Cell-based phenotypic drug-screening identifies luteolin as candidate therapeutic for nephropathic cystinosis

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SIGNIFICANCE STATEMENT

Nephropathic cystinosis (NC) is the most frequent form of cystinosis, a rare lysosomal storage disease (LSD). Kidneys are damaged at early stages of the disease, which presents as renal Fanconi syndrome. The only treatment for cystinosis is oral administration of cysteamine that does not correct the Fanconi syndrome and cannot prevent progression to end-stage kidney disease. This paper describes the identification, through an unbiased approach, of luteolin as a potential treatment for NC. Data obtained in cells derived from cystinotic patients or mouse models, and preclinical studies in cystinotic zebrafish indicate that luteolin corrects aspects of the cystinotic phenotype that are linked to renal Fanconi syndrome. These results provide new perspectives for the treatment of NC and other renal LSDs.
Cell-based phenotypic drug-screening identifies luteolin as candidate therapeutic for nephropathic cystinosis

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Running title: Luteolin for cystinosis

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SIGNIFICANCE STATEMENT

Nephropathic cystinosis (NC) is the most frequent form of cystinosis, a rare lysosomal storage disease (LSD). Kidneys are damaged at early stages of the disease, which presents as renal Fanconi syndrome. The only treatment for cystinosis is oral administration of cysteamine that does not correct the Fanconi syndrome and cannot prevent progression to end-stage kidney disease. This paper describes the identification, through an unbiased approach, of luteolin as a potential treatment for NC. Data obtained in cells derived from cystinotic patients or mouse models, and preclinical studies in cystinotic zebrafish indicate that luteolin corrects aspects of the cystinotic phenotype that are linked to renal Fanconi syndrome. These results provide new perspectives for the treatment of NC and other renal LSDs.

ABSTRACT

Background. Cystinosis is a lysosomal storage disease caused by mutations in the CTNS gene, which encodes for cystinosin, a lysosomal cystine transporter. Defective cystine transport leads to intralysosomal accumulation and crystallization of cystine. The most severe phenotype is nephropathic cystinosis (NC) that manifests during the first months of life with renal Fanconi syndrome. The cystine-depleting agent cysteamine significantly delays symptoms, but cannot prevent progression to end stage kidney disease, and does not treat the Fanconi syndrome, suggesting the involvement of pathways that are unrelated to lysosomal cystine accumulation. Among these, recent data indicate that lysosome-mediated degradation of autophagy cargoes is compromised in cystinosis.

Methods. We performed a high-throughput screening based on an in-cell ELISA assay to identify drugs that reduce the levels of the autophagy-related protein p62/SQSTM1 in cystinotic proximal tubular epithelial cells. The selected hit was therefore tested in preclinical and cell-based model of the disease.
**Results.** Of the 46 positive hits, luteolin was selected based on its efficacy, safety profile and similarity to genistein, which we previously showed to ameliorate other cystinotic cell aspects. Our data show that luteolin improves the lysosome-autophagy degradative pathway, is a powerful antioxidant and has anti-apoptotic properties. Moreover, luteolin stimulates endocytosis and improves the expression of the endocytic receptor megalin.

**Conclusions.** Our data show that luteolin improves defective pathways of cystinosis and may represent a treatment for NC and other renal lysosomal storage diseases.
INTRODUCTION

Nephropathic cystinosis (NC) (MIM 219800) is an autosomal recessive lysosomal storage disease (LSD), caused by severe mutations in the CTNS gene, which encodes cystinosin, a ubiquitously expressed lysosomal cystine-symporter.\(^1\) \(^2\) Defective lysosomal transport of cystine leads to intracellular accumulation and crystallization in all organs.\(^3\) Notably, kidneys, and in particular proximal tubular epithelial cells (PTCs), are affected at early stages of the disease, leading to early onset Fanconi syndrome and inappropriate urinary losses of water, amino-acids, phosphate, bicarbonate, glucose, and low-molecular-weight proteins. Chronic renal failure develops progressively, and most patients reach end stage kidney disease around 10 years of age if not treated with cysteamine. With time, cystine accumulation in other organs causes extra-renal complications, such as hypothyroidism, diabetes mellitus, and myopathy, among others.\(^4\)

Cysteamine is a cystine-depleting agent allowing clearance of cystine from lysosomes and currently represents the only specific treatment for cystinosis. If started early, it significantly delays progression of renal failure, and prevents or delays other complications of the disease.\(^3\) However, cysteamine does not cure cystinosis and does not stop developing the renal Fanconi syndrome. Moreover, patient compliance is often limited by side effects.\(^5\) Hence, efforts have been made to develop new therapies. A first approach has been to develop modified cysteamine molecules or to identify other cystine depleting agents with a better therapeutic profile.\(^6\) Hematopoietic stem cells transplantation has recently emerged as a potential therapy, with promising results in mice.\(^7\) Alternatively, new treatments could target pathways that are not responsive to cysteamine. In particular, mechanisms leading to PTC dysfunction are probably not solely related to cystine accumulation, since renal Fanconi syndrome is not improved by cysteamine. In this respect, recent studies have identified several defects, including enhanced apoptosis,\(^8\)-\(^11\) mitochondrial dysfunction,\(^12\),\(^13\) oxidative stress,\(^14\)-\(^17\) aberrant autophagy,\(^18\)-\(^20\) endo-lysosomal dysfunction,\(^21\),\(^22\) and decreased expression of megalin and cubilin.\(^21\),\(^23\) Among these, altered autophagy probably plays a pivotal role. Accumulation of the autophagy substrate p62/SQSTM1 has been described in human PTCs and in kidney biopsies,
suggesting impaired autophagic flux. Recently, it has been shown that lysosomal dysfunction in primary PTCs obtained from Ctns−/− mice contributes to defective autophagy-mediated clearance of damaged mitochondria. In this hypothesis, defective autophagy, which is unrelated to cystine accumulation, would represent an important target to identify new treatments.

Generally big pharmaceutical company research neglects rare diseases, since the high cost of research and development is not recovered. A potential approach to shorten the timeline for drug discovery and reducing costs is to find new indications for existing drugs. This strategy, defined as “drug repurposing”, takes advantage of the known activities of many drugs approved for human use.

Herein, we used a drug repositioning strategy combined with high-throughput screening (HTS) to identify molecules that reduce the accumulation of p62/SQSTM1 in cystinotic PTCs and restore normal autophagy. Among several positive hits, luteolin emerged as the most interesting candidate. Additional studies showed that this molecule has a good safety profile, improves the lysosome-mediated degradation of the autophagy cargoes, restores lysosomal distribution, stimulates endocytosis in cystinotic PTCs. These results were further validated in-vivo on a previously established zebrafish model of cystinosis.

These insights offer new opportunities for developing treatment for cystinosis and other LSDs.
METHODS

Cell culture and reagents

Conditionally immortalized proximal tubular epithelial cells (ciPTCs), from healthy donor and cystinotic patients were obtained from Radboud University Medical Center, Nijmegen, The Netherlands and cultured as described in Wilmer et al.26 We used ciPTCs bearing the classical homozygous -57kb deletion (CTNS−/− ciPTC). Human cystinotic fibroblasts were kindly provided by laboratorio di Diagnosi Pre e Postnatale delle Malattie Metaboliche, Istituto G. Gaslini, Italy. Fibroblasts were cultured as described.27

Lymphocytes obtained by venous blood from healthy donors and cystinotic patients were collected in preservative-free anticoagulant tubes and then layered onto Histopaque®-1077 solution. After centrifugation at 400 x g for 30min at room temperature, lymphocytes and other mononuclear cells were collected at the plasma/Histopaque®-1077 interface, washed with PBS (Euroclone) and transferred in RPMI (Euroclone) supplemented with 10% FBS (Gibco), 100 units/ml penicillin and 100mg/ml streptomycin (Euroclone). Cells were grown in a humidified atmosphere with 5% CO₂ at 37°C. mPTCs derived from age- and gender-matched Ctns−/− and wild type littermates (C57BL/6 background). Mice were maintained under temperature and humidity-controlled conditions with 12-h light/12-h dark cycles with free access to appropriate standard diet in accordance with the institutional guidelines of National Institutes of Health Guide for the Care and Use of Laboratory Animals. The kidneys were harvested from wild type and Ctns−/− mice (16 weeks) and were taken to generate primary cultures of mPTCs, as previously described.21 Luteolin was purchased from Xi’an, Shaanxi (China), Hanks’ Balanced Salt solution (HBSS), for starvation experiments from Euroclone, TNFα for apoptosis induction, from Peprotech, and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).
RNA extraction, quantitative real-time PCR and Western blotting.

RNA extraction, quantitative real-time PCR and Western blotting are described in supplementary materials.

Fluorescence assays

Fluorescence assays and microscopy analysis are described in supplementary materials.

