CRF grant 'Identification and characterization of the lysosomal transporter involved in cysteamine-mediated cysteine efflux'

Progress Report – July 2009

Research update from the Paris group

Persons working on the project:
- Christine Anne, Inserm scientist
- Cécile Debacker, CNRS technician
- Karolina Marciniak undergraduate student
- Bruno Gasnier, PI

Azita Sharifi (graduate student) participated in some experiments.

Background and objectives

Biochemical studies have previously shown that cysteamine depletes accumulated cystine from cystinotic lysosomes by reacting with cystine to form a mixed cysteine-cysteamine disulfide, which then exits lysosomes through an unidentified lysosomal arginine/lysine transporter named 'system c'.

Our aim is to identify this transporter to help improving current cysteamine treatments.

We previously identified several members of the SLC7 transporter family as potential system c transporters based on immunofluorescence data and, for functionally characterized members, on cysteamine-cysteine competition experiments. CAT2A, CAT2B, an orphan CAT-like protein named SLC7A14 and, to a lesser extent, CAT1 appeared as good system c candidates. However, it is still unknown which of these transporters is (or are) actually involved in cysteamine-mediated cystine lysosomal depletion.

Our efforts during the past 6 months were devoted to developing a reliable in vitro assay to address this issue. The next step would be to decrease or increase expression of the candidates in the in vitro model (using siRNA-mediated silencing or cDNA transfection) to examine whether these manipulations alter the kinetics of lysosomal cystine depletion.

Results

1) Studies on whole cells

As mentioned in our previous report, we first tried to develop a cystine depletion assay in whole cells using readily transfectable cell lines (HeLa, HEK293). Conditions mimicking cystinosis

were artificially created by overloading lysosomes from intact cells with labeled cystine using a tritiated ester precursor ([^{3}H]cystine dimethylester). Subsequently cells were treated with cysteamine to deplete the lysosomal [^{3}H]cystine pool. This approach was abandoned because it proved difficult to get a reproducible cysteamine depletion effect from one experiment to another, presumably because the lysosomal [^{3}H]cystine pool was often masked by other ^{3}H-labeled metabolites.

We thus decided to pursue this approach on cystinotic fibroblasts from patients. In these experiments, the [^{3}H]cystine dimethylester precursor was used merely as a radiotracer to follow the fate of lysosomal cystine, as previously done in the seminal experiments from J.A. Schneider and W.A. Gahl and their co-workers. It should be mentioned that, in contrast with these early studies, we could not use ^{35}S to label the lysosomal cystine pool because of the impossibility to perform radioactive esterification reactions in an academic laboratory and of the prohibitive cost of ordering repeated custom syntheses of a compound labelled with a short half-lived isotope. We thus ordered a custom-made tritiated precursor ([^{3}H]cystine dimethylester) from a company. Cystinotic fibroblasts were kindly provided by C. Antignac.

A representative experiment is shown in the Figure below. As expected, cysteamine triggered efflux of radioactivity from cystinotic fibroblasts pretreated with [^{3}H]cystine dimethylester. However, on one hand, this effect was limited to ~20% of the total amount radioactivity and, on the other hand, substantial efflux of radioactivity was observed in the absence of cysteamine. These disappointing findings presumably reflect the fact that the pool of labeled metabolite is much broader and complex when cystine dimethyl ester is labeled with ^{3}H rather than ^{35}S. For instance, the spontaneous efflux of radioactivity observed in the absence of cysteamine may reflect an efflux of [^{3}H]methanol molecules generated in the lysosome after cleavage of the ester bonds (such a process would be 'silent' if we could have used a ^{35}S-labeled precursor).

