μg/ml 농도의 PNP 조건에서 농도의존적으로 증가하였으며 세포사멸 농도인 6 μg/ml 의 PNP 농도에서는 더욱 현저히 증가되는 발현양상을 보였다. 이러한 결과는 Annexin-V 를 이용한 분석 결과와도 일치하며 P53 에 의한 초기 세포주기 억제기등이 고농도의 PNP 조건에서 세포사멸로 전환되었음을 보여준다. 또한 농도의존적인 bcl-2 발현 감소, bax 발현 증가, 세포질에서의 cytochrome c 검출, caspase-9/-3 and -7 의 활성화 등의 분자적 발현 증거들은 PNP 에 의한 대식세포의 세포사멸 기전이 p53-mitochondrial 경로일 것임을 추정하게 해준다.

I. INTRODUCTION

Nanoparticles are particles with lengths that range from 1 to 100 nanometers in two or three dimensions. Nano-materials received considerable attention because of their potential applications in biology and medicine such as drug-delivery agents, biosensors, or imaging contrast agents (1, 2). However, the fundamental research and knowledge about their interactions with specific cells, mechanism of entry, subcellular compartmentalization, and trafficking throughout the body are still unknown (3). These studies will help understanding the interactions and recognitions of the nanoparticle by the cells at the molecular level (4). While the number of nanoparticle types and their applications continues to increase, studies on their potential toxicity are poorly understood. Particularly, in the medical field, nanoparticles are utilized as diagnostic and therapeutic tools to detect and treat human diseases. Thus, understanding the properties of nanoparticles and their effect on the human body is crucial (1).

Apoptotic cell death is a critical element in defensing against unrestrained cellular proliferation and inducing tumor cell death in many cancer therapies (5-7). The pathways of apoptosis are extremely complicated (8) and characterized by the activation of a family of cell death proteases called the caspases (9). Caspases are the effector molecules of apoptosis in mammals that play a key role in the initiation and execution of apoptosis, necrosis and inflammation (10-12). They are synthesized as inactive precursors or zymogens (procaspases) that need to be proteolytically processed to become active, cleave another caspase and serves to amplify the apoptotic signal. Caspase-9 is the initiator of the mitochondrial pathway and caspase-8 is regarded as the originator of the death receptor-mediated apoptotic pathway. Caspase-3, the most important one of the executioner caspases, is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10) (13).

Two types of apoptotic pathways, intrinsic and extrinsic (or death receptor), have been known (Fig. 1) (14).

The extrinsic apoptotic pathway is initiated by the cell surface death receptors such as TNF1 receptor, Fas, DR3, DR4 and DR5 (15-17) through the formation of the death-inducing-signaling-complex (DISC) (18), leadings to a cascade of activation of caspases such as caspase-8 and caspase-3. The intrinsic pathway is triggered in response to DNA damage, and associated with mitochondrial depolarization and release of cytochrome c from mitochondrial inter-membrane space into the cytoplasm. Cytochrome c, apoptotic protease-activating factor 1 (APAF- 1) and procaspase-9 then form a complex termed "apoptosome" (Fig. 2) (8) , in which caspase-9 becomes activated and promotes activation of caspase-3, caspase-6 and caspase-7 (19). Bcl-2 family members play important roles in regulating mitochondrial integrity and mitochondria-initiated caspase activation (20).

Both intrinsic and extrinsic pathways of apoptosis are associated with each other (21), connected through the cleavage of BID (BH3 interacting-domain death agonist). Another pathway of apoptosis has also been recently recognized which involves perforin/granzyme-A or -B. All three (intrinsic, extrinsic and granzyme B-induced) pathways of apoptosis come together putting cell to death by the activation of cas-pase-3/-7, cell shrinkage, chromatin condensation and fragmentation of chromosomal DNA, degradation of nuclear as well as cytoskeleton proteins (17).

Among many molecules implicated in apoptosis regulation, p53 is an essential molecular which is induced by DNA damage (22, 23). p53 is implicated in the induction of two distinct apoptotic signaling pathways involved in the activation of caspases cascade (Fig. 3) (24).

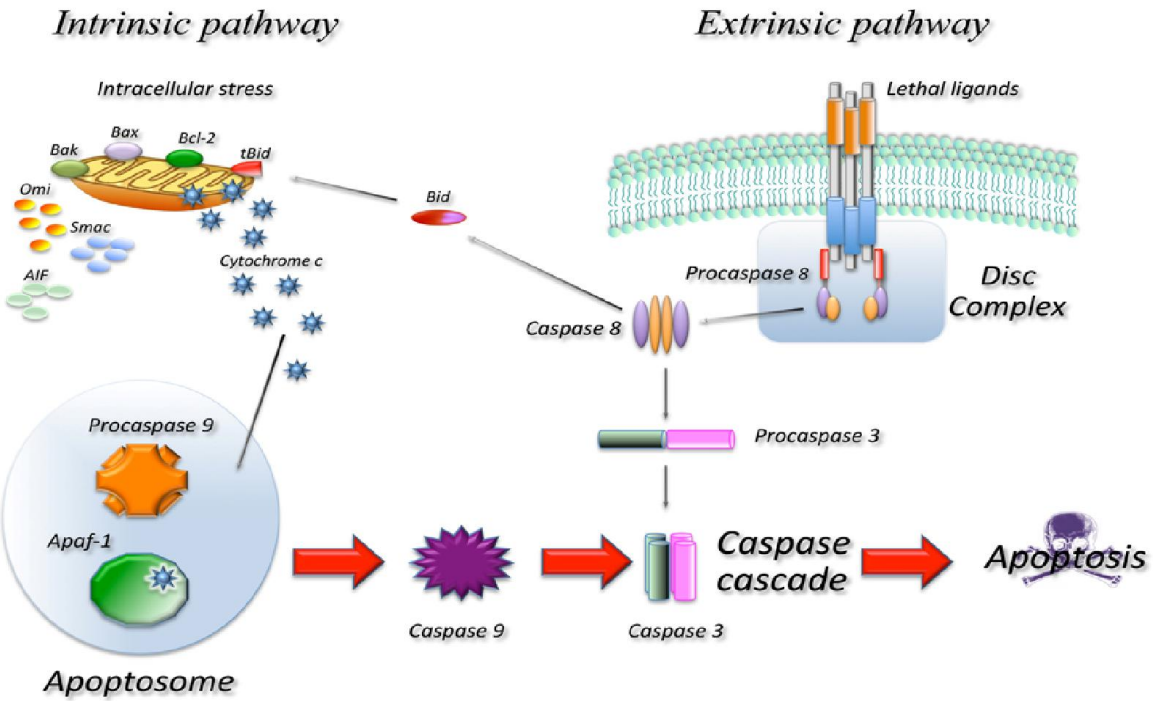

Fig. 1. Schematic representation of the main molecular pathways leading to apoptosis.

