Slc7a9 knockout mouse is a good cystinuria model for antilithiasic pharmacological studies

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Font-Llitjós M, Feliubadalo L, Espino M, Clères R, Mañas S, Frey IM, Puertas S, Colell G, Palomo S, Aranda J, Visa J, Palacín M, Nunes V. Slc7a9 knockout mouse is a good cystinuria model for antilithiasic pharmacological studies. Am J Physiol Renal Physiol 293: F732–F740, 2007.—Cystinuria is a hereditary disorder caused by a defect in the apical membrane transport system for cystine and dibasic amino acids in renal proximal tubules and intestine, resulting in recurrent urolithiasis. Mutations in SLC3A1 and SLC7A9 genes, that codify for rBAT/b0,+AT transporter subunits, cause type A and B cystinuria, respectively. In humans, cystinuria treatment is based on the prevention of calculus formation and its dissolution or breakage. Persistent calculi are treated with thiols [i.e., d-penicillamine (DP) and mercaptoethylpropionylglycine (MPG)] for cystine solubilization. We have developed a new protocol with DP to validate our Slc7a9 knockout mouse model for the study of the therapeutic effect of drugs in the treatment of cystine lithiasis. We performed a 5-wk treatment of individually caged lithium mutant mice with a previously tested DP dose. To appraise the evolution of lithiasis throughout the treatment a noninvasive indirect method of calculus quantification was developed: calculus mass was quantified by densitometry of X-ray images from cystinuric mice before and after treatment. Urine was collected in metabolic cage experiments to quantify amino acids in DP-treated and nontreated, nonlithiasic mutant mice. We have developed a new protocol with DP to validate our Slc7a9 knockout mouse model for the study of the therapeutic effect of drugs in the treatment of cystine lithiasis. We performed a 5-wk treatment of individually caged lithium mutant mice with a previously tested DP dose. To appraise the evolution of lithiasis throughout the treatment a noninvasive indirect method of calculus quantification was developed: calculus mass was quantified by densitometry of X-ray images from cystinuric mice before and after treatment. Urine was collected in metabolic cage experiments to quantify amino acids in DP-treated and nontreated, nonlithiasic mutant mice. We found significant differences between DP-treated and nontreated knockout mice in calculus size and in urinary cystine excretion. Histopathological analysis showed that globally nontreated mutant mice had more severe and diffuse urinary system damage than nontreated knockout mice in calculi size and in urinary cystine excretion. We found significant differences between DP-treated and nontreated, nonlithiasic mutant mice. We have developed a new protocol with DP to validate our Slc7a9 knockout mouse model for the study of the therapeutic effect of drugs in the treatment of cystine lithiasis. We performed a 5-wk treatment of individually caged lithium mutant mice with a previously tested DP dose. To appraise the evolution of lithiasis throughout the treatment a noninvasive indirect method of calculus quantification was developed: calculus mass was quantified by densitometry of X-ray images from cystinuric mice before and after treatment. Urine was collected in metabolic cage experiments to quantify amino acids in DP-treated and nontreated, nonlithiasic mutant mice. We found significant differences between DP-treated and nontreated knockout mice in calculus size and in urinary cystine excretion. Histopathological analysis showed that globally nontreated mutant mice had more severe and diffuse urinary system damage than DP-treated mice. Our results validate the use of this mouse model for testing the efficacy of potential new drugs against cystinuria.

Cystinuria (Omm 22000) is an autosomal disease of renal reabsorption and intestinal absorption of cystine and dibasic amino acids, caused by defects in the amino acid transporter rBAT/b0,+AT. Cystine precipitates in the urinary system to form calculi that can cause obstruction, infection and, ultimately, renal failure (33). Cystinuria is classified into three types according to the urine phenotype in heterozygotes: phenotype I, phenotype non-I, and mixed phenotype. In phenotype I cystinuria, heterozygotes have a normal pattern of amino acid excretion in the urine (recessive inheritance). In phenotype non-I cystinuria, heterozygotes have a variable degree of hyperexcretion of cystine and dibasic amino acids (dominant inheritance with incomplete penetrance). A smaller subset of patients has mixed phenotype cystinuria, which combines phenotype I and non-I mutant alleles (21).