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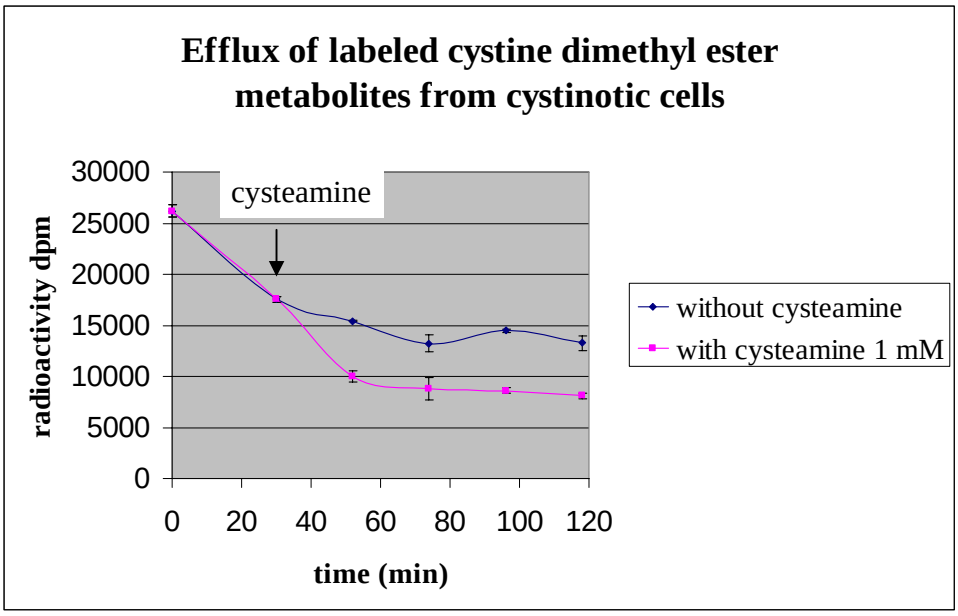
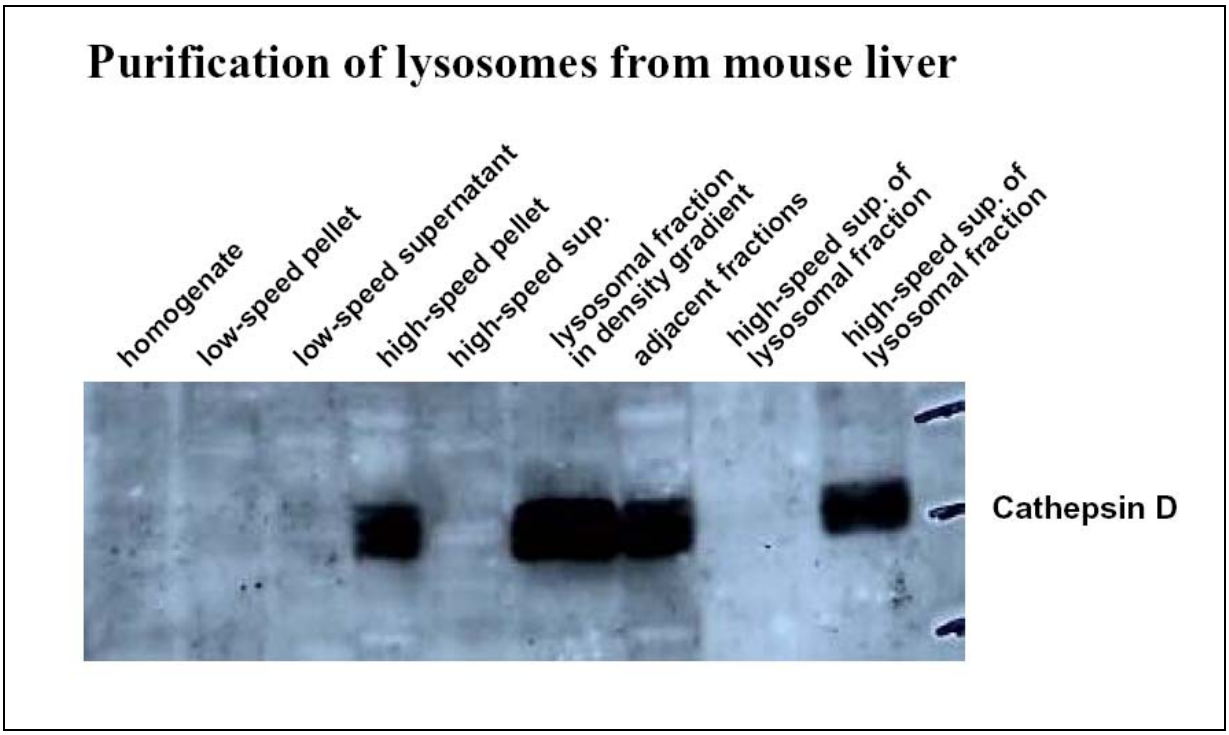


We tried ordering a custom-made methyl ester precursor exclusively tritiated on the cystine moiety to increase the selectivity of lysosomal cystine metabolic labeling. However, only one

company committed itself to doing so, but failed and thus ended up synthesizing a generally-labeled tritiated precursor. It should be mentioned that the partial masking of the lysosomal [^{3}H]cystine pool with other [*G*-^{3}H]cystine dimethylester metabolites not only reduced the relative amount of radioactivity sensitive to the cysteamine treatment, but also hindered measurement of the *time course* of cysteamine-induced radioactivity depletion (see above Figure), a parameter needed to determine accurately the effect of system c candidate silencing or overexpression in subsequent experiments. For these reasons, the whole cell approach was definitely abandoned.

