Renal Glucose Transporters Play a Role in Removal of Cadmium from Kidney Cells Mediated by GMDTC – A Novel Metal Chelator

Xiaojiang Tang (✉ river-t@126.com)
Southern Medical University

Xushen Chen
University at Buffalo

Bo Xiao
University at Buffalo

Qile Zhao
Shanxi Medical University

Wei Hu
Jianersheng (Zhuhai) Pharmtech Co., Ltd

Amber McKenery
University at Buffalo

Zhiyong Zhong
Jianersheng (Zhuhai) Pharmtech Co., Ltd

Xuefeng Ren
University at Buffalo

Research Article

Keywords: Cadmium, GMDTC, Glucose transporter, GLUT2, SGLT2, Kidney

Posted Date: February 14th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2580123/v1

License: ☑️ This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

Cadmium (Cd) is a toxic heavy metal, exposure to which leads to various adverse health effects including chronic kidney damage. Tremendous efforts have been explored in identifying safe chelating agents for removing accumulated Cd from kidney, but with limited success owing to their associated side effects and the ineffectiveness in eliminating Cd from the body. A newly developed chelating agent, sodium (S)-2-(dithiocarboxylato((2S,3R,4R,5R)-2,3,4,5,6-pentahydroxyhexyl) amino)-4(methylthio)butanoate (GMDTC), has been shown to effectively mobilize Cd from kidney. However, the mechanism(s) of removal are unclear, while it has been hypothesized that renal glucose transporters potentially play key roles mainly because GMDTC contains an open chain glucose moiety. To test this hypothesis, we utilized the CRISPR/Cas9 technology and human kidney tubule HK-2 cells, and constructed sodium-dependent glucose transporter 2 (SGLT2) or glucose transporter 2 (GLUT2) gene knockout (KO) cell lines. Our data showed that GMDTC's ability in removing Cd from HK-2 cells was significantly reduced both in GLUT2-/− or SGLT2-/− KO cells, with a removal ratio reduced from 28.28% in the parental HK-2 cells to 7.37% in GLUT2-/− KO cells and 14.6% in SGLT2-/− KO cells. Similarly, knocking out the GLUT2 or SGLT2 gene led to a compromised protective effect of GMDTC in reducing cytotoxicity of HK-2 cells. This observation was further observed in animal studies, in which the inhibition of GLUT2 transporter by phloretin treatment resulted in a reduced efficiency of GMDTC in removing Cd from the kidney. Altogether, our results show that GMDTC is safe and highly efficient in removing Cd from the cells, and this effect is mediated by renal glucose transporters.

Introduction

Cadmium (Cd) is a toxic heavy metal that poses a health risk for humans due to its wide spectrum of deleterious effects and a long elimination half-life of 20 to 30 years [1, 2]. Chronic exposure to Cd, through consumption of contaminated food and water, inhalation of tobacco smoke, and the occupational exposure, is related to various adverse effects, including osteoporosis, diabetes, cardiovascular disease, renal dysfunction, or even cancers [3, 4]. After absorption, Cd is bound to various sulfhydryl-containing molecules (e.g. albumin, low-molecule-weight thiols, metallothionein (MT), and transferrin) in the blood and the formed complexes are mainly taken up by the liver [5, 6]. In the liver, Cd stimulates the synthesis of MT [7, 8], a protein that acts as the precursor of Cd detoxification and is bound to MT to form the Cd-metallothionein (Cd-MT) complex, which is then released to the circulation system. Due to the small size of MTs, the complex is easily filtered through the glomerulus and reabsorbed by the proximal tubular cells, where the Cd-MT complex is endocytosed and degraded in lysosomes into amino acids and free Cd ions [5, 9-12]. When the intracellular loading with Cd ions exceeds the buffering capacity of cytoplasmic MT, liberated Cd ions can immediately start to affect the cell structure and functions, causing pathological changes in renal tubules [11, 13, 14]. Furthermore, Cd is mainly excreted through the urine, and the amount of Cd excreted daily in urine is very low[15]. Thus, with the redistribution of Cd, the kidney is the major target organ for Cd toxicity[16].
Chelating agents have been explored as a major therapy to reduce Cd-induced toxicity for many years[17-19]. Dithiocarbamates (DTC) have been shown to effectively mobilize Cd from animal tissues as indicated by their significantly enhanced biliary excretion of Cd, but not impacting the urinary excretion[20, 21]. An in vivo study has also shown that the combination of 2,3-dimercapto-1-propane sulfonic acid (DMPS) and N-acetyl cysteine (NAC) exhibited a limited benefit in the treatment of Cd intoxication[22]. However, none of these chelators are approved because of their ineffectiveness in Cd removal from the kidneys, or severe side effects, such as flushing, agitation, localized burning at the infusion site [23]. To develop a safe and specific antidote for Cd is critical to meet the need in clinical application and public health interventions.

