The aminoglycoside geneticin permits translational readthrough of the CTNS W138X nonsense mutation in fibroblasts from patients with nephropathic cystinosis

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Abstract
Background Cystinosis is an ultrarare disorder caused by mutations of the cystinosin (CTNS) gene, encoding a cystine-selective efflux channel in the lysosomes of all cells of the body. Oral therapy with cysteamine reduces intralysosomal cystine accumulation and slows organ deterioration but cannot reverse renal Fanconi syndrome nor prevent the eventual need for renal transplantation. A definitive therapeutic remains elusive. About 15% of cystinosis patients worldwide carry one or more nonsense mutations that halt translation of the CTNS protein. Aminoglycosides such as geneticin (G418) can bind to the mammalian ribosome, relax translational fidelity, and permit readthrough of premature termination codons to produce full-length protein.

Methods To ascertain whether aminoglycosides permit readthrough of the most common CTNS nonsense mutation, W138X, we studied the effect of G418 on patient fibroblasts.

Results G418 treatment induced translational readthrough of CTNSW138X constructs transfected into HEK293 cells and expression of full-length endogenous CTNS protein in homozygous W138X fibroblasts.

Conclusions Reduction in intracellular cystine indicates that the CTNS protein produced is functional as a cystine transporter. Interestingly, similar effects were seen even in W138X compound heterozygotes. These studies establish proof-of-principle for the potential of aminoglycosides to treat cystinosis and possibly other monogenic diseases caused by nonsense mutations.

Keywords Cystinosis · Aminoglycoside · Geneticin · Nonsense mutation · Translational readthrough

Introduction
Cystinosis is a rare autosomal recessive disorder caused by mutations of the cystinosin (CTNS) gene, encoding a transmembrane transporter that facilitates cystine efflux from the lysosome [1]. Homozygous CTNS mutations cause intralysosomal cystine accumulation, disturb cellular homeostasis, and drive progressive organ dysfunction. Current treatment involves the sulphydryl drug cysteamine, which chemically reduces cystine to form mixed disulfides that can exit the lysosome via the alternative PQLC2 channel [2, 3]. The resulting cystine depletion slows organ deterioration and delays the need for renal replacement therapy. However, the average life expectancy is approximately 30 years. Thus, there is a clear unmet medical need for children suffering from this devastating disease.

One plausible explanation for the incomplete therapeutic benefit of oral cysteamine is the well-documented problem of therapeutic compliance, particularly among teenagers and young adults, who find the gastrointestinal side effects and offensive odor difficult to accept [4]. However, even among patients who report heroic adherence to the dosing schedule into their teens, renal transplantation is inevitable, suggesting...
there may be consequences of cystinosis that are independent of intralysosomal cystine accumulation. Indeed, recent evidence suggests that there are nonchannel functions of the CTNS protein in proximal tubular epithelial cells (PTECs). Disturbances in endocytosis were identified, in which the reduced expression of brush border multiligand receptors is decreased and, subsequently, there is a delay in trafficking of ligands from the cell surface accompanied by a general disorganization of the lysosomal compartment [5, 6]. Furthermore, Sansanwal and Sarwal [7] demonstrated a defect in autophagic flux in cystinotic cells. The importance of these nonchannel functions of cystinosin to organ deterioration has not been evaluated, but it may be impossible to overcome the ravages of cystinosis by chemical depletion of intralysosomal cystine alone.

In Europe, the most common pathogenic CTNS mutation is a 57-kb deletion encompassing exons 1–10 and a large upstream region [1]. However, about 15% of cystinosis families worldwide harbor a CTNS nonsense mutation [8]. The most common of these is W138X, which was introduced into the French Canadian population from Ireland in the mid-1800s and now accounts for ~50% of cystinotic alleles in the province [9]. This mutation causes a premature termination codon (PTC) in exon 7 of CTNS, resulting in a null allele. PTCs generate truncated proteins and also trigger degradation of the related transcript via nonsense-mediated messenger RNA (mRNA) decay (NMD). It has been known since the 1980s that PTCs can be overcome by some aminoglycoside antibiotics, such as geneticin (G418) and gentamicin [10]. These compounds bind to the mammalian ribosome and inhibit translational termination at PTCs by promoting insertion of near-cognate aminoacyl-transfer RNAs (aa-tRNAs). Thus, it is plausible that aminoglycosides could be used to overcome the nonsense mutations in a significant subset of cystinosis patients.