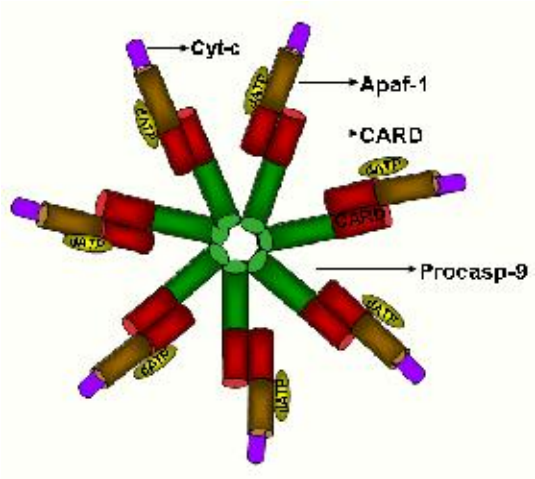

Fig. 2. Apoptosome structure

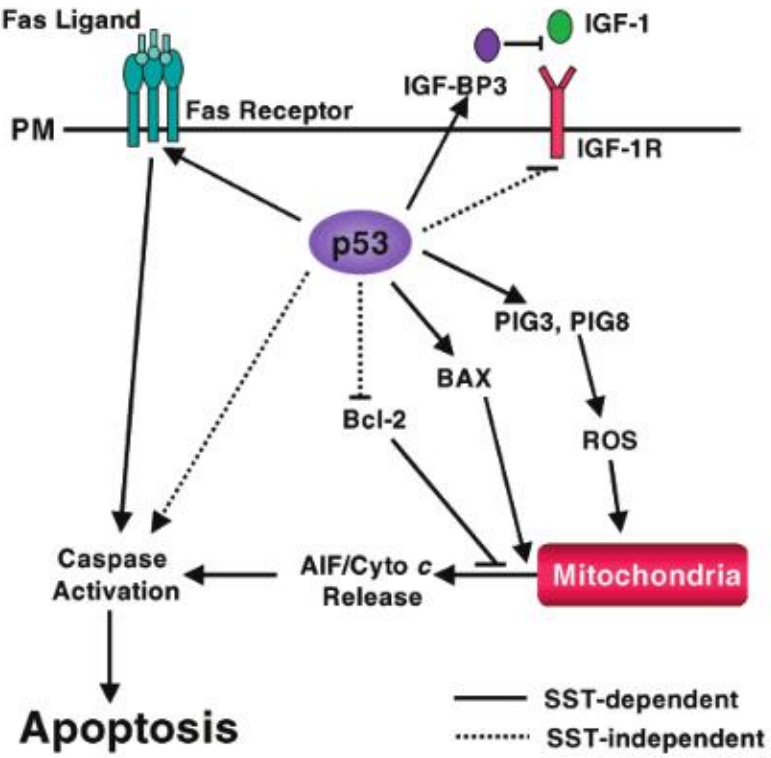

Fig 3. p53-mediated apoptotic signaling. p53-dependent apoptosis is mediated by both sequence-specific transactivation (SST) dependent and SST-independent pathways. Several p53 target genes can promote apoptosis, including the pro-apoptotic BAX protein and the Fas death receptor. BAX facilitates the release of AIF and cytochrome *c* from the mitochondria, thus activating the caspase cascade. p53 inhibits expression of the anti-apoptotic Bcl-2 protein, which normally blocks apoptosis by preventing the release of AIF and cytochrome *c* from the mitochondria.

Under normal conditions, p53 is a short-lived protein. However, the status of p53 is drastically altered when cells are exposed to stress, including DNA damage. Ultimately the activation of p53 leads to cell growth arrest, senescence or apoptosis (25). A family of proteins related to Bcl-2 are involved in both p53-dependent and -independent cell death, at least in part, by releasing cytochrome c from the mitochondria membrane (26-28). p53 may inhibits Bcl-2 function through transactivation of Cdc42 (29), upregulates Bax expression during p53-dependent apoptosis (30) and facilitates the release of the apoptosis-inducing factor and cytochrome *c* from the mitochondria, thus activating the caspase cascade (31).

The cell cycle is divided into four distinct phases (G1, S, G2, and M). Entry into each phase of cell-cycle is regulated by receptor collectives, termed cell-cycle checkpoints. The progression of cells through the cell cycle is promoted by CDKs, which are positively and negatively regulated by cyclins (A, B, D, and E) and CDKIs, respectively (Fig. 4) (32, 33). Cyclin D isoforms (cyclin D1–D3) interact with CDK4 and CDK6 to drive the progression of a cell through G1 phase. The association of cyclin E with CDK2 is active at the G1-S transition and directs entry into S-phase. S-phase progression is directed by the cyclin A/CDK2 complex, and the complex of cyclin A with Cdc2 (also known as cdk1) is important in G2. Cdc2/cyclin B is necessary for the entry into mitosis. Cdk inhibitors fall into two families: INK4 inhibitors and Cip/Kip inhibitors. Both families of Cdk inhibitors play regulatory roles during the G1/S cell cycle checkpoint (34). p21 Waf1/Cip1, a p53 downstream target, is a member of Cip/kip family of Cdk inhibitors. It can bind and inhibit the indicated Cdk or cyclin/Cdk complexes and effectively block the progression of cells from G1 into S-phase (35).

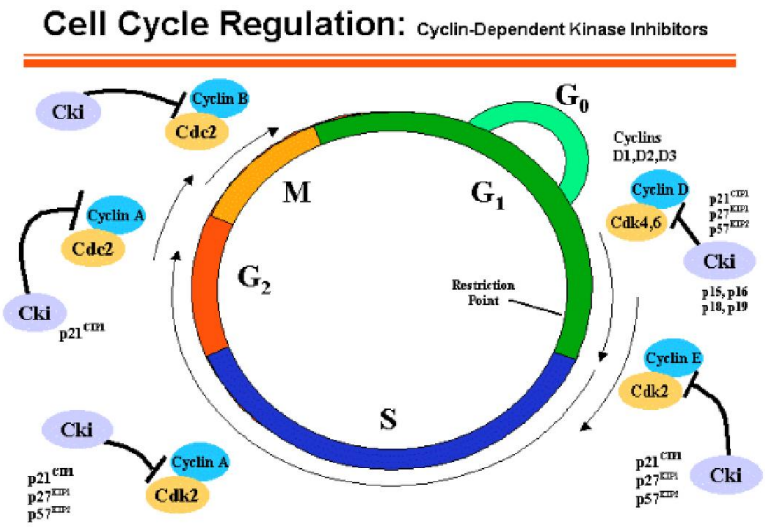

Fig. 4. The cell cycle and its regulation by cyclins, Cdks (cyclin-dependent kinases), and Ckis (cyclin-dependent kinase inhibitors)

Detecting apoptotic cells or monitoring the cell progression to apoptosis is an essential step in developing drugs that regulate apoptosis. Various assays were designed to detect or quantitate apoptotic cells. As typical assays, Annexin V binding, caspase enzyme activity, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling), DAPI staining, DNA gel electrophoresis are commonly used.

Platinum has been known as an anticancer metal drug and its toxicity was well studied (36, 37). It has been used in recent studies as a model drug in developing delivery system (38-40). Platinum nanoparticles (PNPs) were studied as ROS scavenger for anti-inflammatory effect (40-44). In other study, Park et al. reported that PNPs might cause inflammation in mice (45). DNA breaks in cells exposed to water soluble platinum nanoparticles were reported (46). PNPs caused DNA damage and subsequently genotoxic stress (47, 48) on human cells. However, little is known on the toxicity and apoptosis induction by water soluble PNPs. In this study, we investigated cytotoxicity and apoptosis induction of water soluble PNP05 (average size of 5 nm) and PNP30 (average size of 30 nm) on Raw 264.7 cells.

II. MATERIALS AND METHODS

2.1. Reagents

PNPs (platinum nanoparticle 05 or 30) were purchased from Nanopoly Co., Ltd. (Korea). Antibodies of cyclin D1, cleaved caspase-3 and cleaved caspase-7 were obtained from Cell Signaling (Cell Signaling, MA), p38, pJNK1/2, β-actin, cyclin E1 were from AbFrontier (AbFrontier, Korea). Cyclin B1, cdk1, cdk2, cdk4, cytochrome C, Bcl2, Bax, Procaspase 9 and second antibodies were obtained from Santa Cruz (Santa cruz biotechnology Inc., CA). All other chemicals were purchased from Sigma-Aldrich Chemical (Sigma-Aldrich, MO)

2.2. Cell line and cell culture

Raw 264.7 cell line was from Korean cell bank (Korea). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and antibiotics. The cells were incubated under a fully humidified atmosphere and 5 % CO_2 at 37^0C.

2.3. Cytotoxicity assay

Cell viability was determined by using MTT as an indicator of cell viability. The cells were seeded in 96-well plate (10^4 cells/ well) and treated with various concentrations of PNPs (0, 2, 6, 10, 20 μg/ml) for 24 h. After treating, the medium was removed, 200 μl of DMEM medium containing MTT (0.5 mg/ml) was added to each well, followed by a 4 h incubation at 37 ^{0}C. After that, the MTT1 solution was removed and 200 μl of lysis buffer (10 % sodium dodecylsulfate, 0.1 N HCl) was added into each well. The cell dish was incubated for 2 h and the absorbance of each well was read at the wavelength of 540 nm. The data was analyzed using Microsoft Excel.

2.4. Western Blot analysis

PNPs effect on cell cycle proteins and apoptosis relative proteins were investigated by western blotting. Prepared Raw 264.6 cells in 60-mm dishes were treated with 0, 2, 6 μg/ml of PNPs for 24 h. The cell dishes were then washed with PBS and lysed in lysis buffer (Cell Signaling, MA). After centrifugation, protein concentration of supernatants was determined by BCA Kit (PIERCE, IL). 20 μg of total proteins were loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12 % gel for cell cycle proteins: cdk1, cdk2, cdk4, cyclin D1, cyclin B1, cyclin A, cyclin E1 and p53; 15 % gel for cleaved caspases, Bcl2, Bax, cytochrome c); transferred onto PVDF membranes (Millipore, MA). The membranes were blocked with 5 % skim milk in TBS buffer (20 mM Tris-HCl, 136 mM NaCl, pH 7.4) for 2 h and then incubated with primary antibody (1:1000 dilution) in blocking agent at 4 ^{0}C, overnight. After washing with TBS - TBS-T - TBS buffer (3X), the membrane was incubated with secondary antibody (1:10000 dilution) for 2 h at room temperature. Protein bands on membrane were captured on Kodak image station system.

