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Treatment of a methylmalonyl-CoA mutase stopcodon mutation

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ABSTRACT

There are limited treatment options for the metabolic disorder methylmalonic aciduria. The disorder can be caused by nonsense mutations within the methylmalonyl-CoA mutase gene, resulting in the production of a truncated protein with little or no catalytic activity. We used a genomic reporter assay and mouse primary cell lines which carry a stop-codon mutation in the human methylmalonyl-CoA mutase gene to test the effects of gentamicin and PTC124 for stop-codon read-through potential.

Fibroblast cell lines were established from methylmalonic aciduria knockout-stop codon mice. Addition of gentamicin to the culture medium caused a 1.5- to 2-fold increase in mRNA expression of the human methylmalonyl-CoA mutase gene. Without treatment the cells contained 19% of the normal levels of methylmalonyl-CoA mutase enzyme activity which increased to 32% with treatment, suggesting a functional improvement. Treatment with PTC124 increased the amount of human methylmalonyl-CoA mutase gene mRNA by 1.6 ± 0.3-fold and a trend suggesting increased enzyme activity.

The genomic reporter assay, BAC_MMA*EGFP, expresses enhanced green fluorescent protein when read-through of the stop codon occurs. Using flow cytometry, RT-real-time PCR and enzyme assay, read-through was measured. Treatment with PTC124 at 20 μmol/L resulted in a significant increase in enhanced green fluorescent protein, a 2-fold increase in mRNA expression and a trend to a slight increase in enzyme activity.

The clinical relevance of these effects may be tested in mouse models of MMA carrying nonsense mutations in the methylmalonyl-CoA mutase gene. Pharmacological approaches have the advantage of providing a broader effect on multiple tissues, which will benefit many different disorders with similar nonsense mutations.

1. Introduction

Methylmalonic aciduria (MMA) is a metabolic disorder caused by either a defect in the methylmalonyl-CoA mutase enzyme (EC 5.4.99.2) or the cofactor adenosylcobalamin. It is a rare inherited autosomal recessive disorder which is characterized by a build-up of the metabolite methylmalonic acid with secondary biochemical disturbances. Current treatment involves dietary restriction and drugs, to reduce intake and endogenous production of precursors and to help eliminate some produced metabolites. Kidney and/or liver transplantation is also used to reduce the symptoms of the disorder, but the treatment is not able to cure the disorder. Thus new treatment strategies are being investigated.

Up to 14% of MMA mutations are caused by nonsense mutations which cause the introduction of a stop codon, resulting in truncation of the enzyme methylmalonyl-CoA mutase. Stop codon read-through has been examined for the treatment of several diseases including lysosomal storage disorders [1], cystic fibrosis [2], Duchenne muscular dystrophy [3], carnitine palmitoyltransferase 1A deficiency [4] and Menkes disease [5]. G-418 (geneticin) and gentamicin (an aminoglycoside) have been shown to restore expression in a cystic fibrosis cell line carrying a stop codon mutation [2] which has lead to trials in rodents and humans.

Aminoglycoside action unfortunately lacks specificity which would lead to the read-through of many correctly positioned stop codons. Therefore we used the developed BAC_MUT*_EGFP reporter assay to screen other potential compounds. The genomic reporter assay was a HeLa cell line with a bacterial artificial chromosome

Abbreviations: BAC, bacterial artificial chromosome; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethylsulfoxide; EGFP, enhanced green fluorescent protein; MCM, methylmalonyl-CoA mutase; MMA, methylmalonic aciduria; *MUT*, human methylmalonyl-CoA mutase gene; *Mut*, mouse methylmalonyl-CoA mutase gene; PTC, premature termination codon; PTC124, (Ataluren) (3-(5-fluorophenyl)-1,2,4-oxadiazol-3-yl)benzoic acid.

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(BAC) construct containing the R403stop mutation within exon 6, and enhanced green fluorescent protein (EGFP) in-frame with exon 13, of the human methylmalonyl-CoA mutase (MCM) locus (*MUT*) [6]. Zidovudine, adefovir and cisplatin treatment of the genomic reporter assay cell line resulted in increased EGFP reporter produced *in vitro* [6]. These compounds are all approved for clinical use. Cisplatin is used for treatment of some cancers; however its side-effects limit long-term treatment. The read-through produced by cisplatin may be caused by an off target or secondary effect of one of its many intracellular activities.

