Design and Synthesis of Novel Prodrugs for the Treatment of Cystinosis.

Project Report June 2007

Professor Donald Cairns
School of Pharmacy
The Robert Gordon University
Aberdeen

Contents **Page No.**

1.0 Background

Nephropathic cystinosis is a rare autosomal recessive disease characterised by raised intracellular levels of cystine. Symptoms include renal Fanconi syndrome and growth retardation. If untreated, cystinosis results in death from renal failure by the second decade of life. The only treatment for cystinosis is administration of cysteamine, an aminothiol with an offensive taste and smell, which is excreted in breath and sweat causing halitosis and body odour as well as gastric irritation. As a result, patient compliance may be poor.
The purpose of this investigation was the synthesis, characterization and biological evaluation of a series of odourless and tasteless pro-drug forms of cysteamine. It is envisaged that these compounds will improve compliance among cystinotic patients and lead to an increase in patient treatment success and quality of life.

The detailed aims of this project were: -

(i) The synthesis, purification and characterisation of a number of pro-drug derivatives of cysteamine and cystamine that will reduce the cystine burden within cells.
(ii) Evaluation of the *in vivo* activity of synthesized prodrugs using a 'knock-out' mouse model. This work will be carried out in collaboration with Dr C. Antignac, Hôpital Necker-Enfants Malades, Paris, France.[*] **see section 6.0**

1.1 Plan Of Investigation

This project required the synthesis of a number of odourless, tasteless and orally active pro-drug derivatives of cysteamine and cystamine by selected use of the following strategies:

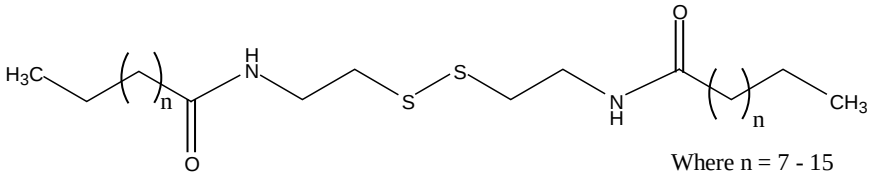
1.1.2 Prodrugs with low aqueous solubility

For a compound to taste unpleasant, it must first dissolve in saliva (for review, see Berge *et al*). The taste of cysteamine may be disguised by production of salts or derivatives which have low water solubility. These derivatives may take the form of embonates, palmitates, oleates and stearates (previously used successfully to disguise the bitter taste of the antibiotics chloramphenicol and erythromycin, Lund *et al*) or suitable ion exchange resin complexes.

1.1.3 Synthetic Chemistry (a)

A number of cystamine and cysteamine derivatives have now been synthesized and characterized by ^{1}HNMR and MS. Their general structures are illustrated in **Figure 1**:

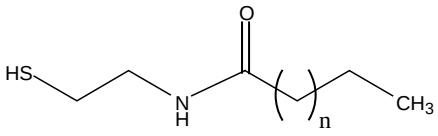
Fatty Amide Derivatives of Cystamine



Where n = 7 - 15

(1)

Fatty Amide Derivatives of Cysteamine



Where n = 7 - 15

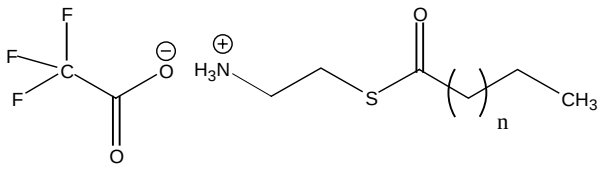
(2)

Figure 1 Examples of amide derivatives.

1.1.4 Pseudopeptide derivatives (b).

A second, more sophisticated, approach was to design a range of cysteamine derivatives with the ability to cross the GI membrane, be transported in the serum to their target cells and decompose to release cysteamine intracellularly. This approach was a refinement of the amino-acid derivatives previously developed by us Tindall *et al*, 2003, Anderson *et al,* 2006. Some of the criticisms directed at the use of amino acids as prodrugs of cysteamine centered around the stability of amino acid derivatives which can breakdown in the gut before intracellular compartmentalisation due to the action of amidase enzymes in the plasma (W.A.Gahl, personal communication). The preparation of pseudopeptide prodrugs to overcome the instability inherent with natural derivatives while retaining enhanced pharmacodynamic properties was underatken. A range of non-toxic derivatives, such as pivaloyl, thioesters and ethoxycarbonyl cysteamine / cystamine were synthesised.

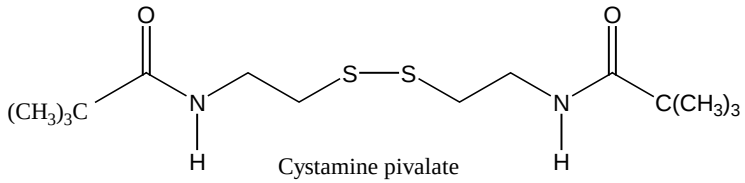
Thioesters



Where n = 7 - 15

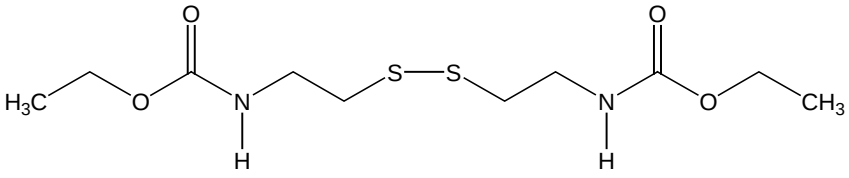
(3)

Figure 2: Examples of Pseudopeptide Derivatives



Cystamine pivalate

(4)



Ethoxycarbonylcystamine

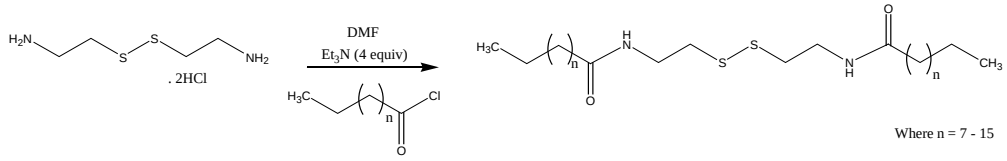
(5)

1.2 General Experimental Procedures

Cystamine Prodrugs

Acid Chloride Route (**Scheme 1**)

To a stirred solution of cystamine dihydrochloride in anhydrous DMF (20 cm^3) at room temperature was added triethylamine (2.02 mol equivalents). The reaction mixture was then allowed to stir for 15 minutes at room temperature. To this was added, drop wise, the relevant fatty acid chloride (2.00 mol equivalents) followed by the addition of triethylamine (2.02 mol equivalents). After addition and with stirring an off-white solid precipitated. To this was added water (100 cm^3) and the solid was then isolated *via* filtration. The solid was then re-precipitated from ethanol at 70°C followed by filtration, which gave a chromatographically pure white solid. The purity was analysed by TLC [DCM : MeOH, 9:1)].
All solids were then dried in a vacuum oven at 65°C.

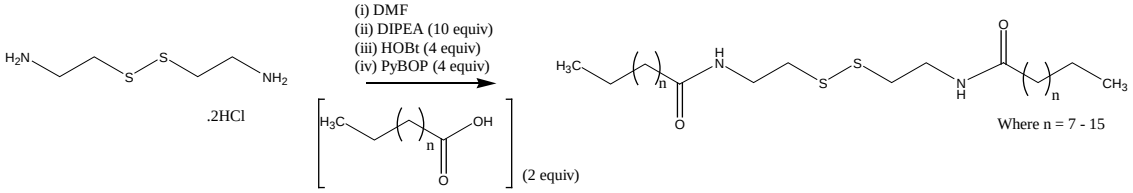


Where n = 7 - 15

Scheme 1

Peptide Coupling Route (**Scheme 2**)

To a stirring solution of cystamine dihydrochloride in anhydrous DMF (20 cm^3) at room temperature was added diisopropylethylamine (2.0 mol equivalents). The reaction mixture was then allowed to stir for 15 minutes at room temperature. To this were added HOBt (5.88 mol equivalents), PyBOP (5.9 mol equivalents), diisopropylethylamine (9.0 mol equivalents) and the relevant fatty acid (6.0 mol equivalents). After addition and with stirring an off-white solid precipitated. To this was added water (150 cm^3) and the solid was then isolated *via* filtration. The solid was then re-precipitated from ethanol at 70°C followed by filtration, which gave a chromatographically pure white solid. The purity was analysed by TLC [DCM : MeOH, 9:1)].
All solids were then dried in a vacuum oven at 65°C.