2.5. FACS analysis of apoptosis using Annexin V

Cells were treated with various concentrations (0, 2, 6, 10 μg/ml) of PNPs for 24 h. The treated cells were harvested by brief trypsinization and washing in PBS. Cells were suspended in Annexin V binding buffer and stained by using Vybrant Apoptosis Assay Kit (Invitrogen, CA). Stained cells were immediately analyzed on Beckman Coulter system (Beckman Coulter, CA) with argon lamp (488 nm). The normal, apoptotic, necrotic populations of cells were expressed as the means of three independent experiments.

2.6. Nuclear staining with DAPI

Cells were seeded in multi-chamber slide (8 chamber slides, 10^4 cells/well) and grow 18 h before treatment. The prepared cells were exposed to various concentrations of PNPs for 24 h. After treatment, the medium were removed and the cells were washed with PBS to fix in 4% paraformaldehyde for 30 min at room temperature. The fixed cells were washed with PBS and stained with DAPI at room temperature in the dark for 15 minutes. The nuclear morphology of the cells was captured on fluorescent microscopy (Zeiss, Germany).

2.7. DNA fragmentation assay

Raw 264.7 cells were seeded in 60 mm plate (10^6 cells/dish) for 18 h and treated with PNPs at various concentrations for 24h. Then treated cells were washed with PBS (phosphate buffered saline) and apoptotic DNA fragments were isolated by using DNA fragmentation apoptosis Kit (BioVision, CA). Extracted DNA was separated in a 1.2 % agarose gel for 30 minutes at 100 voltages and visualized by ethidium bromide. Gel image was taken on Kodak station image system.

2.8. Cell cycle analysis by Flow cytometry

Cells were seeded onto a 60 mm plate (1.5 x 10^6 cells /dish) for 18 h. After treatment with or without PNP30 for 24 h, both floating and adherent cells were collected and suspended in PBS. Then absolute ethanol was slowly added to gain of 75 % ethanol and the cells were kept at -20 ^{0}C for 2 h prior to washing and staining with 400 µl PI solution (10 µg/ml propidium iodide in PBS) in the presence of 200 µg/ml of DNase-free RNase A for 15 min at 37 ^{0}C in the dark. DNA content was analyzed in Beckman-Coulter system with excitation wavelength of 488 nm.

2.9. Determination of cytochrome c release from Mitochondria

Cells were seeded in 60 mm plate with 1,5 x 10^6 cells/well for 18 h. Then, the cells were incubated with 2 μg/ml of PNP30 for 0, 4, 8, 12 hours. Cells were harvested by scraper, and cell pellets were used to extract cytoplasm using Mitochondria/Cytosol Fractionation Kit (Biovision, CA 94043, USA) by following the manufacturer's protocol. Cytochrome c that released from mitochondria into cytosol was detected by Western Blotting.

III. RESULTS

3.1. Cytotoxicity of PNPs on Raw 264.7 cells

The cytotoxic effect of PNPs on Raw 264.7 cells was investigated by using a MTT assay. The cells were treated with different concentrations of PNPs for 24 h as described in Material and Methods. As shown in Figure 5, PNPs suppressed cell viability in a dose-dependent manner. The degree of decrease in cell viability was almost similar between PNP30 and PNP05 showing that both PNP05 and PNP30 are toxic to Raw 264.7 cells.

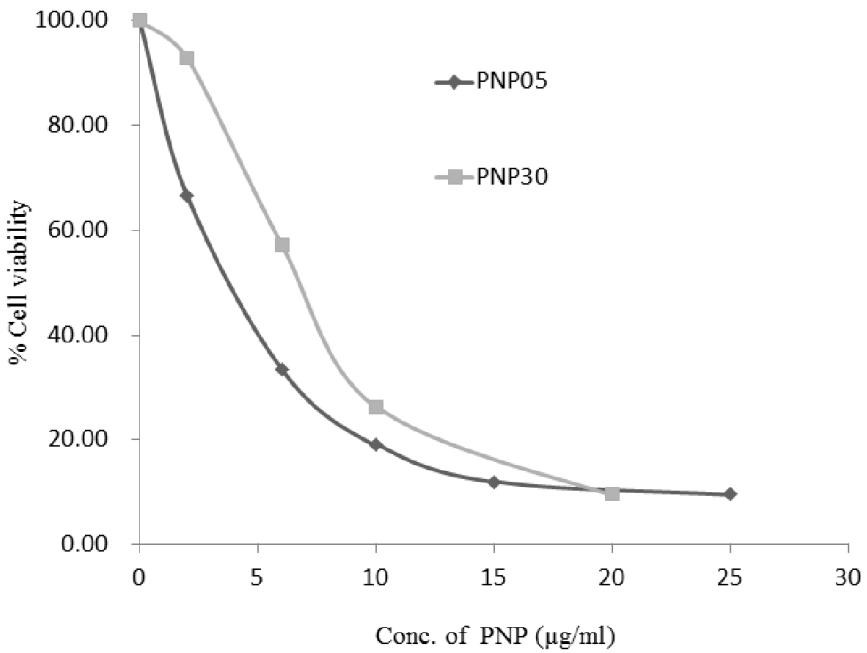

Fig. 5. Cytotoxicity of PNPs on Raw 264.7 cell. Raw 264.7 cells were treated with indicated concentrations of PNPs for 24 h. The percent cell viability was determined by MTT assay. Each value is the average of triplicate. PNP05 and PNP30 were highly toxic on Raw 264.7 cells.

3.2. Morphology of PNP-treated Raw 264.7 cells

Cell morphology was observed after treating cells with 2 μg/ml or 6 μg/ml of PNPs for 24 h. Images of cell morphology were captured on optical contrast phase microscopy at magnification of X200. Morphological changes of Raw 264.7 cells with shrinkage were noticed (Fig. 6). In addition, the density of Raw 264.7 cells became lower.

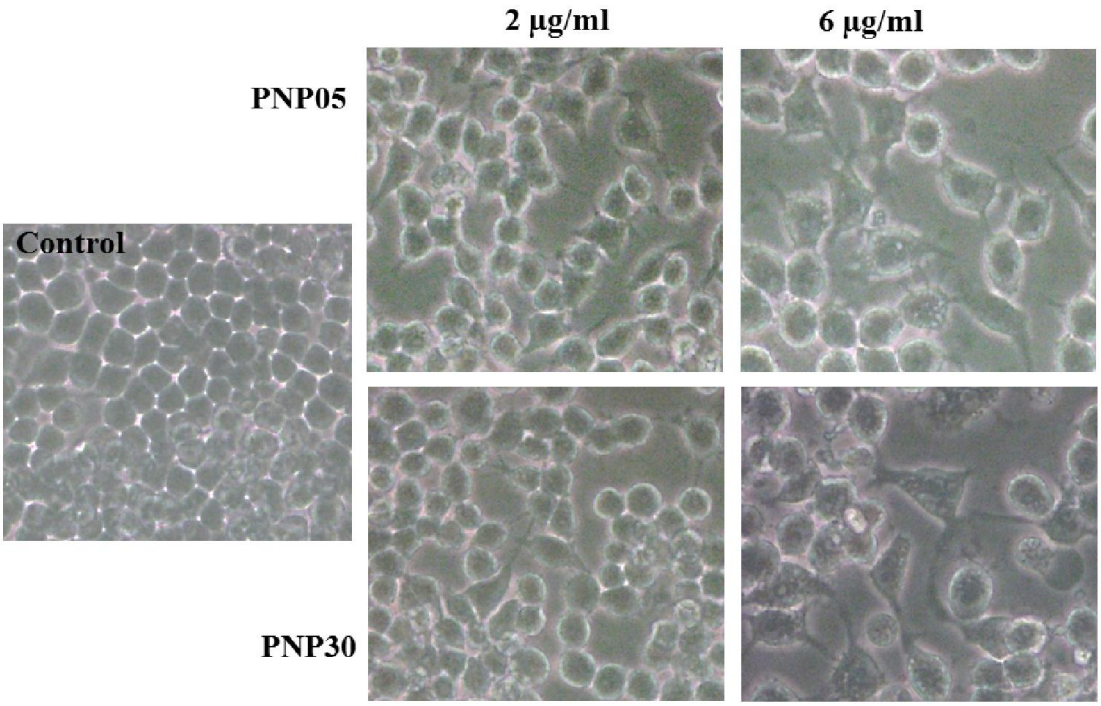

Fig. 6. Effects of PNPs on morphology of Raw 264.7 cells. Raw 264.7 cells were incubated for 24 h in the absence (control) or presence of indicated concentrations of PNPs. Cell morphology was assessed by using a microscopy. The images were captured at X200 of magnification.

