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Multidrug and Toxin Extrusion Proteins Mediate Cellular Transport of Cadmium

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Abstract

Cadmium (Cd) is an environmentally prevalent toxicant posing increasing risk to human health worldwide. As compared to the extensive research in Cd tissue accumulation, little was known about the elimination of Cd, particularly its toxic form, Cd ion (Cd^{2+}). In this study, we aimed to examine whether Cd^{2+} is a substrate of multidrug and toxin extrusion proteins (MATEs) that are important in renal xenobiotic elimination. HEK-293 cells overexpressing the human MATE1 (HEK-hMATE1), human MATE2-K (HEK-hMATE2-K) and mouse Mate1 (HEK-mMate1) were used to study the cellular transport and toxicity of Cd^{2+} . The cells overexpressing MATEs showed a 2 – 4 fold increase of Cd^{2+} uptake that could be blocked by the MATE inhibitor cimetidine. A saturable transport profile was observed with the Michaelis-Menten constant (K_m) of $130 \pm 15.8 \mu\text{M}$ for HEK-hMATE1; $139 \pm 21.3 \mu\text{M}$ for HEK-hMATE2-K; and $88.7 \pm 13.5 \mu\text{M}$ for HEK-mMate1, respectively. Cd^{2+} could inhibit the uptake of metformin, a substrate of MATE transporters, with the half maximal inhibitory concentration (IC_{50}) of $97.5 \pm 6.0 \mu\text{M}$, $20.2 \pm 2.6 \mu\text{M}$, and $49.9 \pm 6.9 \mu\text{M}$ in HEK-hMATE1, HEK-hMATE2-K, and HEK-mMate1 cells, respectively. In addition, hMATE1 could transport preloaded Cd^{2+} out of the HEK-hMATE1 cells, thus resulting in a significant decrease of Cd^{2+} -induced cytotoxicity. The present study has provided the first evidence supporting that MATEs transport Cd^{2+} and may function as cellular elimination machinery in Cd intoxication.

Keywords

multidrug and toxin extrusion protein; cadmium; transporter; toxicity; detoxification; kidney

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Conflict of Interest Statement

The authors declare that they have no financial or personal conflicts of interest that influenced, or could be perceived to have influenced, this work.

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INTRODUCTION

Cadmium (Cd) is a toxic heavy metal which can accumulate in multiple organs, including kidney, liver, testis, lung, and pancreas. In addition to daily life exposure such as foods and cigarette smoking, Cd has been extensively disseminated in the environment as a pollutant due to a strong demand worldwide, particularly in the battery industry. Cd has no known physiological benefit. It may induce mitochondrial damage and culminate in cell death either by apoptosis or necrosis (Thevenod and Lee, 2013b; Thevenod and Lee, 2013a). Emerging evidence has linked chronic low level of Cd exposure to the causes of cancer, cardiovascular diseases, and diabetes (Prozialeck *et al.*, 2008; Edwards and Prozialeck, 2009; Tellez-Plaza *et al.*, 2012; Son *et al.*, 2014).

Kidney has been determined as a major target organ in Cd intoxication (Klaassen *et al.*, 1999; Thevenod and Lee, 2013b; Yang and Shu, 2015). Cd is found both as the free form (Cd^{2+}) and bound to carriers such as albumin, metallothionines (MT), glutathione (GSH), and cysteine (Cys) after absorption from intestine and lung (Klaassen *et al.*, 1999; Zalups, 2000; Wang *et al.*, 2010). All of the Cd-MT, Cd-GSH, Cd-Cys and Cd^{2+} could be filtered through the glomerulus, and reabsorbed by tubular epithelial cells. The bound form Cd-MT could be transported into the proximal tubular cells *via* endocytosis, while the complexes Cd-GSH and Cd-Cys reabsorbed by certain amino acid transporters in the apical membrane of the proximal tubular cells. As for the free Cd^{2+} , several apical membrane transporters, which are responsible for reabsorption of essential metals such as zinc, iron, manganese, and calcium, have been identified as Cd^{2+} transporters (Bannon *et al.*, 2003; Fujishiro *et al.*, 2012; Kovacs *et al.*, 2013; Marchetti, 2013). Recent studies have provided evidence supporting organic cation transporters (OCTs) as basolateral transporters for Cd^{2+} uptake in the proximal tubular cells (Soodvilai *et al.*, 2011; Thevenod *et al.*, 2013). Interestingly, additional transporters such as organic anion transporters (OATs) have also been suggested to play a role in the basolateral uptake of certain bound forms of Cd (Zalups *et al.*, 2004).

