ABCB1 Protects Kidney Proximal Tubule Cells Against Cadmium-Induced Apoptosis: Roles of Cadmium and Ceramide Transport

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Cadmium (Cd2+) damages the kidney proximal tubule (PT) by ceramide-dependent apoptosis and is also a class I carcinogen. Multidrug resistance P-glycoprotein (MDR1, ABCB1) confers resistance to Cd2+ apoptosis, and it has been hypothesized that ABCB1 can directly transport Cd2+ as a mode of cellular protection. Our aim was to investigate the role of ABCB1 in Cd2+ transport and ceramide apoptosis. In rat PT or Madin-Darby canine kidney (MDCK) cells overexpressing ABCB1, ABCB1-dependent efflux of rhodamine 123+ (Rh123+) or 109Cd2+ were determined, and cell death was assayed with MTT, H-33342 nuclear staining, and monolayer integrity by impedance sensing (Electric cell-substrate impedance sensing [ECIS]). ABCB1 inhibitors (PSC833, UIC-2 antibody) did not affect 109Cd2+ efflux in PT cells though Rh123+ transport was blocked. Furthermore, increased ABCB1 expression did not augment 109Cd2+ efflux but attenuated apoptosis by 10–50 μM Cd2+ or 5–25 μM C18-ceramide, which was abolished by PSC833 (1 μM). ECIS measurements of ABCB1-MDCK monolayers exhibited similar effects. Moreover, in ABCB1-MDCK cells, Cd2+-induced ceramide formation, determined by a diacylglycerol kinase assay, was abolished and increased extrusion of nitro-2,1,3-benzoxadiazol-4-yl (NBD)-C6-ceramide, which was abolished by PSC833 (1 μM). ECIS measurements of ABCB1-MDCK monolayers exhibited similar effects. Moreover, in ABCB1-MDCK cells, Cd2+-induced ceramide formation, determined by a diacylglycerol kinase assay, was abolished and increased extrusion of nitro-2,1,3-benzoxadiazol-4-yl (NBD)-C6-ceramide, and NBD-C6-glucosylceramide was observed compared with MDCK cells. Whereas pharmacological block of sphingomyelin synthase (0.1 mM D609) or sphingosine kinase (1 μM dimethylsphingosine), which increase the levels of ceramide and its metabolites, augmented Cd2+-induced apoptosis, Cd2+ apoptosis was significantly decreased not only by prevention of de novo ceramide synthesis (0.1 μM fumonisin B1) but also by inhibition of glucosylceramide synthase (2 μM C6-DGJ). We therefore conclude that Cd2+ efflux is not the mechanism behind ABCB1-mediated protection from Cd2+ apoptosis. Rather, the sphingolipid glucosylceramide may be the proapoptotic substrate extruded by ABCB1.

Key Words: P-glycoprotein; cell death; multidrug resistance; sphingolipids; transition metal.

Multidrug resistance P-glycoprotein is a member of the superfamily of ATP-binding cassette transporters that pumps a broad range of structurally unrelated, hydrophobic, amphiphilic, and cationic xenobiotics out of cells (Gottesman and Ling, 2006). ABCB1 is expressed in the apical membrane of epithelia, including the kidney proximal tubule (PT). ABCB1 is highly upregulated in cancer cells resulting in resistance to chemotherapeutic agents (Gottesman et al., 2002). The expression of ABCB1 can be regulated by a number of stress responses, including nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) (Miller 2010), and also Wnt signaling (Chakraborty et al., 2010; Yamada et al., 2000). Furthermore, upregulation of ABCB1 could be an important step in the transition of normal into carcinogenic cells because evasion of cell death, survival, and proliferation of mutated cells is propagated.

Sphingolipids play important roles in many cellular processes, including cell death, survival, and proliferation (Saddoughi et al., 2008). Apoptotic cell death caused by ceramides (Cer) has been shown to involve Ca2+-activated proteases, calpains (Lee et al., 2007), as well as mitochondrial dysfunction (Siskind et al., 2010) and is thought to be regulated by reactive oxygen species (ROS) (Won and Singh, 2006). Previous studies have demonstrated ABCB1-mediated transport of Cer metabolites, including glucosylceramide (GlcCer) (Eckford and Sharom, 2005; Masuda et al., 2008); yet, it is unclear as to whether this is a cause or consequence of multidrug resistance properties in cancer cells.

Inorganic cadmium (Cd2+) is nephrotoxic and particularly affects the PT S1 segment. In the cell, Cd2+ generates ROS and increases Cer formation (Lee et al., 2007), culminating in cell death by apoptosis if these ROS-mediated stress events are not sufficiently balanced by repair processes (Bork et al., 2010; Thévenod and Friedmann, 1999). Most significantly, Cd2+ is a class I carcinogen that induces cancer of the lung, prostate, and possibly kidneys (Nawrot et al., 2010).