Sodium (S)-2-(dithiocarboxylato ((2S, 3R, 4R, 5R)-2, 3, 4, 5, 6-pentahydroxyhexyl) amino)-4 (methylthio) butanoate (GMDTC) (Fig. 1A) is a new DTC derivative synthesized by our research group. It has strong chelating ability for toxic heavy metals, including Cd$^{2+}$, mercury (Hg$^{2+}$), chromium (Cr$^{6+}$), and lead (Pb$^{2+}$), without significant effects on the status of the essential metals such as sodium (Na$^+$), and potassium (K$^+$), among others [24-26]. However, it is not entirely clear by what mechanism(s) GMDTC removes accumulated Cd from the kidney, although glucose reabsorption pathway has been proposed as GMDTC contains an open chain glucose moiety. The renal glucose reabsorption pathway is the physiological process of renal glucose reabsorption from the glomerular filtrate through proximal tubule epithelial cells into the blood mediated by active sodium-coupled glucose cotransporters (SGLTs) and passive glucose transporters (GLUTs) [27]. Briefly, glucose filtered at the glomerulus is firstly transported by SGLTs into proximal tubule epithelial cells against a concentration gradient coupled with the inward diffusion of sodium ions which is maintained by an Na$^+$/K$^+$ ATPase pump; once the concentration of glucose in epithelial cells has been accumulated to a level above the interstitial one, intracellular glucose diffuses out to the plasma via the facilitative glucose transporters GLUTs in the basolateral membranes [28, 29].

To assist the clinical development of GMDTC as a detoxifying agent for Cd-induced toxicity, here we utilized the CRISPR/Cas9 genome editing technology and constructed glucose transporter 2 (GLUT2$^{-/-}$) and sodium-dependent glucose transporter 2 (SGLT2$^{-/-}$) knockout (KO) human kidney proximal tubule (HK-2) cell lines, two key glucose transporters. We compared the protective effects of GMDTC between these KO cell lines and parental cells upon Cd exposure and examined the involvement of glucose reabsorption pathway in GMDTC-mediated removal of Cd from cells. We further evaluated this in vitro effect in an animal model. Our results supported the involvement of glucose reabsorption pathway in Cd excretion from the kidney by GMDTC, mechanistically supporting the therapeutic potential of GMDTC used for kidney damage and other health effects caused by Cd exposure.

Materials And Methods

2.1. Chemicals and Reagents

GMDTC (purity 95%-97%, molecular weight 433 g/mol) was synthesized by WuXi AppTec (Tianjin, China). Representative HPLC chromatogram of GMDTC is shown in Supplementary Fig. 1. Cadmium chloride
(CdCl\(_2\), purity ≥ 99.99%, molecular weight 183.32 g/mol) was purchased from Alfa Aesar (Tewksbury, MA, USA), and phloretin (purity ≥ 99%) was from Sigma-Aldrich (St. Louis, MO). The chemicals were freshly prepared and dissolved in 0.9% saline and passed through 0.22 µm filter prior to treating cells or animals. The alamarBlue™ Cell Viability Reagent was obtained from ThermoFisher Scientific (Waltham, MA, USA). The human kidney proximal tubule HK-2 (CRL-2190) cell line was obtained from American Type Culture Collection (Manassas, VA, USA).

2.2. Cell Culture

HK-2 cells were cultured in DMEM/F12 medium containing a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12. Cell culture media were supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin (100 U/mL penicillin and 100 μg/mL streptomycin). Cells were incubated at 37 °C in an atmosphere of 5% CO\(_2\) and 95% humidity.

2.3. Targeting Strategy

The target genes are solute carrier family 2 member 2 (SLC2A2) and solute carrier family 5 member 2 (SLC5A2), which encode GLUT2 and SGLT2 proteins, respectively. We used CRISPR/Cas 9 gene editing system to generate GLUT2\(^{-/-}\) or SGLT2\(^{-/-}\) KO HK-2 cell lines. A total of 4 single-guide RNAs (sgRNAs) (Table 1), two for each target gene, were designed and purchased from Thermo Fisher Scientific (Waltham, MA, USA) with high on-target scores.

Table 1. SgRNAs for generation of KO HK-2 cell lines.

<table>
<thead>
<tr>
<th>SgRNAs</th>
<th>Target gene</th>
<th>Chr</th>
<th>Sequence</th>
<th>PAM(^a)</th>
<th>Strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT2 sgRNA 1</td>
<td>SLC2A2</td>
<td>3</td>
<td>CTACCGACAGCCTATTCTAG</td>
<td>TGG</td>
<td>-</td>
</tr>
<tr>
<td>GLUT2 sgRNA 2</td>
<td>SLC2A2</td>
<td>3</td>
<td>CACCGATATACATAGGAACC</td>
<td>AGG</td>
<td>+</td>
</tr>
<tr>
<td>SGLT2 sgRNA 1</td>
<td>SLC5A2</td>
<td>16</td>
<td>ATTGTCAATCAGGGCCTTCT</td>
<td>GGG</td>
<td>-</td>
</tr>
<tr>
<td>SGLT2 sgRNA 2</td>
<td>SLC5A2</td>
<td>16</td>
<td>AAGCGCTTGCGCAGGTACTG</td>
<td>TGG</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) PAM: protospacer adjacent motif.

2.4. Analysis of Off-target Effects

The Cas-OFFinder algorithm (http://www.rogenome.net/cas-offinder/) was used to identify potential off-target sites (NGG PAMs with up to 3 mismatches) in Homo sapiens (GRCh38/hg38). Cas-OFFinder outcomes were further filtered to identify the most problematic off-targets with the fewest mismatches. It is reported that any sgRNAs with more than 3 mismatches have a low risk of causing off-target effects, and 96% of 2 bp mismatches do not function\[30\]. Seed region (the 10-12 bp closest to the PAM site) is not the most important for binding and any mismatches in this region, and further reduces the possibility
of off-target effects[31]. Therefore, problematic off-target sites were defined as off-targets with up to 1 mismatch in the seed region and up to 2 mismatches in the non-seed region with an NGG PAM. Approximately 400 base pairs of sequence flanking each off-target site was amplified by PCR for further cleavage efficiency analysis to determine off-target effect. The potential off-target sites are shown in (Supplemental Table 1).