Two genes responsible for cystinuria have been identified so far. Mutations in SLC3A1, located in chromosome 2p16.3–21 and encoding rBAT, cause mainly phenotype I cystinuria (4, 5), except for one mutation, dupE5-E9, that often associates with phenotype non-I (21). Mutations in SLC7A9, located in chromosome 19q12–13.1 and encoding the rBAT-associated subunit b0,+AT, cause mainly phenotype non-I cystinuria (18) and also some phenotype I cases (13, 21, 22, 30). Due to this phenotypic variability, a new classification based on genetics was proposed: type A due to mutations in SLC3A1 and type B due to mutations in SLC7A9 (13). The possibility that type AB, with one mutation in each of the above-mentioned genes, would produce the disease, is left open but is unlikely (21). The rBAT/b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsible for the b0,+AT heteromeric complex is responsibl...
regular follow-up and optimal pharmacotherapy significantly increase stone-free intervals (28). Human cystinuria is treated by preventing the urolithiasis through high fluid intake, a low-salt diet (<2 g NaCl/day), moderate reduction of protein intake (<0.8 g/day), and urine alkalization to pH values of at least 7.5 (with potassium citrate, or sodium bicarbonate in severe renal insufficiency cases) to maximize cystine solubility, and if this fails, by the dissolution or breakage of calculi (25, 28, 34). Calculi are removed by surgical nephrolithotomy (large stones), percutaneous nephrolithotripsy, intracorporeal lithotripsy and, in the case of recently formed calculi, by extracorporeal shock wave lithotripsy. These procedures, mostly minimally invasive, carry the risk of progressive renal function impairment (28). The pharmacological approach is based on oral administration of sulfhydryl molecules able to displace the redox equilibrium between cystine (insoluble) and cysteine (soluble), by forming a soluble complex with cysteine. Patients who persistently develop stones are frequently treated with oral sulfhydryl agents like D-penicillamine (DP) and α-mercapto propionylglycine (1, 10). Although these agents are quite effective, they have multiple side effects that often cause discontinuation of treatment (8, 11).

β,β-Dimethylcysteine (DP), is a structural analog of cysteine, which in humans, apart from cystinuria, has been used for the treatment of a variety of diseases including Wilson’s disease, rheumatoid arthritis, scleroderma, and heavy metal poisoning (26). The dose used for the treatment of cystinuria patients are adjusted according to the urinary excretion of cystine (27). The dosage in children is 20–40 mg·kg⁻¹·day⁻¹ given in two doses, which is important as cystine concentration in the tubules increases during the night (1, 7, 20). DP is associated with a wide spectrum of adverse effects and toxicities, including copper and pyridoxine depletion, proteinuria, glomerulonephritis, and nephrotic syndrome (12, 26, 28). α-Mercapto propionylglycine seems to have significantly fewer side effects than DP and is preferred by most physicians (32). The dose of D-penicillamine administered to mice was calculated based on the human therapeutic dose. The conversion of the recommended dose from humans (1, 7) to mice through the body surface (for a human of 70 kg, 1.84 m²; for a mouse of 25 g, 97.5 cm²) is the following: 30 mg·kg⁻¹·person⁻¹ corresponds to 450 mg·kg⁻¹·mouse⁻¹. Doses administered in this study were 450 and 900 mg DP·kg⁻¹·day⁻¹.

**Palatability test.** In previous studies, no significant differences were found among liquid intake of control mice (Slc7a9+/+), heterozygotes (Slc7a9+/−), and mutants (Slc7a9−/−) (data not shown), so control mice were used for the palatability test. To check the palatability of DP, 18 Slc7a9−/− female mice of 2–3 mo were caged in groups of three for 1 wk to allow mice to adapt to water with a slightly different taste. Of the six resulting cages, three had water in the drinking bottles, and the other three had water with 14.9 mM DP.

**Animals and experimental design of DP treatment.** The first day of the study, 35 Slc7a9−/− lithiasic mice were analyzed by X-ray to select those with calculi in the urinary bladder within a size range that could be accurately measured and with no calculi in the kidney, which could cause additional injuries and higher suffering and mortality. Finally, 20 lithiasic mice were selected.