Pertinently, it was discovered almost 20 years ago that Cd2+ causes upregulation of ABCB1 (Murakami et al., 1991). Moreover, decreased Cd2+ toxicity was demonstrated in kidney PT cells overexpressing ABCB1 as a consequence of
ROS-dependent NF-κB activation (Thevenod et al., 2000), most likely as a defense mechanism against cytotoxicity induced by Cd²⁺. The simplest mechanism to account for ABCB1-mediated abrogation of Cd²⁺ toxicity is direct Cd²⁺ efflux by ABCB1, as has been proposed by several studies (Achard-Joris et al., 2005; Broeks et al., 1996; Endo et al., 2002; Kimura et al., 2005). In this study, increased ABCB1 expression and transporter activity protect against cell death induced by Cd²⁺ but not by increased Cd²⁺ efflux out of cells. Instead, ABCB1 upregulation abolishes Cd²⁺-induced Cer formation, protects against toxicity induced by exogenous C₆-Cer, and increases efflux of nitro-2,13-benzoxadiol-4-yl (NBD)-C₆-Cer and NBD-C₆-GlCer, indicating that ABCB1 mediates efflux of Cd²⁺-induced proapoptotic Cer and/or its metabolites to attenuate their cellular levels and protects cells from undergoing cell death.

MATERIALS AND METHODS

Cell culture and inhibitors. Immortalized cells from the S1 segment of rat kidney PT (WKPT-0293 Cl.2, WKPT-1292 Cl.8, and SKPT-0193 Cl.2) (Woost et al., 1996) were cultured as previously described (Lee et al., 2007). WKPT-0293 Cl.2 cells were used at passages 33–34. Madin-Darby canine kidney (MDCK) cells stably overexpressing human MDR1 were obtained from Dr Michael M. Gottesman and will be henceforth referred to as ABCB1-MDCK cells. MDCK cells were cultured in high glucose Dulbecco’s Modified Eagle Medium + GlutaMax I + 25mM HEPES supplemented with 10% fetal bovine serum and 1% antibiotics (all from Invitrogen, Karlsruhe, Germany) at 37°C in a humidified incubator with 5% CO₂. Experiments with CdCl₃ (CdCl₂) (Merck Chemicals Ltd, Nottingham, U.K.) and N-hexanoyl-ß-erythro-sphingosine (C₆-ceramide; Avanti Polar Lipids, Inc., Alabaster, AL) were performed in serum-free medium (SF). Unless otherwise indicated, cells were grown for 2 days prior to treatment. Inhibitors used were PSC833 (a kind gift from Sanofi-Aventis, Basel, Switzerland), MK571 (Biomol, Hamburg, Germany), and β-actin (Sigma-Aldrich) antibodies were used at 1:200, 1:250, and 1:5000, respectively. Densitometry analysis was performed using TINA v2.09 (Raytest GmbH, Straubenhardt, Germany), and signals were normalized to loading controls.

109Cd²⁺ kinetic transport studies. Cellular 109Cd²⁺ uptake (specific activity 1.5 MBq/μg Cd³⁺; QSA Global, Braunschweig, Germany) was performed according to a previously described protocol (Erfurt et al., 2003), with some modifications. Briefly, confluent monolayers were washed twice with HBSS before Cd³⁺ incubation. The concentration of CdCl₃ was adjusted in HBSS and labeled with 109Cd²⁺ to give a final activity of 18.5 kBq/μL. At specific time points, monolayers were washed and solubilized overnight. 109Cd²⁺ content was determined using a Cobra II Auto-Gamma counter (Packard Instrument Company, Meriden, CT). To demonstrate the presence of a membrane bound and of a cytosolic pool of Cd²⁺, cell monolayers were washed three times with HBSS + 2mM ethylene glycol-bis-(2-aminoethyl)-N,N,N’-tetraacetic acid (EGTA) and harvested following 60-min incubation at 37°C. Homogenization was performed in a cell disruption chamber (Parr Instrument Company, Moline, IL) by nitrogen pressure cavitation (1000 psi for 10 min at 4°C). A crude membrane fraction was obtained by high-speed ultracentrifugation (150,000 x g for 45 min) in a Beckman Optima TLX tablettocupentrifuge, and 109Cd²⁺ radioactivity in pellet and supernatant was determined. 109Cd²⁺ uptake into WKPT-0293 Cl.2 cells was temperature dependent (Supplementary figs. 1A and 1B) and mainly transported into a cytosolic pool (Fig. 1A). For efflux studies, cells were first loaded with 109Cd²⁺ for 60 min (as described above) ± inhibitors or drug solvents (0.1% vol/vol). The medium was then replaced with HBSS containing 2mM EGTA and inhibitors to create an extracellular sink for the released Cd²⁺. At specified time points, 109Cd²⁺ was determined in the extracellular medium and in solubilized cells. Efflux, which was also temperature sensitive, was determined as pmol per well (Supplementary fig. 1B) or as a percentage of total cellular 109Cd²⁺ (Fig. 1B).

NBD-C₆-ceramide transport assays. Transport of Cer labeled with fluorescent NBD was performed according to a previously reported protocol with slight modifications (van Helvoort et al., 1996). Cell monolayers grown in 24-well plates were washed thrice with HBSS without bicarbonate + 20mM HEPES, pH 7.4 + 1% BSA (wt/vol) (HBSS-HB) and incubated with 5μM NBD-C₆-Cer (Avanti Polar Lipids, Inc.) diluted in HBSS-HB for 45 min at 37°C. For NBD-C₆-GlCer (1μM; Avanti Polar Lipids, Inc.), cells were washed and loaded in modified HBSS without BSA. After two further washes, cells were incubated in 200 μL HBSS-HB at room temperature to promote back-exchange of sphingolipids on the cell surface with gentle shaking for 0–60 min. At each time point, the extracellular medium was transferred to a black 96-well plate and both the intracellular and extracellular NBD fluorescence was determined at 485/535
Aberthold Mithras LB940. NBD lipid efflux into the extracellular compartment was calculated as a percentage of total NBD fluorescence.