2.5. Cell Transfection

Briefly, HK-2 cells were seeded in 24-well plates at a density of $1.0 \times 10^4$ cells/well for further transfection using Lipofectamine™ CRISPRMAX™ Transfection Reagent (Invitrogen, Carlsbad, CA, USA). SgRNAs (240 ng of each) were mixed with TrueCut™ Cas9 Protein v2 (Invitrogen, Carlsbad, CA, USA) to form the ribonucleoprotein (RNP) complex, and the transfection procedure was performed according to the manufacturer's instructions. After 2 days incubation at 37 °C, cells were rinsed with PBS and genomic DNA was extracted for cleavage efficiency analysis with T7 endonuclease I assay.

2.6. T7 Endonuclease 1 (T7E1) Assay

The genomic sequences containing on and off target regions mediated by each sgRNA were polymerase chain reaction (PCR) amplified using the primers listed in Table 2. PCR products were purified using E.Z.N.A® Cycle Pure Kit (Omega Bio-tek, Norcross, GA, USA). The purified PCR products were then mixed with 1 μL T7E1 buffer and were denatured and annealed for heteroduplex formation. Reaction amplicons were treated with 0.5 μL T7 endonuclease 1 (New England Biolabs, Ipswich, MA, USA) at 37 °C for 15 minutes, following the addition of 1.7 μL of 0.22 M ethylene diamine tetraacetic acid (EDTA) to stop the reactions. Finally, the digested products were analyzed with 1.5% agarose gel electrophoresis and images were attained by Bio-Rad Gel Doc imaging system. Band intensities were calculated using ImageJ. Cleavage efficiency was calculated according to the equation: Fraction Cleaved = sum of cleaved band intensities / (sum of the cleaved and parental band intensities); Cleavage Efficiency = 1− [(1−Fraction Cleaved) ½].

Table 2. Primers used in PCR analysis to check on-target and off-target effects caused by specific sgRNAs.
<table>
<thead>
<tr>
<th>SgRNAs</th>
<th>Primers (Forward oligo)</th>
<th>Primers (Reverse oligo)</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT2 sgRNA 1</td>
<td>TACTCAACTGTAGAAGCTCC</td>
<td>GGGCATGCAGAAATGTAG</td>
<td>618</td>
</tr>
<tr>
<td>(on target)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLUT2 sgRNA 2</td>
<td>AGCTCTAAAGTTAAGACCTGGGC</td>
<td>CTACAACTGCATATGAGGTC</td>
<td>615</td>
</tr>
<tr>
<td>(on target)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGLT2 sgRNA 1</td>
<td>AATCCAGGGGTGCTAGTTAGC</td>
<td>ATGTTGGCTGGGTCTCCC</td>
<td>600</td>
</tr>
<tr>
<td>(on target)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGLT2 sgRNA 2</td>
<td>GACACAAGGGTTGGTGCTTTGTT</td>
<td>TCATCATAACCCCTCGAGGTC</td>
<td>608</td>
</tr>
<tr>
<td>(on target)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLUT2 sgRNA 1</td>
<td>AGCCTGGTGTGTTGTTGAAGG</td>
<td>GACTGGCTTCAAACTGGCT</td>
<td>402</td>
</tr>
<tr>
<td>(off target)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGLT2 sgRNA 2</td>
<td>ACAGAGGTGTTGCTCTGCA</td>
<td>TTGTGGGCTGGCTGTTCA</td>
<td>475</td>
</tr>
<tr>
<td>(off target)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AGGGCCTGGTCTTGACT</td>
<td>GGAGCTGGGGAACCATCAAT</td>
<td>453</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAGAGGGAGGGCAAGTTGAA</td>
<td>584</td>
</tr>
</tbody>
</table>

### 2.7. Clone Isolation and Tracking of Indels by Decomposition (TIDE) Analysis

The transfected cells were subject to single-cell sorting into 96-well plates to obtain single clones using FACSARia III Cell Sorter (BD bioscience, USA). When the clonal cells in each well reached confluency, genomic DNAs encompassing the CRISPR/Cas9 target sites were extracted and PCR amplified. The purified PCR products were then submitted for Sanger sequencing. Sequence traces of target-specific PCR products attained from Sanger sequencing were analyzed with the TIDE webtool (https://tide.nki.nl/) to determine the spectrum and frequency of targeted mutations generated.

### 2.8. Western Blotting

To analyze the protein expression of the gene knockout cell lines, the cells were lysed using the M-PER™ Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA). The protein concentration was determined using Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Total protein amount of 20 µg for each sample was separated using 10% SDS-PAGE gel electrophoresis and transferred to a PVDF membrane. After blocking, the following primary antibodies were used: Anti-SGLT2 antibody (1:1,500, Abcam, ab137207); Anti-Glucose Transporter GLUT2 antibody [5D1] (1:1,500, Abcam, ab85715); Anti-β-Actin antibody (1:500,000, Sigma-Aldrich, A1978). As secondary
antibodies, Goat Anti-Rabbit IgG H&L (HRP) (1:10,000, Abcam, ab205718) and Goat Anti-Mouse IgG H&L (HRP) (1:10,000, Abcam, ab205719) were utilized. The signals were detected with the Clarity™ Western ECL Substrate (Bio-Rad, Hercules, CA, USA).