Three groups of mice from 2 to 4 mo of age were individually caged: group A [10 Slc7a9−/− lithiasic mice from both genders (3 males and 7 females)] without treatment; group B [10 Slc7a9−/− lithiasic mice from both genders (2 males and 8 females)] treated with DP; and group C [10 Slc7a9−/− mice from both genders (3 males and 7 females)] without treatment as controls. On day 20 (after treatment with 450 mg·DP·kg⁻¹·mouse⁻¹·day⁻¹) and 34 (at the end of the whole treatment), mice from groups A and B were analyzed by X-ray again.

Liquid intake, appearance, and behavior of mice were monitored daily. The drinking solution was also changed daily to ensure the stability of the DP. The animals were weighed four times during the experiment: at days 0, 20, 27, and 34. A mouse from group B showed symptoms of suffering during the experiment and was killed and excluded from the statistical analysis. On day 35, all the animals were killed, and calculi, kidneys, liver, and bladder were extracted to evaluate the possible damage caused by calculi and/or the drug.

**Calculi detection.** Calculi in the urinary system were detected by X-ray radiography (at 28 kV, 16 mA/s) in a Senima HF apparatus (focus of 0.1–0.3 mm), which is a suitable device for obtaining an accurate image of radiopaque calculi in small animals. All mutant mice received general anesthesia using inhalational isofluorane 1–3% (Forane, Abbot) and were individually placed sideways to obtain full body X-ray plaques. The Service of Medical Physics and Radiological Protection from ICO-IDIBELL performed a densitometric study with the Compact E.O.S. Classic revealing device (AGFA) and the specific films used in this study (FUJI Medical X-ray film UM-MAHC for mammography); the films were exposed with a densitometer (PTW SensiX) and read in a PTW DensiX densitometer. The densitometric curve showed linearity between optical densities of 0.7 and 3.1, with a regression coefficient of 0.997. All mean optical density values obtained from densitometric analysis of X-ray plaques in this study were within this range (nonsaturated zone). Necropsy of all mice was performed at the end of the procedure, and calculus mass was obtained directly by weight.

**Calculi mass analysis.** A noninvasive indirect method to measure cystine calculus mass during the treatment was developed. The densitometry of the calculi in the X-ray plaques (following a similar principle used to measure bone density in medical centers) can appraise the mass of the calculus as a function of the intensity of the grey at each point and the total surface of the calculus in the image. A calibrated high-resolution GS-800 densitometer (Bio-Rad) and Quantity One software (Bio-Rad) were used to measure the calculi in the X-ray plaques. The software removes the background from the contour of the calculus or group of calculi and provides the parameter of adjusted mass (AM) that is an accurate measure (in arbitrary units).
of the calculi mass: AM = area (mean of grey at calculus – mean of intensity at contour).

Metabolic cage experiments. X-rays were performed in mutant mice of 3–4 mo to select mice without calculi, because cystine calculi could interfere with urine cystine concentration. Two groups of six nonlithiasic Slc7a9+/− mice were caged individually for 7 days in metabolic cages (Tecniplast) with food and drink ad libitum. After a 2-day adaptation period, 24-h urine was collected on 2 consecutive days, and amino acid excretion was analyzed. Then, a 4-day, 450-mg DP/kg mouse−1day−1 treatment was started, and after 2 days of treatment, 24-h urine was collected again for 2 consecutive days. On the last day, X-rays were performed again to check that none of the mice had started to form calculi during the experiment.

Urine samples were collected individually in tubes with 0.5–1 ml of filtered mineral oil (M8410, Sigma) to minimize urine evaporation. To preserve amino acids and prevent degradation, we also added 200 µl of a solution of thymol (10% in isopropanol). Thymol (38492, Fluka) has antimicrobial, antifungal, and antioxidant activities and does not interfere with later amino acid analysis. Samples were frozen for their conservation until the analysis. For analysis of cystine, ornithine, lysine, and arginine, thawed urine was diluted 25-fold with LiOH (16 M) to solubilize possible cystine crystals, and the diluted urine was mixed with Li-citrate sample dilution buffer, pH 2.20 (1:1).