Reverse transcriptase PCR. Total RNA was isolated, reverse transcribed to complementary DNA, and PCR was executed for rat abcb1a and gapdh as previously described (Chakraborty et al., 2010). Other primer pairs used are as follows: rat abca1 forward 5'-GCTCCTGCTGAAATACCG-3', rat abca1 reverse 5'-TGAGGGACGATTCCACAT-3' (Luo et al., 2010); canine abca1 forward 5'-GGGACTCTGGCCCAAGAGCTGT-3', canine abca1 reverse 5'-TCTTCGTCCGTGCCGTTGCC-3', canine gapdh forward 5'-AACAT-CATCCCTGCTCCAC-3', and canine gapdh reverse 5'-GAC-CACCTGGTCTCAGTGT-3' (Chu et al., 2009). Densitometry analysis was performed using TINA v2.09. Signals were corrected for loading and normalized to controls.

Statistical analyses. Unless indicated otherwise, data were expressed as means ± SE of at least three individual experiments. Uptake and efflux data were curve fitted by nonlinear regression using SigmaPlot 8.0 (SPSS Inc., Chicago, IL). Pairwise comparisons were performed by Student’s unpaired t-test, and multiple comparisons were performed using one-way ANOVA with Tukey’s or Dunnett’s post hoc tests. p < 0.05 was considered statistically significant.

RESULTS

ABCB1 Does Not Mediate Efflux of Cd²⁺ from Kidney PT Cells

The inhibition of effluxed Rh123⁺ (33.2 ± 3.2% after 30 min) by the potent ABCB1 blocker, PSC833 (1 μM), a cyclosporine A derivative (Thevenod et al., 2000), demonstrated the presence of functional ABCB1 in rat WKPT-0293 Cl.2 cells. It has been hypothesized that ABCB1 protects against Cd²⁺-induced cell death of kidney PT cells by directly pumping out Cd²⁺ (Endo...
Subcellular fractionation demonstrated that the majority of $^{109}$Cd$^{2+}$ radioactivity is found in the cytosol ($>50\%$) with little membrane bound ($<13\%$) after 60-min incubation (Fig. 1A). As shown in Figure 1B, efflux of $^{109}$Cd$^{2+}$ ($22.0 \pm 5.2\%$) into the extracellular medium of control cells after 15 min was not affected by PSC833. Similarly, functional UIC-2 antibody, which specifically blocks ABCB1-mediated transport (Mechetner and Roninson, 1992) at the extracellular side, had no effect on $^{109}$Cd$^{2+}$ efflux ($20.2 \pm 5.3\%$) in comparison to a nonfunctional ABCB1 antibody, C219. Similar results were obtained after 30 min (data not shown). Moreover, both inhibitors did not increase net uptake of Cd$^{2+}$ at $37^\circ C$, which would be expected if the efflux component were mediated by ABCB1 (data not shown). In addition, no correlation between ABCB1 protein levels and $^{109}$Cd$^{2+}$ efflux but a positive correlation between ABCB1 expression and PSC833-sensitive Rh123$^+$ efflux was observed in PT cell lines with different levels of ABCB1 expression (Supplementary fig. 1D; Fig. 1C). Taken together, these data provide evidence that Cd$^{2+}$ is not transported by ABCB1.

The multidrug resistance protein (MRP2; ABCC2) has been postulated to transport transition metals complexed to glutathione (Kepler et al., 1998) in kidney PT, which express MRP2 in their apical membranes. The MRP inhibitor MK571 (IC$_{50}$ 3–5 $\mu$M) had no effect on both $^{109}$Cd$^{2+}$ uptake (data not shown) and efflux (Fig. 1B), suggesting that MRPs are also not responsible for Cd$^{2+}$ efflux in WKPT-0293 Cl.2 cells.

**Role of ABCB1 Overexpression on Cd$^{2+}$ Efflux and Cell Death in Kidney Cells**

ABCB1 is a target gene of Wnt signaling via the transcriptional activator complex $\beta$-catenin/TCF4 (Chakraborty et al., 2010; Thevenod et al., 2007). To better govern ABCB1 levels in WKPT-0293 Cl.2 cells, we introduced $\Delta N$-$\beta$-catenin, which is not degraded and therefore increases TCF4 transcriptional activity. Immunoblotting demonstrated that Wnt signaling components significantly increased ABCB1 expression by 1.77 $\pm$ 0.26 fold (Fig. 2A). Similarly, ABCB1a messenger RNA increased by $\sim1.3$-fold in $\Delta N$-$\beta$-catenin compared with empty vector cells (Supplementary fig. 2A). TCF4-wt transfection
exhibited comparable results (data not shown). Rh123\(^{+}\) efflux was also increased in \(\Delta N\)-\(\beta\)-catenin and TCF4-wt cells compared with empty vector control demonstrating that ABCB1 transport function, which is integral to its multidrug resistance properties, is concomitantly increased (Supplementary fig. 2B).

