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Cisplatin, one of the most effective anticancer drugs used in the treatment of ovarian cancer, causes DNA damage and leads to apoptosis. Caspases, a family of cysteine proteases, are essential for the induction of apoptosis. Initiator caspases activate effector caspases to trigger apoptosis. Caspase-2 can function as both an initiator and effector caspase although there are controversies regarding its role in DNA damage-induced apoptosis. Caspase-2 is the only caspase constitutively located in the nucleus, although its function there is unknown. In the present study we have investigated if caspase-2 is important during cisplatin-induced apoptosis and whether cisplatin treatment affects the localization of caspase-2. Caspase-2 depletion suggested that caspase-2 acts upstream of caspase-9 in cisplatin-induced apoptosis. We also made a novel observation that rottlerin, an inhibitor of DNA damage-induced apoptosis, specifically downregulates caspase-2 via the ubiquitin proteasome-mediated pathway. We further show that cisplatin induces caspase-2 translocation out of the nucleus.

Moreover, translocation of caspase-2 is important for cisplatin-induced cell death.

INVOLVEMENT OF CASPASE-2 IN CISPLATIN-INDUCED

CELL DEATH IN 2008 OVARIAN CANCER CELLS

Brett T. Adkins, B.S.

APPROVED:

Alexandra Basu

Major Professor

Lance Berg

Committee Member

Committee Member

University Member

Graduate Advisor

Chair, Department of Molecular Biology and Immunology

Dean, Graduate School of Biomedical Sciences

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THESIS

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Brett T. Adkins, B.S.

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CHAPTER 1

INTRODUCTION

Ovarian cancer is the 5th leading cause of cancer-related death among women in the United States (www.cancer.org). An estimated 22,000 cases of ovarian cancer were diagnosed in the United States in 2007. Although the incidence rate of ovarian cancer has decreased by 0.7% per year since 1985, approximately 15,000 deaths from this cancer occurred in 2007 (www.cancer.org). Women, older than 55, a positive family history, nullipara, or prescribed hormone replacement therapy or fertility drugs all have an increased risk of developing ovarian cancer. There are no obvious early symptoms of ovarian cancer and less than 1/5 of cases are diagnosed before the cancer has metastasized (www.NIH.gov, www.cancer.org). While, a vaccine against virally-induced ovarian cancer was approved by the Food and Drug Administration, thousands of new cases continue to emerge due to late diagnosis. Thus, a better understanding of the biochemical pathways involved in ovarian cancer therapies may lead to a more efficacious treatment and an increased chance of survival.

Cisplatin

Structure and action of cisplatin. Platinum compounds such as *cis*-diamine dichloloroplatinum(II) or cisplatin (Figure 1) are important anticancer agents used in the treatment of cancers, such as ovarian, lung, bladder and testicular cancers [1-3]. Cisplatin, one of the most effective and potent chemotherapeutic drugs, was synthesized in 1847, but its anticancer activity was not discovered until the 1960's [2].

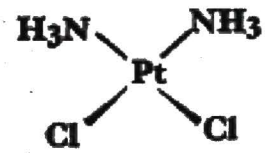

Figure 1: Structure of cisplatin [2]

The effectiveness of cisplatin is hampered clinically by renal toxicity and development of resistance. The precise mechanism of action of cisplatin still remains unknown, although it is widely accepted that cisplatin damages DNA by forming DNA adducts. Once in aqueous solution, the –Cl group is replaced by a water molecule generating a positively charged electrophile. This molecule is believed to be the active species reacting with nucleophilic sites to form DNA adducts. It is estimated that only 1% of intracellular cisplatin reacts with nuclear DNA forming intra- and inter-strand cross-links (Figure 2). The most common adduct is an intrastand cross-link between adjacent guanines [4]. DNA adducts are thought to be the cytotoxic lesion induced by cisplatin which lead to apoptosis.

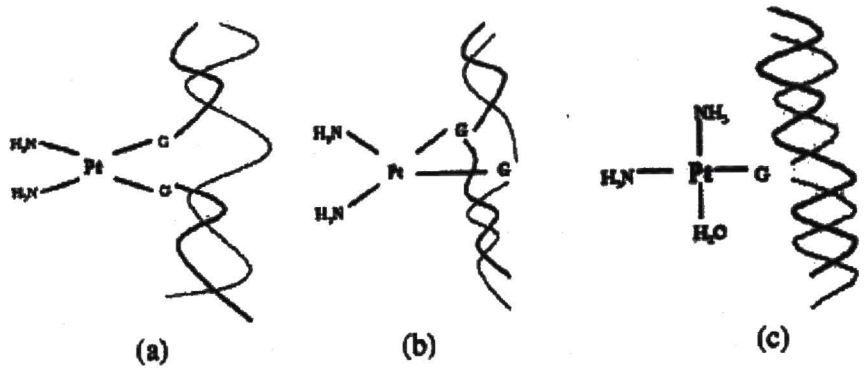

Figure 2: Product formed from cisplatin and DNA: (a) intrastrand cross-links; (b) interstrand cross-links; (c) monoadducts [2]

Cellular responses to cisplatin-induced DNA damage. Cisplatin-induced lesions are repaired by the nucleotide-excision repair pathways. However, the cisplatin adducts inhibit replication and transcription and may induce replication-mediated double strand breaks [2, 4, 5]. After detection of a double-strand break, cellular sensors activate repair mechanisms or stimulate apoptosis if the damage is too severe [5]. Special proteins detect double-strand breaks and signal apoptosis by phosphorylating p53, stabilizing it. Stable p53 blocks proliferation by upregulating p21, which prompts G1 to S cell cycle arrest [5]. Cisplatin lesions also induce apoptosis by activating Jun N-terminal kinase and p38 kinase. The sustained activation of these kinases transcriptionally activates the gene that encodes the TNF receptor superfamily member 6 (FAS) ligand [5]. Although the mechanism(s) where by cisplatin kills cells is incompletely understood, activation of the caspase cascade is ultimately necessary in arresting cancer cell proliferation and tumor growth by apoptosis.

Apoptosis

Apoptosis is a genetically controlled process of cellular self-destruction that functions in normal cellular homeostasis, embryonic development, and elimination of unwanted, transformed or damaged cells [4, 6, 7]. Apoptosis requires the activation of energy-requiring intracellular machinery and effects single cells asynchronously [3]. Apoptosis is a highly regulated process maintained by several signal transduction pathways and loss of apoptosis plays a crucial role in the regulation of tumor development [8, 9]. Apoptosis is characterized by various morphological and biochemical alterations. These alterations include cell shrinkage, plasma membrane blebbing, loss of cell-cell contact, chromatin condensation, nuclear DNA fragmentation and recognition by phagocytic cells [3, 4, 9]. It is commonly accepted that damage to DNA or other cellular stress leads to initiation of the apoptotic response [9]. Apoptosis is divided into three distinct stages. In the initiation stage, the stimulus is received followed by initiation of several pathways responding to the stimulus. This is followed by the effector stage where the decision to live or die is made based upon the ratio of pro and anti- apoptotic pathways initiated. The last stage of apoptosis, which is the irreversible execution phase, is characterized by auto digestion of various proteins and DNA cleavage. A conserved feature of the third stage is the specific degradation of a series of proteins by a family of proteases called caspases [4].

Caspases

General overview. Caspases, a family of cysteine aspartic acid proteases, play an essential role in the regulation and execution of apoptosis. Caspases are synthesized as inactive enzyme precursors and require activation during programmed cell death. Caspases consist of an N-terminal prodomain and a linker peptide, which is flanked by a large and small subunit [10, 11]. Depending on the structure of the prodomain, caspases are classified as an initiator or effector. Caspases with large prodomains of 150-200 amino acids are initiator caspases (-2, -8, -9, -10) and caspases with short prodomains of 20-30 amino acids are effector caspases (-3, -6, -7) [12]. The large prodmains of initiator caspases contain structural motifs, which belong to the death domain super family. This superfamily consists of protein interaction domains, the death effector domain (DED) and the caspase recruitment domain (CARD). DED and CARD domains, possessed by initiator caspases, are responsible for recruiting the initiator caspase to the adaptor protein complex, resulting in proteolytic activation of initiator caspases [10] (Figure 3).

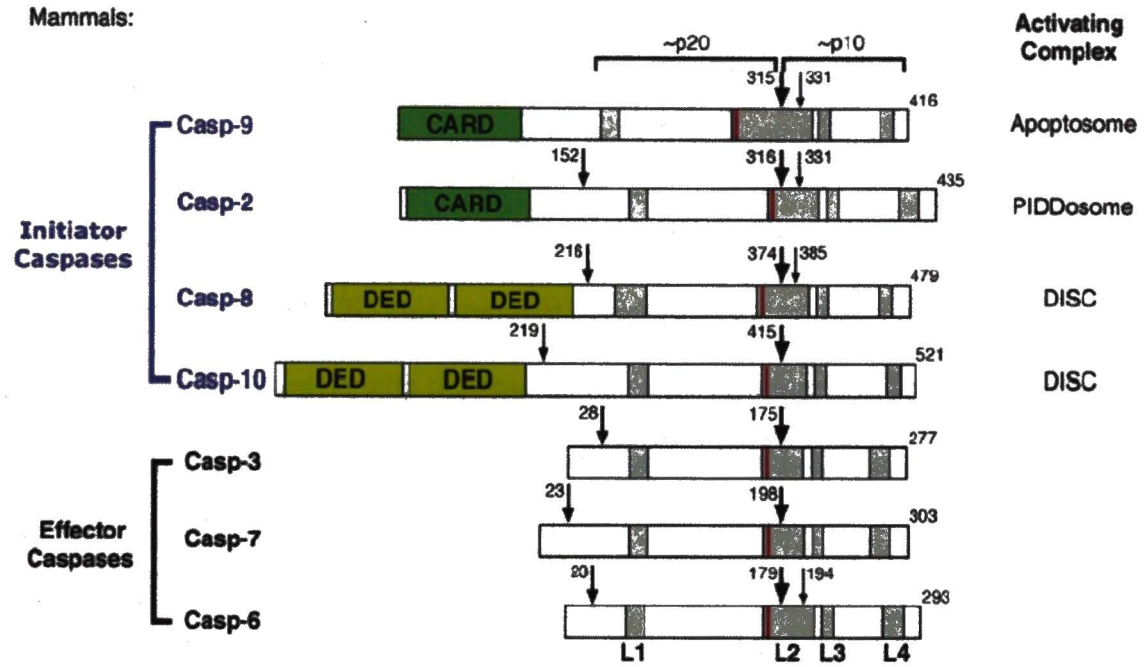

Figure 3: Structure of initiator and effector caspases [13]

Intrinsic and extrinsic pathways. Caspase activation can be initiated through either the death receptor-mediated extrinsic pathway or the mitochondria-mediated intrinsic pathway [14, 15] (Figure 4). The extrinsic pathway is activated by stimulation of death receptors, such as the tumor necrosis factor-α (TNF) receptor superfamily. Binding of ligand to the receptor causes multiple receptors to aggregate on the target cell surface. Clustering of the receptor death domain recruits the adaptor protein Fas-associated death domain (FADD). FADD recruits initiator caspase-8 to form the death-inducing signaling complex (DISC) [3]. Caspase-8 is autocatalytically activated and in turn activates effector caspase-3 and the caspase cascade, which results in apoptosis. Alternatively, cellular stress, such as DNA damage triggers the mitochondrial cell death pathway [13]. Outer

mitochondrial membrane permeability is increased, which promotes the release of cytochrome c and other apoptogenic factors [16]. Once released into the cytosol, cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1). The N-terminus of Apaf-1 contains a caspase recruitment domain (CARD) that oligomerizes upon binding to cytochrome c and dATP [9]. This complex is known as the apoptosome. The CARD domain of Apaf-1 interacts with the prodomain of caspase-9 and activates the initiator caspase-9. Active caspase-9 activates effector caspase-3 and the caspase cascade resulting in cell death [13].

