Mitochondrial antibiograms in personalized medicine

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Received August 27, 2012; Revised and Accepted December 3, 2012

Some ribosomal antibiotics used in clinical practice to fight pathogenic bacteria can provoke serious adverse drug reactions in patients. Sensitivity to the antibiotics is a multifactorial trait but the genetic variation of sensitive individuals to off-target effects of the drugs might be one of the factors contributing to this condition. Thus, the protein synthesis apparatus of mitochondria is similar to that of bacteria because of its endosymbiotic origin and, therefore, mitochondrial ribosomes are frequently unintended off-targets of these antibiotics. Because of the limitations of epidemiologic studies of pharmacogenomics, we constructed 25 transmitochondrial cell lines using platelets from individuals belonging to high-frequency European mitochondrial DNA (mtDNA) haplogroups and grew them in the absence or presence of commonly used ribosomal antibiotics. Next, we analyzed the mitochondrial synthesis of proteins and the mitochondrial oxygen consumption to ascertain whether some side effects of ribosomal drugs are due to their interaction with particular mtDNA haplogroup-defining polymorphisms. The amount of mitochondrial translation products, the p.MT-CO1/succinate dehydrogenase subunit A ratio and the ratio of respiratory complex IV quantity to citrate synthase (CS)-specific activity were significantly lower, after the treatment with linezolid, in cybrids harboring the highly frequent m.3010A allele. These results suggest that mitochondrial antibiograms should be implemented for at least the most frequent mitochondrial ribosomal RNA (rRNA) polymorphisms and combinations of polymorphisms and the most frequently used ribosomal antibiotics. In this way, we would obtain individualized barcodes for antibiotic therapy, avoid the side effects of the antibiotics and enable appropriate personalized medicine.

New sequence accession numbers: Genbank JX081995-JX082003 and JX092102.

INTRODUCTION

Adverse reactions associated with marketed drugs are estimated to be between the fourth and sixth leading cause of death in the USA and an important reason for hospitalization, leading to increased health care costs (1). Although the basis for drug-related toxicity of the ribosomal inhibitors is unknown, several lines of evidence point to mitoribosomes as the Achilles heel of ribosomal antibiotics (2).

A particular antibiotic binds to slightly different ribosomal pockets in somewhat different ways. In fact, this contributes to its selective effect on bacteria without affecting human beings (2–4). Some rare mutations in bacterial ribosomal RNAs (rRNAs) make them more similar to the human ones; decrease the binding of a particular antibiotic and cause resistance to the antibiotic (4). It is also possible that equally rare mutations in mitochondrial rRNAs (mt-rRNAs) could make them more similar to the bacterial rRNA, thus increasing susceptibility to a particular antibiotic and provoking toxic effects in individuals (5,6). Because of its endosymbiotic origin, the protein synthesis apparatus of the mitochondrion is very similar to that of bacteria, and mitochondria are frequently unintended off-targets of different ribosomal antibiotics used to fight bacteria. Thus, the human mitochondrial DNA (mtDNA) m.1555A>G/MT-RNR1 transition in the decoding site of the 12S rRNA increases its similarity to the bacterial small subunit rRNA, the binding of aminoglycosides to the

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drug-binding pocket and susceptibility to aminoglycoside-induced miscoding. The result can be non-syndromic hearing loss (7). However, this rare human mutation is completely fixed in Bornean and Sumatran orangutan species (8), and because these species diverged 400 000 years ago, this means that m.1555A>G is an ancient mutation in orangutans. Old age and high frequency are two criteria usually used to rule out an mtDNA mutation as pathological (9), but we have recently found that aminoglycosides negatively affect the orangutan oxidative phosphorylation (OXPHOS) function by decreasing the synthesis of mtDNA-encoded polypeptides and the amount and activity of respiratory complex IV (CIV). These drugs also diminish the growth rate of orangutan cells (8).