2) Studies on purified lysosomes

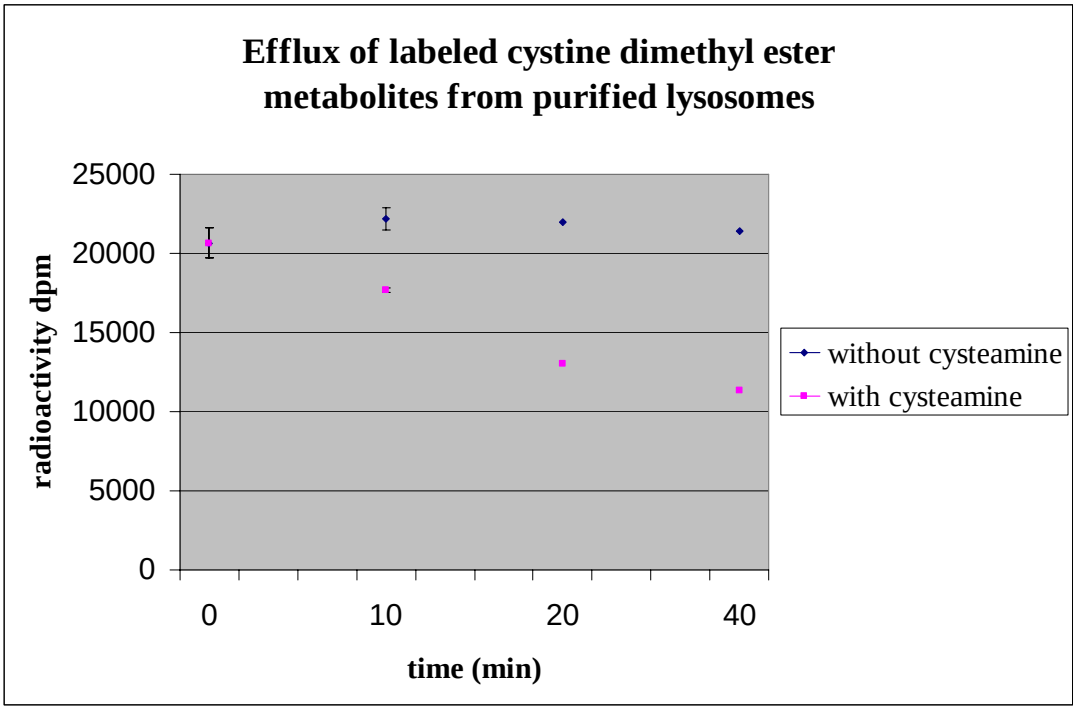
To overcome these difficulties, we decided to simplify our assay and measure cystine depletion on purified lysosomes. Because higher amounts and purer lysosomes can be purified from tissue rather than cultured cells, this approach was initiated using mouse liver as starting material. The purification protocol was defined after a 3-day stay of C. Anne in the laboratory of Prof. M. Jadot in Namur (Belgium), an known expert in lysosome purification. The figure bellow illustrates the enrichment of a lysosomal marker, cathepsin D, during a representative purification made in Paris.



We then used these purified lysosomes to assay the cystine-depleting effect of cysteamine by radiotracer flux experiments. Lysosomes were incubated with [*G*-^{3}H]cystine dimethylester for 60 min, washed, and the radioactivity egress was measured in the presence or absence of 1 mM cysteamine using a filtration assay. It should be mentioned that we used liver tissue from wild-type mice or rats in this set of experiments. However, the concentration of [*G*-^{3}H]cystine

dimethylester was adjusted to overload endogenous cystinosin without triggering an osmotic burden to the lysosome (cleavable permeant esters are sometimes used to disrupt lysosomes).