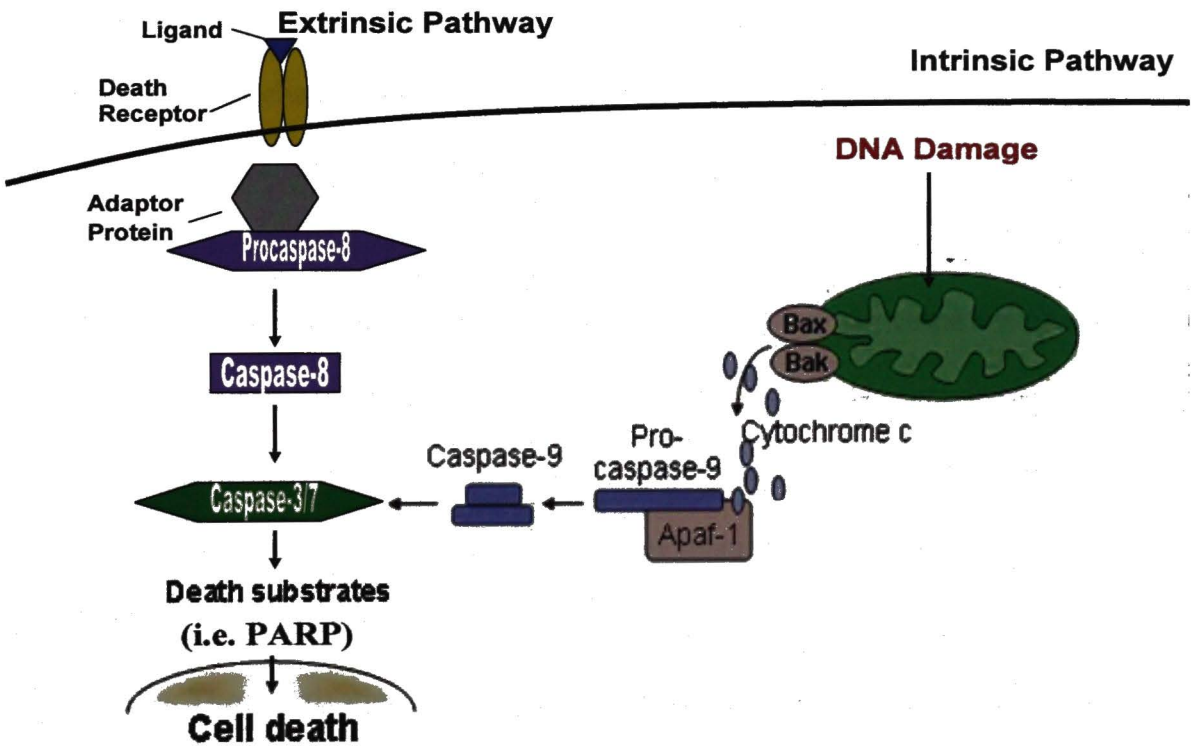

Figure 4: Extrinsic and Intrinsic apoptotic pathways
Adapted from www.dkfz.de/en/phd-program/images/Krebshelf.jpg

Effector caspase activation. The definition of activation varies greatly between effector and initiator caspases. The cleavage of effector caspase by an initiator caspase enhances the catalytic activity of the effector caspase several-fold. In solution, effector procaspases are homodimers with the active site conformationally blocked. Activation of an effector procaspase requires cleavage after a specific aspartate residue, which causes heterotetramer formation, $p17_2$-$p12_2$ mediated by their initiator caspase [10]. Formation of the heterotetramer causes release of the short prodmain. The heterotetramer is formed by two heterodimers aligning in a head to tail configuration; active sites are located at opposite ends of the heterodimer and are formed by residues of the p17 and p12 subunits [10]. Effector caspases remain homodimers through the cleavage of the prodomain and the activating intra-chain cleavage of the linker region allows rearrangement of the active site into its activated form [18, 19]. Effector caspase activation is an irreversible event and the cleavage of critical cellular proteins by the effector caspases results in apoptosis.

Initiator caspase activation. Unlike effector caspases, initiator caspases are mainly monomers in solution and are inactive in this monomeric state. Current research indicates that interaction with an activating complex substantially increases the catalytic activity of an initiator caspase [13, 19]. The three main initiator caspases (-2, -8, -9) are activated by PIDDosome, death inducing signaling complex (DISC) and the apoptosome, respectively. These activating complexes trigger the caspase cascade by activating the initiator caspases. Although exact mechanisms are not thoroughly understood, the induced proximity mechanism in the form of proximity-driven dimerization is the model suggested by recent data. The induced-proximity model theorizes that initiator caspases

autocatalytically cleave and thus activate themselves when brought into close proximity to each other [18]. However, caspase-9 activation is mediated by the apoptosome independently of intrachain cleavage. Also, the initiator caspases are inactive as monomers and active as dimers, thereby suggesting dimerization is a possible method of activation [13]. The proximity-driven dimerization model suggests that the activating complexes promote the homodimerization of initiator caspases and possibly their activation due to the high local concentrations of procaspase zymogens [13, 20, 21]. However, dimerization is not directly known to activate initiator caspases, an alternate method of activation has been proposed. The induced conformation model provides several alternative hypotheses of initiator caspase activation [13]. This model attributes caspase activation to the apoptosomes' ability to change the active site conformation. While, the two models vary on their mechanism of activation, dimerization of zymogens and active site conformation, both models propose that alteration of activation site conformation leads to caspase activation [13].

Substrate specificity. Caspases are indispensable to initiation of the apoptotic cascade; consequently substrate recognition is highly specific. Caspase substrate selectively is determined by binding pockets formed by four active-site loops on each caspase. Caspases recognize a contiguous four amino acid sequence in their substrate termed, P4-P3-P2-P1. As their name implies, caspases cleave after aspartic acid residues, which almost always reside at the P1, C-terminal residue [12, 17]. Preference for the P2 and P4 positions varies greatly among initiator and effector caspases and is thought to contribute to their substrate specificity [12]. The P3 position is most often a glutamate in known

caspases. In addition to these four residues, caspase-2 requires the presence of a P5 residue. Catalytic efficiency is increased 35-fold when leucine occupies the binding groove of the P5 site [17]. Inhibitors mimic caspase recognition motifs and competitively bind to caspases.

Caspase inhibitors. Given the fact that caspases are an essential event leading to apoptosis, multiple inhibitors have been naturally developed to regulate the proteolytic action of caspases. Additionally, some inhibitors can signal caspases for degradation through the ubiquitin proteasome-mediated pathway [12]. Baculovirus protein, p35, is a pan-caspase inhibitor. Crystal structures show that p35 binds to caspase-8 and inhibits activation through a protected thioester bond with the catalytic residue of caspase 8 [10, 12]. Another family of specific caspase inhibitors is known as inhibitors of apoptosis proteins (IAPs). IAPs are thought to be the main control point of apoptosis regulation and may also determine the threshold above which caspase activation is rapidly engaged [17]. IAPs specifically inhibit initiator caspase-9 and effector caspases -3, and -7 [10]. The functional unit of the IAP family is the baculoviral IAP repeat (BIR) [17]. IAPs are composed of eight members with X-linked inhibitor of apoptosis (XIAP) being the most widely studied. XIAP contains three BIR domains. The linker region between the first and second BIR targets the caspase -3/-7 active site and the third BIR domain is specific for inhibiting caspase-9 activity [10]. Furthermore, IAP can be inhibited by second mitochondria-derived activator of caspase (SMAC)/DIABLO proteins, which antagonize caspase inhibition and promote apoptosis [10, 12].

Caspase-2

General Overview. Caspase-2 was described by two groups in 1994 and is the most evolutionarily conserved among all caspases [22-24]. Caspase-2 defies traditional categorization as either an initiator or effector caspase because it has properties of both groups. As an initiator, caspase-2 shares sequence homology with initiator caspase-9 and has a long prodomain that contains a CARD [25]. As an effector, caspase-2 has the VDVAD (Val-Asp-Val-Ala-Asp) substrate specificity of effector caspase -3, and -7 [26]. Initially, caspase-2 was not studied as heavily as other initiator caspases, primarily due to the lack of phenotype in caspase-2 deficient mice. The caspase-2 null mouse is characterized by an increase number of oocytes due to inhibition of apoptosis during development [17]. However, recent data has implicated caspase-2 involvement in DNA damage-induced apoptosis [25] and stress induced apoptosis [20, 27].

The human *CASP-2* gene encodes two main isoforms. The two different mRNA species, caspase-2L (long isoform), and caspase-2S (short isoform), are derived from alternative splicing in the 3' end of caspase-2 pre-mRNA [28, 29]. Caspase-2S arises from the inclusion of the 61-base pair exon 9, which introduces a premature stop codon. The short isoform contains only the p17 region [25]. Overexpression of caspase-2L has been shown to induce apoptosis; on the other hand, overexpression of caspase-2S was shown to suppress cell death [22]. Expressions of the alternatively spliced forms are tissue specific as well as cell lineage and stimulus dependent. However, current evidence alludes to potential positive and negative regulatory role of caspase-2.

Caspase-2 localization. Subcellularly, procaspase-2 has been found in the cytosol, mitochondria and in the Golgi complex [7, 25, 30]. Interestingly, procaspase-2 is the only procaspase present constitutively in the nucleus [30-33]. Nuclear localization of procaspase-2 is mediated by two nuclear localization signals in the prodomain [31]. The implication of nuclear localization remains unclear since procaspase-2 with mutated nuclear localization sequence can still be processed and further induce apoptosis in the cytoplasmic compartment [31]. Nuclear localization of caspase-2 may suggest that it acts as a detector of DNA damage.

Caspase-2 activation. Activation of caspase-2 is another black box. As mentioned, caspase-2 activation is thought to occur through association with a large adaptor protein complex, PIDDosome [34]. The PIDDosome is a 670-kDa complex comprised of RAIDD (RIP-associated Ich-1/Ced-3 homologue with a death domain), PIDD (p53-induced protein with a death domain) and procaspase-2 [34]. RAIDD, an adaptor molecule and primarily a cytoplasmic protein, contains a CARD and DD domain. RAIDD associates with caspase-2 through its CARD domain and with PIDD through its DD [21, 34]. Full-length PIDD (Figure 5) is a 100-kDa protein containing seven leucine-rich repeats, two ZU-5 domains and a C-terminal DD [35]. PIDD is processed at two sites. Cleavage at S446 falls between the ZU-5 domains and generates PIDD-N (48kDa) and PIDD-C (51kDa) fragments [36]. Upon stimulation, PIDD-C is further cleaved at S588 resulting in PIDD-CC (37kDa) fragment [36].

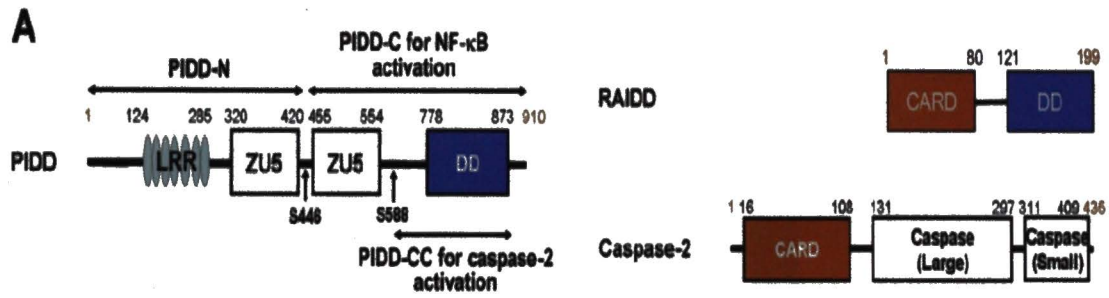

Figure 5: Domain organization of the PIDDosome components [36]

PIDD is unique in that the amount of auto-processing of PIDD determines if it will act in a pro- or antiapoptotic fashion. Upon DNA damage, PIDD-C recruits RIP-1 and NF-kappa-B essential modulator (NEMO) and ultimately activation of anti-apoptotic NF-κB [35]. Alternatively, processing of PIDD-C into PIDD-CC results in a proapoptotic cascade of events. PIDD-CC contains the C-terminal DD, which interacts with RAIDD through DD. The interaction between the PIDD DD and RAIDD DD form the oligomeric platform of the PIDDosome [36]. The PIDDosome facilitates the oligomerization and activation of caspase-2. The PIDDosome complex (Figure 6) is formed by seven RAIDD, five PIDD-CC and seven procaspase-2 molecules, but only the seven RAID DD and five PIDD DD form the ologmeric platform [36]. Dimerization is required for activation of caspases; the RAIDD DD:PIDD DD complex mediates oligomerization, while the RAIDD CARD: caspase-2 CARD complex is responsible for caspase-2 recruitment [36]. The recruitment of seven molecules into close proximity allows dimerization and thus activation of caspase-2.

