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Mitochondrial pharmacogenomics: barcode for antibiotic therapy

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Ribosomal RNA (rRNA)-targeting drugs inhibit protein synthesis and represent effective antibiotics for the treatment of infectious diseases. Given the bacterial origins of mitochondria, the molecular and structural components of the protein expression system are much alike. Moreover, the mutational rate of mitochondrial rRNAs is higher than that of nuclear rRNAs, and some of these mutations might simulate the microorganism's rRNA structure. Consequently, individuals become more susceptible to antibiotics, the mitochondrial function is affected and toxic effects appear. Systems are available to analyze the interaction between antibiotics and mitochondrial DNA genetic variants, thus making a pharmacogenomic approach to antibiotic therapy possible.

Antibiotics and ribosomal RNAs

Antibiotics represent an impressive chemical arsenal for therapeutic intervention against pathogens. Only those compounds altering fundamental cell processes, with important negative consequences for pathogen survival, and with no side-effects in humans will be considered as potentially useful antibiotics. Approximately 40% of antibiotics interfere with bacterial protein biosynthesis [1] and target functionally important sites on the ribosome. The ribosomes are ribozymes because the most important functions are carried out by the ribosomal RNA (rRNA). Thus, the decoding site, the peptidyl transferase center (PTC) and the protein exit tunnel are formed by rRNAs, and most ribosomal antibiotics interact primarily with these rRNAs [2–4].

Protein synthesis is a key and universal process, and the high evolutionary conservation of functional sites within rRNAs, targeted by ribosomal drugs, implies limitations with respect to selectivity and toxicity [5]. Still, comparisons of rRNA sequences from bacteria and eukaryotes have shown subtle differences in these locations, despite their conservation, and these minute differences might lead to drug selectivity [3]. Thus, a single nucleotide can determine the selectivity of drugs affecting protein synthesis [5]. However, a multitude of different nucleotides located within the rRNA participate in the binding of a drug to its target region; correspondingly, several different nucleotide substitutions can be associated with selectivity and toxicity of the drug [5]. As a result, the same antibiotic might bind in different modes to slightly different ribosomal pockets, and this can be influenced not only by the often-conserved nucleotides of a functional site targeted by an antibiotic but also by the less-conserved peripheral rRNA residues [4].

Some mutations in the rRNAs of microorganisms modify the antibiotic-binding site and yield acquired drug resistance. Although the acquisition of a resistance mutation often imposes a toll on the fitness of the resistant microorganism, compensatory mechanisms can alleviate this and block the reversion to the sensitive phenotype [6]. Some resistance mutations in bacteria are present in human wild-type rRNA and can account for the antibiotic selectivity [7]. Therefore, the development of new antibiotics to deal with these resistant microorganisms will be hampered because they could increase their mitochondrial toxicity. Moreover, if bacterial rRNA mutations can induce resistance to an antibiotic, human rRNA mutations that mimic bacterial wild-type rRNA nucleotide could increase toxicity and be responsible for side-effects after the antibiotic therapy. Thus, knowledge of the

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interaction of particular antibiotics with the different human rRNAs is crucial to avoid antibiotic toxicity.

Human cells contain cytosolic and mitochondrial ribosomes. The mitochondrial genome originated from an eubacterial ancestor, and the mitochondrial DNA (mtDNA)-encoded rRNAs (mt-rRNAs 12S and 16S) are much more similar to the prokaryotic ones (16S and 23S rRNAs) than to the nuclear-encoded ones (18S and 28S rRNAs) [8]. Because eubacteria cause most infectious diseases and antibiotic therapy has been developed to fight them, mt-rRNA sequence variation should be taken into account to avoid antibiotic toxicity.

OXPHOS, mtDNA, mitoribosomes and mitochondrial rRNAs

Mitochondria are intracellular organelles that contain several compartments: outer membrane, intermembrane space, inner membrane and matrix. In the inner membrane is located the oxidative phosphorylation (OXPHOS) system, which includes the electron transport chain (ETC) and the ATP synthase (complex V) (Fig. 1a) and which is the key for the survival of the cell and for accommodating the environment conditions. In fact, external signals, in the form of nutrients and oxygen, interact at the OXPHOS level and trigger intracellular responses mediated by second messengers, such as the red-ox state or the ATP, Ca^{2+} and reactive oxygen species (ROS) levels, and can modify the expression of many nuclear and mitochondrial genes.