ABCB1 protects cells from undergoing cell death and therefore higher ABCB1 expression should lead to increased cell survival even if \(\text{Cd}^{2+}\) itself is not extruded. To that end, we determined \(\text{Cd}^{2+}\) cytotoxicity by MTT assay after 6 h in subconfluent cells. In empty vector cells, 25\(\mu\)M \(\text{Cd}^{2+}\) caused 16.7 ± 2.4% cell death. This was significantly reduced by −40% to 9.9 ± 1.4% in \(\Delta N\)-\(\beta\)-catenin cells. Comparably, cell death by 50\(\mu\)M \(\text{Cd}^{2+}\) was also reduced by −40% in \(\Delta N\)-\(\beta\)-catenin cells (Fig. 2B). But when \(^{109}\text{Cd}^{2+}\) efflux was determined in \(\Delta N\)-\(\beta\)-catenin cells, no significant change could be seen (Fig. 2C), even though ABCB1-mediated Rh123\(^{+}\) transport is augmented, further evidencing that \(\text{Cd}^{2+}\) transport by ABCB1 is not the mechanism behind the increased cell survival.

Activation of Wnt signaling also transactivates target genes aside from ABCB1 that are involved in cell proliferation and survival, such as cyclin D1 and c-Myc (Chakraborty et al., 2010; Thevenod et al., 2007) and could account for the observed protective effects against \(\text{Cd}^{2+}\) toxicity. Hence, \(\text{Cd}^{2+}\) efflux and toxicity were examined in MDCK cells stably transfected with ABCB1 (Supplementary fig. 3A). ABCB1 expression appeared to be rate limiting for Rh123\(^{+}\) (2 or 50 \(\mu\)g/ml) efflux because it quickly saturated in MDCK cells, but not in cells with ABCB1 overexpression (Supplementary fig. 3B). Furthermore, Rh123\(^{+}\) (2 \(\mu\)g/ml) efflux was blocked by PSC833, which indicates that transport is mediated by functional ABCB1 (Supplementary fig. 3C).

Similar to \(\Delta N\)-\(\beta\)-catenin cells, cell death induced by low \(\text{Cd}^{2+}\) was drastically attenuated in ABCB1-MDCK cells. In control MDCK cells, 38.5 ± 3.7%, 62.0 ± 4.3%, and 81.4 ± 2.8% toxicity by 10, 20, and 50\(\mu\)M \(\text{Cd}^{2+}\), respectively, after 6 h was observed by MTT assay using subconfluent cells (Fig. 3A). Only 10.2 ± 5.0% and 27.9 ± 2.1% cell death could be observed for 10 and 20\(\mu\)M \(\text{Cd}^{2+}\), respectively (Fig. 3A) that were highly significant. The lack of protection at 50\(\mu\)M \(\text{Cd}^{2+}\) could be a limitation of the assay because an almost maximal toxicity was reached by MDCK cells or due to activation of different death pathways not affected by ABCB1 (see below and Lee et al., 2006).

In another approach, electrical properties of confluent MDCK and ABCB1-MDCK monolayers were measured using ECIS (see “Methods” section) where capacitance reflects the conducting surface area of the electrodes: once cells attach, spread, and proliferate, the conducting surface area, i.e., the capacitance of the electrodes, decreases. Similarly, when cells die and detach from the electrode surface, capacitance increases. As shown in Figure 3B, nontreated cells remained stable during the entire experiment. However, \(\text{Cd}^{2+}\)-treated MDCK cells began to detach from the monolayer at ~35 h after \(\text{Cd}^{2+}\) application and this process accelerated as time progressed. Conversely, the capacitance of ABCB1-MDCK cells remained low in the 60th hour following \(\text{Cd}^{2+}\) addition, indicating little cell loss. Quantitative analysis of the curves following initiation of detachment showed that the change in capacitance of \(\text{Cd}^{2+}\)-treated MDCK cells (1.18 ± 0.24 nF/h) was significantly higher than that of \(\text{Cd}^{2+}\)-treated ABCB1-MDCK cells (0.44 ± 0.18 nF/h) (Fig. 3B).

This protection against \(\text{Cd}^{2+}\) toxicity could not be attributed to direct extrusion of \(\text{Cd}^{2+}\) by ABCB1 as parental MDCK and ABCB1-MDCK cells showed virtually no difference in \(^{109}\text{Cd}^{2+}\) efflux at 10\(\mu\)M (Fig. 3C) or 100\(\mu\)M \(\text{Cd}^{2+}\) (Supplemental fig. 3D) though ABCB1 expression is much higher in ABCB1-MDCK cells (Supplementary fig. 3A). Moreover, PSC833 did not affect \(^{109}\text{Cd}^{2+}\) efflux (data not shown). One could further argue that differences in expression of intracellular levels of the \(\text{Cd}^{2+}\)-detoxifying protein, metallothionein, could account for the decreased cell death observed in ABCB1 overexpressing cells. Immunoblotting demonstrated low levels of metallothionein in MDCK and ABCB1-MDCK cells (Supplementary fig. 3E). These observations were confirmed by immunofluorescence staining (data not shown). Taken together, these data provide decisive evidence that ABCB1 does not transport \(\text{Cd}^{2+}\).