As compared to the comprehensive understanding in Cd accumulation, its elimination is poorly recognized. Since an extremely long half-life of Cd has been observed both in rodents and humans (10–30 years) (Thomas *et al.*, 1980; Jarup and Akesson, 2009), an elimination mechanism, while presumably being not as efficient as those for uptake and accumulation, could be critical to Cd detoxification. As a cellular protective mechanism, endogenous thiol-containing groups such as GSH, Cys and MT sequester most Cd present in the cell. Cd^{2+} could be released from those degradable bound complexes and there is equilibrium between the bound and the free forms of Cd in the cell. The released Cd^{2+} might be either chelated by thiol-containing groups or transported out of the cell. Hence we postulated that the efflux transporters resided in the apical side of the renal tubular epithelial cells for Cd^{2+} , if any, might serve as a detoxification mechanism. Inspiringly, Endo and colleagues have found that efflux of Cd^{2+} occurred *via* a proton antiport exchanger ($\text{Cd}^{2+}/\text{H}^{+}$ antiport) (Endo *et al.*, 1998a; Endo *et al.*, 1998b). Moreover, the identification of OCTs as basolateral Cd^{2+} transporters also provides a clue that the organic cation transporters in the apical membrane may be involved in transport of Cd^{2+} (Soodvilai *et al.*, 2011; Thevenod *et al.*, 2013).

Multidrug and toxin extrusion proteins (MATEs) are located in the apical membrane in hepatocytes and renal proximal tubules (Otsuka *et al.*, 2005; Masuda *et al.*, 2006). Functioning as organic cation transporters, MATEs are responsible for elimination of various, structure unrelated endogenous and exogenous compounds in the kidney. The present study was to determine whether Cd²⁺ was a substrate of MATEs. In particular, since the free Cd²⁺ is mainly responsible for Cd-induced cytotoxicity, we sought to test whether MATEs could ameliorate such cytotoxicity by transporting Cd²⁺ out of cells.

Materials and Methods

Materials

[¹⁴C]-metformin (1.0 mCi, 90 mCi/mmol) was purchased from Moravak Biochemicals. Cd Chloride was purchased from Sigma-Aldrich (St. Louis, MO). Cd Standard (1000 µg/mL in 2% HNO₃) was purchased from SPEXCertiPrep Inc. (Metuchen, NJ). Indium Standard (100 µg/mL in 2% HNO₃) was purchased from ULTRA Scientific Inc. (N. Kingstown, RI). Dulbecco's Modified Eagle's medium (DMEM), PBS buffer, Opti-MEM reduced serum medium, Lipofectamine 2000, hygromycin, and fetal bovine serum (FBS) were purchased from Invitrogen. All other reagents were commercial available. All the reagents used for Inductively Coupled Plasma mass Spectrometry (ICP-MS) were of trace ICP-MS grade.

Cell Lines

The generation of HEK-293 cell lines stably expressed human MATE1 (HEK-hMATE1) and human MATE2-K (HEK-hMATE2-K) using the Flp-In system (Invitrogen) has been described previously (Li *et al.*, 2013). In brief, the cDNAs of hMATE1 and hMATE2-K were constructed into pcDNA5 empty vector, and the stable cell lines were established by selection against hygromycin (75 µg/mL). Transient transfection was used to overexpress mouse MATE1 in HEK-293 cells (HEK-mMATE1) according to manufacturer's instruction (Lipofectamine 2000, Invitrogen). The overexpression of the MATE transporters in HEK-293 cells was confirmed by real-time PCR and functional tests.

Cell Culture

HEK-293 cells were cultured in DMEM supplemented with 10% FBS, 100 µg/mL streptomycin, and 100 U/mL penicillin, and maintained in 75 cm² plastic flask under 37°C in a humidified atmosphere with 5% CO₂.