In the mitochondrial matrix, there are several molecules of supercoiled, closed, circular DNA known as mtDNA. This DNA encodes for 13 fundamental OXPHOS subunits (Fig. 1b) found from bacteria to humans. As an example, the seven mtDNA-encoded complex I subunits are the only components of the membrane arm of bacterial complex I, and the three mtDNA-encoded complex IV subunits are the only ones present in bacterial complex IV [9]. The rest of the mitochondrial proteins are encoded in the nucleus, synthesized in the cytosol and imported into the mitochondria. During evolution, the DNA from the endosymbiont decreased its size because redundant genes were lost, many other genes of the primitive bacteria were transferred to the nuclear DNA and the remaining ones were reduced in size [10]. Thus, the mitochondrial ribosomes (mitoribosomes) lack the 5S rRNA, their proteins are now nuclear encoded, and the size of the mitochondrial 12S (954 nucleotides) and 16S (1558 nucleotides) rRNA genes (MT-RNR1 and MT-RNR2) is approximately half as large as the bacterial rRNA genes [11]. However, both rRNAs are mitochondrially encoded in all the sequenced mtDNAs and encode for the most important rRNA domains, such as the decoding site or the PTC.

Human 55S mitoribosomes contain two subunits of 28S and 39S. The small one comprises 12S rRNA and 28 proteins, and the large contains the 16S rRNA and 48 proteins. Surprisingly, the rRNA:protein ratio in mitoribosomes is different (1:2) to that in cytosolic and bacterial ribosomes (2:1). Mitoribosomes contain higher protein content and lack several of the major rRNA structures of bacterial ribosomes. Despite being encoded in the nuclear chromosomes, the mitoribosome proteins evolve faster than those of cytosolic ribosomes [11]. It is possible that the faster evolution by oxidative mutagenesis of the mtDNA enables the mt-rRNAs to compensate for new changes in the mitochondrial ribosome proteins [12].

