Title: A pre-clinical drug study using Cysteamine/Everolimus combination therapy to treat cystinosis knock-out rats
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Background:
Using cystinotic induced pluripotent stem cells, we have discovered that a combination treatment of Cysteamine and Everolimus (a rapamycin-derivative used clinically in cancer treatments) can ameliorate the cystinotic phenotype. This novel drug combination may have therapeutic potential in humans but needs further investigation in a relevant pre-clinical animal model. As the previously reported mouse and zebrafish models of cystinosis are not ideal for drug studies, we have generated a rat model of cystinosis that more closely resembles the human disease and has an earlier onset of disease symptoms compared to these other models. In this project, we propose to perform a **preclinical drug study** in our rat model of cystinosis to investigate whether the novel combination treatment of Cysteamine and Everolimus ameliorates the cystinotic phenotype. If successful, the proposed combination treatment studies will pave the way for testing a new therapy in cystinosis patients with the potential to improve health outcomes and quality of life.

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 Cysteamine and Everolimus ameliorates the cystinosis-like phenotype. To achieve this, we proposed the following Aims:

Aim 1: Determine the optimal dose of Cysteamine that achieves a 50% rescue of the cystinotic phenotype in *Ctns* KO rats (yr 1)

Aim 2: Determine the optimal dose and schedule of Everolimus delivery (yr 1)

Aim 3a: Evaluate Cysteamine and Everolimus drug-drug interactions in *Ctns* KO rats (yr 1-2)

Aim 3b: Evaluate whether treating *Ctns* KO rats with Cysteamine and Everolimus provides greater therapeutic benefit than Cysteamine alone (yr 2)

Progress to date:

Aim 1: <u>Determine the optimal dose of Cysteamine that achieves 50% rescue of the cystinotic phenotype in the *Ctns* KO rat</u>

Overview of aim: We set out to determine the optimal dose of Cysteamine which would result in a 50% reduction in cystine levels in our *Ctns* rats in order to ensure that we can detect a beneficial effect of Everolimus when we use them in combination. The original doses selected based on other studies were 30, 60 and 120mg/kg. To avoid possible high levels of variability in delivering the drug in drinking water we deliver Cysteamine in an edible jelly pill that is fed to the rats twice a day.
We first sought to determine the optimal dose of cysteamine that the rats can tolerate by feeding the rats three doses of cysteamine over the course of 10 days and recording the percentage of jelly eaten to completion (Figure 1). Next, we measured the amount of cystine in white blood cell, polymorphonuclear leucocytes (PMNs) and kidney tissue using HPLC-MS/MS (Figures 2 and 3). The dose that maintains tissue cystine levels at 50% will be used for combination studies.

Results of aim 1:

1) Determining the optimal dose of Cysteamine that the rats can tolerate

Cysteamine containing jellies were made by adding 1g Raspberry jelly powder and 1g Gelatine with 10 ml of milliQ water and heating to 50 °C to dissolve. The mixture is cooled to 40 °C before addition of Cysteamine to avoid heat effects. Vehicle jellies contain no Cysteamine. Rats are pre-conditioned to eat jellies by feeding them with jellies containing no drug weekly for 3 weeks prior to initiation of drug study. During the experiment, rats were fed Cysteamine containing jellies twice daily, (6am and 6pm) over the course of 10 days and the percentage of jelly eaten was recorded. We found early on that 120 mg/kg was too toxic for the rats and they refused to eat it, therefore, we reduced the concentration of the highest dose to 90 mg/kg. In order to detect a measurable reduction in cystine levels we need the rats to eat the majority of the jellies containing Cysteamine. As can be seen in figure 1, there was a high degree of aversion to eating the jelly containing Cysteamine at the higher concentrations of 60 and 90 mg/kg with rats eating 50% or less of the jelly as the experiment progressed most likely due to the gastrointestinal toxicity induced by Cysteamine. We saw good compliance at 30 mg/kg in the majority of animals and chose this concentration as the upper limit of Cysteamine to be used.