Here, we show that G418 normalizes CTNS mRNA levels, restores full-length CTNS protein, and reduces pathologic cystine accumulation in patient fibroblasts harboring the W138X mutation.

Materials and methods

Cell culture and collection

Fibroblasts were grown in DMEM (Gibco no. 11995-065 or Corning no. 10-013-CV) with 10% FBS (Wisent no. 080-450). Ten milligrams/mL genetinic stock (G418, Gibco no. 10131027) was added to give final concentrations as stated. Experiments were performed when cells reached 70–80% confluency, with an incubation time of 48 h. Trypsinization was performed using 0.25% trypsin and 2.2 mM EDTA (Corning no. 25-053-CI), cells were washed three times with PBS, and then pellets were snap frozen in an ethanol bath. Samples were stored at −80 °C until preparation for mRNA, protein, or cystine analysis.

qPCR

mRNA was extracted using the Zymo Research Quick-RNA MiniPrep kit (no. R1054) and stored at −80 °C. Complementary DNA (cDNA) was generated using iScript Reverse Transcriptase Supermix for RT-qPCR (Bio-Rad, no. 170-8841) from 500 ng RNA. One microtiter cDNA was added for qPCR with the SsoFast EvaGreen Supermix with Low ROX (Bio-Rad no. 172-5211) using the following primers: hCTNS Fwd GCAGTCACGCTGGTCAAGTA, Rev AGAAGGATGAGAATG; hGAPDH Fwd GAGTCGACGGATTTGGGTGT, Rev GATCTCGC TCCTTGGAAGATG; and hB2M Fwd AGATGAGT ATGCCTGCGTGT, Rev GCTTACATGTCTCGATCCCA CTTA.

Western immunoblotting

Cells were harvested from culture flasks and lysed with lysis buffer (8 M urea/4% SDS/40 mM Tris (pH 6.8)/0.1 mM EDTA). Thirty to fifty micrograms of protein from each sample was assayed via SDS-PAGE followed by Western blot analysis. PVDF membranes (GE Healthcare) were blocked with 5% nonfat milk/PBST and probed overnight with a primary antibody anti-CTNS (LSBio, no. LS-C157668 (C-terminal region of CTNS); Abnova, M09 (N-terminal region of CTNS)] and anti-actin (Sigma, no. A5441), followed by secondary antibody incubation for 1 h with ECL α-rabbit IgG-HRP (GE Healthcare). All antibodies were used at concentrations recommended by the manufacturer. Bands were visualized using ECL 2 Western Blotting Substrate (Thermo Scientific Pierce).

Plasmid construction

The pcDNA3.1-CTNS and pcDNA3.1-CTNS-DsRed plasmids were kindly provided by Dr. F. Emma (Bambino Gesù Children’s Hospital and Research Institute, Rome, Italy).

The generation of these plasmids has been previously described [11]. The pcDNA3.1-kozakCTNS was generated by PCR utilizing the primers kozakctns1 5′-GCTC GGATCCGCCGGCACCATGATAAGGAATTGGCT GACTATTTTATC-3′ (BamHI site underlined and Kozak sequence in bold) and hCTNSs-msc(R) 5′-GCTG GCCACCGGCTCATAAC-3′ (MscI site underlined). The PCR product was digested with BamHI and MscI then cloned into pcDNA3.1-CTNS.

Plasmids pcDNA3.1-CTNSW138X and pcDNA3.1-CTNSW138X-DsRed were constructed by PCR-mediated
mutagenesis with primers kozakcns1 and hCTNSbsu361(R) 5′-CACCTGAGGGTAGAAGGAGATGGATCAGGCCAC-3′ (W138X mutation site underlined). The PCR products were ligated into the plasmid backbone previously described.