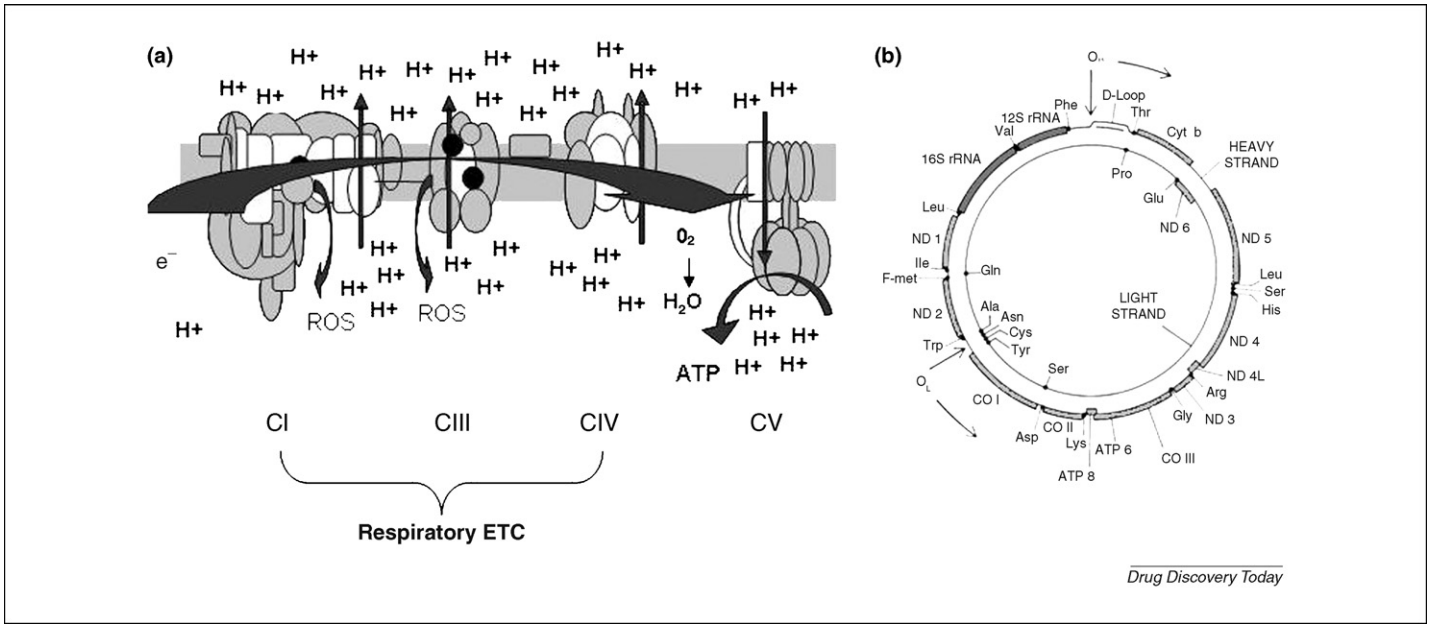

FIGURE 1

Scheme of the oxidative phosphorylation system and mitochondrial genome. **(a)** Oxidative phosphorylation system. The respiratory complexes (CI, NADH: ubiquinone oxidoreductase; CIII, Ubiquinol: cytochrome *c* oxidoreductase and CIV, cytochrome *c* oxidase), plus ubiquinone and cytochrome *c* (components of the ETC), transfer electrons to finally reduce oxygen to water. These complexes pump protons to the intermembrane space, creating an electrochemical gradient. When protons come back to the mitochondrial matrix through CV, ATP is synthesized. Occasionally, electrons can be donated to oxygen in CI and CIII, thus producing reactive oxygen species (ROS). Moreover, this gradient can be used for other purposes, such as thermogenesis, apoptosis, protein or substrate import and maintenance of cellular calcium levels. **(b)** Genetic map of human mitochondrial DNA (mtDNA). MtDNA encodes seven (ND 1–6 and ND 4L) of the 46 subunits of complex I, 1 (Cyt b) of the 11 subunits of complex III, 3 (CO I–III) of the 13 proteins that compose complex IV and 2 (ATP 6 and 8) of the 16 subunits of complex V, plus 22 transfer RNAs (tRNAs), defined by the three-letter code, and two rRNAs (12S and 16S rRNAs) required for the expression of the mtDNA-encoded OXPHOS subunits.

Antibiotics, side-effects and mitochondrial protein synthesis

Antibiotic therapy is based on selective toxicity, meaning that the microorganism but not the host should be affected. However, because of the bacterial origin of mitochondria, some antibiotics could also act on mitochondrial protein synthesis and, therefore, have side-effects for human beings. Thus, chloramphenicol and oxazolidinones, acting on the PTC, produce myelosuppression [13,14] and cause lactic acidosis and optic and peripheral neuropathy, phenotypes frequently found in mitochondriopathies [15,16]. By using human tissues or cells, after treatment with these antibiotics, cell growth, mitochondrial mass, respiratory complex activities, levels of mtDNA-encoded p.MT-CO1 and CO2 subunits, and mitochondrial protein synthesis are decreased, and these parameters tend to return to normal when the antibiotic is removed [17–19]. Cell growth inhibition does not happen in rho0 cells, which are not dependent on OXPHOS energy [14]. Another inhibitor of the large-subunit rRNA, erythromycin, also impairs cell growth in galactose medium and mitochondrial protein synthesis and might have accelerated a bioenergetics crisis in a patient with Leber's hereditary optic neuropathy disease [20]. Moreover, resistance to this antibiotic is sometimes cytoplasmically inherited [21].

Tetracyclines and aminoglycosides, acting on the small-subunit rRNA, can provoke side-effects in humans. Tetracycline treatment has been shown to produce immunosuppressive effects and to affect cytochrome oxidase activity in concanavalin-A-simulated thymocytes [22] and -HepG2 cells [23]. Aminoglycosides are both nephrotoxic and ototoxic. Although renal impairment is, in general, mild and reversible, ototoxicity is irreversible [24,25].