3.3. Nucleus fragmentation by PNPs

Nuclear fragmentation and condensation are one of the characteristics of apoptosis. To confirm that PNPs induce apoptotic cell death, Raw 264.7 cells were treated with PNPs for 24 h at different concentrations and then the nuclei of treated cells were stained with DAPI for the observation by fluorescent microscopy (Fig. 7). Nuclei of control cells were small with intact DNA as normal morphology. However, after treating with PNPs, nuclei of treated-cells became bigger and/or divided into small parts which are typical characteristic of apoptosis.

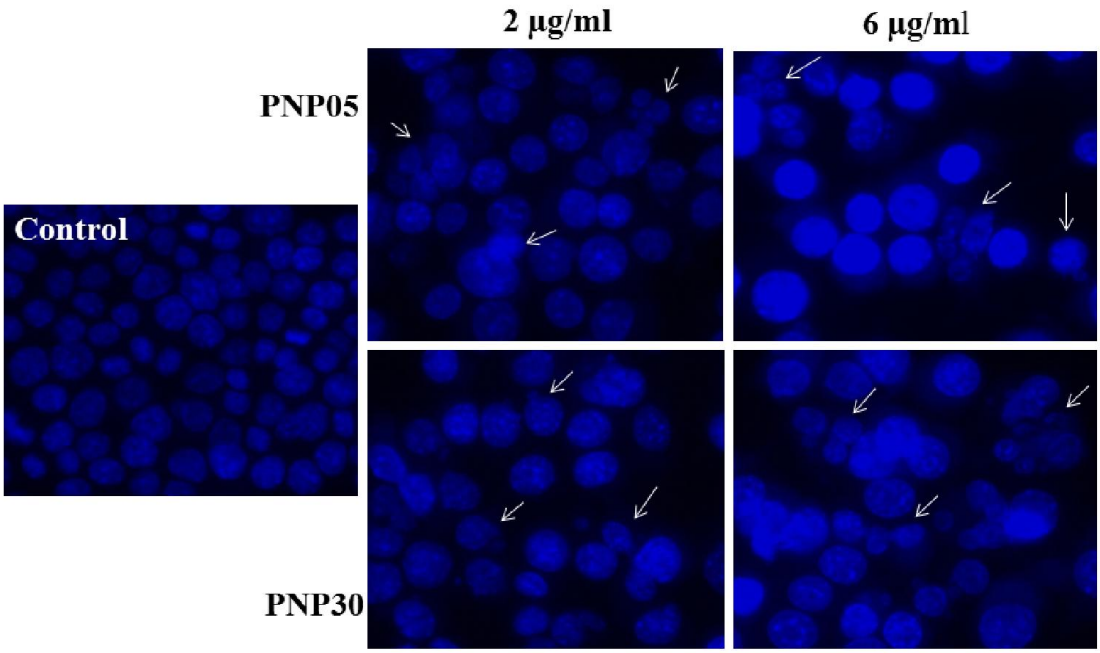

Fig. 7. Visualization of nuclei after exposing Raw 264.7 cells to PNPs. Raw 264.7 cells were incubated with PNPs at indicated for 24 h. Cells were stained with DAPI and observed under a fluorescent microscopy. The images were captured at X400 of magnification.

3.4. PNPs induce an increase in the Annexin V-binding on Raw 264.7 cells

Viable cells maintain an asymmetric distribution of different phospholipids between the inner and outer leaflets of the plasma membrane. However, this distribution changes during apoptosis. In particular, phosphatidylserine (PS) relocates from the cytoplasmic face to the outer leaflet of the plasma membrane and extent of PS exposure can distinguish apoptotic cells from non-apoptotic cells. The Annexin-V binding assay is based on the relocation of PS to the outer cell membrane. Annexin-V binding can be observed under two conditions. The first condition is observing cells midway through the apoptosis pathway. PS translocates to the outer leaflet of the cell membrane. The second condition is observings very late apoptosis or necrotic and membrane permeabilization state. This membrane permeabilization allows Annexin-V to enter into cells and bind to phosphatidylserine on the cytoplasmic face of the membrane. Viable cells (non-apoptotic cells) are negative to Annexin V and PI, early apoptotic cells are positive to Annexin V but not to PI, and late apoptotic cells are positive to both Annexin V and PI. In our experiment, apoptosis was investigated by double staining treated-cells with Annexin-V and propidium iodide. The cells were treated with PNPs for 24 h before staining and analyzing by Beckman Coulter system. We found that apoptotic cell populations (early apoptosis plus late apoptosis) were increased in a dose-dependent manner under the conditions of PNPs treatments. PNP05 strongly induced apoptosis (44.73 % at 2 μg/ml to 91.85 % at 10 μg/ml) and PNP30 also showed similar effect (23.40 % at 2 μg/ml to 80.50 % at 10 μg/ml) (Fig. 8). The data in Figure 8 confirmed both of PNP05 and PNP30 actually induced cell death by apoptosis.

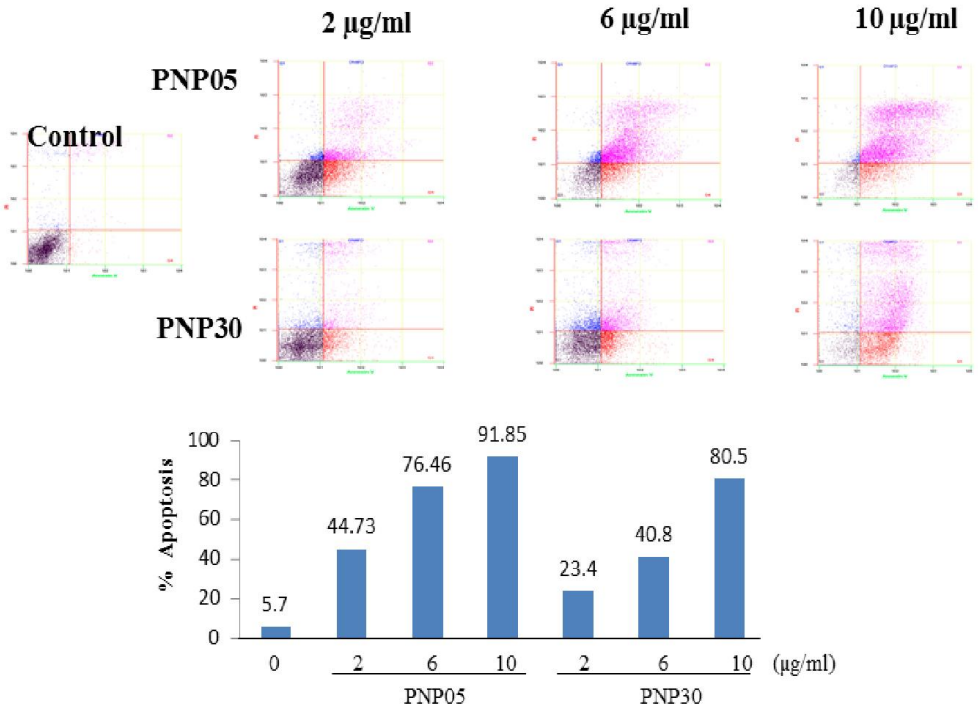

Fig. 8. PNPs induce an increase in the Annexin V-binding Raw 264.7 cells. Raw 264.7 cells were treated with indicated concentration of PNPs for 24 h, stained with Alexa Fluor® 488 annexin V (Vybrant® Apoptosis Assay Kit) and analyzed by flow cytometry by Beckman-Coulter system. Each value is the average of two independent experiments.

3.5. PNPs induce DNA fragmentation

During the process of apoptosis, the activation of caspase cascades results in the inactivation of DNA repair system and internucleosomal cleavage of DNA, generating DNA fragment (called apoptotic DNA ladder). To confirm that PNPs induce apoptotic cell death, the DNA fragmentation analysis was carried out. As shown in Figure 9, characteristic "DNA ladder" bands were clearly visualized by agarose gel electrophoresis stained with ethidium bromide under ultraviolet illumination, after incubating cells for 24 h with indicated concentrations of PNP05 and PNP30. Both PNP05 and PNP30 showed the similar effects on DNA fragmentation.