Cd²⁺ Cellular Uptake and Quantification

The cellular uptake experiments were performed in the 24-well plates coated with poly-D-lysine. The protocol has been described elsewhere with minor modification (Li *et al.*, 2013; Muller *et al.*, 2013). In order to characterize MATE function in the heterogeneously expression system (HEK-293 cells), uptake studies were performed under an artificial intracellular acidic environment established by pre-incubation with NH₄Cl. 3.5×10⁵ cells were seeded in each well and incubated for 18–24 hours to reach confluence. Once ready, the cells were washed by pre-warmed K⁺ based buffer (KBB, 140 mM KCl, 0.4 mM KH₂PO₄, 0.8 mM MgSO₄, 1.0 mM CaCl₂, 25 mM glucose, and 10 mM HEPES, pH 7.4), then incubated in KBB buffer containing 30mM NH₄Cl for 15 minutes at 37°C, thereafter

incubated for 5 minutes in NH_4Cl -free KBB buffer which were replaced with the KBB buffer containing different concentrations of Cd^{2+} with or without MATE inhibitors for different periods of time. The uptake was stopped by adding 750 μL ice-cold KBB buffer, and the wells washed 3 times by KBB buffer of room temperature. After thorough aspiration of KBB buffer, 200 μL of nitric oxide (67–70%, Sigma-Aldrich, St. Louis, MO) was added to each well and the plate then shaken for 15 minutes. Thereafter, 100 μL of cell lysate was transferred to a 2 ml tube and incubated under 56°C for at least 4 hours. 20 μL internal standard (Indium, 100 $\mu\text{g}/\text{mL}$) and 1880 μL 2% nitric oxide was then added into each tube to make a final volume of 2 ml which was ready for quantification by ICP-MS (Agilent 7700). Cellular protein levels in parallel wells were determined by a bicinchoninic acid (BCA) protein assay kit (Bio-Rad Co. Hercules, CA). The protein levels were used to normalize the Cd^{2+} concentration values determined from ICP-MS analysis.

Metformin Cellular Uptake

The protocol for performing metformin uptake study is similar to that for Cd^{2+} as described above. The substrate metformin (50 μM : 10 μM [^{14}C]-metformin plus 40 μM non-radioactive metformin) was incubated with or without MATE inhibitors to probe cellular MATE activities. The reaction was stopped by adding ice-cold KBB and the cells were washed 3 times with KBB. The cells were shaken for another 15 minutes after adding 300 μL of cell lysis buffer (PBS with 1% triton X-100). 250 μL of cell lysate was then added to the scintillation tube which was pre-loaded with 3 ml Biodegradable Counting Cocktail buffer (Fisher Scientific Inc., Pittsburgh, PA). The radioactivity was counted by a liquid scintillation analyzer (PerkinElmer, Tri-Carb 2910 TR) normalized by the protein level in the lysate.

Determination of Cell Viability

The HEK-293 cells overexpressing a MATE transporter and mock cells were plated at a density of $3.5 \times 10^5/\text{well}$ in 24-well plates in a serum-containing medium for 18–24 hours. After grown to confluent, Cd^{2+} treatment was started with varied length of incubation periods. MATE transporters are bi-directional *per se*. Depending on the direction of their driving force of proton gradient, MATEs can either take up the substrates into the cell as uptake transporters or transport them out of the cell as efflux transporters. We analyzed Cd^{2+} cytotoxicity with two treatment protocols. First, the cells were intra-acidified by pre-incubation with NH_4Cl -containing KBB buffer as described above. The acidified cells of which the expressed MATE transporters are supposed to take up their substrates were then treated by different concentration of Cd^{2+} for 30 or 60 minutes with or without MATE inhibitors. After incubation, the uptake medium was aspirated, and the cells were washed once by PBS and then incubated in serum-containing medium. After incubation for another 18 hours, the cell viability was determined by using the cell counting kit-8 (CCKi-8, Enzo Life Science Inc.) according to manufacturer's instruction. In brief, 200 μL of the cell counting medium (10% of CCKi-8 in the serum-containing medium) was added into each well. After incubation under 37°C for 30 to 60 minutes, the medium was transferred to a 96-well and absorbance was measured at 450 nm. The second treatment protocol was followed to test if MATE transporters could efflux Cd^{2+} out of the cell and reduce Cd^{2+} -induced cytotoxicity. The cells were firstly intra-acidified and treated with Cd^{2+} as described above.

After this Cd preloading period, the cells were washed by KBB buffer for 3 times, then changed to Krebs-Ringer HEPES (KRH) buffer (125 mM NaCl, 4.8 mM KCl, 1.2 mM CaCl_2 , 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 25 mM HEPES, 5.6 mM glucose, pH 7.4) with or without known MATE inhibitors. After 12 hours of incubation during which preloaded Cd^{2+} could be transported out of the cell, cell viability was assayed as described above.