PTC124 (Altaluren) (3-[5-(2-fluorophenyl)-[1,2,4]oxadiazol-3-yl]-benzoic acid) was found using a high throughput screen [7]. This compound induces production of full-length functional CFTR protein in a mouse model of cystic fibrosis [8] and has been tested for various disorders caused by stop-codon mutations.

A transgenic mouse model (MMA stop codon mice) carrying the human R403stop mutation on *MUT* was developed carrying a mutation identified in an individual with mut^0 MMA which resulted from a single base change of C → T in exon 6 of *MUT* (producing a TGA stop codon) [9]. This MMA knockout-stop codon mouse model has similar metabolite biochemistry to the human disorder.

In humans one of the major limitations of using aminoglycosides as drugs is their high nephrotoxicity and ototoxicity [10,11], which would be particularly detrimental to MMA patients because nephrotoxicity occurs as part of the natural progression of the disorder. Thus we aim to examine the potential of PTC124 for the alleviation of MMA in mouse model fibroblast cell lines and a genomic reporter assay. The advantage of using such developed lines, over fibroblast lines obtained from patients, is that we can isolate and specifically examine the single mutation of interest. Using a stably integrated BAC, rather than cDNA constructs, ensures necessary flanking regulator sequences are present.

2. Materials and methods

2.1. Fibroblast cell line treatment and analysis

Fibroblast cell lines were established from skin of transgenic knockout mouse pups carrying a TGA stop codon insertion in the human *MUT* gene 'MMA knockout-stop codon mice' ($Mut^{-/-}MUT^{Stop+/-}$) [9]. The R403stop mutation had been identified within exon 6 of a MMA patient [12]. Two cell lines were used to investigate the effects of gentamicin treatment on human MCM expression. Cell culture conditions for gentamicin treatment were as follows: Cells were grown to ~70% confluence then gentamicin was added to the medium DMEM (Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin/0.1 mg/ml streptomycin) at concentrations of between 0 and 800 μg/mL and incubated at 37 °C in an atmosphere of 5% CO_2. Media was changed after 24 h and again after 48 h (fresh gentamicin added), and then the cells were harvested for analysis at 72 h.

2.2. mRNA expression

Total mRNA was isolated using the QIAGEN RNeasy minikit (Qiagen, Hilden, Germany). The Superscript II First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) was used to generate cDNA. Real time RT-PCR was performed using the ABi7300 Real Time PCR system with SYBRgreen PCR Master Mix (Applied Biosystems, Foster City, CA). *MUT* mRNA expression was detected using the primers hmMUT-F (5′-TTCTATAAGGACAACATTAAGGCTGGTC-3′) and hmMUT-R (5′-CAATAGCAACTCCAGCCATTCC-3′). Expression was normalized to *ACTB* (human beta actin) (forward 5′-AGGCACCA GGGCGTGAT-3′ and reverse 5′-TCGCCCACATAGGAATCCTT-3′). *MUT* mRNA primers bind to the methylmalonyl-CoA mutase cDNA sequence prior to the stop codon mutation. The results were analyzed using the "delta-delta comparative Ct method" and presented as fold change in mRNA levels in cells treated with different compounds, relative to untreated cells.

2.3. Methylmalonyl-CoA mutase activity

MCM activity was determined using the method of [^{14}C] propionate incorporation into trichloroacetic acid-precipitable material [13]. MCM protein levels were not measured due to the lack of a specific antibody to MCM.

2.4. Genomic reporter assay generation

A genomic reporter was produced as previously described [6]. Briefly, a BAC containing the entire human *MUT* locus (RP11-463L20, accession number AL590668) was modified to include the R403stop mutation [12]. The gene for EGFP was added in-frame with exon 13 of the *MUT* locus to create the EGFP reporter construct, BAC_MUT*_EGFP. The construct was then electroporated into HeLa cells to produce the genomic reporter assay . The genomic reporter assay was grown in DMEM and incubated at 37 °C in an atmosphere of 5% CO_2. The clone in this work was the same as Clone 1 of the original publication [6].