Roles of Ceramides and ABCB1 in \(\text{Cd}^{2+}\)-Induced Cell Death

We recently reported that \(\text{Cd}^{2+}\) increases the sphingolipid Cer (Lee et al., 2007), which is postulated to be a substrate of ABCB1 in its oxidized form or glycosylated form (Eckford and Sharom, 2005; Masuda et al., 2008). Next, we investigated the effect of ABCB1 on increased Cer induced by \(\text{Cd}^{2+}\). \(\text{Cd}^{2+}\) increased Cer in MDCK cells by almost 60% after 3 h, which is in accordance with our previous observations in WKPT-2093 Cl.2 cells (Lee et al., 2007). In contrast, Cer were augmented by only ~18% in ABCB1-MDCK cells following the same \(\text{Cd}^{2+}\) exposure (Fig. 4A). Interestingly, the basal level of Cer in ABCB1-MDCK was noticeably but insignificantly lower than that in MDCK cells (0.31 ± 0.04 vs. 0.49 ± 0.07\(\mu\)M Cer). To further delineate the role of Cer in \(\text{Cd}^{2+}\)-induced cell death, we used the Fusarium mycotoxin FB1, a potent inhibitor of Cer synthase (IC\(_{50}\) = 0.1\(\mu\)M), and thereby blocks de novo sphingolipid biosynthesis (see Fig. 7) (Lee et al., 2007). Cell death measurement using H-33342 and ethidium bromide staining shows that 20\(\mu\)M \(\text{Cd}^{2+}\) for 6 h increased apoptosis by ~5.5-fold in MDCK cells compared with a ~1.6-fold increase in ABCB1-MDCK cells (Fig. 4B). In the presence of FB1, apoptosis by \(\text{Cd}^{2+}\) was abolished in MDCK cells, indicating that de novo Cer contributes to cell death induced by \(\text{Cd}^{2+}\), as previously shown in WKPT-0293 Cl.2 cells (Lee et al., 2007). There was no significant effect of FB1 ± \(\text{Cd}^{2+}\) in ABCB1-MDCK cells, which could be explained by the lower intracellular Cer levels in these cells (see Fig. 4A).

To mimic increased Cer levels by \(\text{Cd}^{2+}\), we applied a cell-permeable short-chain Cer, C\(_{2}\)-Cer, which is metabolized to more physiologically relevant long-chain Cer (Chapman et al.,...
FIG. 3. Protection of Cd\textsuperscript{2+} toxicity in ABCB1-MDCK cells is not associated with increased Cd\textsuperscript{2+} efflux. (A) Cd\textsuperscript{2+} cytotoxicity after 6 h of ~40% confluent cells was determined by MTT assay (n = 7). (B) Electrical properties of confluent cell monolayers measured with ECIS (see “Methods” section). Graphs represent three independent experiments. Data from individual curves were analyzed over a period of 30 h following start of capacitance increase in response to Cd\textsuperscript{2+} (n = 3). Statistics using Student’s unpaired t-test compare ABCB1-MDCK to MDCK cells. (C) Efflux of \textsuperscript{109}Cd\textsuperscript{2+} from MDCK and ABCB1-MDCK cells (n = 3–4). Total cellular \textsuperscript{109}Cd\textsuperscript{2+} (100%) (in pmol \textsuperscript{109}Cd\textsuperscript{2+} per well): MDCK, 339.5 ± 20.3; ABCB1-MDCK, 418.4 ± 5.7.
Similar to previous studies in PT cells (Lee et al., 2007), C₆-Cer increased cell death in MDCK cells after 6-h exposure (Fig. 5A), where the maximum concentration tested of 50 μM C₆-Cer induced 42.3 ± 5.3% cell death (n = 5). Under the same conditions, only 25.1 ± 2.8% cell death was observed in ABCB1-MDCK cells (p < 0.05).

Experiments with C₆-Cer conducted in ΔN-β-catenin–transfected PT cells, where ABCB1 expression is elevated (Fig. 2A), showed similar results. C₆-Cer (5 μM) caused 13.9 ± 2.2% toxicity in empty vector cells, whereas when nondegradable ΔN-β-catenin was introduced, toxicity was highly significantly attenuated to 3.5 ± 1.7% (Fig. 5B).

FIG. 4. Ceramide formation and role of de novo ceramide synthesis in apoptosis by Cd²⁺ in ABCB1-MDCK cells. (A) Total ceramide levels were determined from cells exposed to 20 μM Cd²⁺ for 3 h (n = 4). Statistics compare Cd²⁺-treated to control cells. (B) MDCK and ABCB1-MDCK cells were incubated in 0.5% serum for 24 h and then with or without FB₁ for 24 h in 0.5% serum followed by FB₁ ± Cd²⁺ in SFM for 6 h. Apoptosis and necrosis was determined by H-33342 and ethidium bromide staining (n = 4). Statistical analyses using one-way ANOVA compare treatments to control conditions as well as FB₁ + Cd²⁺ to Cd²⁺.
Similar effects were observed with 25 μM C₆-Cer (data not shown).

To underline the role of ABCB1 in Cd²⁺- and C₆-Cer-induced toxicity, apoptotic cell death with PSC833 was determined. Control and PSC833-only cells exhibited negligible apoptosis (< 1%). As shown in Figure 5C, 10 and 25 μM C₆-Cer increased apoptosis by about 3.8- and 6.9-fold in MDCK cells, respectively, which is similar to the effect of 20 μM Cd²⁺ (~4.0-fold) after 6 h, whereas apoptosis was not increased in ABCB1-MDCK cells exposed to C₆-Cer or Cd²⁺. Preincubation with PSC833 had no further effect on apoptosis in MDCK cells, but it completely reversed protection against apoptosis by C₆-Cer or Cd²⁺ in ABCB1-MDCK cells, restoring apoptosis rates to those in MDCK cells.