Screening of the Prestwick Chemical Library in ciPTCs

The Prestwick Chemical Library was purchased from Prestwick Chemical (Illkirch-Graffenstaden, France) and consisted of a collection of 1200 off-patent small molecules, 95% of which are approved for human use (FDA, EMA and other agencies). Twenty-four h after seeding in 96 well plates, each molecule of the library was added to the ciPTCs at final concentration of 10μM, using the epMotion ® 5075 automated pipetting system.

We included 8 wells with Ctrl ciPTCs as positive control, and 8 wells with CTNS⁻/⁻ ciPTCs as negative, in column 1 and 12 of each plate, respectively, both treated with dimethyl sulfoxide (DMSO) 0.1% v/v, the vehicle in which compounds are dissolved. After 24h, we evaluated p62/SQSTM1 protein levels, normalized for number of cells, spectrophotometrically, by in-Cell ELISA assay (in Cell-ELISA color detection kit, ThermoFisher Scientific), according to manufacturer’s instructions. The values of p62/SQSTM1 under treatment with each drug of the library were normalized respect to the average of controls (p62/SQSTM1 level in CTNS⁻/⁻ ciPTCs) of the same plate.

Measurement of intracellular and mitochondrial reactive oxygen species (ROS)

Cellular ROS generation was detected by CellROX® Deep Reagent (C10422, ThermoFisher Scientific) and by CellROX® Green Reagent (C10444, ThermoFisher Scientific), while mitochondrial ROS were detected by MitoSOX ™ Red Mitochondrial Superoxide Indicator (M36008,
ThermoFisher Scientific), according to the manufacturer’s instructions. Protocol details are described in supplementary materials.

**Endocytosis assays**

HA-Meg4 binding assay and BSA uptake were performed as described in supplementary materials.

**Fish maintenance and breeding**

*Ctns*−/− and wild type adult zebrafish were raised at 28.5°C, on a 14/10-hour light/dark cycle under standard aquaculture conditions. After mating, every 20–30 fertilized embryos were transferred per well of a 6-well plate having approximately 5ml of clean egg water (Instant Ocean Sea Salts, 60μg/ml) and methylene blue (0.5ppm). Embryos were incubated at 28.5°C in the dark and the medium was refreshed daily. Drugs were dissolved in the swimming water with the required concentrations starting from 48h post fertilization (hpf). Every day the debris was removed, and dead embryos were sorted out and counted. Survival was monitored over the first 144hpf. Hatching rates were calculated in surviving embryos at 48, 72 and 96hpf, while deformity rates were evaluated at 96hpf. All larval experiments were performed between the 3rd and the 6th days post fertilization (dpf). Animal care and experimental procedures were conducted in accordance with the ethical committee guidelines for laboratory animal experimentation at KU Leuven and the reference European directive (DIRECTIVE 2010/63/EU) on the protection of animals used for scientific research.

**Evaluation of apoptosis in zebrafish larvae**

Cell death was evaluated by Caspase-3 enzyme activity, as described in supplementary materials.
Immunohistofluorimetric staining of larval renal proximal tubules for megalin, early endosomal antigen-1 (EEA1) and Ras-related protein-11 (Rab11)

Five dpf wild type and ctns-/- zebrafish larvae (either non-treated or treated with 100µM luteolin) were fixed and stained for megalin, EEA1 and Rab11 antibody, as described in supplementary materials.

Statistical analysis

To monitor the performance of the screening, we used the $Z'$-factor statistical parameter. $Z'$-factor was evaluated by using the formula $Z' = 1 - 3 \times (\sigma_p + \sigma_n) / |\mu_p - \mu_n|$, where $\sigma_p$ and $\sigma_n$ are the standard deviations of positive or negative sample, and $\mu_p$ and $\mu_n$ represent the averages. GraphPad Prism software was used for all statistical analyses. The results are expressed as means ± SEM and they are representative of at least three independent experiments, unless otherwise specified in the figure legends. Differences between experimental groups were evaluated using one-way analysis of variance (ANOVA) followed by Bonferroni when appropriate. When only two groups were compared, unpaired or paired two-tailed Student’s t-tests were used for numerical data, while Fisher's exact test and Chi-square test were used for categorical data as appropriate. Significant differences were given as *p<0.05, **p<0.01 or ***p<0.001.
RESULTS

Cell-based drug screening for molecules that reduce p62/SQSTM1 levels

p62/SQSTM1 protein levels were analyzed in ciPTCs obtained from a healthy donor (Ctrl) and a cystinotic patient with a homozygous -57kB CTNS deletion (CTNS-/- ciPTC). In-Cell ELISA experiments showed significantly increased levels in cells derived from cystinotic patient, compared to those obtained from healthy donor (Figure 1A). mRNA levels were unchanged (Supplementary figure 1A). Increased accumulation of p62/SQSTM1 in cystinosis was further confirmed by Western blot (Figure 1B). Notably, treatment with 100μM cysteamine for 24h did not modify significantly p62/SQSTM1 levels (Figure 1B), despite significant decrease in cystine levels (Supplementary figure 1B), indicating that the accumulation of this protein is not directly related to lysosomal cystine accumulation.

After inhibition of mRNA synthesis for 24h with actinomycin D, p62/SQSTM1 protein levels remained higher in CTNS-/- ciPTCs, compared to Ctrl, indicating that the accumulation was not related to higher mRNA transcription (Supplementary figure 1C-D). Moreover, p62/SQSTM1 levels decreased significantly in Ctrl after 4h starvation in HBSS, while they remained higher in CTNS-/- ciPTCs (Supplementary figure 1E), suggesting impaired autophagic flux.

This was further supported by bafilomycin and by chloroquine treatments that increased p62/SQSTM1 protein levels in Ctrl but had no effect on the already elevated levels of CTNS-/- ciPTCs (Supplementary figure F-G). Of notice, increased levels of p62/SQSTM1 are specific of ciPTCs and are not increased in other cell types, such as lymphocytes and fibroblasts obtained from cystinotic patients (Supplementary figure 1H).

Quantitative in-Cell ELISA was performed to identify drug that decrease p62/SQSTM1 levels in cystinotic cells. The Z’-factor value for the in-cell ELISA assay was 0.62±0.06, demonstrating the robustness of the assay. Screening was performed in triplicates on CTNS-/- ciPTCs, using the Prestwick chemical library (Figure 1C). Using a threshold for positivity of 50% reduction in p62/SQSTM1 levels above levels in Ctrl cells, we identified 46 positive compounds (Supplementary
After discarding molecules with a low therapeutic range and with a restricted topical use, six molecules were retained for additional studies (Figure 1D-E). Among these, luteolin emerged as a particularly interesting candidate.

All together these results showed that degradation of the autophagic substrate p62/SQSTM1 is compromised in ciPTCs. Drug-screening revealed luteolin as the best candidate to rescue this phenotype.

**Luteolin has a favorable toxicity profile**

The effect of luteolin on p62/SQSTM1 levels was analyzed in *CTNS<sup>−/−</sup>* ciPTC using a dose-response inhibition model, which revealed a half maximal inhibitory concentration (IC<sub>50</sub>) of 127µM (Supplementary figure 2A). The toxicity profile was also analyzed on ciPTCs showing an IC<sub>50</sub> of 170µM and 189µM for Ctrl and *CTNS<sup>−/−</sup>* ciPTC, respectively (Supplementary figure 2B). In mPTCs, no substantial changes in cell viability after 24h were noticed at 20µM and 10-15% cell death was observed at 50µM (Supplementary figure 2C). Further, luteolin toxicity profile was tested *in-vivo* using the *ctns<sup>−/−</sup>* zebrafish. Survival was analyzed for 96h after exposing wild type and *ctns<sup>−/−</sup>* embryos to different concentrations of luteolin in the swimming water at 48hpf. Luteolin had no significant effect on embryos survival at concentrations of 100 and 500µM (Figure 2 A-B). Moreover, luteolin improved delayed hatching and deformity rates of mutated embryos (Figure 2 C-F).

These data showed that luteolin has a good safety profile in preclinical model of cystinosis.

**Luteolin rescues autophagic defects in cystinosis**

Cell immunofluorescence (Figure 3A-B) and Western blot (Supplementary figure 3A-B) further confirmed the effects of luteolin on p62/SQSTM1 levels in cystinotic ciPTCs (Figure 3A) and mPTCs (Figure 3B).
To further analyze autophagy, we measured LC3-II levels in mPTCs (Figure 3C). By Western blotting LC3-II levels, in Ctns−/− mPTCs were significantly increased compared to wild type animals. Luteolin significantly decreased LC3-II levels. Thereafter, we transfected CTNS−/− ciPTCs with LC3 cDNA expressing the tandem RFP/GFP fluorescent tag. In this system, green fluorescence is quenched when vesicle pH decreases, indicating fusion with the lysosomal compartment. As shown in Figure 3D, the percentage of yellow positive structures (autophagosomes) was significantly higher in cystinotic cells, while the percentage of RFP-only positive structures (autolysosomes) was significantly lower. This was restored by luteolin treatment (Figure 3D).

