Genetic disruption of Abl nuclear import reduces renal apoptosis in a mouse model of cisplatin-induced nephrotoxicity

P Sridevi¹, MK Nhiayi¹ and JYJ Wang*¹

DNA damage activates nuclear Abl tyrosine kinase to stimulate intrinsic apoptosis in cancer cell lines and mouse embryonic stem cells. To examine the in vivo function of nuclear Abl in apoptosis, we generated Abl-μNLS (μ, mutated in nuclear localization signals) mice. We show here that cisplatin-induced apoptosis is defective in the renal proximal tubule cells (RPTC) from the Abl+/− mice. When injected with cisplatin, we found similar levels of platinum in the Abl+/+ and the Abl+/− kidneys, as well as similar initial inductions of p53 and PUMA expression. However, the accumulation of p53 and PUMA could not be sustained in the Abl+/− kidneys, leading to reductions in renal apoptosis and tubule damage. Co-treatment of cisplatin with the Abl kinase inhibitor, imatinib, reduced the accumulation of p53 and PUMA in the Abl+/+ but not in the Abl+/− kidneys. The residual apoptosis in the Abl+/− mice was not further reduced in the Abl+/−; p53−/− double-mutant mice, suggesting that nuclear Abl and p53 are epistatic to each other in this apoptosis response. Although apoptosis and tubule damage were reduced, cisplatin-induced increases in phospho-Stat-1 and blood urea nitrogen were similar between the Abl+/+, the Abl+/− and the Abl+/− kidneys, indicating that RPTC apoptosis is not the only factor in cisplatin-induced nephrotoxicity. These results provide in vivo evidence for the pro-apoptotic function of Abl, and show that its nuclear localization and tyrosine kinase activity are both required for the sustained expression of p53 and PUMA in cisplatin-induced renal apoptosis.

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The ubiquitously expressed Abl tyrosine kinase contains three nuclear localization signals (NLS), a nuclear export signal (NES), and undergoes nucleocytoplasmic shuttling in response to cell adhesion or DNA damage. The cytoplasmic Abl is activated by growth factors, cytokines, extracellular matrix proteins, and microbial infections to regulate a wide variety of actin-based cellular processes. The nuclear Abl is activated by DNA damage to regulate gene expression and DNA repair. Previous cell-based studies have shown that DNA damage stimulates the nuclear accumulation and the activation of Abl tyrosine kinase through ATM and DNA-PK-dependent mechanisms, and that Abl stimulates apoptosis by activating the p53-family of transcription factors. Furthermore, nuclear entrapment can convert the oncogenic BCR-ABL tyrosine kinase into a death inducer. The in vivo demonstration of the apoptosis function of Abl has been hampered by the neonatal lethality of the Abl-knockout mice. The Abl−/− mice are runt and suffer from many developmental defects that make them unsuitable for the study of stress-induced cell death. We have shown that Abl knockout interferes with the neuronal apoptosis in the Rb-knockout embryos. However, that result did not provide evidence for the pro-apoptotic function of Abl in DNA damage response. As DNA damage signaling is initiated in the nucleus, we generated the Abl-μNLS (μ, mutated in the NLS) allele by knockin substitution mutations of 11 Lys and Arg with Gin in the three NLS in the mouse Abl gene (Supplementary Figure s1). In embryonic stem (ES) cells, the Abl-μNLS mutation blocked nuclear import and reduced Bax activation by DNA damage. We have since achieved germline transmission of the Abl-μNLS allele and show here that the Abl+/− mice are protected from cisplatin-induced renal apoptosis.

We chose cisplatin-induced nephrotoxicity as an experimental model because it is a toxic side effect in platinum-based therapies of ovarian, testicular, and other cancers. The epithelial cells of the renal proximal tubules (RPTC) are particularly sensitive to cisplatin due to an active uptake mechanism, and these cells undergo cisplatin-induced apoptosis. Previous studies using mouse genetic models have identified a number of mechanisms underlying cisplatin-induced nephrotoxicity, including intrinsic apoptosis, mitochondrial reactive oxygen species (ROS) production, and inflammation. In this study, we provide evidence that the nuclear import and the tyrosine kinase activity of Abl are also required for cisplatin to induce RPTC apoptosis in the mice.