Scheme 2

Cysteamine Prodrugs (**Scheme 3**)

Synthesis of ^{t}Boc-Cysteamine

To a stirring solution of cysteamine hydrochloride in anhydrous DCM (25 cm^3) was added diisopropylethylamine (1.0 mol equivalents) and the reaction mixture allowed to stir for 20 minutes. To this was added tBoc anhydride (1.1 mol equivalents). The reaction mixture was stirred for a further 1.5 hours. TLC analysis was performed (DCM:EtOH 9:1, followed by Iodine tank). The solution was then partitioned between DCM and water. The DCM extracts were then washed with water (3 x 50 cm^3), dried with $MgSO_4$, filtered and evaporated to dryness. This gave a clear gum.

Synthesis of N-^{t}Boc-S-Fmoc-Cysteamine

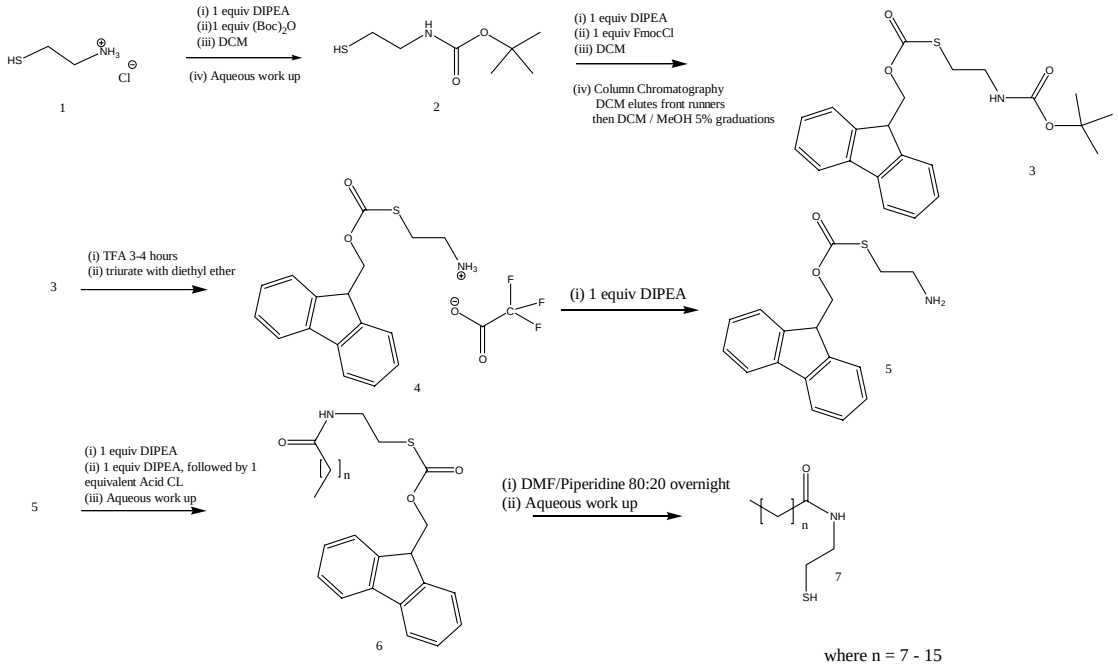
To a stirring solution of ^{t}Boc-Cysteamine in anhydrous DCM (50 cm^3) was added diisopropylethylamine (1.0 mol equivalent) and the reaction stirred for 15 minutes. To this was added 9-fluorenylmethyl chloroformate (1.0 mol equivalent) and the reaction stirred for 2.5 hours. After TLC analysis the solution was partitioned between DCM and water. The DCM extracts were then washed with water (3 x 50 cm^3), dried with $MgSO_4$, filtered and evaporated to a low volume. The solution was applied to a silica gel chromatography column (4 x 30 cm^3) prepared with DCM. The column was initially eluted with the same solvent. The eluent was then changed to DCM : MeOH (9:1) when the major product was eluted. Fractions containing the major product were combined, filtered and evaporated to give a clear/yellow oil.

Synthesis of S-Fmoc-Cysteamine Trifluoroacetate salt

The ^{t}Boc protected compound, N-^{t}Boc-S-Fmoc-Cysteamine, was dissolved in trifluoroacetic acid (5 cm^3) at room temperature. After 3.5 hours, the solvent was evaporated and the resulting solid re-evaporated with ethanol (3 x 20 cm^3). Addition of diethyl ether gave an off white precipitate that was filtered and dried over $CaCl_2$. The product was chromatographically homogenous by TLC [DCM : MeOH (9:1)].

Synthesis of N-Acyl-S-Fmoc-Cysteamine

To a stirred solution of S-Fmoc-Cysteamine Trifluoroacetate salt in DMF (25 cm^3) was added diisoprpylethylamine (1.0 mol equivalent) and the reaction mixture was stirred for a 20 minutes. To this was added, drop wise, the relevant fatty acid chloride and the reaction stirred for a further 1.5 hours. After stirring an off-white solid precipitated. To this was added water (100 cm^3) and the solid isolated *via* filtration.

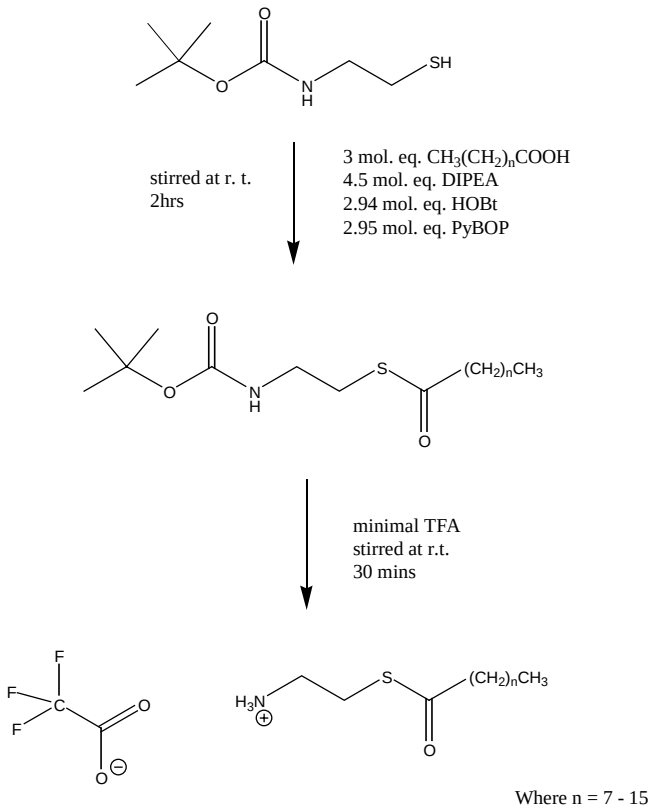


Scheme 3

Synthesis of Thioesters (**Scheme 4**)

To a stirring solution of N^{t}Boc cysteamine in anhydrous DMF (20 cm^3) at room temperature was added diisopropylethylamine (1.0 mol equivalents). The reaction mixture was then allowed to stir for 15 minutes at room temperature. To this were added HOBt (2.94 mol equivalents), PyBOP (2.95 mol equivalents), diisopropylethylamine (4.5 mol equivalents) and the relevant fatty acid (3.0 mol equivalents). After

addition and with stirring an off-white solid precipitated. To this was added water (150 cm^3) and the solid was then isolated *via* filtration. The solid was then re-precipitated from ethanol at 70°C followed by filtration, which gave a chromatographically pure white solid. The purity was analysed by TLC [DCM : MeOH, 9:1)].
All solids were then dried in a vacuum oven at 65°C.



Scheme 4

1.3 Compound Library

The synthetic routes employed enabled the generation of the compound library shown in **table 1**.