Representative data are shown below for an experiment performed on wild-type mouse liver lysosomes. As illustrated, in the absence of cysteamine, the amount of radioactivity present in lysosomes remained stable over a time span of 40 min whereas, in contrast, the presence of cysteamine induced a clear efflux of radioactivity with a half-life of ~15 min. After longer incubations, cysteamine depleted radioactivity with a maximal extent of 50% the initial amount (not shown), presumably because the [^{3}H]methanol generated in the lysosome by cleavage of the methyl ester bonds (and representing half of all H atoms) remained associated with the lysosomal membrane in our experimental conditions.



Next objectives

We now have a clear, and reproducible (not shown), biochemical assay to measure the cystine-depleting effect of cysteamine. Our next goal is to repeat to apply this assay on lysosomes purified from CAT-defective mice to identify the transporter involved in this process. Because the above assay requires a high number (20) of 12-week old mice for a single experiment, we are currently trying to scale down our protocol.

Progress Report Mainz Group

(Ellen Closs, Jean-Paul Boissel and Isabel Jaenecke)

In the third grant period, we

- further characterized the transport properties of the chimera between the human cationic amino acid transporter hCAT-2A and the putative lysosomal "system c" transporter SLC7A14, referred to as hCAT2/A14-BK (see scheme in Fig. 1).
- investigated the subcellular distribution of the chimera
- made new constructs to localize protein regions responsible for lysosomal localization
- made new constructs to try to force the protein into the plasma membrane

Characterization of the transport properties of the hCAT2/A14-BK chimera

Our preliminary results had shown that the hCAT2/A14-BK chimera (Fig. 1), containing the domain of SLC7A14 that corresponds to the so called "functional domain" of the CAT proteins, exhibits transport properties similar to the lysosomal transport system c in human skin fibroblasts. In addition, we found that SLC7A14 is prominently expressed in these cells (data not shown). This supports the notion that SLC7A14 may indeed represent the lysosomal system c.

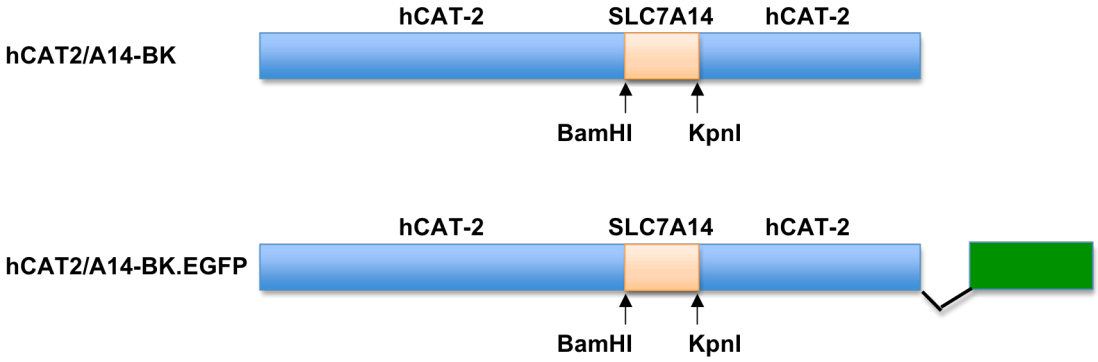


Figure 1: Scheme of the chimera between hCAT-2 and SLC7A14
The upper scheme shows the protein domain of SLC7A14 (red) introduced into the hCAT-2A backbone (blue). The lower scheme shows the same chimera fused to EGFP at its C-terminus.

Further experiments revealed the following transport properties of the chimera:

1) In agreement with system c, the chimera exhibited a high apparent K_M for L-arginine that was similar to the apparent K_M of hCAT-2A and significantly higher than the apparent K_M of hCAT-1.

2) Transport by the chimera was moderately trans-stimulated, in contrast to hCAT-2A which showed no trans-stimulation and hCAT-1 which exhibited a stronger trans-stimulation.

3) Transport by the chimera was pH-dependent, similar to hCAT-2A and different from hCAT-1, which was pH-independent. We further showed, that the corresponding chimeras hCAT-2/1 and hCAT-1/2A (containing the "functional domain" of hCAT-1 in the hCAT-2

backbone and vice versa) exhibit pH-independence and -dependence, respectively. This provides evidence that the pH-dependence is indeed determined by the "functional domain". SLC7A14 is thus likely to be also pH-dependent. As discussed by Pisoni et al. (1), the observed pH-dependence (lower activity at pH 5 compared to pH 7.5) points rather to a function of SLC7A14 as an import then an export transporter for cationic amino acids in lysosomes. We wondered if the pH-dependence might be altered in the absence of trans-substrate. However, in efflux experiments, the pH-dependence of the chimera as well as of hCAT-1 and hCAT-2A were the same in the absence or presence of trans-substrate.