These observations of the ancient and fixed orangutan m.1555G allele raised questions about the effects on human health of the interactions between ribosomal antibiotics and mt-rRNA alleles similar to this one in orangutans. In the human mtDNA, ancient and highly frequent single-nucleotide polymorphisms (SNPs) define mtDNA haplogroups (groups of phylogenetically related mtDNA genotypes). Nearly half of the western European population belongs to mtDNA haplogroup H, defined by SNPs, including m.2706A in the MT-RNR2 gene that encodes the 16S rRNA. Subhaplogroup H1 encompasses approximately half of the H individuals and is characterized by the SNP m.3010A in the 16S rRNA. This SNP also defines subhaplogroup J1 (Table 1 and Supplementary Material, Fig. S1). The m.2706 and m.3010 positions are very close to the ribosomal peptidyl transferase center (PTC) (Supplementary Material, Fig. S2) (10). Several antibiotics, such as chloramphenicol and linezolid, bind to the bacterial PTC. Some patients treated with linezolid develop side effects, such as lactic acidosis, myelosuppression or neuropathy that are consistent with OXPHOS dysfunction (11). In fact, a decrease in mitochondrial protein synthesis by linezolid has been previously reported (12–17). Moreover, sequencing the mt-rRNAs of four patients with lactic acidosis after linezolid treatment suggested that SNPs at positions m.2706 and m.3010 might be associated with a higher susceptibility to linezolid (18,19). However, other nuclear DNA (nDNA) genetic and environmental differences together with the high frequency of these mtDNA SNPs made it difficult to draw any conclusions.

To ascertain whether some side effects of linezolid are due to its interaction with these mtDNA SNPs, we constructed transmitochondrial cell lines from different mtDNA haplogroups and analyzed diverse mitochondrial parameters in the absence or presence of this inhibitor of protein synthesis.

Table 1. Association between combinations of 16S rRNA polymorphisms and mtDNA haplogroups

<table>
<thead>
<tr>
<th>Haplogroup</th>
<th>m.2706</th>
<th>m.3010</th>
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<tr>
<td>H non-H1</td>
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<td>Uk</td>
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RESULTS

The inhibition of mitochondrial protein synthesis by linezolid depends on the mtDNA haplogroups

We first measured the effect of linezolid on mitochondrial protein synthesis. The linezolid dose used in clinical practice is 600 mg every 12 h. The steady-state peak serum concentrations (44.5–80.0 μM) are reached 0.5–2 h after oral administration. Plasma linezolid concentrations remain above the minimum inhibitory concentration 90 (MIC90) for susceptible target pathogens (11.9 μM) for the majority of the 12 h dosing interval (20). To be sure that the linezolid concentrations used in our work could be found in the cell environment of the patients’ tissues, and therefore, have physiological relevance, we grew our cybrids at five different linezolid concentrations (10, 5, 2.5, 1 and 0.1 μM). Then, we determined the p.MT-CO1 levels normalized to the succinate dehydrogenase subunit A (SDHA) amount. A concentration of 10 μM linezolid decreased the p.MT-CO1/SDHA ratio by 50% (Fig. 1A). Moreover, there was a significant negative correlation (r = −0.94, P = 0.013) between linezold concentrations and p.MT-CO1/SDHA ratios (Fig. 1A), confirming the effect of clinically relevant linezolid concentrations on mitochondrial protein synthesis.

Next, to examine the effect of the mtDNA genetic background on the inhibition of mitochondrial protein synthesis by linezolid, we divided the 20 cybrid cell lines according to the mtDNA haplogroup. A decrease in the p.MT-CO1/SDHA ratio as a function of the linezolid concentration was observed for each haplogroup (Fig. 1A). However, haplogroups T and J1 were apparently more resistant and susceptible, respectively, to the linezolid effect than the other groups. At 2.5 μM linezolid, the mtDNA haplogroup and the p.MT-CO1/SDHA ratio were not independent variables (P = 0.0034, ANOVA) (Fig. 1A). Because the effect of the mtDNA genetic background was clearly observed for 2.5 μM linezolid, we used this antibiotic concentration for the next analyses.

To test whether the linezolid effect was specific for p.MT-CO1 or extended to all mtDNA-encoded polypeptides, we performed mitochondrial protein synthesis assays. We found no significant differences in the amount of mitochondrial translation products among mtDNA haplogroups (Fig. 1B and Supplementary Material, Fig. S3). However, we observed a pattern similar to that found for the p.MT-CO1/SDHA ratios. Thus, haplogroups H non-H1 and T were more resistant, and haplogroups H1 and J1 more susceptible to the inhibitory effect of linezolid (Fig. 1B). Very often, when mitochondrial protein synthesis is estimated by metabolic labeling in cultured cells with radioactive methionine, no decrease in the protein synthesis rate can be detected for mtDNA pathologic mutations affecting genes involved in this process (21). Interestingly, we detected a positive and significant correlation (r = 0.518, P = 0.0181) between the amount of mitochondrial translation products determined by metabolic labeling and the p.MT-CO1/SDHA ratio determined spectrophotometrically.