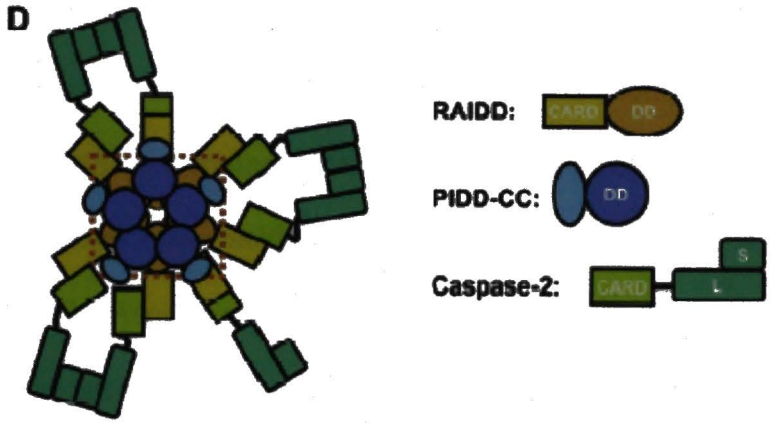

Figure 6: A model of the PIDDosome complex [36]

Caspase-2 processing is not required for activation, rather dimerization in the PIDDosome is the initial activation step. Dimerization of procaspase-2 increases baseline activity and allows autocatalytic intra-chain cleavage [37]. Fully processed caspase-2 promotes a more stable dimer formation and significantly increases catalytic activity [37]. The crystal structure of dimeric caspase-2 in solution reveals a unique disulfide bond linking the two monomers [38]. It was initially thought the disulfide bond at the dimerization interface was a novel mechanism to stabilize the dimerization of caspase-2. However, a mutagenic study showed the bond was insignificant in the ability of caspase-2 to dimerize [37].

Caspase-2 substrates. Only a few substrates of caspase-2 have been identified. Other than itself, caspase-2 is known to cleave glogin-160, αII-spectrin , PKCδ, and Bid [25, 30, 39, 40].

Caspase-2 cleaves glogin-160 at ESPD (Glu-Ser-Pro-Asp), a site unique to caspase-2 [25]. Mutational analysis of the C-terminal aspartic acid of golgin-160 abolished

caspase-2 mediated cleavage and slowed Golgi disassembly during apoptosis. Thus, it is proposed that the Golgi complex may play a role in pro-apoptotic signal transduction.

α II-spectrin is a component of the membrane cytoskeleton and is thought to be involved in membrane blebbing during apoptosis [41]. αII-spectrin is cleaved at DETD (Asp-Glu-Thr-Asp) by caspase-2 as well as capsase-3. Proteolysis at αII-spectrin occurs early in the apoptotic cascade and leads to the destabilization of the spectrin network. The identification of αII-spectrin as a caspase-2 substrate suggests that in addition to its inhibitor role, caspase-2 also acts as an effector caspase.

Protein kinase C (PKC)-δ is believed to function as a proapoptotic protein and its activation is thought to be required for DNA damage-induced apoptosis [42, 43]. It has been reported that caspase-2 can mediate PKCδ cleavage and activation [40]. In turn, PKCδ activates downstream targets and leads to apoptosis.

Bid is a proapoptotic member of the Bcl2 family of proteins [6]. Bid activates Bax/Bak, which in turn induces mitochondrial outer membrane permeabilization and subsequent cytochrome c release leading to apoptosis. Bid has been identified as a substrate for caspase-2 as well as caspse-3 and -8 [39, 44]. The caspase cleavage site of Bid, confirmed by mutational analysis, is LQTD (Leu-Gln-Thr-Asp), which corresponds most closely with the preferred cleavage site of caspase-8 [39]. This finding suggests another way in which activated caspase-2 may induce apoptosis.

Caspase-2 substrate specificity. As can be seen from the above information, caspase-2 has widely varying substrate specificity. Initially, caspase-2 was grouped with other initiator caspases said to have a preference for the DEXD (Asp-Glu-X-Asp) motif, more

recent data suggests a requirement of a fifth residue for efficient substrate cleavage and a 35-fold increase of the LDESD (Leu-Asp-Glu-Ser-Asp) motif compared to the DEXD motif [38]. The specific mechanism by which caspase-2 activates apoptosis remains unclear. Caspase-2 is unable to directly process or activate effector caspases -3, -6, or -7 nor initiator caspase -8 or -9 [44]. Thus, caspase-2 may induce apoptosis independent of the caspase cascade through one of the substrates noted above or through interaction with a yet unidentified substrate.

Since caspase-2 has been shown to have characteristics of effector and initiator caspases and it is the only caspase which is constitutively localized to the nucleus, I have examined in what role caspase-2 acts and have monitored its localization in response to cisplatin-induced apoptosis. Given that caspase-2 is an important regulator of DNA damage-induced apoptosis, the present study was undertaken to better understand how caspase-2 is involved in cisplatin-induced cell death pathway.

CHAPTER 2

MATERIALS AND METHODS

Materials:

Opti-minimal essential medium (OPTI-MEM) and Roswell Park Memorial Institute (RPMI) 1640 were obtained from Invitrogen (Carlsbad, Ca). MG132 was obtained from Calbiochem (San Diego, CA). Cisplatin was from Sigma (St. Louis, MO). siRNA against caspase-2,-3,-7,-8,-9 and non-targeting siRNA were obtained from Dharmacon (Lafayette, CO). Monoclonal antibody to GAPDH was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibody to caspase-2 and PARP, and polyclonal antibody to caspase-9 were purchased from BD Pharmingen (San Diego, CA). Polyclonal antibody to caspase-3 and monoclonal antibody to caspase-8 were obtained from BioSource/Invitrogen (Carlsbad, CA). Horseradish peroxidase-conjugated goat anti-mouse and donkey anti-rabbit antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Polyvinylidene difluoride membrane was from Millipore (Bedford, MA), and enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL). Lipofectamine 2000

transfection reagent and fluorescent secondary antibodies (anti-mouse Alexa 488, anti-rabbit Alexa 488, anti-mouse Alexa 568, and anti-mouse Alexa 647), MitoTracker® Deep Red 633, DAPI were obtained from Molecular Probes/Invitrogen (Eugene, OR).

Cell culture:

2008 cells were maintained in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum and 2 mM glutamine. Cells were kept in a humidified incubator at 37^0C with 95% air and 5% CO_2.

siRNA Methodology:

Control non-targeting siRNA or siRNAs against caspase-2 were introduced into 2008 cells using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, cells were seeded one day before transfection. Lipofectamine 2000 and siRNA diluted in Opti-minimal essential medium (MEM) were mixed gently at a ratio of 300 ng lipofectamine 2000 : 133 ng siRNA and incubated at room temperature for 15–20 min. Culture medium was replaced with Opti-MEM and 100 μl of siRNA:lipofectamine 2000 complexes were added to cells. After 4–6 h, fresh culture medium was added to cells. Forty-eight hours following transfection, cells were treated as indicated.

Immunoblot analysis:

Following treatment with cisplatin, rottlerin, and/or MG132, cells were collected, washed twice with phosphate-buffered saline and swelled in lysis buffer containing (0.15 mM NaCl, 10 mM Tris-HCL, pH 7.4, 1 mM EDTA, 0.5% Nonidet-40, 1% Triton X-100, 0.2mM sodium vanadate, 1 mM DTT, 1:500 dilution of protease and phosphotase inhibitor cocktail). Protein was determined by the Bradford [45]. Method using bovine serum albumin as a standard. Cell lysates were electrophoresed in 7.5% - 15% gradient sodium dodecyl sulfate (SDS)-polyacrylamide gels (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was analyzed for protein expression by immunoblotting. Immunoblot analyses were performed with 1:1000 dilution of indicated antibodies. The proteins were visualized using enhanced chemiluminescence detection kit from Amersham, per manufacturers' protocol.

Immunofluorescence and Hoechst staining:

Cells were seeded overnight in 8- well chamber slides from Nunc (Naperville, IL). After indicated treatment the mitochondria of live cells was stained by MitoTracker® Deep Red. Cells were fixed for 20 min in 2% paraformaldehyde at RT. Cells were then permeabilized by incubation in 0.5% Triton X-100 for 7 min at 4°C. Nonspecific binding was impeded by diluting 1° and 2° antibodies (1:100) and (1:800) respectively in blocking buffer (2% bovine serum albumin diluted in phosphate-buffered saline. 1° and 2° antibodies were incubated at 4° overnight and at room temperature for 60 min, respectively. The endoplasmic reticulum marker, Calnexin, was a gift from Dr. Ma.

Nuclei staining was performed by a 10 min incubation with Hoechst 33342 (.5μg/ml in blocking buffer) at room temperature. The cells were washed at RT with phosphate-buffered saline 3 x 5 min between all steps. Stained cells were mounted with Aquamount from Polysciences (Warrington, PA) and examined under a LSM 510 META confocal laser scanning microscope or an Axiovert 40 CFL inverted transmitted-light microscope (Zeiss, Göttingen, Germany). Fluorescent secondary antibodies (anti-mouse Alexa 488, anti-rabbit Alexa 488, anti-mouse Alexa 568, and anti-mouse Alexa 647) were purchased from Molecular Probes (Carlsbad, CA).

CHAPTER 3

RESULTS

3.1 Cisplatin treatment causes induction of p53, cleavage of PARP and activation of caspase-2 in ovarian cancer 2008 cells

Cisplatin is a DNA damaging agent used in the treatment of ovarian cancer. It has been reported that the activation of caspase-2 requires p53 [7]. DNA damage induces the tumor suppressor protein, p53, resulting in apoptosis. p53 regulates expression of p53-inducible protein with a death domain (PIDD) which is part of the PIDDosome complex necessary for the activation of procaspase-2 [7, 35]. To determine the importance of caspase-2 in cisplatin-induced apoptosis in ovarian cancer 2008 cells, we first treated 2008 cells with increasing concentrations of cisplatin for 24 h and monitored p53 induction and cleavage of PARP. During apoptosis, activation of caspase-3 and -7 leads to the cleavage of poly (ADP-ribose) polymerase (PARP) from its 115-kDa full length to the 85-kDa fragment. Although PARP cleavage is an irreversible process, the presence of a cleavage fragment is not guarantee cells are undergoing apoptosis. However, PARP cleavage is frequently used to monitor apoptosis. Figure 1 shows a concentration-dependent induction of p53 by cisplatin treatment and cleavage of PARP.

Caspases are synthesized in the cell as inactive procaspases [46]. Site-specific cleavage of the procaspases results in the removal of the prodomain and activates the caspase. Therefore, decrease in procaspase-2 or an increase in cleavage fragments were monitored by Western blotting to determine activation of caspase-2 by cisplatin. Figure 2 shows that the processing of procaspase-2 into its 35-kDa and 14-kDa fragments was increased in a concentration dependent manner.

These experiments demonstrate that cisplatin-induced DNA damage in 2008 cells is associated with an increase in caspase-2 processing/activation.

3.2 Caspase-2 acts as an apical caspase to inhibit cisplatin-induced apoptosis

Based upon the previous study, caspase-2 may be involved in DNA damage-induced apoptosis. However, caspase-2 activation could be a secondary effect of cisplatin treatment. Therefore, to determine the importance of caspase-2 in cisplatin-induced apoptosis caspase-2 was depleted using siRNA against caspase-2. Control experiment was performed by transfecting non-targeting siRNA. Figure 3A shows that transfection of caspase-2 siRNA into 2008 cells effectively depleted caspase-2. Figure 3B shows that upon knockdown of caspase-2 induction of p53 and PARP cleavage in response to cisplatin was also decreased.