4) Pisoni et al. identified cationic amino acid derivatives that interfere with transport by system c, but not sytem y^+ (1). In accordance with these observations, transport of the chimera, but not of hCAT-1, hCAT-2B and hCAT-3 (the system y^+ transporter expressed in skin fibroblasts) was inhibited by ε–trimethyl-L-lysine. However, the inhibition of the chimera was less pronounced than reported for system c (34% versus 50%). Transport by hCAT-2A (which is not expressed in skin fibroblasts) was inhibited to a similar extend as the chimera. The strongest inhibitor of system c identified by Pisoni et al. (1), α-N-methyl-L-arginine (synthesized for our experiments by Tocris Bioscience, Bristol, UK), did not inhibit the chimera nor CAT-1 or CAT-2A. Further experiments are necessary to find out if inhibition by α-N-methyl-L-arginine occurs at a protein region outside the functional domain.

Protein expression and plasma membrane localization of the hCAT2/A14-BK chimera

We found that the transport activity of the chimera was very variable in different experiments. Thus, the expression period (between cRNA injection and experiment) was extended to 3 days, instead of 2 days usually used for the hCAT proteins. To assess the amount of total and cell surface protein expressed in the oocytes, EGFP was fused to the C-terminus of the chimera (see Fig. 1) and the expression of this fusion protein was compared to the expression of a corresponding hCAT-2A.EGFP fusion protein. Fluorescent micrographs showed exclusive plasma membrane localization of both fusion proteins. In addition, Western blot analysis of total and biotinylated cell surface proteins revealed similar expression of both fusion proteins in the plasma membrane.

Protein regions responsible for lysosomal localization

Our preliminary results showed that the hCAT-2/SLC7A14-BK chimera was the only chimera that mediated transport of cationic amino acids across the plasma membrane of oocytes. To assess protein expression and plasma membrane localization, a series of EGFP fusion proteins was constructed that will be characterized in the next funding period (Fig. 2). These include SLC7A14 truncated at the N- and C-termini (which are extended compared to the hCAT proteins), as well as partial deletion of two large loops between predicted transmembrane domains 9/10 and 10/11 (to mimic the structural architecture of hCAT-2A, and the other CAT proteins).
Furthermore, fusion constructs between SLC7A14, 4F2, and DsRed were constructed to try to force plasma membrane targeting of SLC7A14 (Fig. 3).