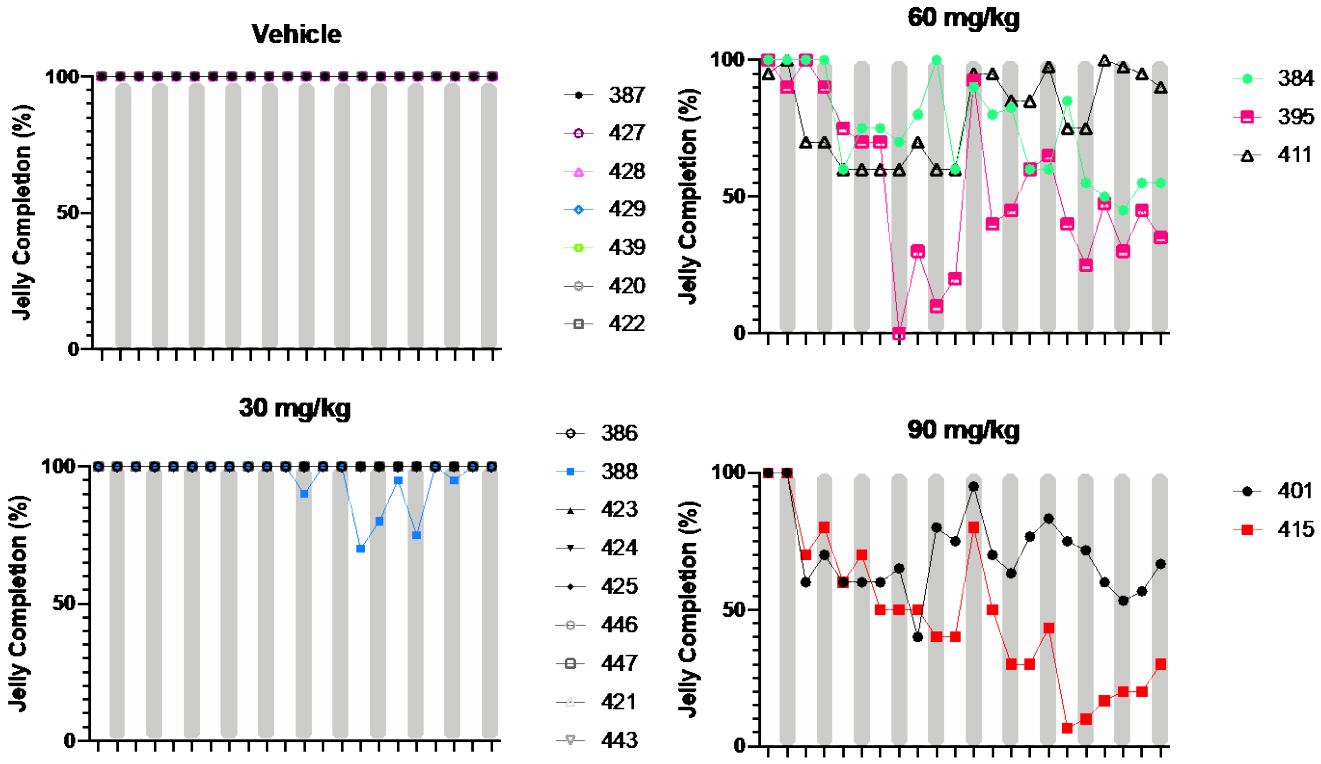

Figure 1: Determining optimal dose of Cysteamine

Graphs showing the percentage of jelly with or without Cysteamine that was eaten to completion over the course of 10 days. Numbers indicate individual animals. White bar = 6am feed. Grey bar = 6 pm feed.

2) Measuring cystine levels in PMNs and kidney tissue following treatment with Cysteamine

In order to determine whether the Cysteamine is having a therapeutic effect on the cystine levels in the *Ctns* rats we first measured the amount of cystine in Polymorphonuclear leucocytes (PMNs) with and without Cysteamine treatment. Animals were fed three doses of cysteamine (7.5, 15 and 30 mg/kg) based on the results from the previous experiment. Blood was collected from each animal before treatment (baseline) and following 10-day treatment with Cysteamine or vehicle only jellies. Following extensive optimisation of the protocol to isolate PMN cells, we found that the levels of cystine in the PMNs did not reduce as expected (figure 2). This is most likely due to the timing of the experiment

where the blood was collected 5 hrs after the final treatment and the fact that polymorphonuclear leucocytes harbouring cystine are short living cells (≈12 hours); therefore, we may have missed the therapeutic effects of Cysteamine. To overcome this we are now performing a specific time course experiment whereby we will measure cystine in PMNs at 0, 15, 30, 60, 120, 240, 480 mins post-treatment (see future work for more details).

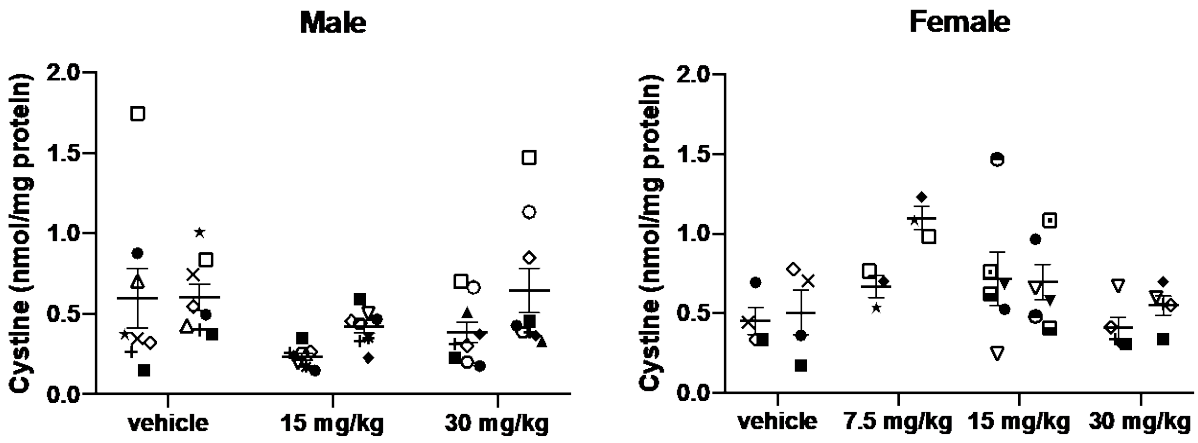

Figure 2: Amount of cystine (nmol/mg of protein) in PMNs at baseline and after 10 days of dosing. Each unique symbol represents one animal. Two-way ANOVA performed all data are plotted mean ± SEM. No significance was found.