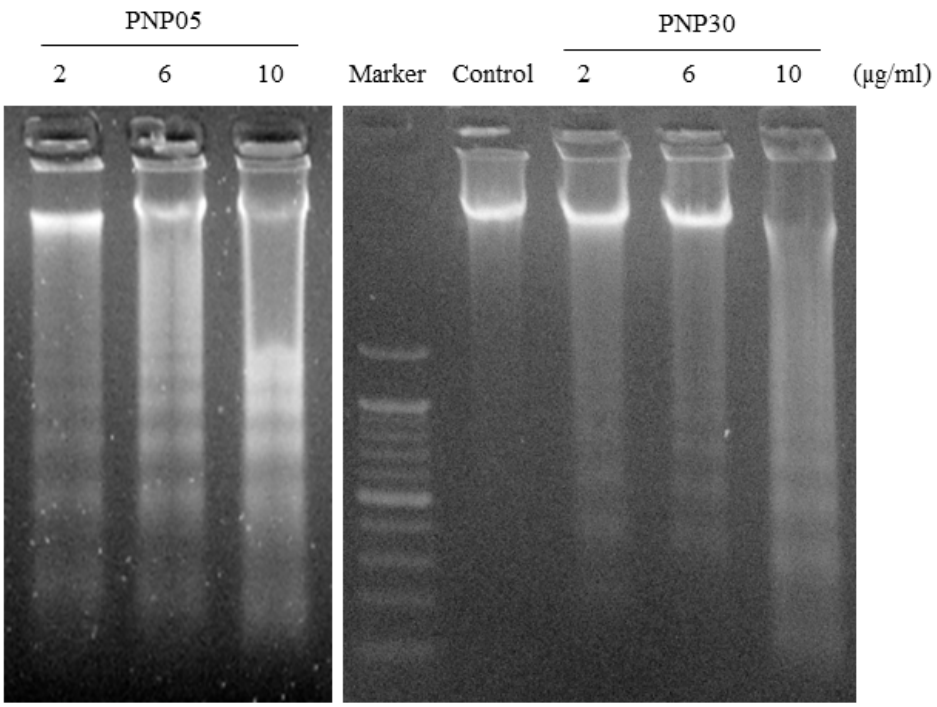

Fig. 9. PNPs cause DNA laddering on Raw 264.7 cells. Raw 264.7 cells were incubated with media alone (control) or with indicated concentration of PNPs for 24 h. DNA was isolated and electrophoresed on a 1.2 % agarose gel.

3.6. Induction of caspase activation by PNPs

Caspases play a critical role in the execution phase of apoptosis. In apoptosis, pro-caspases are processed by proteolytic cleavage to be active enzymes. Caspase 3 is considered to be the most important executioner caspases and activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10) (13). Pro-caspase-3 is cleaved into active caspase-3, and then caspase 3 cleaves pro-caspase-6 and pro-caspase-7. Therefore, activated forms of caspase 3 and caspase 7 are significant apoptotic protein markers. In our investigation, both caspases were dramatically activated by PNPs in dose-dependent manner (Fig. 10), compared to untreated cells. Since PNP05 and PNP30 showed the similar effects on the death of Raw 264.7 cells, we only used PNP30 on following other experiments.

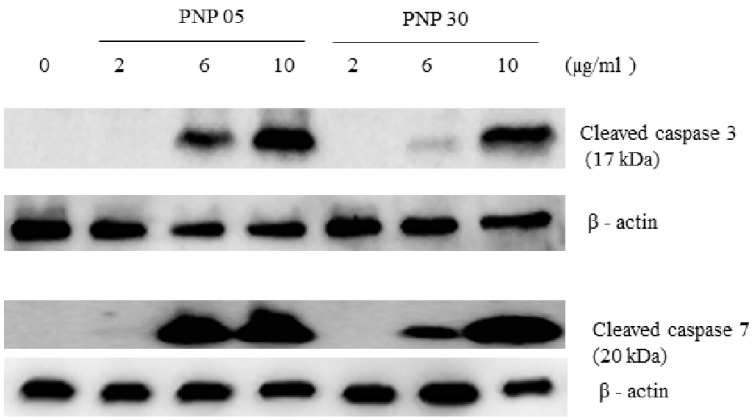

Fig. 10. PNPs induced the activation of caspase-3, -7 on Raw 264.7 cells. Raw 264.7 cells were treated with different concentrations of PNPs for 24 h. Total cell lysates (40 μg) were separated on 15 % SDS–PAGE and detected by Western blotting.

3.6. Effect of PNP on the cell cycle regulation

To examine whether the growth inhibition observed in the cell viability assay is involved in cell cycle arrest or not, we further evaluated cell cycle progression using fluorescence activated cell sorting analysis, after treating cells with different concentrations of PNP30 for 24 h and staining with propidium iodide (PI) (Fig. 11).

Figure 11 showed that in the Raw 264.7 cells treated with 2 μg/ml of PNP30, the percentage of S phase cell population decreased with the corresponding increase in the population of G2/M phase. The number of cells at G2/M phase was increased from 11.7 % to 18.3 % at 2 μg/ml, but both of S and G2/M phases decreased at 6 μg/ml. Instead the number of cells at sub-G1 phase was dramatically increased from 0.25 % to 21.14 % indicating that PNP30 initially prolonged or arrested cells at G2/M phase at low concentration probably to repair damaged DNA, and underwent to cell death through apoptotic pathway when the errors are beyond repair.

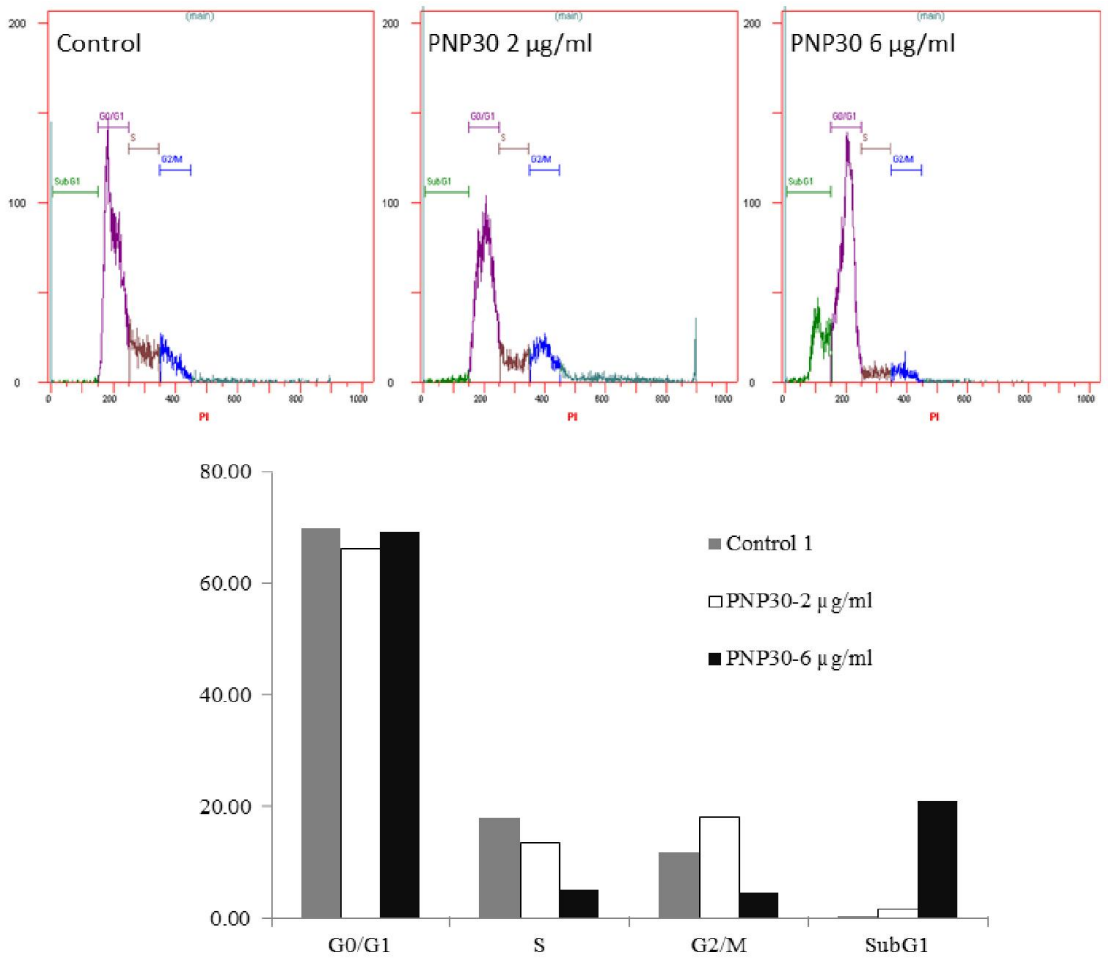

Fig. 11. Effect of PNP on cell cycle regulation. Raw 264.7 cells were treated with indicated concentrations of PNP30 for 24 h. The cells were stained with Propidium Iodide (PI) and evaluated cell cycle progression by fluorescence activated cell sorting analysis.