These data demonstrated that the autophagic flux is impaired in ciPTCs and mPTCs. Luteolin efficiently rescued autophagic defects in these cells.

**Luteolin protects from apoptosis**

Increased susceptibility to apoptosis is a known phenotype of cystinotic cells. We therefore investigated the possible protective role of luteolin against apoptosis in Ctrl and CTNS−/− ciPTCs. Cells were pre-treated with luteolin for 6h and apoptosis was subsequently induced by exposure to 30 ng/ml tumor necrosis factor alpha (TNFα) and 2.5μg/ml actinomycin D for 18h as previously described. Levels of cleaved poly-ADP-ribose polymerase (PARP-1) (Figure 4A) and cleaved caspase-3 (Supplementary figure 4A) were assessed by Western blotting. As shown, CTNS−/− ciPTCs had increased activation of both PARP-1 and caspase-3 after apoptotic stimuli, compared to Ctrl ciPTCs.

Luteolin pre-treatment significantly protected from apoptosis in stimulated cells.

Similarly, apoptosis was tested in ctns−/− zebrafish by measuring caspase-3 enzyme activity in larval homogenates (Figure 4B). Increased apoptotic rate was apparent at 5 dpf. Exposure to 100 and 500μM luteolin reduced apoptosis significantly in ctns−/− larvae.
These data confirmed increased susceptibility of cystinotic cells and zebrafish model to apoptosis and highlighted the ability of luteolin to rescue this phenotype.

**Luteolin ameliorates oxidative stress in PTCs**

Thereafter, we investigated the antioxidant effect of luteolin in ciPTCs using the CellROX® probe. Basal cellular ROS levels were 2.2-fold higher in \( CTNS^{-/-} \) ciPTC, compared to Ctrl cells. As expected, ROS levels increased significantly (7.5-fold) in \( CTNS^{-/-} \) ciPTCs after exposure to \( tert \)-butyl-hydroperoxide (Figure 5A). Pre-treatment with 50\( \mu \)M luteolin for 24h decreased cellular ROS generation by approximately 80% in both Ctrl and \( CTNS^{-/-} \) ciPTCs. These findings were further confirmed under basal conditions on mPTCs by fluorescence microscopy. The CellROX signal decreased to control levels upon treatment with 50\( \mu \)M luteolin for 24h (Figure 5B). We further monitored mitochondrial oxidative stress, by MitoSOX™ probe. No significant difference was found in basal mitochondrial ROS levels in \( CTNS^{-/-} \) ciPTC, compared to Ctrl cells, nevertheless, 50\( \mu \)M luteolin neutralized mitochondrial ROS production (Supplementary figure 4B). In \( CTNS^{-/-} \) ciPTC, the exposure to \( tert \)-butyl-hydroperoxide increased mitochondrial ROS levels (3.0-fold), revealing a higher sensitivity of \( CTNS^{-/-} \) ciPTCs. Pre-treatment with 50\( \mu \)M luteolin for 24h decreased mitochondrial ROS generation by approximately 80% in both Ctrl and \( CTNS^{-/-} \) ciPTCs (Supplementary figure 4B).

All together these data indicated that luteolin significantly rescued the increased sensitivity to oxidative stress of both human and murine cystinotic PTCs.

**Luteolin improves lysosome dynamics and processing in cystinotic PTCs**

Lysosome-associated membrane protein 1 (LAMP1) positive structures were analyzed by fluorescent microscopy, after exposure to 50\( \mu \)M luteolin for 24h. As shown in Figure 6A, distribution of enlarged perinuclear lysosomes in cystinotic cells were prevented by luteolin
treatment, re-establishing a pattern of lysosomal distribution similar to control cells. This was observed both in human ciPTCs and in mPTCs. More specifically, after luteolin treatment the fraction of perinuclear lysosome decreased on average from 54% to 40% in $\text{CTNS}^{-/-}$ ciPTCs (Figure 6A) and from 44% to 22% in mPTCs (Figure 6B). In addition, cathepsin-D expression was analyzed in mPTCs. The proteolytic generation of the 32kDa mature cathepsin D was lower in $\text{Ctns}^{-/-}$ mPTCs, compared to wild type cells. Luteolin treatment restored the expression of mature cathepsin-D (Figure 6C).

Serial block face scanning electron microscopy of the proximal region of the pronephros of $\text{ctns}^{-/-}$ zebrafish larvae revealed an increase in the size of lysosomes compared to wild type, as reported previously.²⁵ Treatment of the $\text{ctns}^{-/-}$ larvae with luteolin (100$\mu$M for 72h) partially rescued this phenotype, with a significant reduction in lysosome size compared to untreated larvae, although the lysosomes were still larger than those of wild type larvae (Figure 7).

These data showed that luteolin reverts impaired lysosomal dynamics and morphology in cystinosis.

**Luteolin restored megalin expression and enhanced protein reabsorption**

We then evaluated surface expression of the endocytic receptor megalin, *in-vitro* and *in-vivo*. We first used the HA-Meg4 mini-receptor construct (an accepted surrogate for full-length megalin), transiently expressed in ciPTCs. Fluorescence intensity was measured after cell incubation on ice with anti-HA antibody. $\text{CTNS}^{-/-}$ ciPTCs showed a significant reduction in relative HA-Meg4 surface levels compared to $\text{Ctrl}$ cells (0.43±0.04 in $\text{CTNS}^{-/-}$ compared to 1.18±0.10 in $\text{Ctrl}$ cells), which was significantly improved by luteolin treatment (0.73±0.07; Figure 8A). Expression of endogenous megalin was also assessed in $\text{Ctrl}$ and $\text{CTNS}^{-/-}$ ciPTCs. Fluorescence intensity was measured after staining with anti-megalin antibody. As shown in Supplementary figure 5A, $\text{CTNS}^{-/-}$ ciPTCs showed significant reduction of expression of endogenous megalin compared to $\text{Ctrl}$ cells, which was rescued by luteolin treatment. *In-vivo* studies were carried out on 5dpf $\text{ctns}^{-/-}$ zebrafish larvae. As shown in Figures 8 B-D, megalin expression was grossly altered in mutated animals, with most of the signal...
located in subapical and intracellular vesicular structures. After treatment with luteolin, megalin expression was largely restored in the brush border membranes of ctns−/− larvae tubular cells (Figure 8 B-D), while it was unchanged in wild type larvae (data not shown). Further, luteolin treatment partially restored in ctns−/− larvae defective expression of the early endosomal marker EEA1 and of Rab11, which marks preferentially recycling endosomes (Supplementary figure 5B). Receptor-mediated endocytosis of albumin was investigated in mPTCs. We found a reduction (~85%) of fluorescent BSA uptake in Ctns−/− mPTCs, compared to wild type cells (Figure 8E). Pre-treatment with 50µM luteolin for 24h increased BSA uptake in both wild type and Ctns−/− mPTCs. These data showed that trafficking of megalin to the plasma membrane is compromised in cystinosis. Luteolin efficiently rescued localization of megalin to the plasma membrane and improved reabsorption of endocytic cargoes.

**DISCUSSION**

NC is caused by lack of functional cystinosin, a cystine/H⁺ symporter that allows lysosomal cystine efflux. Cysteamine is currently the only therapy available for this disease, allowing clearing of cystine from lysosomes.29 For many years, enhanced cell oxidation has been considered a cornerstone of the disease.14, 16, 17, 30, 31 Although results of various studies are difficult to harmonize, several experimental data show impaired response of cystinotic cells to oxidative stimuli.17, 32 A leading hypothesis for many years has been that the Cys/CySS redox state unbalance, secondary to cystine retention in lysosomes, impairs glutathione synthesis.31, 32, 33 This hypothesis was substantiated by lower glutathione levels in several cystinotic cell lines,14, 33, 34, 35 and by improvement of glutathione levels after treatment with N-acetyl-cysteine or with cysteamine,31, 33 which replete the cytoplasmic pool of cysteine from the external milieu or from the lysosomal compartment, respectively. More recent data however, offer an alternative or complementary explanation, by demonstrating that lysosomal dysfunction in cystinosis compromises autophagy-mediated clearance of damaged mitochondria in PTC, which are a potent source of ROS.20 In this context, part of the beneficial effects
of luteolin, a natural flavonoid present in various fruits and vegetables,\textsuperscript{36} may be explained by its antioxidant properties,\textsuperscript{37} similarly to the positive effects on renal lesions reported in \textit{Ctns}^{-/} mice treated with other mitochondria–targeted ROS scavengers, such as MitoTempo\textsuperscript{®} and mitoquinone.\textsuperscript{20,38} The antioxidant effects of luteolin are related to its capacity to scavenge oxygen and nitrogen reactive species.\textsuperscript{39} In ROS-insulted primary cultured neurons for example, luteolin has been shown to decrease endogenous free radicals, to improve mitochondrial membrane potential and to ameliorate mitochondrial viability.\textsuperscript{40}

