Title: Evaluation of a novel drug combination treatment of CF10 and Everolimus for nephropathic cystinosis in a new cystinotic rat model
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Background:
The lives of cystinosis patients could be improved by developing 1) a better variant of cysteamine that is more tolerable with less side-effects and 2) alternative therapies that target other pathways affected in cystinosis, such as autophagy, that are likely to play a role in the kidney failure but are not corrected by cysteamine alone. Towards these goals, we have used rodent models of cystinosis to develop a pro-drug version of cysteamine (CF10) that can be delivered in high doses with few side-effects, and found that the mTOR inhibitor Everolimus can ameliorate aspects of the cystinotic Fanconi syndrome. This work made use of a new rat model of cystinosis (Hollywood et al., 2022) that we have developed that has a phenotype that closely resembles the human disease. In this project we will **test the hypothesis that CF10/ low dose Everolimus combination therapy provides a more effective treatment for cystinotic rats than cysteamine/low dose Everolimus combination**. Specifically, we will determine if this new drug treatment has the potential to minimise the unpleasant side-effects seen with cysteamine, reduce the frequency and level of dosing, and determine if it is more effective at slowing, and potentially stopping the decline in kidney function. This preclinical study will provide the justification, and inform the appropriate dosing regime, for future human clinical trials.

The overall goal of this project is to conduct preclinical therapeutic drug intervention studies in *Cystinosin (Ctns)* knock-out (KO) rats to determine whether a combination treatment of CF10 and Everolimus is more efficacious at ameliorating the cystinosis phenotype than Cysteamine and Everolimus. To achieve this, we propose the following Aims:

Aim 1: Assess the long-term effectiveness of low dose Everolimus on renal function in *Ctns*$^{-/-}$ rats

Aim 2: Assess the effects of a Cysteamine and low dose Everolimus combination treatment on the renal defects in Ctns-/- rats.

Aim 3a: Evaluate CF10 and Everolimus drug-drug interactions in cystinotic rats.

Aim 3b: Assess the effects of a CF10 and Everolimus combination treatment on the renal defects in *Ctns*$^{-/-}$ rats.

Progress to date:

Aim 1: Assess the long-term effectiveness of low dose Everolimus on renal function in *Ctns*$^{-/-}$ rats

Overview of aim:
Rationale: As part of our previous CRF funded study, we have previously performed a high dose 6-month treatment study with Everolimus delivered twice a week to cystinotic rats. Our data showed that at this dose we see a dramatic decrease in weight gain. However, there was clear benefit to other parameters measured such as total protein, albumin, calcium and phosphate and kidney weight and health. Therefore, the goal of this aim is to assess the therapeutic effectiveness of Everolimus in *Ctns*$^{-/-}$ rats by performing a 6-month treatment study with a low dose. We anticipate that the lower dose of Everolimus will still have the beneficial effects seen at the higher dose without the negative effects on animal weight.

Results of aim 1:

Treatment of *Ctns* KO rats with low dose everolimus for 6-months

We previously tested a high dose of Everolimus delivered alone and in combination with Cysteamine and observed an adverse effect on weight gain and wound healing. Following discussions with cystinosis clinicians we decided to lower the dose of Everolimus and deliver daily. To evaluate the therapeutic potential of low dose Everolimus in preventing the onset of Fanconi syndrome and kidney decline, *Ctns* KO rats (n = 6/group/sex) were dosed once daily with Everolimus delivered in jelly pills from 1-month of age to 6-months. Blood and urine were collected at six time-points: 1 month baseline (prior to the start of dosing) and at 2, 3, 4, 5 and 6 months of age. At each time-point, urine output and water intake were measured, and body weights were recorded weekly. At 6-months the animals were humanely culled, and tissues collected. Kidney weights were recorded, and tissues were processed for cystine measurements and for further analysis by H&E and immunohistochemistry.

In cystinosis rats treated with low dose Everolimus for 6 months, there was no adverse effect on weight gain compared to vehicle controls. This is in stark contrast to the high dose of Everolimus which caused a dramatic reduction in weight gain over time in these animals.

When we looked at Fanconi syndrome (FS) markers such as polydipsia, polyuria and excretion of FS markers; protein, glucose and calcium we observed no difference between the high and low dose of Everolimus suggesting that the low dose of Everolimus exhibits the same benefits as the high dose. Likewise, no differences were observed in other FS markers such as albumin, creatinine, urea and phosphate, data not shown.

For cystine levels, low dose Everolimus significantly reduced kidney and lung cystine levels in these tissues to a similar extent as high dose Everolimus. Gross kidney morphology was slightly better in low dose Everolimus treated animals. Excitingly, histological scoring revealed that low dose Everolimus exhibited a superior reduction in all parameters measured when compared to high dose Everolimus and vehicle controls.

Taken together, these results suggest that low dose Everolimus is sufficient to exhibit the same benefits as high dose without the unwanted adverse effect on weight gain.