3.7. PNP induces p53, p21 expression.

A large body of data suggest that in response to DNA damage or growth factor withdrawal, p53 protein expression increases (49) and cell growth is suppressed by cell-cycle arrest and apoptosis in certain cell types (50-52). The cell cycle regulation and the DNA repair function of p53 are largely executed by transactivation of p53-response genes such as p21/Waf1/Cip1 (53, 54). A previous data showed that PNPs caused DNA damage and p53-mediated growth arrest in human cells (55). Our current data showed that PNPs induced cell cycle arrest at G2/M phase at 2 μg/ml of PNP and apoptosis at 6 μg/ml of PNP on Raw 264.7 cells. To investigate whether the p53-associated mechanism of cells death or not, we further examined the expression of p53 and p21. PNPs-treated Raw 264.7 cells clearly increased the expression level of both p53 and p21 Waf1/Cip1 (p21) proteins (Fig. 12).

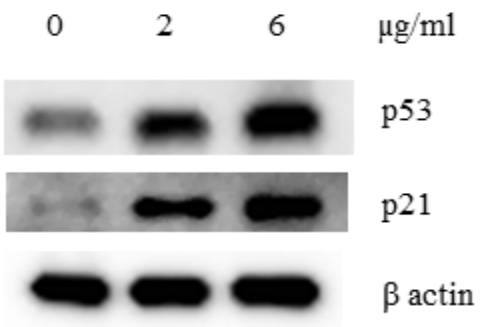

Fig. 12. PNP induces p53, p21 activation. Raw 264.7 cells were treated with indicated concentrations of PNP30 for 24 h. Total cell lysates (20 μg) were loaded on 15 % SDS-PAGE and detected by Western blotting.

3.8. Effect of PNP on cell cycle proteins

Because cell cycle is tightly driven by a family of proteins called CDKs (cyclin-dependent kinases) (56), and these kinases are positively regulated by cyclins (A, B, D, E) and negatively regulated by CDKIs (cyclin-dependent kinase inhibitors) (57), we investigated the expression of these proteins by western blot analysis. Western blotting was performed using antibody against cdk4, cdk2, cdk1 (cdc2), cyclin D1, cyclin E1, cyclin A, cyclin B1 (Fig. 13).

Figure 13 showed that PNP significantly altered the level expression of cyclins and Cdks. The level of cyclin E1, cyclin D1, cyclin B1, cdc2 (cdk1) decreased but the level of cdk4, cdk2 and cyclin A almost unchanged at 2 μg/ml of PNP. However, the expressions of those proteins were dramatically decreased at 6 μg/ml of PNP treatment.

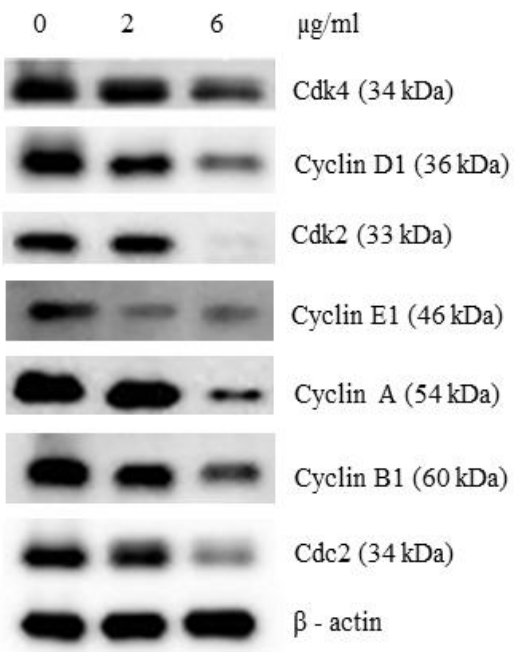

Fig. 13. Effect of PNP on cell cycle proteins. Raw 264.7 cells were treated with indicated concentrations of PNP30 for 24 h. Total cell lysates (20 μg) were separated on 15 % SDS-PAGE and detected by Western blotting.

3.9. PNP induces cytochrome c release, activates caspase-9, down-regulates Bcl2 and up-regulates Bax protein

The release of cytochrome c from mitochondria is a central event in the "intrinsic" apoptotic pathway (23, 24). To determine whether apoptosis induced by PNP involves mitochondrial pathway or not, the release of cytochrome c into cytosol was investigated after treating Raw 264.7 cells with 6 μg/ml of PNP30 for different time by Western Blot (Fig. 14).

Our result showed that the release of cytochrome c from mitochondria into cytosol was increased in a time-dependent manner. The release of cytochrome c from mitochondria results in the formation of the apoptosome and the activation of caspase 9. To confirm this, the expression of pro-caspase 9 in PNP-treated Raw 264.7 cell was examined (Fig. 15).

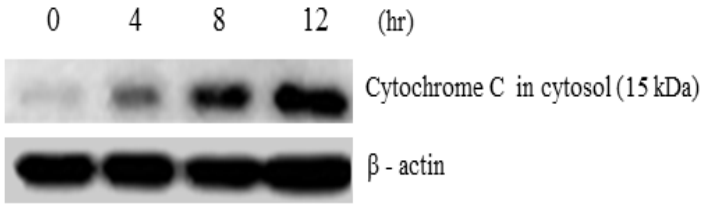

Fig. 14. Effect of PNP on cytochrome c release. Raw 264.7 cells were incubated with 6 μg/ml of PNP30 for 0, 4, 8, 12 hours. Cytoplasm was extracted by using Mitochondria/Cytosol Fractionation Kit. Cytochrome c released into cytoplasm was detected by Western Blotting.

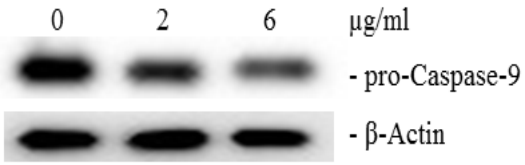

Fig. 15. Effect of PNP on pro-caspase-9 expression. Raw 264.7 cells were treated with indicated concentrations of PNP30 for 24 h. Total cell lysates (20 μg) were separated on 15 % SDS-PAGE and detected by Western blotting.

The expression of pro-caspase-9 was significantly decreased indicating that caspase 9 was activated, subsequently activating caspase-3 and caspase 7. This is consistent with our previous results of caspase-3/-7 expression in Figure 10.

The intrinsic apoptotic pathway is dominated by the Bcl-2 family of proteins, which governs the release of cytochrome c from the mitochondria (58, 59). Some of these proteins such as Bcl-2 and Bcl-XL are anti-apoptotic, while others such as Bax, Bad or Bid are pro-apoptotic. The sensitivity of cells to apoptotic stimuli depends on the balance of pro- and anti-apoptotic Bcl-2 proteins.

We found that PNP30 significantly down-regulated the expression of Bcl2 and up-regulated of Bax (Fig. 16), facilitating the release of apoptosis-inducing factor and cytochrome c from the mitochondria and leading to activation of the caspase cascade (31).

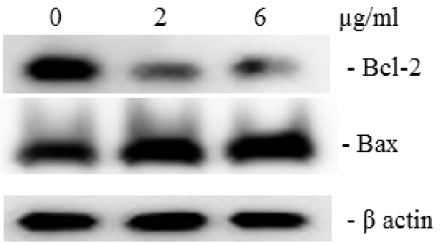

Fig. 16. Effect of PNP on the expression of Bcl2, Bax proteins. Raw 264.7 cells were treated with indicated concentrations of PNP30 for 24 h. Total cell lysates (20 μg) were loaded on 15 % SDS-PAGE and detected by Western blotting.

IV. DISCUSSION AND CONCLUSION

In this study, we investigated the toxicities of PNPs on Raw 264.7 cells and their apoptosis induction with respect to the cell cycle regulation, mitochondrial pathway and caspase dependence. PNP05 and PNP30 were toxic on Raw 264.7 cells (Fig. 5). Cytotoxicity of PNP triggered the cell cycle arrest at G2/M phase at a low dose (Fig. 11) and induced programed cells death at a high concentration. The evidences of apoptosis were confirmed by morphological changes (Fig. 6), nuclei fragmentation (Fig. 7), increased population of apoptotic cell by Annexin V assay (Fig. 8), DNA ladder bands (Fig. 9), and the activation of caspase-3/-7 (Fig. 10).