2.9.  Cell Viability Assays

HK-2 cells with or without CRISPR/Cas9 targeted editing were seeded in 96-well plates at a density of 1.0 x 10^4 cells/well in 100 µL of medium (composition described in the section of ‘Cell Culture’), and then incubated 24 h before treatments. In the GMDTC and Cd toxicity experiments, the cells were treated with various concentrations of GMDTC or CdCl₂ for 24 h. In the GMDTC rescue experiment, the cells were pre-treated with 250 µM CdCl₂ for 2 h to allow for accumulation of Cd in cells, washed twice with phosphate buffered saline (PBS), replaced with fresh culture medium to eliminate extracellular Cd, and then treated with various concentrations of GMDTC for 24 and 48 h, respectively. Cell viability was measured using the alamarBlue assay. After treatments, 1 mM alamarBlue reagent was added into each well and incubated for 4 h. Fluorescence was recorded on a BioTek microplate reader using a fluorescence excitation wavelength of 560 nm (excitation range is 540-570 nm) and an emission of 590 nm (emission range is 580-610 nm). Cell viability was calculated relative to control.

2.10.  Animal and Treatment

Pathogen-free male NIH Swiss mice (17-20 g) were purchased from Guangdong Medical Laboratory Animal Center, China (GDMLAC, National certification No. 2006A015). All animals were provided a standard diet and housed in an approved facility with climate control and a 12 h light/12 h dark cycle. Mice were administered CdCl₂ via intraperitoneal (i.p.) injection for five consecutive days with a dose of 6 µmol/kg body weight (Fig. 1B). Thirty-five days post treatment, mice were used for experiments. The detailed animal grouping and treatment are shown in the Fig. 1B. Briefly, thirty-six Cd²⁺-laden mice were randomly divided into 6 subgroups, then treated with GMDTC of low (108 mg/kg body weight per day) or high (433 mg/kg body weight per day) concentration and/or GLUT2 inhibitor (phloretin) at a dose of 10 mg/kg body weight per day for 14 days, respectively. GMDTC and phloretin were administered via i.p. injection and oral gavage, respectively. In the combined treatment groups, phloretin was administered 30 min prior to GMDTC. Typical serum biochemical parameters (alanine aminotransferase, aspartate aminotransferase, creatinine, blood urea nitrogen, total bilirubin, and uric acid), indicating hepatic and renal functions, were analyzed to evaluate impact of high dose (433 mg/kg body weight) GMDTC on mice. As compared to control group, no significant changes were observed in high dose GMDTC group (Supplementary Table 2). During the last 24 h of treatment, urine samples were collected to determine the Cd excretion level. At the end of treatment, mice were euthanized individually in a CO₂ chamber and kidney tissues were collected for Cd measurements. A counterpart control group was administrated 0.9% saline instead of CdCl₂, GMDTC or phloretin. The doses of chemicals used for animal treatment were based on our previous study [25]. Animal experiments complied with the Guide for the Care and Use of Laboratory Animals, and the Animal Care and Use Committee of GDMLAC in China approved animal test protocols (#00062324).
2.11. Measurement of Urinary Cd\(^{2+}\) by Graphite Furnace Atomic Absorption Spectroscopy (GFAAS)

Concentrations of Cd\(^{2+}\) in urine were measured by a GFAAS (PerkinElmer Pinnacle 900T, Waltham, MA, USA) as previously described[25]. Briefly, a hollow cathode lamp operated at the current of 6.0 mA and a wavelength of 228.8 nm with a spectral bandpass of 0.8 nm was used. After digested in 0.5 mL 70% nitric acid at 70 °C, the urine samples and standard samples were diluted five times with a solution containing 0.5% nitric acid and 0.1% TrionX-100. Ten microliter of the solution was injected into the graphite furnace for analysis. Urinary Cd\(^{2+}\) concentration was calculated using the following equation:

\[ \text{Urinary Cd (µg/g, cr.)} = \frac{Cd (\text{µg/L}) \times \text{Dilution Factor}}{\text{Urinary Creatine (g/L)}} \]

2.12. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) Analysis

HK-2 cells with or without CRISPR/Cas9 targeted editing were treated in a similar manner as GMDTC rescue cell viability experiments. The amount of 2 × 10\(^6\) cells were seeded in a 100-mm plate with 10 mL complete medium for overnight culturing before treatments. On the following day, cells of about 70% confluence were first pretreated with 250 µM CdCl\(_2\) for 2 h, washed twice with PBS, and then replaced with fresh culture medium including 0 or 1500 µM GMDTC for another 24 h culturing. After incubation, cells were washed twice with PBS and collected by trypsin treatment. Total cell numbers were checked by TC20 Automated Cell Counter (Bio-rad, Hercules, CA, USA). Cell pellets were collected by centrifuge and digested in 100 µL concentrated 70% nitric acid (HNO\(_3\)) for 2 h at 100 °C. After digestion, mixtures were diluted in 5 mL 2% HNO\(_3\), and filtered before analysis of Cd using ICP-MS. In a similar fashion, Cd in the kidney was prepared and analyzed by ICP-MS as described previously[25]. Briefly, the whole right kidney of each animal was collected, weighted (about 200 - 300 mg) and dried at 80 °C for 1.5 h. The dried samples were then digested in 0.5 mL of concentrated 70% HNO\(_3\) at 70 °C for overnight, followed by dilution in 5 mL 2% HNO\(_3\). Cd\(^{112}\) was quantified using authentic metal standards (AACD1, Inorganic Ventures, Lakewood, NJ, USA) and Indium\(^{115}\) (AAIN1, Inorganic Ventures, Lakewood, NJ, USA) was added as internal standard. ICP-MS was performed at the Chemistry Instrumentation Center at the University at Buffalo. Cd\(^{2+}\) content in cells and kidney samples were calculated using the following equation:

\[ \text{Cd in cells (ng/10}^6\text{ cells)} = \frac{Cd (\text{µg/L}) \times \text{Dilution Factor} \times 5 \text{ mL}}{\text{Cell Numbers (10}^6\text{)}} \]

\[ \text{Cd in kidney (µg/g)} = \frac{Cd (\text{µg/L}) \times \text{Dilution Factor} \times 5 \text{ mL} \times 0.001}{\text{Weight of Kidney (g)}} \]
2.13. Statistical Analysis

All data are presented as mean ± SD. One-way ANOVA and post-hoc Tukey Tests were conducted using the GraphPad Prism software (GraphPad Software, San Diego, CA) to analyze the differences between the groups. Mann-Kendall test was used to assess the significance of trend in rescuing effects mediated by GMDTC along with increasing concentrations in Cd-treated cell lines. It was considered statistically significant when the $P$-value was less than 0.05.

**Results**

3.1. Generation of $\text{GLUT2}^{-/-}$ or $\text{SGLT2}^{-/-}$ KO HK-2 cell lines with CRISPR/Cas9 gene editing system

We utilized the CRISPR/Cas9 gene editing tool to construct $\text{GLUT2}^{-/-}$ and $\text{SGLT2}^{-/-}$ KO cell lines. Two sgRNAs were designed for each gene. The gene editing activity of each sgRNA was evaluated using the T7E1 assay. In addition to the multiple bands produced by sgRNA 1 of SGLT2, the cleaved bands by the T7E1 enzyme showed that insertion or deletion mutations (indel) were introduced into the genomes (Supplementary Fig. 2A). The gene modification efficiencies were 62.90% and 36.43% for GLUT2 sgRNA 1 and 2, respectively, and 27.02% for SGLT2 sgRNA2. It is proposed that pool cells with high cleavage efficiency are suitable for further analysis [32]. Thus, transfected cells with GLUT2 sgRNA 1 and SGLT2 sgRNA 2 were selected to isolate single clones for further validation and proliferation. Then, we analyzed the nucleotide sequences of the PCR products of targeted regions in single clones, and further confirmed the successful indel mutation of the intended $\text{GLUT2}$ and $\text{SGLT2}$ gene locus by both sgRNAs used (Supplementary Fig. 2B & C, Supplementary Fig. 3A & B). Next, to investigate the type of mutations in more detail, Sanger sequencing files of PCR products of single clones were analyzed by the TIDE assay. The TIDE analysis results showed that $\text{GLUT2}^{-/-}$ single clone 1 ($\text{GLUT2}^{-/-}$-S1) exhibited 23 base deletions, and the percentage of sequences with this deletion was 98.4%, while 96.3% of sequences in $\text{SGLT2}^{-/-}$ single clone 14 ($\text{SGLT2}^{-/-}$-S14) showed 8 base deletions (Supplementary Fig. 2D). Off-target effect is a general concern for the use of CRISPR/Cas9 technology. PCR products of potential off-target sites were subjected to T7E1 enzyme detection (Supplementary Fig. 4) and Sanger sequencing (Supplementary Fig. 3C & D1-3). The results showed that CRISPR/Cas9 does not cause non-specific
mutagenesis at high-risk regions as suggested by Cas-OFFinder tool, and each sgRNA is highly specific for intended target site. Furthermore, western blotting results showed that the CRISPR/Cas9 editing had resulted in significant loss of GLUT2 and SGLT2 in GLUT2−/− and SGLT2−/− KO cell lines, respectively (Supplementary Fig. 2E). Though residual protein expression was observed probably due to translation reinitiation leading to N-terminally truncated target proteins or skipping of the edited exon leading to protein isoforms with internal sequence deletions [33], frameshift mutations could still lead to full disruption of the protein despite no or weak nonsense-mediated decay of target mRNAs [34]. Collectively, these results indicate that GLUT2−/− and SGLT2−/− KO HK-2 cell lines were successfully generated by the CRISPR/Cas9 system.