Statistical Analysis

GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA) was used to simulate transport kinetics and conduct statistical analysis. All of the experiments were repeated for at least 3 times, in each of which at least triplicate measurements were performed. Data from a representative experiment are shown and presented as Mean $\pm$ standard deviation (SD). For Cd uptake kinetics, the apparent $V_{\max}$ and K_m values were determined by fitting the data to the Michaelis-Menten equation. The values of half maximal inhibitory concentration (IC_{50}) were obtained by nonlinear regression with the inhibition data. Background uptake in the control cells was subtracted before fitting the data. A two-tail Student's t -test was used when statistically comparing between two groups, while one-way analysis of variance (ANOVA) followed by *post hoc* Turkey comparison was used when comparing between more than two different groups. $P < 0.05$ was considered as statistically significant.

RESULTS

Cd^{2+} is a substrate of MATEs *in vitro*

By using the HEK-293 cells stably transfected with an empty vector (HEK-C), human MATE1 (HEK-hMATE1), human MATE2-K (HEK-hMATE2-K), and the HEK-293 cells transiently transfected with mouse MATE1 (HEK-mMATE1) and the empty vector (HEK-Mock), we sought to determine whether Cd^{2+} was a substrate of these MATE transporters. Firstly, the cells were incubated in KBB buffer containing 50 μM Cd^{2+} with or without a known MATE inhibitor cimetidine (10 μM) for 20 minutes. The rate of Cd^{2+} uptake was about 2 to 4-fold in the MATE-overexpressing cells as compared to the control cells, and Cd^{2+} uptake contributed by MATEs could almost be blocked by addition of 10 μM of cimetidine (Fig. 1). We then performed a series of time-dependent and dose-dependent kinetic studies to further characterize Cd^{2+} uptake by different MATEs. The uptake of 5 μM Cd^{2+} in HEK-hMATE1 was linear up to 120 minutes, while the uptake seemed to be saturated for 50 μM Cd^{2+} when the uptake time was longer than 30 minutes (Data not shown). A saturable uptake profile was seen for all three MATE transporters in the dose-dependent Cd^{2+} uptake studies (Fig. 2). The $V_{\max}$ and K_m values were determined as $350 \pm 20.8 \text{ pmol (mg protein)}^{-1} \text{ min}^{-1}$ and $130 \pm 15.8 \mu\text{M}$ for HEK-hMATE1; $363 \pm 29.3 \text{ pmol (mg protein)}^{-1} \text{ min}^{-1}$ and $139 \pm 21.3 \mu\text{M}$ for HEK-hMATE2-K; and $271 \pm 19.2 \text{ pmol (mg protein)}^{-1} \text{ min}^{-1}$ and $88.7 \pm 13.5 \mu\text{M}$ for HEK-mMATE1, respectively (Table 1).

Cd^{2+} is an inhibitor of metformin uptake mediated by MATEs *in vitro*

The ability of Cd^{2+} to inhibit the uptake of metformin, a typical substrate for MATE transporters, was examined in HEK-MATEs and control cells. A range of Cd^{2+} (from 0.01 μM to 250 μM) was co-incubated with 50 μM metformin for 5 minutes in KBB buffer to determine the inhibitory potency of Cd^{2+} on MATEs (Fig. 3A–C). As expected, Cd^{2+} could

be an inhibitor of the three MATE transporters. The inhibitory effect was not due to any apparent Cd^{2+} -induced cytotoxicity as the cell viability was not affected under the same incubation conditions (Fig. 3D–F). MATEs-mediated metformin uptake could be inhibited by Cd^{2+} over the concentration range from 5 μM to 250 μM , with the calculated IC_{50} values of $97.5 \pm 6.0 \mu\text{M}$ for HEK-hMATE1, $20.2 \pm 2.6 \mu\text{M}$ for HEK-hMATE2-k, and $49.9 \pm 6.9 \mu\text{M}$ for HEK-mMate1, respectively (Table 1).