Table 1 Compound Library

Compound Name (common)	Prodrug / Compound Description
Cysteamine Decanoate	C_{10} fatty amide of cysteamine
Cysteamine Palmitate	C_{16} fatty amide of cysteamine
Cysteamine Stearate	C_{18} fatty amide of cysteamine
Cysteamine Myrisolate Thioester	C_{14} thioester of cysteamine
Cysteamine Palmitate Thioester	C_{16} thioester of cysteamine

Cysteamine Stearate Thioester	C_{18} thioester of cysteamine
Cysteamine Tridecanoate Thioester	C_{13} thioester of cysteamine
Cystamine Decanoate	C_{10} fatty amide of cystamine
Cystamine Oleiate*	C_{18} unsaturated fatty amide of cystamine
Cystamine γ-Linolenate**	C_{18} unsaturated fatty amide of cystamine
Cystamine Elaidioate***	C_{18} unsaturated fatty amide of cystamine
Cystamine Eruciate	C_{22} unsaturated fatty amide of cystamine
Cystamine Linolenate****	C_{18} unsaturated fatty amide of cystamine
Cystamine Laurate	C_{12} fatty amide of cystamine
Cystamine Tridecanoate	C_{13} fatty amide of cystamine
Cystamine Pentatdecanoate	C_{15} fatty amide of cystamine
Cystamine Palmitate	C_{16} fatty amide of cystamine
Cystamine Heptadecanoate	C_{17} fatty amide of cystamine
Cystamine Stearate	C_{18} fatty amide of cystamine
Cystamine Nonadecanoate	C_{19} fatty amide of cystamine
Cystamine Benzoylate	Experimental chromophoric amide of cystamine
Cystamine Succinate	Potential multi-dose prodrug starting material
Cystamine Pantethinate	Cystamine-pantothenic acid conjugate
Cystamine Pivolate	Pseudopeptide Derivatives
Cystamine Ethoxycarbonate	Pseudopeptide Derivatives

* cis-9-octadecenoic acid conjugate.
** cis,cis,cis-9,12,15-Octadecatrienoic acid conjugate.
*** trans-9-octadecanoic acid conjugate.
**** cis-9,cis-12-Octadecatrienoic acid conjugate.

2.0 Drug Solubility Studies

A library of novel water insoluble prodrugs has been synthesised, however their low aqueous solubility caused difficulties for quantitative *in vitro* evaluation.
Several methods to enhance solubility were investigated:

(i) Use of various solvents and co-solvents were explored (e.g. aqueous ethanol, DMSO/Water and DMSO/Ethanol).

(ii) The use of different concentrations of β-cyclodextrin were evaluated, however only a very small increase in aqueous solubility was observed. The drug/CD interaction was modelled in-house using molecular modelling techniques to attempt to predict the physicochemical features required for solubility.

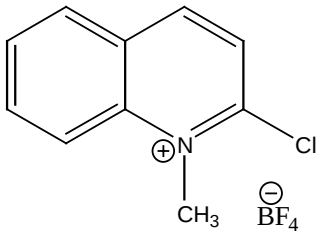
(iii) The use of solubility enhancers such as ultrasonic baths and sonic probe were evaluated, but they did not result in a measurable increase in solubility.

All of the synthesised compounds showed limited solubility in DMSO, with the unsaturated prodrugs proving more soluble than their saturated counterparts. None of the synthesised prodrugs smell strongly, most have no discernible odour whatsoever with a few showing a faint 'chemical' smell. Only two compounds have been evaluated for taste (DC) and were tasteless.

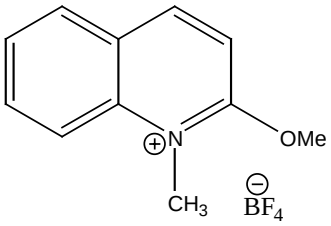
Surprisingly, when solubility studies were repeated with ethanol, the solubility of the prodrugs increased. Concentrations of 1000-4000μM were achieved and as a result, ethanol will now be used for manufacture of stock solutions for all future cell work. Solubility data are presented as Appendix 3.

3.0 Quantitative HPLC determination of cysteamine and biologically relevant thiols.

A number of methods for the quantitative detection of thiols are available in the literature (Chwatko, *et al,* Ivanov *et al*, Camera *et al*, Kusmierek, *et a*l, De Graaf-Hess, *et al*, Bald, *et al*).
Using a combination of these methods, a novel assay for the *in-vitro* determination of cysteamine and cystine has been established. This reverse phase HPLC method employs a thiol specific, UV tagging agent synthesized and characterized in our laboratories. The structures of the tagging agents are shown below. All compounds have been fully characterized by ^{1}H NMR, ^{13}C NMR and MS.



(6)



(7)

HPLC detection and quantification of various thiol containing compounds, including homocysteine, glutathione and cysteamine has been demonstrated **Figure 3** (tagged with chromophore **7**).

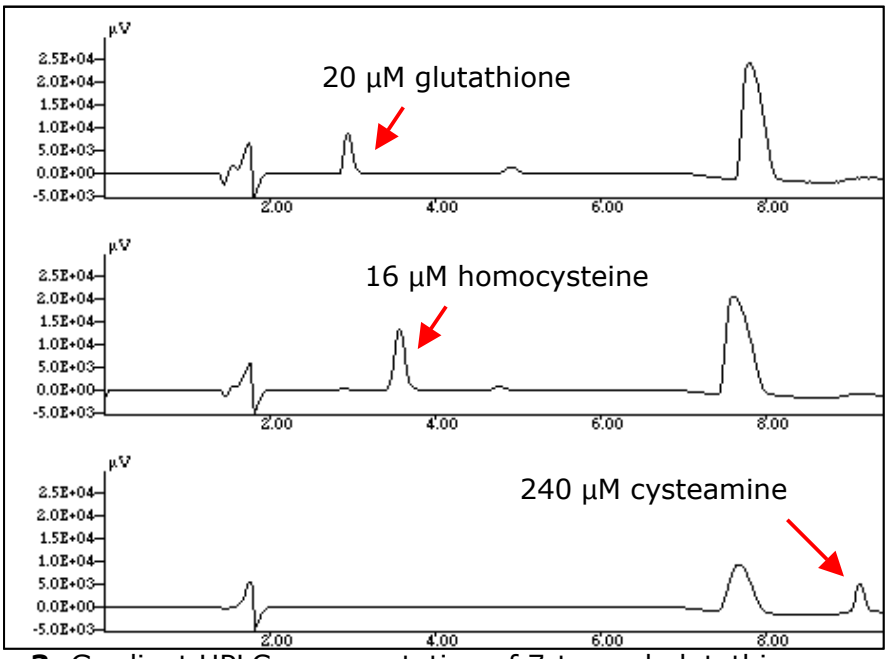


Figure 3: Gradient HPLC representation of 7-tagged glutathione, homocysteine and cysteamine

Cysteamine tagged with **6** elutes with a retention time (RT) of 2.8 minutes [Demonstrated at 5.4µM and 10.8µM cysteamine], **Figure 4**.

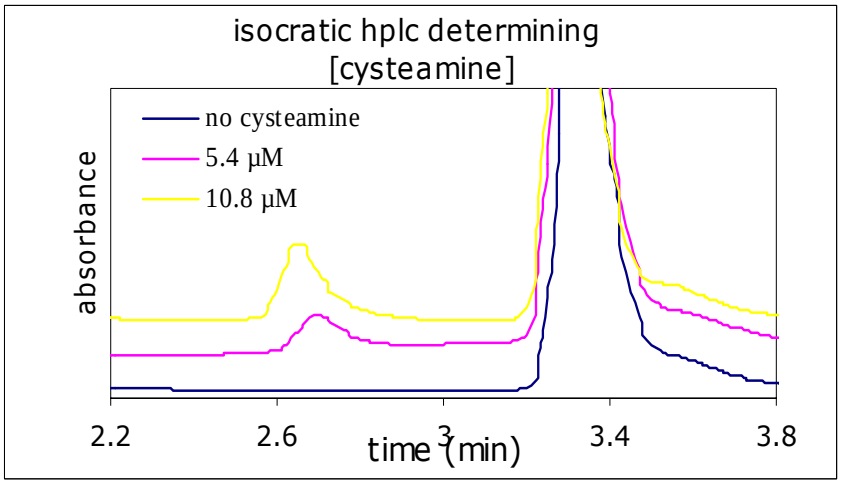


Figure 4. Isocratic HPLC chromatograph of 6-Tagged cysteamine.