Caspase-9 is believed to be the apical caspase during DNA damage-induced cell death whereas caspase-2 can function as both apical and executioner caspase. To determine if caspase-2 acts as an apical caspase, we examined caspase-9 processing in caspase-2 depleted cells. Figure 4 shows that cisplatin treatment caused a decrease in

procaspase-9 and an increase in processed forms. However, knockdown of caspase-2 inhibited processing of caspase-9.

These experiments show that caspase-2 depletion by siRNA decreased cisplatin-induced apoptosis and the induction of p53. Moreover, caspase-2 functions upstream of caspase-9 during cisplatin-induced apoptosis.

3.3 Effect of rottlerin on caspase-2 processing in 2008 cells

Protein kinase C-δ (PKCδ) is a proapoptotic protein and its activation is thought to be required for DNA damage-induced apoptosis. Rottlerin is a PKCδ inhibitor; our lab has shown that rottlerin acts upstream of caspase-9 to inhibit cisplatin-induced apoptosis. Given that caspase-2 acts upstream of caspase-9 and rottlerin inhibits DNA damage-induced apoptosis prior to caspase-9 processing; we pretreated 2008 cells with rottlerin prior to treatment with cisplatin to determine if rottlerin inhibits DNA damage-induced apoptosis upstream of caspase-2. We made a novel observation that rottlerin specifically induced downregulation of caspase-2 but not of caspase-7, -8 or -9 (Figure 5). Furthermore, rottlerin treatment reduced cisplatin-induced cell death as seen by a decrease in caspase-2 processing and PARP cleavage (Figure 6).

I have shown that caspase-2 acts in an initiator caspase role during cisplatin-induced apoptosis. Moreover, depletion of caspase-2 inhibited cisplatin-induced apoptosis. This set of experiments demonstrated that downregulation of initiator caspase-2 with a chemical inhibitor, rottlerin, may reduce cisplatin-induced cell death.

3.4 Rottlerin downregulates caspase-2 by the proteasome-mediated pathway

To determine the mechanism of caspase-2 downregulation by rottlerin, we examined the effect of proteasome inhibitor on rottlerin-induced caspase-2 downregulation. MG132, a proteasome inhibitor, was found to inhibit rottlerin-induced caspase-2 downregulation (Figure 7).

Since MG132 was able to inhibit the rottlerin-induced downregulation of caspase-2, we examined if inhibition of caspase-2 downregulation by MG132 can prevent antiapoptotic effect of rottlerin. Figure 8 shows that MG132 alone enhanced cisplatin-induced apoptosis as determined by the cleavage of PARP. However, rottlerin inhibited cisplatin-induced apoptosis even when caspase-2 downregulation was prevented by pretreatment of cells with MG132. Thus, lack of caspase-2 downregulation was not sufficient to trigger cisplatin-induced apoptosis.

3.5 Cisplatin altered intracellular localization of caspase-2

Caspases are localized to specific subcellular compartments according to their cellular responses. Caspase-2 is unique because it is the only caspase constitutively localized to the nucleus [31, 32]. Specialized localization allows caspases to interact with their upstream activating complex or downstream substrate(s). To determine the importance of caspase-2 localization on cisplatin-induced apoptosis we first monitored caspase-2 localization following cisplatin treatment by immunofluorescence using a confocal microscope (Zeiss LSM 510 Meta).

Figure 9A shows that caspase-2 is constitutively localized throughout the cell, including the nucleus. A time course study was used to determine if cisplatin effects caspase-2 localization. Treatment with cisplatin caused a time-dependent increase in translocation of caspase-2 to the region around the nucleus (Figure 9B). Thus, although caspase-2 is constitutively localized in the nucleus it translocates out of the nucleus into the perinuclear region upon DNA damage.

Organelle markers and organelle-specific antibodies were used to determine if caspase-2 was associated with specific intracellular components after cisplatin-induced translocation. Since DNA damage-induced apoptosis affects mitochondrial cell death pathway we used MitoTracker® Deep Red to determine if caspase-2 is localized in the mitochondria. In addition, since caspase-2 is translocated to the perinuclear region following cisplatin treatment, we used calnexin as a marker for endoplasmic reticulum (ER) to determine if caspase-2 is translocated to ER. Although there was some co-localization of caspase-2 with the mitochondria (Fig. 10A) caspase-2 appears to associate with the ER (Fig. 10B, C).

3.6 Caspase-2 distribution is altered by MG 132 treatment

Since MG132 inhibited rottlerin-induced caspase-2 downregulation but did not reverse the antiapoptotic effect of rottlerin, we monitored the localization of caspase-2. Cells were pretreated with or without rottlerin and/or MG132 and then treated with cisplatin. To determine the effect of rottlerin and MG132 upon caspase-2 levels and localization, the same microscope settings were used for all images within this set of

experiments. Control cells show ubiquitous caspase-2 localization as previously seen (Figure 11A). Consistent with Western blotting experiments (Figure 5), rottlerin induced downregulation of caspase-2 as seen by a decrease in fluorescence intensity (Figure 11B). MG132 alone did not affect caspase-2 translocation but the extent of perinuclear localization of caspase-2 was increased in cisplatin-treated cells (Figure 11C), this is consistent with increase in cisplatin-induced PARP cleavage by MG132 (Figure 8). When cells were treated with rottlerin and MG132 the nuclear distribution of caspase-2 was increased. Interestingly, cisplatin failed to induce translocation of caspase-2 to the perinuclear region when cells were treated with rottlerin and MG132 prior to cisplatin treatment (Figure 11D).

Thus, lack of perinuclear localization of caspase-2 may explain why rottlerin retained an antiapoptotic effect in the presence of MG132.

CHAPTER 4

DISCUSSION

Caspase-2 was the first caspase identified in the apoptotic signaling pathway. Caspase-2 is an important regulator of DNA damage-induced apoptosis. This study showed that caspase-2 is necessary for cisplatin-induced apoptosis in ovarian cancer 2008 cells. We also made a novel observation that rottlerin, an inhibitor of DNA damage-induced apoptosis, specifically downregulates caspase-2 via the ubiquitin-mediated proteasome pathway. We further show that cisplatin induces caspase-2 translocation out of the nucleus. Moreover, translocation of caspase-2 is important for cisplatin-induced cell death.

There are controversies regarding the involvement of caspase-2 in DNA damage-induced apoptosis. Caspase-2 is distinctive in that it contains characteristics of initiator (CARD domain) and effector (substrate specificity) caspases [30]. It is unclear whether caspase-2 acts as an initiator or effector caspase in DNA damage induced apoptosis. In this study we concentrate of the role of caspase-2 as an initiator caspase and concede that caspase-2 may play a role as an effector caspase in this same system. We found that

knockdown of caspase-2 by siRNA decreased processing of caspase-9, suggesting that caspase-2 functions upstream of caspase-9. Caspase-9 is considered the apical caspase involved in DNA damage-induced cell death [47-50]. One study showed the dependence of caspase-2 activation upon caspase-9 and -3 activity [47]. However, the above mentioned study was conducted in a cell-free system and the mechanism of action may differ in vitro. Furthermore, caspase-2 processing has been reported in MCF7 cells which lacks functional caspase-3 [51]. Other studies treated with a chemotherapeutic drug at a concentration that was later found to directly stimulate mitochondrial damage bypassing the caspase cascade [48, 52]. Thus, the sequence of caspase activation in response to apoptotic stimuli can vary with cell type, model system, and treatment conditions. This study suggests that caspase-2 acts as an initiator caspase in cisplatin-induced cell death.

Rottlerin has been shown to protect against DNA damage-induced apoptosis [53]. Prior data from our lab has shown that rottlerin inhibited processing and activation of caspase-9 [54]. Because the present study has shown that caspase-2 acts upstream of caspase-9; we wanted to investigate if rottlerin regulates caspase-2 activation. We made a novel observation that rottlerin specifically induced caspase-2 downregulation; thus it can be inferred that the inhibition of processing and activation of downstream caspase-9 is an effect of caspase-2 downregulation.

Since rottlerin is believed to be a protein kinase C-δ (PKCδ) inhibitor, we expected that rottlerin-induced caspase-2 downregulation would occur via the PKCδ pathway. However, reports have indicated that rottlerin functions on targets other than

PKCδ [55-60]. A recent report from our lab showed that rottlerin induced downregulation of caspase-2 through a PKCδ-independent pathway [61]. We found that the proteasome inhibitor, MG132, was able to prevent rottlerin-mediated downregulation of caspase-2, suggesting that rottlerin triggers caspase-2 downregulation through the proteasome mediated pathway. Since MG132 prevents caspase-2 downregulation by rottlerin, we expected that pretreatment with MG132 would reverse the protective effect of rottlerin on cisplatin-induced cell death. Intriguingly, we found that MG132 had no effect on rottlerin-mediated protection against cisplatin-induced cell death. Taken together, these results propose that rottlerin induces downregulation of caspase-2 through a proteasome mediated pathway. Furthermore, downregulation of caspase-2 by rottlerin cannot explain its antiapoptotic function during cisplatin-induced apoptosis.

It has been described that caspases localize to different subcellular compartments in order to better interact with upstream activators and/or downstream substrates [30, 62]. Therefore, we examined the localization of caspase-2 during cisplatin treatment. We observed nuclear localization of procaspase-2 in 2008 cells, consistent with previous reports [31-34]. Upon DNA damage by cisplatin, we found that caspase-2 translocated to a region outside of the nucleus (the perinuclear region). A recent reported suggested translocation of caspase-2 to the cytoplasm upon cisplatin treatment [63]. However, this report did not state how they determined caspase-2 was localized in the cytoplasm after cisplatin treatment. We conducted additional analysis to further determine the localization of the caspase-2 after cisplatin-induced translocation. Endoplasmic

reticulum (ER) and mitochondrial staining revealed co-localization of caspase-2 with both organelles.

Our study showed that cisplatin induced caspase-2 translocation to the ER. In addition to DNA damage, cisplatin has also been reported to induce calpain activation in enucleated cells (cytoplasts), which is thought to be associated with ER stress [64]. A study has shown caspase-2 to be the apical caspase processed after ER stress [27]. Our results clearly show a co-localization of caspase-2 with the ER after cisplatin treatment. Cisplatin may be inducing ER stress independent of DNA damage. The ER stress could induce activation of caspase-2 in addition to the DNA damage induced activation of caspase-2. The result would be two sources of caspase-2 processing; the amplified activation of caspase-2 would lead to an increase in the amount of mitochondrial permeabilization and induction of apoptosis [65]. Alternatively, active caspase-2 may be somehow directed to the ER after DNA damage-induced activation.

Several studies have implicated caspase-2 to be required for induction of the mitochondrial pathway in etoposide and heat shock-induced apoptosis[66-70]. Studies have shown caspase-2 to be necessary for the cleavage and activation of Bid [6, 71]. Moreover, it has been recently shown in A-549 and U2-OS cell lines that caspase-2 independently integrates Bax into the mitochondrial membrane and works in concert with the oncoprotein, c-Myc, to activate Bax and thus initiate the intrinsic pathway following cisplatin treatment [72]. Results from our study support the above reports in that upon DNA damage-induced apoptosis, a portion of the caspase-2 translocated out of the

nucleus colocalizes with the mitochondria, presumably to induce the mitochondrial pathway of apoptosis.

Another noteworthy observation of these studies was that caspase-2 remained nuclear in response to cisplatin treatment in 2008 cells that were pretreated with rottlerin. Furthermore, rottlerin prevents caspase-2 translocation even when downregulation of caspase-2 has been inhibited by MG132 treatment. This suggests that the effect of rottlerin on translocation is independent of inducing caspase-2 downregulation. These findings imply that the lack of caspase-2 perinuclear translocation may explain why rottlerin retained its antiapoptotic effect. The nuclear retention does not allow caspase-2 interaction with the mitochondria and the ER thereby suggesting that caspase-2 translocation is necessary for DNA damage induced apoptosis.