Samples were filtered through centrifugal filter devices with 3-kDa molecular weight cut-off (Nanosep, Pall, East Hills, NY) before analysis, and the injection volume was 75 µl. Amino acids were separated by ion-exchange chromatography with a Biochrom BC30 amino acid analyzer (Biochrom, Cambridge, UK) employing a Li-citrate buffer system and ninhydrine postcolumn detection. Creatinine concentrations in urine were determined with the Creatinin Liquicolor Jaffé reagent (Rolf Greiner BioChemica, Flacht, Germany) according to the manufacturer’s instructions.

Histology. Animals were killed with CO2, and tissues were fixed with 10% formol ≥O/N at room temperature. Paraffin sections of 4 µm were stained with hematoxylin/eosin or with hematoxylin/periodic acid-Schiff, examined with an Olympus BX60 microscope, and photographed using a Color View III digital camera and Cell B digital software. Histological analysis was performed by a blinded observer.

Statistical methods. Data were summarized, compared, and expressed as means ± SE. Analysis of variance (ANOVA) (31) methods were used to determine differences across groups and to assess the effect of treatment on weight and liquid intake. All pairwise comparisons were done through a t-test, whereas Tukey’s test was used for those comparisons which included more than two groups. Statistical significance was defined if the null hypothesis could be rejected at the P < 0.05 level. All the analyses were carried out using the R statistical package (39).

RESULTS

DP palatability test. A palatability test was performed on Slc7a9+/− mice to analyze liquid intake during DP administration. The group with water drank 17.0 ± 0.8 ml/cage (means ± SE) and the group with water+DP (14.9 mM) drank 16.5 ± 0.9 ml/cage. The evolution of the body weight of mice and the possible side effects of DP administration were also monitored during the palatability test. The fact that no side effects were detected in the palatability test and that there were no significant differences in the intake between both groups, nor in the body weight of mice (data not shown), allowed us to design the procedure with DP administration in the drinking water without the need to add sweeteners (sucrose) or pH correctors.

Body weight and liquid intake monitoring of mice during DP treatment. Three groups of ten mice of 2–4 mo were studied for 5 wk (see MATERIALS AND METHODS). Mice in group B received 450 mg DP·kg−1·day−1 during the first 3 wk and 900 mg DP·kg−1·day−1 for the last 2 wk. All mice were weighed individually at the beginning of the experiment, and at 3, 4 and 5 wk (Fig. 1A). There were no significant differences in body weight between groups A and B. However, the control group had an average weight significantly higher from the beginning of the experiment compared with mutant groups (P < 0.001).

Significant differences in liquid intake among the three groups were also found (Fig. 1B). Mice in group C drank less than those of groups A and B (P < 0.001) throughout the experiment. During the first 3 wk, mice in group C drank less than mutants (groups A and B) (P < 0.001), but after the doubling of the dose in group B (last 2 wk), no statistically significant differences between mice of groups B and C were found since day 24 (P = 0.1) (Fig. 1B).

Evolution of calculi size during DP treatment. To appraise the evolution of cystine calculi mass during the course of DP
treatment, a noninvasive indirect method of quantification was developed (see MATERIALS AND METHODS), based on densitometry of the X-ray plaques, which offers an indication of the mass of the calculi as a function of their surface and opacity (Fig. 2). This system provides the parameter of AM that is a measure of the calculi mass.

All mice from both lithiasic groups were analyzed with this indirect imaging analysis system. The X-ray images taken throughout the experiment from the bladder of four representative mice from each mutant group are shown (Fig. 3). Calculi size evolved in a different way between both groups, even though each mouse had different calculi numbers, sizes and shapes: the calculi size kept growing in group A, while in group B it remained nearly the same throughout the treatment.

At the end of the treatment, all animals were killed and the calculi of both lithiasic groups were extracted and weighed. The stone weights were compared with the calculated AM obtained from the indirect mass quantification. The linear relationship between AM and calculi weight shows that each unit increase in calculi mass leads to a 0.2889 unit increase in AM with 96.77% precision of this estimation (Fig. 4). The strong correlation ($r = 0.98$) found between the weight of the calculi collected by necropsy at day 35 and their AM at day 34 demonstrates that this method can be satisfactorily used to monitor changes in stone mass in the Slc7a9 knockout model during in vivo experiments.