2.5. PTC124 treatment

Using a genomic reporter assay (BAC_MUT*_EGFP) we tested the effect of the stopcodon read-through compound PTC124. Cells were treated with PTC124 (1 and 20 μmol/L) for 72 h before being collected and analyzed for EGFP expression (mRNA using real time RT-PCR and protein via flow cytometry) and MCM enzyme activity. Stock PTC124 (Selleckchem Co, Shanghai, China) was dissolved in DMSO (1 mmol/L) prior to dilution in media, with a final DMSO concentration of 2% on the cells.

A third fibroblast cell line established from a transgenic knockout pup ($Mut^{-/-}MUT^{Stop+/-}$) was also tested with PTC124. Cells were treated with PTC124 (20 μmol/L) for 72 h before being collected and analyzed for *MUT* expression (forward 5′-GCTACAG-GATTTGCTGATCTTGGT-3′; reverse 5′-CATCCGCATCCACAGCCT-3′) by real time RT-PCR and for MCM enzyme activity.

2.6. Flow cytometry

After treatment, genomic reporter assay cells were trypsinized, washed and analyzed. Flow cytometry was performed using a LSR II flow cytometer (Becton–Dickinson, Franklin Lakes, NJ) and analyzed using the FACSDiva Software Package, Version 1.4 (Becton–Dickinson, Franklin Lakes, NJ). Median peak fluorescence was determined for live cells of each sample.

2.7. Statistical analysis

Data expressed as mean (±SEM). Analyses were performed using a two-sample *t*-test. Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Treatment of fibroblast cell lines with gentamicin

It was found that the presence of gentamicin in culture medium over a concentration range of 600–800 μg/mL caused a 1.5- to

2-fold increase in mRNA expression of the human *MUT* gene in two fibroblast cell lines established from MMA knockout-stop codon mice, $Mut^{-/-}MUT^{Stop+/-}$ (Fig. 1).

Without treatment the cells contained 19% the normal levels of MCM enzyme activity (Fig. 2). When the cell line was treated with 800 μg/mL gentamicin for 72 h, MCM activity increased to 32%, suggesting a functional improvement. Addition of cobalamin did not enhance MCM activity (data not presented).

3.2. Treatment of BAC_MMA*EGFP cells with PTC124

The genomic reporter assay, BAC_MMA*EGFP, expressed EGFP when read-through of the stop codon within the *MUT* gene occurred. Using flow cytometry an increase in median peak fluorescence was measured when the reporter cell line was treated with 1 or 20 μmol/L PTC124 (Fig. 3). PTC124 treatment at 20 μmol/L resulted in a significant increase in median peak fluorescence of approximately 75% compared to untreated cells, showing that the cells expressed EGFP via read-through of the reporter. The diluent (2% DMSO) does not alter EGFP median peak fluorescence. HeLa cells do not express EGFP, which was not altered with PTC124 treatment (as determined by flow cytometry and mRNA levels). In our *in vitro* system DMSO caused the cells to adhere to the plate more than normal, which was enhanced by PTC124, resulting in increased incubation times required to collect the cells.

When EGFP mRNA expression levels were determined for the PTC124 treated genomic reporter assay, they showed no change at 24 h incubation, however there was a 2-fold increase at 72 h incubation.

MCM activity was determined for the cell lines treated with PTC124. HeLa cell line activity did not change with PTC124 treatment (92 ± 36 pmol propionate/mg protein/18 h). Whilst the BAC_MMA*EGFP genomic reporter assay showed a 33% increase in activity (from 130 ± 61 to 173 ± 69 pmol propionate/mg protein/18 h, $n = 5$), however this increase was not statistically significant.

3.3. Treatment of fibroblast cell lines with PTC124

Treatment of a MMA knockout-stop codon pup fibroblast cell line ($Mut^{-/-}MUT^{Stop+/-}$) with PTC124 increased the amount of *MUT* mRNA by 1.6 ± 0.3-fold ($n = 3$) compared to untreated cells or cells treated with 2% DMSO. The MMA knockout-stop codon pup fibroblast cell line showed a 25% increase in MCM activity (from 55 ± 11 to 69 ± 6 pmol propionate/mg protein/18 h, $n = 5$); however, this increase was not statistically significant. The control cell line activity did not change with PTC124 treatment (94 ± 23 pmol propionate/mg protein/18 h).