¹Division of Hematology-Oncology, Department of Medicine, Moores UCSD Cancer Center, UCSD School of Medicine, University of California, San Diego, La Jolla, CA, USA

*Corresponding author: JYJ Wang, Division of Hematology-Oncology, Department of Medicine, Moores UCSD Cancer Center, School of Medicine, University of California, San Diego, 3855 Health Sciences Drive, Room 4328, La Jolla, CA 92093-0820, USA. Tel: 858 534 6253; Fax: 858 534 2821; E-mail: jywang@ucsd.edu

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Abbreviations: AKI, acute kidney injury; ANOVA, analysis of variance; BUN, blood urea nitrogen; ES, embryonic stem; HIC, immunohistochemistry; LMB, leptomycin B; MEF, mouse embryonic fibroblasts; MOMP, mitochondrial outer membrane permeabilization; NES, nuclear export signal; NLS, nuclear localization signal; PAS, Periodic Acid Schiff; PT, proximal tubule; ROS, reactive oxygen species; RPTC, renal proximal tubule cells; TKE, total kidney extracts; TUNEL, terminal deoxynucleotidyltransferase dUTP nick end labeling; μNLS, mutated in nuclear localization signals

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Results

RPTCs from Abi$^{+/\mu}$ mice are resistant to cisplatin-induced apoptosis. We succeeded in germline transmission of the Abi$^{+/NLS}$ allele (Supplementary Figure s1) and found that the Abi$^{+/\mu}$ mice are healthy and fertile (Table 1). In mice, the knockout of the Abi-related gene (Arg/Abi2) alone is well tolerated, but the combined loss of Abi and Arg caused early embryonic lethality. We found that homozygous mutations of Abi$^{+/NLS}$ and Arg caused late embryonic lethality due to brain developmental defects, indicating that Arg is required for the proper development of the Abi$^{+/\mu}$ mice (not shown). Together, these breeding results show that nuclear Abi is not essential to mouse development, and that defect in the cytoplasmic Abi function causes neonatal lethality. In the Abi$^{+/\mu}$ mouse embryo fibroblasts (MEFs), only cytoplasmic Abi was detected (Figure 1a). Treatment with leptomycin B (LMB), which inhibits nuclear export, caused Abi nuclear accumulation in the Abi$^{+/\mu}$ but not in the Abi$^{+/\mu}$ MEFs (Figure 1a). We also prepared primary cultures of RPTC and confirmed their identity by the expression of Megalin (Lrp2, an endocytosis receptor for protein reabsorption) (Figure 1c). In the Abi$^{+/\mu}$ RPTC, Abi was diffusely localized in the cytoplasm and the nucleus, whereas only cytoplasmic Abi was detected in the Abi$^{+/\mu}$ RPTC (Figure 1d). Cisplatin treatment induced nuclear accumulation of Abi but not Abi$^{+/NLS}$ in the Abi$^{+/\mu}$ and the Abi$^{+/\mu}$ RPTC, respectively (Figure 1d). Cisplatin also induced DNA fragmentation in RPTC, and this response was reduced in the Abi$^{+/\mu}$ RPTC (Figure 1e). This result is consistent with the previous finding that cisplatin-induced apoptosis was reduced in the Abi$^{+/\mu}$ ES cells.

Table 1 Genotypes of pups from breeding 10 pairs of Abi$^{+/\mu}$/heterozygous mice

| Genotype$^{a}$ | No. of pups$^{b}$ | % expected frequency | % observed frequency |
|---------------|-----------------|---------------------|---------------------|
| Abi$^{+/\mu}$ | 68              | 25                  | 30.9                |
| Abi$^{+/\mu}$ | 88              | 50                  | 40.0                |
| Abi$^{+/\mu}$ | 64              | 25                  | 29.1                |

$^{a}$Abi genotype, wild type (+), or $\mu$/NLS (\mu) was determined as described in Materials and Methods, and Supplementary Figure s1