A second distinctive feature of cystinotic cells is their propensity to undergo apoptosis when stimulated with intrinsic or extrinsic stimuli.\textsuperscript{8} The underlying mechanisms have been attributed to abnormal cysteinylation of protein kinase C\textgreek{d} by cystine released from disrupted lysosomes.\textsuperscript{9} Importantly, cysteamine restores normal susceptibility to apoptosis,\textsuperscript{8} in theory by preventing lysosomal cystine accumulation and lysosomal rupture.\textsuperscript{9} We observed that luteolin also reduces sensitivity to apoptosis in pre-clinical models of cystinosis. However, luteolin has no effect on cystine storage (Supplementary figure 1B). Cysteamine has been used for the treatment of cystinosis since the 1980’s and has substantially ameliorated the outcome of patients. Despite its efficacy in lowering lysosomal cystine however, cysteamine cannot cure the disease, and does not prevent some aspects of the renal disease, namely the renal Fanconi syndrome.\textsuperscript{41} These observations suggest the involvement of other cellular pathways in the pathophysiology of NC, in addition to the engulfment of the lysosomal compartment with cystine.\textsuperscript{42,43} Therefore, research in this field is increasingly focusing on new therapies aimed at correcting cell phenotypes that are not restored by cysteamine. These include, for example, abnormal lysosomal distribution,\textsuperscript{22} LAMP2 mislocalization,\textsuperscript{19} mTORC1 dysregulation,\textsuperscript{44} impaired chaperon-mediated autophagy,\textsuperscript{19} or abnormal level of p62/SQSTM1 in renal tubular cells.

Defective lysosome-autophagy degradation pathways have been recently linked to epithelial dysfunction\textsuperscript{20} and to the renal Fanconi syndrome, often the first clinical manifestation of the disease.
The present work shows that luteolin ameliorates several of the above-mentioned features. This molecule (3’,4’,5,7-tetrahydroxyflavone) was identified by drug repositioning, an approach that is particularly valuable for rare diseases because it shortens the duration and cost of drug development. To this end, we selected the Prestwick chemical library that contains 1200 molecules, most of which have been licensed for human use, to perform a HTS based on a semi-automated in-cell ELISA assay. Our phenotypic target was p62/SQSTM1 in PTCs, which was used as a marker of altered autophagy. This choice was based on the recent demonstration that autophagy is heavily impaired in cystinotic cells, a feature that is unresponsive to cysteamine as shown in our experiments. In addition, our results show that luteolin restores the distribution of lysosomes in cystinotic PTC, improves megalin expression, and enhances endo-lysosomal trafficking processes. In immune cells, luteolin has also been shown to inhibit cytokine expression, NFkB activation, and TLR4 signaling at micromolar concentrations. The exact mechanisms by which luteolin exerts its polyhedral effects are incompletely understood.

Impaired autophagy is a key feature in lysosomal storage diseases, including cystinosis, and is secondary to altered delivery of cargoes to the engulfed lysosomal compartment. Sansanwal et al. had already observed increased levels of p62/SQSTM1 in cystinotic renal PTCs, and in renal biopsies obtained from cystinotic patients. Importantly, this finding is not ubiquitous and is restricted to some cells, including renal proximal tubular cells. For example, Napolitano et al. observed normal macro-autophagy and normal autophagic flux in cystinotic fibroblasts. Similarly, we also observed comparable p62/SQSTM1 levels in lymphocytes and fibroblasts obtained from cystinotic patients and from control subjects. These data suggest that lack of cystinosin determines tissue and cell-specific effects.

Our data show that luteolin significantly improves the autophagic flux in cystinotic PTCs, resulting in decreased p62/SQSTM1 accumulation. Luteolin has also been shown to increase the number of autolysosomes and to promote autophagy in human cutaneous squamous carcinoma cells. These effects were blocked by chloroquine, an inhibitor of the last steps of the autophagic process.
Similarly, luteolin was also found to protect mice from traumatic brain injury, an effect that was attributed to its anti-inflammatory properties, but was also associated with increased number of LC3-positive cells and decreased levels of p62/SQSTM1.

In addition, the distribution of lysosomes normalized after treatment with luteolin in our cell models, most likely because of improved functionality, as indicated by mature cathepsin D levels. Recently, Janssens et al. showed that inhibition of megalin-mediated endocytosis efficiently prevents accumulation of cystine and delays progression of kidney disease in Ctns-/- mice. In these experiments, ablation of megalin was used as a strategy to block uptake of proteins, whose degradation further increases cystine contents in Ctns-/- mice. In cystinosis, defective expression of megalin has been well documented, including in reports by the same authors. Lack of megalin expression reflects impaired intracellular trafficking and membrane protein recycling, similarly to what is observed in other proximal tubular defects characterized by low molecular weight proteinuria, such as Lowe syndrome and Dent disease. In addition, lack of megalin expression may also indicate cell dedifferentiation, as shown in mPTCs by Raggi et al. Therefore, and irrespective of the underlying mechanism, restored expression of megalin after in-vitro and in-vivo treatments in our experimental settings indicates that luteolin may be efficient in treating the renal Fanconi syndrome of cystinosis and is not at odds with the report by Janssens et al.

In conclusion, luteolin was selected among positive hits of a HTS aimed at improving autophagy because of its additional effects on oxidation, apoptosis and inflammation, all of which are altered in cystinosis. Moreover, luteolin belongs to the flavonoid family, similarly to genistein, which we recently showed to ameliorate lysosomal cystine content and lysosomal compartment distribution in cystinosis, probably through stimulation of the TFEB pathway. Flavonoids have been proposed for the treatment of other LSDs and luteolin has been shown to have a very good safety profile in humans. Taken together, our results indicate that luteolin may be valuable for the treatment of cystinosis, in particular to ameliorate aspects of the disease that are not improved by cysteamine and establish a proof-of-principle to begin preclinical studies in-vivo.
AUTHOR CONTRIBUTIONS


All authors drafted, revised and approved the final version of the paper.

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Supplementary materials and methods

Supplementary references

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Figure 2

A

Survival in wild type embryos

B

Survival in ctns−/− embryos

C

Hatching rate in wild type

D

Hatching rate in ctns−/−

E

Deforomefity rates in wild type

F

Deforomefity rates in ctns−/−
**Figure 3**

A. **p62/SQSTM1 - Hoechst**

B. **p62/SQSTM1 - DAPI**

C. LC3-I and LC3-II protein levels in wild type and Ctns<sup>−/−</sup> cells treated with luteolin.

D. **RFP-GFP-LC3**

% Autophagosomes and % Autolysosomes with and without luteolin treatment.
Figure 5

A. CellROX intensity plot showing the effect of TBHP (300 μM, 1 hour) and luteolin (50 μM) treatment on wild type and Ctnnb1−/− MEFs. 

B. Fluorescence intensity (mean per cell) of CellROX in wild type and Ctnnb1−/− MEFs treated with luteolin (50 μM) and/or TBHP (300 μM, 1 hour).
Figure 6

A

LAMP1/Hoechst

Ctrl

CTNS<sup>-/-</sup>

B

LAMP1/DAPI

wild type

CTNS<sup>-/-</sup>

C

luteolin

CtsD

β-actin

Mature CtsD / β-actin (relative to wild type)
Figure 7

Wild type vs. ctns^−^ untreated and treated with luteolin. The graph shows the comparison of lysosome diameter (μm) between wild type and ctns^−^ under untreated and treated conditions. The data is represented with a scatter plot and the significance levels are indicated with asterisks (*** for p-value ≤ 0.001 and n.s. for non-significant).
Figure 8

A) Ctrl and CTNS−/−

B) Megalin and EEA1

C) Relative cellular expression (%)

D) Relative EEA1 expression (%)

E) untreated and luteolin

Legend:

- Ctrl
- CTNS−/−
- untreated
- luteolin

Statistical significance:

- ** p < 0.01
- *** p < 0.001
Figure legends

Figure 1: Cell-based phenotypic drug screening for compounds that reduce p62/SQSTM1 levels
(A) p62/SQSTM1 protein levels were analyzed in ciPTCs from a healthy donor (Ctrl) and a cystinotic patient (CTNS^-/-) by in-cell ELISA. Levels of p62/SQSTM1 were normalized by janus green cell stain. Data are shown as fold change of untreated Ctrl. Mean values ± SEM from 7 independent experiments are reported. Student’s t test ***p<0.001 relative to untreated Ctrl. (B) Western blotting and densitometric analysis of p62/SQSTM1 and β-actin levels in Ctrl and CTNS^-/- ciPTC after treatment with 100µM cysteamine for 24h. Histogram shows levels of p62/SQSTM1 normalized to those of β-actin and reported as relative to untreated Ctrl ratios. Data are shown as mean ± SEM from 3 independent experiments. Student’s t test *p<0.05; NS not statistically significant relative to untreated Ctrl and CTNS^-/- ciPTC. (C) p62/SQSTM1 levels were analyzed in CTNS^-/- ciPTCs after treatment with DMSO (vehicle, negative control) or 10µM of each drug of the Prestwick chemical library (red and blue dots). Ctrl cells were used as positive control (black dots). The threshold for significance (red dashed line) was set at 50% reduction in the relative increased p62/SQSTM1 levels in CTNS^-/- ciPTCs (blue line) compared to Ctrl cells (black line). This threshold highlights 46 positive hits (red spots). Levels of p62/SQSTM1 were normalized to janus green cell stain signal. Data are represented as mean value of 3 independent experiments. (D, E) Diagram represents process of selection of 6 candidate drugs. The screening yielded 46 positive hits, of which 9 drugs were excluded because they are registered for topical use only, 14 for severe side effects, and 17 because of their low therapeutic range.