Future direction of this study should focus on the functional significance of the specific localization of caspase-2 following cisplatin treatment. This can be achieved many ways, caspase-2 with a mutated nuclear import or export sequence, or caspase-2 with a mutated ER targeting sequence. Mutating caspase-2 would determine what effect caspase-2 is playing in the organelle for which the trafficking sequence has been mutated. Förster resonance energy transfer (FRET) analysis could be used to look at the interaction of caspase-2 and a specific organelle. A GFP-tagged caspase-2 construct would also allow live cell staining with specific organelle markers and time lapse photos could be taken to follow the translocation after cisplatin treatment. ER directed caspase-2 localization could also deduce if caspase-2/ER co-localization is independent of cisplatin-induced apoptotic response or an effect of ER stress-induced apoptosis. FRET analysis

can be employed to show whether caspase-2 is activated before or after cisplatin induced translocation. This study would determine the location of activation and further resolve the caspase-2 signaling pathway. Taken together, our results show that caspase-2 acts as an apical caspase in 2008 ovarian cancer cells in response to cisplatin-induced apoptosis. Another intriguing finding is that caspase-2 is retained in the nucleus after rottlerin treatment even when its downregulation is blocked by MG132, which may be the reason for its antiapoptotic effect.

CHAPTER 5

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CHAPTER 6

FIGURE LEGENDS AND FIGURES

Figure 1. Cisplatin increased p53 levels and processing of PARP. 2008 cells were treated with 5μM and 10μM cisplatin for 24 hours. Western blot analyses were performed with monoclonal antibodies to p53, PARP as described in "Materials and Methods." GAPDH was used to control for loading differences.

Figure 2. Cisplatin increased processing of procaspase-2. 2008 cells were treated with 5μM and 10μM cisplatin for 24 hours. Western blot analyses were performed with monoclonal antibodies to procaspase-2 as described in "Materials and Methods." GAPDH was used to control for loading differences.

Figure 3. Caspase-2 depletion decreased cisplatin-induced apoptosis. A) 2008 cells were transfected with control siRNA or siRNA targeted against caspase-2. B) Caspase-2 depleted cells were treated with 2.5μM and 5μM cisplatin for 16 h. Western blot analysis was performed with monoclonal antibodies to procaspase-2, p53, PARP as described in "Materials and Methods." GAPDH was used to control for loading differences.

Figure 4. Caspase-2 acts as an initiator caspase in cisplatin-induced apoptosis. 2008 cells were transfected with control siRNA or siRNA targeted against caspase-2. Caspase-2 depleted cells were treated with 2.5μM and 5μM cisplatin for 16 h. Western blot analysis was performed with monoclonal antibody to procaspase-2 and with polyclonal antibody to procaspase-9 as described in "Materials and Methods." GAPDH was used to control for loading differences.

Figure 5. Rottlerin specifically induced procaspase-2 downregulation. 2008 cells were treated with 10μM rottlerin for 16 h. Western blot analyses were performed with monoclonal antibodies to procaspases-2, -8, and polyclonal antibodies to procaspases-7, -9 as described in "Materials and Methods." GAPDH was used to control for loading differences.

Figure 6. Rottlerin decreased cisplatin-induced apoptosis. 2008 cells were treated with 10μM rottlerin for 16 h. Western blot analyses were performed with monoclonal antibodies to procaspase-2, PARP, polyclonal antibody to procaspase-9 as described in "Materials and Methods." GAPDH was used to control for loading differences.

Figure 7. Inhibition of rottlerin-induced procaspase-2 downregulation by MG132. 2008 cells were treated with 5μM MG132 for 30 minutes prior to treating with 10μM rottlerin for 60 minutes. Western blot analyses were performed with monoclonal antibodies to procaspases-2, -8, and polyclonal antibodies to procaspases-7, -9 as

described in "Materials and Methods." GAPDH was used to control for loading differences.

Figure 8. Inhibition of caspase-2 downregulation by MG132 did not prevent antiapoptotic effect of rottlerin. 2008 cells were treated with 5μM MG132 for 30 minutes prior to treating with 10μM rottlerin for 60 minutes. Cells were then treated with 10μM cisplatin for 16 h. Western blot analyses were performed with monoclonal antibodies to procaspase-2 and PARP as described in "Materials and Methods." GAPDH was used to control for loading differences.

Figure 9. Cisplatin altered intracellular localization of caspase-2. Confocal laser scanning microscopy of 2008 cells were treated with 20μM cisplatin for indicated time followed by staining with antibody against caspase-2 (green) and the nuclear stain DAPI (blue) as described in "Materials and Methods."

Figure 10. Cisplatin induced caspase-2 translocation to the mitochondria and endoplasmic reticulum. A) Confocal laser scanning microscopy of 2008 cells that were treated with 20μM cisplatin for 8 h followed by staining of mitochondrial marker MitoTracker (red), the nuclear stain DAPI (blue), and staining with antibody against caspase-2 (green). B) Confocal laser scanning microscopy of 2008 cells that were treated with 20μM cisplatin for 8 h followed by staining with the nuclear stain DAPI (blue), staining with antibody against caspase-2 (green), and calnexin (red). C) Enlargement of Figure 10B as described in "Materials and Methods."

Figure 11. Perinuclear localization of caspase-2 is important for cisplatin-induced apoptosis. A) Confocal laser scanning microscopy of untreated 2008 cells. B) 2008 cells were treated with 10μM rottlerin for 60 minutes. C) 2008 cells were treated with 5μM MG132 for 30 minutes. D) 2008 cells treated with 5μM MG132 for 30 minutes prior to treating with 10μM rottlerin for 60 minutes. Indicated cells were then treated with 20μM cisplatin for 8 h followed by staining with antibody against caspase-2 (green) and the nuclear stain DAPI (blue) as described in "Materials and Methods."

Figure 12. Diagram of rottlerin and MG132 effect on cisplatin-induced translocation of caspase-2. Cisplatin treatment caused DNA damage and induced p53. PIDDosome complex activated caspase-2, caspase-2 then translocated out of the nucleus and colocalized with the mitochondria and ER. Rottlerin treatment downregulated caspase-2 and inhibited DNA damage-induced apoptosis. MG132 prevented rottlerin induced caspase-2 downregulation, but did not

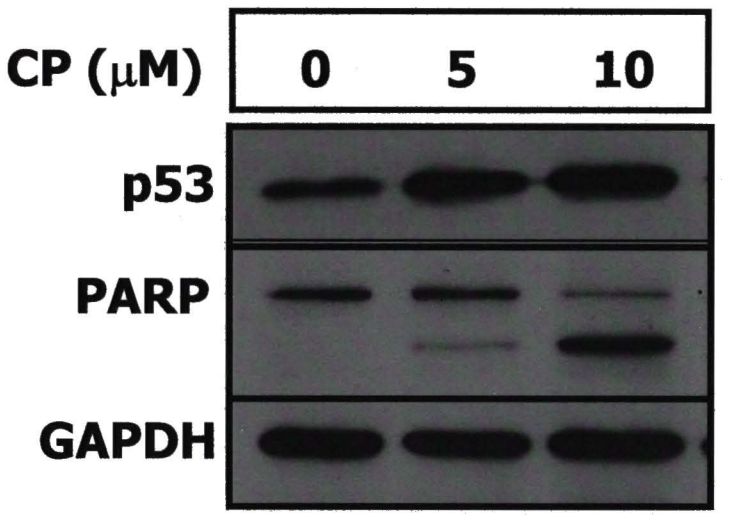


Figure 1. Cisplatin increased p53 levels and processing of PARP.

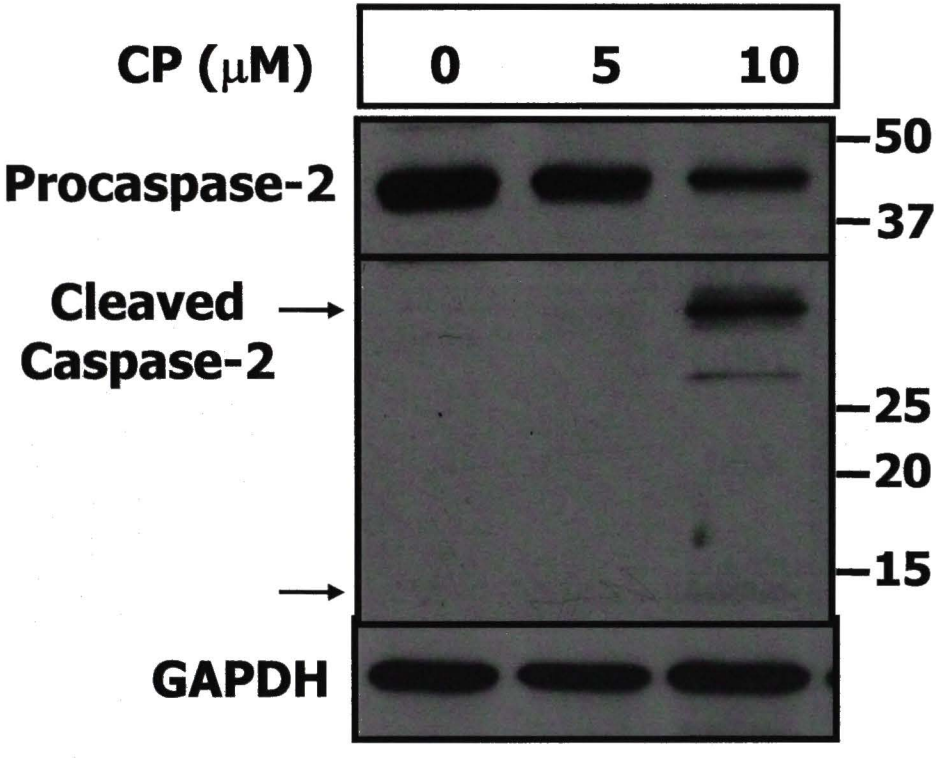


Figure 2. Cisplatin increased processing of procaspase-2.

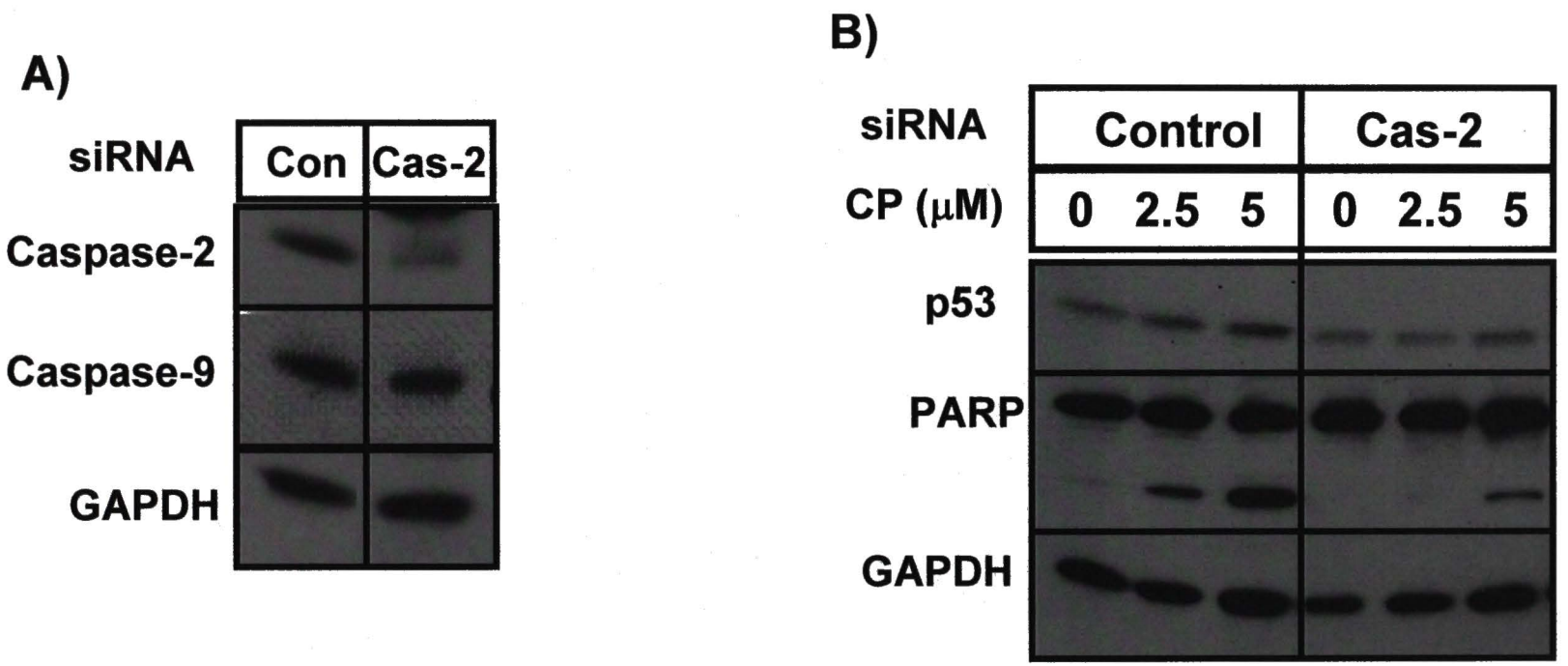


Figure 3. Caspase-2 depletion decreased cisplatin-induced apoptosis.