**Cell transfection**

HEK293 cells were grown to ~80% confluency then transfected with pcDNA3.1-CTNS\(^{W138X}\)-DsRed using Lipofectamine 2000 (Thermo Fisher Scientific) and Opti-MEM (Gibco no. 11058021). One microliter of transfection reagent was used per 2 μg of plasmid. After 24 h, cells were treated with G418 as described then stained with DAPI and observed using a Zeiss LSM780 laser scanning confocal microscope.

CTNS\(^{57\text{kb} \text{Del/57\text{kb} \text{Del}}}\) fibroblasts were grown to ~80% confluency then transfected with pcDNA3.1-CTNS\(^{W138X}\) using FuGENE HD transfection reagent (Roche Applied Science) at a DNA/transfection reagent ratio of 1:3 according to the manufacturer’s instructions. After 48 h, cells were treated with G418 as described and harvested for Western immunoblotting.

**Intracellular half-cystine measurement**

Cell pellets were resuspended in 130 μL of 30 μM homocysteine solution and sonicated in Covaris microTUBE AFA Fiber Pre-Slit Snap-Cap tubes (no. 520045) using the Covaris S220 sonicator (settings: peak power 140, duty factor 0.0, cycles/bursts 50). The lysate was then transferred to a 1.5-mL Eppendorf tube containing a further 170 μL of homocysteine solution and centrifuged for 10 min at 12,000×g. The supernatant was collected and snap frozen in an ethanol bath. Samples were then stored at ~80 °C. Half-cystine (cysteine) levels were determined by HPLC analysis of the supernatant using fluorescent detection. Total protein was measured using the BioBasic Better BCA Protein Assay kit (no. SK3051-500), as per the manufacturer’s instructions (96-well plate format). Half-cystine results were corrected to total protein, and values obtained from normal fibroblast samples were subtracted from cystinotic cell samples to show only pathologic accumulation from cystine, not free cysteine.

**Results**

**CTNS\(^{W138X/W138X}\) fibroblasts display the molecular phenotypes of cystinosis**

To study the CTNS\(^{W138X}\) mutation, we assembled a panel of fibroblast lines from patients with nephropathic cystinosis and normal controls. In Fig. 1a, the W138X, 57-kb deletion, and 1035insC mutations are illustrated in comparison to the wild-type CTNS transcript. The cell lines used in this study are listed in Fig. 1b along with the genotype for each CTNS allele. Two homozygous nonsense mutant CTNS\(^{W138X/W138X}\) cell lines (WG1012 and WG1896) were found to have an average of 38% and 14% of normal levels of CTNS mRNA,
respectively (Fig. 2a); CTNS protein was nearly undetectable in the CTNS<sup>W138X/W138X</sup> lines (Fig. 2b). In fibroblasts from a cystinosis patient bearing the homozygous CTNS deletion (CTNS<sup>57kbDel/57kbDel</sup>), CTNS mRNA (data not shown) and CTNS protein were undetectable (Fig. 2b).

**G418 induces translational readthrough in CTNS<sup>W138X/W138X</sup> fibroblasts**

After treatment with G418 for 48 h, CTNS transcript levels increased to within the normal range in both CTNS<sup>W138X/W138X</sup> lines, WG1012 and WG1896 (Fig. 3a). Furthermore, G418 induced endogenous CTNS protein expression in both cell lines, detectable by immunoblotting with an antibody targeting either an N-terminal (WG1896) or C-terminal (WG1012) epitope in CTNS (Fig. 3b). G418 did not induce endogenous CTNS expression in CTNS<sup>57kbDel/57kbDel</sup> fibroblasts (Fig. 3b) and had no effect on CTNS protein levels in wild-type fibroblasts (data not shown).