Recent results have demonstrated that these drugs do not show any significant change in the cell growth profile of human umbilical vein endothelial cells following incubation for 24 hours using an Alamar blue assay. Representative growth curves are presented as **Figure 9**.

4.0 Preliminary Toxicology Studies (MCF-7)

The cytotoxic effect of a range of bis-acylated fatty amides of cystamine was evaluated against MCF-7 cell lines grown in culture (**Figures 5 and 6**). Stock solutions of 30μM in 100% DMSO were used [final concentration of prodrug 0.3μM; 1%DMSO]. The data suggest a slight decrease in cell viability in the presence of drug (approximately 10%).

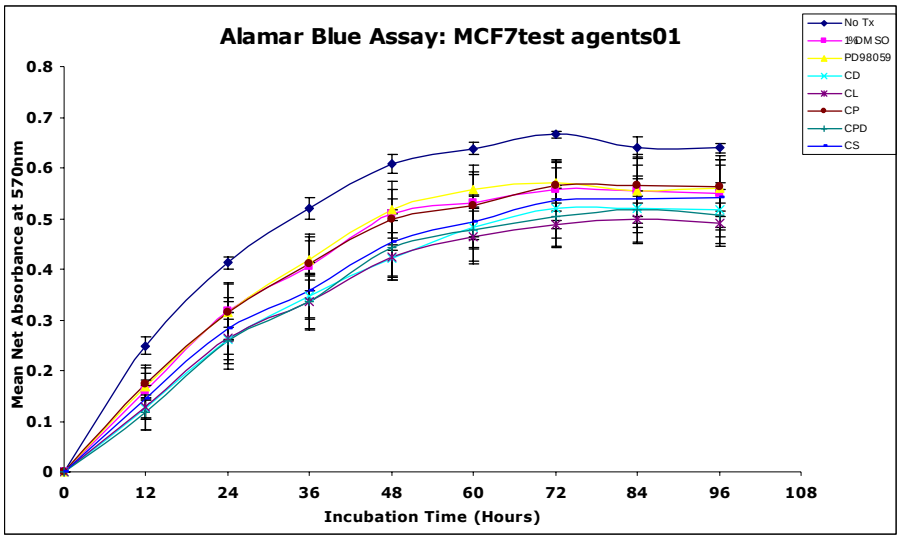


Figure 5: Alamar blue assay for Cystamine Decanoate (CD), Cystamine Pentadecanoate (CPD), Cystamine Stearate (CS), Cystamine Laurate (CL), and Cystamine Palmitate (CP). [PD98059 represents cells in the presence of an inhibitor, this does not relate to the cystinosis study].

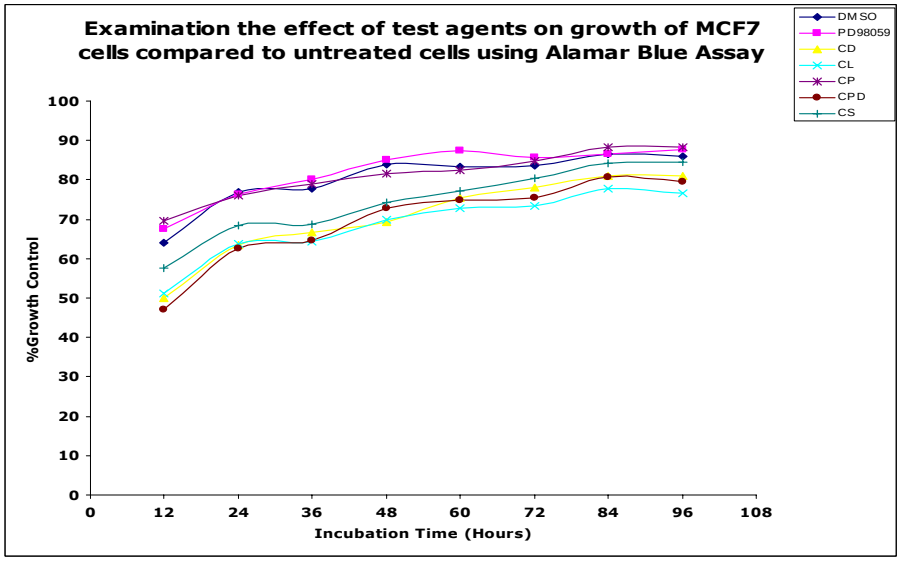


Figure 6: Effect of test agents on growth of MCF7 cells compared to untreated cells. Cystamine Decanoate (CD), Cystamine Pentadecanoate (CPD), Cystamine Stearate (CS), Cystamine Laurate (CL), and Cystamine Palmitate (CP). [PD98059 represents cells in presence of an inhibitor, this does not relate to the cystinosis study].

However, when the percentage growth inhibition was plotted against the inhibition experienced in the presence of 1% DMSO only (**Figure 7**) the inhibition observed was not significantly different to that of the vehicle alone, suggesting that the major inhibition observed was due to the presence of 1% DMSO i.e. the synthesised prodrugs were no more toxic than the DMSO alone.

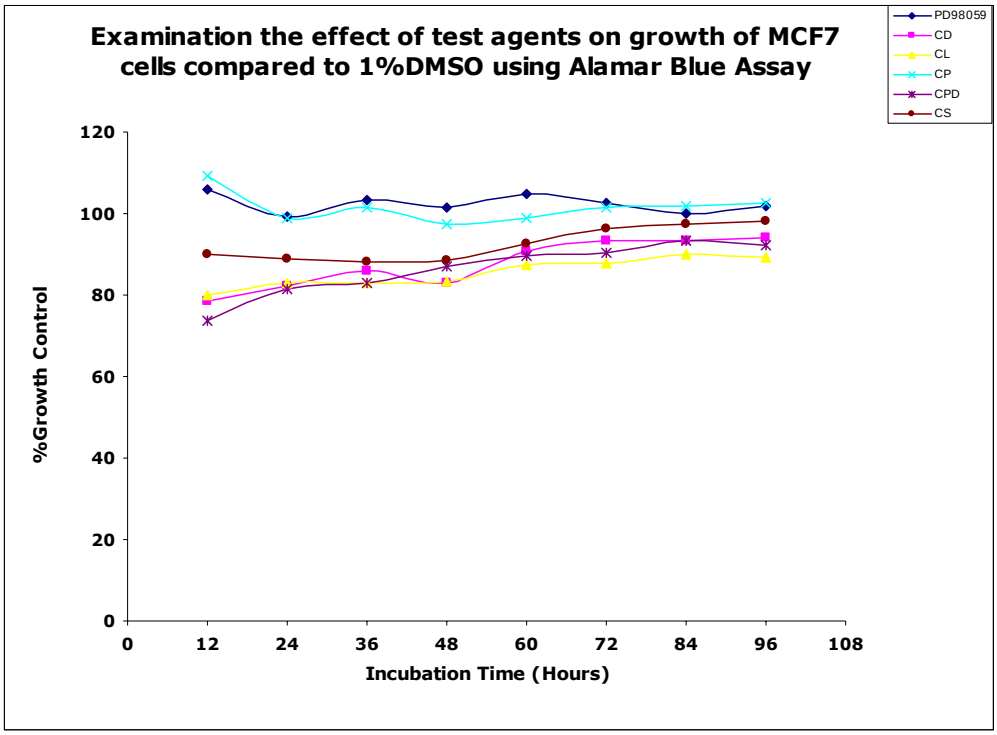


Figure 7: Effect of test agents on growth of MCF7 cells compared to 1%DMSO. Cystamine Decanoate (CD), Cystamine Pentadecanoate (CPD), Cystamine Stearate (CS), Cystamine Laurate (CL), and Cystamine Palmitate (CP). [PD98059 represents cells in presence of an inhibitor, this does not relate to the cystinosis study].

4.1 Increasing Drug Concentrations

Using stock solutions of 30μM in 100% DMSO, [final concentration of prodrug 1.5μM; 5%DMSO] an attempt was made to explore the maximum acceptable concentration of prodrug and vehicle. The cytotoxic effect of a range of bis-acylated fatty amides of cystamine was evaluated against MCF-7 cell lines and the results displayed in **Figure 8.**

Significant inhibition of cell growth was observed, but the presence of prodrug did not result in a significant difference when compared to 5% DMSO on its own. It was therefore concluded that the growth inhibition observed was due to the cytotoxic action of the comparatively high concentration of DMSO.