Thus the AM mean of group A (nontreated) at day 0 was 26.4 ± 4.7, while that of group B (DP-treated) was 21.2 ± 2.8 (Fig. 5A). These groups at day 0 did not show any significant differences in AM. The inferred mass at the beginning of the experiment was 83.6 ± 16.1 and 65.6 ± 9.6 mg for group A and B, respectively, while after the treatment, the calculi weight of group A was higher (140.8 ± 16.9 mg) than that of group B (65.4 ± 10.3 mg), with these differences being statistically significant ($P = 0.00154$) (Fig. 5B). After 3 wk of

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Fig. 2. X-ray plaque of a lithiasic mouse. The contour of the stones applied to quantify densitometry with Quantity-One software is shown in white. The densitometry of the calculi of X-ray plaques can appraise the mass of the calculi as a function of the intensity and surface of the calculi in the image. The background from the contour of the stones was subtracted.

Fig. 3. Image illustrating the evolution of the cystine stones in the knockout mouse model treated with water (group A) and with D-penicillamine (group B). On average, in group A the calculi kept on growing, while in group B the D-penicillamine prevented the growth of the stones.

Fig. 4. Correlation between the calculi weight at day 35 and their adjusted mass calculated from the X-ray plaques (day 34). The regression line and its parameters are shown.
Fig. 5. A: evolution of the adjusted mass of calculi throughout the experiment. The mean and SE of each group are shown. B, Lithiasic mice without treatment; grey squares, lithiasic mice treated with d-penicillamine: 450 mg·kg⁻¹·day⁻¹ in the first 3 wk and a double dose in the last 2 wk. B: calculi weight of all animals after treatment obtained from direct measurement. The mean of each group is indicated as a bar. Group A, without treatment; group B, treatment.

treatment with 450 mg DP·kg⁻¹·day⁻¹, the AM of group A had increased 13.4 ± 1.5, while that of group B had only increased 2.5 ± 1.1 (P = 0.000021). After 2 wk of additional treatment with 900 mg DP·kg⁻¹·day⁻¹, the AM of group A had still increased 2.9 ± 1.6, while that of group B had reduced 2.3 ± 1.0 (P = 0.014). Thus, in total, a variation in AM of 16.2 ± 2.1 in group A and of 0.2 ± 1.4 in group B (P = 0.0000087) was observed in the 5-wk experiment. It was not possible to analyze whether there were differences in the evolution of the calculi in both sexes, because there were few males, but they did not seem to follow any different trend to females.

Urinary phenotype of Slc7a9 knockout mice after DP administration. The main phenotypic trait of cystinuria, besides calculi, in humans and in this knockout model, is the urinary hyperexcretion of cystine, lysine, arginine, and ornithine. The therapeutic mechanism of DP consists of cystine-cysteine equilibrium displacement to produce adducts of DP-cysteine (27, 32). To check the decrease in cystine excretion by DP treatment, urine amino acid excretion was measured in six Slc7a9⁺/⁻ male mice with no X-ray-visible calculi, which were housed in metabolic cages for 7 days. Before the treatment, the mice showed a massive urine hyperexcretion of cystine and dibasic amino acids. During DP treatment, the mice showed a statistically significant lower hyperexcretion of cystine (P = 0.002) but the same hyperexcretion for ornithine (P = 0.36), lysine (P = 0.73), and arginine (P = 0.67) (Fig. 6). Urine excretion of other amino acids was within the normal range and not affected by DP treatment (data not shown). Urinary DP in urine samples from treated mice was present in the disulfide form only: urinary concentration of DP-disulfide obtained from the six nonlithiasic Slc7a9 knockout mice in two consecutive days was 1.1 ± 0.1, 1.3 ± 0.2, 3.0 ± 0.4, 4.7 ± 1.3, 4.9 ± 0.1, and 8.2 ± 0.2 mM. The urinary cysteine-DP-disulfide levels could not be quantified because we could not have reference substances for the mixed disulfides. As the color yield with ninhydrin derivatization varies with the substrate, the reference substance is indispensable for quantification. No cysteine or free DP was detected in these samples.

