Metabolic effects of bezafibrate in mitochondrial disease

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Abstract

Mitochondrial disorders affect 1/5,000 and have no cure. Inducing mitochondrial biogenesis with bezafibrate improves mitochondrial function in animal models, but there are no comparable human studies. We performed an open-label observational experimental medicine study of six patients with mitochondrial myopathy caused by the m.3243A>G MTTL1 mutation. Our primary aim was to determine the effects of bezafibrate on mitochondrial metabolism, whilst providing preliminary evidence of safety and efficacy using biomarkers. The participants received 600–1,200 mg bezafibrate daily for 12 weeks. There were no clinically significant adverse events, and liver function was not affected. We detected a reduction in the number of complex IV-immunodeficient muscle fibres and improved cardiac function. However, this was accompanied by an increase in serum biomarkers of mitochondrial disease, including fibroblast growth factor 21 (FGF-21), growth and differentiation factor 15 (GDF-15), plus dysregulation of fatty acid and amino acid metabolism. Thus, although potentially beneficial in short term, inducing mitochondrial biogenesis with bezafibrate altered the metabolic signature of mitochondrial disease, raising concerns about long-term sequelae.

Keywords bezafibrate; mitochondria; mitochondrial disorder; mitochondrial DNA; mitochondrial encephalomyopathy

Introduction

Mitochondrial diseases are genetically determined metabolic disorders of oxidative phosphorylation (OXPHOS) caused by mutations of mitochondrial DNA (mtDNA) or nuclear genes encoding mitochondrial proteins (Wallace, 2018). As a group, they affect ~1 in 5,000 (Gorman et al, 2015). The clinical features involve tissues with high-energy demands, especially striated muscle and the nervous system. Mitochondrial diseases cause substantial morbidity and have no cure. Several new strategies are being developed as possible treatments for mitochondrial disorders. These include protein replacement; small molecules including antioxidants, amino acids and nucleotide supplementation; cell therapy; and gene manipulation (Nightingale et al, 2016). Although pre-clinical studies have seen the greatest progress, several are now under evaluation in clinical trials (Steele et al, 2017). One approach is to induce mitochondrial biogenesis, which is effective in animal models of mitochondrial diseases (Ahola-Erkkila et al, 2019; Visconi et al, 2011; Cerutti et al, 2014; Peralta et al, 2016). Several compounds show promise in pre-clinical studies, including agonists of AMP-protein activated kinase (Visconi et al, 2011; Peralta et al, 2016), peroxisome proliferator-activated receptor (PPAR) (Yatsuga & Suomalainen, 2012; Besit et al, 2017), situins (Cerutti et al, 2014) and Nir2 (Hayashi et al, 2017). Of these, bezafibrate has the most extensive pre-clinical evidence of efficacy in animal models (Dillon et al, 2012) and patient cell lines (Hofer et al, 2014). Bezafibrate has a favourable side-effect profile in humans but has been noted to cause liver toxicity in rodents, including mouse models of mitochondrial disease (Visconi et al, 2011;
Dillon et al., 2012; Yatsuga & Suomalainen, 2012). We, therefore, designed an open-label, investigator-led, non-randomised experimental medicine study to evaluate the effects of bezafibrate in patients with mitochondrial disease. We incorporated detailed molecular, biochemical and metabolomic profiling alongside clinical measures. Our primary aim was to evaluate the safety of inducing mitochondrial biogenesis with bezafibrate in patients with the m.3243A>G MTTLI mutation. Our secondary aim was to provide preliminary evidence of efficacy to power a subsequent randomised controlled trial.

