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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