3.2. Compromised protective effects of GMDTC in Cd-treated GLUT2−/− and SGLT2−/− KO HK-2 cell lines

As shown in Supplementary Fig. 5A, at concentrations up to 5,000 μM, GMDTC did not cause significant cytotoxicity on HK-2 cells post 24h treatment. We choose 1,500 μM GMDTC as the highest dose of GMDTC used in our experiment. At this dose, there was no significant impact on cell viability in all three cell lines, HK-2, GLUT2−/− KO and SGLT2−/− KO cells (Fig. 2A). We examined the rescue ability of GMDTC in HK-2 cells pretreated with CdCl₂ because Cd-induced renal toxicity is largely due to the accumulation of Cd in proximal tubule cells in the kidney. Therefore, as an effective chelator to prevent Cd-induced kidney cell cytotoxicity, the chelator must be able to remove the accumulated Cd²⁺ from the cell interior. All three cell lines were pretreated with 250 μM CdCl₂ (LC50 of 2-h acute CdCl₂ treatment, Supplementary Fig. 5B) for 2 h. After washing the cells and the addition of new medium, various concentrations of GMDTC were administered and cells were further incubated for 24 h. The administration of GMDTC resulted in a significantly dose-dependent improvement of HK-2 cell viability at 24-h post treatment, and HK-2 cell viability with 1,500 μM GMDTC treatment is 1.32-fold higher when compared to HK-2 cells treated with CdCl₂ alone (Fig. 2B). Furthermore, the fold change of HK-2 cell viability, indicating rescue effect of GMDTC treatment, demonstrated a monotonic upward trend along with increasing concentrations of GMDTC (Mann-Kendall trend test P<0.01). In comparison, the rescuing ability of GMDTC for HK-2 cells from Cd²⁺-induced cytotoxicity was significantly reduced in GLUT2−/− and SGLT2−/− KO cells at almost all compared doses (Fig. 2B, Supplementary Fig. 6). In addition, no dose-dependent effect was observed in both GLUT2−/− and SGLT2−/− KO cell lines (Fig. 2B). We further measured Cd levels inside of CdCl₂-exposed cells with or without GMDTC administration at 1,500 μM by ICP-MS. The amount of Cd in HK-2 cells treated with Cd alone was as high as 327.23 ng/10⁶ cells and was significantly decreased to 234.61 ng/10⁶ cells after 24-h GMDTC rescue treatment (Fig. 2C). The removal of Cd was 28.3% (Fig. 2D). Although GMDTC’s effect in removing Cd from the cells was evident in each GLUT2−/− or SGLT2−/− KO cell line (Fig. 2D), the efficiency was significantly decreased in each KO cell lines, 7.4% in GLUT2−/− KO cells and 14.6% in SGLT2−/− KO cells, respectively, compared to that of HK-2 cells (28.28%).

3.3. The involvement of renal glucose reabsorption pathway in GMDTC-mediated Cd removal in vivo
To further examine the role of kidney glucose reabsorption pathway in GMDTC-mediated cleaning Cd in vivo, we treated Cd-laden mice with GLUT2 inhibitor (phloretin) in addition to GMDTC administration. As expected, GMDTC treatment had a significant effect in removing Cd from the kidneys, and significantly increased the excretion of Cd in the urine (Table 3). This effect was observed more significantly in animals given high dose of GMDTC. At low dose of GMDTC treatment group, when the activity of GLUT2 was inhibited by phloretin, the Cd level in kidney was largely comparable between phloretin+GMDTC\textsuperscript{low}+Cd group and the GMDTC\textsuperscript{low}+Cd group. The excretion of Cd from urine was significantly decreased in phloretin+GMDTC\textsuperscript{low}+Cd group (Table 3). The Cd level in the kidneys was increased in phloretin+GMDTC\textsuperscript{high}+Cd group compared to the GMDTC\textsuperscript{high}+Cd group while the difference was not significant ($P=0.0543$) due to high variance of the data (Table 3). However, urinary Cd level in phloretin+GMDTC\textsuperscript{high}+Cd group was further increased, which was significantly higher than the urinary level in the GMDTC\textsuperscript{high}+Cd group (Table 3).

Table 3. Kidney and 24h urinary cadmium levels in Cd laden mice after GMDTC treatment with or without phloretin administration. (mean ± SD, n=6)

<table>
<thead>
<tr>
<th>Group</th>
<th>GMDTC (mg/kg)</th>
<th>Phloretin (mg/kg)</th>
<th>Kidney Cd (μg/g)</th>
<th>Kidney Cd Decorporation Rate (%)</th>
<th>Urinary Cd (μg/g Cr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0.02 ± 0</td>
<td>-</td>
<td>0.00 ± 0</td>
</tr>
<tr>
<td>Cd</td>
<td>0</td>
<td>0</td>
<td>24.22 ± 5.91</td>
<td>-</td>
<td>9.42 ±7.15</td>
</tr>
<tr>
<td>Cd+phloretin</td>
<td>0</td>
<td>10</td>
<td>20.73 ± 5.94</td>
<td>14.42</td>
<td>18.23±15.53</td>
</tr>
<tr>
<td>Cd+GMDTC\textsuperscript{low}</td>
<td>108</td>
<td>0</td>
<td>19.57 ± 5.82</td>
<td>19.21</td>
<td>91.62±62.47**</td>
</tr>
<tr>
<td>Cd+GMDTC\textsuperscript{high}</td>
<td>433</td>
<td>0</td>
<td>13.53 ± 5.71**</td>
<td>44.17</td>
<td>207.28±195.86**</td>
</tr>
<tr>
<td>Cd+GMDTC\textsuperscript{low}+phloretin</td>
<td>108</td>
<td>10</td>
<td>18.44 ± 5.64</td>
<td>23.88</td>
<td>32.41±17.21*&amp;</td>
</tr>
<tr>
<td>Cd+GMDTC\textsuperscript{high}+phloretin</td>
<td>433</td>
<td>10</td>
<td>16.16 ± 5.82*</td>
<td>33.31</td>
<td>298.94±170.96**</td>
</tr>
</tbody>
</table>

Note: Compared to Cd group, "*" $p<0.05$, "**" $p<0.01$ Compared to Cd+GMDTC\textsuperscript{low} group, "&" $p<0.05$.