We next measured the level of cystine in the kidney tissue of these animals. Importantly, as seen in figure 3 there is a reduction in the amount of cystine in males at 30 mg/kg following 10 days of treatment however due to animal outliers this was not significant. The effects were less obvious in female animals however there is a trend towards reduction at all doses. It is worth noting that 10 day treatment may not be long enough to see a significant reduction in the kidney tissues and longer treatment may result in better reduction.

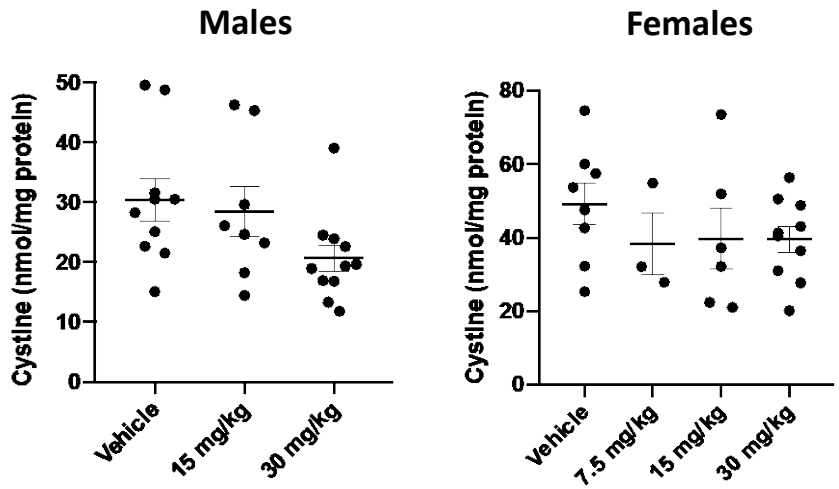

Figure 3: Amount of cystine (nmol/mg of protein) in kidney tissue following 10 days of dosing. Two-way ANOVA performed all data are plotted mean ± SEM. No significance was found.

Future Work:
Based on the results gathered so far a maximal dose of 30 mg/kg of Cysteamine will be used going forward. At this dose we do see a reduction in cystine levels in the kidney tissues of *Ctns* rats which is promising. To better understand whether delivery of Cysteamine in jelly pills is reaching sufficient levels in the blood of these animals to be therapeutic we are in the process of performing a time course PK study in the *Ctns* rats. Blood will be collected at 0, 15, 30, 60, 120, 240, 480 mins post-treatment and cysteamine levels measured using HPLC-MS/MS. We will also measure the level of cystine in the PMNs in parallel and this information will allow us to determine how much Cysteamine is in the blood following treatment in both sexes and whether the animals are reaching a desired therapeutic threshold in the blood to lower cystine when Cysteamine is delivered in jelly pills. If the results show that delivery with jelly pills is insufficient then we will repeat this study using subcutaneous injection of Cysteamine.

Aim 2 is to determine the optimal dose and schedule of Everolimus delivery and this will begin shortly. To determine whether Everolimus is having a therapeutic effect on mTOR signalling we have optimised a western blot readout to measure the level of a downstream target of mTOR, p70S6K in kidney tissue and are optimising a similar readout in blood (figure 4).

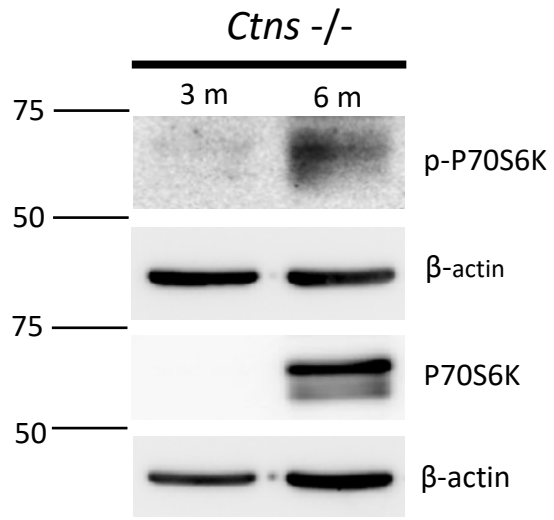

Figure 4: Western blot of total and phosphorylated p70S6k in 3- and 6-month-old *Ctns* rat kidney tissue.