Enhancement of Cd^{2+} cytotoxicity by MATEs-mediated uptake

To examine whether MATEs-mediated uptake of Cd^{2+} would translate to alteration of Cd^{2+} -induced cytotoxicity, cell viability was compared between HEK-MATEs cells and control cells. After being treated with 100 μM Cd^{2+} in KBB buffer for 60 minutes, the cells were then incubated in serum-containing culture medium for another 18 hours for manifest of Cd^{2+} toxicity. The viability of HEK-hMATE1, HEK-hMATE2-k and HEK-mMate1 cells was significantly lower than that of control cells (Fig. 4A–B). When co-treated with the MATE inhibitor cimetidine (10 μM), the enhanced cytotoxicity was almost abolished in HEK-hMATE1 and HEK-mMate1 cells. As for the HEK-hMATE2-K cells, while we did not see a full recovery, the cell viability was increased from 10% to 35% by cimetidine co-treatment. The increase of Cd^{2+} -induced cytotoxicity in HEK-MATEs cells was also found with lower levels of Cd^{2+} exposure (Fig. 4C–D). All the cells were incubated with Cd^{2+} from 5 μM to 100 μM and the cell viability was analyzed as described above. The HEK-MATE cells showed a significantly decreased viability in all of the concentrations tested except 5 μM . A representative image of the enhanced cytotoxicity by Cd^{2+} treatment in HEK-hMATE1 cells was shown in Figure 4E.

Decrease of Cd^{2+} cytotoxicity by MATEs-mediated efflux

When the intracellular cytoplasm was artificially acidified, the membrane MATEs served as uptake transporters as described above. However, the MATEs may physiologically efflux their substrates out of the cells, giving their cellular location of apical membrane and the proton gradient across the renal proximal tubules. In other words, they could transport Cd^{2+} out of the cell as a protection mechanism from Cd^{2+} -induced cytotoxicity. To prove this concept, the HEK-hMATE1 cells were preloaded with Cd^{2+} as described in the Methods. The cells were then incubated in a physiologically relevant efflux buffer (KRH buffer) with or without MATE inhibitors. The MATE inhibitors could significantly increase intracellular Cd^{2+} concentrations, and enhanced Cd^{2+} -induced cytotoxicity (Fig. 5). We also conducted similar studies with the classical substrate metformin. The presence of MATE inhibitors could significantly decrease the extracellular metformin concentrations while increase intracellular ones (Data now shown). These results supported that the MATE transporters could serve as cellular detoxification machinery by excretion of Cd^{2+} .

DISCUSSION

The issue of Cd pollution comes along with human industrial civilization and is now a major global environmental challenge. The notion of identifying the mechanism of Cd elimination is appealing, especially since Cd is a cumulative toxicant with an extremely long biological half-life in the body. Such a mechanism could be critical to the understanding of Cd toxicity

and the associated diseases such as cancer, cardiovascular diseases, and diabetes. In the present study, we have provided the first evidence supporting that Cd^{2+} is a substrate of MATE transporters. In particular, our results have indicated that MATE function is a determinant of cellular response to Cd^{2+} treatment. Combined with previous findings (Thevenod *et al.*, 2013), the present study has suggested that the organic cation transport system, consisting of the basolateral OCTs and the apical MATEs in epithelial cells such as renal proximal tubules, serves as a detoxification mechanism underlying Cd^{2+} elimination from the kidney.

As a critical organ in xenobiotic disposition, the kidney highly expresses xenobiotic transporters. Multidrug resistance protein 1 (MDR1), breast cancer resistance protein (BCRP), multi-drug resistance protein 2 (MRP2), and multidrug and toxin extrusion proteins (MATE1 and MATE2-K) are among those major transporters for xenobiotic efflux in the apical membrane of human renal tubular epithelia cells (Konig *et al.*, 2013). Specifically, MDR1 and MATEs are more frequently involved in the apical transport of cationic compounds (Zhou, 2008; Cascorbi and Haenisch, 2010; Nies *et al.*, 2011). P-glycoprotein, encoded by *MDR1* gene, was previously characterized to be a transporter of Cd^{2+} and its activity was characterized as a determinant of Cd^{2+} -induced toxicity (Thevenod *et al.*, 2000; Kimura *et al.*, 2005). However, recent findings have indicated that Cd^{2+} is not a substrate of P-glycoprotein and the previously observed protective effect by this transporter was not due to Cd^{2+} efflux (Lee *et al.*, 2011). Different from most efflux transporters which belong to the ATP-binding cassette (ABC) transporter family, MATEs use an outward proton gradient as the driving force to transport substrates out of the cell (substrate/ H^+ antiport). Two MATE isoforms are expressed in the apical membrane of human kidney proximal tubules, hMATE1 and hMATE2-K, encoded by *SLC47A1* and *SLC47A2* genes respectively, while only one isoform (mMate1) is found in mouse kidney which is encoded by *Slc47a1* gene (Li *et al.*, 2011). A wide overlap of substrate spectrum has been recognized between OCTs and MATEs (Nies *et al.*, 2011; Yonezawa and Inui, 2011; Muller *et al.*, 2013), which function collaboratively to eliminate their substrates in the kidney. Inspiringly, both Soodvilai *et al* and Thévenod *et al* have previously demonstrated the involvement of OCTs in mediating Cd transport across the basolateral membrane into the renal proximal tubular cells (Soodvilai *et al.*, 2011; Thevenod *et al.*, 2013). This drove us to examine the interaction between Cd and MATEs.