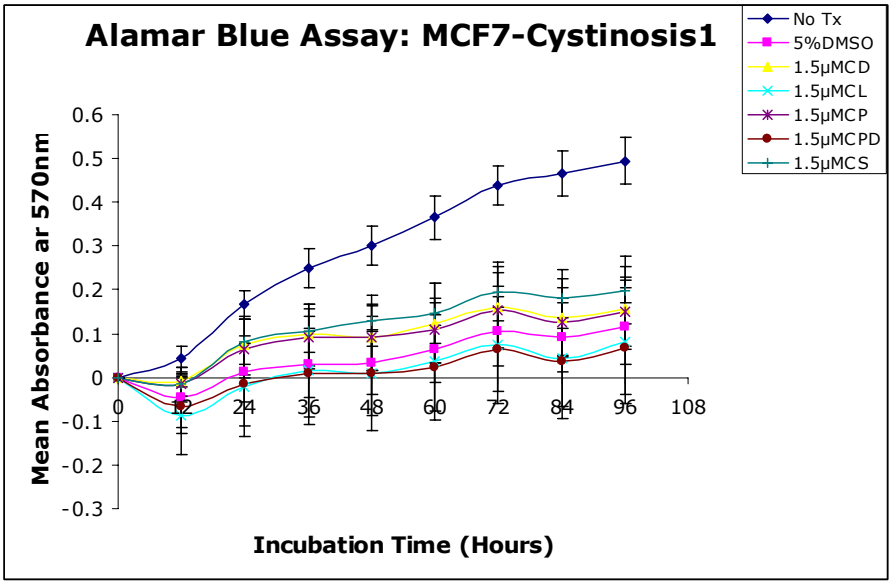


Figure 8: Alamar blue assay for Cystamine Decanoate (CD), Cystamine Pentadecanoate (CPD), Cystamine Stearate (CS), Cystamine Laurate (CL), and Cystamine Palmitate (CP).

5.0 Determination of cytotoxicity (Vehicle = Ethanol; HUVEC cells)

Recent results have demonstrated that these drugs do not show any significant change in cell growth profile of human umbilical vein endothelial cells following incubation for 24 hours using an Alamar blue assay. Representative growth curves are presented as **Figure 9**; full results see Appendix 2.

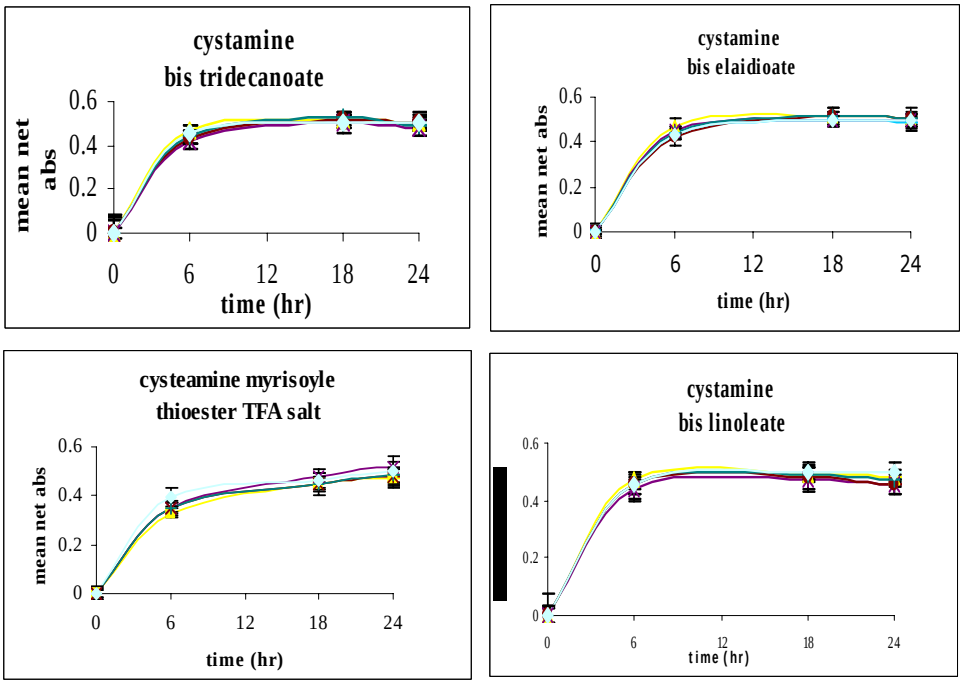


Figure 9: Alamar blue proliferation assay of HUVE cells following exposure to various concentrations of pro-drug in 1% ethanol solution. No significant change in cell growth was determined at 24 hours as determined by Mann-whitney non-parametric analysis.
Key: yellow = 0 μM , dark purple = 5 μM, dark red = 10 μM, sea green = 20 μM and turquoise = 50 μM.

6.0 Conclusions

The project '*Design and Synthesis of Novel Prodrugs for the Treatment of Cystinosis*', funded by CRN in 2005 has achieved solid progress although is running slightly behind schedule. Delays have occurred due to refurbishment work on the research laboratories at RGU and because the post doctoral worker originally assigned to carry out the work was appointed to a lectureship within the School of Pharmacy. He does, however, remain closely involved with the project. Additional staff have been appointed and a graduate research student will start in summer 2007.
The chemistry section of the work has been very successful. A number of novel prodrugs have been synthesised in good yield and we now have a library of > 50 compounds. The discovery chemistry is now essentially over and we do not envisage undertaking the synthesis of any new compounds during this current round of funding.
The synthesised compounds are odourless and the small numbers evaluated are tasteless. A number of the compounds exhibit very low aqueous solubility. This was envisaged in their design, but does cause problems with formulation and testing. The formulation of these prodrugs is supported by another source and the data in Appendix 3 are presented for completeness.
In the course of the project a novel quantitative reverse phase HPLC assay for thiols has been developed and will be used for the assay of prodrug activity.
The synthesised compounds have been evaluated for cytotoxicity and found to be non-toxic to 50 μM in MCF7 and HUVEC cultures.
Determination of the ability of the compounds to deplete cells of cystine will form the next stage of the project.
Following comments made at the SRB meeting in Salt Lake City and in your letter of 31st August concerning the cost of *in vivo* studies, funds of approximately £5k from the initial award are held in reserve for the *in vivo* assay.
My colleague, Dr Kay and I have submitted abstracts of some of this work to the British Pharmaceutical Conference in September 2007. If our papers are accepted, I will forward the abstracts and completed texts to CRN for approval prior to publication.

7.0 References

Anderson, R.J., Cairns, D., Cardwell, W.A., Case, M., Groundwater, P.W., Hall, A.G., Hogarth, A.L., Jones, A.L., Meth-Cohn, O., Suryadevara, P., Tindall, A., Thoene, J.G. (2006), *Letters in Drug Design and Discovery*, 3 (5), 336-345.

Bald, E., Glowacki, R., (2001) *J. Liq. Chrom. & Rel. Technol.,* 24 1323

Berge, S.M., Bighley, L.D., Monkhouse, D.C., *Selection of Pharmaceutical salts* J. Pharm. Sci. 1977, **66**, 1-19

Camera, E., and Picardo, M. (2002), *J. Chromatogr. B* 781 181.

Chwatko, G., Bald, E., (2002), *J. Chromatogr. A* 949 141.

De Graaf-Hess, A., Trijbels, F., Blom, H., (1999), *Clinical Chemistry* 45 2224.

Ivanov, A.R., Nazimov, I.V., Baratova, L.A., (2000), *J. Chromatogr. A* 870, 433.

Kusmierek, K., Glowacki, R., Bald, E., (2005), *Anal. Bioanal. Chem.* 382 231.

Lund, W. (Ed) The Pharmaceutical Codex, The Pharmaceutical Press, London, 1994.

Tindall, A., Anderson, R.J., Cardwell W.A. and Cairns, D “Prodrugs for the Treatment of Cystinosis” J. Pharm. Pharmacol, 2003, Suppl., 182

W.A. Gahl, Personal communication.