Figure 2: Luteolin has a favorable toxicity profile and improves hatching rates and deformity rates in ctns^-/- zebrafish larvae. (A-B) Survival rates in wild type and ctns^-/- embryos and larvae after 0, 100 or 500 µM luteolin. Data are reported as mean ± SEM (n=3 independent experiments). (C-D) Hatching rates in surviving wild type and ctns^-/- embryos evaluated at 48, 72 and 96hpf with
0, 100 or 500µM of luteolin. (E-F) Deformity rates in wild type and ctns⁻/⁻ larvae after 0, 100 or 500µM luteolin. The total numbers of embryos evaluated for survival, hatching and deformity rates were 97 embryos per group of wild type and 350 embryos per group of ctns⁻/⁻ zebrafish. Luteolin was administered at 48hpf in all experiments dissolved in the swimming water with the specified concentrations. **p<0.01 and ***p<0.001 relative to the untreated ctns⁻/⁻ zebrafish using Fisher’s test.

Figure 3: Luteolin rescues autophagic defects in cystinosis. (A) Representative images of Ctrl and CTNS⁻/⁻ ciPTCs after treatment with 50µM luteolin for 24h. After fixing, cells were stained with anti-p62/SQSTM1 antibody (red) and Hoechst (nuclei, blue). Scale bar is 10µm. Graph represents number of p62/SQSTM1 positive structures per cells (n>70 cells from 3 independent experiments, one-way ANOVA followed by Bonferroni’s post hoc test, *p<0.01 and ***p<0.001 and NS not statistically significant relative to untreated Ctrl or CTNS⁻/⁻ cells). (B) Representative images of wild type and Ctns⁻/⁻ mPTC after treatment with 50µM luteolin for 24h. After fixing, cells were stained with anti-p62/SQSTM1 antibodies (red) and DAPI (nuclei, blue). Scale bar is 10µm. Graph represents number of p62/SQSTM1 structures per cell (n>214). One-way ANOVA followed by Bonferroni’s post hoc test, *p<0.01 and ***p<0.001 relative to untreated wild type or Ctns⁻/⁻ cells. (C) Western blotting and densitometric analyses of LC3 and β-actin protein levels in mPTCs after treatment with 50µM luteolin for 24h. Data are shown as mean ± SEM (n=3 independent experiments), Student’s t test *p<0.05; **p<0.01 and NS not statistically significant relative to untreated wild type or Ctns⁻/⁻ mPTC. (D) Representative images of Ctrl and CTNS⁻/⁻ ciPTCs transfected with RFP-GFP-LC3. After 24h from transient transfection, cells were left untreated or treated with 50µM luteolin for 24h. Scale bar is 10µm. GFP and RFP signals were analyzed by confocal microscopy. Percentage of autophagosomes (yellow vesicles) and percentage of autolysosomes (red-only vesicles) were calculated based on the ratio between the number of yellow and red-only puncta, respectively, and the total number of autophagosomes (number of yellow + red-only puncta). Data are reported as mean
SEM (n>70 cells from 5 independent experiments). One-way ANOVA followed by Bonferroni’s post hoc test, **p<0.01 and *p<0.05 relative to untreated Ctrl and CTNS⁻/⁻ ciPTCs.

**Figure 4: Luteolin reduces sensitivity to apoptosis in CTNS⁻/⁻ ciPTCs and in ctns⁻/⁻ zebrafish larvae.** (A) Western blotting and densitometric analysis of PARP-1 and β-actin protein levels in Ctrl and CTNS⁻/⁻ ciPTCs left untreated or pre-treated with 50µM luteolin for 6h. After luteolin pre-treatment, where indicated apoptosis was induced by 18h treatment with 30ng/ml TNF-α and 2.5µg/ml actinomycin D (actD). Histogram shows levels of cleaved PARP-1 normalized to those of β-actin and reported as relative to untreated Ctrl ratios. Data are shown as mean ± SEM from 3 independent experiments. Student’s t test *p<0.05; **p<0.01 and NS not statistically significant relative to untreated or TNF-α/actD treated Ctrl and CTNS⁻/⁻ ciPTCs. (B) Quantitation of caspase-3 enzyme activity (RLU/µg protein) in homogenates of 120 hpf wild type and ctns⁻/⁻ zebrafish larvae either untreated or treated with 5, 10, 50, 100 and 500µM luteolin. Twenty larvae were homogenized in each pellet and at least three replicates were performed per condition. Student’s t test *p<0.05; **p<0.01 and ***p<0.001 relative to untreated ctns⁻/⁻ condition.

**Figure 5: Luteolin treatment reduces oxidative stress in cystinotic cells.** (A) Ctrl and CTNS⁻/⁻ ciPTCs were left untreated or pre-treated with 50µM luteolin for 24h. Levels of cellular ROS were then analyzed both at basal conditions and after 2h exposure to 100µM tert-butyl- hydroperoxide (TBHP) by CellRox staining and flow cytometry. Diagram shows CellRox mean intensities normalized to those of untreated Ctrl. Data are presented as mean ± SEM from 9 independent experiments. Student’s t test *p<0.05; **p<0.01 and ***p<0.001 relative to untreated or TBHP treated Ctrl and CTNS⁻/⁻ ciPTCs. (B) Representative images and quantification of CellRox (green)
staining in wild type and Ctns<sup>-/-</sup> mPTCs under basal condition or treatment with 50µM luteolin for 24h. Scale bar is 10µm. CellRox fluorescence intensities per cell are reported; means and SEM are shown in red (n>100 cells, one-way ANOVA followed by Bonferroni’s post hoc test ***p<0.001 and NS not statistically significant relative to untreated wild type or Ctns<sup>-/-</sup> cells).

**Figure 6: Luteolin treatment rescues morphology and degradation capacities of lysosomes in cystinotic cells.** (A) Representative images of Ctrl and CTNS<sup>-/-</sup> ciPTCs left untreated or treated with 50µM luteolin for 24h. After fixing, cells were stained with anti-LAMP1 antibody (green) and Hoechst (nuclei, blue). Scale bar is 10µm. Fraction of perinuclear lysosomes was quantified in at least 5 randomly selected fields (red circles) per condition. Mean ± SEM are shown by column bars. One-way ANOVA followed by Bonferroni’s post hoc test, ***p<0.001 relative to untreated Ctrl or CTNS<sup>-/-</sup> ciPTCs. (B) Representative images of wild type and Ctns<sup>-/-</sup> mPTCs under basal condition or treatment with 50µM luteolin for 24h. After fixing, cells were stained with anti- LAMP1 antibody (red) and Dapi (nuclei, blue). Scale bar is 10µm. Fraction of perinuclear lysosomes was quantified in at least 5 randomly selected fields (red circles) per condition. Mean ± SEM are shown by column bars. One-way ANOVA followed by Bonferroni’s post hoc test, *p<0.05 and **p<0.01 relative to untreated wild type or Ctns<sup>-/-</sup> mPTCs. (C) Western blotting and densitometric analysis of cathepsin D (CtsD) and β-actin protein levels from wild type and Ctns<sup>-/-</sup> mPTCs, left untreated or treated with 50µM luteolin for 24h. Histogram shows levels of mature cathepsin D (33 kDa) normalized to those of β-actin and reported as relative to control wild type ratios (n=3 independent experiments). Student’s t test *p<0.05 and **p<0.01 relative to untreated wild type or Ctns<sup>-/-</sup> mPTCs.

**Fig. 7: Luteolin restores impaired morphology of lysosomes in the pronephros of ctns-/- larvae.**
Representative block face scanning EM images of the proximal tubule of 5dpf wild type and ctns<sup>-/-</sup> larvae after treatment without or with luteolin (100µM for 72h). Arrowheads indicate lysosomes
greater than 1.5µm diameter. Arrows indicate lysosomes less than 1.5 µm diameter. Scale bar is 5µm. Diameter of lysosomes was quantified in 5 randomly selected fields (n= 80 lysosomes from 4 tubules of 2 larvae/each experimental condition). Mann-Whitney U test ***p<0.001 and NS not statistically significant relative to untreated wild type or ctns−/− larvae.