To confirm that G418 induces translational readthrough of the CTNS W138X nonsense mutation, we transiently transfected HEK293 cells with an expression plasmid containing wild-type CTNS-DsRed or mutant CTNS<sup>W138X-DsRed</sup> fusion cDNA. DsRed fluorescence was detected in cells transfected with CTNS-DsRed but not CTNS<sup>W138X-DsRed</sup>. After 48 h of treatment with 200 μg/mL G418, DsRed fluorescence was easily detected (Fig. 4a). A high-powered image (∗× 1000) confirmed that DsRed fluorescence was intracellular (Fig. 4b).

CTNS<sup>W138X</sup> was transiently transfected into CTNS57kbDel/57kbDel fibroblasts and treated with 400 μg/mL of G418 for 24 h. CTNS translational readthrough was demonstrated by immunoblotting with a C-terminal CTNS antibody (Fig. 4c).

**Treatment of CTNS<sup>W138X/W138X</sup> fibroblasts with G418 reduces intracellular cystine levels**

To confirm that the protein resulting from G418-induced readthrough functions as a cystine transporter, we measured intracellular half-cystine levels in the CTNS<sup>W138X/W138X</sup> fibroblast lines WG1012 and WG1896 after 48 h of treatment with 200 μg/mL G418. Half-cystine levels were reduced to 28% of untreated levels in WG1012 cells and to 44% of untreated levels in WG1896 cells (Fig. 5a).

To compare the magnitude of this effect with that of cysteamine, the current treatment for cystinosis, we first examined cell cystine reduction (WG1012 cells) in response to various concentrations of cysteamine (5–100 μM). Langman et al. [12] noted that the maximum reduction in half-cystine occurs about 1 h after the cysteamine peak in serum following an oral dose. Therefore, we measured fibroblast half-cystine levels after 1 h of cysteamine treatment. Reduction of half-cystine level achieved by 200 μg/mL G418 (7.6 nmol/mg protein) was comparable to that achieved by 50 μM cysteamine after 1 h (6.7 nmol/mg protein = 36% of untreated level) (Fig. 5b).

**G418 promotes translational readthrough from a single W138X allele in compound heterozygous cystinotic fibroblasts**

The majority of cystinotic patients with a CTNS nonsense mutation are unlikely to be homozygous but most often harbor some other types of mutation on the trans allele. To ascertain whether G418 induces sufficient CTNS expression to reduce pathologic accumulation of cystine, we tested the effect of G418 on two compound heterozygous fibroblast cell lines (CTNS<sup>W138X/57kbDel</sup> and CTNS<sup>W138X/1035insC</sup>). Both untreated cell lines expressed CTNS mRNA at reduced levels compared...
to normal fibroblasts. In the presence of 200 μg/mL G418 for 48 h, both compound heterozygotes normalized CTNS mRNA levels into the normal range (Fig. 6a, b). Furthermore, G418 reduced cell cystine levels to 36% of untreated baseline in CTNS^{57kbDel/57kbDel} fibroblasts, G418 had no significant effect on CTNS mRNA (data not shown) or cell cystine (Fig. 6d).

**Discussion**

Untreated patients bearing the homozygous CTNS^{W138X/W138X} nonsense mutations are clinically indistinguishable from those with homozygous CTNS deletions; leukocyte cystine levels are similar, both groups develop renal Fanconi syndrome in the first year of life, and both develop progressive renal dysfunction requiring renal replacement therapy after about 10–11 years. In fibroblast cell lines from two CTNS^{W138X/W138X} homozygotes, we show that CTNS protein is nearly undetectable and found no basal CTNS translation from expression plasmids bearing the CTNS^{W138X} mutation when transfected into human fibroblasts. Thus, the CTNS W138X nonsense mutation functions as a null allele without significant residual activity. In the presence of G418 (200–400 μg/ml), we observed translation of full-length CTNS protein from exogenous CTNS^{W138X} expression plasmids and restoration of endogenous CTNS protein, detected by immunoblotting with a C-terminal anti-CTNS antibody. This demonstrates the ability of the aminoglycoside, G418, to induce translational readthrough of the most common nonsense mutation (W138X) causing cystinosis in humans.