These studies were further complemented by ECIS measurements. Coincubation with PSC833 did not influence cell detachment and disruption of MDCK monolayer integrity induced by 25 μM Cd²⁺ (Fig. 5D). Conversely, in ABCB1-MDCK monolayers, which were barely damaged by 25 μM Cd²⁺ alone, PSC833+Cd²⁺ dramatically increased detachment of cells, confirming the apoptosis measurements (see Fig. 5C). Taken together, these data attest that ABCB1 can also confer protection against cell death caused by the sphingolipid, Cer, indicating that increased levels of this toxic compound or its metabolites induced by Cd²⁺ may be extruded by ABCB1 to ensure cellular survival.

**Transport of NBD-Ceramides by ABCB1 and Effect of Inhibition of Ceramide-Metabolizing Enzymes on Cd²⁺ Toxicity**

To demonstrate that Cer and/or its metabolites are transported by ABCB1, we monitored the extrusion of NBD fluorescently labeled C₆-Cer or C₆-GlcCer. As shown in Figure 6A, MDCK cells loaded with NBD-C₆-Cer extruded 10.4 ± 0.6% NBD fluorescence after 15 min. At the same time point, ABCB1-MDCK cells extruded 24.6 ± 2.7% NBD fluorescence (p < 0.01, n = 4). Moreover, PSC833 (1 μM) abolished augmented
extrusion of NBD fluorescence in ABCB1-MDCK cells (Supplementary fig. 3F, top), indicating that NBD-sphingolipid efflux is mediated exclusively by ABCB1. Similar to NBD-C₆-Cer, NBD-C₆-GlcCer extrusion was increased in ABCB1-MDCK cells (Fig. 6B) and was abolished by preincubation with 1μM PSC833 (Supplementary fig. 3F, bottom). After 15-min incubation, 26.1 ± 1.1% NBD fluorescence was released from MDCK cells, whereas ABCB1-MDCK cells expelled 34.3 ± 0.3% NBD fluorescence (p < 0.01, n = 3) (Fig. 6B), supporting our hypothesis that Cer and/or its metabolite GlcCer may be the toxic molecules pumped out of cells by ABCB1 to prevent Cd²⁺ apoptosis.

Cer can be converted to sphingomyelin by sphingomyelin synthase (SMS) and to GlcCer by glucosylceramide synthase (GCS) or is degraded to sphingosine and to sphingosine-1-phosphate by sphingosine kinase (SK) (see Fig. 7). Hence, to identify the toxic Cer metabolite extruded by ABCB1, we tested inhibitors of the enzymatic reactions in Cer metabolism (Table 1). Addition of the SK inhibitor, DMS, which blocks the Cer degradation pathway, augmented Cd²⁺ cytotoxicity (Table 1). Similar effects were seen with the SMS inhibitor D609. Though D609 also blocks phosphatidylcholine-specific phospholipase C, it increased Cer by almost twofold in WKPT-0293 Cl.2 cells at 0.1mM (Torchalski and Thévenod, unpublished data). In comparison to other GCS inhibitors, such as PDMP and PPMP, C₉DGJ is more selective and does not affect glucosidase enzymes (Andersson et al., 2000). Application of C₉DGJ to WKPT-0293 Cl.2 cells attenuated Cd²⁺ cytotoxicity by ~50%, suggesting that GlcCer is toxic and inhibition of its formation increases cell survival. Hence, DMS, D609, and C₉DGJ should all result in increased Cer levels, whereas only C₉DGJ should prevent GlcCer formation. But only C₉DGJ was able to reduce Cd²⁺-induced cell death, which argues in favor of GlcCer being the toxic compound extruded by ABCB1.
Kidney damage is often a result of chronic exposure to low amounts of Cd\(^{2+}\) present in the environment and food chain, which leads to subsequent accumulation in the kidney. The Cd\(^{2+}\) concentrations employed here (10–50 \(\mu\)M) were chosen to represent Cd\(^{2+}\) levels after long-term exposure to small amounts of Cd\(^{2+}\). This is based on the following rationale: the Cd\(^{2+}\) concentrations are still relatively low considering that Cd\(^{2+}\) binds to various intracellular proteins as well as to membranes, therefore the free Cd\(^{2+}\) concentrations are estimated to be in the submicromolar range. Furthermore, as Cd\(^{2+}\) accumulates in the human body after long-term exposure to low Cd\(^{2+}\) concentrations, higher concentrations will be reached in the cells and these may approximate the concentrations chosen in the present study. In this study, we have demonstrated through numerous experimental approaches that though increased ABCB1 confers protection against Cd\(^{2+}\)-induced cell death, this cannot be attributed to ABCB1-mediated efflux of Cd\(^{2+}\). Rather, Cer, an important proapoptotic signaling molecule, through its metabolism to GlcCer appears to mediate Cd\(^{2+}\)-induced cell death and is effluxed by ABCB1 to contribute to cell survival.