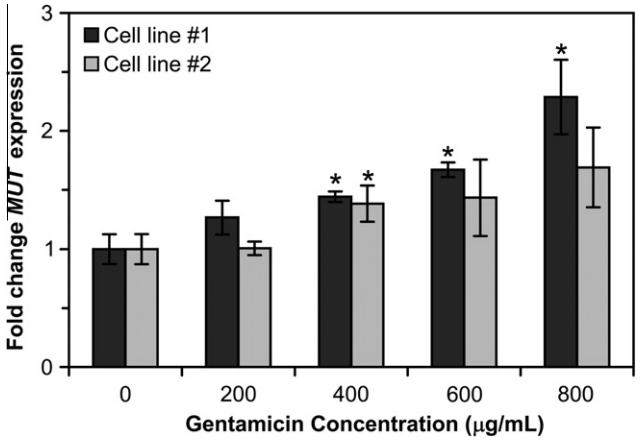

Fig. 1. Real Time RT-PCR results showing fold change in *MUT* expression relative to untreated cells in two different mouse fibroblast cell lines in the presence of gentamicin (between 0 and 800 μg/mL) ($n = 5$). Cell lines #1 and #2 were established from MMA knockout-stop codon mice ($Mut^{-/-}MUT^{Stop+/-}$). $^*p < 0.05$ no gentamicin vs. gentamicin treated.

4. Discussion

In this study, we attempted to rescue the function of a nonsense mutation of MCM using gentamicin and PTC124 in the aim of causing read-though to produce the full length protein and thus increase active methylmalonyl-CoA mutase enzyme levels.

4.1. Treatment of fibroblast cell lines with gentamicin

Clinically, gentamicin has been used as a stop codon read-through compound to treat several disorders [14,1,15]. In primary

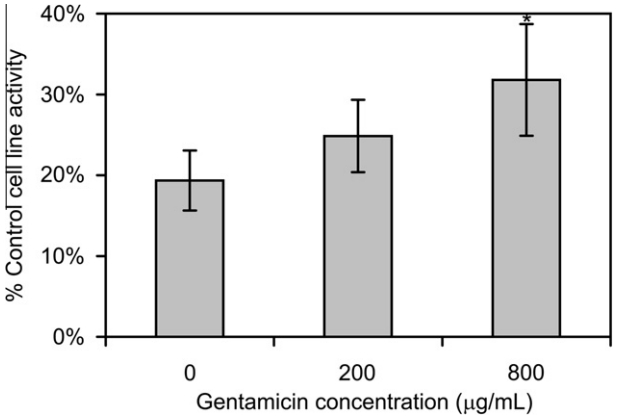

Fig. 2. Methylmalonyl-CoA mutase activity in a mouse fibroblast cell line in the presence of gentamicin (between 0 and 800 μg/mL) ($n = 3$). The cell line was established from MMA knockout-stop codon mice ($Mut^{-/-}MUT^{Stop+/-}$) and compared to control mice carrying the mouse mutase gene. Gentamicin (800 μg/mL) increases the enzyme activity from approximately 20% to 30% of the control cell line (92 ± 36 pmol propionate/mg protein/18 h). $^*p < 0.05$ no gentamicin vs. 800 μg/mL gentamicin treated.

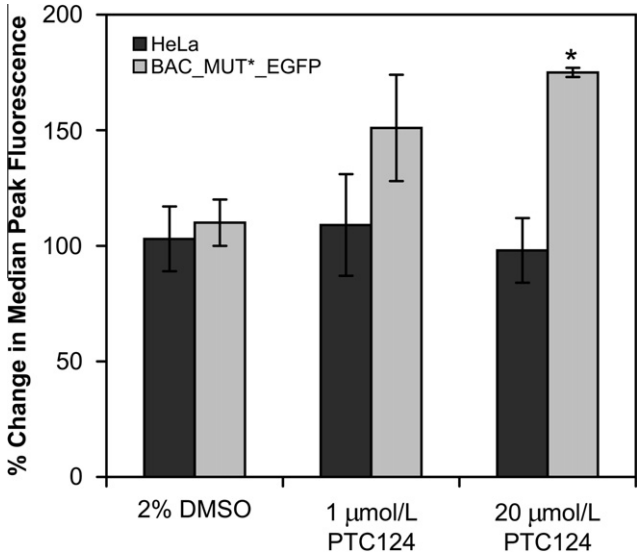

Fig. 3. Treatment of the BAC_MUT*EGFP genomic reporter assay with PTC124. Comparison of median peak fluoresce of EGFP compared to control untreated cells ($n = 6$). The BAC_MUT*EGFP cells treated with PTC124 showed an increase in EGFP produced by read-through of the defective *MUT* gene. $^*p < 0.05$ between BAC_MUT*-EGFP cells and HeLa cells (treated with 20 μmol/L PTC124).