Results

Participants and treatment

We studied six unrelated adults with mitochondrial myopathy due to the m.3243A>G MTTLI mutation in skeletal muscle (50-84% heteroplasmy, four female, mean age 50 years, range 44-57), identified from local clinics and the UK MRC Mitochondrial Disease Cohort. All had clinical evidence of proximal weakness on bedside testing (Medical Research Council grade 4+ or less). Four had diabetes and deafness, but no other clinical features of mitochondrial disease (Table 1). The entry and exclusion criteria are shown in Appendix Tables S1 and S2. The standard dose of bezafibrate used to treat dyslipidaemia in the UK is 200 mg three times daily (TDS). Based on pre-clinical toxicology and efficacy studies in rodents, we calculated an equivalent dose range (Appendix Table S3) and treated with 200 mg TDS for 6 weeks, then 400 mg TDS in the absence of clinical toxicity. The study design is summarised in Fig 1. Needle muscle biopsies were performed with ethical approval and patient consent.

Safety and tolerability

All six completed the study and treatment regime. There was no change in mean body mass index (BMI) during the treatment (Appendix Table S4). Although there was a trend towards a reduction in non-fasting triglyceride levels after 12 weeks of treatment, the mean level across all participants did not change significantly (Appendix Tables S5). One serious adverse event was reported: acute abdominal pain due to constipation which resolved overnight. This is a recognised complication of both bezafibrate treatment and mitochondrial disease. Four had mild hypoglycaemia leading to a reduction in the insulin dose in the three patients with insulin-treated diabetes. The other patient with mild hypoglycaemia had glucose intolerance under dietary control. The liver function tests remained normal. Three had a reversible reduction in estimated glomerular filtration rate (Fig EV1). FGF-21 and GDF-15 levels were elevated at baseline and increased further at 6 then 12 weeks with increased dose (Figs 2A and EV2) irrespective of gender, body mass index, body surface area or diabetes mellitus (Fig EV3). Unsupervised k-means clustering of the normalised serum metabolomics data separated the pre- and post-treatment groups (Group 1 vs. Groups 2 and 3; Fig EV4A), but there were no differences between weeks 6 and 12 (Fig EV4B). Investigation of the differentially regulated metabolites (Fig 2B, Dataset EV1) and pathways (Fig 2C, Dataset EV2) highlighted changes in amino acid metabolism and tricarboxylic acid cycle intermediates (TCA cycle) between 0 and 6 and 12 weeks of treatment (Fig EV4C and D).

Biochemistry and genetics

Targeted analysis of acyl-carnitines as a surrogate of β-oxidation showed a decrease in the levels of C3, C8, C14:1, C16:1, C18:1 and C20 consistent with the induction of fatty acid oxidation on treatment (Figs 2D, and EV4E and F). At baseline, skeletal muscle complex 1 activity was low and complex III and IV activities were high or similar to control values relative to citrate synthase (Figs 2E and EV5A). Mean enzyme activities, including citrate synthase, did not change on treatment (Fig 2E), although there was considerable variation (Figs 2E, and EV5A and B). Quadruple immunofluorescence showed a reduction in the number of complex IV-deficient muscle fibres after treatment (P-value = 0.046, Fig 3A and B, Appendix Table S6, not detectable by Western blotting, Appendix Fig S1). Hierarchical cluster analysis and unsupervised k-means clustering of the skeletal muscle RNA-seq transcripts did not discriminate different groups before and after treatment (Fig 3C, Appendix Fig S2). Surprisingly, the analysis of differentially expressed transcripts (Dataset EV3) and Reactome Pathway Analysis (Dataset EV4) showed a significant decrease in genes involved in oxidative phosphorylation, including MT-ND1, MT-ND2, MT-ND4, MT-ND5, and MT-CYB (Fig 3D and E). However, there was no change in the average mtDNA copy number in skeletal muscle with treatment (Appendix Fig S3). The mean percentage m.3243A>G mtDNA heteroplasmy in blood, urinary sediment and skeletal muscle did not change with treatment, although there was considerable variation in urinary sediment measurements (Fig 3F).

