Biochemical Pharmacology 79 (2010) 137–146

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Proteasome inhibitors prevent cisplatin-induced mitochondrial release of apoptosis-inducing factor and markedly ameliorate cisplatin nephrotoxicity

Ling Liu [b], Cheng Yang [b], Christian Herzog [b], Rohit Seth [b], Gur P. Kaushal [a,b,c,*]

[a] Central Arkansas Veterans Healthcare System, Little Rock, AR 72205, United States
[b] University of Arkansas for Medical Sciences, Department of Medicine, Little Rock, Arkansas 72205, United States
[c] University of Arkansas for Medical Sciences, Department of Biochemistry, Little Rock, Arkansas 72205, United States

ARTICLE INFO

Article history:
Received 13 April 2009
Accepted 14 August 2009

Keywords:
AIF
Mitochondria
Apoptosis
Mcl-1
Bax
PS-341
MG-132
Acute kidney injury

ABSTRACT

We demonstrate the effect of proteasome inhibitors in mitochondrial release of apoptosis-inducing factor (AIF) in cisplatin-exposed renal tubular epithelial cells (LLC-PK_1 cells) and in a model of cisplatin nephrotoxicity. Immunofluorescence and subcellular fractionation studies revealed cisplatin-induced translocation of AIF from the mitochondria to nucleus. Mcl-1, a pro-survival member of the Bcl-2 family, is rapidly eliminated on exposure of renal cells to cisplatin. Proteasome inhibitors PS-341 and MG-132 blocked cisplatin-induced Mcl-1 depletion and markedly prevented mitochondrial release of AIF. PS-341 and MG132 also blocked cisplatin-induced activation of executioner caspases and apoptosis. These studies suggest that proteasome inhibitors prevent cisplatin-induced caspase-dependent and -independent pathways. Overexpression of Mcl-1 was effective in blocking cisplatin-induced cytochrome *c* and AIF release from the mitochondria. Downregulation of Mcl-1 by small interfering RNA promoted Bax activation and cytochrome *c* and AIF release, suggesting that cisplatin-induced Mcl-1 depletion and associated Bax activation are involved in the release of AIF. Expression of AIF protein in the mouse was highest in the kidney compared to the heart, brain, intestine, liver, lung, muscle, and spleen. In an *in vivo* model of cisplatin nephrotoxicity, proteasome inhibitor MG-132 prevented mitochondrial release of AIF and markedly attenuated acute kidney injury as assessed by renal function and histology. These studies provide evidence for the first time that the proteasome inhibitors prevent cisplatin-induced mitochondrial release of AIF, provide cellular protection, and markedly ameliorate cisplatin-induced *acute kidney injury*. Thus, AIF is an important therapeutic target in cisplatin nephrotoxicity and cisplatin-induced depletion of Mcl-1 is an important pathway involved in AIF release.

Published by Elsevier Inc.

1. Introduction

Nephrotoxicity or toxic acute kidney injury (AKI) is one of the major side effects of cisplatin chemotherapy used for several solid tumors [1]. Cisplatin preferentially accumulates in the kidney and is associated with damage to proximal tubular epithelial cells, primarily in the S3 segment. Although mitochondrial dysfunction is an important component of renal tubular epithelial cells (RTEC) injury in cisplatin nephrotoxicity [2] the precise mechanism of cisplatin-induced mitochondrial apoptotic pathway is not entirely understood.

Apoptosis-inducing factor (AIF) is an evolutionarily-conserved flavoprotein that resides exclusively in the intermembrane space of the mitochondria [3,4]. In response to a specific apoptotic stimulus, AIF is released from the mitochondria and is translocated to the nucleus [3,5]. The presence of two nuclear localization signals enables AIF to translocate to the nucleus [4,6]. When in the nucleus, AIF associates with chromatin and the nuclear protein cyclophilin A [7] and causes nuclear chromatin condensation, DNA fragmentation, and cell death [3]. Following mitochondrial release and translocation to the nucleus, AIF promotes DNA fragmentation and apoptosis independently of caspases [3]. Recent evidence suggests that AIF also plays a role in maintaining the mitochondrial respiratory function in normal physiological conditions [8,9]. Mitochondrial AIF protects cells from oxidative stress by detoxification of reactive oxygen species that are normally released by the respiratory chain. AIF is also involved in the complex I function of the respiratory chain in the mitochondria [8–10] but is not a part of complex I [9]. Therefore, it is conceivable that translocation of AIF from the mitochondria to the nucleus not only can cause nuclear chromatinolysis and DNA fragmentation, but also that the subsequent lack of it in the mitochondria can simultaneously

* Corresponding author at: Central Arkansas Veterans Healthcare System, 4300 West 7th St., Little Rock, AR 72205, United States. Tel.: +1 501 257 5834; fax: +1 501 257 5827.
E-mail address: Kaushalgurp@uams.edu (G.P. Kaushal).

0006-2952/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.bcp.2009.08.015

compromise oxidative phosphorylation. This implies that agents that can block the mitochondrial release of AIF will protect cells from the proapoptotic role of AIF.

We previously demonstrated that, along with the release of cytochrome *c*, significant apoptosis-inducing factor (AIF) is also released from the mitochondria upon exposure of RTEC to cisplatin [11]. Cisplatin-induced apoptosis is contributed by both caspase-3-dependent and caspase-3-independent mechanisms [12]. This is consistent with our studies on cisplatin-induced activation of all executioner caspases and the mitochondrial release of AIF [11] that is translocated to the nucleus and promotes DNA fragmentation independent of caspases. We have recently shown that proteasome inhibitors MG-132 or lactacystin markedly prevented cisplatin-induced apoptosis in cultured RTEC [13] suggesting that proteasome inhibition is a potential target in cisplatin nephrotoxicity. In fact in a related study, the proteasome inhibitor, lactacystin has been shown to attenuate renal ischemia-reperfusion injury [14], but the precise cellular mechanism in the protection by proteasome inhibition is not known.

In cisplatin injury to RTEC, we have demonstrated that proteasome inhibitors blocked not only cisplatin-induced mitochondrial release of cytochrome *c* and subsequent caspase activation and apoptosis, but also prevented cisplatin-induced loss of the pro-survival Bcl-2 family member Mcl-1 [13]. We showed that Mcl-1 was highly susceptible to cisplatin injury and was rapidly depleted on exposure of RTEC to cisplatin. Cisplatin-induced loss of Mcl-1 resulted from posttranslational degradation by the ubiquitin-proteasome system rather than by transcriptional inhibition [13]. Overexpression of Mcl-1 or prevention of cisplatin-induced Mcl-1 depletion by the proteasome inhibitors provided protection from cisplatin-induced apoptosis [13]. These studies together suggest that proteasome-mediated Mcl-1 elimination is crucial in cisplatin-induced apoptosis via the mitochondrial apoptotic pathway. However, it is not known whether proteasome-mediated Mcl-1 degradation controls AIF release from the mitochondria.

Although proteasome inhibitor MG-132 prevented cisplatin-induced caspase activation and apoptosis, it was of interest to examine the role of proteasome inhibitors in preventing caspase-independent apoptosis and providing protection from cisplatin nephrotoxicity. The present study was therefore undertaken to determine whether (i) proteasome inhibitors including recently developed selective and potent inhibitor PS-341 (also called as Bortezomib or Velcade) [15] play a role in preventing cisplatin-induced mitochondrial release of AIF; (ii) proteasome-mediated Mcl-1 depletion is involved in cisplatin-induced mitochondrial release of AIF; and (iii) proteasome inhibition confers protection in cisplatin nephrotoxicity *in vivo*.

2. Materials and methods

2.1. Cell culture and reagents

LLC-PK_1 cells (porcine kidney proximal tubular epithelial cells) are the RTEC used in the present study. These were purchased from American Type Culture Collection (ATCC, Manassas, VA) and were cultured as described in our previous studies [11]. Cultures were maintained in a humidified incubator with 5% CO_2–95% air at 37 °C and were provided fresh medium at 48–72-h intervals. Cells were plated at a density of 5×10^4 cells/cm^2 in six-well plates. When cells were ~80% confluent, cisplatin was added to cultures to a final concentration of 50 μM. Ovarian cancer cells A2780 were obtained from Sigma and cultured according to the supplier's instructions. Antibodies to AIF (sc-13116), α-tubulin (sc-8035), and α-actinin (sc-15335) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to active caspase-3 (catalog no. 9664) was obtained from Cell Signaling Technology (Beverly, MA). Proteasome inhibitor MG-132 was from Calbiochem (San Diego, CA) and PS-341 was from Millennium Pharmaceuticals. Cell transfection reagents, primers, and one-step RT-PCR kit were from Invitrogen (Carlsbad, CA). Plasmid preparation kit, nucleotide purification kit, and RNeasy RNA purification kit were from Qiagen (Valencia, CA). All other chemicals were from Sigma (St. Louis, MO) or Fisher Scientific (Hampton, NH).