Complete translation of CTNS protein does not guarantee clinically relevant restoration of its lysosomal channel function. The wobble in codon recognition that is induced by G418 permits insertion of the native amino acid, tryptophan, which has been shown to be a common near-cognate insertion at UGA PTCs after readthrough [13–16]. However, nonnative tRNA inclusions might also occur that could diminish channel function. Importantly, we show that G418 restores enough
a functional CTNS protein to reduce fibroblast half-cystine within 24 h; thus, cystine efflux from lysosomes must be in excess of the rate at which cystine is being generated. To understand whether this might be clinically relevant, we compared the effect of G418 to that of cysteamine in mutant fibroblasts. Therapy with oral cysteamine at doses of 325 mg/m² every 6 hr reduce leukocyte cystine to about 15–20% of untreated baseline levels and has been shown to delay progressive renal insufficiency and slow deterioration of other organs in nephropathic cystinosis [2, 17, 18]. At this dose, peak serum cysteamine (at 72 min) is up to 50 μM and achieves maximal depletion of leukocyte cystine about 48 min thereafter [12]. We found that reduction of cystine using 200 μg/mL G418 was comparable to that of 50 μM cysteamine (~30% of untreated baseline) in vitro, suggesting a clinically relevant effect.

The primary impact of a PTC is to stall translation prematurely and release an unstable, truncated protein. However, failure to displace nuclear proteins during the pioneer round of translation recruits NMD machinery, which causes mRNA decapping and transcript decay. We found that CTNS transcript levels were reduced in CTNSW138X/W138X patient fibroblasts. Interestingly, the effects of G418 on translational readthrough were accompanied by normalization of CTNS transcript levels, suggesting that PTC-induced transcript decay was fully suppressed by the drug. By permitting insertion of a near-cognate tRNA at a PTC, G418 averts the arrest of translation that would otherwise lead to assembly of the NMD complex and transcript degradation.

Although reduction of intralysosomal cystine by cysteamine is associated with clinical benefit, recent studies suggest that there may be a variety of nonchannel functions of CTNS which cannot be restored by chemical depletion of intralysosomal cystine [5, 7, 19, 20]. Thus, aminoglycoside readthrough of CTNS nonsense mutations has the potential to achieve clinical benefit beyond what is possible with cysteamine. However, the toxicity of G418 precludes its use in humans. Helip-Woolley et al. [21] found that another aminoglycoside, gentamicin (300 μg/ml), induces readthrough of exogenous CTNSW138X-GFP in HEK293 cells and reduced intracellular cystine in cystinotic fibroblasts after 15 days. However, no reduction was observed after 48 h because the nonsense mutation readthrough effect of gentamicin is relatively weak compared to that of other aminoglycosides. Furthermore, renal and ototoxicities still make it unsuitable for long-term therapy at the doses that would be required. Recently, Eloxx Pharmaceuticals generated a series of novel aminoglycoside derivatives and systematically screened them for retention of nonsense mutation readthrough properties, excluding those with high affinity binding to the prokaryotic (and, presumably, the mitochondrial) ribosome [22–25]. Some compounds with a high ratio of translational readthrough to prokaryotic binding affinity (NB84 and ELX-02) have been tested in animal models of genetic human disease [26, 27]. Our studies suggest that if these compounds...
Fig. 5 Effect of G418 on pathologic cystine accumulation in CTNS<sup>W138X/W138X</sup> fibroblasts. Half-cystine was measured after 24-h exposure to G418 (200 μg/mL) and normalized to total cell protein in a WG1012 (n = 6; unpaired one-tailed t test) and WG1986 CTNS<sup>W138X/W138X</sup> fibroblasts (n = 3; unpaired one-tailed t test). b Half-cystine measured in WG1012 cells after 1-h exposure to various concentrations of cysteamine (n = 3; unpaired two-tailed t test). *p < 0.05, **p < 0.01.