Among other factors, tissue-specific side-effects of an antibiotic might also be because of the physiological particularities of a given tissue. For example, OXPHOS provides most of the cell ATP, but energy demands differ from one tissue to another. The nervous system is very dependent on mitochondrial energy, and optic neuropathy and sensorineural hearing loss are very common manifestations of mitochondrial syndromes owing to mtDNA mutations [26]. Curiously, toxic optic neuropathy and ototoxicity are sometimes found after the administration of different antibiotics, such as macrolides [27] or chloramphenicol [28].

Antibiotics and rare mt-rRNA genetic variants

The mutational rate of the mtDNA is much higher than that of nuclear DNA, and mt-rRNAs are probably the Achilles heel of ribosomal antibiotics [29]. Therefore, the population variation in the mt-rRNAs, simulating the prokaryote rRNA, might introduce variability in the toxicity of these drugs. How is it possible, then, that toxicity produced by antibiotics often remains undetected until a large number of patients have been exposed? If toxicity depends on particular genetic variants, the frequency of these variants will determine the prevalence of adverse events, and it might not be revealed by even a large Phase III trial. As an example, mtDNA is maternally inherited and evolves along human lineages, accumulating new mutations. Thus, the latest mutations originated in mtDNA will be very recent and rare in the population and will affect only one individual or pedigree. The m.3096T>C/MT-RNR2 transition has been observed once in the analysis of 2959 mtDNA sequences (0.034%) [30]. If an antibiotic produced an adverse event in 0.034% of the patients, it would be necessary to expose more than 30,000 patients before the probability of the event became evident [31]. This transition (Fig. 2) is equivalent to Ec.2609T>C in *Escherichia coli*. This position, located less than 4.4 Å away from the 11-OH and 12-OH groups of the