**Fig. 8: Luteolin restores endocytic defects in cystinosis.** (A) Ctrl and CTNS−/− ciPTCs were transiently transfected with HA-Meg4. After 4-6h, transfection medium was replaced by full medium or 50µM luteolin for 24h. The cell surface exposure of HA–Meg4 was measured through binding at 4°C (cell surface HA–Meg4) of an anti-HA mouse antibody. The total amount of HA–Meg4 expressed was measured using an anti-HA rabbit antibody (total HA–Meg4) in permeabilized HA–Meg4 cells. The arrows indicate plasma membrane localization of HA-Meg4. Scale bar is 20µm. Histogram shows ratios of cell surface/total HA-Meg4 fluorescence intensity (n> 65 cells for each experimental condition). Student’s t test **p<0.01; ***p<0.001 and NS not statistically significant relative to untreated Ctrl and CTNS−/− ciPTCs. (B) Representative confocal images of the protein expression of the multiligand receptor megalin (green) and the early endosomal antigen-1 (EEA1, red) in wild type larvae, untreated ctns−/− larvae and luteolin (100µM) treated ctns−/− larvae. Scale bar is 5µm in all images. Quantitation of peri-luminal fluorescent intensity at the level of the proximal tubules of megalin (C, n=8 larvae/group) and EEA1 (D, n= 4 larvae/group). Relative fluorescence intensities in quantitation graphs were referred to the untreated ctns−/− group, which was considered as 100%. Student’s t test *p<0.05; **p<0.01 and ***p<0.001. (E) Representative images of BSA uptake (red) in wild type and Ctns−/− mPTCs under basal condition or treatment with 50µM luteolin for 24h. Scale bar is 10µm. Number of BSA positive structures per cell was quantified; mean and SEM are shown in red (n>300 cells, one-way ANOVA followed by Bonferroni’s post hoc test, ***p<0.001 relative to untreated wild type or Ctns−/− mPTCs).
SUPPLEMENTARY MATERIALS

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Supplementary figure 5: Luteolin treatment restores endocytic defects in CTNS⁻/- ciPTCs and ctns⁻/- zebrafish larvae.

Supplementary materials and methods

Supplementary references
Supplementary figure 1

A

B

C

D

E

F

G

H

ScholarOne support: 888-503-1050
Supplementary figure 2

A

B

C

Cell Viability (%)

Cell Viability (%)

Cell Viability (%)

luteolin concentration (log M)

luteolin concentration (log M)

luteolin concentration (μM)

wild type

CTNS−/−
Supplementary figure 3

A

B

for Peer Review

Journal of the American Society of NEPHROLOGY

ScholarOne support: 888-503-1050
Supplementary figure 4

A

<table>
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<tr>
<th></th>
<th>Ctrl</th>
<th>CTNS&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
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<td>+</td>
</tr>
<tr>
<td>Caspase-3</td>
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<td>β-actin</td>
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</table>

B

Graph showing comparative analysis of mRNA and protein levels.
Supplementary figure 5

A

B

Megalin

Rab11

Merge

wild type

cnts/-

cnts/-
luteolin

Relative Rab11 expression [%]

No treatment

No treatment

Luteolin (100µM)

wild type

cnts/-
Supplementary figure legends

Supplementary figure 1: Levels of p62/SQSTM1 are specifically increased in human cystinotic PTC. (A) p62/SQSTM1 mRNA levels were analyzed by real-time qPCR in ciPTCs from healthy donor (Ctrl) and cystinotic patient (CTNS⁻/⁻). Differences in p62/SQSTM1 mRNA levels between Ctrl and CTNS⁻/⁻ ciPTCs are not statistically significant (NS). Data are shown as mean ± SEM from 4 independent experiments. (B) Levels of cystine, normalized to mg of total proteins and reported as relative to untreated Ctrl, were measured in Ctrl and cystinotic CTNS⁻/⁻ ciPTCs after treatment without or with 100µM cysteamine or 50µM luteolin for 24h. Data are shown as mean ± SEM from 6 independent experiments. Student’s t test *p<0.05; **p<0.01 and NS not statistically significant relative to untreated Ctrl and CTNS⁻/⁻ ciPTCs. (C) p62/SQSTM1 levels were analyzed by in-Cell ELISA in Ctrl and CTNS⁻/⁻ ciPTCs after treatment with 2.5µg/ml actinomycin D for 24h. p62/SQSTM1 levels were normalized by janus green cell stain. Data are presented as mean ± SEM from 3 independent experiments, Student’s t test *p<0.05; **p<0.01 and NS not statistically significant relative to untreated Ctrl and CTNS⁻/⁻ ciPTCs.

Western blotting and densitometric analysis of p62/SQSTM1 and β-actin protein levels in Ctrl and CTNS⁻/⁻ ciPTCs after treatment with (D) 2.5µg/ml actinomycin D (actD) for 24h, (E) Hank’s salt (HBSS) for 4h, (F) 250nM bafilomycin A1 for 4h. Histograms show levels of p62/SQSTM1 normalized to those of β-actin and reported as relative to untreated Ctrl ratios. Data are shown as mean ± SEM from at least 4 independent experiments. Student’s t test *p<0.05; **p<0.01 and NS not statistically significant relative to untreated Ctrl and CTNS⁻/⁻ ciPTCs. (G) p62/SQSTM1 levels were analyzed by in-Cell ELISA in Ctrl and CTNS⁻/⁻ ciPTCs after treatment with 100µM chloroquine for 24h. p62/SQSTM1 levels were normalized by janus green cell stain. Data are presented as mean ± SEM from 4 independent experiments, Student’s t test *p<0.05 and NS not statistically significant relative to untreated Ctrl and CTNS⁻/⁻ ciPTCs. (H) Densitometric analysis of p62/SQSTM1 and β-actin protein levels in lymphocytes from 4 healthy donors (Ctrl) and 8 cystinotic patients (CTNS⁻/⁻).
Histogram shows levels of p62/SQSTM1 normalized to those of β-actin. Data are presented as mean ± SEM. Analysis of p62/SQSTM1 levels by in-Cell ELISA in fibroblasts from two healthy donors (Ctrl #1 and Ctrl #2) and two cystinotic patients (CTNS⁻/⁻ #1 and CTNS⁻/⁻ #2). Levels of p62/SQSTM1 were normalized by janus green cell staining and shown as mean ± SEM from 3 independent experiments, Student’s t test NS not statistically significant relative to Ctrl cells.

Supplementary table 1 List of the 46 best hits yielded by HTS

<table>
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<td>Benzbithine benzylpenicillin</td>
<td>Misoprostol</td>
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<td>Betahexol chloride</td>
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<td>Kanamycin A sulfate</td>
<td>Zalcitabine</td>
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</table>

Supplementary figure 2: Toxicity profile of luteolin. (A) p62/SQSTM1 levels were analyzed by in-Cell ELISA in CTNS⁻/⁻ ciPTCs after treatment with increasing doses of luteolin (0; 5; 10; 20; 40; 50; 80 and 100μM) for 24h. p62/SQSTM1 levels were normalized by janus green cell stain. Data are presented as mean ± SEM from 3 independent experiments. Half maximal inhibitory concentration...
(IC₅₀) obtained is 127μM. (B) Cell viability was measured by Presto blue staining in Ctrl (black line) and CTNS⁻/⁻ (red line) ciPTCs after 24h treatment with increasing doses of luteolin (4.5μM; 9μM; 18μM; 37μM; 75μM; 150μM; 300μM; 600μM; 1200μM). Values were normalized to those of untreated Ctrl or CTNS⁻/⁻ ciPTCs. Normalized values are reported as mean ± SEM from 3 independent experiments. Half maximal inhibitory concentration (IC₅₀) obtained is 170μM and 189μM for Ctrl and CTNS⁻/⁻ ciPTC, respectively. (C) Cell viability as measured by MTT assay in wild type and Ctns⁻/⁻ mPTCs after treatment with 0, 10, 20 and 50μM luteolin for 24h. Values were normalized to those of untreated wild type and Ctns⁻/⁻ PTC. Data are reported as mean ± SEM from 3 independent experiments. Student’s t test *p<0.05 and **p<0.01 relative to untreated wild type and Ctns⁻/⁻ PTC.

Supplementary figure 3: Treatment with luteolin reduces p62/SQSTM1 levels in both human and mouse PTCs. (A) Western blotting and densitometric analysis of p62/SQSTM1 and β-actin protein levels in Ctrl and CTNS⁻/⁻ ciPTCs after treatment with 50μM luteolin for 24h. Histogram shows levels of p62/SQSTM1 normalized to those of β-actin and reported as relative to Ctrl ratios. Data are shown as mean ± SEM (n=7 independent experiments), Student’s t test *p<0.05 and **p<0.01 relative to untreated Ctrl or CTNS⁻/⁻ ciPTCs⁻/⁻. (B) Western blotting and densitometric analysis of p62/SQSTM1 and β-actin protein levels in wild type and Ctns⁻/⁻ mPTCs after treatment with 50μM luteolin for 24h. Histogram shows levels of p62/SQSTM1 normalized to those of β-actin and reported as relative to wild type ratios. Data are shown as mean ± SEM (n=3 independent experiments), Student’s t test *p<0.05 and **p<0.01 and NS not statistically significant relative to untreated wild type or Ctns⁻/⁻.