Appendix 1

Experimental Details

Acid Chloride Route

To a stirred solution of cystamine dihydrochloride (1.0 g, 4.4 mmol) in anhydrous DMF (20 cm^3) at room temperature was added triethylamine (0.90 g, 8.9 mmol). The reaction mixture was then allowed to stir for 15 minutes. To this was added, drop wise, stearoyl chloride (2.7 g, 0.88 mmol) followed by the addition of triethylamine (0.90 g, 8.9 mmol). After addition and with stirring an off-white solid precipitated. To this was added water (100 cm^3) and the solid isolated *via* filtration. The solid was then re-precipitated from ethanol at 70°C followed by filtration, which gave a chromatographically pure white solid. The purity was analysed by TLC [DCM : MeOH, 9:1)]. All solids were then dried in a vacuum oven at 65°C. Yield 2.6 g (86%).

Found:

Mp. 115°C

The ^{1}HNMR spectrum (pyridine, C_5D_5N at 55°C) (270MHz) had δ: 0.9 (6H, t, 2 x C$\underline{H}_3$); 1.1-1.5 (56H, br.s, C$\underline{H}_2$'s); 1.8 (4H, m, 2 x COCH$_2$C$\underline{H}_2$); 2.4 (4H, t, 2 x COC$\underline{H}_2$CH$_2$); 3.1 (4H, t, 2 x SC$\underline{H}_2$); 3.8 (4H, m, 2 x NHC$\underline{H}_2$); 8.4 (2H, br.s., 2 x N$\underline{H}$CO)

The EI/CI mass spectrum had: 685 (100%) (M + H^+); M, 684.

IR (KBr) ν_{max} cm^{-1} 3298 (N-H); 1635 (C=O); 1219 (C-N).

Peptide Coupling Route

To a stirring solution of cystamine dihydrochloride (1.0 g, 4.4 mmol) in anhydrous DMF (20 cm^3) at room temperature was added diisopropylethylamine (1.14 g, 8.8 mmol). The reaction mixture was then allowed to stir for 15 minutes. To this were added HOBt (3.96 g, 25.9 mmol), PyBOP (13.52 g, 26 mmol), diisopropylethylamine (5.17 g, 40 mmol) and then decanoic acid (4.55 g, 26.4 mmol). After addition and with stirring an off-white solid precipitated. To this was added water (150 cm^3) and the solid isolated *via* filtration. The solid was then re-precipitated from ethanol at 70°C followed by filtration, which gave a chromatographically pure white solid. The purity was analysed by TLC [DCM : MeOH, 9:1)]. All solids were then dried in a vacuum oven at 65°C. Yield 1.8 g (88%).

Found:

Mp. 108°C

The ^{1}HNMR spectrum (pyridine, C_5D_5N at 55°C) (270MHz) had δ: 0.9 (6H, t, 2 x C$\underline{H}_3$); 1.1-1.5 (24H, m, C$\underline{H}_2$'s); 1.8 (4H, q, 2 x COCH$_2$C$\underline{H}_2$); 2.4 (4H, t, 2 x COC$\underline{H}_2$CH$_2$); 3.1 (4H, t, 2 x SC$\underline{H}_2$); 3.8 (4H, m, 2 x NHC$\underline{H}_2$); 8.4 (2H, br.s., 2 x N$\underline{H}$CO)

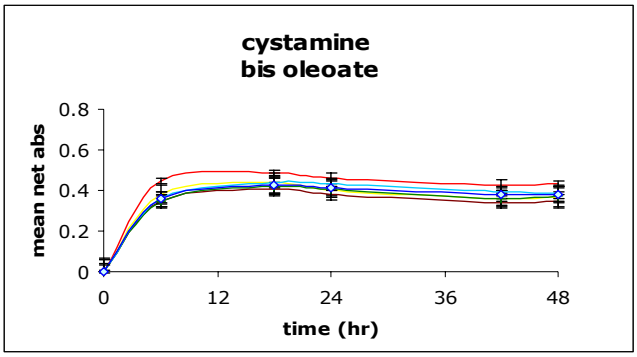
The electrospray (+) mass spectrum had: m/z 483 (M + Na) (100%); 943 (M + 2Na) (50%); ES (-) 495 (M + Cl) (70%); M, 460.

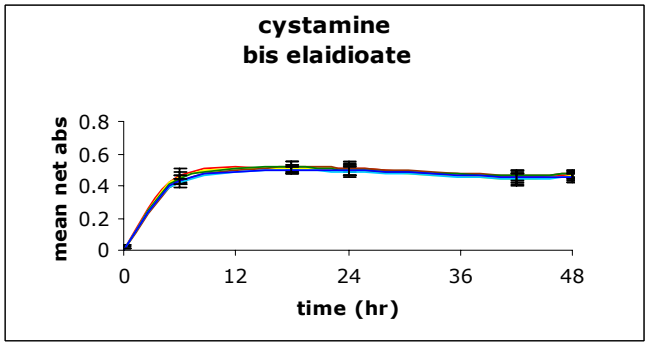
IR (KBr) ν_{max} cm^{-1} 3301 (N-H); 1636 (C=O).

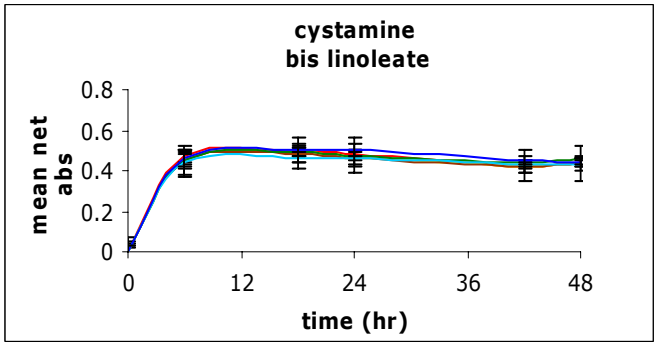
Appendix2

Unsaturated compounds:

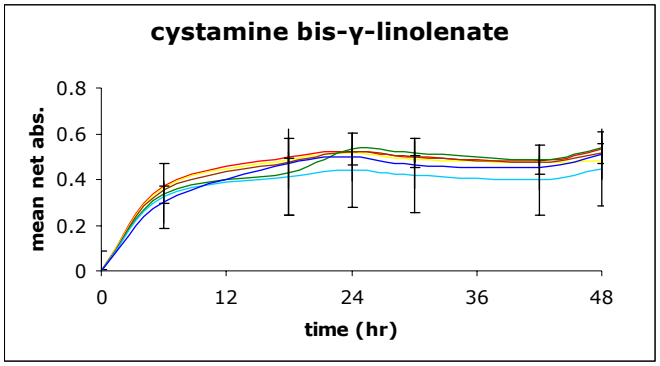
Key: 0 µM = red ; 2 µM = yellow ; 10 µM = brown
20 µM = green ; 40 µM = sky blue ; 50 µM = blue

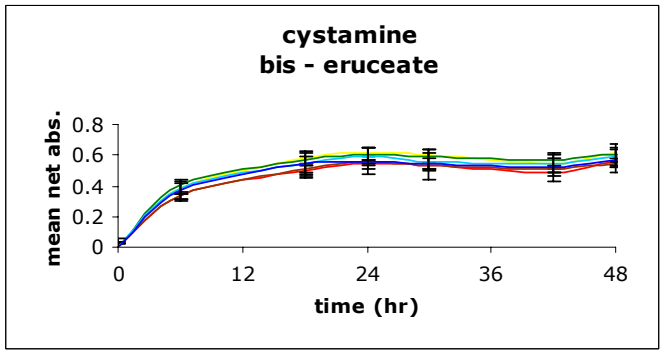






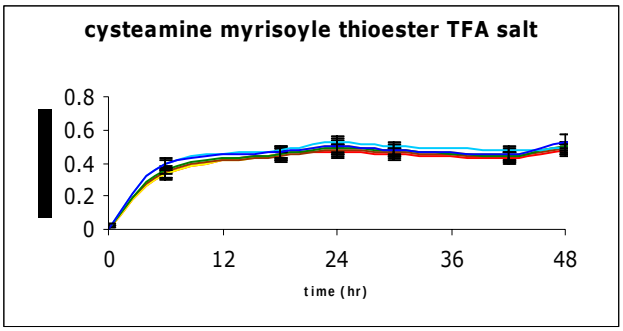
Key: 0 µM = red ; 2 µM = yellow ; 10 µM = brown
20 µM = green ; 40 µM = sky blue ; 50 µM = blue