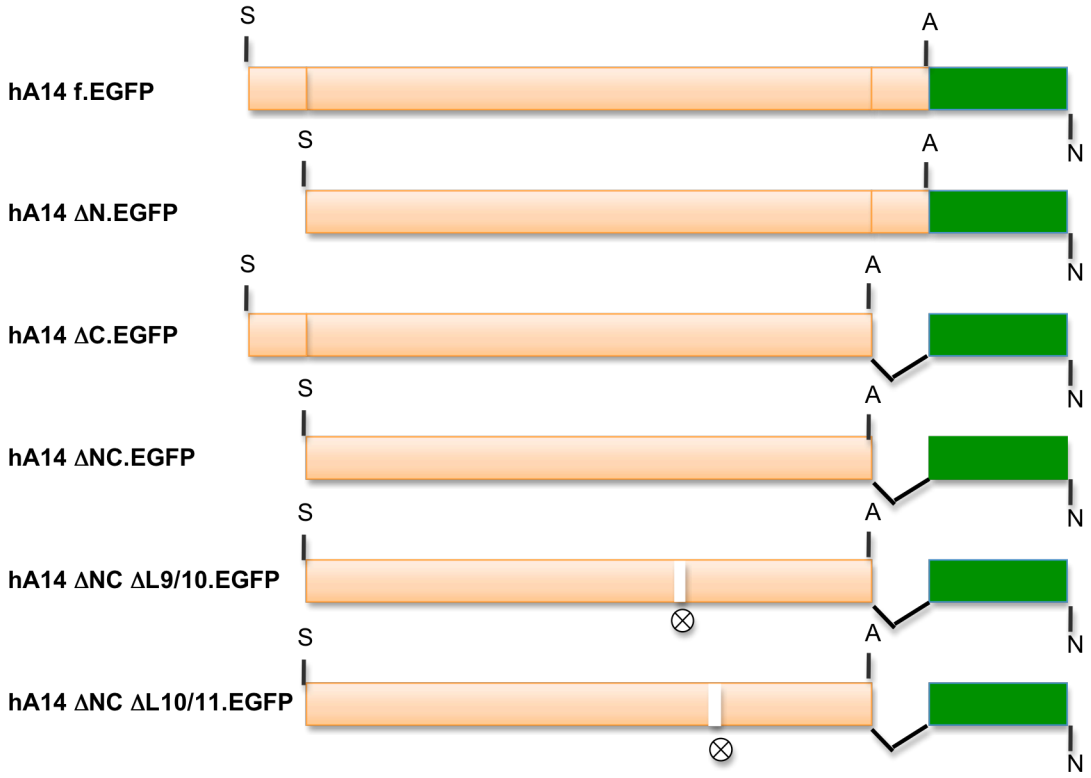


Figure 2: Schemes of SLC7A14-EGFP-fusion proteins to be tested
The schemes show fusion proteins between EGFP (green) and SLC7A14 (red) with truncated N- and C-termini as well as partial deletion of two large loops between predicted transmembrane domains 9/10 and 10/11 as indicated. These will be tested for plasma membrane localization and transport activity

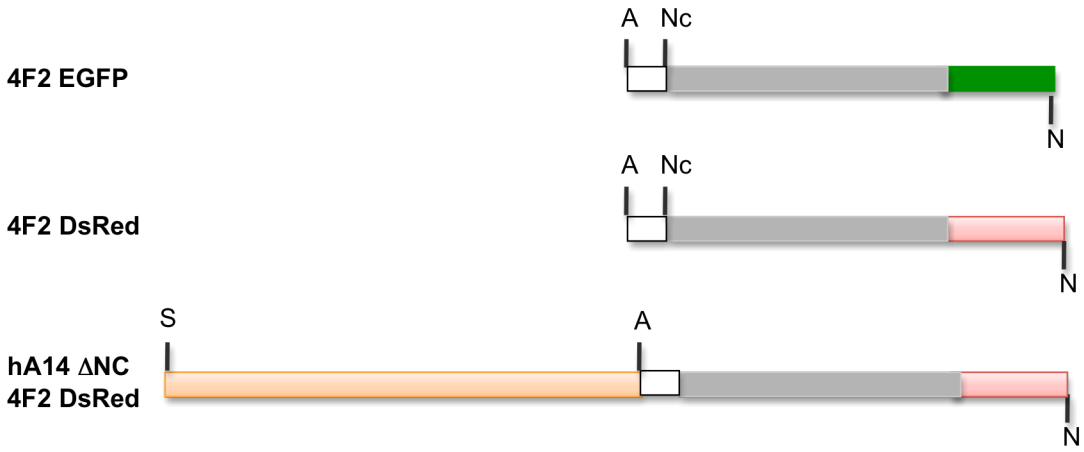


Figure 3: Schemes of SLC7A14-4F2-EGFP-fusion proteins to be tested
The schemes show fusion proteins between the membrane protein 4F2 (CD98, gray) and either EGFP (green) or DSRed (dark red) that will be used as marker for membrane localization. A fusion protein between SLC7A14 with truncated N- and C-termini (red), 4F2 (gray) and DsRed (dark red) was constructed to try to force SLC7A14 into the plasma membrane.

Research focus for the next 6 months:

Characterization of new SLC7A14-EGFP and SLC7A14-4F2-EGFP fusion proteins

The new fusion proteins will be tested in the oocyte expression system for membrane localization (using either fluorescent microscopy or biotinylation of surface proteins) and transport activity.

Measurements of lysosomal transport

In collaboration with the group of Bruno Gasnier in Paris, we are in the process to set up a protocol to measure lysosomal transport directly. Using human cell lines with stable overexpression of SLC7A14.EGFP, this will enable us to study the function of the protein in is native environment.

Reference

1. Pisoni, R.L., J.G. Thoene, R.M. Lemons, and H.N. Christensen. 1987. Important differences in cationic amino acid transport by lysosomal system c and system y+ of the human fibroblast. *J Biol Chem* 262:15011-15018.