2.2. Immunofluorescence localization of AIF and active caspase-3

Cells were grown on sterile glass coverslips and treated with 50 μM cisplatin for 16 h in the presence or absence of 5 μM MG-132 and 1 μM PS-341. Following treatments, cells were washed in PBS and fixed in 2% paraformaldehyde in PBS for 15 min. After being washed twice in PBS, cells were permeabilized for 1 h in blocking buffer containing 1% BSA, 1% goat serum, 0.1% saponin, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 2 mM NaV_2O_5 in PBS. The cells were then incubated with mouse monoclonal anti-AIF antibody (1:200) or rabbit anti-caspase-3 (cleaved) antibody (1:200) for 1 h in a 37 °C humidified incubator. After three washes in washing buffer containing 1% bovine serum albumin and 0.1% saponin in PBS, the cells were incubated at 37 °C in a humidified incubator for 1 h with a 1:500 dilution of Alexa Fluor-conjugated secondary antibody (goat anti-mouse Alexa Fluor 488 for AIF and goat anti-rabbit Alexa Fluor 594 for cleaved caspase-3) in blocking solution and again washed with washing buffer. The nuclei were stained with 0.5 μg/ml of 4′,6′-diamidino-2-phenylindole (DAPI) for 5 min, and the cells were washed twice in washing buffer. Coverslips were then mounted on slides using antifade mounting medium (Molecular Probes). Localization of AIF, active caspase-3, and morphological changes of the nuclei were analyzed using a Zeiss deconvolution microscope.

2.3. Subcellular fractionation

2.3.1. Isolation of mitochondria

Mitochondria were isolated by sucrose density gradient centrifugation essentially as described [16] and used in our previous studies [11] with minor modifications. Briefly, cells were washed with PBS containing 1 mM EDTA and resuspended in isotonic homogenization buffer supplemented with 1× protease inhibitor mixture (Sigma). Cells were broken with 30–35 strokes of a Dounce homogenizer (Wheaton), and the suspension was centrifuged at 2000 × *g* in an Eppendorf centrifuge at 4 °C. The supernatant was then centrifuged at 13,000 × *g* at 4 °C for 10 min. The pellet was resuspended in 1 ml of homogenization buffer and layered on top of a discontinuous sucrose gradient consisting of 20 ml of 1.2 M sucrose, 10 mM HEPES, pH 7.5, 1 mM EDTA, and 0.1% bovine serum albumin on top of 17 ml of 1.6 M sucrose, 10 mM HEPES, pH 7.5, 1 mM EDTA, and 0.1% bovine serum albumin. The samples were centrifuged at 27,000 rpm for 2 h at 4 °C using a Beckman SW28 rotor. Mitochondria recovered at the 1.6–1.2 M sucrose interface were washed and resuspended in homogenization buffer. This procedure results in the mitochondrial preparation with very little contamination of other organelles. Contamination of mitochondria in the nuclear fraction was determined by immunoblotting for cytochrome oxidase subunit IV, an integral membrane protein of the mitochondria.

2.3.2. Isolation of nuclear fraction

The nuclei were prepared by a previously described procedure [11]. Briefly, LLC-PK_1 cells were gently scraped using the rubber policeman, harvested by centrifugation, and washed twice with PBS. The cells were resuspended in homogenization buffer containing 210 mM mannitol, 70 mM sucrose, 1 mM EDTA,

10 mM HEPES, pH 7.5; supplemented with 1× protease inhibitor mixture (Sigma); and homogenized with 30–35 strokes of a Dounce homogenizer (Wheaton). To establish the number of stokes for cell permeabilization, the trypan blue exclusion method (0.4% trypan blue solution in PBS diluted 1:10 with cell suspension), which discriminates between permeabilized (stained) cells and intact cells (unstained), was used. The suspension was then transferred to Eppendorf centrifuge tubes and centrifuged at 500 × *g* for 10 min to pellet nuclei and unbroken cells. The nuclear fraction was adjusted to the final concentration of 0.25 M sucrose and 0.35% Triton X-100 and layered on top of a discontinuous sucrose density gradient prepared with 0.32, 0.8, and 1.2 M sucrose in a Beckman centrifuge. The tubes were centrifuged at 40,000 × *g* for 2 h. The nuclei were recovered at the interface of 0.8 and 1.2 M sucrose. Samples were stored at −70 °C before being used for Western blot analysis.

2.4. Immunoprecipitation and Western blots

Immunoprecipitation was carried out to determine activation of Bax. Cells were harvested, washed once in cold phosphate-buffered saline, and lysed in a buffer P (10 mM HEPES pH 7.4, 150 mM NaCl, 1% CHAPS supplemented with complete protease inhibitors (Roche Applied Science)) for 30 min on ice. Thereafter, 400 μl cleared lysates were incubated with Bax 6A7 monoclonal antibody (556467, BD PharMingen, San Diego, CA) and 50 μl (50% slurry) protein G-sepharose beads (17-0618-01, GE Healthcare, Piscataway, NJ) in an overhead rotator at 4 °C overnight. After extensive washing with buffer P the immunoprecipitated complex were resolved on 12% NuPAGE Bis–Tris Gel (Invitrogen, Carlsbad, CA). Western blot was performed by using Bax (B-9) antibody (sc-7480, Santa Cruz Biotechnology, Santa Cruz, CA). For Western blot analysis in general, proteins were subjected to SDS–PAGE, transferred to a Transblot membrane, and blotted with specific antibodies. Blots were developed and detected by enhanced chemiluminescence.

2.5. Overexpression of Mcl-1

Transfection was carried out with Lipofectamine 2000 (Invitrogen) as per manufacturer's recommendation with plasmid DNA (3xFLAGCMV/Mcl-1, kindly provided by Dr. X. Wang). Plasmid DNA without the insert was used as a control. LLC-PK_1 cells growing at 70–80% confluence were transfected with Mcl-1 plasmid DNA or empty vector for 36 h and then treated with 50 μM cisplatin.

2.6. RNA interference

RTECs were plated in a six-well plate with complete medium. When the cells were 70% confluent, old medium was replaced with fresh medium without serum and antibiotics. Cells were transfected with Mcl-1 siRNA (sc-35877, Santa Cruz Biotechnology, Santa Cruz, CA) according to manufactures protocol and as described in our studies [17]. Briefly, cells were transfected with 480 pmols Mcl-1 or control scrambled siRNA-A (sc-37007, Santa Cruz Biotechnology, Santa Cruz, CA) in 10 cm culture dishes for 24 h.

2.7. Animals and induction of cisplatin-induced AKI

Ten-week-old C57BL/6 male mice weighing 20–25 g were obtained from the Jackson Laboratories (Bar Harbor, ME, USA) and maintained on a standard diet with free access to water. Animal protocol to develop a mice model of cisplatin-induced AKI complied with the standards for care and use of animal subjects, and was approved by the Institutional Animal Care and Use Committee and Institutional Safety Committee. Mice ($n = 6$) were given a single intraperitoneal injection of cisplatin (20 mg/kg b.w.) or MG-132 (2 mg/kg b.w.) 1 h before the injection of cisplatin (20 mg/kg b.w.). Control animals ($n = 6$, in each group) were administered either 5% DMSO in saline (50 μl/mice) or MG-132 (2 mg/kg b.w.) in 5% DMSO in saline. Kidneys were harvested at 1, 2, 3, 4, and 5 days for renal function and histology. Blood was collected for BUN and serum creatinine level determinations. BUN and creatinine were determined using a diagnostic kit from International Bio-Analytical Industries Inc. (Boca Raton, FL, USA).

2.8. Kidney histology

Kidney tissue was fixed in phosphate-buffered 4% formalin (pH 7.4) for 24 h and then embedded in paraffin. Sections were cut into 5 μm-thick slices and used for staining with periodic acid-Schiff stain (PAS). Tubular injury including brush-border loss, tubule dilatation, tubule necrosis, and tubule cast formation after cisplatin injection was evaluated in PAS-stained sections using a semiquantitative scale ranging from 0 to 4 in which no injury (0), mild (1), moderate (2), severe (3), and to very severe (4) were assigned. The slides were evaluated and scored in a blinded fashion to the treatment by a renal pathologist.

2.9. Statistical analyses

Results are mean ± SEM. Comparison between values was determined by the Student's *t* test. A *P* value of less than 0.05 was considered significant.