In the present study, we tested the hypothesis that MATEs could transport Cd^{2+} by using HEK-MATEs and control cells. A two- to four-fold increase in the uptake of Cd^{2+} was shown in HEK-MATEs cells as compared to control cells, which could be then fully abolished by the MATE inhibitor cimetidine (Fig. 1 & 2), indicating that Cd^{2+} is a substrate of MATEs. Moreover, when metformin was used as the substrate, the function of MATEs was dose-dependently inhibited by Cd^{2+} (Fig. 3A–C). It should be pointed out that even though Cd^{2+} is cytotoxic, it did not cause any apparent toxicity in HEK-MATE cells at concentrations up to 250 μM with a short period of incubation for cellular uptake, under which the metal ion was clearly characterized as a substrate and an inhibitor of the MATEs (Fig. 3D–F). With the increasing concentrations of Cd, as expected, HEK-MATE cells exhibited an enhanced sensitivity to Cd^{2+} -induced cytotoxicity as compared to the control cells, which could also be partially or fully abolished by the MATE inhibitor cimetidine

(Fig. 4). While species differences in the recognition of a substrate are common for organic cation transporters (Minematsu and Giacomini, 2011), similar transporter kinetics of Cd^{2+} between hMATEs and mMATE1 were obtained in the present study. The extremely long biological half-life and the toxicity associated with Cd make it unethical to perform prospective human studies in order to ascertain the role of MATEs in Cd elimination and detoxification. Our data have suggested that mouse might be an appropriate *in vivo* model for this purpose. The genetic mouse models of MATE1 deficiency have been available (Li *et al.*, 2011). With our present *in vitro* evidence supporting Cd^{2+} as a substrate of MATEs, it would be interesting to compare the toxicokinetics and toxicity of Cd^{2+} in the mice with different MATE function.

Three human MATE isoforms, namely hMATE1, hMATE2 and hMATE2-K, have been identified. hMATE2 has been reported to have no expression on the cell surface but possess transporter activity (Komatsu *et al.*, 2011). It would be interesting to examine whether hMATE2 has the ability to intracellularly retain Cd^{2+} in future studies. As for hMATE1 and hMATE2-K, although they share substrates, hMATE1 usually exhibits a higher intrinsic activity in transporting those substrates as compared to hMATE2-K *in vitro* in cell models (Kajiwara *et al.*, 2012; Muller *et al.*, 2013). Data from our lab also indicated that there was only 4–5 fold of uptake increase by hMATE2-K in comparison to more than 20-fold of uptake increase by hMATE1 when using metformin as the substrate to characterize MATE function in HEK293 cells (data not shown). It should be noted, however, that the cellular surface levels of transporter proteins, which affects the $V_{\max}$ of uptake, were not accurately determined when comparing the activities of these MATEs in the heterogeneous overexpression cell models. In the present study, in addition to $V_{\max}$, similar K_m values were obtained for the uptake of Cd^{2+} by hMATE2-K and hMATE1. The IC_{50} values are also in the same magnitude while using Cd^{2+} to inhibit the uptake of metformin in HEK-hMATE2-K and HEK-hMATE1 cells, suggesting similar affinity of Cd^{2+} to the two MATEs. Both hMATE2-K and hMATE1 could be a determinant of Cd^{2+} -induced cytotoxicity in our cellular measurements *in vitro*. We postulate that hMATE1 along with hMATE2-K might play a role in Cd^{2+} elimination and detoxification in human kidney.