mouse fibroblasts established from the "humanized" mouse model of MMA (MMA knockout-stop codon mouse) which does not express endogenous mouse MCM, the presence of gentamicin in culture medium increased expression of the human *MUT* gene. There is also an indication that this mRNA is translated, to a similar degree, into a functional improvement in MCM activity. Whilst high concentrations of gentamicin were toxic to the cultured cells, a dose of 800 μg/mL was found to be effective, similar to our *in vitro* genomic reporter assay [6].

These results show that the primary cell lines containing the stop-codon mutation can be treated with aminoglycosides and this can potentially be translated to therapies for MMA patients with similar mutations. For treatment of cystic fibrosis an *in vitro* gentamicin dose of 600 μg/mL was converted to a patient dose of 10 mg/kg (daily intravenous administration) [16]. This dosage scheme could be translated to MMA patients, however the side effects of gentamicin includes issues with the kidney, which is already compromised in MMA patients. Thus we are investigating other possible candidates for treatment of stop-codon mutations.

4.2. Treatment of BAC_MMA*EGFP cells with PTC124

Recently we used a genomic reporter assay to investigate different compounds for stop codon read-through. We found that the compounds zidovudine, adefovir and cisplatin were able to increase the production of *MUT* mRNA [17], and may have stop codon read-through capacity [6]. PTC124 is another stop codon read-through drug showing great potential. Here we found that PTC124 was able to increase expression of the reporter gene (EGFP) to a similar level as gentamicin, however it did not increase the enzyme activity as significantly. PTC124 has been shown to have up to 12-fold read through ability without the side effects of gentamicin [7]. Whilst we were unable to reproduce the amount of read through, the results are promising with an increase in EGFP mRNA and protein, and a trend toward increased MCM enzyme activity.

4.3. Treatment of fibroblast cell lines with PTC124

We trialed PTC124 on primary mouse cell lines from a MMA knockout-stop codon mouse and were able to detect modest increases in mRNA levels and enzyme activity. A small increase in the amount of enzyme in the system may actually be enough to reduce the metabolic burden of the disorder. Such approaches may be particularly beneficial during intercurrent metabolic decompensations, where all aspects of management need to be optimized.

The effects of PTC124 have been variable for different researchers. For example when patient cells with nonsense mutations causing carnitine palmitoyltransferase 1A deficiency were treated, PTC124 was found to increase enzymatic activity [4]. Recently, PTC124 was trialed for long-QT syndrome type 1 mutations by transfection of constructs carrying stop codon mutations into human embryonic kidney-293 cells [18]. Whilst gentamicin and G418 were able to effect production of full length protein, PTC124 was unable to produce the read-through. Thus the ability of PTC124 is currently unpredictable and may depend on the target and the system in which it is tested. Our results are very promising for the investigated mutation which causes mut^0 MMA and provide additional evidence for proof of principle that PTC124 is a potential therapeutic agent for treating patients with any genetic condition that results from a nonsense mutation.

4.4. Future directions

Further characterization of the effects of gentamicin and PTC124 treatment on this model of MMA *in vitro* will include development of a specific antibody against human MCM, to enable investigation of protein expression by Western blotting and immunohistochemisty, as well as further studies of enzyme activity. In addition, *in vivo* treatment of our various animal models with PTC124 and other stop codon read through compounds will also be trialed.

5. Conclusions

PTC124 has been trialed *in vitro* for the treatment of MMA using a human genomic reporter assay and transgenic mouse fibroblast cells (both with a known MMA stop codon mutation) and found to have some ability to cause production of active MCM. While the potential use of gentamicin and PTC124 as treatments for MMA patients would obviously be limited to those in which the disorder has arisen as a result of interruption of the gene with a premature stop codon, the results suggest that these drugs or similar compounds may represent a potential therapy or supplementary treatment.

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