Clinical measures

Mean submaximal exercise testing parameters did not change after treatment. Actigraphy showed no change in accelerometry, activity or sleep cycles with treatment. There was no difference in the skeletal muscle 31P-MRS phosphocreatine recovery time (t1/2, PCr), adenosine diphosphate recovery time (t1/2, ADP), or the ratio (ADP/PCr) with treatment (Appendix Fig S4). However, treatment was associated with an increase in end-systolic volume and end-systolic

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<tr>
<th>Table 1. Clinical characteristics of the six patients with the m.3243A&gt;G MTTLI mutation and mitochondrial myopathy.</th>
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<td>MIDD: mitochondrially inherited diabetes and deafness; NMDAS, Newcastle Mitochondrial Disease Adult Scale.</td>
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https://www.readcube.com/articles/10.15252/emmm.201911589?no_additional_a... 04/03/2020
Figure 1. Summary of the study design.
Metabolic effects of bezafibrate in mitochondrial disease

Figure 2.
Metabolic effects of bezafibrate in mitochondrial disease

Figure 2. Metabolic effects of bezafibrate in patients with the m.3243A>G MTTL1 mutation. W0, W4, and W12 refer to the week of study.
A. Serum FGF-21 and GDF-15 levels before, during and after treatment (two-sided Wilcoxon signed-rank test, P-value = 0.031 in all significant cases) (see Fig EV2 for details where P1-P6 refer to the individual patients).
B. Volcano plot showing differences in metabolite levels between 0 and 6 and 12 weeks of treatment. Metabolites in histidine, alanine, aspartate and glutamine metabolism, and the TCA cycle pathways are annotated at 10% false discovery rate (see Dataset EV2 for the whole list of differentially regulated metabolites). Although some other metabolites showed a more pronounced difference, these did not fit into a recognised pathway. Metabolite labels are colour-coded according to the identified KEGG pathways (dark red = "Alanine, aspartate and glutamate metabolism", dark green = "Histidine metabolism" and dark blue = "Citrate cycle (TCA cycle)" with transition colour code if the metabolite belongs to more than one KEGG pathways).
C. Scatterplot depicting P-values from the metabolites Pathway Enrichment Analysis (x-axis) and impact values from Pathway Topology Analysis (y-axis) (see Dataset EV2 for details). KEGG pathways at 10% adjusted Holm-Bonferroni P-value are highlighted.
D. Volcano plot of asyl-carnitine metabolites between 0 and 6 and 12 weeks of treatment with highlighted differential metabolites at 10% FDR.
E. Respiratory chain complex and citrate synthase activity in skeletal muscle before and after 12 weeks of treatment. No significant differences were detected before and after 12 weeks of treatment by using the two-sided Wilcoxon signed-rank test with an empirical level of significance.

Data Information: in (a) and (f), horizontal dotted lines denote the mean values in healthy age-matched controls. In (f), solid horizontal lines and bars represent the mean ± SD of the technical replicates at each time point. Horizontal lines on (a) and (f) refer to statistical comparisons of the mean values, where ns = non-significant, "P-value ≤ 0.05.

index (Fig 3G). The mean questionnaire scores did not change following treatment, including the NMDAS and muscle-specific scores (Appendix Table S7). There was no correlation between the level of m.3243A>G heteroplasmacy or clinical features and the clinical or biochemical responses to treatment.

Discussion

As expected, we detected evidence of on-target effects of bezafibrate through the induction of fatty acid β-oxidation (Bonvento et al, 2010) and a trend towards decreased triglyceride levels. However, even using doses higher than typically used to treat dyslipidaemia, bezafibrate had a minimal impact on markers of mitochondrial biogenesis. Similar to other studies using PPAR agonists, we observed dose-dependent increases in serum GDF-15 and FGF-21 (Inagaki et al, 2007; Yatsuga & Suomalainen, 2012), which have been proposed as biomarkers of mitochondrial disease (Lehtonen et al, 2016). This raises the possibility that treatment led to an exacerbation of the mitochondrial pathology in patients with the m.3243A>G mutation, although given the lack of specificity of GDF-15 and FGF-21, it is also plausible that the changes we observed were due to the direct effects of bezafibrate on metabolism independent of oxidative phosphorylation.