Physiologically, MATEs are located at the apical membrane of proximal tubules in the kidney where the substrates can transport out of these cells by using the outward proton gradient as the driving force. In order to characterize the transport kinetics of MATEs, a pre-acidified approach was used to reverse the transport direction *in vitro* in the HEK-MATE cells (Fig. 1–4), by which the intracellular accumulation of substrate could be quantified and the transport kinetics could be determined. Nevertheless, in order to prove the concept that MATEs could reduce Cd^{2+} -induced cytotoxicity by efflux of Cd^{2+} , the cells were pre-loaded with Cd^{2+} . Like the uptake of Cd^{2+} that could be inhibited by cimetidine, the efflux of pre-loaded Cd^{2+} was significantly blocked by this inhibitor, as well as two additional MATE inhibitors nefazodone and propafenone in HEK-hMATE1 cells. These inhibitors resulted in increased intracellular accumulation of Cd^{2+} as compared to the control treatment (Fig. 5). We also observed an increase in cytotoxicity as expected with the use of nefazodone and propafenone in HEK-hMATE1 cells. However, the increase of Cd^{2+} accumulation in the cells by cimetidine did not translate into an increased cytotoxicity. One possible explanation is that the pharmacological action of cimetidine might counter toward Cd^{2+} -induced

cytotoxicity, since it has been reported as an antioxidant and even a metal binding agent (Lambat *et al.*, 2002; Ahmadi *et al.*, 2011). We would like to point out that the effects of cimetidine on Cd²⁺-induced cytotoxicity might be dependent on different experimental conditions (protective in Fig. 4 vs. insignificant effect in Fig. 5). Cimetidine was only incubated for a short period of time with Cd²⁺ to inhibit its uptake in Figure 4. However, the compound was present in the culture for 12 hours to inhibit the efflux of preloaded Cd²⁺ (Fig. 5), likely allowing the manifestation of its pharmacological counteraction to Cd²⁺ cytotoxicity. Further efforts are needed to demonstrate whether MATEs indeed function *in vivo* to eliminate Cd²⁺ in the kidney.

Several factors should be considered while interpreting our data. First of all, it should be pointed out that our data did not rule out the role of other possible cellular elimination machinery including additional efflux transporters since a great portion of Cd²⁺ transport was observed in control cells. Moreover, the bound forms are the major species in the distribution of Cd among different tissues in the body. It is not clear whether those bound forms can interact with MATEs as well. Given the characteristic toxicokinetics (extremely long half-time and tissue accumulation tendency) of Cd in mammals, it is likely that MATEs may be not efficient mechanism of overall Cd elimination from the body. Instead, they might be a critical local mechanism of free Cd²⁺ equilibrium, contributing to Cd-induced toxicity in certain tissues such as the kidney with high levels of MATE expression. In addition, Cd²⁺ was characterized as a substrate of MATEs in our heterogeneous overexpression cell models using stable (for hMATE1 and hMATE2-K) or transit (for mMate1) transfection, hence, the V_{max} values obtained from this study might be reflective of overexpressed transporter proteins and irrelevant to physiological conditions. The importance of MATE function in Cd²⁺ elimination and detoxification has yet to be validated under more physiologically relevant conditions, and ideally in the more complex *in vivo* settings such as using Mate-knockout mouse model.

In conclusion, our study has provided the first evidence to support that MATE transporters are involved in cellular transport and detoxification of Cd²⁺. Since MATE transporters are highly polymorphic in human populations and their function is important in the disposition of xenobiotics including clinically used drugs, our findings will be of significance for the identification of factors that influence individual susceptibility to Cd toxicity and the interaction among drugs, environmental toxins and other xenobiotics.

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Highlights

- Cadmium is an environmentally prevalent toxicant.
- Little was known regarding the elimination and detoxification of cadmium.
- Cadmium ion is here demonstrated as a substrate of MATE transporters.
- MATEs may function as cellular elimination machinery in cadmium detoxification.

Figure 1. Uptake of Cd²⁺ by MATEs with or without the MATE inhibitor cimetidine
 Confluent cells were grown in 24-wells plate and incubated with 50 μM Cd²⁺ at 37 °C for 20 minutes with or without 10 μM of cimetidine. **A.** Uptake of Cd²⁺ (50 μM) in control HEK293 cells stably expressing an empty vector (HEK-C), HEK-hMATE1 and HEK-hMATE-2K stable cells. **B.** Uptake of Cd²⁺ (50 μM) in HEK293 cells transiently transfected with the empty vector (HEK-Mock) or mouse Mate1 (HEK-mMate1). Means ± SD of four measurements from a representative experiment are shown. ###*P* < 0.001 as compared to control cells (HEK-C or HEK-Mock); ****P* < 0.001 as compared to no cimetidine treatment. ns, no significance.