Discussion
Cd is one of the most toxic heavy metals that human beings can be exposed to at work and in the industrial environment[35, 36]. Once absorbed, Cd mainly circulates to the kidneys and accumulates in the proximal tubular cells throughout life with a half-life of several decades[37, 38]. Once the accumulation of Cd reaches certain levels, it can lead to chronic damage to the kidneys and cause irreversible deleterious consequences[11, 39, 40]. Therefore, to protect against Cd-induced renal toxicity, treatments aiming at limiting Cd accumulation and enhancing its removal from the kidneys have attracted great interest. Our previous work showed GMDTC was low in toxicity in vivo and exhibited a strong chelating ability with toxic heavy metals, including Cd, and a high efficiency in removing Cd from kidneys. In addition, given the hydrophilic characters of GMDTC and the formed GMDTC-Cd complexes, we have hypothesized that it is likely that they are transported through active transport mechanisms, the renal glucose transportation pathway, instead of a passive transport mechanism[25]. In this study, we constructed cell models by knocking out glucose transporters (GLUT2 and SGLT2) and demonstrated the impact of renal glucose transporters in GMDTC-mediated removal of Cd in vitro and in vivo.

Chelators, the main type of metal removal agents, have been studied intensively as potential therapeutic drugs for Cd-induced renal toxicity [17-19]. However, there are currently no approved chelation therapies available due to their severe side effects and disappointing ineffectiveness in removing Cd from the kidney[41, 42]. Although Fulgenzi et al. have reported that EDTA chelation therapy significantly increased urinary elimination of Cd based on a study of 379 patients with neurodegenerative diseases [43], EDTA could cause nephrotoxicity especially with repeated high-dose treatment (above 75 mg/kg) in subjects with a previous history of kidney damage [44] and increase the risk of renal dysfunction [45]. Dimercaprol (British Anti-Lewisite, BAL) and its analogues meso-2, 3-dimercaptosuccinic acid (DMSA) are also used as antidotes for heavy metal poisoning[46]. In contrast, BAL may increase burdens of kidney and liver, decrease the level of patients’ survival and even enhance nephrotoxicity[47]. A randomized trial, in which 396 children were given DMSA at the dose of 1,050 mg/body surface area (m²)/day for 7 days, showed that DMSA did not lower blood Cd in children with background exposure[48]. Combination therapy with chelating agents and other substances has been proposed in the management of heavy metal toxicity[41, 49]. But overall, there is a general dearth of studies to develop chelators that may be employed for the treatment of Cd toxicity.

Our data showed GMDTC administration could significantly reduce intracellular Cd level in HK-2 cells pretreated with Cd, and the removal of Cd in 24 hour was 28.3%. This reduction of Cd level inside of HK-2 cells led to a reduced toxicity and an increase of cell viability in a dose-dependent manner. In GLUT2−/− or SGLT2−/− KO HK-2 cell models, cells were similarly pretreated with Cd before GMDTC administration. However, GMDTC treatment under most concentrations could not improve the cell viability when compared to KO cells treated with Cd alone, as observed in the GMDTC rescuing experiment. The reduced effects of GMDTC in protecting cytotoxicity from Cd exposure was supported by the evidently reduced removal ratio of Cd from cells. The removal ratio was reduced to 7.4% and 14.6% in HK-2 cells with GLUT2 or SGLT2 gene knocked out, respectively. In view of the hydrophilic characters of GMDTC and the formed GMDTC-Cd complexes as well as the open chain glucose moiety harbored by GMDTC, we
hypothesize the transportation process of GMDTC and GMDTC-Cd is comparable with glucose. Firstly, GMDTC filtered at the glomerulus of nephron is actively transported into the proximal tubule epithelial cells via SGLT2 against a concentration gradient coupled with the inward diffusion of sodium ions which is maintained by an Na⁺/K⁺ ATPase pump; in renal tubule cells, GMDTC binds free Cd ions to form GMDTC-Cd complexes; driven by concentration gradient, intracellular GMDTC-Cd diffuses out to the plasma facilitated by the facilitative glucose transporters GLUT2 in the basolateral membranes; the majority of GMDTC-Cd complexes is consequently excreted from the body through urine following glomerular filtration. Although our research measures Cd not GMDTC and GMDTC-Cd complex directly in the constructed model cells and kidneys of mice, the data does suggest that knocking down of the SGLT2 and GLUT2 affects the Cd cytotoxicity and the deposition of Cd, which is compatible with our hypothesis. However, we also noticed that the GMDTC's effects in removing Cd from the cells were not completely abolished. One possible explanation is that GMDTC can enter cells by multiple mechanisms, such as other glucose transporters (e.g., SGLT1, sodium-dependent glucose transporter 1). Studies have shown that a compensatory increase in SGLT1-mediated glucose reabsorption occurs when SGLT2 is inhibited in mice[50, 51]. In addition, the chemical structure of GMDTC contains not only a glucose motif, but also a methionine group. GMDTC could utilize amino acid transporter entering cells. Considering that there could be more mechanisms involved in the transportation of GMDTC entering the cells, we conducted an animal study by blocking GLUT2 transporter using phloretin, so blocking the transportation route of GMDTC out of the cells. The in vivo results are not as clear as the in vitro data. GMDTC's effects in removing Cd from the kidney were clearly demonstrated at both low and high dose. However, blocking of the GLUT2 transporter has less impact on the Cd level in the kidneys. Only the high GMDTC dose group showed a higher Cd in the kidney after phloretin treatment with a borderline significant level (P=0.054). Although, as projected, the urinary Cd level was significantly lower in the low GMDTC dose group post phloretin treatment, phloretin treatment further increased the urinary Cd level in the high GMDTC dose group. Altogether, while the results are not all consistent, particularly the in vivo data, we believe that the constructed GLUT2⁻/⁻ and SGLT2⁻/⁻ knockout cell models provide convincing evidence supporting the involvement of renal glucose transporter in GMDTC-mediated cleaning of Cd from cells.

