Evaluation of Novel Prodrugs for the Treatment of Nephropathic Cystinosis

Dr Rachel Knott, Dr Graeme Kay and Professor Donald Cairns

The School of Pharmacy and Life Sciences,
The Robert Gordon University,
Schoolhill,
Aberdeen
UK
AB10 1FR

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 course at which 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). These results have indicated that cystamine decanoate is able to reduce the level of intracellular cystine and the results of this are summarised in the text below.

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. While the proof of concept is now demonstrated we now wish to identify the effect of the novel prodrug after shorter periods of time (≤ 24 hours) and compare its efficacy to that of cysteamine, the current oral therapy.

Further work: During the remaining 6 months of funding we wish to evaluate more of the novel prodrugs in our cystinotic cell model. In order to continue this work we have submitted a grant to CRN for consideration to fund this work for an additional24 months funding. This would enable us to characterise the apoptotic status of the cells following addition of the prodrug and enable us to develop a human cell CTNS knockout which would greatly facilitate the screening of further prodrugs.

INTRODUCTION

Cystinosis is caused by a mutation in the gene encoding cystinosin which is a lysosomal membrane transporter[1]. Defects in this transporter protein result in a build up of cystine in the lysosomes causing progressive cell damage, apoptosis and cell death[2]. 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[3]. 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.

METHOD

Cell culture: Cystinotic fibroblasts were purchased from Coriel Laboratories (GM00008) and cultured using MEM supplemented with 15% FCS, 2mM L-glutamine, 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 L-glutamine, 1U/ml penicillin and 1µg/ml streptomycin . The cells were grown at 37ºC in a humidified atmosphere of 5% CO2 /95% room air and were expanded when they reached approximately 80% confluence.

Detection of cystinosin: Cystinotic fibroblasts and HUVEC were seeded into chamber slides and grown to confluence. The respective growth medium was removed and the monolayers were washed three times in phosphate buffered saline, left to air dry, fixed in 4% paraformaldehyde and stored at -20ºC until required for immunocytochemistry. The primary antibody raised against a synthetic peptide of cystinosin (Lifespan Biosciences) was added to the cells followed by a biotinylated anti-rabbit antibody (Molecular probes). Specific binding to cystinosin was visualised with the addition of streptavadin alkaline phosphatase and application of the appropriate substrate. Positive staining was evident as a red colour.

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.

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 either the novel prodrug (50µM), cysteamine (50µM) or vehicle control (1% ethanol) were added to the flask. After 48 hours incubation the cells were harvested and the level of lysosomal cystine was determined using a novel reverse-phase HPLC as previously described[4].

RESULTS

The cystinosin protein was visualised as a red colour in HUVEC (Fig. 1a) localised predominately in the perinuclear region with some staining dispersed throughout the cytoplasm. Staining was also evident in cystinotic fibroblasts (Fig. 1b) although the distribution of the protein was markedly different with diffuse staining throughout the cytoplasm.

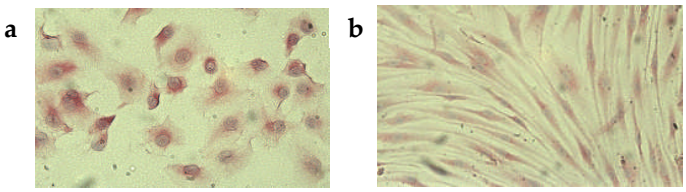

Figure 1: Immunocytochemical detection of cystinosin in HUVEC (a) and cystinotic fibroblasts (b)

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.

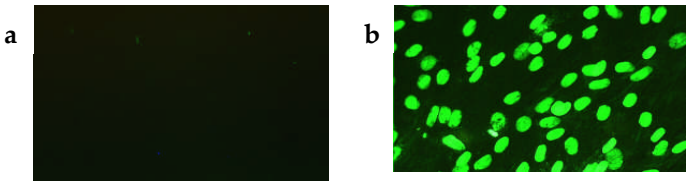

Figure 2: Detection of apoptotic HUVEC (a) and cystinotic fibroblasts (b) using YOPRO

The level of cystine in the lysosomes of cystinotic fibroblasts was determined following incubation with cysteamine or the novel prodrug cystamine decanoate and compared to a vehicle control. The level of significance of the findings was determined by the Student's t test (n=7). Cysteamine ($p<0.01$ ***) and cystamine decanoate ($p<0.01$**) were able to significantly reduce the level of lysosomal cystine (Figure 3).

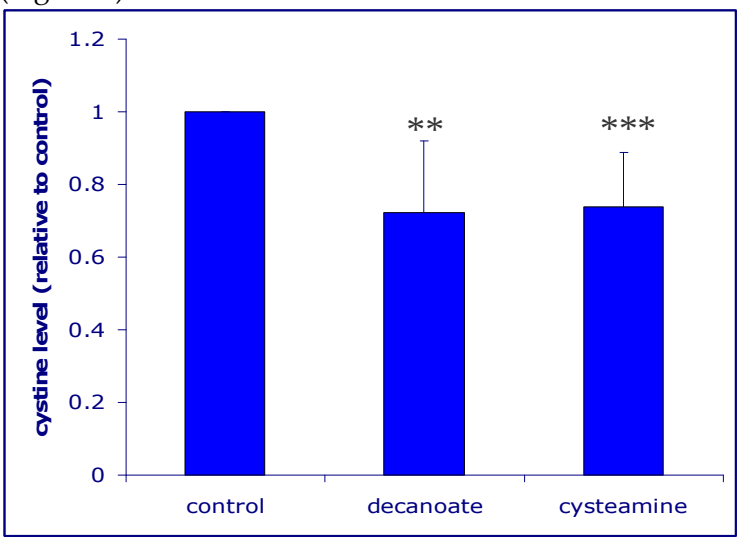

Figure 3: Lysosomal level of cystine in cystinotic fibroblasts following 48 hour treatment with cysteamine and cystamine decanoate as determined by reverse-phase HPLC. Results are calculated as level of cystine/protein and are presented relative to the control group.

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

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