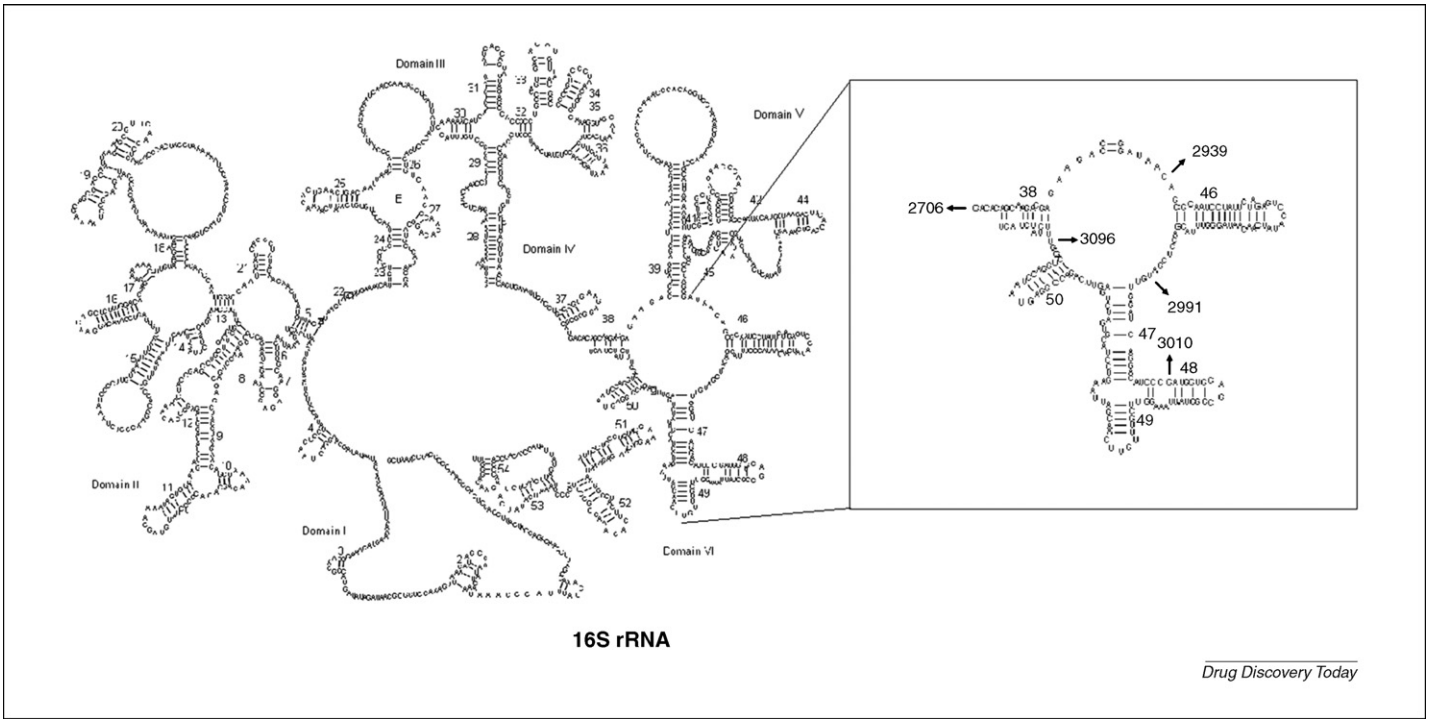

FIGURE 2

Mitoribosome large-subunit rRNA. The secondary structure of the 16S rRNA is represented, highlighting the peptidyl transferase center (PTC) and the nucleotide positions discussed in the text.