$^{b}$Total number of pups = 200; male = 115; female = 105

Cisplatin-induced p53 response is blunted in Abi$^{+/\mu}$ kidneys. We then compared the in vivo apoptotic response in the Abi$^{+/\mu}$ and the Abi$^{+/\mu}$ kidneys in a model of cisplatin-induced nephrotoxicity. We observed similar platinum levels in the Abi$^{+/\mu}$ and the Abi$^{+/\mu}$ kidneys at 24 and 48 h (Figure 2a), showing that the renal uptake, accumulation, and removal of platinum were not affected by the Abi$^{+/NLS}$ mutation. In both the Abi$^{+/\mu}$ and the Abi$^{+/\mu}$ mice, cisplatin injection induced nuclear p53 expression in the kidney tissues (Figures 2b and c). Nuclear p53 signal was not detectable in cisplatin-treated p53$^{-/-}$ mice, demonstrating specificity of this immunohistochemistry (IHC) assay (Figure 2b). At 30 h after cisplatin injection, we found similar numbers of nuclear p53-positive cells in the Abi$^{+/\mu}$ and Abi$^{+/\mu}$ renal tissues (Figure 2c, left). At this time point, co-injection with the Abi kinase inhibitor imatinib did not significantly alter the numbers of nuclear p53-positive cells in the Abi$^{+/\mu}$ or the Abi$^{+/\mu}$ renal tissues (Figure 2c, left). At 48 h after cisplatin injection, the number of nuclear p53-positive cells increased in the Abi$^{+/\mu}$ but not in the Abi$^{+/\mu}$ kidneys (Figure 2c, right). At the 48-h time point, co-injection with imatinib significantly reduced nuclear p53-positive cells in the Abi$^{+/\mu}$ but not in the Abi$^{+/\mu}$ kidney tissues (Figure 2c). The IHC results were confirmed by immunoblotting of total kidney extracts. The levels of p53 protein at 30 h after cisplatin injection were similar in the total extracts from the Abi$^{+/\mu}$ and the Abi$^{+/\mu}$ kidneys, but p53 protein levels were reduced by 48 h in the Abi$^{+/\mu}$ kidneys (Figure 2d, Supplementary Figure s2b). Co-injection with imatinib did not affect the p53 levels at 30 h in the Abi$^{+/\mu}$ or the Abi$^{+/\mu}$ kidneys (Figure 2d, left). However, co-injection with imatinib reduced the p53 levels in the Abi$^{+/\mu}$ but not in the Abi$^{+/\mu}$ kidneys at the 48-h time point (Figure 2d, right). We found that the p53 mRNA was also upregulated in the Abi$^{+/\mu}$ and Abi$^{+/\mu}$ kidneys after cisplatin injection (Figure 2e). The induction of Tp53 transcription could be due to the activation of protein kinase C-delta (PKC$\delta$). Interestingly, the Tp53 mRNA levels were also reduced at the 48-h time point in the Abi$^{+/\mu}$ kidneys (Figure 2e). Taken together, these results show that the induction of p53 expression by cisplatin is not affected by the Abi$^{+/NLS}$ mutation. However, the continued accumulation of p53 in cisplatin-damaged renal tissues required the nuclear import and the kinase activity of Abi.

An important regulator of the p53 protein levels is the Mdm2 E3-ubiquitin ligase, which suppresses the accumulation of p53 at steady state. In response to DNA damage, a number of pathways are activated to disrupt the p53-Mdm2 interaction, leading to the accumulation of p53. The activated p53 then stimulates the transcription of Mdm2 to establish a negative feedback loop that controls the extent and duration of the p53 response. We found similar basal levels of Mdm2 mRNA in the untreated Abi$^{+/\mu}$ and the Abi$^{+/\mu}$ kidneys, and similar increases after cisplatin injection (Figure 2e). This result showed that nuclear Abi was not required for cisplatin to stimulate Mdm2 transcription. In total kidney extracts from the Abi$^{+/\mu}$ mice, the Mdm2 protein levels were elevated after cisplatin injection (Figure 2d, Supplementary Figure s2c). However, in the Abi$^{+/\mu}$ kidneys, the basal levels of Mdm2 protein were higher and cisplatin injection did not increase the Mdm2 protein despite upregulation of its mRNA (Figure 2d, Supplementary Figure s2c). Treatment of mice with imatinib alone did not affect the basal levels of Mdm2 protein or mRNA in the Abi$^{+/\mu}$ or the Abi$^{+/\mu}$ kidneys (Figures 2d and e; Supplementary Figure s2c). Co-injection with cisplatin plus imatinib also had no effects on the Mdm2 mRNA (Figure 2e). Those results suggest that the Abi–Mdm2 interaction would be lost in the Abi$^{+/\mu}$ mice, and this defect could have accounted for the blunted accumulation of the p53 protein in the Abi$^{+/\mu}$ kidneys. It has
also been reported that Abl can phosphorylate Mdm2 at Y-394. However, because the kinase-defective Abl could stabilize p53, pY394-Mdm2 is not likely to be required for Abl to stimulate p53 expression. Our finding that imatinib reduced Mdm2 protein in cisplatin-treated Abl+/+ and in the Abl/+ kidneys indicates that the cytoplasmic Abl tyrosine kinase,
or another enzyme that is inhibited by imatinib, may regulate the expression of Mdm2 protein in response to DNA damage.