**ABCB1 Does Not Transport Cd\(^{2+}\)**

To date, there has been some evidence to suggest that ABCB1 may be involved in direct Cd\(^{2+}\) transport out of cells. First, deletion of the ABCB1 homologue in the nematode Caeenorhabditis elegans decreased cellular resistance to Cd\(^{2+}\) (Broeks et al., 1996) and then Cd\(^{2+}\) resistance was found to be associated with overexpression of ABCB1 in mammalian cells (Thevenod et al., 2000). More recently, the group of Sakata has investigated the effect of ABCB1 inhibitors on Cd\(^{2+}\) accumulation (Endo et al., 2002) and transepithelial transport (Kimura et al., 2005) in LLC-PK1 kidney cells. Though the effects were modest, Kimura et al. observed a decrease in basolateral-to-apical Cd\(^{2+}\) transport in the presence of ABCB1 inhibitors and increased intracellular Cd\(^{2+}\) accumulation in ABCB1 overexpressing LLC-PK1 cells (Kimura et al., 2005). Finally, human ABCBI expressed in an Escherichia coli tolC mutant strain with hypersensitivity to Cd\(^{2+}\) conferred Cd\(^{2+}\) resistance as well as reduced Cd\(^{2+}\) accumulation (Achard-Joris et al., 2005). In contrast, \(^{109}\)Cd\(^{2+}\) efflux in the present study was neither affected by well-known potent inhibitors of ABCB1 (Fig. 1) nor the overexpression of ABCB1 (Figs. 2 and 3). The discrepancy between the data from our and Sakata’s studies could lie in the experimental design because we studied \(^{109}\)Cd\(^{2+}\) efflux directly. The data of Sakata et al. would indicate that ABCB1 is a high-affinity Cd\(^{2+}\) transporter (they used 1 \(\mu\)M Cd\(^{2+}\)), but our previous work and the present study indicate that ABCB1 protects against Cd\(^{2+}\) toxicity at concentrations between 5 and 50\(\mu\)M.

To inhibit ABCB1, PSC833 and UIC-2 monoclonal antibody were applied (Fig. 1B) (Mechetner and Roninson, 1992). As a negative control, C219 was used because it binds to intracellular epitopes near the ATP-binding domains of ABCB1 and therefore does not interact with ABCB1 function in intact cells. Both inhibitors (Fig. 1B), as well as ABCB1 overexpression (Fig. 3C), had no effect on \(^{109}\)Cd\(^{2+}\) efflux. Taken together, these observations argue against a role for ABCB1 in directly transporting Cd\(^{2+}\) ions out of the cell. Moreover, efflux of Cd\(^{2+}\)-GSH complexes by MRP transporters were also ruled out, as MK571 had no effect on Cd\(^{2+}\) efflux (Fig. 1B), which is in accordance with a recent study by L’Hoste et al. in mouse PT cells (L’Hoste et al., 2009). Nevertheless, \(^{109}\)Cd\(^{2+}\) efflux is evident. Possible candidates for Cd\(^{2+}\) transport in kidney cells may include human organic...
cation transporter 2 (Thévenod et al., unpublished data), copper ATPases (Adle et al., 2007), or cystic fibrosis transmembrane conductance regulator (L’Hoste et al., 2009).

The Wnt signaling pathway, which is crucial for development, has been implicated in the propagation of carcinogenesis as a result of aberrant stimulation. We have previously demonstrated that Cd\(^{2+}\): (1) activates the Wnt signaling pathway (Chakraborty et al., 2010; Thevenod et al., 2000) and (2) augments expression of ABCB1, which was associated with a decrease in apoptosis (Thevenod et al., 2000) that has been corroborated by others (Huynh-Delerme et al., 2005). Here, we have directly linked active Wnt signaling (by transfection of TCF4-wt or β-catenin) with protection against Cd\(^{2+}\)-induced apoptosis through increased ABCB1 expression (Fig. 2). Cd\(^{2+}\) is known to disrupt binding between E-cadherin and β-catenin (Prozialeck and Lamar, 1997), causing its release into cytosol and nucleus where it activates TCF4 transcriptional activity (Chakraborty et al., 2010). In addition to Wnt signaling, Cd\(^{2+}\) may modulate ABCB1 activity through

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**TABLE 1**

**Effect of Sphingolipid Metabolism Inhibitors on Cd\(^{2+}\) Toxicity in WKPT-0293 Cl.2 Cells**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>+ Inhibitor</th>
<th>+ Cd(^{2+})</th>
<th>+ Inhibitor + Cd(^{2+})</th>
<th>Significance</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMS</td>
<td>−2.3 ± 1.5%</td>
<td>16.8 ± 2.8%</td>
<td>35.6 ± 6.0%</td>
<td>0.006</td>
<td>5</td>
</tr>
<tr>
<td>D609</td>
<td>11.2 ± 5.6%</td>
<td>8.8 ± 1.6%</td>
<td>74.1 ± 2.5%</td>
<td>0.000</td>
<td>7</td>
</tr>
<tr>
<td>C(_9)DGJ</td>
<td>−2.8 ± 2.3%</td>
<td>14.8 ± 2.2%</td>
<td>6.9 ± 1.5%</td>
<td>0.016</td>
<td>11</td>
</tr>
</tbody>
</table>

Note. Cytotoxicity (% control) was determined by MTT assay following 1-h 50µM Cd\(^{2+}\) exposure. Inhibitor preincubations: 1µM DMS for 30 min in SFM; 0.1mM D609 in SFM, pH 7 for 30 min; C\(_9\)DGJ (2µM) in standard culture medium for 2 days. Significance column compares inhibitor + Cd\(^{2+}\) to Cd\(^{2+}\) only using one-way ANOVA.
induction of stress response proteins, such as NF-κB and heat shock proteins (Bertram et al., 1996; Thevenod et al., 2000). These reports detailing increased cell survival as a consequence of Cd²⁺-induced ABCB1 expression are significant considering the fact that Cd²⁺ is a well-established carcinogen, causing tumor formation in various organs (Joseph, 2009), such as lung and kidney.