3. Results

3.1. Proteasome inhibitors block AIF translocation to nucleus

Immunofluorescence analysis of control and cisplatin-treated LLC-PK_1 cells revealed translocation of AIF from the mitochondria into the nucleus of many cells (Fig. 1A). As shown, control cells reveal exclusively mitochondrial punctate staining for AIF with absolutely no nuclear staining. Cisplatin-treated cells show nuclear staining of AIF indicating translocation of AIF from the mitochondria. Staining with DAPI revealed many cisplatin-induced condensed and fragmented nuclei that co-stained with AIF. When cells were treated for 30 min with proteasome inhibitors PS-341 or MG-132 prior to the cisplatin exposure and immunostained with anti-AIF antibody no nuclear staining for AIF was observed (Fig. 1A) suggesting that PS-341 or MG-132 blocked translocation of AIF to the nucleus. PS-341, a selective and potent proteasome inhibitor (also known as Bortezomib or Velcade) is a dipeptide boronate (pyrazylcarbonyl-phenylalanyl-leucyl-boronate) originally developed at ProScript and now distributed by Millennium Pharmaceuticals [15]. MG-132 (z-Leu-Leu-Leu-CHO) is a potent and cell permeable proteasome inhibitor. We have used structurally different proteasome inhibitors to ensure that the effect is not specific to only one kind of inhibitor. To examine whether PS-341 blocks cisplatin-induced caspase-3 activation we performed immunostaining with antibody to the active caspase-3. Most of the cisplatin-induced apoptotic nuclei displayed localization for active caspase-3 which was prevented by PS-341 (Fig. 1B). Activation of other executioner caspases (caspase-6 and caspase-7) was also blocked by PS-341 or MG-132 (not shown). Western blot analysis of cytosolic and nuclear fractions from control and cisplatin-treated LLC-PK_1 cells also revealed active caspase-3 in the nucleus and this nuclear localization was markedly prevented by treatment with PS-341 or MG-132 (Fig. 1C). These studies demonstrate that the proteasome inhibitors prevent cisplatin-induced AIF translocation as well as

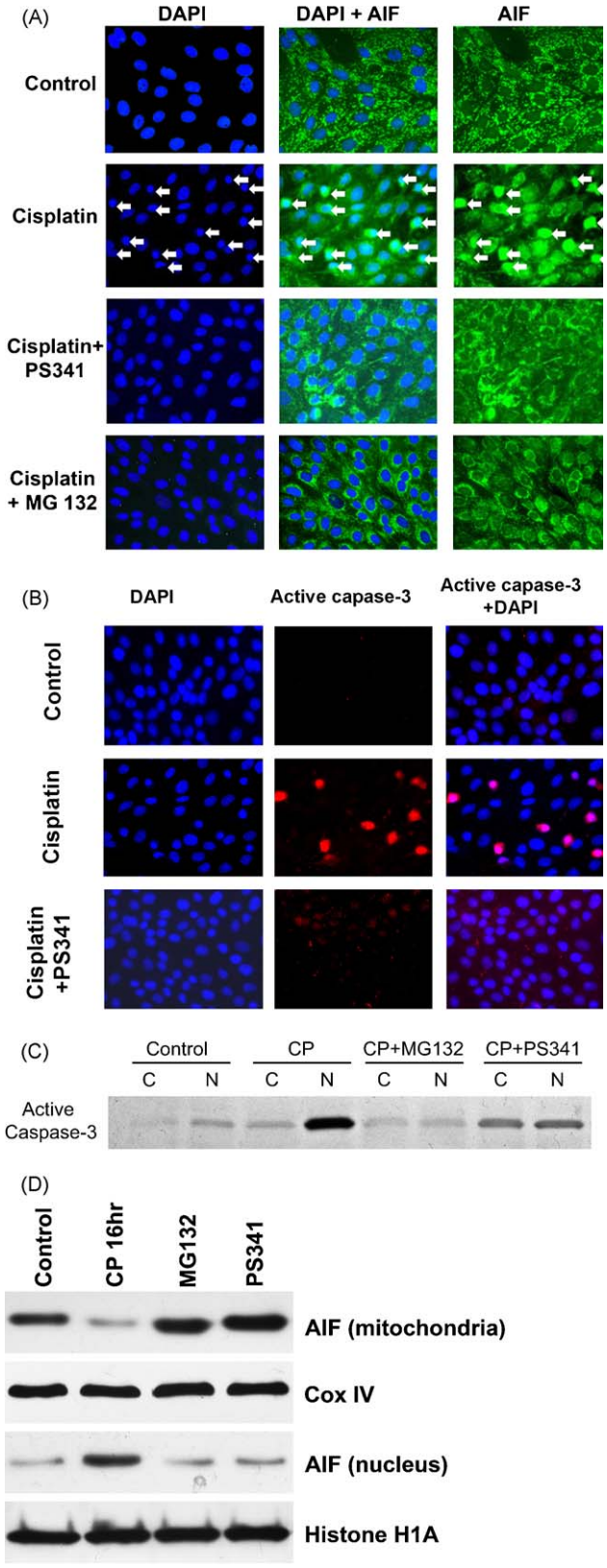

Fig. 1. (A) PS-341 and MG-132 block cisplatin-induced AIF translocation. Cells were untreated or treated with 50 μM cisplatin in the presence and absence of 1 μM PS-341 or 5 μM MG-132 or for 16 h. MG-132 was added 30 min prior to the treatment with cisplatin. Cells were stained with anti-AIF (*green*) followed by DAPI (*blue*) for nuclei, and images were overlaid as shown. Control cells show clear staining with DAPI for intact nuclei (*blue*) and clear punctate staining for AIF (*green*) exclusively in the mitochondria without nuclear staining. Cisplatin-treated cells show fragmented and condensed nuclear staining with DAPI (*blue*) and prominent staining for nuclear AIF (*green*) that co-localizes with nuclei (*blue*). (B) PS-341 blocks cisplatin-induced activation of caspase-3. Cells were untreated or treated with 50 μM cisplatin in the presence and absence of 1 μM PS-341 for 12 h. Cells were stained with anti-active caspase-3 (*red*) antibody followed by DAPI (*blue*) for nuclei, and images were overlaid as shown. (C) Active caspase-3 in nuclear fraction and effect of PS-341 and MG-132. Cells were untreated or treated with 5 μM MG-132 or 1 μM PS-341 for 30 min prior to the treatment with 50 μM cisplatin for 16 h. Cytosolic and nuclear fractions were isolated as described under Section 2 and we loaded 10 μg protein in each lane for the nuclear fractions and 50 μg protein in each lane for the cytosolic fractions. Western blot was done using antibody to active caspase-3. Control lanes are untreated cells. (D) Analysis of the AIF translocation by subcellular fractionation. Cells were treated with MG-132 or PS-341 for 30 min prior to the treatment with 50 μM cisplatin. Mitochondrial and nuclear fractions were isolated as described under Section 2 and 10 μg of mitochondrial proteins, 10 μg of nuclear proteins, and 50 μg of cytosolic proteins were electrophoresed and immunoblotted for AIF. Blots for cytochrome *c* oxidase subunit IV, and histone H1A confirm equivalent loading for the mitochondrial and nuclear fractions, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

caspase activation. Western blot analysis of the subcellular fractions also revealed translocation of AIF from mitochondria to the nuclei (Fig. 1D). In control cells, AIF was primarily present in the mitochondrial fraction, and a very small amount was detected in the nuclear fraction. In cisplatin-treated cells, mitochondrial AIF was considerably reduced and was predominantly detected in the nuclei. In the presence of proteasome inhibitor MG-132 or PS-341, AIF was primarily in the mitochondria and very little was detected in the nuclei (Fig. 1D). Blots for cytochrome *c* oxidase subunit IV and histone H1A were included to confirm equivalent loading for the mitochondrial and nuclear fractions, respectively. There was some cross-contamination that became evident when mitochondrial marker cytochrome *c* oxidase subunit IV was assessed in the nuclear fractions, and nuclear marker histone H1A was assessed in mitochondrial fractions by immunoblotting (not shown).

3.2. Proteasome inhibitors prevent cisplatin-induced activation of caspases in renal epithelial cells but enhance caspase activation in cancer cells

We have previously shown that proteasome inhibitor MG-132 blocked cisplatin-induced caspase-3 activation in renal tubular epithelial cells [13]. In this study, the effect of proteasome inhibitors PS-341 and lactacystin was also examined on caspase-3 activation in a time-course manner. Proteasome inhibitors alone did not alter basal caspase-3 activity (data not shown). Cisplatin-induced caspase-3 activation was blocked by the proteasome inhibitors in a time-dependent manner (Fig. 2A). Nuclear staining with propidium iodide revealed that proteasome inhibitors PS-341 or lactacystin provided protection to cells from cisplatin-induced nuclear fragmentation and condensation (Fig. 2B) as we have previously observed with MG-132 in RTEC [13]. PS-341 or MG-132 also inhibited activation of other executioner caspases (not shown). In contrast to the effect in renal tubular epithelial cells, the proteasome inhibitors enhanced cisplatin-induced caspase-3 activation in an ovarian cancer cell line A2780. MG-132 alone has no effect on LLC-PK1 cells whereas it caused enhanced activation of caspase-3 in ovarian cancer cells (Fig. 2C, A and B). A recent study showed that the proteasome inhibitor PS-341 enhanced cisplatin uptake and cell killing in a synergistic manner in 2008 ovarian cancer cells [55].