An important target gene of p53 in apoptosis is PUMA. At 30 h after cisplatin injection, similar levels of PUMA induction were found in the Abl+/+ and Abl+/m kidneys extracts and tissues (Figure 2d; Supplementary Figures S2a and d). However, PUMA levels were reduced in the Abl+/m kidneys by 48 h (Figure 2d, right). We found that the PUMA RNA was similarly induced and maintained in the Abl+/+ and the Abl+/m kidneys (Figure 2e). Furthermore, imatinib co-treatment did not affect the levels of PUMA RNA (Figure 2e). These results show that nuclear import of Abi and its kinase activity are not required for cisplatin to activate the transcription of the PUMA gene in the p53+/+ genetic background. However, these results suggest that PUMA protein expression could not be sustained despite the upregulation of its mRNA in the Abl+/m kidneys.
Reduction in apoptosis and proximal tubule damage in Abl\textsuperscript{+/−} kidneys. The expression of p53 and PUMA\textsubscript{a} did not cause DNA fragmentation assessed by TUNEL (terminal deoxynucleotidyl-transferase dUTP nick end labeling) staining. However, TUNEL-positive nuclei became detectable at 48 h (Figure 3a). In keeping with reduced apoptosis of the explanted RPTC (Figure 1e), the numbers of TUNEL-positive cells were significantly lower in the Abl\textsuperscript{+/−} than in the Abl\textsuperscript{+/+} kidneys (Figures 3a and b). Co-injection with imatinib reduced TUNEL-positive cells in the Abl\textsuperscript{+/−} but not in the Abl\textsuperscript{+/+} kidneys (Figures 3a and b). The reduced TUNEL staining correlated with the reduced expression of p53 and PUMA\textsubscript{a} (Figure 2). At 72 h after cisplatin injection, TUNEL staining became unreliable due to the accumulation of protein casts in the renal tissues (Figure 3c). We therefore assessed the proximal tubule (PT) damage by PAS (Periodic Acid Schiff) staining, which reacts with the brush borders of PT epithelial cells and the glycoprotein aggregates (casts) that accumulate in the damaged PT.\textsuperscript{32} In the Abl\textsuperscript{+/−} renal tissues, PAS-positive casts (*) and droplets (arrows) were readily detected at 72 h (Figures 3c and d); by comparison, these PAS-positive features were significantly lower in the Abl\textsuperscript{+/+} tissues (Figures 3c and d). Imatinib treatment alone did not alter the PAS staining or the PT morphology in renal tissues (Figures 3c and d). However, co-injection with cisplatin plus imatinib significantly reduced the number of PAS-positive protein casts in the Abl\textsuperscript{+/−} but not in the Abl\textsuperscript{+/+} kidneys (Figures 3c and d). These results showed that the reduced apoptosis (48 h) correlated with reduced PT damage (72 h) in the Abl\textsuperscript{+/+} kidneys. Therefore, cisplatin-induced renal apoptosis and PT damage require the nuclear import and the tyrosine kinase activity of Abl.

No further reduction of renal apoptosis by combining Abl-\muNLS with p53-null mutations. It has been demonstrated that cisplatin-induced renal apoptosis requires p53.\textsuperscript{33} We therefore compared the apoptotic response in the Abl\textsuperscript{+/−} and the p53\textsuperscript{−/−} kidneys. To compare littermates, we first generated the Abl\textsuperscript{+/−}; p53\textsuperscript{+/−} mice. Intercrossing of these compound heterozygotes generated p53\textsuperscript{−/−}; Abl\textsuperscript{+/−} male, but not female, pups at the expected Mendelian frequency (Table 2), indicating female-specific developmental defects of the double mutants. Interestingly, we found that the renal apoptotic response to cisplatin was reduced to similar low levels in the Abl\textsuperscript{+/−} and the p53\textsuperscript{−/−} single-mutant mice (Figure 4c). These lower levels of TUNEL staining were not further reduced in the Abl\textsuperscript{+/−}; p53\textsuperscript{−/−} double-mutant kidneys.
We also measured PT damage by PAS staining at 72 h and found that the levels of cast-positive tubules were similarly reduced in each of the single- and the double-mutant kidneys (Figures 4b and d). Moreover, there were no significant differences among the Abf\textsuperscript{+/+}, p53\textsuperscript{−/−}, the Abf\textsuperscript{+/−}; p53\textsuperscript{−/−}, and the Abf\textsuperscript{−/−}; p53\textsuperscript{−/−} kidneys in cisplatin-induced PT damage (Figures 4b and d). These results show that the nuclear Abl is as important as p53 in cisplatin-induced renal apoptosis and tubule damage, and that the nuclear Abl and p53 are epistatic to each other in this apoptotic response.