Supplementary figure 4: Luteolin treatment reduces sensitivity to apoptosis and mitochondrial ROS in cystinotic cells. (A) Western blotting and densitometric analysis of caspase-3 and β-actin protein levels in Ctrl and CTNS⁻/⁻ ciPTCs left untreated or pre-treated with 50μM luteolin for 6h. After luteolin pre-treatment, where indicated apoptosis was induced by 18h treatment with 30ng/ml TNF-
α and 2.5µg/ml actinomycin D (actD). Histogram shows levels of cleaved caspase-3 normalized to those of β-actin and reported as relative to untreated Ctrl ratios. Data are shown as mean ± SEM from 4 independent experiments. Student’s t test *p<0.05; **p<0.01 and NS not statistically significant relative to untreated or TNF-α/actD treated Ctrl and CTNS−/− ciPTCs. (B) Ctrl and CTNS−/− ciPTCs were left untreated or pre-treated with 50µM luteolin for 24h. Levels of mitochondrial ROS were then analyzed both at basal conditions and after 2h exposure to 100µM tert-butyl-hydroperoxide (TBHP) by MitoSOX probe and flow cytometry. Diagram shows MitoSOX mean intensities normalized to those of untreated Ctrl. Data are presented as mean ± SEM from 4 independent experiments. Student’s t test *p<0.05; **p<0.01 and NS not statistically significant relative to untreated or TBHP treated Ctrl and CTNS−/− ciPTCs.

Supplementary figure 5: Luteolin treatment restores endocytic defects in CTNS−/− ciPTCs and ctns−/− zebrafish larvae. (A) Representative images of Ctrl and CTNS−/− ciPTCs after treatment with 50µM luteolin for 24h. After fixing, cells were stained with anti-megalin antibody (green) and Hoechst (nuclei, blue). Scale bar is 20µm. Relative fluorescence intensities per cell are shown in the graph (n>40 cells from 3 independent experiments for each condition). Mean ± SEM are shown in red. One-way ANOVA followed by Bonferroni’s post hoc test, *p<0.05; ***p<0.001 relative to untreated Ctrl or CTNS−/− ciPTCs. (B) Representative confocal images of the protein expression of the multiligand receptor megalin (green) and Ras-related protein-11 (Rab11, red) in wild type larvae, untreated ctns−/− larvae and luteolin (100µM) treated larvae. Scale bar is 5µm in all images. Quantitation of peri-luminal fluorescent intensity at the level of the proximal tubules of Rab11 (n=4 larvae/group). Relative fluorescence intensities in quantitation graphs were referred to the untreated ctns−/− group, which was considered as 100%. Student’s t test **p<0.01 and ***p<0.001.
SUPPLEMENTARY MATERIAL AND METHODS

Antibody

The following antibodies were used in this study for human cells experiments: rabbit anti-Caspase-3 (9662 Cell Signaling), mouse anti-p62/SQSTM1 (sc-28359 Santa Cruz Biotechnology), mouse anti-LAMP1 (sc-20011 Santa Cruz Biotechnology), rabbit anti-LC3 (NB100-2220 Novus), rabbit anti-megalin antibody (ab76969 Abcam), rabbit anti Poly [ADP-ribose] polymerase 1 (PARP-1) (9542 Cell Signaling), mouse anti-HA Tag (32-6700 Thermofisher), rabbit anti HA-Tag (3724 Cell signaling), mouse anti-β-actin (AM4302 Ambion).

The following antibodies were used for mPTC experiments: rabbit anti-LC3 (PM036, MBL), rabbit anti-p62/SQSM1 (PM045, MBL); goat anti-Cathepsin-D (sc-6486, Santa Cruz Biotechnology); mouse anti-β-actin (A2228, Sigma-Aldrich). The following antibodies were used for zebrafish experiments: rabbit anti-megalin (kindly provided by Michele Marino, University of Pisa, Italy) and goat anti-EEA1 (Santa Cruz Biotechnology, Dallas, TX, USA), mouse anti-Rab11 (BD Biosciences Pharmingen, San Jose, CA, USA).

RNA extraction and quantitative real-time PCR

Total RNA was extracted by TRIzol reagent (Ambion, Life Technologies, Foster, CA) and cDNA was synthesized using the Euro-Script RT-PCR kit (EuroClone, Milano, Italy) according to the manufacturer’s instructions. Quantitative PCR assays were performed by FAST STAR SYBR Green Master (Roche) using the following primers for SQSTM1 gene: Forward: 5’-CAGATGGAGTGGATAC-3’ and Reverse: 5’-CTGGAGTACCTGTA-3’. Gene expression data were determined using the 2^ΔΔCt method and normalized using human glyceraldehyde-3-phosphate dehydrogenase, (GAPDH) with following primers Forward: 5’-AGGTCAAGTCAACCGATT-3’; Reverse: 5’-GCCCAATACGACCAATCCG -3’.

Western blotting
Proteins were extracted lysing the cells in ripa buffer, containing proteases and phosphatases inhibitor (Thermo scientific), sonicated for 2min and centrifuged for 10min at 13,000rpm at 4°C. Protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). Proteins were separated under reducing condition by electrophoresis on 4-15% gradient gels (Bio-Rad) and immunoblotted onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk (cell signaling) diluted in Tris-buffered saline, 0.1% Tween 20 and incubated overnight with primary antibodies. Following the incubation with horseradish peroxidase secondary antibody conjugate IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoblots were developed with LiteAblot EXTEND (EuroClone, Milan, Italy) and acquired with the ChemiDoc XRS System (Bio-Rad). Quantification of relative density of each band normalized to β-actin, with Image Lab software (Bio-Rad).

**Immunofluorescence**

For immunofluorescence assay, ciPTCs were grown on glass coverslips, fixed with 4% paraformaldehyde (PFA) and permeabilized for 30min with phosphate-buffered saline containing 0.05% saponin, 0.5% bovine serum albumin, 50mM NH4Cl and incubated for 2h with appropriate primary antibody. After repeated washing with PBS, the slides were incubated for 30 min with the suitable fluorophore-conjugated Alexa secondary antibodies (Molecular probe). Nuclei were stained by Hoechst 33342, trihydrochloride, trihydrate (Invitrogen). At least 5 images per condition were acquired on a Nikon Eclipse E600 microscope (Nikon Instruments, Melville, NY) equipped with epifluorescence optics. p62/SQSTM1 number per cells was analyzed by a dedicated pipeline created with the open-source cell image analysis software CellProfiler™. LAMP1 distribution was evaluated with ImageJ software (National Institute of Health, Bethesda, MD), calculating total number of LAMP1 structure per cells and number of LAMP1 structures in defined peri-nuclear region (area within 10 µm of the nucleus).
For immunofluorescence, the mPTCs were fixed for 10min with 4% paraformaldehyde in PBS, quenched with 50mM NH4Cl and permeabilized for 30min in blocking buffer solution containing 0.1% Triton X-100 and 0.5% BSA dissolved in PBS. Subsequently, mPTCs were incubated overnight with the appropriate primary antibodies at 4°C. After repeated washing with PBS, the slides were incubated for 30min with the suitable fluorophore-conjugated Alexa secondary antibodies (Invitrogen), mounted with the Prolong Gold Anti-fade reagent, and analyzed by using a Leica SP8 confocal laser scanning microscope (Center for Microscopy and Image Analysis, University of Zurich). The quantitative cell image analyses were performed using the open-source cell image analysis software CellProfiler™. The pipeline “Cell/particle counting and scoring the percentage of stained objects” was used to score the numbers of LAMP1 and p62/SQSTM1 positive vesicles. The specific module “Measure-Object-Intensity-Distribution” was used to score the number of LAMP1 positive structures contained into perinuclear region (area within 10µm of the nucleus).