This study is not without potential issues. Our repeated sequencing data confirms the completely knockout of the SGLT2 and GLUT2 genes in the HK-2 cells. However, western blotting results indicate there are measurable SGLT2 and GLUT2 protein. We couldn't determine whether these detectable proteins are SGLT2 and GLUT2 protein or not. In addition, the knocking down of SGLT2 and GLUT2 in HK-2 cells apparently affect the accumulation of Cd in the cell. The reason is unknown at this time. The animal study did not deliver as clear results as the in vitro experiments, and the specificity of phloretin's effects in blocking GLUT2 transporter is not satisfied and the blocking effects are not complete. We are in the process of obtaining SGLT2 knockout mice and of generating kidney GLUT2 conditional knockout mice. A HPLC-based method to measure intracellular GMDTC-Cd complex in cell and in tissue samples is under development. We expect that these knockout mouse models and targeted analyses of GMDTC-Cd complex in cell and tissue samples should provide more defined information regarding the role of glucose transporters in GMDTC-mediated Cd removal in vitro and in vivo.
In conclusion, we show that GMDTC is a relative safe with low toxicity and can remove Cd inside of cells and from kidney, supporting GMDTC as an effective chelator in protecting Cd-caused chronic renal toxicity. The ability of GMDTC to protect cells from toxicity was significantly decreased in GLUT2 and SGLT2 gene KO cells, suggesting that renal glucose transportation pathway is involved in the GMDTC-mediated Cd removal from kidney cells.

**Abbreviations**

Cd, Cadmium; Cd-MT, Cd-metallothionein; DMEM, Dulbecco’s Modified Eagle Medium; DMPS, 2,3-dimercapto-1-propane sulfonic acid; DTC, Dithiocarbamates; EDTA, ethylene diamine tetraacetic acid; FBS, fetal bovine serum; GFAAS, graphite furnace atomic absorption spectroscopy; GLUT2, glucose transporter 2; GMDTC, sodium (S)-2-(dithiocarboxylato((2S,3R,4R,5R)-2,3,4,5,6-pentahydroxyhexyl) amino)-4(methylthio)butanoate; ICP-MS, inductively coupled plasma mass spectrometry; KO, knockout; MT, metallothionein; NAC, N-acetyl cysteine; PAM, protospacer adjacent motif; PBS, phosphate buffered saline; RNP, ribonucleoprotein; SGLT2, sodium-dependent glucose transporter 2; sgRNA, single-guide RNA; SLC2A2, solute carrier family 2 member 2; SLC5A2, solute carrier family 5 member 2; T7E1, T7 endonuclease 1; TIDE, tracking of indels by decomposition.

**Declarations**

**Acknowledgements**

ICP-MS was completed at the UB Chemistry Instrument Center, State University of New York at Buffalo. Cell viability analysis, DNA gel imaging, western blot imaging and single clonal cell isolation in this study was performed using instruments at the Optical Imaging and Analysis Facility, School of Dental Medicine, State University of New York at Buffalo. We also thank Dr. Andrew McCall for expert assistance with single clonal cell isolation.

**Funding**

This work was supported by the National Natural Science Foundation of China (81872571 to X.T.), the Support Scheme of Guangzhou for Leading Talents in Innovation and Entrepreneurship (No. 2019013 to X.T.), the Zhuhai Industry-University Research Cooperation Project (ZH22017001210086PWC to W.H.), the Guangdong Basic and Applied Basic Research Foundation (2021A1515010771 to Z.Z.), and the SUNY Research Seed Grant Program (RSG201080.3 to X.R.).

**Competing Interests**

The authors declare the following competing financial interest(s): GMDTC and its clinical application are under protection by the patent ZL200510035377, and the patent 201811185272.8. All authors have no other competing financial interests to declare.

**Author Contributions**

References


17(4):435-441.


**Figures**
Figure 1

GMDTC Chemical Structure and Schematic Procedure of Mice Treatment. (A) Chemical structure of Sodium (S)-2-(dithiocarboxylato((2S,3R,4R,5R)-2,3,4,5,6 pentahydroxyhexyl)amino)-4-(methylthio)butanoate (GMDTC). (B) Grouping and experimental steps of Cd\textsuperscript{2+}-laden mice in the study of the impact of inhibiting renal glucose transporter GLUT2 on GMDTC’s effect in removing Cd\textsuperscript{2+} from kidney.
Figure 2

Compromised protection of GMDTC to Cd-induced cytotoxicity cannot protect cells from cadmium-induced cytotoxicity in KO cells. (A) HK-2, HK-2/GLUT2−/− and HK-2/SGLT2−/− cells treated with 1500 μM GMDTC exhibited no significant difference on cell viability compared to control after 24 h. (B) Cell viability analysis of all three cell lines, HK-2, HK-2/GLUT2−/− and HK-2/SGLT2−/− cells post GMDTC treatment. (C) Cd levels among three cell lines with Cd exposure and after GMDTC administration; (D) Cadmium removal ratio after GMDTC rescue. Data are mean ± SD of 3 independent experiments. HK-2 vs. HK-2/GLUT2−/−cells: “*”p 0.05, “**”p 0.01; HK-2 vs. HK-2/SGLT2−/−: “^”p 0.05, “^^”p 0.01.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterialSubmittedtoBTER.pdf