MtDNA-encoded CIV polypeptides are the catalytic subunits of CIV and are also required for the assembly of this electron transport chain (ETC) complex. Although there were no...
Figure 1. Effect of linezolid on different OXPHOS parameters according to the mtDNA haplogroup. OXPHOS parameters were determined in each cybrid cell line growing without or with antibiotic. The control value obtained for each cybrid without the antibiotic was expressed as 100%, and the value from the treated cells was expressed as a percentage of the control value. (A) p.MT-CO1/succinate dehydrogenase subunit A (SDHA) ratio. Dashed lines represent the mean of 20 cybrid cell lines. There is a significant and negative correlation ($r = -0.94$, $P = 0.013$) between linezolid concentrations and p.MT-CO1/SDHA ratios. The p.MT-CO1/SDHA ratio was dependent on the mtDNA haplogroup at 2.5 μM linezolid ($P = 0.0034$, ANOVA). The asterisk denotes that the p.MT-CO1/SDHA ratio was significantly lower than that from haplogroup T ($P \leq 0.0473$, Fisher’s protected least significant difference [PLSD]). (B) Levels of mtDNA-encoded polypeptides. (C) CIV-specific activity/CS-specific activity ratio. (D) CIV quantity/CS-specific activity ratio. This ratio was dependent on the mtDNA haplogroup ($P = 0.0296$, ANOVA). The asterisk and square denote that this ratio was significantly lower than that from haplogroup T ($P < 0.0443$, Fisher’s PLSD) or H non-H1 ($P \leq 0.0213$, Fisher’s PLSD), respectively. (E) Oxygen consumption normalized by CS-specific activity.
significant differences in the ratios of the specific activities of CIV/citrate synthase (CS) among the mtDNA haplogroups, the pattern of higher ratios for cybrids from haplogroups H non-H1 and T and lower for haplogroups H1 and J1 (Fig. 1C) was again observed. The same pattern was also observed for the ratios of CIV quantity/CS-specific activity, and there was a positive and significant correlation (r = 0.595, P = 0.0047) between these two ratios. However, there were significant differences (P = 0.0296, ANOVA) among the mtDNA haplogroups (Fig. 1D).

Respiratory complexes, with mtDNA-encoded subunits, transport electrons through the ETC to finally reduce oxygen to water. Therefore, we next evaluated the effect of linezolid on the oxygen consumption in cybrids from different mtDNA haplogroups, but we did not find any significant differences (Fig. 1E). However, the pattern previously observed was maintained; thus, haplogroups T and H non-H1 showed higher oxygen consumption than haplogroups H1 and J1. Moreover, oxygen consumption positively and significantly correlated with the ratio of specific activities of CIV/CS (r = 0.667, P = 0.0009) and with the ratio of CIV quantity/CS-specific activity (r = 0.483, P = 0.0299).

Two mitochondrial 16S rRNA polymorphisms modify susceptibility to linezolid

To verify that the differential effect of linezolid on mitochondrial protein synthesis was mediated by polymorphisms at the m.2706 and m.3010 positions and not a nonspecific response due to other SNPs defining these mtDNA haplogroups, we took two different approaches. First, we determined the p.MT-CO1/SDHA ratio after treatment with paromomycin (2.8 mM). This antibiotic binds the mitochondrial 12S rRNA in the ribosomal subunit opposite to the locations of linezolid binding and these SNPs. It was previously shown that exposure to this concentration of paromomycin caused an 8% average increase in doubling time of control cell lines (22). However, we did not find any differences in the p.MT-CO1/SDHA ratio among the mtDNA haplogroups (Fig. 2A).

Next, to confirm the pivotal role of these SNPs, we constructed five more cybrids from mtDNA haplogroup Uk. This haplogroup contains 19 SNPs, including those at the m.2706 and m.3010 positions, far from H1. However, although haplogroup Uk shares the positions m.2706 and m.3010 with haplogroup T, it is 27 SNPs away from this haplogroup (Supplementary Material, Fig. S1). Interestingly, the p.MT-CO1/SDHA ratios of cybrids from haplogroup Uk, for all linezolid concentrations, were very similar to those of haplogroup T. At 2.5 μM linezolid, haplogroups Uk and T were significantly different from H1 and J1 (Fig. 2B). These results suggest that the two SNPs, m.2706 and m.3010, were responsible for the modulation of linezolid susceptibility.