Determination of mature autophagosome

Tandem fluorescently tagged RFP-GFP-LC3 (ptfLC3), in which LC3 is expressed as a fusion protein with both GFP and RFP in tandem1 was a gift from Tamotsu Yoshimori (Addgene plasmid Cat # 21074). ciPTCs were transfected with ptfLC3 vector by Lipofectamine LTX and Plus Reagent (Invitrogen) according to the manufacturer’s instructions. After 16h of transient transfection, ciPTC were treated with 50µM luteolin (Xi’an, Shaanxi, China) for indicated time. After fixing, the glass coverslips were acquired by confocal microscopy using Olympus Fluoview FV1000 and FV10-ASW4.1 software (Olympus Corporation, Tokyo, Japan). Images were processed with ImageJ software (National Institute of Health, Bethesda, MD). The percentage of autophagosomes (yellow vesicles) and the percentage of autolysosomes (red-only vesicles) were calculated based on the ratio between the number of yellow and red-only puncta, respectively, and the total number of autophagosomes (number of yellow + red-only puncta).
Endocytosis assays

HA-tagged Meg4 plasmid and pcDNA3-RAP were kindly provided by Marzolo MP (Universidad Católica de Chile, Santiago, Chile). ciPTCs were transiently transfected with HA-Meg4 and pcDNA3-RAP a megalin chaperone, to facilitate the transport of newly synthesized Meg4 to the cell surface. After 4-6h, transfection medium was replaced by full medium or 50µM luteolin for 24h. Cells were serum starved for 2h at 37°C in serum-free DMEM F12, then washed twice in cold PBS with 1% BSA chilled to 4°C and incubated with the anti-HA rabbit antibody for 1h on ice. The unbound antibody was rinsed off with cold DMEM. After binding assays, the cells were fixed, permeabilized, and stained with an anti-HA mouse, followed by goat anti-mouse Alexa-Fluor-555 (to detect total levels of expressed HA-Meg4) and anti-rabbit Alexa-Fluor-488 antibody (to detect bound anti-HA antibody).

The endocytic capacity of mPTCs was examined by measuring albumin uptake. mPTCs, previously starved for 1h in HBSS, were incubated with 100µg/mL Alexa555-BSA (A34786, Thermo Fisher Scientific) diluted in cell culture medium without FBS supplementation, for 15min at 37°C. After washing three times in PBS and once in acid solution (150mM NaCl, 10mM acetic acid, pH 3.5), the mPTCs were fixed in 4% PFA for 10min (room temperature). After three times washing in PBS, mPTCs were counterstained with 1µM DAPI for 5min, mounted with the Prolong Gold Anti-fade reagent and processed for confocal microscopy.

Measurement of intracellular reactive oxygen species (ROS)

Generation of intracellular or mitochondrial ROS in ciPTC, were detected by CellRox® Deep Reagent (Thermo Fisher Scientific) or by MitoSOX™ Red Mitochondrial Superoxide Indicator (M36008, ThermoFisher Scientific), respectively. ciPTCs were untreated or pre-treated for 22h with 50µM luteolin, before addition of 100µM tert-Butyl hydroperoxide (Thermo Fisher Scientific). Two h later, the cells were pulsed with 2.5µM CellRox® Deep Reagent or 5µM MitoSOX™ and the
plate were incubated for 30 min at 37°C in the dark. Once washed twice with phosphate-buffered saline (PBS) and detached by trypsin-EDTA (Euroclone), the cells were immediately analyzed by flow cytometry BD FACSCanto II System; (BD Biosciences, San Jose, CA, USA). Ten thousand data points were collected for each sample and analyzed with FACSDiva software (BD Biosciences, San Jose, CA, USA). To detect ROS in mPTC, the cells were pulsed with 5 µM CellROX® Green Reagent (C10444, Thermo Fisher Scientific) for 10 min in live cell imaging at 37°C. After washing three times, the cells were subsequently analyzed by confocal microscopy. The fluorescence intensity was quantified by using ImageJ Shortcut software.

**Viability assays**

Cell viability assays in ciPTCs and in mPTCs treated with different concentration of luteolin, were performed by Presto Blue® reagent (Thermo Fisher Scientific) and Thiazolylbluetetrazolium bromide (MTT) reagent respectively, in according with manufacturer’s instruction.

**LC-MS determination of cystine**

For cystine measurements, the cells were sonicated in the presence of 10 mmol/l N-ethylmaleimide. The protein fraction was precipitated by the addition of 10% 5-sulfosalicylic acid and measured using the Bio-Rad Protein Assay. The supernatant (50 µl) was spiked with 50 µL of the internal standard solution (Cystine d6) and vortexed for 5 sec; then the mixture was extracted with 200 µl of acetonitrile, vortexed at least 30 sec, and then centrifuged at 13,000 rpm for 9 min. Liquid chromatography and mass spectrometry analysis was performed by a UHPLC Agilent 1290 Infinity II 6470 (Agilent Technologies) equipped with an ESI-JET-STREAM source operating in the positive ion (ESI+) mode. The software used for controlling this equipment and analyzing data was MassHunter Workstation (Agilent Technologies). The separation column was InfinityLab Poroshell 120 HILIC 1.9 µm 100x2.1 mm (Agilent Technologies). Method validation was performed based on the US Food and Drug Administration (FDA) guideline for industry bioanalytical method validation (FDA 2013).
and European Medicines Agency (EMA) guideline (EMA 2011). The validation of the assay was performed including selectivity, specificity, linearity and limit of quantification, accuracy and precision, matrix effects and recovery, and stability.

**Evaluation of apoptosis in zebrafish larvae**

**Caspase-3 enzyme activity.** At 5dpf, wild type and *ctns*<sup>−/−</sup> larvae (untreated or treated with 5, 10, 50, 100 or 500µM of luteolin) were homogenized in RIPA buffer. Twenty larvae were homogenized per 300µl of the buffer and at least three replicates were performed for each condition. After centrifugation (10min, 4°C, 12,000g), supernatants were preserved at −20°C until time of analysis. Caspase-3 enzyme activity were assayed in 1/100 dilution of the supernatant of each homogenate using a commercial luciferase-based assay (Caspase-Glo® 3/7, Promega, Madison, WI, USA) according to manufacturer’s protocol. Luminescence was measured using the FlexStation-3 microplate reader (Molecular Devices LLC, San Jose, CA, USA). Enzyme activities were expressed in luminescence units (RLU)/µg protein for each sample, and averages were compared with the untreated *ctns*<sup>−/−</sup> condition.

**Immunohistofluorometric staining of larval renal proximal tubules for megalin, early endosomal antigen-1 (EEA1) and Ras-related protein-11 (Rab11)**

Five dpf wild type and *ctns*<sup>−/−</sup> (either non-treated or treated with 100µM luteolin) zebrafish larvae were fixed using 4% PFA overnight at 4°C (n= 8 larvae per group). In short, larvae were washed 3 x 5min in PBS then incubated in 30% sucrose at 4°C for a maximum of 3 days. Larvae were then embedded in 15% cold-water fish gelatin/15% sucrose, oriented in parallel in cryosection molds and frozen rapidly over a metal surface on dried ice. Frozen larvae were cut within 24h using the Leica CM3050 S cryotome (Leica Microsystems, Wetzlar, Germany). Sections were stained for a combination of rabbit anti-megalin antibody and goat anti- EEA1 antibody or a combination of rabbit anti-megalin antibody and mouse anti-Rab11 antibody. Slides were kept overnight at 4°C then washed and
incubated for 3h at room temperature with the secondary antibody. Secondary antibodies were Alexa-488 anti-rabbit (1:200), Alexa 594 anti-sheep (1:600) and Alexa 594 anti-mouse (1:600) for megalin, EEA1 and Rab11, respectively (Thermo Fisher Scientific, Waltham, MA, USA). Images were taken using confocal microscopy (Leica SP5, Leica Microsystems, Wetzlar, Germany).

**Block face scanning electron microscopy**

Samples were prepared and imaged with the help of the EM Facility at the Faculty of Life Sciences, University of Manchester, UK as previously described.3 Briefly, five days post fertilization larvae were fixed in 2.5% glutaraldehyde, 4% formaldehyde in 0.1 HEPES, pH 7.2, before high density staining for serial block face imaging based on Williams et al.4 This involved en-bloc staining with reduced osmium, thiocarbohydrazide, osmium, uranyl acetate and lead aspartate, followed by dehydration in staged ethanol (30-100%) and embedding in Epon 812 Hard formulation. Following embedding the samples were mounted on 3view pins and trimmed to locate the pronephros. A Gatan 3view mounted within a FEI Quanta 250FEG was used to collect images every 5 µm. Images were of each pronephros were collected with a field of view selected to encompass the extent of the pronephros (6000x6000 to 8000x8000 pixels) with 12nm pixel resolution. Inverted back-scattered electron images were collected at high vacuum with an accelerating voltage of 3.8kV, and a 2µs dwell time. Images were batch converted to tifs in Digital Micrograph and analyzed using ImageJ.

**References**


Cell-based phenotypic drug-screening identifies luteolin as candidate therapeutic for nephropathic cystinosis

OUTCOME
Luteolin improves defective pathways of cystinosis.

METHODS
1) Drug screening in cystinotic proximal tubular cells (PTCs) to identify new therapeutics which reduce the levels of the autophagic substrate p62/SQSTM1.

2) Validation of the best positive hit in several disease models:
   - hPTCs
   - mPTCs
   - zebrafish

CONCLUSION
Luteolin may represent a treatment for nephropathic cystinosis and other renal lysosomal storage diseases.

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