Following bezafibrate treatment, we also saw an alteration of metabolic pathways involving glutamine metabolism and the tricarboxylic acid (TCA) cycle (Buzkova et al, 2018), as well as an increase in the level of several amino acids including histidine, alanine, aspartate and N-acetyl-aspartate (NAA) (Thompson Legault et al, 2015). Glutamine is an abundant circulating amino acid and has an anaplerotic role, replenishing TCA cycle intermediates, which drive the mitochondrial respiratory chain to generate reducing equivalents (Mullen et al, 2014; Chen et al, 2018). In the context of mtDNA mutations, glutamine is reduced by the TCA cycle leading to the synthesis of aspartate, which is essential for nucleotide synthesis (Chen et al, 2018). Thus, increased amino acid levels, including glutamate, could be part of a compensatory response to the primary OXPHOS defect (Buzkova et al, 2018; Chen et al, 2018). Similar findings have been observed for aspartate, with effects on reductive carboxylation (Chen et al, 2018; Sullivan et al, 2018). Likewise, increased histidine has been observed in models of mitochondrial disease (Vo et al, 2007; Chen et al, 2018), where it can act as a source of folate (Mao et al, 2004) and TCA cycle pre-intermediates such as glutamate, which are then degraded to α-ketoglutarate (Alfano et al, 1996). It is therefore possible that bezafibrate provokes or enhances an endogenous compensatory response to the primary metabolic defect that resembles a fasting response (Crooks et al, 2014; Tan et al, 2017), despite the patients being well-nourished throughout, and with no associated weight loss. However, given the observed increase in all three amino acids, it remains puzzling that, overall, the TCA metabolites decreased on treatment. The reasons for this are not clear, but could reflect the effects of increased aspartate levels, which are known to inhibit α-ketoglutarate utilisation (Chen et al, 2018), with knock-on effects on other TCA cycle intermediates.

Our study was not designed to detect significant changes in clinically meaningful parameters, but the data will provide a useful basis for future power calculations. It is encouraging that we observed an improvement in parameters of cardiac function on MRI that are known to be impaired in patients with m.3243A>G (Hollingsworth et al, 2012). The clinical significance of these findings is unclear at present, but they support the use of cardiac MRI as a biomarker to monitor treatment in mitochondrial diseases (Steele et al, 2017), which is sensitive to changes over a short period and correlates with the underlying biochemical defect in skeletal muscle (Hollingsworth et al, 2012).

Importantly, we observed considerable variability in several proposed biomarkers, including exercise physiology and m.3243A>G heteroplasmacy in urinary sediments, explaining why these do not always correlate with clinical severity. On the other hand, being sensitive to changes at the single-cell level, quadrupole immunofluorescence shows promise when compared bulk measures of respiratory chain protein abundance and function in skeletal muscle. We did not observe a similar change in either the respiratory chain activity or protein abundance on Western blots, probably because the changes only affected a small proportion of the individual muscle fibres, and were thus not detectable in an analysis of a muscle homogenate. Thus, although the quadrupole immunofluorescence may be a sensitive biomarker indicative of a treatment response, the clinical significance of these findings remains to be determined.
Figure 3.
It should be noted that our results are based on observations in a specific subgroup of patients with mitochondrial disease due to the m.3243A>G mutation. However, they do suggest that bezafibrate should be used with caution to treat dyslipidaemia in patients with mitochondrial disorders. Future studies inducing mitochondrial biogenesis through PPAR activation should proceed with caution, with careful monitoring for possible toxicity. Measuring blood metabolomic biomarkers, FGF-21, and GDF-15 in addition to serum creatinine kinase would provide some reassurance if levels do not change on treatment.