The nucleotide at the m.3010 position has a significant effect on the inhibition of mitochondrial protein synthesis by linezolid

The previous results already suggested that the effects of the SNPs at the m.2706 and m.3010 positions on the inhibition of mitochondrial protein synthesis by linezolid were different. To evaluate the contribution of each SNP to linezolid susceptibility, we compared all the studied variables between cybrids harboring: (1) m.2706G (non-H lineages) or m.2706A (haplogroup H, including H1 and non-H1) and (2) m.3010G (non-H1 and non-J1 lineages) and m.3010A (haplogroups H1 plus J1). There were no significant differences between cybrids from haplogroup H and the rest (Fig. 3A). However, the amount of mitochondrial translation products, the p.MT-CO1/SDHA ratio and the ratio of CIV quantity to CS-specific activity were significantly higher in cybrids with m.3010G (Fig. 3B).

Interaction of these polymorphisms with other PTC antibiotics

Different antibiotics will bind the same ribosomal target in slightly different ways. The binding of different antibiotics to slightly different targets might produce different results. To examine the effect of these mt-rRNA SNPs on other PTC antibiotics, we chose chloramphenicol. This antibiotic produces side effects similar to those of linezolid. Serum concentrations of chloramphenicol should be maintained at 46.4–92.8 μM to ensure an adequate therapeutic level and avoid toxicity (23). Because 2.5 μM linezolid represents a 21.0% of the lower limit of its therapeutic range, we used a chloramphenicol concentration of 10 μM (21.6% of the lower limit of its therapeutic range) to assess the effects of this antibiotic. There were no significant differences (P = 0.053) in the p.MT-CO1/SDHA ratio among the mtDNA haplogroups, but this ratio was significantly lower in cybrids from haplogroup H1 than in cybrids from haplogroups H non-H1, Uk and T (Fig. 4).

DISCUSSION

The mutation rate of human mtDNA is very high (9). If the mutations affect the antibiotic binding sites in the mt-rRNA genes and modify the susceptibility to these drugs, they can have medical consequences.

The m.2939C>A transversion in the MT-RNR2 gene was isolated in a chloramphenicol-resistant human cell line (24). The m.2991T>C/MT-RNR2 transition has been described in three different chloramphenicol-resistant human cell lines (24–26). In contrast, the m.1494C>T and m.1555A>G transitions in the MT-RNR1 gene increase the susceptibility to aminoglycosides (27,28) and although they are pathologic mutations, they are not very rare. Thus, m.1494C>T has been reported in 24 Chinese and Spanish pedigrees showing non-syndromic deafness (29,30). Moreover, the frequency of the m.1555A>G mutation in English or Australian populations is approximately one in 500 individuals (31,32) and one in 600 in the Chinese population (33). An analysis of 2460 complete mtDNA sequences showed that 315 out of 2512 mitochondrial rRNA (12S and 16S mt-rRNAs) positions were variable (10). It is possible that some of these rRNA polymorphisms affect interactions with antibiotics, although they show a high population frequency.

In fact, here we show that the m.3010G>A polymorphism, present in ~25% of the western European population
(haplogroups H1 and J1), rebuilds a base pair in the mitochondrial 16S rRNA helix 48, decreases the synthesis of mtDNA-encoded polypeptides in cells treated with linezolid and might be responsible in part for the side effects associated with this antibiotic. This polymorphism also defines mtDNA haplogroup D4, which represents 14.9% of the Han Chinese population (34) and is present in many Asian and Native American populations. Moreover, m.3010G>A also defines the African mtDNA haplogroup L2a1c that encompasses, for example, 3.9% of the Lake Chad Basin population (35) and is present in many other African populations. Thus, the m.3010G>A transition is found, at moderately high frequencies, all around the world. Sometimes this change will be the only MT-RNR polymorphism, but on other occasions it will be accompanied by another variant or variants that might affect its interaction with PTC antibiotics. This might explain why the m.2706G>A transition contributes to the large differences in the p.MT-CO1/SDHA ratios of cybrids from haplogroups H1 and J1 treated with chloramphenicol.