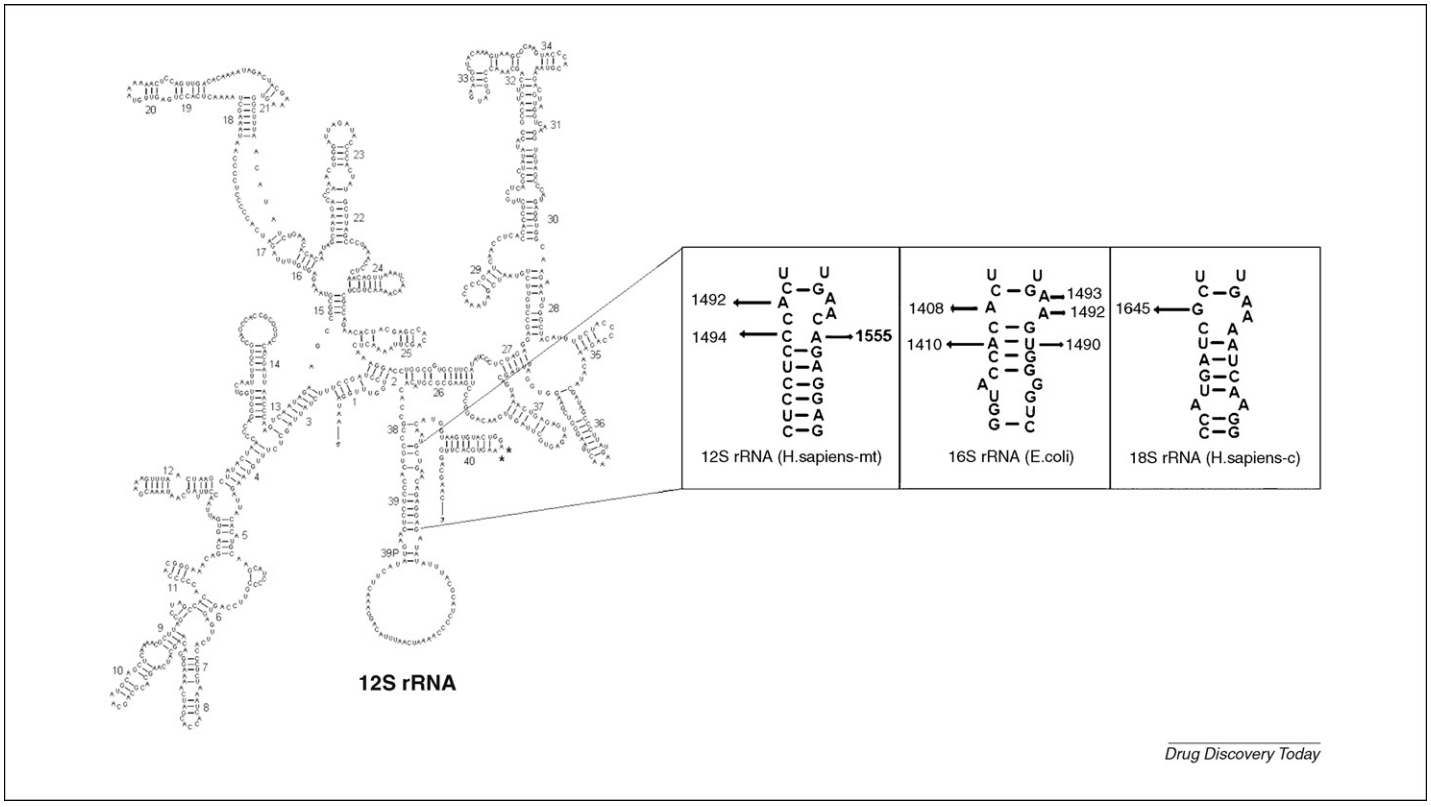

FIGURE 3

Mitoribosome small-subunit rRNA. The secondary structure of the 12S rRNA is represented, highlighting the decoding site. A comparison of this site in bacterial, cyto- and mitoribosomes is shown. Nucleotide positions discussed in the text are indicated. Asterisks in loop 40 denote methylated positions.

lactone ring from the macrolides [2], facilitates the shift of the antibiotic to the high-affinity site [32], decreases the minimal inhibitory concentration, and increases affinity to erythromycin and azithromycin. This slightly increases sensitivity to these antibiotics [33].

Another example is familial susceptibility to aminoglycosides. During decoding, a minihelix is formed between the messenger RNA (mRNA) codon and the cognate aminoacyl-transfer RNA (tRNA) anticodon. The two conserved adenines (Ec.1492A and Ec.1493A) of the A-site, folded back within the helix ('off' state), are flipped out and interact with the cognate codon–anticodon minihelix ('on' state). This conformational change determines the continuation of translation. The aminoglycosides stabilize the 'on' state even in the absence of the cognate tRNA–mRNA complex, the affinity of this site for a non-cognate tRNA increases, efficient discrimination is prevented and proteins with wrong sequences are produced [34,35]. The aminoglycoside selectivity is largely because of a single nucleotide position within the rRNA. The cytoribosomes are insensitive to the aminoglycosides because of the presence of a c.1645G [36] (Fig. 3). The sensitivity of the prokaryote ribosomes is due to the presence of an Ec.1408A in *E. coli*'s 16S rRNA, and the bacteria can become resistant to aminoglycosides by mutating this position to the eukaryotic nucleotide (Ec.1408A>G) [29]. Human mitochondrial 12S rRNA has an A in the equivalent position, m.1492A, and is sensitive to aminoglycosides. Moreover, two mt-rRNA mutations have been associated with familial aminoglycoside-related hearing loss (m.1494C>T and m.1555A>G) [37,38]. The equivalent positions in the bacterial 16S rRNA, which form a Watson–Crick base pair (WCbp), are important for aminoglycoside binding. Wild-type mitochondria lack the WCbp, but the pathologic mutations rebuild it and increase its similarity to its eubacterial homologue, enabling aminoglycoside binding [39–41]. If these mutations were deleterious when associated with aminoglycosides, they would have been phenotypically neutral until the beginning of the antibiotics era. As an example, deafness associated with m.1555A>G is very common in Spain owing to the acquisition of the mutation from a common maternal ancestor. Because pedigrees with this mutation were not related in the four previous generations, the female ancestor in whom the mutation appeared cannot be very recent, the selection against this mutation must be low and it could be transmitted for many generations [42]. Something similar happened for the m.1494C>T mutation. The transition was found in a normal individual from a population study and defines a small cluster in mtDNA haplogroup A [43]. Therefore, it cannot be very pathologic and antibiotics, or other factors, are probably necessary to trigger the hearing loss phenotype.