Histopathological analysis. After the 5-wk DP treatment, the two kidneys, bladder, and a piece of liver from all mice from the three groups were extracted and fixed. Before fixation, the kidneys were sectioned longitudinally to extract calculi, in case they had developed. The calculi in the bladder were also extracted, and both organs were weighed. The three organs of each animal were stained with hematoxylin/eosin. Additionally, kidneys were stained with hematoxylin/periodic acid-Schiff, which allows a more accurate observation of renal tubules.

There were no significant differences in kidney weight among the three groups. However, the weight of all lithiasic mice bladders was significantly higher than that of the controls (P < 0.001), although DP-treated compared with nontreated mice weight did not show significant differences (Table 1).

Histopathological analysis of liver from the three groups showed that none of the mice had infectious, neoplastic, or other macroscopic alterations in the liver, so all could be used for histological study (data not shown). For the histopathological analysis of kidneys and bladders, the following parameters were appraised: from each kidney (left and right separately) tubular dilatation, tubular necrosis/fibrosis/regeneration, glomerulonephritis, glomerular dilatation, pelvic infiltration, interstitial fibrosis, and interstitial chronic nephritis. Infiltration, destrucrization, and epithelial loss were appraised in bladders. Some or all the aforementioned renal and bladder histological alterations were found in different degrees (Figs. 7 and 8).

Tukey tests for multiple comparisons of means were performed to compare histological damage among the three groups (Table 2). Only two mice of the control group presented

Table 1. Kidneys and bladder weight of mice of the 3 groups after the 5-wk experiment

<table>
<thead>
<tr>
<th>Group Genotype</th>
<th>DP Dose, mg·kg⁻¹·day⁻¹</th>
<th>n</th>
<th>Bladder Weight, mg</th>
<th>Left Kidney Weight, mg</th>
<th>Right Kidney Weight, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>A  Slc7a9⁻/⁻</td>
<td>140 ± 31</td>
<td>9</td>
<td>169 ± 38</td>
<td>176 ± 26</td>
<td></td>
</tr>
<tr>
<td>B  Slc7a9⁻/⁻</td>
<td>450</td>
<td>9</td>
<td>112 ± 35</td>
<td>165 ± 42</td>
<td></td>
</tr>
<tr>
<td>C  Slc7a9⁻/⁻</td>
<td>10</td>
<td>10</td>
<td>26 ± 8</td>
<td>159 ± 28</td>
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Values are means ± SE, n. No. of mice: DP, d-penicillamine. A Tukey test to compare bladder, left kidney, and right kidney mean weights by group was performed. Statistically significant differences for bladder weight between groups C and A (P < 0.001) and C and B (P < 0.001) were detected. There were no statistically significant differences in left and right kidney weights among the groups.
moderate pelvic infiltration, and one of them also presented mild chronic interstitial nephritis (Fig. 8). Both lithiasic groups showed significant differences compared with the control group. The DP-treated lithiasic mice also showed significant differences compared with nontreated lithiasic mice: urinary system damage was significantly less severe and/or frequent in DP-treated mice with the exception of glomerulonephritis and glomerular fibrosis, which was more severe and frequent in DP-treated mice than in nontreated mice (Figs. 7, A, B, D and Fig. 8). Tubular lesions were almost absent in DP-treated mice, while most of the nontreated mice had mild tubular dilatation, necrosis, and/or fibrosis (Fig. 7, E, G, H and Fig. 8). Nontreated mice also had more severe and frequent chronic interstitial nephritis than DP-treated (Fig. 7, A, B, D and Fig. 8). Bladder infiltration was similar in both lithiasic groups, but nontreated mice had higher bladder destructurization and epithelial loss than DP-treated mice (Fig. 8).

DISCUSSION

Animal models for human diseases have proven their value for obtaining insight into pathogenic mechanisms and for testing therapeutic drugs and innovative treatment protocols (2, 29, 41, 42). Here, we present an in vivo protocol to test the efficacy of potential antilithiasic drugs in the Slc7a9 knockout cystinuria model (17). We set up the conditions for administration of thiol drugs in drinking water and for determination of calculi mass by X-ray imaging and densitometric quantification in Slc7a9 knockout mice.