3.3. Mcl-1 overexpression blocks AIF translocation to nucleus

The mechanism of cisplatin-induced mitochondrial release of AIF is not known. Mcl-1 is an antiapoptotic member of the Bcl-2 family that plays an important role in cell survival. We previously showed that among the antiapoptotic members of the Bcl-2 family,

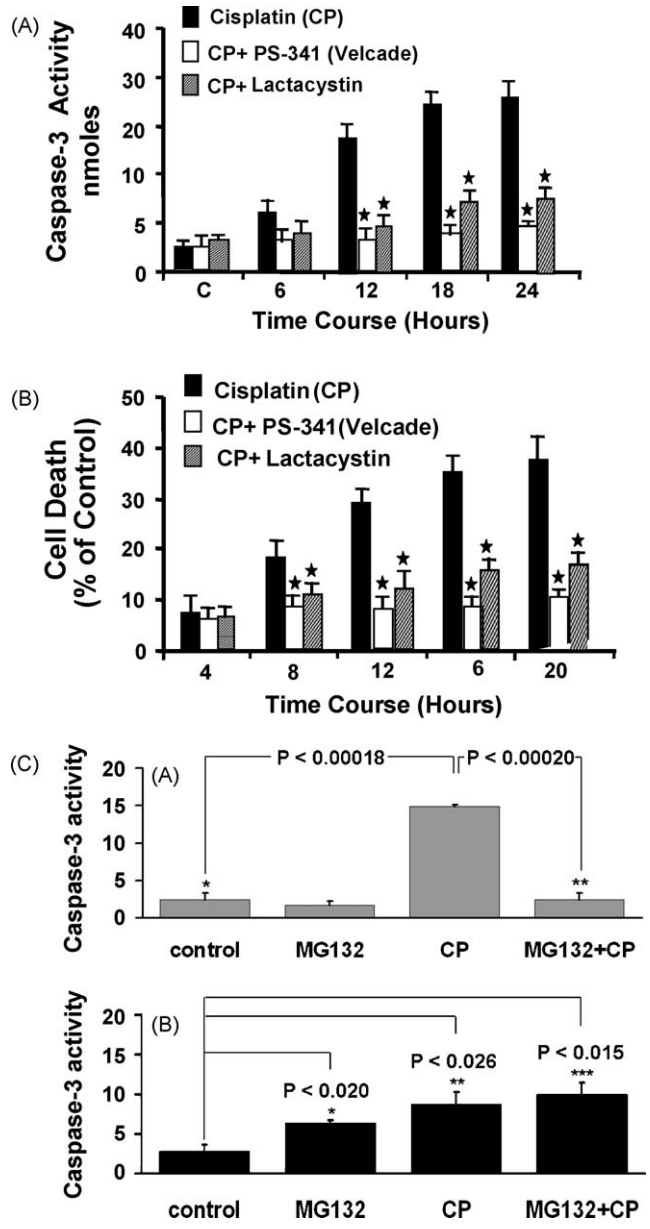

Fig. 2. (A) Time-course of inhibition of cisplatin-induced caspase-3 activation by PS-341 (Velcade) and lactacystin in RTEC. LLC-PK1 cells were treated with 50 μM cisplatin (CP) at various times, as indicated, in the presence and absence of proteasome inhibitors PS-341 (1 μM) or lactacystin (5 μM). Cell lysates (100 μg protein) were analyzed for caspase-3 activity with DEVD-AMC as the substrate [13]. The liberated AMC moiety was determined with a fluorescent spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength of 460 nm as we described previously [6]. Results are means ± SE ($n = 5$; $^*P < 0.002$ compared with CP-treated cells). (B) Time-course of inhibition of cisplatin-induced cell apoptosis by the proteasome inhibitors Velcade and lactacystin in RTEC. LLC-PK1 cells were treated with 50 μM cisplatin (CP) for varying periods of time in the presence and absence of proteasome inhibitor 1 μM PS-341 (Velcade) or 5 μM lactacystin as indicated. Following incubations, cell apoptosis was determined by DAPI staining. Results are mean ± SE, $n = 4$; $^*P < 0.001$ compared with CP-treated cells. (C) Effect of MG132 on LLC-PK1 and A2780 ovarian cancer cells. (A) LLC-PK_1 cells were grown to 70% confluency and untreated or treated with either 5 μM MG-132, 50 μM cisplatin or both MG-132 and cisplatin and harvested after 12 h. Caspase-3 activity was determined using the fluorogenic substrate DVED-AMC and changes in fluorescence were recorded in a Spectramax M5 microplate reader at Ex 380 nm/Em 460 nm. Bars represent mean ± SE ($n = 3$), P is 0.00018 for cisplatin versus control (*) and 0.0002 for cisplatin versus sample treated with both MG132 and cisplatin (**) as shown. (B) A2780 ovarian cancer cells were grown to 70% confluency and untreated or treated with either 5 μM MG-132, 50 μM cisplatin or both MG-132 and cisplatin and harvested after 12 h. Caspase-3 activity was determined using the fluorogenic substrate DVED-AMC and changes in fluorescence were recorded in a Spectramax M5 microplate reader at E_x 380 nm/E_m 460 nm. Bars represent mean ± SE ($n = 3$), P is 0.02 for MG132 versus control (*), 0.026 for cisplatin versus control (**), and 0.015 for sample treated with both MG132 and cisplatin versus control (***) as shown.

Mcl-1 expression is rapidly declined in RTEC in response to cisplatin and proteasome inhibitors MG-132 or lactacystin prevented cisplatin-induced rapid loss of Mcl-1 resulting in its accumulation in cells [13]. Thus we tested whether Mcl-1 is capable of preventing the mitochondrial release of AIF. We overexpressed full-length Mcl-1 by transient transfection of LLC-PK_1 cells and examined cisplatin-induced mitochondrial release of AIF. Transient transfection with *Mcl1* expression plasmid (p3xFLAGCMV/MCl-1) resulted in high expression levels of Mcl-1 in LLC-PK_1 cells (Fig. 3A). Cells carrying the control plasmid without the Mcl-1 insert showed Mcl-1 expression at the same level as untransfected cells. Overexpression of Mcl-1 markedly blocked cisplatin-induced release of AIF from the mitochondria (Fig. 3B) suggesting that cisplatin-induced Mcl-1 depletion is involved in the AIF release. As shown in Fig. 3B, cisplatin-induced cytochrome *c* release was also blocked by the proteasome inhibitors. Moreover, following cisplatin-induced depletion of Mcl-1, both MG-132 and PS-341 accumulate Mcl-1 (Fig. 3C). These studies suggest that Mcl-1 controls AIF release.

3.4. Mcl-1 depletion promotes Bax activation and AIF release

Recent studies provide evidence that Bax activation plays an important role in mitochondrial release of AIF [18–20]. Therefore, we first tested whether Mcl-1 depletion results in Bax activation in RTEC. Specific depletion of Mcl-1 in RTEC was achieved by using Mcl-1 specific siRNA. Mcl-1 siRNA effectively down-regulated Mcl-1 expression as scrambled siRNA did not change the expression of Mcl-1 (Fig. 4A). Upon activation Bax undergoes N-terminal conformational change, which can be monitored by immunoprecipitation using conformation-specific antibody, anti-Bax 6A7 and subsequently detection of Bax in the immunoprecipitates by Western blots using normal Bax antibody. Mcl-1 depletion by its siRNA treatment or cisplatin exposure markedly resulted in the Bax activation (Fig. 4B). Also, the proteasome inhibitor MG-132 was quite effective in preventing Bax activation (Fig. 4B). In addition, downregulation of Mcl-1 resulted in release of both AIF and cytochrome *c* (Fig. 4A). Taken together, these studies demonstrate that Mcl-1 depletion promotes Bax activation and release of mitochondrial AIF and the proteasome inhibitor MG132 prevents Bax activation.

3.5. Effect of the proteasome inhibitor MG-132 on cisplatin-induced mitochondrial release of AIF and morphological and functional renal damage in a mouse model of cisplatin nephrotoxicity

Expression of AIF protein in various organs in a C57BL/6 mouse strain revealed that AIF in the kidney is highest among the heart, brain, intestine, liver, lung, muscle, and spleen (Fig. 5A). Proteasome inhibitor MG-132 markedly prevented cisplatin-induced mitochondrial release of AIF in the kidney cortices (Fig. 5B). We then assessed the role of proteasome inhibition in the pathogenesis of cisplatin nephrotoxicity by measuring renal function and kidney histology. We administered MG-132 intraperitoneally 1 h before the cisplatin injection. The effect of MG-132 on cisplatin-induced impairment of renal function was determined by blood urea nitrogen (BUN) and creatinine values at 1, 2, and 3 days. At 3 days after cisplatin treatment, the mice showed marked deterioration in renal function as reflected in increased levels of BUN and serum creatinine. Intraperitoneal administration of 2 mg/kg body weight (b.w.) MG-132 significantly reduced cisplatin-induced elevated levels of both BUN and serum creatinine (Fig. 5C). As shown,

