Evaluation of Novel Prodrugs for the Treatment of Nephropathic Cystinosis

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Summary

Background: The design and synthesis of novel prodrugs which are an odourless, and tasteless oral therapy for the treatment of cystinosis has been carried out and we are now evaluating their efficacy. As a result of the support received from CRN we have appointed a post-doctoral research fellow to evaluate the efficacy of novel pro-drugs and have now demonstrated that at least one of these prodrugs, cysteamine decanoate, is able to deplete levels of intracellular cystine. These results were recently published (McCaughan et al., 2008). We are now determining the time frame that the prodrug is able to deplete the cystine in comparison to cysteamine and evaluating the efficacy of other candidate compounds.

Results: In the first 6 months of this project we have consolidated our initial observations by investigating shorter time periods of exposure of cystinotic cells to the novel prodrug (48 hours) and in the subsequent 6 months have evaluated the effect of cysteamine decanoate after 24 hours. The results indicated that the prodrug was able to significantly reduce the cystine content compared to vehicle control within the same time period. We have also stained the cell with YoPRO to determine their apoptotic status which indicated that although the cells were actively proliferating that there was a high intensity of staining. This is coincident with a significantly higher level of activity of caspase 3/7 compared to a healthy endothelial cell line. The presence of the prodrug did not significantly reduce the level of caspase 3/7 activity.

Conclusions: These data support our hypothesis that the novel prodrug is able to reduce intra-lysosomal cystine while at the same time offering the prospect of an odourless and tasteless oral therapy. The cystinotic fibroblasts showed significant levels of caspase 3/7 when compared to healthy cells but this was not significantly lowered by the presence of the prodrug.

INTRODUCTION

Cystinosis is caused by a mutation in the gene encoding cystinosin which is a lysosomal membrane transporter. Defects in this transporter protein result in a build up of cystine in the lysosomes causing progressive cell damage, apoptosis and cell death. Current therapy involves regular administration of cysteamine which is an aminothiol with a characteristically offensive taste and smell resulting in halitosis, body odour and gastric irritation. Patient compliance is poor and this is attributable to the adverse side-effects and the regularity of administration. We have synthesised a library of prodrugs with the aim of improving the pharmacodynamic properties of cysteamine with a corresponding increase in patient compliance. We report here on the results of one of these prodrugs, cystamine decanoate with respect to the reduction of cystine in the lysosomes over a 24 and a 72 hour period. We also report on data from other candidate prodrugs and on the use of the level of caspase 3 & 7 level in treated and untreated cystinotic fibroblasts.

METHOD

Cell culture: Cystinotic fibroblasts were purchased from Coriel Laboratories (GM00008) and cultured using MEM supplemented with 15% FCS, 2mM Lglutamine, 1U/ml penicillin and 1µg/ml streptomycin. Human umbilical vein endothelial cells (HUVEC) were purchased from The European Collection of Cell Cultures (ECACC) and cultured using GMEM supplemented with 10% FCS, 2mM Lglutamine, 1U/ml penicillin and
1g/ml streptomycin. The cells were grown at 37°C in a humidified atmosphere of 5% CO2/95% room air and were expanded when they reached approximately 80% confluence.

**Cystine determination:** Cystinotic fibroblasts were seeded into 75cm² flasks (Nunc) and were used between passage 12 and 20 when they were a minimum of 80% confluent. At this point the novel prodrug, cysteamine or vehicle control was added to the respective flask. After 24 hours incubation the cells were harvested and the level of lysosomal cystine was determined using a novel reverse-phase HPLC as previously described4.

**Labelling of apoptotic cells:** Cystinotic fibroblasts and HUVEC were seeded in chamber slides at a density of 0.25 x 10⁵ cells/ml. On reaching 80% confluence, the media was removed and replaced with fresh media containing Yo-PRO (1 in 10,000 dilution). The chamber slides were then returned to the incubator for 30 minutes, before removing the media and rinsing three times in ice cold phosphate buffered saline.

**Detection of caspase 3/7 activity:** Cystinotic fibroblasts were cultured in 25cm² flasks for 48 hours in the respective test conditions highlighted in table 1. Cells were collected by scraping into 1ml PBS. Cell counts were performed by means of trypan blue dye exclusion, and 6 x 10⁴ cells/100μl added in triplicate to a black polystyrene 96 well plate, along with triplicate 100μl cell-free media to act as a background control. Subsequently, 100μl of Apo-ONE® homogenous caspase 3/7 solution (Promega, USA) was added to each well containing cell sample or cell-free media. Caspase 3/7 activity was read as fluorescent emission (excitation 499 nm/emission 521nm) on a plate reader (Biotek) at intervals of 15 minutes.

**RESULTS**

The prodrug cysteamine decanoate is able to significantly reduce the level of cystine in cystinotic fibroblasts when compared with a vehicle control after 24 hours incubation to a comparable level to that achieved by Cystagon (Figure 1).

Cystinotic fibroblasts stained intensely in the presence of YoPRO when compared to healthy cells. As can be seen in figure 2 this results in a dramatic difference when compared to healthy cells. Apoptotic cells were visualised as a green fluorescent stain reflecting the enhanced membrane permeability associated with apoptosis. HUVEC showed no evidence of apoptosis (Fig. 2a) while the majority of cystinotic fibroblasts showed intense nuclear stain. Figure 2b.
Caspase 3/7 enzyme activity was then measured. Cystinotic fibroblasts demonstrated the highest level of activity when compared to both HUVEC and HUVEC following treatment with 0.4% H$_2$O$_2$ (Figure 3).

The cystinotic fibroblasts were then treated with the prodrugs and the level of caspase 3/7 was determined and some of the data is presented in Figure 4. Although there was no significant difference in the level of caspase 3/7 activity in cystinotic cells treated with prodrug when compared with the control, it was also noted that at this time point cysteamine also did not significantly reduce enzyme activity. Further investigation demonstrates a high degree of variability in the effectiveness of Cystagon and the prodrug in reducing caspase 3/7 and there are a clearly a wide range of factors that may contribute to this variation which are relevant to our drug screening programme.

CONCLUSION AND FUTURE WORK This work has demonstrated that the novel prodrug cystamine decanoate is able to deplete lysosomal cystine in cystinotic fibroblasts after 48 hours with a comparable efficacy to the therapeutic standard. Work is continuing to further characterise the efficacy of this compound and others from a library of novel prodrugs and to determine the effect of the prodrugs on the stage and incidence of apoptosis in cultures of cystinotic fibroblasts.

REFERENCES