We determined that the intake of doses up to 900 mg DP·kg$^{-1}$·mouse$^{-1}$·day$^{-1}$ dissolved in the drinking water did not reduce liquid intake of mice, so we could perform oral administration of the drug in drinking water, minimizing stress and manipulation of the animals, and avoiding problems associated with the administration by esophageal gavage for pro-
longed periods (irritation, inflammation of the gastrointestinal tract, and risk of esophagus perforation and death of the animal). Moreover, the administration with gavage has to be a high dose once a day, in contrast to continuous administration of the drug in drinking water, which is very important for the efficiency of the treatment. Control mice drank less than lithiasic mice throughout the experiment. A possible explanation would be that mutant mice with calculi tend to drink more than controls, maybe to fill their bladder to suffer less rubbing by the stones, or maybe to dilute the four hyperexcreted amino acids.

To quantify calculi mass, a volume measurement was not the most suitable because the shape of the stones is very diverse, and an important error in applying any type of volume-based calculation would be committed. Moreover, there are often several stones that overlap in the X-ray, increasing the error of calculation would be committed. Moreover, there are often several stones that overlap in the X-ray, increasing the error of measure. For these reasons, we have developed an imaging system that allows a quantitative follow-up of lithiasis evolution in mice by densitometric analysis of the calculi in radiographic plaques. This noninvasive indirect method has proven to be very accurate for monitoring changes in cystine calculi mass and a very useful tool for in vivo lithiasis follow-up studies.

For amino acid excretion quantification, we used ion-exchange chromatography. High-performance liquid chromatography and other chromatographic techniques can distinguish thiol drug from cystine and cysteine, but often the sample preparation leads to disruption of thiol drug-cysteine complex (3, 35). Ion-exchange chromatography is a widely used clinical technique for analysis of urinary amino acids (23). It can resolve cysteine, cystine, and DP as well as other mixed disulfides that can be formed with DP. The redox state of the analyzed compounds is not altered during sample preparation. It is therefore highly unlikely that thiols bound to protein via disulfide bonds could dissociate under these conditions. In the urine samples of DP-treated mice analyzed, all thiols were detected in the oxidized form: DP-disulfide excretion was in the range of 1–8 mM, and the cysteine-DP complex was also detected but no reduced forms were detected.

During the treatment with DP, there were no significant differences in body weight among both Slc7a9<sup>−/−</sup> lithiasic groups. However, the control group had an average body weight significantly higher than mutant groups from the beginning of the experiment. Previous data also indicated that lithiasic mutant mice in this genetic background had significantly lower weight than control mice (data not shown), whereas nonlithiasic mutant mice have weights similar to the controls (17). The most probable explanation, then, is that the lithiasic mice suffered sporadic weight loss due to lower protein intake attributable to pain induced by obstruction and friction of stones.

The 3-wk treatment with 450 mg DP·kg<sup>−1</sup>·day<sup>−1</sup> has demonstrated very significant differences in calculi evolution between DP-treated and nontreated mice. In the absence of the drug, the calculi mass increased rapidly, while the DP almost halted their growth. Moreover, 4-day administration of the same DP dose specifically decreased cystine excretion by 50%, indicating that DP may prevent cystine calculi growth by lowering the excess of cystine in the renal tubule. Doubling the DP dose for an additional 2 wk reduced calculi size, although in this period of time the calculi did not dissolve completely. This dose is still far lower than the LD<sub>50</sub> in mice.