PNPs were known to cause DNA damage (60), induce genotoxic stress (48) and arrest cell cycle progress (47). Up-regulation of both p21 and p53 resulted in cell-cycle arrest and apoptosis (55, 61). Recent studies showed that p53 and p21 are necessary to maintain G2 arrest following DNA damage (54, 61). The mechanism of p53-dependent G2 arrest involves an initial inhibition of cyclin B1/Cdc2 activity by p21 and a subsequent reduction of cyclin B1 and Cdc2 protein levels (54, 62, 63). The reduced expression of cyclin B1/Cdc2 is mediated in part by p53-dependent repression of the cyclin B1 and Cdc2 promoters (54, 61). Normal mammalian cellular proliferation is tightly regulated at each phase of cell cycle by activation and deactivation of a series of cell cycle molecules such as cyclins, cyclin-dependent kinases (Cdks), and Cdk inhihitors (CdkIs). Cdks are relatively small proteins and binds to a cyclin. Without cyclin, Cdk has little kinase activity; only the cyclin-Cdk complex is an active kinase. In mammalian cells, Cdc2 (Cdk1) with its partners cyclin A2 and B1 can drive the cell cycle (64). In our study, PNP treatment on cell increased the expression of p53 and p21 dramatically (Fig.12), indicating that PNP induced DNA damage. Furthermore, western blotting of cell cycle related proteins revealed that the expression of

cell cycle proteins (cdk4, cyclin D1, cdk2, cyclin E1, cyclin A, cyclin B1, Cdc2) were slightly decreased at 2 μg/ml of PNPs but dramatically decreased at 6 μg/ml (Fig. 13). Noticeably, although the cell cycle proteins were almost totally suppressed at 6 μg/ml of PNP, the expression level of p53 still maintained high suggesting that p53 not only involved in cell cycle arrest but also apoptosis. In addition, flow cytometry study on cell cycle analysis revealed that cell cycle was shifted from G2/M arrest at low concentration of PNP (2 μg/ml) (Fig. 11) into sub G1 phase at high concentration of PNP (6 μg/ml) suggesting that p53 protein expression increased to respond to PNP-induced DNA damage repair and apoptosis through both mechanisms: cell cycle arrest at low concentration of PNP for DNA repair and apoptosis at high concentration of PNP when the DNA errors is beyond repair. Namely, cells skipped DNA repair to go straight toward apoptosis.

p53 has been shown to specifically repress transcription from a number of genes such as Bcl-2, MAP4 and a number of viral promoters and transactivate Bax in some cell types (66). A previous report showed that p53-mediated apoptosis under cellular stress could induce the transcription of many pro-apoptotic genes through mitochondrial pathway (65) which is initiated with Mitochondrial Outer Membrane Permeabilization (MOMP). MOMP is mainly controlled by Bcl-2 family members such as Bax, Bad, Bcl2. The ratio of Bax and Bcl-2 expression is the determining factor for the induction of apoptosis. In our results, the ratio of Bax/Bcl-2 protein expression was significantly increased (Fig. 16), supporting that PNP induces apoptosis in pathway related with the disruption of mitochondria function. The increase in p53 protein level, corresponding down-regulation of anti-apoptotic proteins Bcl2, and up-regulation of pro-apoptotic protein Bax might have led to changing the ratio of Bax/Bcl-2, stimulated the release of cytochrome c from mitochondria into cytosol (Fig. 14), subsequently leading to the activation of caspase 9 (Fig. 15), which further activated downstream executioner caspases, such as caspase-3/-7 (Fig. 10), and finally resulted in program cell death.

Taking together, our study revealed that PNPs induced DNA damage, inhibited the growth and survival of Raw 264.7 cells through cell cycle arrest and apoptosis induction. The signaling pathway involved in p53-mitochondria pathway was shown as simplified in Fig. 17.

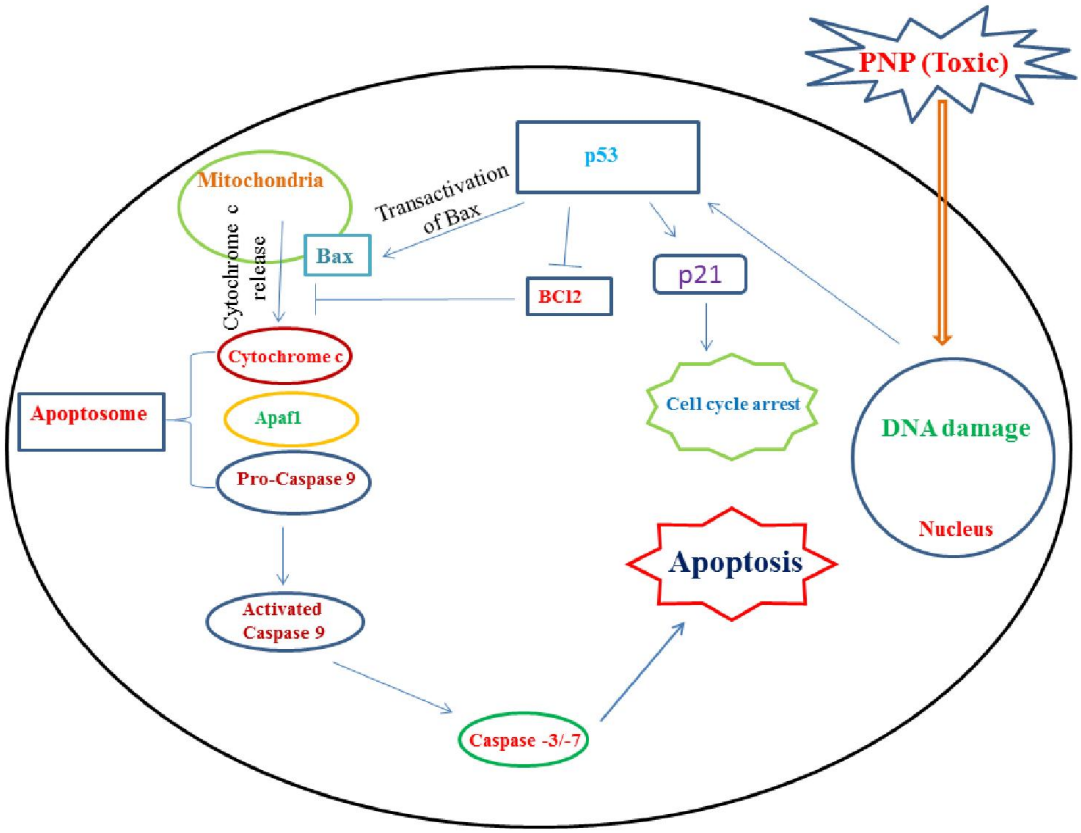

Fig.17. Scheme of PNP-induced apoptotic pathways

REFERENCES

1. Lewinski N, Colvin V, Drezek R. Cytotoxicity of nanoparticles. Small 2008;4:26-49.

2. Shukla R, Bansal V, Chaudhary M, Basu A, Bhonde RR, Sastry M. Biocompatibility of gold nanoparticles and their endocytotic fate inside the cellular compartment: a microscopic overview. Langmuir 2005;21:10644-10654.

3. Bhattacharyya S, Kudgus RA, Bhattacharya R, Mukherjee P. Inorganic nanoparticles in cancer therapy. Pharm Res;28:237-259.

4. Bhattacharyya S, Bhattacharya R, Curley S, McNiven MA, Mukherjee P. Nanoconjugation modulates the trafficking and mechanism of antibody induced receptor endocytosis. Proc Natl Acad Sci U S A;107:14541-14546.

5. Soini Y, Paakko P, Lehto VP. Histopathological evaluation of apoptosis in cancer. Am J Pathol 1998;153:1041-1053.

6. Barnes DM, Camplejohn RS. P53, apoptosis, and breast cancer. J Mammary Gland Biol Neoplasia 1996;1:163-175.

7. White E. Life, death, and the pursuit of apoptosis. Genes Dev 1996;10:1-15.

8. Rajesh P. Rastogi RaRPS. APOPTOSIS: MOLECULAR MECHANISMS AND PATHOGENICITY. EXCLI Journal 2009;8:155-181.

9. Martin SJ, Green DR. Protease activation during apoptosis: death by a thousand cuts? Cell 1995;82:349-352.

10. Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell 2004;116:205-219.

11. Ghavami S, Hashemi M, Ande SR, Yeganeh B, Xiao W, Eshraghi M, et al. Apoptosis and cancer: mutations within caspase genes. J Med Genet 2009;46:497-510.

12. Green DR, Ferguson T, Zitvogel L, Kroemer G. Immunogenic and tolerogenic cell death. Nat Rev Immunol 2009;9:353-363.

13. Sakahira H, Enari M, Nagata S. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 1998;391:96-99.

14. Favaloro B, Allocati N, Graziano V, Di Ilio C, De Laurenzi V. Role of apoptosis in disease. Aging (Albany NY);4:330-349.

15. Haupt S, Berger M, Goldberg Z, Haupt Y. Apoptosis - the p53 network. J Cell Sci 2003;116:4077-4085.

16. Yan N, Shi Y. Mechanisms of apoptosis through structural biology. Annu Rev Cell Dev Biol 2005;21:35-56.

17. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol 2007;35:495-516.

18. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998;281:1305-1308.