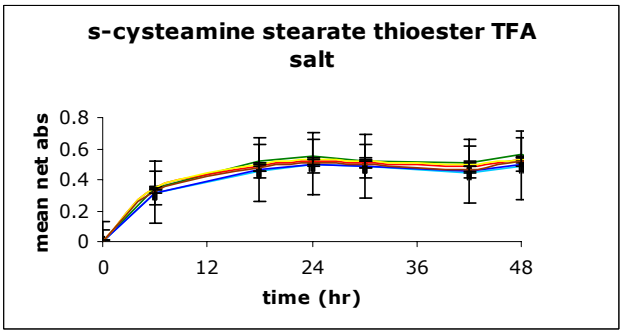


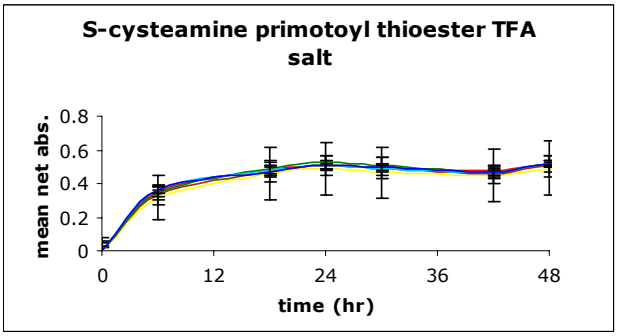


Thioesters:

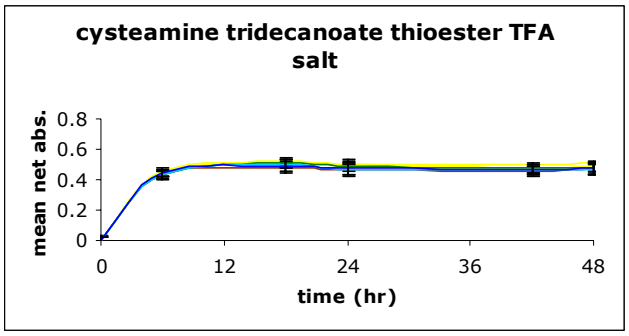
Key: 0 µM = red ; 2 µM = yellow ; 10 µM = brown
20 µM = green ; 40 µM = sky blue ; 50 µM = blue





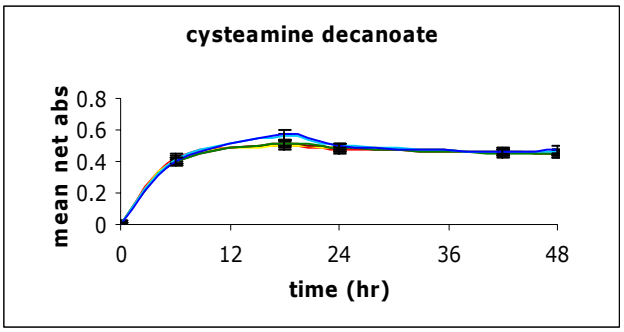


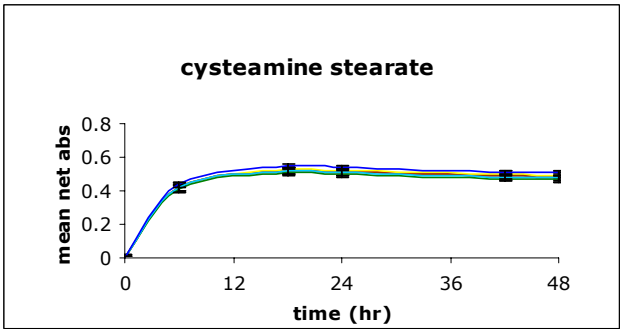
Key: 0 µM = red ; 2 µM = yellow ; 10 µM = brown
20 µM = green ; 40 µM = sky blue ; 50 µM = blue

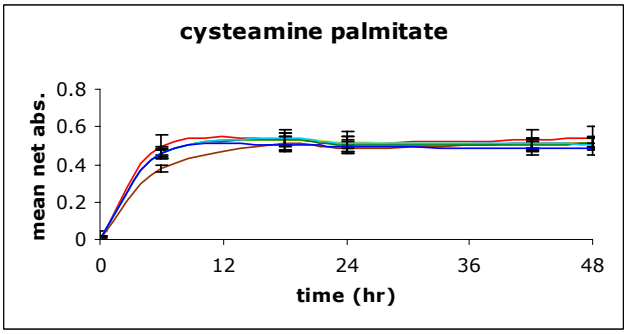


Cysteamine derivatives:

Key: 0 µM = red ; 2 µM = yellow ; 10 µM = brown
20 µM = green ; 40 µM = sky blue ; 50 µM = blue

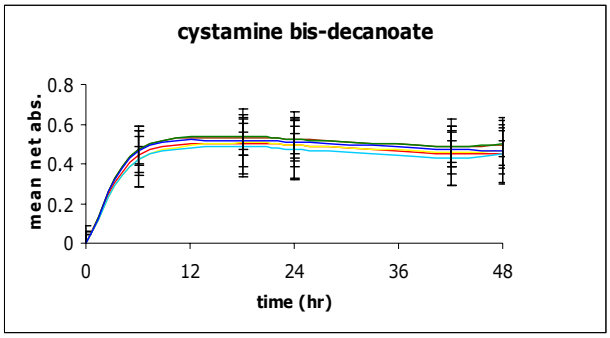


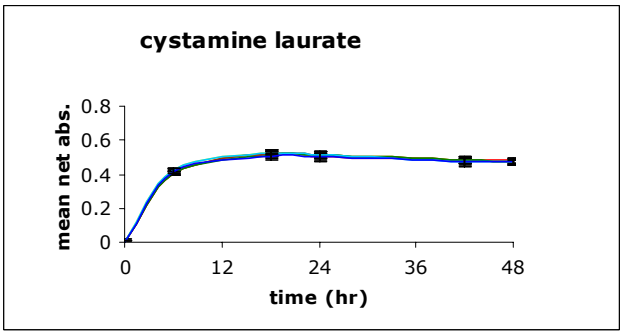


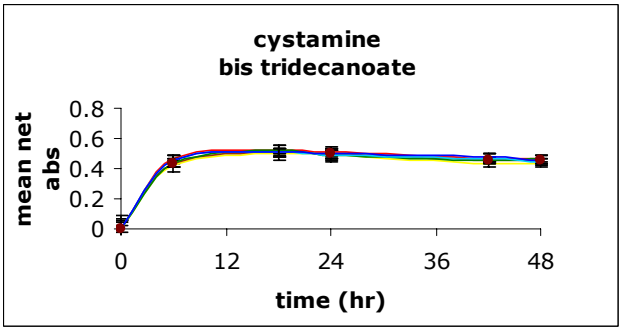


Saturated compounds:

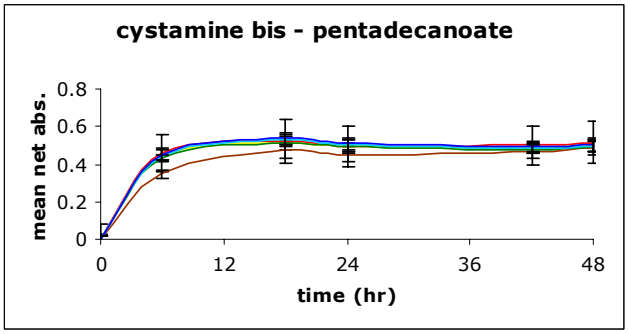
Key: 0 µM = red ; 2 µM = yellow ; 10 µM = brown
20 µM = green ; 40 µM = sky blue ; 50 µM = blue