Fig. 6 Effect of G418 on compound heterozygous CTNS fibroblasts harboring a W138X allele. Fibroblasts from patients with compound heterozygous CTNS mutations that include one W138X allele were incubated for 48 h with 200 μg/mL G418. RT-qPCR analysis of CTNS in a CTNS<sup>W138X/1035insC</sup> (n = 4; unpaired two-tailed t test) and b CTNS<sup>W138X/57kbDel</sup> (n = 4; unpaired two-tailed t test) fibroblasts (***p < 0.001). Intracellular half-cystine levels in c CTNS<sup>W138X/1035insC</sup> (*p = 0.02) and CTNS<sup>W138X/57kbDel</sup> (n = 3; *p < 0.01, unpaired two-tailed t test) fibroblasts and d CTNS<sup>57kbDel/57kbDel</sup> fibroblasts (n = 3; unpaired two-tailed t test, not significant).
exert PTC readthrough effects comparable to G418 without appreciable toxicity, they could be of interest in the treatment of cystinosis.

While homozygous CTNS WT138X/W138X patients are relatively common among French Canadians, most CTNS nonsense mutations worldwide occur in compound heterozygosity with a deletion, missense, or frameshift mutation. Importantly, we noted that G418 reduces cell cystine in fibroblasts from CTNS compound heterozygotes. CTNS transcript levels in CTNS WT138X/57kbDel or CTNS WT138X/1035insC (frameshift) cells were normalized, and pathologic cystine accumulation was reduced to about 47% of untreated baseline (average of the two compound heterozygote cell lines) by 200 μg/ml G418. This effect is slightly less than that in the two W138X homozygous fibroblast lines (reduction to 36% of untreated baseline), suggesting that slightly higher doses of aminoglycoside might be needed in compound heterozygotes than in homozygotes, but that a clinically relevant reduction of cellular cystine is still achievable.

Children with nephropathic cystinosis usually exhibit renal Fanconi syndrome in the first year of life, but physical atrophy of the proximal tubule (swan neck deformity) is not seen until the second year [28]. This suggests that there might be a window of opportunity to avert irreversible proximal tubular injury if translational readthrough therapy could be started shortly after diagnosis. The heavy flux of tubular protein targeted to lysosomes of the proximal tubule might require a correspondingly high rate of lysosomal cystine efflux and a higher level of CTNS readthrough compared to other tissues. On the other hand, aminoglycosides are concentrated in proximal tubular cells about 25-fold above serum levels [29, 30]. Thus, the G418 concentrations used to reduce cystine accumulation in fibroblasts might be more effective at inducing CTNS W138X readthrough in proximal tubules.

**Summary**

We demonstrate that the aminoglycoside, G418, induces translational readthrough of the CTNS W138X premature termination codon and generates sufficient functional CTNS protein to reduce pathologic cystine accumulation in homozygous and compound heterozygous patient fibroblasts. While G418 toxicity precludes use in human cystinosis patients, our study establishes proof-of-principle for the potential of recently developed nontoxic aminoglycosides to treat a subset of cystinosis patients. We speculate that PTC readthrough drugs might be applicable to a variety of monogenic renal diseases beyond cystinosis.

**Author’s contributions** EJB participated in the experimental design; performed the cell culture, qPCR, cell transfection, and intracellular half-cystine measurements; analyzed the results; and participated in the manuscript preparation.

LLC participated in the experimental design; performed the cell culture, qPCR, Western immunoblotting, plasmid construction, and intracellular half-cystine measurements; analyzed the results; and participated in the manuscript preparation.

REK participated in the experimental design.

JHS performed the cell transfection.

RL performed the cell culture.

DMI participated in the experimental design.

PG participated in the experimental design, data analysis, and manuscript preparation.

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**Compliance with ethical standards**

**Ethics compliance** All primary cell lines used in this article were subject to IRB approval from the RI-MUHC REB (protocol: 2018-2922).

**Conflict of interest** The authors declare that they have no conflicts of interest.

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