We also examined the levels of p53, PUMA\textsubscript{\textalpha}, and cleaved caspase-3 (ΔC3) in total kidney extracts from the single and the double mutants at the 72-h time point (Figure 4e, Supplementary Figures s3a–c). In the Abf\textsuperscript{+/+}; p53\textsuperscript{−/−} kidneys, we observed variable, but consistent, reductions in p53, PUMA\textsubscript{\textalpha}, and ΔC3. In the Abf\textsuperscript{+/−}; p53\textsuperscript{−/−} kidneys, we found reduced ΔC3 in kidneys with high levels of PUMA\textsubscript{\textalpha} in some mice (Figure 4e, Supplementary Figures s3b and c). Although PUMA was identified as a target gene of p53,\textsuperscript{31} the transcription of PUMA can be stimulated by other factors including p63, p73, NF-kB, FOXO, and SMAD4.\textsuperscript{34–38} Previous studies have shown that cisplatin activates the Abl tyrosine kinase to stimulate p63 and p73.\textsuperscript{39–41} The wild-type level of PUMA\textsubscript{\textalpha} expression in some of the Abf\textsuperscript{+/−}; p53\textsuperscript{−/−} kidneys could, therefore, be driven by the Abl-p63 or the Abl-p73 pathways. Previous studies have also shown that the cytoplasmic p53 can stimulate mitochondrial outer membrane permeability transition.\textsuperscript{39–41} The loss of this cytoplasmic p53 function might explain the reduced ΔC3 formation in those Abf\textsuperscript{+/−}; p53\textsuperscript{−/−} kidneys that expressed wild-type levels of PUMA\textsubscript{\textalpha}. In other words, both the PUMA\textsubscript{\textalpha} and the cytoplasmic p53 are required for cisplatin to induce caspase-3 cleavage in the mouse kidneys.\textsuperscript{39–41} In the kidneys of the Abf\textsuperscript{+/−}; p53\textsuperscript{−/−} double mutants, PUMA\textsubscript{\textalpha} and ΔC3 were further reduced (Figure 4e, Supplementary Figure s3b and c), consistent with the epistatic interaction of nuclear Abl and p53 in cisplatin-induced renal apoptosis. This result also indicated that other caspase-independent pathways\textsuperscript{42} might account for the residual TUNEL positivity in the double-mutant kidneys.

Table 2 Genotypes of pups from breeding 40 pairs of Abf\textsuperscript{+/−}, p53\textsuperscript{−/−} compound heterozygous mice

| Genotype\textsuperscript{a} | No. of pups\textsuperscript{b} | % expected frequency | % observed frequency | Male to female ratio |
|---------------------------|-------------------------------|---------------------|---------------------|---------------------|
| Abf\textsuperscript{+/+} p53\textsuperscript{−/−} | 62 | 6.25 | 9.2 | 1.3 |
| Abf\textsuperscript{+/−} p53\textsuperscript{−/−} | 99 | 12.5 | 14.6 | 1.1 |
| Abf\textsuperscript{−/−} p53\textsuperscript{−/−} | 60 | 6.25 | 8.9 | 1.1 |
| Abf\textsuperscript{+/+} p53\textsuperscript{−/−} | 96 | 12.5 | 14.2 | 0.9 |
| Abf\textsuperscript{+/−} p53\textsuperscript{−/−} | 181 | 25 | 26.7 | 1.0 |
| Abf\textsuperscript{−/−} p53\textsuperscript{−/−} | 71 | 12.5 | 10.5 | 1.8 |
| Abf\textsuperscript{+/+} p53\textsuperscript{−/−} | 35 | 6.25 | 5.2 | 1.7 |
| Abf\textsuperscript{+/−} p53\textsuperscript{−/−} | 49 | 12.5 | 7.2 | 2.1 |
| Abf\textsuperscript{−/−} p53\textsuperscript{−/−} | 24 | 6.25 | 3.5 | 5.0 |

\textsuperscript{a}Abf genotype: wild type (++) or µNLS (−); p53 genotype: wild type (+) or null (−), was determined as described in Materials and Methods, and Supplementary Figure s1