### Table 2. Adjusted <i>P</i> values of Tukey test for multiple comparisons of means to measure histological damage differences among the 3 groups

<table>
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<tr>
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<th>B-A</th>
<th>C-A</th>
<th>C-B</th>
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<tbody>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
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<tr>
<td>Tubular dilatation</td>
<td>0.015003</td>
<td>0.012124</td>
<td>1.000000</td>
</tr>
<tr>
<td>Tubular necrosis/fibrosis</td>
<td>0.003903</td>
<td>0.001782</td>
<td>0.985371</td>
</tr>
<tr>
<td>Tubular regeneration</td>
<td>0.933743</td>
<td>0.348735</td>
<td>0.574844</td>
</tr>
<tr>
<td>Glomerular fibrosis/glomerulonephritis</td>
<td>0.013288</td>
<td>0.017324</td>
<td>0.000002</td>
</tr>
<tr>
<td>Glomerular dilatation</td>
<td>0.415039</td>
<td>0.250889</td>
<td>0.954788</td>
</tr>
<tr>
<td>Pelvic lesions</td>
<td>0.998747</td>
<td>0.653822</td>
<td>0.697964</td>
</tr>
<tr>
<td>Interstitial fibrosis</td>
<td>0.075388</td>
<td>0.065828</td>
<td>1.000000</td>
</tr>
<tr>
<td>Chronic interstitial nephritis</td>
<td>0.072645</td>
<td>0.00008</td>
<td>0.016268</td>
</tr>
<tr>
<td>Bladder infiltration</td>
<td>0.988604</td>
<td>0.000198</td>
<td>0.000401</td>
</tr>
<tr>
<td>Bladder destructurization</td>
<td>0.586927</td>
<td>0.007801</td>
<td>0.088293</td>
</tr>
<tr>
<td>Epithelial loss</td>
<td>0.184145</td>
<td>0.031308</td>
<td>0.699794</td>
</tr>
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</table>

A, Slc7a9<sup>−/−</sup> lithiasic mice without treatment; B, Slc7a9<sup>−/−</sup> lithiasic mice treated with DP; C, Slc7a9<sup>−/−</sup> mice without treatment, as controls. Statistically significant differences are shown in bold.
which in acute oral administration is 8,900 mg/kg in females and 8,419 mg/kg in males (24).

Histopathological analysis showed that globally nontreated mutant mice had more urinary system damage than DP-treated mice, indicating that DP is effective not only in reducing urinary cystine excretion and calculi mass but also in maintaining renal structure integrity. Kidney size did not show significant differences among the three groups. All Slc7a9<sup>−/−</sup> lithic mouse bladders were significantly greater in size and weight than the control bladders, due to the hypertrophy produced by calculi growth. However, bladder size did not show significant differences between both lithic mutant groups, even though nontreated mice had a higher number and/or size of stones in their bladders. In previous studies of this model, several organs (skin, spleen, thymus, skeletal muscle, heart, lung, stomach, intestine, liver, pancreas, brain, and eye) of the three possible genotypes were studied histopathologically and were unaltered (data not shown).

DP-treated mutant mice had very mild renal tubular and bladder damage, with the exception of a mild and moderate glomerulonephritis and glomerular fibrosis, in contrast to nontreated mice that presented very intense and diffuse urinary system damage. These data are in agreement with several clinical studies of DP in humans that report membranous glomerulonephritis as the main side effect in patients treated with DP for different diseases (reviewed in Ref. 26). All these data demonstrate that the Slc7a9 knockout mouse is an excellent model for the study of antilithic pharmacological activity in cystinuria.

Despite the increase in our understanding of the underlying pathomechanisms of cystinuria, patients still form recurrent stones and have to undergo repeated interventions with increasing risk of renal insufficiency. Dietary and pharmacological metaphylaxis may lower the frequency of recurrent stones but are often not practiced (28). In humans, pharmacological treatment with thiol drugs, although quite effective, is reserved for the most severe cases (when cystine excretion exceeds 3 mmol/day), due to the number and severity of their side effects in a significant number of patients (7, 11, 28), entailing that 69 and 31% of the patients do not tolerate the treatment with DP and MPPG, respectively; this limits treatment success (32).

The fact that many patients suffer from renal insufficiency as a result of recurrent stone formation and repeated urological interventions, together with thiol drug side effects, justifies an effort for the improvement of pharmacological treatment of cystinuria. Therefore, we attained the goal of developing an animal model protocol with which to study the effect of drugs on urinary cystine lithiasis to find a better treatment to prevent or reduce cystine calculi with fewer side effects than those of drugs currently used.

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GRANTS

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REFERENCES


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