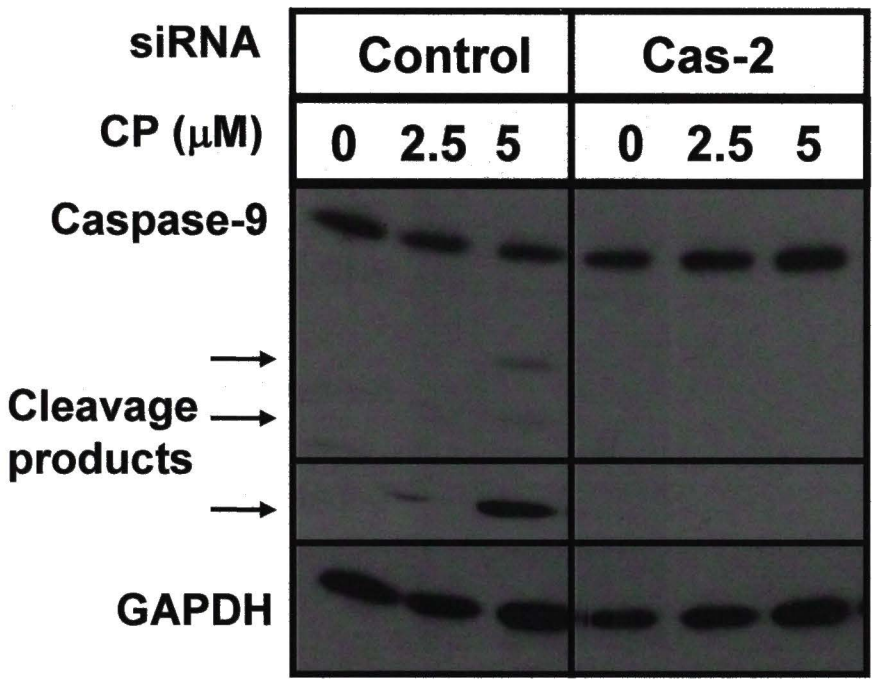


Figure 4. Caspase-2 acts as an initiator caspase in cisplatin-induced apoptosis.

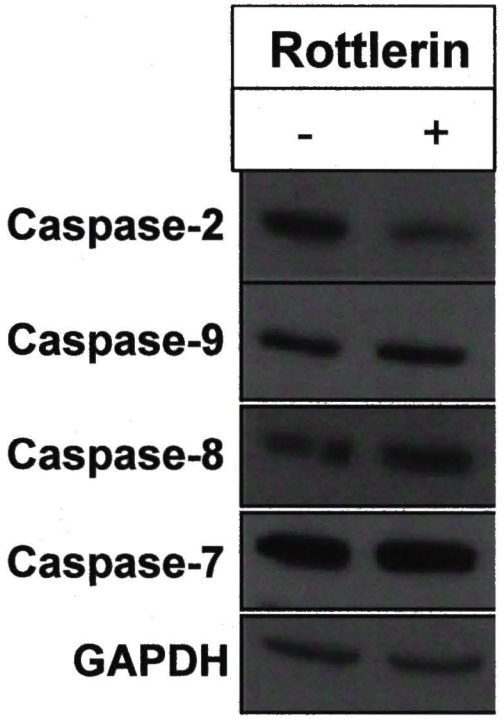


Figure 5. Rottlerin specifically induced procaspase-2 downregulation.

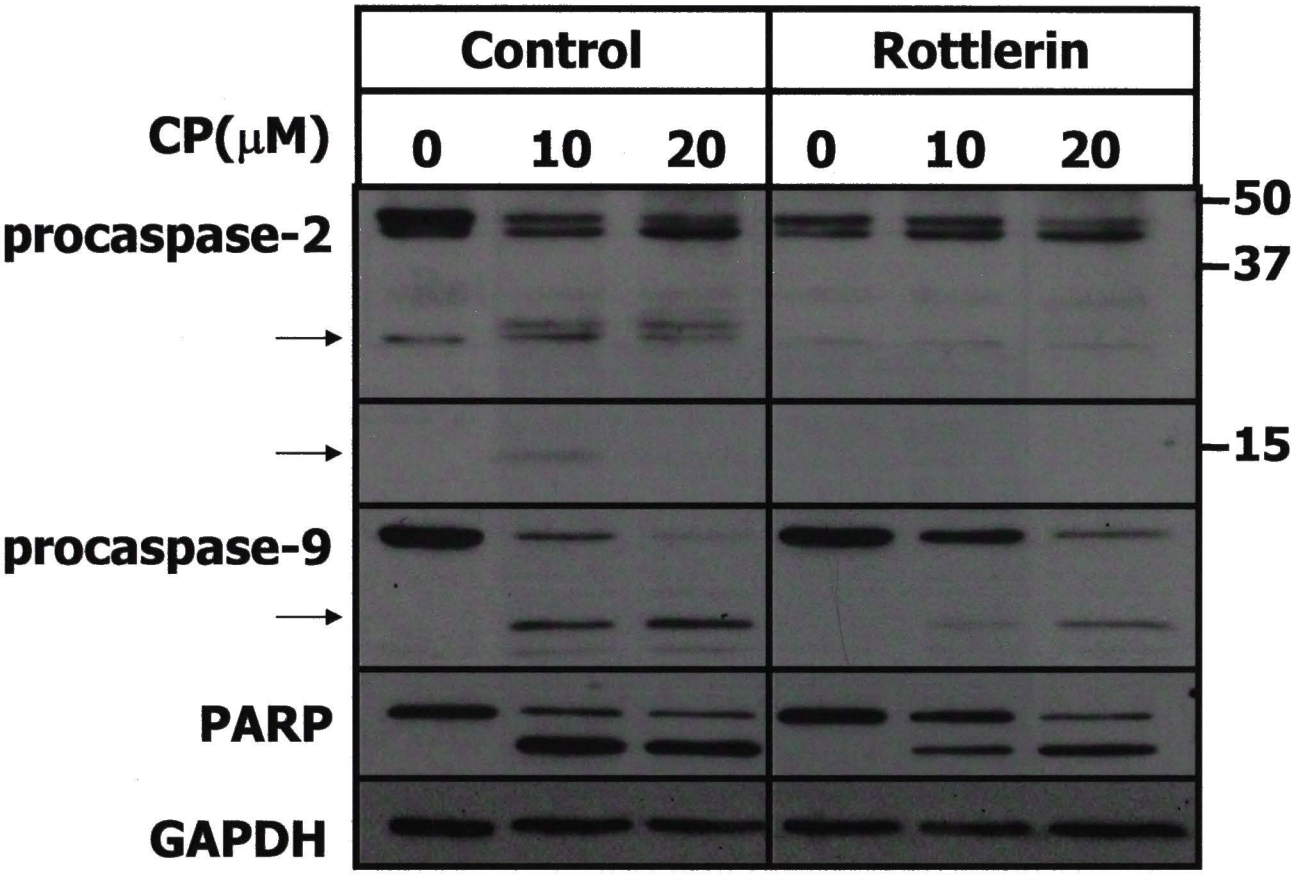


Figure 6. Rottlerin decreased cisplatin-induced apoptosis.

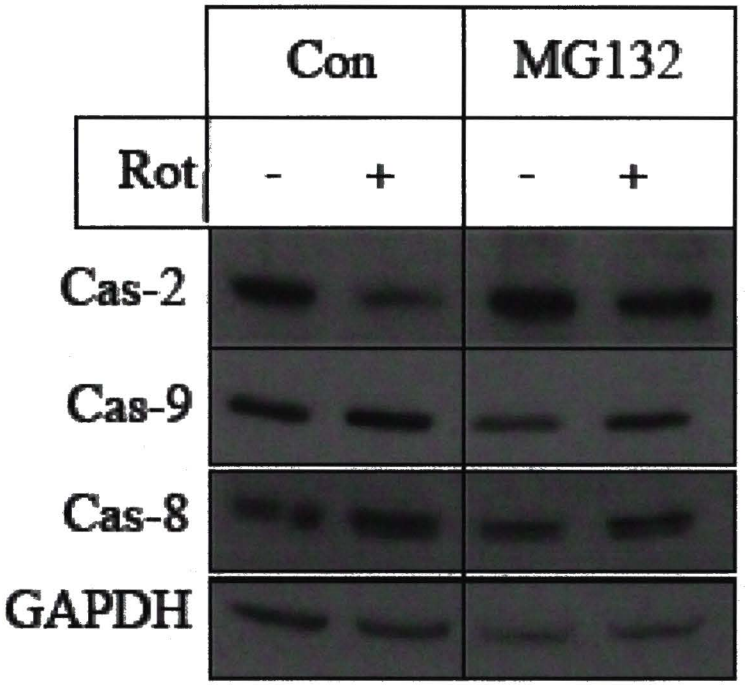


Figure 7. Inhibition of rottlerin induced caspase-2 downregulation by MG132.

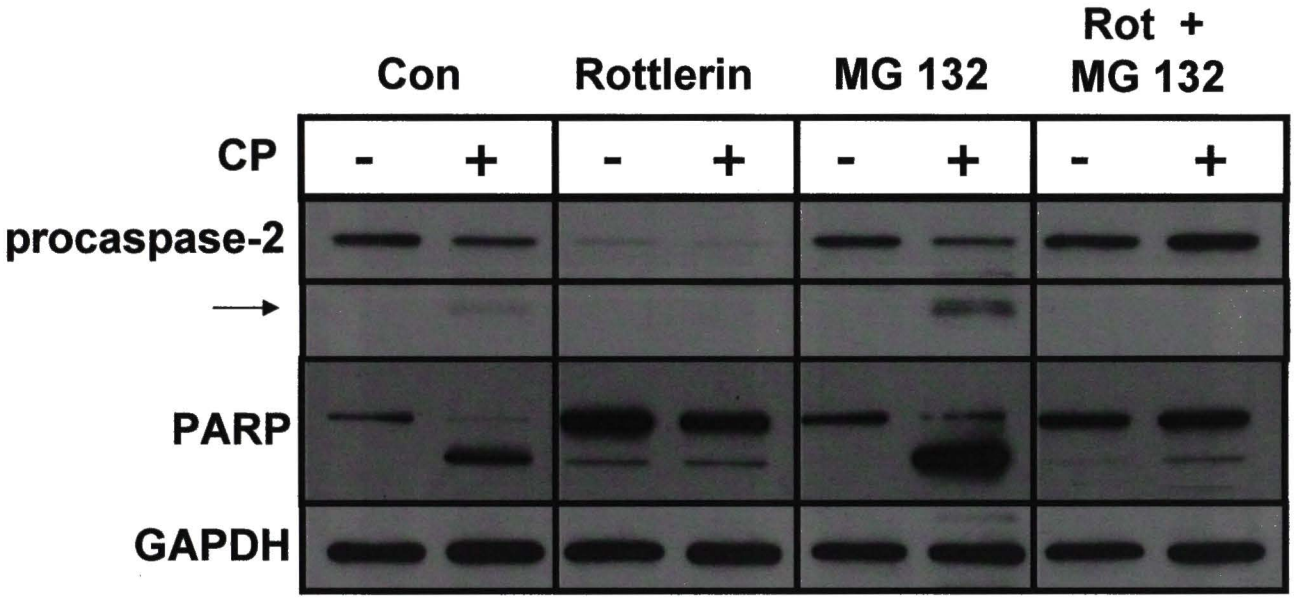


Figure 8. Inhibition of caspase-2 downregulation by MG132 did not prevent antiapoptotic effect of rottlerin.