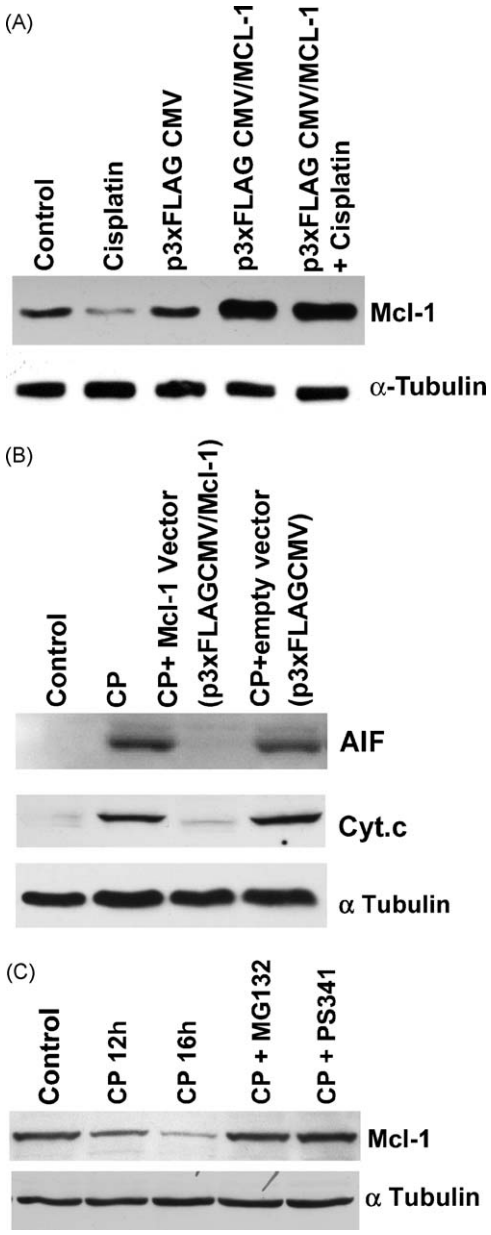

Fig. 3. (A) Overexpression of Mcl-1. LLC-PK$_1$ cells growing at 70–80% confluence were transfected transiently with Mcl-1 expression plasmid p3xFLAGCMV10/MCL-1 or the plasmid without the insert as described in the Materials and Methods section. Cell lysates (50 μg protein) were subjected to Western blot and filters were probed with an antibody to Mcl-1. As shown, α-tubulin was used as a loading control. (B) Overexpression of Mcl-1 blocks cisplatin-induced mitochondrial release of AIF. Cells were transfected with Mcl-1 expression plasmid p3xFLAGCMV10/Mcl-1 or the plasmid without the insert. After 36 h of transfection cells were treated with 50 μM cisplatin for 12 h. Cell lysates (50 μg protein) were subjected to Western blot and filters were probed with specific antibodies to AIF, cytochrome *c* and α-tubulin. Control is LLC-PK$_1$ cells without Mcl-1 vector and cisplatin. (C) Proteasome inhibitors MG-132 or PS-341 prevent cisplatin-induced depletion of Mcl-1. LLC-PK$_1$ cells were treated with 50 μM cisplatin for varying lengths of time in the presence and absence of 5 μM MG-132 or 1 μM PS-341. Cell lysates (50 μg protein in each lane) were analyzed by Western blot analysis with specific antibodies to Mcl-1 and α-tubulin. α-Tubulin was used as a control.

MG-132 alone did not effect either BUN or creatinine values. We also examined the effect of MG-132 on cisplatin-induced histological damage by staining kidney sections with periodic acid-Schiff stain (PAS). Histological assessment following cisplatin treatment revealed severe tubular damage characterized by extensive loss of tubular epithelial cells, loss of brush-border membranes, tubular dilation, cellular desquamation, and proteinaceous casts of the tubular cells (Fig. 5D, part C). Extensive loss of tubular epithelial cells in the proximal tubules is generally attributed to both necrosis and apoptosis. Proteasome inhibitor MG-132 ameliorated most of the cisplatin-induced tubular injury. The glomeruli maintained their normal structure upon cisplatin treatment. Animals treated with cisplatin and MG-132 resulted in considerable improvement in histology as reflected in significant protection from the severe tubular injury (Fig. 5D, part D). MG-132 alone had no effect on tubular injury (Fig. 5D, part B) and the kidney histology is similar to that of normal kidneys of mice administered with 5% DMSO in saline (Fig. 5D, part A). Semiquantitative evaluation of kidney sections showed a tubular necrosis score of 0.06 ± 0.02 in kidneys from mice treated with 5% DMSO in saline alone, 0.05 ± 0.04 in kidneys from mice treated with MG-132 alone, 3.9 ± 0.6 in kidneys of 3 days cisplatin-treated mice, and 0.5 ± 0.07 in mice treated with 3 days cisplatin and MG-132. The statistical differences were significant between kidneys of cisplatin-treated mice and either 5% DMSO in saline or cisplatin plus MG-132 treated mice ($n = 6$, $P < 0.005$). These studies thus demonstrate that proteasome inhibitor MG-132 provides significant protection from cisplatin-induced AKI.

4. Discussion

Our studies demonstrate that AIF is released from the mitochondria upon exposure of RTEC to cisplatin and this release is effectively blocked by the proteasome inhibitors. We provided evidence that cisplatin-induced rapid depletion of Mcl-1, an antiapoptotic member of the Bcl-2 family, was involved in mitochondrial release of AIF. Increased accumulation of Mcl-1 by proteasome inhibitors or overexpression of Mcl-1 prevented cisplatin-induced mitochondrial release of AIF. Knockdown of Mcl-1 levels by RNA interference resulted in the release of AIF and induction of apoptosis. These studies suggest that AIF release is regulated by Mcl-1 in the mitochondria. We previously reported that the proteasome-ubiquitin pathway of protein degradation is involved in the cisplatin-induced Mcl-1 depletion in RTEC [13]. These studies showed that the MG-132 blocked cisplatin-induced decline in Mcl-1, resulting in the recovery and accumulation of Mcl-1. Mcl-1 overexpression or accumulation of Mcl-1 by MG-132 prevented cisplatin-induced caspase activation and apoptosis [13]. Many previous studies have demonstrated cisplatin-induced production of reactive oxygen species (ROS). Therefore, proteasome is a likely candidate which can be affected by ROS.

Previous studies using non-renal cells have documented that rapid depletion of Mcl-1 in response to cytotoxic signals is a critical step that promotes cell death [21–28,13]. We have shown that, in cisplatin injury to RTEC, Mcl-1 is a target of the ubiquitin-proteasome system [13]. In non-renal cells, Mcl-1 is targeted for ubiquitin-proteasome-mediated degradation following DNA damage [27,29,30], anoxia [21] and growth factor withdrawal [24]. In these studies, proteasome inhibitors or overexpression of Mcl-1 provided significant protection from cell death.

Although Mcl-1 is a bonafide antiapoptotic member of the Bcl-2 family of proteins its specific role in regulating the mitochondrial pathway of apoptosis has recently emerged [31,32]. The survival function of Mcl-1 is conferred upon its interaction and binding to proapoptotic proteins including Bid, Bim, Noxa, Puma, and Bak [31–33]. In addition, Mcl-1 is able to block Bax function at the mitochondria downstream of Bax activation and translocation [34,35]. Loss of Mcl-1 contributes to activation of the mitochondrial death pathway [36] while increased Mcl-1 level contributes to suppression of the mitochondrial apoptosis pathway [37]. Ultraviolet irradiation-induced depletion of Mcl-1 in HeLa cells resulted in mitochondrial-dependent induction of apoptosis [27].