Figure 2. Dose-dependent uptake of Cd^{2+} by MATE transporters

HEK293 cells overexpress human (h) MATE1, hMATE2-K, mouse (m) Mate1, or empty vector, as described in Figure 1 legend. The HEK-hMATE1 (A), HEK-hMATE2k (B), HEK-mMate1 (C), and HEK-C (A & B) or HEK-Mock (C) cells were incubated with different concentration of CdCl_2 under 37°C for 10 minutes. Cd was detected by a mass spectrometry method. Rate of Cd uptake was expressed as pmol/mg protein/min. Means $\pm$ SD of triplicate measurements from a representative experiment are shown.

Figure 3. Inhibition of uptake of metformin into HEK-MATEs cells by Cd²⁺ treatment HEK-hMATE1 (A), HEK-hMATE2-K (B), HEK-mMate1 (C), and control cells HEK-C (A & B) and HEK-Mock (C), were incubated with metformin (50 μM) in the absence and presence of different concentration of Cd²⁺ under 37°C for 5 minutes. The curves were obtained by nonlinear regression fitting using GraphPad software. Cell toxicity was also determined by viability test after 5-minute incubation of different concentrations of Cd²⁺ in HEK-hMATE1 (D), HEK-hMATE2-K (E) and HEK-mMate1 (F). Means ± SD of three to six measurements from a representative experiment are shown. ***P* < 0.01, ****P* < 0.001. ns, no significance.

Figure 4. Cd²⁺-induced cytotoxicity in HEK-MATE and control cells

A & B: Effects of the MATE inhibitor cimetidine and Cd²⁺ on cell viability. HEK-C, HEK-hMATE1, HEK-hMATE2-K, HEK-Mock and HEK-mMate1 were incubated with Cd²⁺ (100 μM) with or without cimetidine (10 μM) for 30 minutes under 37°C. **C & D:** Dose-dependent Cd²⁺-induced cell toxicity. HEK-C, HEK-hMATE1, HEK-Mock and HEK-mMate1 were incubated in different concentrations of Cd²⁺ for 60 minutes under 37°C. **E:** Images of HEK-hMATE1 and HEK-C cells treated with 25 μM or 50 μM Cd²⁺ for 60 minutes at 37 °C. A–D: Data were normalized against measurements without Cd²⁺ or cimetidine treatment. Means ± SD of three to six measurements from a representative experiment are shown. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. ns, no significance. A–E: After Cd treatment, the cells were incubated in normal serum-containing medium for 18 hours until the cell viability was assayed by the cell counting kit-8.

Figure 5. Protective role of hMATE1 in Cd²⁺-induced cytotoxicity

A: Effect of three MATE inhibitors on Cd²⁺-induced cytotoxicity in hMATE1 cells pre-loaded with Cd²⁺. **B:** The MATE inhibitors show no toxicity when incubated with the cells alone. **C:** Effect of the MATE inhibitors on Cd²⁺ accumulation in hMATE1 cells pre-loaded with Cd²⁺. Means $\pm$ SD of three to six measurements from a representative experiment are shown. * $P < 0.05$, *** $P < 0.001$ compared to control (no inhibitor) group; ns, no significance.

Table 1**Kinetic parameters of interaction between cadmium ion (Cd^{2+}) and MATE transporters**

To calculate Michaelis-Menten parameters (V_{max} and K_m), HEK293 cells overexpressed human (h) MATE1, hMATE2-K, or mouse (m) Mate1 were incubated with different concentration of Cd^{2+} under 37°C for 10 minutes, as described in Methods and Fig. 2 legend. To calculate IC_{50} , metformin ($50\text{ }\mu\text{M}$) was used as the substrate of MATEs and the cells were incubated with metformin in the absence and presence of different concentration of Cd^{2+} under 37°C for 5 minutes as described in Methods and Fig.3 legend.

Parameters	hMATE1	hMATE2-K	mMate1
V_{max} (pmol/mg protein/min)	350 ± 20.8	363 ± 29.3	271 ± 19.2
K_m (μM)	130 ± 15.8	139 ± 21.3	88.7 ± 13.5
IC_{50} (μM)	97.5 ± 6.0	20.2 ± 2.6	49.9 ± 6.9