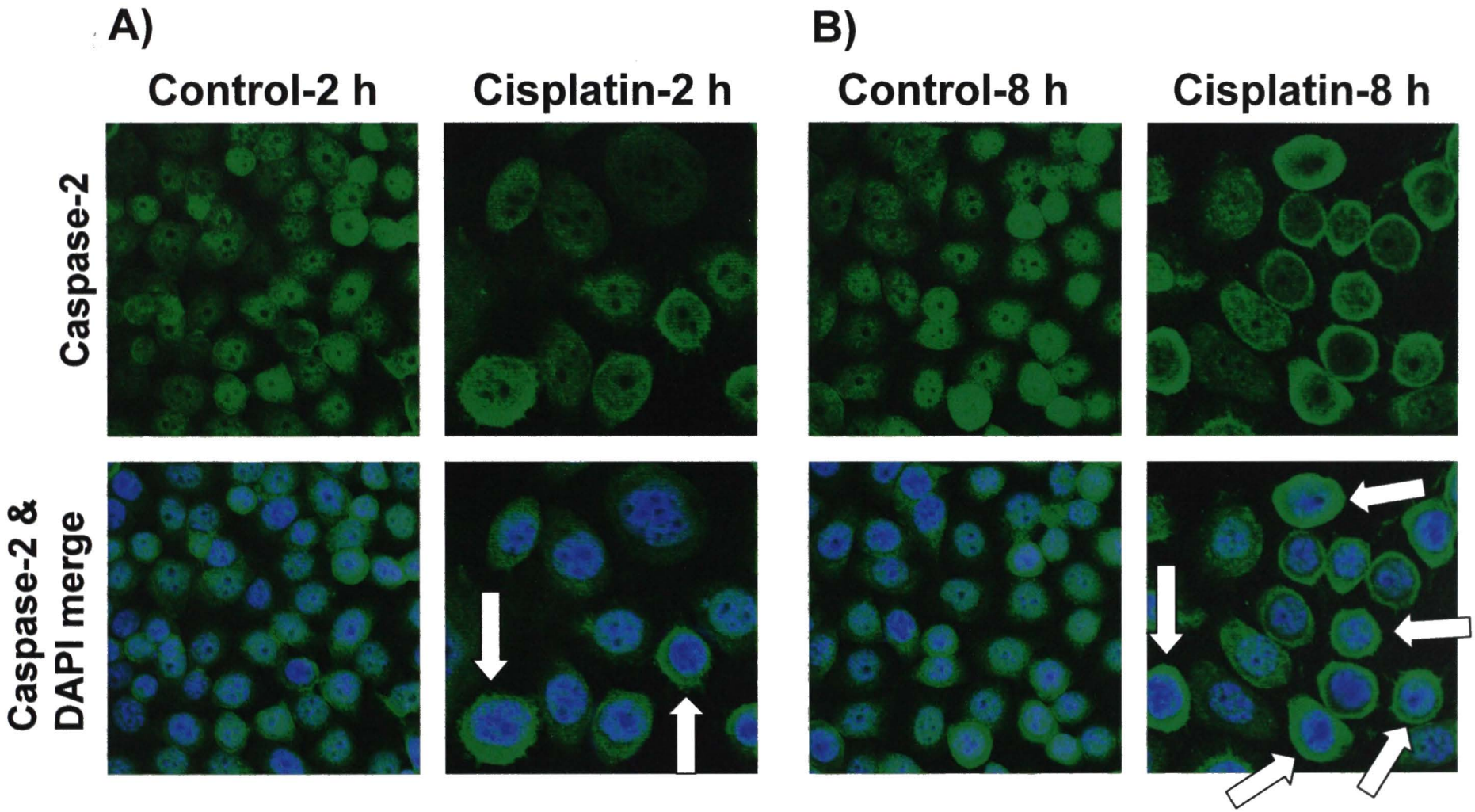


Figure 9. Cisplatin altered intracellular localization of caspase-2.

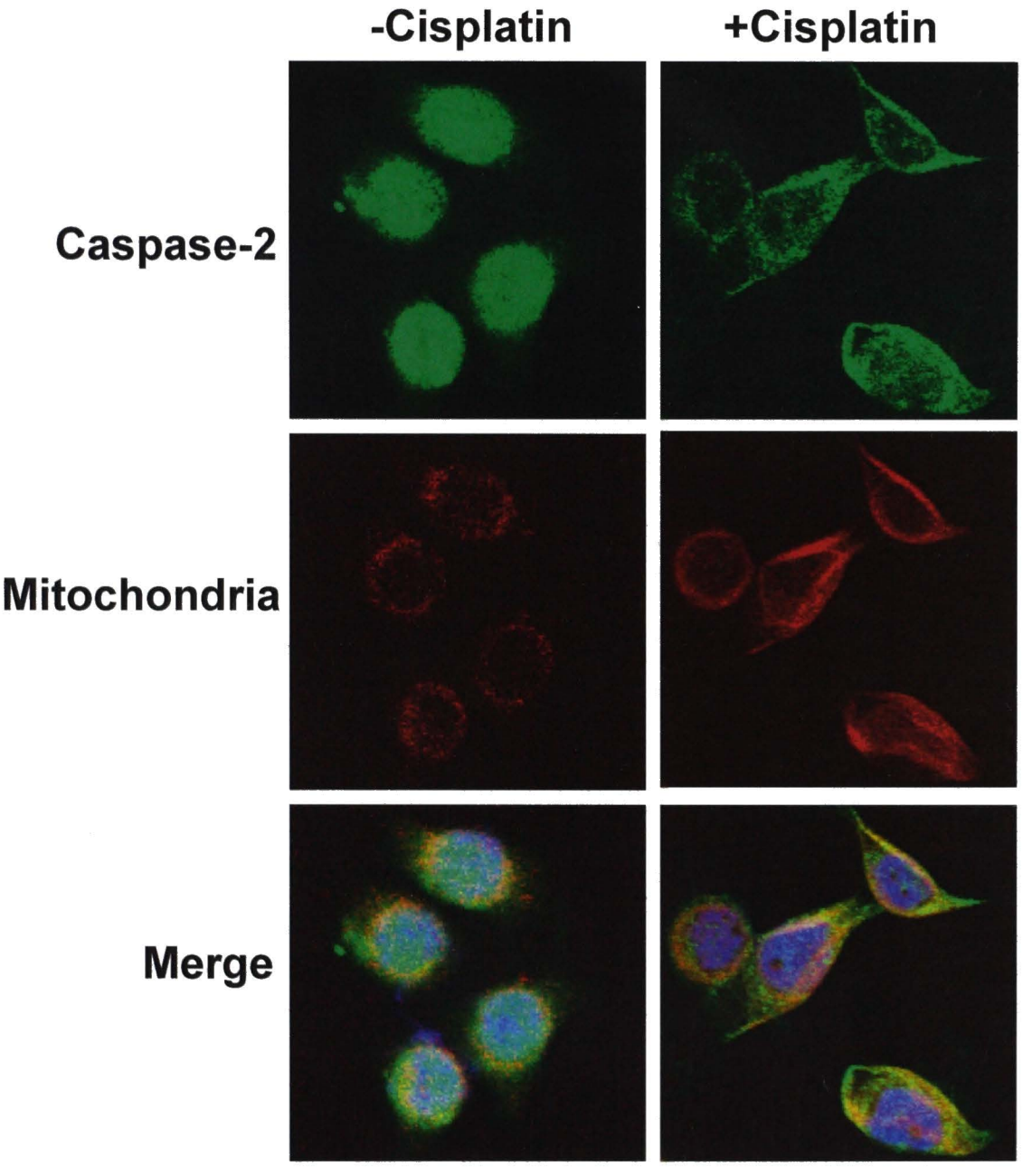


Figure 10A. Cisplatin induced caspase-2 translocation to the mitochondria.

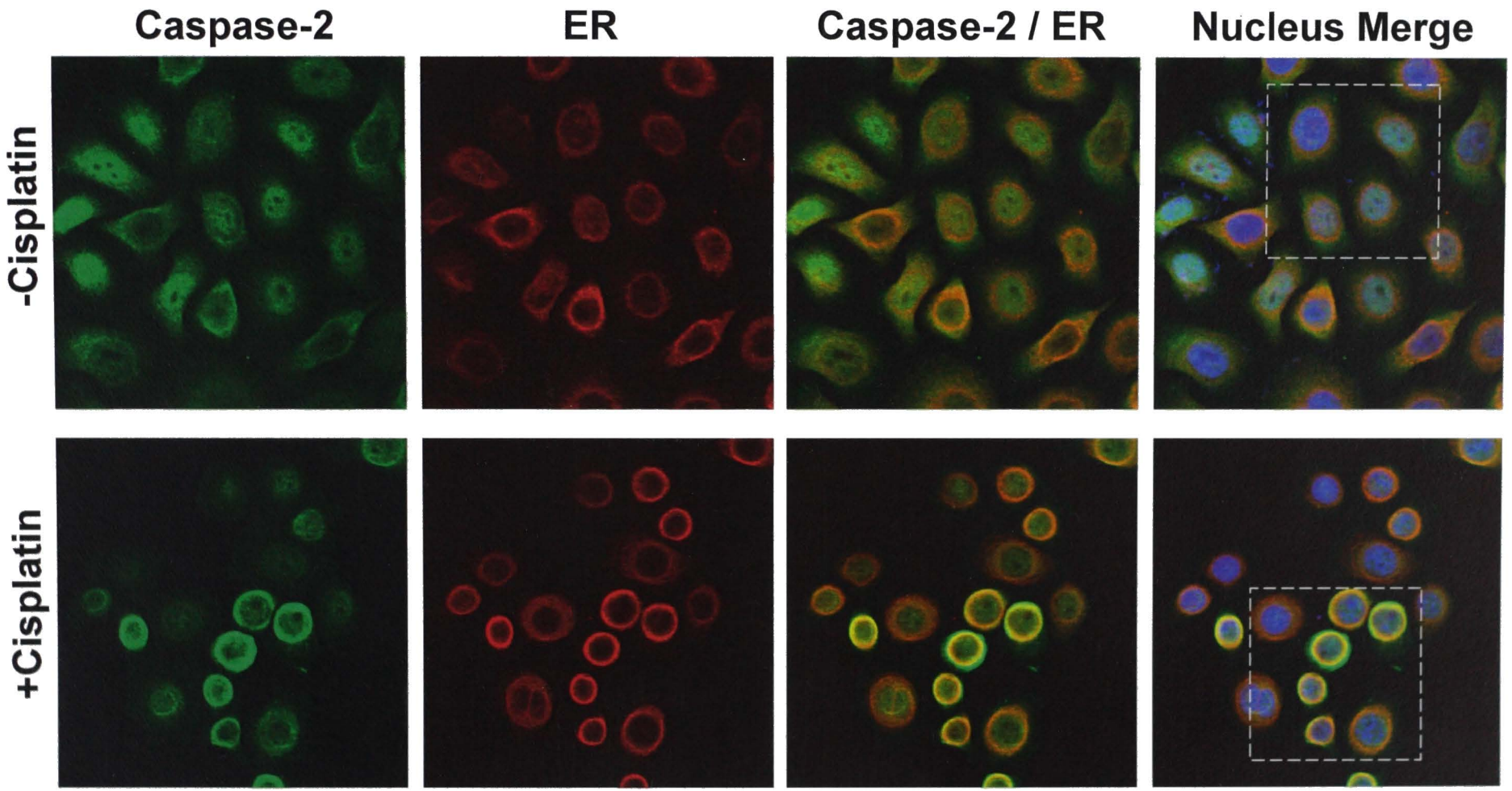


Figure 10B. Cisplatin induced caspase-2 translocation to the endoplasmic reticulum.