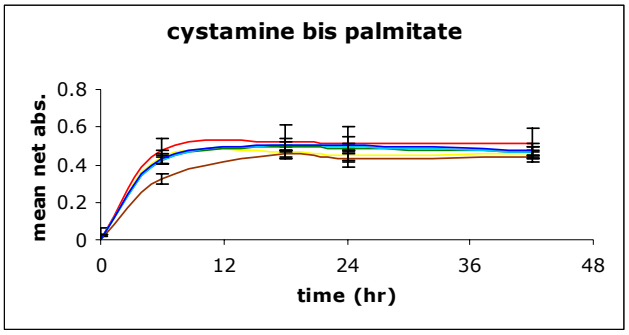


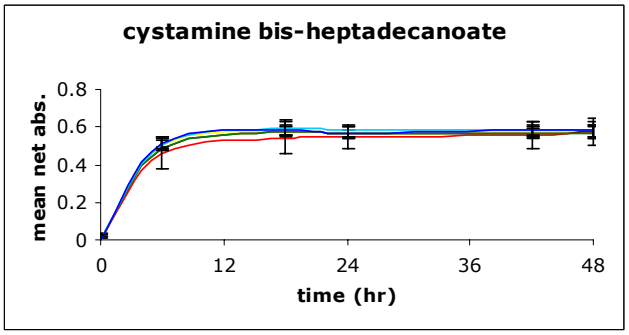




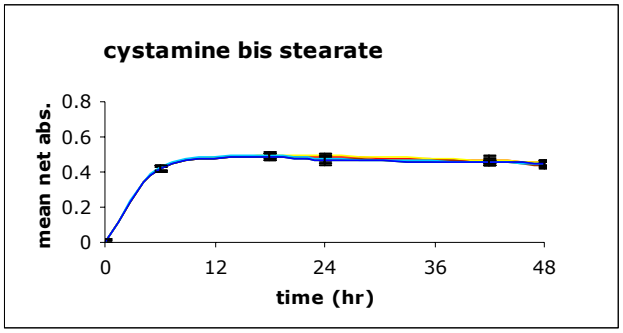
Key: 0 µM = red ; 2 µM = yellow ; 10 µM = brown
20 µM = green ; 40 µM = sky blue ; 50 µM = blue

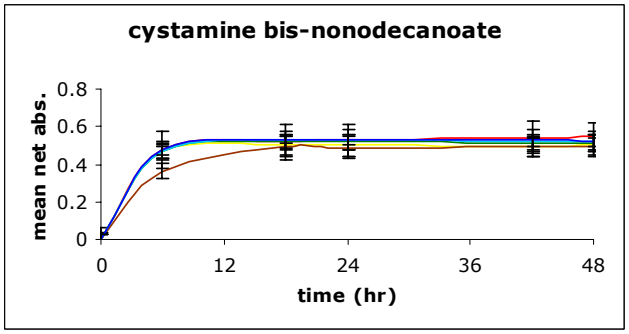






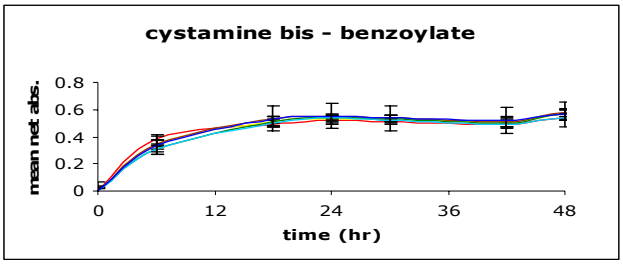
Key: 0 µM = red ; 2 µM = yellow ; 10 µM = brown
20 µM = green ; 40 µM = sky blue ; 50 µM = blue

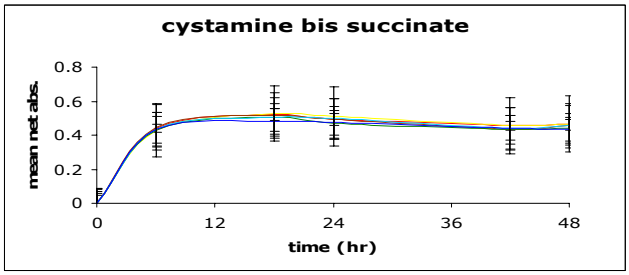




Additional derivatives of cystamine:

Key: 0 µM = red ; 2 µM = yellow ; 10 µM = brown
20 µM = green ; 40 µM = sky blue ; 50 µM = blue





Appendix 3

Table 2 Solubility in Ethanol and Effect of Temperature

Prodrug Concentration (μM)	**Solubility in Ethanol and Effect of Temperature**					
	Cystamine Stearate	Cystamine Palmitate	Cystamine Laurate	Cystamine Penta-decanoate	Cystamine Decanoate	Cystamine Elaidioate
100	Cloudy suspension at 21°C, 37°C and 44°C.	Soluble. Clear solution at 21°C, 37°C and 44°C.	Soluble. Clear solution at 21°C, 37°C and 44°C.	Soluble. Clear solution at 21°C, 37°C and 44°C.	Soluble. Clear solution at 21°C, 37°C and 44°C.	Soluble. Clear solution at 21°C, 37°C and 44°C.
200	Cloudy suspension at 21°C, 37°C and 44°C	Soluble. Clear solution at 37°C and 44°C. Cloudy solution at 21°C.	Soluble. Clear solution at 21°C, 37°C and 44°C.	Soluble. Clear solution at 21°C, 37°C and 44°C.	Soluble. Clear solution at 21°C, 37°C and 44°C.	Soluble. Clear solution at 21°C, 37°C and 44°C.
300	Cloudy suspension at 21°C, 37°C and 44°C.	Part soluble. Precipitation at 21°C. Clear solution at 37°C and 44°C.	Soluble. Clear solution at 21°C, 37°C and 44°C.	Soluble. Clear solution at 21°C, 37°C and 44°C.	Soluble. Clear solution at 21°C, 37°C and 44°C.	Part soluble. Clear solution at 37°C and 44°C. Precipitation occurred at 21°C.
500	-	Part soluble. Precipitation at 21°C and 37°C Clear solution at 44°C	Soluble. Clear solution at 21°C, 37°C and 44°C.	Soluble. Clear solution at 21°C, 37°C and 44°C.	Soluble. Clear solution at 21°C, 37°C and 44°C.	Part soluble. Precipitation at 21°C and 37°C. Clear solution 44°C.

Prodrug Concentration (µM)	**Solubility in Ethanol and Effect of Temperature**					
	Cystamine Stearate	Cystamine Palmitate	Cystamine Laurate	Cystamine Penta-decanoate	Cystamine Decanoate	Cystamine Elaidioate
1000	-	Precipitation at 21°C, 37°C and 44°C.	Part soluble. Precipitation at 21°C. Clear solution at 37°C and 44°C.	Part soluble. Precipitation at 21°C. Clear solution at 37°C and 44°C.	Soluble. Clear solution at 21°C, 37°C and 44°C.	Part soluble. Precipitation at 21°C. Clear solution 37 and 44°C.
1500	-	-	Part soluble. Precipitation at 21°C. Clear solution at 37 and 44°C.	Part soluble. Precipitation at 21°C and 37°C. Clear solution 44°C.	Soluble. Clear solution at 21°C, 37°C and 44°C.	Part soluble. Precipitation at 21°C and 37°C. Clear solution at 44°C.
2000	-	-	Part soluble. Precipitation occurred at 21°C and 37°C.Clear solution at 44°C.	Part soluble. Precipitation at 21°C. Clear solution at 37° and 44°C.	Soluble. Clear solution at 21°C, 37°C and 44°C.	Cloudy suspension at 21°C, 37°C and 44°C.
2500	-	-	-	Part soluble. Precipitation at 21°C. Clear solution at 37°C.	Soluble. Clear solution at 21°C, 37°C and 44°C.	-

Concentration (µM)	**Solubility in Ethanol and Effect of Temperature**					
	Cystamine Stearate	Cystamine Palmitate	Cystamine Laurate	Cystamine Penta-decanoate	Cystamine Decanoate	Cystamine Elaidioate
2500 (continue)	-	-	-	Clear solution at 44°C.	-	-
3000	-	-	-	-	Soluble. Clear solution at 21°C, 37°C and 44°C.	-
3500	-	-	-	-	Soluble. Clear solution at 21°C, 37°C and 44°C.	-
4000	-	-	-	-	Soluble. Clear solution at 21°C, 37°C and 44°C.	-