\textsuperscript{b}Total number of pups = 677; male = 376; female = 301

Reduction in renal apoptosis did not ameliorate nephrotoxicity. In patients, nephrotoxicity is routinely assessed by blood urea nitrogen (BUN) and serum creatinine, which are delayed biomarkers for kidney injury, as these levels reflect the glomerular filtration rate.\textsuperscript{43,44} Consistent with it being a delayed marker, we detected significant increases in the BUN levels only at the 72-h time point (Supplementary Figure s4a). We then measured the BUN levels across the nine genotypes generated by crossing the Abf\textsuperscript{+/−}; p53\textsuperscript{−/−} mice, and found that BUN increases in all mice (Figure 5a). The variations in the BUN increases were statistically insignificant (Figure 5a). The serum-creatinine levels were also similarly increased between the Abf\textsuperscript{+/−} and the Abf\textsuperscript{−/−} mice (Supplementary Figure s4b). At a lower dose of cisplatin, the increases in BUN were again found to be similar between the Abf\textsuperscript{+/−} and the Abf\textsuperscript{−/−} mice (Supplementary Figure s4c). These results suggest that the kidneys were still injured in the Abf\textsuperscript{−/−} mice. We therefore measured the levels of Kim-1 (Kidney Injury Molecule-1) RNA, which is consistently upregulated in different models of kidney injury\textsuperscript{44,45} and found similar levels of Kim-1 induction by cisplatin in the Abf\textsuperscript{+/−} and Abf\textsuperscript{−/−} kidneys (Supplementary Figure s4d).

A recent study has shown that cisplatin activates Stat-1 phosphorylation in the cochlea of rats and that administration of Stat-1-siRNA blocked cisplatin-induced hearing loss measured by the auditory brainstem response threshold.\textsuperscript{46} The phosphorylation of Stat-1 leads to activation of downstream targets such as iNos and Cox-2, which induce inflammation to cause hair cell damage.\textsuperscript{46} We found that Stat-1 was expressed at comparable levels in the Abf\textsuperscript{+/−} and the Abf\textsuperscript{−/−} kidneys (Figure 5b). Injection with cisplatin stimulated Stat-1 phosphorylation to a low level at 30 h and a higher level at 48 h; and the increases in phosphor-Stat-1 (pStat1) were similar between the Abf\textsuperscript{+/−} and Abf\textsuperscript{−/−} kidneys (Figure 5b). Furthermore, co-injection with imatinib did not affect the increase in pStat1 in the Abf\textsuperscript{+/−} or the Abf\textsuperscript{−/−} kidneys (Figure 5b). These results show that cisplatin-induced Stat-1 phosphorylation was similarly upregulated in the Abf\textsuperscript{+/−} and the Abf\textsuperscript{−/−} kidneys. The pStat1 and possibly other cisplatin-activated pathways could explain the kidney injury detected in the apoptosis-defective Abf\textsuperscript{−/−} mice (Figure 5c).

Discussion

The study of the Abf\textsuperscript{−/−}NLS mice has provided the first in vivo evidence for the pro-apoptotic function of Abl, and shown that nuclear Abl is as important as p53 in cisplatin-induced renal apoptosis. Furthermore, this in vivo study has generated new insights on the nuclear Abl tyrosine kinase function in apoptosis. First, our previous study of the Abf\textsuperscript{−/−} ES cells\textsuperscript{7} and this study of the Abf\textsuperscript{−/−} kidneys have shown that the induction of p53 expression by cisplatin does not require Abl nuclear import. Instead, we found that nuclear Abl is required for the continued accumulation of p53. Second, the study of the Abf\textsuperscript{−/−} kidneys has indicated an unexpected role for nuclear Abl in the regulation of Mdml protein expression. Third, we have found that nuclear Abl is required for the sustained expression of PUMA\textsubscript{\textalpha} protein in cisplatin-induced apoptosis.
The nuclear Abl-dependent p53 accumulation is consistent with the previous findings that Abl interacts with Mdm2 to block p53 degradation and that the Abl-mNLS mutant protein is defective in binding Mdm2. Besides the p53 protein, we found that its mRNA was also upregulated by cisplatin in the kidneys. A previous study has shown that DNA damage upregulates Tp53 transcription through PKCδ, which is activated by cisplatin in the mouse.
Several studies have suggested an interdependent activation of Abl and PCK by ROS. Although the initial upregulation of the Tp53 mRNA was not affected by Abl- m NLS, its levels were reduced at a later time, indicating that the PKC activity might be lowered in the Abl m/m kidneys. An important pro-apoptotic target gene of p53 is PUMA, which is a BH3-only protein that sequesters the anti-apoptotic Bcl2-family members to stimulate Bax and Bak-dependent mitochondrial outer membrane permeabilization (MOMP). We show here that nuclear Abl is not required for cisplatin to induce PUMA mRNA. However, nuclear Abl is required for the sustained expression of the PUMA protein. This finding is completely unexpected and indicates a novel function for nuclear Abl in the regulation of PUMA translation or protein stability. Interestingly, we found that cisplatin could still induce PUMA expression in the Abl m/m; p53 m/m kidneys, but PUMA expression was lost in the kidneys of the double-mutant mice (Abl m/m; p53 m/m). The upregulation of PUMA in the Abl m/m; p53 m/m mice is likely to be mediated by p63 and/or p73, as previous studies have established a redundant role of these three transcription factors in PUMA expression.