The number and frequency of similar, detrimental mt-rRNA SNPs could be very high because it is possible that these SNPs were phenotypically neutral in the absence of antibiotics, and because ribosomal antibiotics only began to be used in the 1940s, negative selection has not had time to remove these genetic variants from the population even if the interactions with the drugs were deleterious. Therefore, all these observations suggest that mitochondrial antibiograms should be implemented for at least the most frequent mt-rRNA polymorphisms and combinations of polymorphisms and the most frequently used ribosomal antibiotics. In this way, we would obtain something similar to individualized barcodes for antibiotic therapy, avoid the side effects of the antibiotics...
and enable appropriate personalized medicine (5,6). The positive and significant correlation that we have found between the results of the laborious and semiquantitative metabolic labeling technique and the easy and quantitative p.MT-CO1/SDHA ratio suggests that, for high-throughput analyses, the latter procedure can replace the former.

MATERIAL AND METHODS

Cybrids and growth conditions

To homogenize nuclear and environmental factors, we used transmitochondrial cell lines (cytoplasmic hybrids or cybrids) with the osteosarcoma 143B rho0 nuclear background (36). Twenty cybrids were constructed by fusing platelets, with mitochondria and mtDNA, from selected individuals (36). Twenty cybrids were also built (Table 1 and Supplementary Material, Fig. S1). Thus, these cybrid cell lines only differed in the mtDNA haplogroup. OXPHOS parameters were determined in each cybrid without the antibiotic expressed as 100%, and the value from the treated cells was expressed as a percentage of the control value. Asterisk, triangle and square denote that this ratio was significantly lower than that from haplogroup H non-H1, Uk and T (P < 0.0200, Fisher’s PLSD).

Figure 4. Effect of chloramphenicol on the p.MT-CO1/SDHA ratio according to the mtDNA haplogroup. OXPHOS parameters were determined in each cybrid cell line growing without or with antibiotic. The control value obtained for each cybrid without the antibiotic was expressed as 100%, and the value from the treated cells was expressed as a percentage of the control value. Asterisk, triangle and square denote that this ratio was significantly lower than that from haplogroup H non-H1, Uk and T (P < 0.0200, Fisher’s PLSD).

Genetic analysis

Most of the mtDNA sequences were obtained by using previously described methods (38,39). The Genbank accession numbers are FJ178379, HM103354–HM103363, JN635298, JN635300–JN635302, JX081995–JX082000, JX092102. Three sequences (JX082001–JX082003) were determined following the protocols from GeneChip® Human Mitochondrial Resequencing Array 2.0 (Affymetrix, Santa Clara, CA, USA). To locate mutations, the human revised Cambridge reference sequence was used (NC012920).

Biochemical analysis

Oxygen consumption in intact cells was analyzed using the high-resolution oxygraph OROROBOS®, according to the previously defined protocols (38), although in this case, this parameter was measured using the galactose growth medium without FBS.

The specific activity and quantity of CIV were measured by using Mitoprofile® Human Complex IV Activity and Quantity kit from Mitosciences (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. To correct for potential differences in the cell fraction occupied by the mitochondria, we normalized CIV-specific activities and quantities to the specific activity of CS, an nDNA-encoded enzyme of the mitochondrial matrix. The mitochondrial inner membrane mtDNA-encoded p.MT-CO1 and the nDNA-encoded SDHA polypeptide levels were measured using the MitoBiogenesis® In-Cell ELISA kit from Mitosciences (Invitrogen) according to the manufacturer’s instructions. These variables, together with the previous ones and the protein levels, were assayed following previously described protocols in a NovoStar MBG Labtech (Offenburg, Germany) microplate instrument (39,40). The observed p.MT-CO1/SDHA ratio significantly correlates (r = 0.93, P = 0.0044) with the expected one from a mix of different amounts of rho0 (0, 20, 40, 60, 80 and 100%) and rho- (100, 80, 60, 40, 20 and 0%) cells.

The mitochondrial protein synthesis was analyzed as described previously with minor modifications (38). Three bands, corresponding to p.MT-ND5, p.MT-CO3 and p.MT-ND3 polypeptides (upper, middle and lower part of the gel, respectively) were selected for quantification.

Statistical analysis

The statistical package StatView 6.0 was used to perform all of the statistics. OXPHOS parameters were determined in each cybrid cell line growing without or with antibiotic. The control value obtained from each cybrid without the antibiotic was expressed as 100%, and the value from the treated cells was expressed as a percentage of the control value. Data for mean, standard deviation and sample size are presented. The normal distribution was assessed by the Kolmogorov–Smirnov test. One-way ANOVA and protected least significant differences (PLSDs) were performed for haplogroups’ analyses. For comparing polymorphisms, the unpaired two-tailed t-test was used. Correlations were determined by a z-score. The P-values <0.05 were considered statistically significant.