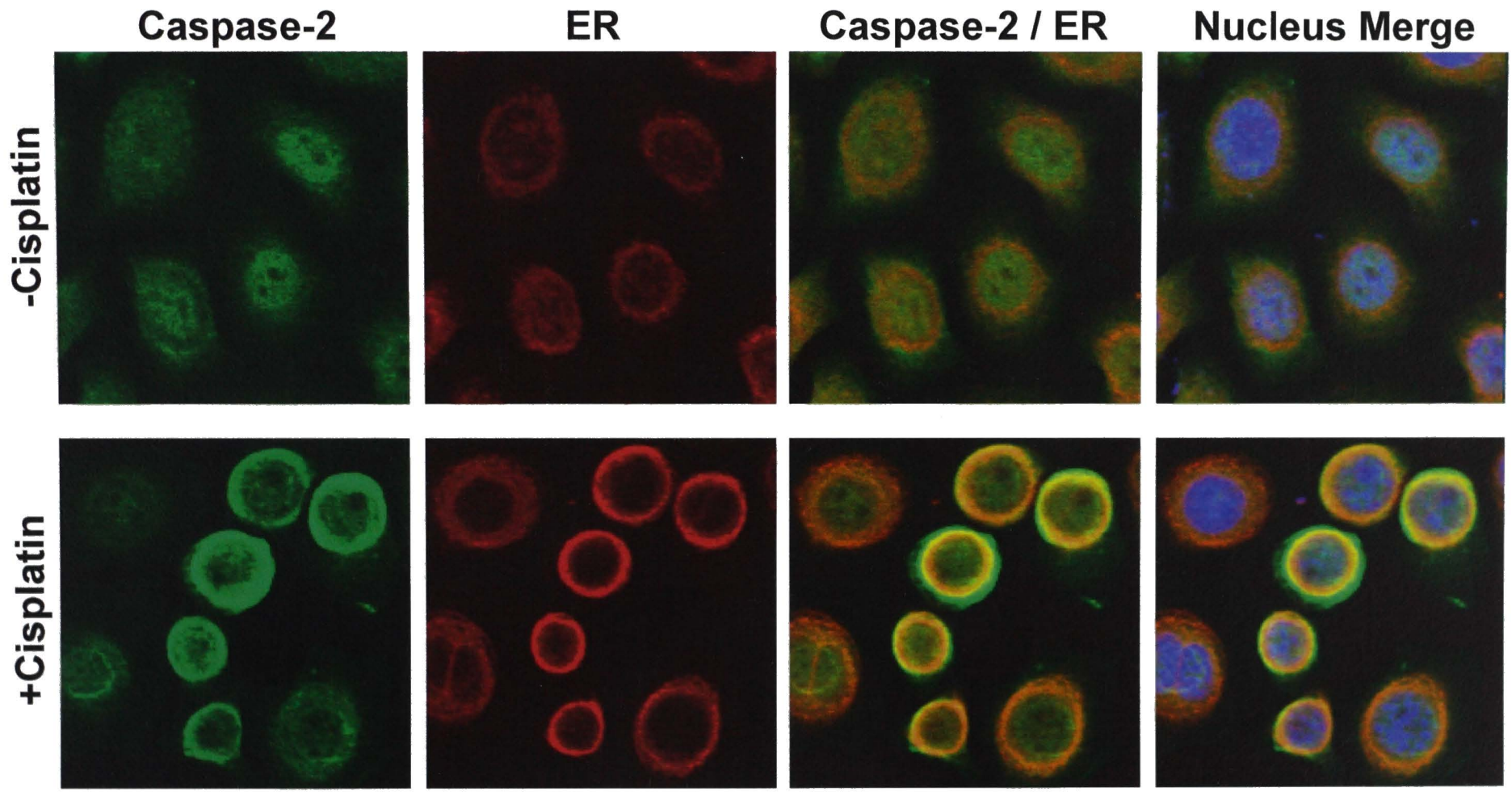


Figure 10C. Enlarged view of 10B

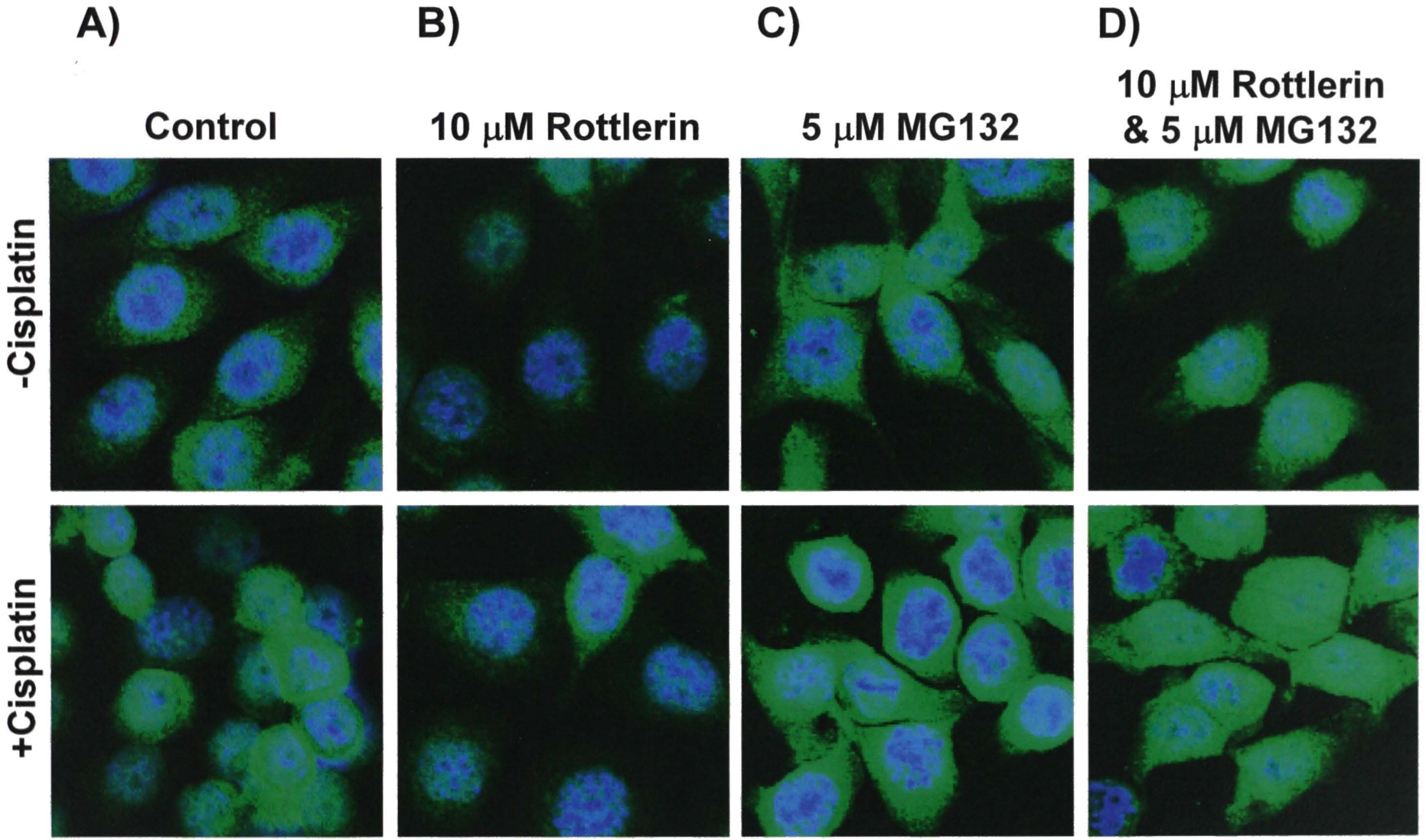
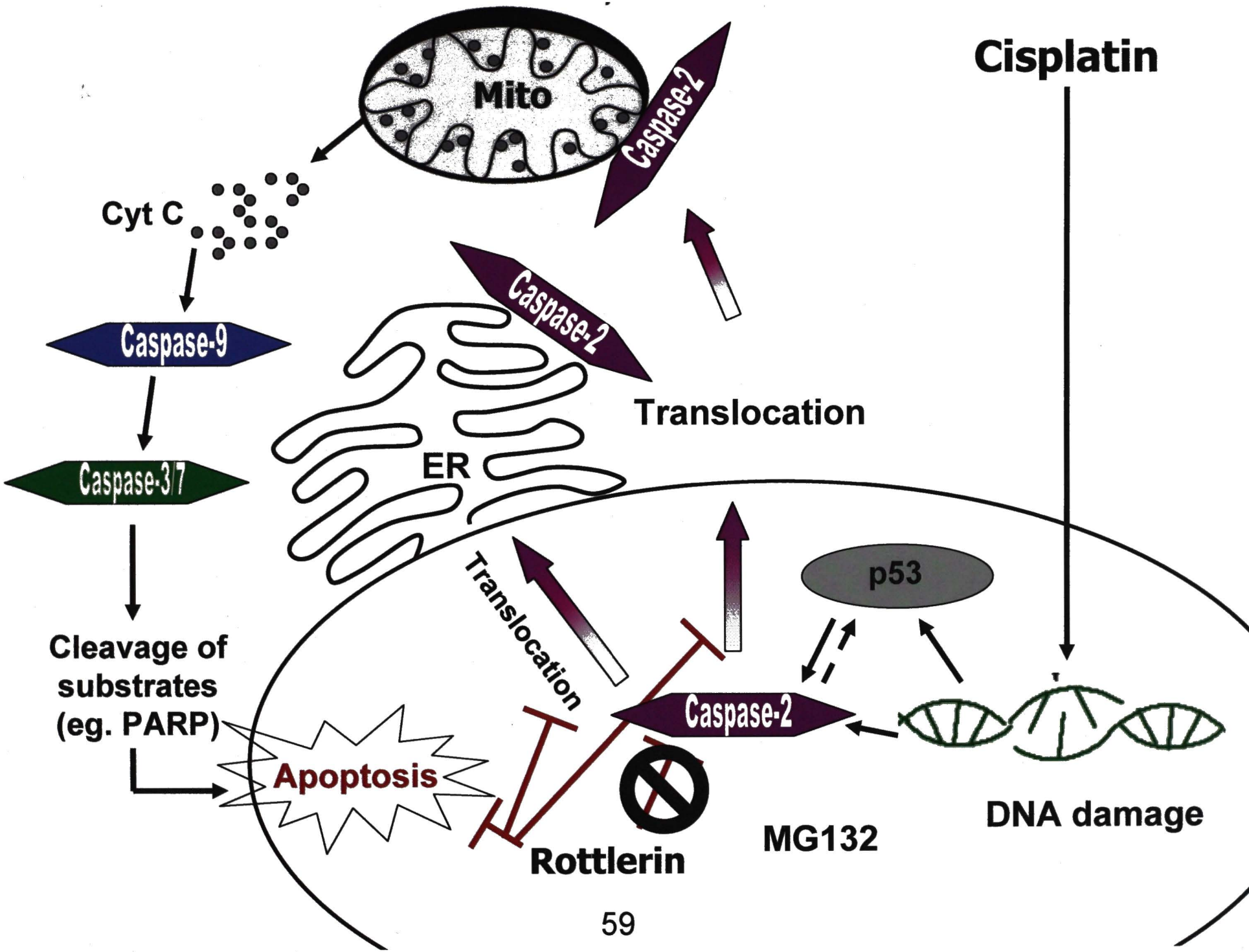


Figure 11. Perinuclear localization of caspase-2 is important for cisplatin-induced apoptosis.


Mito
Caspase-2
Cisplatin
Cyt C
Caspase-9
Caspase-2
Translocation
ER
Caspase-3/7
p53
Translocation
Cleavage of substrates (eg. PARP)
Caspase-2
Apoptosis
DNA damage
Rottlerin
MG132