This study also shows that p53 and nuclear Abl-dependent apoptotic response is not the only factor in cisplatin-induced nephrotoxicity. The pathophysiology of acute kidney injury involves epithelial cell death, interstitial inflammation, small vessel obstruction, and local ischemia. Other than apoptosis, cisplatin also induces oxidative stress, necrosis, autophagy, and inflammation in the renal tissues. In the Abl m mice, injection with cisplatin stimulated the expression of Kim-1, which encodes a transmembrane glycoprotein that is upregulated in response to kidney injury caused by many different agents. Furthermore, we found that cisplatin induced the phosphorylation of Stat-1 in the kidneys of the Abl m/m and Abl m/m mice. As Stat-1 has been shown to be required for cisplatin to cause ototoxicity (hearing loss) in the rats, the activation of Stat-1 could have contributed to cisplatin-induced nephrotoxicity in the Abl m/m mice. Therefore, a concerted strategy that targets the nuclear Abl and the Stat-1 pathways may be required to ameliorate nephrotoxicity associated with platinum-based cancer therapy.
Materials and Methods

Materials. Pharmacological hospital-grade cisplatin (1 mg/ml in saline) was from APP Pharmaceuticals (Schaumburg, IL, USA). Imatinib mesylate, rabbit anti-p53 antibody (IHC and western blotting), goat anti-PUMAα antibody (IHC and western blotting) were from Santa Cruz Biochemicals (Santa Cruz, CA, USA), Mdm2 antibody was from Calbiochem (EMD Millipore Biosciences, Billerica, MA, USA); pSTAT1 (Y701) antibody was from Cell Signaling Technology Inc. (Danvers, MA, USA); and STAT-1 antibody was from BD Biosciences (San Jose, CA, USA). Type-2 Collagenase, L-glutamine, nonessential amino acids, and DMEM F12 media were from Invitrogen (Carlsbad, CA, USA). Soybean trypsin inhibitor and hydrocortisone were from Sigma Aldrich (St Louis, MO, USA). Dextrose, glycine, 70-μm mesh cell culture sieve and fetal bovine serum (FBS) were from Fisher Scientific (Pittsburg, PA, USA). HBSS, Insulin Transferrin Selenium (ITS), and sodium pyruvate were from Cellgro (Manassas, VA, USA). Penicillin-streptomycin stock solution and Fungizone were from Mediatech (Manassas, VA, USA).

Genotyping primers. The Abi genotypes were determined from tail DNA by PCR using the following primers: forward, 5’-TGTTGCCACCTCGTGTGACAT-3’, reverse, 5’-GATGGCGCTCGAGAACAAATCTCA-3’ (Supplementary Figure s1). Genotyping of the p53-null allele was according to Jacks et al.56

Mice. Breeding, handling, and experimentation with mice were according to protocols approved by The Institutional Animal Care and Use Committees of University of California at San Diego. Embryonic stem cells with one Abi\(^{+/−}\) (μ) allele containing the neocytin-resistant gene (neo) cassette flanked by lox sites were previously described.7 Following germline transmission of this Abi\(^{+/−}\)-Neo-lox\(^{−}\) allele, female were bred with male Abi\(^{+/−}\)-Neo-lox\(^{−}\) mice. Male Abi\(^{+/−}\)-Neo-lox\(^{−}\) and Abi\(^{−/−}\)-Neo-lox\(^{−}\) mice were previously described. 7 Following germline transmission of this Abi\(^{−/−}\)-Neo-lox\(^{−}\) allele, female were bred with male Abi\(^{−/−}\)-Neo-lox\(^{−}\) mice to generate a breeding colony of Abi\(^{−/+}\)-Neo-lox\(^{−}\) mice.