19. Nicholson DW, Thornberry NA. Apoptosis. Life and death decisions. Science 2003;299:214-215.

20. Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T, et al. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell 2001;8:705-711.

21. Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. Nat Rev Cancer 2002;2:277-288.

22. Hansen R, Oren M. p53; from inductive signal to cellular effect. Curr Opin Genet Dev 1997;7:46-51.

23. Chen X, Ko LJ, Jayaraman L, Prives C. p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. Genes Dev 1996;10:2438-2451.

24. Stewart ZA, Pietenpol JA. p53 Signaling and cell cycle checkpoints. Chem Res Toxicol 2001;14:243-263.

25. Giaccia AJ, Kastan MB. The complexity of p53 modulation: emerging patterns from divergent signals. Genes Dev 1998;12:2973-2983.

26. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, et al. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 1997;275:1129-1132.

27. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 1997;275:1132-1136.

28. Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT, Thompson CB. Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. Cell 1997;91:627-637.

29. Thomas A, Giesler T, White E. p53 mediates bcl-2 phosphorylation and apoptosis via activation of the Cdc42/JNK1 pathway. Oncogene 2000;19:5259-5269.

30. Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 1995;80:293-299.

31. Sionov RV, Haupt Y. The cellular response to p53: the decision between life and death. Oncogene 1999;18:6145-6157.

32. Pestell RG, Albanese C, Reutens AT, Segall JE, Lee RJ, Arnold A. The cyclins and cyclin-dependent kinase inhibitors in hormonal regulation of proliferation and differentiation. Endocr Rev 1999;20:501-534.

33. Zafonte BT, Hulit J, Amanatullah DF, Albanese C, Wang C, Rosen E, et al. Cell-cycle dysregulation in breast cancer: breast cancer therapies targeting the cell cycle. Front Biosci 2000;5:D938-961.

34. Sherr CJ, Roberts JM. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev 1995;9:1149-1163.

35. Pietenpol JA, Stewart ZA. Cell cycle checkpoint signaling: cell cycle arrest versus apoptosis. Toxicology 2002;181-182:475-481.

36. Cepeda V, Fuertes MA, Castilla J, Alonso C, Quevedo C, Perez JM. Biochemical mechanisms of cisplatin cytotoxicity. Anticancer Agents Med Chem 2007;7:3-18.

37. Jordan P, Carmo-Fonseca M. Molecular mechanisms involved in cisplatin cytotoxicity. Cell Mol Life Sci 2000;57:1229-1235.

38. Zhou D, Xiao H, Meng F, Zhou S, Guo J, Li X, et al. Layer-by-Layer Assembled Polypeptide Capsules for Platinum-Based Pro-Drug Delivery. Bioconjug Chem 2012.

39. Li GQ, Chen XG, Wu XP, Xie JD, Liang YJ, Zhao XQ, et al. Effect of dicycloplatin, a novel platinum chemotherapeutical drug, on inhibiting cell growth and inducing cell apoptosis. PLoS One 2012;7:e48994.

40. Wang X, Guo Z. Targeting and delivery of platinum-based anticancer drugs. Chem Soc Rev 2012;42:202-224.

41. Rehman MU, Yoshihisa Y, Miyamoto Y, Shimizu T. The anti-inflammatory effects of platinum nanoparticles on the lipopolysaccharide-induced inflammatory response in RAW 264.7 macrophages. Inflamm Res 2012;61:1177-1185.

42. Nomura M, Yoshimura Y, Kikuiri T, Hasegawa T, Taniguchi Y, Deyama Y, et al. Platinum nanoparticles suppress osteoclastogenesis through scavenging of reactive oxygen species produced in RAW264.7 cells. J Pharmacol Sci 2011;117:243-252.

43. Yoshihisa Y, Honda A, Zhao QL, Makino T, Abe R, Matsui K, et al. Protective effects of platinum nanoparticles against UV-light-induced epidermal inflammation. Exp Dermatol 2010;19:1000-1006.

44. Onizawa S, Aoshiba K, Kajita M, Miyamoto Y, Nagai A. Platinum nanoparticle antioxidants inhibit pulmonary inflammation in mice exposed to cigarette smoke. Pulm Pharmacol Ther 2009;22:340-349.

45. Park EJ, Kim H, Kim Y, Park K. Intratracheal instillation of platinum nanoparticles may induce inflammatory responses in mice. Arch Pharm Res 2010;33:727-735.

46. Gehrke H, Pelka J, Hartinger CG, Blank H, Bleimund F, Schneider R, et al. Platinum nanoparticles and their cellular uptake and DNA platination at non-cytotoxic concentrations. Arch Toxicol 2011;85:799-812.

47. Asharani PV, Xinyi N, Hande MP, Valiyaveettil S. DNA damage and p53-mediated growth arrest in human cells treated with platinum nanoparticles. Nanomedicine (Lond) 2010;5:51-64.

48. Pelka J, Gehrke H, Esselen M, Turk M, Crone M, Brase S, et al. Cellular uptake of platinum nanoparticles in human colon carcinoma cells and their impact on cellular redox systems and DNA integrity. Chem Res Toxicol 2009;22:649-659.

49. Agarwal ML, Taylor WR, Chernov MV, Chernova OB, Stark GR. The p53 network. J Biol Chem 1998;273:1-4.

50. Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML, et al. Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature 1993;362:849-852.

51. Debbas M, White E. Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. Genes Dev 1993;7:546-554.

52. Wagner AJ, Kokontis JM, Hay N. Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21waf1/cip1. Genes Dev 1994;8:2817-2830.

53. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, et al. WAF1, a potential mediator of p53 tumor suppression. Cell 1993;75:817-825.

54. Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, et al. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science 1998;282:1497-1501.

55. Asharani PV, Xinyi N, Hande MP, Valiyaveettil S. DNA damage and p53-mediated growth arrest in human cells treated with platinum nanoparticles. Nanomedicine (Lond);5:51-64.

56. Goldie JH, Coldman AJ. A mathematic model for relating the drug sensitivity of tumors to their spontaneous mutation rate. Cancer Treat Rep 1979;63:1727-1733.

57. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 1999;13:1501-1512.

58. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2002;2:647-656.

59. Kuwana T, Mackey MR, Perkins G, Ellisman MH, Latterich M, Schneiter R, et al. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell 2002;111:331-342.

60. Porcel E, Liehn S, Remita H, Usami N, Kobayashi K, Furusawa Y, et al. Platinum nanoparticles: a promising material for future cancer therapy? Nanotechnology 2010;21:85103.

61. Taylor WR, DePrimo SE, Agarwal A, Agarwal ML, Schonthal AH, Katula KS, et al. Mechanisms of G2 arrest in response to overexpression of p53. Mol Biol Cell 1999;10:3607-3622.

62. Innocente SA, Abrahamson JL, Cogswell JP, Lee JM. p53 regulates a G2 checkpoint through cyclin B1. Proc Natl Acad Sci U S A 1999;96:2147-2152.

63. Badie C, Bourhis J, Sobczak-Thepot J, Haddada H, Chiron M, Janicot M, et al. p53-dependent G2 arrest associated with a decrease in cyclins A2 and B1 levels in a human carcinoma cell line. Br J Cancer 2000;82:642-650.

64. Satyanarayana A, Kaldis P. Mammalian cell-cycle regulation: several Cdks, numerous cyclins and diverse compensatory mechanisms. Oncogene 2009;28:2925-2939.

65. Chipuk JE, Maurer U, Green DR, Schuler M. Pharmacologic activation of p53 elicits Bax-dependent apoptosis in the absence of transcription. Cancer Cell 2003;4:371-381.

66. Miyashita T, and Reed, J.C. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 1995;80:293-299.

ACKNOWLEDGEMENT

First and foremost, I would like to express my sincere gratitude to my great supervisor, Prof. Hoon Yoo who brought me into the new exciting field of biotechnology. His kindness, encouragement and enthusiasm have been inspirited me throughout my studies. He willingly shared the knowledge and giving advices not only in research but also in living in Korea. I also wish to express my sincere thanks to all professors at College of Dentistry, Chosun University for their teaching me during my graduate training.

I am very much grateful to Dr. Le Thanh Do for his guidance, enlightening discussions and his kind help during working time in Korea.

I would like to thanks to Prof. Iel-Soo Bang and Prof. Sang-Gun Ahn for helping me during my thesis.

I also would like to express my thanks to members of Oral Microbiology, Oral Pathology and Oral Histology Departments for helping me in my study.

Thanks you to all my Vietnamese and Korean friends for their encouragement, friendly helping me during my stay in Korea.

Finally, I would like to express my special thankful to my parents, my family and my friends for their love, support, and encouragement in every moment of my life.

Korea, October, 2013

Ta Thi Loan