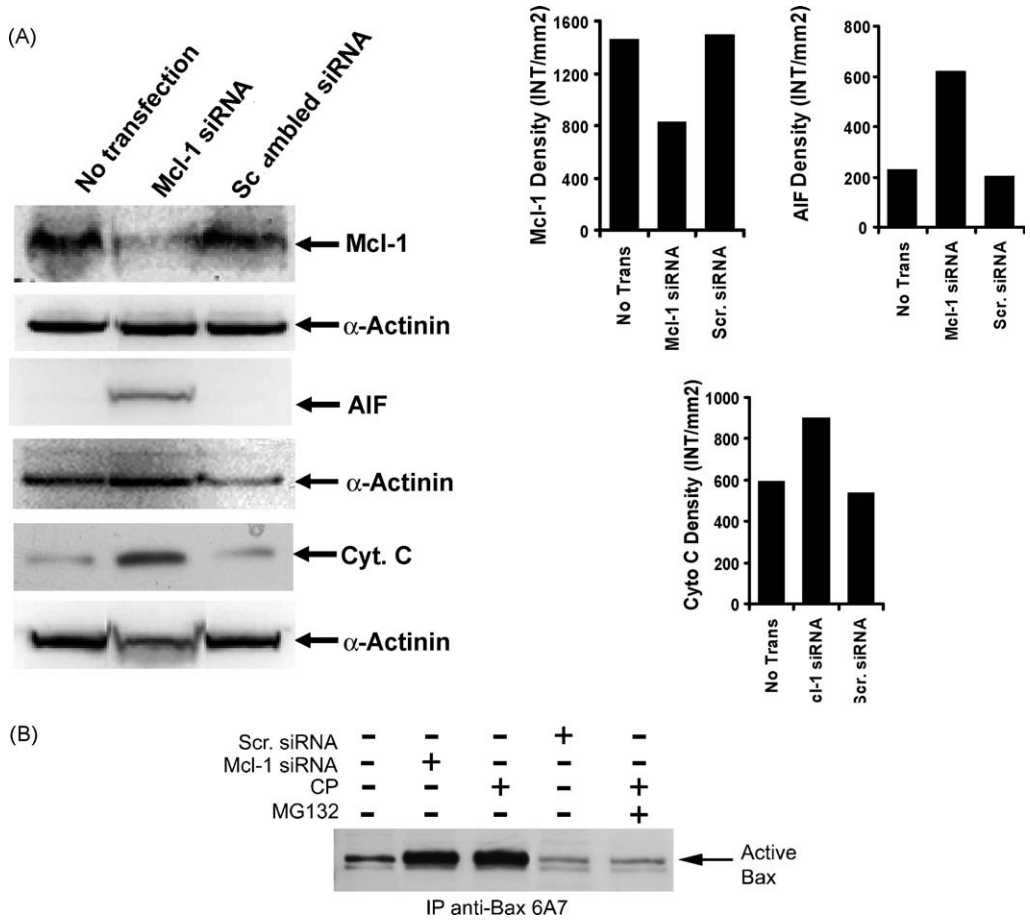

Fig. 4. Downregulation of Mcl-1 activates Bax and promotes mitochondrial AIF and cytochrome *c* release. (A) Downregulation of Mcl-1 by siRNA promotes AIF and cytochrome *c* release. *Left panel:* Cells were transfected with 480 pmols Mcl-1 or control scrambled siRNA (sc-37007, Santa Cruz Biotechnology, Santa Cruz, CA) in 10 cm culture dishes for 24 h. Cell lysates (50 μg protein in each lane) were analyzed by Western blot analysis with specific antibodies to Mcl-1, AIF, cytochrome *c*, and α-actinin using specific antibodies. *Right panels*: Quantitative analysis by densitometry of the protein levels of Mcl-1, AIF, and cytochrome *c* from the Western blot shown in the left panel. (B) Downregulation of Mcl-1 activates Bax and MG-132 prevent Bax activation. Cells were transfected with Mcl-1 siRNA or treated with 50 μM cisplatin or 50 μM cisplatin and 5 μM MG132 for 12 h. Cell lysates were immunoprecipitated with Bax 6A7 monoclonal antibody (Bax conformation-specific antibody), as described in the "Materials and Methods" sections. After washing the immunoprecipitated complex was resolved on 12% NuPAGE Bis–Tris Gel and blotted with Bax antibody as shown. In this experiment, scrambled siRNA (sc-37007, Santa Cruz Biotechnology, Santa Cruz, CA) was used as a control to show the specific effect of Mcl-1 siRNA.

Depletion of Mcl-1 can therefore free the proapoptotic BH3-only proteins that can directly activate Bax and Bak which promote mitochondrial membrane permeabilization. In a model of cycloheximide-induced apoptosis, depletion of Mcl-1 was crucial in activation of Bim, Bax and Bak [38].

Bax plays a crucial role in the mitochondrial release of AIF [18–20]. Our study and that of others have shown that Bax is activated in cisplatin injury to RTEC [2,39,40] it is likely that cisplatin-mediated Mcl-1 depletion may contribute to Bax activation and subsequent release of AIF. The mechanism underlying the release of AIF from the mitochondrial intermembrane space is not yet fully understood. A recent study has suggested that this process requires the cooperative upstream action of calpains and Bax [18]. Calpain may be involved in the cleavage of the mitochondrial membrane-associated AIF [41] while Bax facilitates the mitochondrial membrane permeabilization that is necessary for AIF release. In *Bax* (−/−) cells AIF is not released from mitochondria to the cytosol in response to alkylating DNA damage-mediated cell death induced by N-methyl-N′-nitro-N′-nitrosoguanidine [18]. A recent study has also shown that overexpression of hsp70 inhibited Bax activation and reduced mitochondrial release of AIF [42].

The expression AIF in the mouse kidney is highest compared to other various organs. Because of the role of AIF in the mitochondria for oxidative phosphorylation, the release of AIF from the mitochondria in cisplatin nephrotoxicity will not only compromise oxidative phosphorylation in the kidney but also will result in nuclear chromatinolysis and DNA fragmentation. Thus AIF is an important therapeutic target in cisplatin nephrotoxicity and perhaps for other diseases of the kidney. Therefore, an agent that can block the mitochondrial release of AIF will protect kidneys from the proapoptotic role of AIF. Some tissues such as lung, spleen, and intestine have relatively less abundance of AIF than kidney, heart, and liver. At present we do not know why muscle has relatively less abundance of AIF than in the heart and kidney. It is not known whether different homologs are present in muscle or in organs with less-abundant AIF. Our study demonstrated that proteasome inhibitor MG-132 markedly attenuated cisplatin nephrotoxicity. The protective effects of proteasome inhibitors have been demonstrated in many experimental models of cellular injury. In the kidney, the proteasome inhibitor lactacystin attenuated the development of ischemic acute renal failure in rats [14,43]. Lactacystin markedly suppressed ischemia/reperfusion-induced severe lesions, such as tubular necrosis, proteinaceous casts in tubuli, and medullary congestion [14]. The proteasome inhibitors ameliorated myocardial ischemia-reperfusion injury and preserved regional myocardial contractile function, significantly reduced the size of myocardial infarction, and prevented acute toxicity [44,45]. The proteasome inhibitor ablated