**Is Cd²⁺-Induced Ceramide the Toxic Moiety Extruded by ABCB1?**

The ATP-binding cassette transporter family is well known in their lipid transporting properties. For example, ABCA1 transports cholesterol, ABCB4 transports phospholipids, and ABCA7 and ABCC1 (MRP1) transport sphingolipids (Borst and Elferink, 2002) (ABCA1 was expressed in MDCK and ABCB1-MDCK cells at very low and comparable levels; data not shown). The sphingolipid, Cer, either in its glycosylated (Eckford and Sharom, 2005; van Helvoort et al., 1996) or oxidized (Masuda et al., 2008) form, in addition to phospholipids (Romsicki and Sharom, 2001), are substrates of ABCB1. Previous evidence for ABCB1 supported our decision to focus solely on investigating the role of ABCB1 in mediating Cer transport (Eckford and Sharom, 2005; Thevenod et al., 2000; van Helvoort et al., 1996). Levels of intracellular Cer can be regulated by four distinct mechanisms as follows: (1) regeneration to/from sphingomyelin, (2) de novo synthesis from palmitoyl CoA and serine, (3) degradation to sphingosine, and (4) conversion to GlcCer and higher glycosphingolipids (Fig. 7). Cd²⁺ could increase Cer formation by inducing mechanisms (1) and (2) or by inhibiting (3) and (4). To the best of our knowledge, the effect of Cd²⁺ on Cer metabolizing enzymes has not been investigated. We have previously reported that 50μM Cd²⁺ increases Cer levels in WKPT-0293 Cl.2 cells after ≥3 h. Cer appeared to originate from the de novo synthesis pathway because low nontoxic concentrations of the Cer synthase inhibitor, FB₁, could prevent Cer increase as well as apoptotic cell death induced by Cd²⁺ (Lee et al., 2007).

Given the molecular mechanism of ABCB1 function (Aller et al., 2009), Cer and/or its metabolites seem to be much more plausible substrates than Cd²⁺ ions because they reside at the membrane surface and are therefore more readily available for mobilization through the “vacuum cleaner” and/or “flippase” actions of ABCB1. Indeed, growing evidence indicates that Cer and/or its metabolites can be transported by ABCB1 across the membrane from the inner to the outer leaflet (Eckford and Sharom, 2005; Masuda et al., 2008). Furthermore, GlcCer has been postulated to play an important role in mediating multidrug resistance through ABCB1 in tumor cells because it can be reversed through inhibition of GCS (Olshefski and Ladisch, 2001). In these cases, conversion of Cer to GlcCer would permit ABCB1 to extrude the toxic Cer in the form of GlcCer culminating in increased cell survival. However, this hypothesis has been contested by others (Tepper et al., 2000).

Our data from NBD-Cer transport studies along with toxicity assays using C₃DGDJ indicate that GlcCer itself is the toxic form expelled by ABCB1 (Fig. 6, Table 1). Because NBD-C₆-Cer is in part metabolized to NBD-C₆-GlcCer and NBD-C₆-sphingomyelin in MDCK cells (van Meer et al., 1987), one could argue that sphingomyelin is also a candidate for the toxic moiety transported by ABCB1. However, the SMS inhibitor D609 drastically augmented Cd²⁺-induced cell death (Table 1) demonstrating that sphingomyelin cannot be the toxic moiety that is expelled by ABCB1.

Our preliminary results suggest that GlcCer is the toxic Cer metabolite generated during Cd²⁺ toxicity and is extruded by ABCB1 rather than Cer (Fig. 6; Table 1), which is supported by a recent study (Eckford and Sharom, 2005). Though sphingolipids may be removed from the cell by ABCB1 to promote survival, it is still evident that a substantial amount of Cd²⁺ is effluxed by ABCB1-independent means (Fig. 1.). Hence, the outcome of this study raises important questions for future work: (1) How does GlcCer cause toxicity? (2) How are Cer metabolizing enzymes affected by Cd²⁺? (see Fig. 7) (3) Which transport pathways mediate Cd²⁺ efflux? and (4) Is ABCB1 also protective against apoptosis induced by other toxic metals, such as Ni²⁺, Cr⁶⁺, or As³⁺, by efflux of proapoptotic GlcCer?

In summary, we have established that ABCB1 is not an efflux pathway for Cd²⁺ ions out of the cell as a mechanism to promote cell survival. Instead, intracellular Cer and GlcCer levels appear to be modulated by ABCB1 that could occur by direct efflux of GlcCer. Future work will focus on determining the sphingolipid moiety that is transported by ABCB1 to further delineate the mechanism of its antiapoptotic actions and subsequently progression of Cd²⁺ carcinogenesis.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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