Preparation of mouse RPTCs. Mouse RPTC were prepared as described.58 Briefly, mouse renal cortices were minced in ice-cold dissection solution (10 mM glucose, 5 mM glycine, 1 mM alanine, 15 mM HEPES, pH 7.4, 150 mM NaCl with osmolality at 325 osmol/kg H2O), digested for 30 min at 37°C with type-2 collagenase (0.1% w/v in DS) and filtered through 70-μm mesh cell culture sieves. The PT fragments that did not pass through the 70-μm sieves were resuspended by flushing the sieve in the reverse direction with warm DS (37°C) containing 1% (w/v) bovine serum albumin (BSA). The PT fragments were then cultured at 37°C with 5% CO2 in maintaining medium containing 1:1 DMEM/F12, 1% FCS, 15 mM HEPES, 2 mM L-glutamine, 50 mM hydrocortisone, 5 μg/ml each of insulin and transferrin, 50 mM sodium pyruvate, nonessential amino acids, penicillin 100 IU/ml, and streptomycin 100 μg/ml, pH 7.4. The medium was changed every 2 days. For immunofluorescence staining, RPTC were directly seeded onto acid-washed and poly-L-lysine-coated coverslips. The percentage of RPTC in these cultures was >80%, as determined by immunofluorescence staining for the RPTC marker megalin.

Preparation of mouse kidneys. Mouse kidneys were homogenized on ice in RIPA buffer (25 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.1% SDS, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail). The lysates were sonicated and centrifuged at 12,000 r.p.m. for 10 min. The supernatant was quantified using Bio-Rad Lowry protein assay reagent, and 30–50 μg lysates were loaded per lane of SDS polyacrylamide gels. Western blotting was performed by standard protocols.

RNA analysis. Total RNA was extracted from frozen kidneys using RNA extraction kit (Qiagen, Germantown, MD, USA) and converted to cDNA using ABI kit, following the manufacturer’s protocol (Life Technologies, Carlsbad, CA, USA). The primer sets used were as follows: GAPDH (forward: 5’-TGATGACATCAA GAAGTGGTGAAG-3’, reverse: 5’-TCCCTGAGGCGCATGAGCCAT-3’), p53 exon 3 (forward: 5’-ACCTCTGAGGCGGATGCTG-3’, reverse: 5’-CCTGCA CATAACAGCTTGGC-3’), PUMA exon 3 (forward: 5’-CCTACAGCTCCCTG TCCAGC-3’, reverse: 5’-CAACCGCCGAGTACGCGCGGCG-3’), Mdm2 exon 10 (forward: 5’-CATGCAATGAAATGACTCTCCC-3’, reverse: 5’-AAGCCCCGACAACTGATGT-3’), reverse: 5’- CTCTTACGATAGAACGATGAGAG-3’). Total RNA without reverse transcription was used as control to rule out genomic DNA PCR.

Histology and IHC. For assessment of kidney tissue injury, 5-μm sections were stained with PAS. Tubular protein casts were counted in at least eight random fields in the PAS-stained sections. For p53 and PUMA IHC, sections were deparaffinized, dehydrated, and antigen retrieval achieved by heating in Retrievagen (A pH 6.0) to BD biosciences, at 95°C for 15 min. The sections were allowed to cool down, and immunostaining was performed using Dako DAB-IHC kit (Dako Technologies, Carpinteria, CA, USA), following the manufacturer’s protocol to stain the antigen brown. Hematoxylin was used to counterstain the nuclei blue.

BUN and serum-creatinine measurement. Nephrotoxicity was assessed by BUN and serum creatinine levels using QuantiChrom Di-Ur500 urea assay kit and QuantiChrom CT500 creatinine assay kit, respectively (Bioassay Systems, Hayward, CA, USA).

TUNEL assay. TUNEL was performed on tissue sections using Trevigen TACS-XL–Blue in situ apoptosis detection kit (Trevigen, Gaithersburg, MD, USA). Nuclei were counterstained with nuclear fast red. Sections were photographed (× 200), and apoptotic nuclei and apoptotic bodies in at least eight representative fields were counted per mouse. TUNEL labeling was performed on RPTC cells using Takara In situ Apoptosis Detection Kit. RPTC nuclei were stained with DAPI, and TUNEL-positive cells were counted in six fields out of a total of around 200 nuclei.

Measurement of tissue platinum. Whole kidneys were boiled, weighed, finely minced, transferred into conical tubes containing 215 μl of concentrated nitric acid, and digested overnight at 95°C. The digested tissues were diluted with 3 ml of buffer containing 0.1% Triton X-100, 1.4% v/v concentrated nitric acid and 1 ppb Indium. The digested samples were subjected to inductively coupled plasma/mass spectroscopy (ICP-MS) to determine the platinum content.

Statistical methods. For statistical analysis, Prism 5.0 software (GraphPad Software, San Diego, CA, USA) was used. The statistical significance, when only two groups were compared, was assessed using Bonferroni post-test analysis was used to compare the effects of the different treatments (cisplatin versus cisplatin + IM) on the two Abi genotypes.

Conflict of Interest

The authors declare no conflict of interest.

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