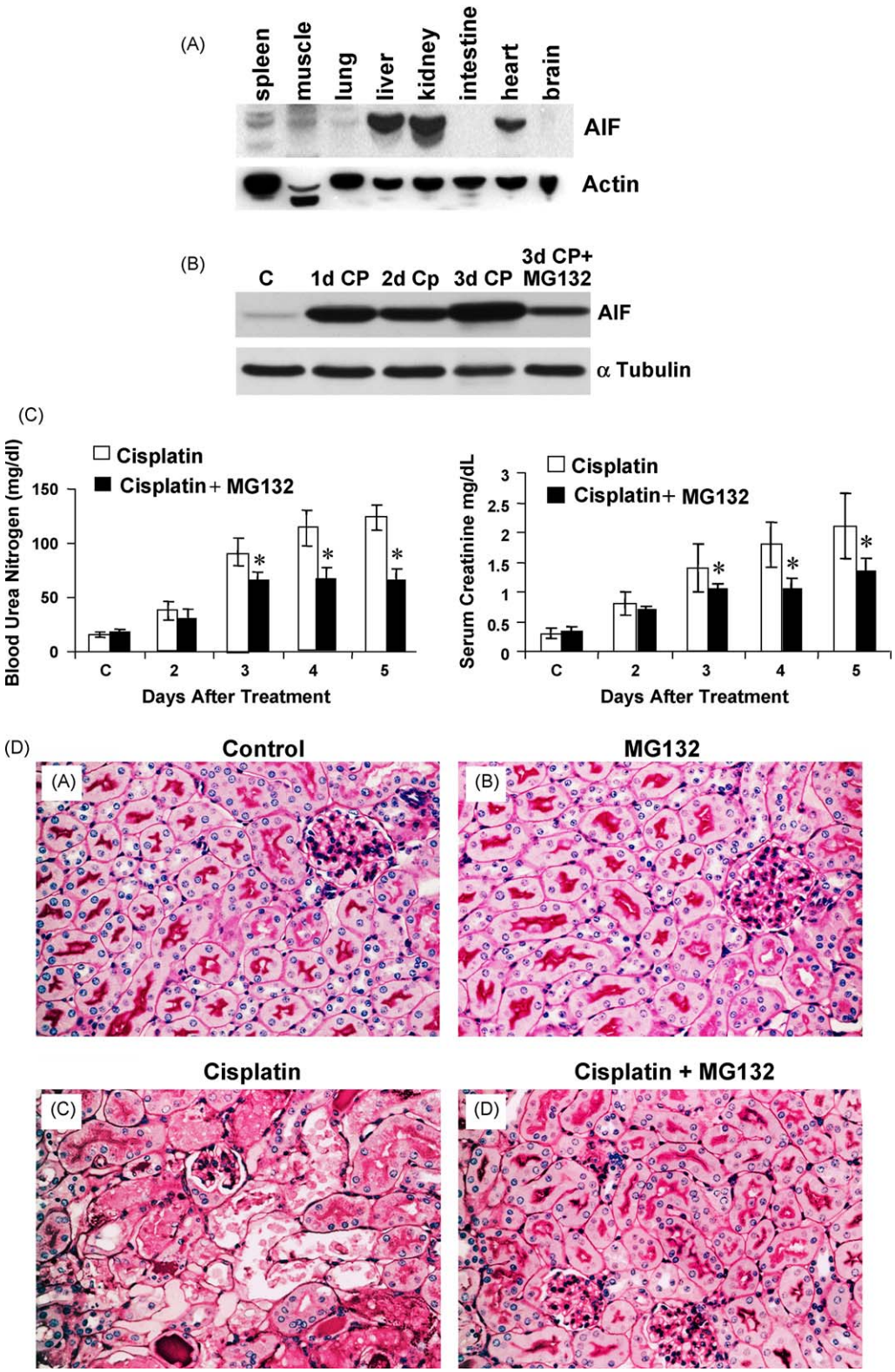

Fig. 5. (A) Expression AIF protein in the mouse organs. Organs from a 10 weeks old mouse (strain c57Bl/6) were isolated and the tissues were homogenized in a lysis buffer containing protease inhibitor cocktail. The tissue homogenates (100 μg protein) from various organs as shown were subjected to Western blot using specific AIF antibody. As shown, β-actin was used as a loading control. (B) Effect of proteasome inhibitor MG-132 on the mitochondrial release of AIF in a mouse model of cisplatin nephrotoxicity. A mouse model of cisplatin nephrotoxicity was developed as described in Section 2. Mice were injected intraperitoneally with 20 mg/kg body weight cisplatin in saline. Proteasome inhibitor MG-132 (2 mg/kg body weight in 5% DMSO in saline) was administered 30 min prior to the administration of cisplatin (3d + MG132). Control mice were administered 5% DMSO in saline. Kidneys from mice were removed at 0 day (control, c), one day (1d), two days (2d), and three days (3d). Cytosolic fractions from kidney cortices were prepared and 100 μg protein samples were subjected to Western blot analysis using AIF antibody. (C) Effect of proteasome inhibitor MG-132 on blood urea nitrogen (BUN) and serum creatinine in cisplatin-induced acute renal failure. Mice were injected intraperitoneally with either, 20 mg/kg body wt. cisplatin or cisplatin with MG-132 for 2d, 3d, 4d and 5d period. At the indicated times, (A) blood was urea nitrogen, $n = 6$, $^*P < 0.01$ and (B) serum creatinine $n = 5$, $^*P < 0.02$ were determined using a diagnostic kit from International Bio-Analytical Industries Inc. (Boca Raton, FL, USA). D. Effect of proteasome inhibitor MG-132 on cisplatin-induced histological damage in a mouse model of cisplatin nephrotoxicity. Kidneys from mice were removed three days after intraperitoneal administration of 5% DMSO in saline (A), 2 mg/kg body weight

liver injury induced by intestinal ischemia-reperfusion [46]. Another study showed that the proteasome inhibitor blocked ischemia-induced cell death, provided neuroprotection in focal ischemic brain injury [47], and significantly reduced infarct volume in a focal model of cerebral ischemia [10]. In a rat model of embolic focal cerebral ischemia, co-administration of the proteasome inhibitor with recombinant human tissue plasminogen activator significantly reduced infarct volume and improved neurologic recovery [48]. The precise mechanisms underlying the protective effects of proteasome inhibitors in these experimental models of cellular injury are yet to be determined.

Cisplatin is a chemotherapeutic drug for solid tumors and one of the major side effects of this drug is nephrotoxicity [1,2]. Thus, it is important that any agent that prevents its nephrotoxicity does not inhibit its tumoricidal effect. At present it is not known whether protection from cisplatin nephrotoxicity affects the tumor response to cisplatin in patients with tumors or in experimental models of animals with tumors. However, accumulative evidence shows that proteasome inhibitors are effective in killing cancer cells while sparing normal cells [49–51]. A proteasome inhibitor has been shown to sensitize tumor cells to cisplatin in ovarian cancer cells [52] and synergize with cisplatin in the killing of head and neck squamous cell carcinoma cells [53] and pancreatic cancer cells [54]. A recent study demonstrated that the proteasome inhibitor PS-341 enhanced cisplatin uptake and cell killing in a synergistic manner in 2008 ovarian cancer cells [55]. Our study showed that the proteasome inhibitor MG-132 enhanced cisplatin-induced caspase-3 activation in an ovarian cancer cell line A2780. Thus a unique opportunity exists that the proteasome inhibitors may not only enhance cisplatin-induced tumoricidal activity but may also prevent the side effects of nephrotoxicity. Future studies will therefore, evaluate whether proteasome inhibitor synergizes cisplatin-induced tumoricidal activity while providing a therapeutic effect by protecting cisplatin-induced nephrotoxicity in a tumor bearing animal model of ovarian cancer or other solid tumors.

In summary, the present study demonstrated that the proteasome inhibitors MG-132 and PS-341 prevented cisplatin-induced mitochondrial release of AIF, caspase activation, and apoptosis. Cisplatin-induced mitochondrial release of AIF was mediated by Mcl-1 which is highly susceptible to proteasome-ubiquitin-mediated degradation in cisplatin injury to renal epithelial cells. The mechanism of cisplatin-induced mitochondrial release of AIF further revealed that rapid depletion of Mcl-1 in cisplatin injury resulted in Bax activation and subsequent AIF release. The ability of proteasome inhibitors to upregulate Mcl-1 in renal cells prevented Bax activation and associated mitochondrial release of AIF. Our studies further demonstrated that the mouse kidneys expressed highest amounts of AIF among various organs and the proteasome inhibitors markedly prevented AIF release and decline in renal function in a mouse model of cisplatin nephrotoxicity.

Acknowledgements

This work was supported by VA Merit grant to GPK. The authors thank Ms. Cindy Reid for technical editing assistance.

References

[1] Arany I, Safirstein RL. Cisplatin nephrotoxicity. Semin Nephrol 2003;23:460–4.
[2] Pabla N, Dong Z. Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. Kidney Int 2008;73:994–1007.
[3] Modjtahedi N, Giordanetto F, Madeo F, Kroemer G. Apoptosis-inducing factor: vital and lethal. Trends Cell Biol 2006;16:264–72.
[4] Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, et al. Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 1999;397:441–6.
[5] Joza N, Susin SA, Daugas E, Stanford WL, Cho SK, Li CY, et al. Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature 2001;410:549–54.
[6] Mate MJ, Ortiz-Lombardia M, Boitel B, Haouz A, Tello D, Susin SA, et al. The crystal structure of the mouse apoptosis-inducing factor AIF. Nat Struct Biol 2002;9:442–6.
[7] Cande C, Vahsen N, Kouranti I, Schmitt E, Daugas E, Spahr C, et al. AIF and cyclophilin A cooperate in apoptosis-associated chromatinolysis. Oncogene 2004;23:1514–21.
[8] Pospisilik JA, Knauf C, Joza N, Benit P, Orthofer M, Cani PD, et al. Targeted deletion of AIF decreases mitochondrial oxidative phosphorylation and protects from obesity and diabetes. Cell 2007;131:476–91.
[9] Vahsen N, Cande C, Briere JJ, Benit P, Joza N, Larochette N, et al. AIF deficiency compromises oxidative phosphorylation. EMBO J 2004;23:4679–89.
[10] Buchan AM, Li H, Blackburn B. Neuroprotection achieved with a novel proteasome inhibitor which blocks NF-kappaB activation. Neuroreport 2000;11:427–30.
[11] Seth R, Yang C, Kaushal V, Shah SV, Kaushal GP. p53-dependent caspase-2 activation in mitochondrial release of apoptosis-inducing factor and its role in renal tubular epithelial cell injury. J Biol Chem 2005;280:31230–9.
[12] Cummings BS, Schnellmann RG. Cisplatin-induced renal cell apoptosis: caspase 3-dependent and -independent pathways. J Pharmacol Exp Ther 2002;302:8–17.
[13] Yang C, Kaushal V, Shah SV, Kaushal GP. Mcl-1 is downregulated in cisplatin-induced apoptosis, and proteasome inhibitors restore Mcl-1 and promote survival in renal tubular epithelial cells. Am J Physiol Renal Physiol 2007;292:F1710–7.
[14] Itoh M, Takaoka M, Shibata A, Ohkita M, Matsumura Y. Preventive effect of lactacystin, a selective proteasome inhibitor, on ischemic acute renal failure in rats. J Pharmacol Exp Ther 2001;298:501–7.
[15] Nencioni A, Grunebach F, Patrone F, Ballestrero A, Brossart P. Proteasome inhibitors: antitumor effects and beyond. Leukemia 2007;21:30–6.
[16] Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S, Lauper S, et al. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. J Cell Biol 1999;144:891–901.
[17] Yang C, Kaushal V, Haun RS, Seth R, Shah SV, Kaushal GP. Transcriptional activation of caspase-6 and -7 genes by cisplatin-induced p53 and its functional significance in cisplatin nephrotoxicity. Cell Death Differ 2008;15:530–44.
[18] Moubarak RS, Yuste VJ, Artus C, Bouharrour A, Greer PA, Menissier-de Murcia J, et al. Sequential activation of poly(ADP-ribose) polymerase 1, calpains, and Bax is essential in apoptosis-inducing factor-mediated programmed necrosis. Mol Cell Biol 2007;27:4844–62.
[19] Rashmi R, Kumar S, Karunagaran D. Human colon cancer cells lacking Bax resist curcumin-induced apoptosis and Bax requirement is dispensable with ectopic expression of Smac or downregulation of Bcl-XL. Carcinogenesis 2005;26:713–23.
[20] Whiteman M, Chu SH, Siau JL, Rose P, Sabapathy K, Schantz JT, et al. The pro-inflammatory oxidant hypochlorous acid induces Bax-dependent mitochondrial permeabilisation and cell death through AIF-/EndoG-dependent pathways. Cell Signal 2007;19:705–14.
[21] Brunelle JK, Shroff EH, Perlman H, Strasser A, Moraes CT, Flavell RA, et al. Loss of Mcl-1 protein and inhibition of electron transport chain together induce anoxic cell death. Mol Cell Biol 2007;27:1222–35.
[22] Craig RW. MCL1 provides a window on the role of the BCL2 family in cell proliferation, differentiation and tumorigenesis. Leukemia 2002;16:444–54.
[23] Cuconati A, Mukherjee C, Perez D, White E. DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells. Genes Dev 2003;17:2922–32.
[24] Derouet M, Thomas L, Cross A, Moots RJ, Edwards SW. Granulocyte macrophage colony-stimulating factor signaling and proteasome inhibition delay neutrophil apoptosis by increasing the stability of Mcl-1. J Biol Chem 2004;279:26915–21.
[25] Derouet M, Thomas L, Moulding DA, Akgul C, Cross A, Moots RJ, et al. Sodium salicylate promotes neutrophil apoptosis by stimulating caspase-dependent turnover of Mcl-1. J Immunol 2006;176:957–65.
[26] Inoshita S, Takeda K, Hatai T, Terada Y, Sano M, Hata J, et al. Phosphorylation and inactivation of myeloid cell leukemia 1 by JNK in response to oxidative stress. J Biol Chem 2002;277:43730–4.
[27] Nijhawan D, Fang M, Traer E, Zhong Q, Gao W, Du F, et al. Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. Genes Dev 2003;17:1475–86.
[28] Piret JP, Minet E, Cosse JP, Ninane N, Debacq C, Raes M, et al. Hypoxia-inducible factor-1-dependent overexpression of myeloid cell factor-1 protects hypoxic cells against tert-butyl hydroperoxide-induced apoptosis. J Biol Chem 2005;280:9336–44.
[29] Warr MR, Acoca S, Liu Z, Germain M, Watson M, Blanchette M, et al. BH3-ligand regulates access of MCL-1 to its E3 ligase. FEBS Lett 2005;579:5603–8.