Materials and Methods

In overview, the following data were collected: (i) Measures of safety and tolerability at 0, 6, and 12 weeks including adverse events, haematological and biochemical blood tests, creatine kinase; FGF-21, GDF-15 and serum metabolomics; (ii) respiratory chain enzyme activity in skeletal muscle at baseline and 12 weeks; (iii) percentage of cytochrome c oxidase (COX) muscle fibres by histochemistry, quantification of respiratory chain complexes I and IV proteins NDUF8 and COX1 by quadruple fluorescence immunohistocytochemistry (Rocha et al, 2015) and Western blotting, all at 0 and 12 weeks; (iv) muscle RNA-seq at 0 and 12 weeks; (v) percentage heteroplasmy of the m.3243A>G in blood and urinary sediment at 0, 6, and 12 weeks, and muscle at 0 and 12 weeks; total mtDNA copy number in skeletal muscle at 0 and 12 weeks; (vi) submaximal exercise testing. $^{31}$P-magnetic resonance spectroscopy ($^{31}$P-MRS) in skeletal muscle, cardiac function by MRI, accelerometry and activity, timed up and go (TUG), and the following questionnaires: International Physical Activity Questionnaire (IPAQ), Newcastle Mitochondrial Disease Adults Scale (NMDAS), the Newcastle Mitochondrial Disease Quality of Life Score (NMQ), the Fatigue Impact Score (FIS), all at 0 and 12 weeks. See the Expanded View document for method details and citations.

Detailed methods

**Serum FGF-21 and GDF-15**

Blood samples were collected in a serum-separating tube at times 0, 6, and 12 weeks of the study. Samples were centrifuged at 5,000 g for 10 min, and the resulting serum was snap frozen. FGF-21 and GDF-15 were measured by ELISA from BioVendor and R&D systems, respectively, following manufacturer’s instructions.

**Serum metabolomics analysis**

Sample preparation, QA/QC and UltraHig Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS) were performed at Metabolon© as well as compound identification, quantification and data curation as described previously (Shih et al, 2014). A total of 867 different metabolites were measured on human serum from six patients over three-time points at 0 (untreated), 6 and 12 weeks (treated). Among the measured metabolites, only four were partially characterised (untargeted), whilst known (targeted) metabolites spanned a wide range of biochemical classes (“superpathways”) including 187 “Amino Acid” (22%), 27 “Carbohydrate” (3%), 28 “Cofactors and Vitamins” (3%), 11 “Energy” (1%), 409 “Lipid” (47%), 37 “Nucleotide” (4%), 37 “Peptide” (4%) and 127 “Xenobiotics” (15%). Normalisation and imputation of serum metabolites were performed as previously described (Shih et al, 2014; Kruusiek et al, 2015). Briefly, data normalisation (N) consisted of the following steps: (N.1) partially characterised metabolites and metabolites belonging to “Xenobiotics” biochemical class were removed from the analysis, reducing the number of targeted metabolites to 736; (N.2) each metabolite raw value was rescaled to have median 1 to adjust for variation due to instrument run-day tuning differences; (N.3) a log transformation with base 10 was applied to all the metabolites; (N.4) after transformation, data points lying more than four standard deviations from the mean of each metabolite concentration were excluded. For the imputation (I) of missing values, we employed the KNN-TN method (Shah et al, 2017), which consists of the following steps: (I.1) Estimation of the detection level (DL) of the machine to be the minimum observed value for the whole dataset; (I.2) maximum likelihood estimation (MLE) of $\mu_m$ and $\sigma_m$, assuming that each metabolite $m$ ($m = 1, \ldots, 736$) follows a left-truncated (on the DL) Gaussian distribution with mean $\mu_m$ and standard deviation $\sigma_m$; (I.3) standardisation of each metabolite using the MLEs of $\mu_m$ and $\sigma_m$; (I.4) for each metabolite $m$ with a missing value in sample $i$, detection of its $K$ closest metabolites (which have an observed value for their $i^{th}$ sample) using the K-nearest neighbours (KNN) algorithm; (I.5) imputation of the missing value with a weighted average of the $K$ values found in (I.4). The weights are functions of the Pearson correlations between the metabolite with missing values and its $K$