MG-132 in 5% DMSO in saline (B), 20 mg/kg body weight cisplatin (C), and cisplatin and MG-132 (D). (A) Kidneys from mice treated with 5% DMSO in saline showed normal morphology with intact RTEC and well preserved brush-border membranes. (B) Mice treated with MG132 alone showed normal RTEC morphology. (C) Kidneys from mice treated with cisplatin alone showed excessive loss of RTEC, tubular dilation, intratubular debris, and cast formation. (D) Kidneys from mice treated with cisplatin and MG-132 showed relatively normal tubular morphology with no loss of RTEC and preservation of brush-border membranes.

[30] Zhong Q, Gao W, Du F, Wang X. Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis. Cell 2005;121:1085–95.
[31] Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene 2007;26:1324–37.
[32] Zhuang J, Brady HJ. Emerging role of Mcl-1 in actively counteracting BH3-only proteins in apoptosis. Cell Death Differ 2006;13:1263–7.
[33] Day CL, Smits C, Fan FC, Lee EF, Fairlie WD, Hinds MG. Structure of the BH3 domains from the p53-inducible BH3-only proteins Noxa and Puma in complex with Mcl-1. J Mol Biol 2008;380:958–71.
[34] Germain M, Milburn J, Duronio V. MCL-1 inhibits BAX in the absence of MCL-1/BAX Interaction. J Biol Chem 2008;283:6384–92.
[35] Gillissen B, Essmann F, Hemmati PG, Richter A, Oztop I, Chinnadurai G, et al. Mcl-1 determines the Bax dependency of Nbk/Bik-induced apoptosis. J Cell Biol 2007;179:701–15.
[36] Han J, Goldstein LA, Gastman BR, Rabinowich H. Interrelated roles for Mcl-1 and BIM in regulation of TRAIL-mediated mitochondrial apoptosis. J Biol Chem 2006;281:10153–6.
[37] Wang X, Chen W, Zeng W, Bai L, Tesfaigzi Y, Belinsky SA, et al. Akt-mediated eminent expression of c-FLIP and Mcl-1 confers acquired resistance to TRAIL-induced cytotoxicity to lung cancer cells. Mol Cancer Ther 2008; 7:1156–63.
[38] Adams KW, Cooper GM. Rapid turnover of mcl-1 couples translation to cell survival and apoptosis. J Biol Chem 2007;282:6192–200.
[39] Jiang M, Wei Q, Wang J, Du Q, Yu J, Zhang L, et al. Regulation of PUMA-alpha by p53 in cisplatin-induced renal cell apoptosis. Oncogene 2006;25:4056–66.
[40] Nagothu KK, Bhatt R, Kaushal GP, Portilla D. Fibrate prevents cisplatin-induced proximal tubule cell death. Kidney Int 2005;68:2680–93.
[41] Polster BM, Basanez G, Etxebarria A, Hardwick JM, Nicholls DG. Calpain I induces cleavage and release of apoptosis-inducing factor from isolated mitochondria. J Biol Chem 2005;280:6447–54.
[42] Ruchalski K, Mao H, Li Z, Wang Z, Gillers S, Wang Y, et al. Distinct hsp70 domains mediate apoptosis-inducing factor release and nuclear accumulation. J Biol Chem 2006;281:7873–80.
[43] Takaoka M, Itoh M, Kohyama S, Shibata A, Ohkita M, Matsumura Y. Proteasome inhibition attenuates renal endothelin-1 production and the development of ischemic acute renal failure in rats. J Cardiovasc Pharmacol 2000;36:S225–7.
[44] Campbell B, Adams J, Shin YK, Lefer AM. Cardioprotective effects of a novel proteasome inhibitor following ischemia and reperfusion in the isolated perfused rat heart. J Mol Cell Cardiol 1999;31:467–76.
[45] Pye J, Ardeshirpour F, McCain A, Bellinger DA, Merricks E, Adams J, et al. Proteasome inhibition ablates activation of NF-kappa B in myocardial reperfusion and reduces reperfusion injury. Am J Physiol Heart Circ Physiol 2003;284:H919–26.
[46] Yao JH, Li YH, Wang ZZ, Zhang XS, Wang YZ, Yuan JC, et al. Proteasome inhibitor lactacystin ablates liver injury induced by intestinal ischaemia-reperfusion. Clin Exp Pharmacol Physiol 2007;34:1102–8.
[47] Williams AJ, Dave JR, Tortella FC. Neuroprotection with the proteasome inhibitor MLN519 in focal ischemic brain injury: relation to nuclear factor kappaB (NF-kappaB), inflammatory gene expression, and leukocyte infiltration. Neurochem Int 2006;49:106–12.
[48] Zhang L, Zhang ZG, Zhang RL, Lu M, Adams J, Elliott PJ, et al. Postischemic (6-hour) treatment with recombinant human tissue plasminogen activator and proteasome inhibitor PS-519 reduces infarction in a rat model of embolic focal cerebral ischemia. Stroke 2001;32:2926–31.
[49] Almond JB, Cohen GM. The proteasome: a novel target for cancer chemotherapy. Leukemia 2002;16:433–43.
[50] Milano A, Iaffaioli RV, Caponigro F. The proteasome: a worthwhile target for the treatment of solid tumours? Eur J Cancer 2007;43:1125–33.
[51] Orlowski RZ, Kuhn DJ. Proteasome inhibitors in cancer therapy: lessons from the first decade. Clin Cancer Res 2008;14:1649–57.
[52] Yunmbam MK, Li QQ, Mimnaugh EG, Kayastha GL, Yu JJ, Jones LN, et al. Effect of the proteasome inhibitor ALLnL on cisplatin sensitivity in human ovarian tumor cells. Int J Oncol 2001;19:741–8.
[53] Li C, Li R, Grandis JR, Johnson DE. Bortezomib induces apoptosis via Bim and Bik up-regulation and synergizes with cisplatin in the killing of head and neck squamous cell carcinoma cells. Mol Cancer Ther 2008;7:1647–55.
[54] Nawrocki ST, Carew JS, Pino MS, Highshaw RA, Dunner Jr K, Huang P, et al. Bortezomib sensitizes pancreatic cancer cells to endoplasmic reticulum stress-mediated apoptosis. Cancer Res 2005;65:11658–66.
[55] Jandial DD, Farshchi-Heydari S, Larson CA, Elliott GI, Wrasidlo WJ, Howell SB. Enhanced delivery of cisplatin to intraperitoneal ovarian carcinomas mediated by the effects of bortezomib on the human copper transporter 1. Clin Cancer Res 2009;15:553–60.