Despite the low frequency of each of the mutations that potentially increase antibiotic susceptibility, together they might be an important health problem. In fact, an analysis of 2460 mtDNA sequences and Mitomap (http://www.mitomap.org) has shown that 315 of the 2512 mt-rRNA positions (12.5%) are polymorphic [44], although most of them will not affect the interaction with antibiotics.

Antibiotics and common mt-rRNA genetic variants

Measurable signs of hearing loss are found in 20% of patients receiving aminoglycosides [24], although the frequency of the

previously mentioned m.1494C>T and m.1555A>G mutations is low. Therefore, other mt-rRNA variants with higher population frequencies could behave as susceptibility factors. Many mitochondrial genetic variants are ancient mutations that survived and expanded in populations. These polymorphisms define mitochondrial haplogroups (clusters of phylogenetically related mitochondrial genotypes) and are relatively frequent in human populations. Thus, m.827A>G and m.1095T>C mutations are found in different aminoglycoside-related hearing loss pedigrees [45,46], but these mutations define important mtDNA haplogroups [30,44].

Which mt-rRNA population polymorphisms will affect the interaction with antibiotics? As we have seen, antibiotics bind in functionally important positions. Frequently, in the absence of the antibiotic, the bacterial biological efficiency is decreased in the resistant genotype, meaning that this genotype has a phenotypical effect. In fact, this approach has been proposed for the finding of new rRNA targets [47]. Therefore, the association of a human phenotype with mt-rRNA variants could be a way to look for genetic variants that might affect the interaction with antibiotics. For example, the position m.2706A defines several small clusters of haplogroups L0, D4, U1811 and J1c and the whole haplogroup H. Although the last one is not the oldest of the Western Eurasian haplogroups, it is paradoxically the most frequent (approximately 45%). Moreover, this haplogroup has been associated with increased survival after sepsis and it has been suggested that this nucleotide position could have a subtle effect on mitochondrial protein synthesis, which is unmasked during sepsis [48]. The m.2706A>G transition has been proposed as predisposing individuals to linezolid-associated lactic acidosis [49]. Interestingly, this nucleotide is located at helix 38 of the mitochondrial 16S rRNA, very close to the PTC (Fig. 2). A potential mitochondrial resistance, originated by this nucleotide, to antibiotics used in intensive care units might be responsible for the increased survival of patients with this haplogroup because recovery after sepsis is directly related to physiological reserve, and this is crucially dependent on mitochondrial function [48]. Another 16S rRNA mutation that might influence susceptibility to antibiotics is the m.3010G>A variant. This mutation defines several clusters in the haplogroups L2a, L3, C, R* and U3 and major mitochondrial subhaplogroups, such as H1, J1 and D4. The last two of these have been shown to be over-represented in centenarians [50]. This variant rebuilds a base pair in a short stem (helix 48), which is part of the ribosomal A-site (Fig. 2). Mutations in this stem confer resistance to avilamycin and evernimicin [51]. Moreover, this m.3010G>A transition has been found in one individual that developed lactic acidosis after treatment with linezolid [49].

Because the side-effects are not very frequent, although antibiotics are widely used and mitochondrial haplogroups show high frequencies in the populations, other factors must be involved in generating toxic effects.

Mitochondrial susceptibility to antibiotics is probably a multifactorial phenotype

Streptomycin affects mitochondrial translation in cells with the HeLa cervical carcinoma nuclear background and the mtDNA m.1555G>A mutation [52], but it does not have this effect on cells with the 143B osteosarcoma nuclear background and the same mtDNA mutation [53]. Therefore, the nuclear genetic background will probably influence the toxicity produced by antibiotics. As an example, TFB1M is a mitochondrial transcription factor and it is closely related to rRNA methyltransferases. It has been shown that TFB1M expression in *E. coli* lacking the Ksg rRNA methyltransferase restores dimethylation of two adjacent adenine residues in a stem–loop structure of the bacterial 16S rRNA and reverses resistance to the antibiotic kasugamycin [54]. These residues, 28 nucleotides downstream from the pathogenic m.1555G>A mutation, are evolutionarily conserved in the mitochondrial 12S rRNA [55] (Fig. 3).

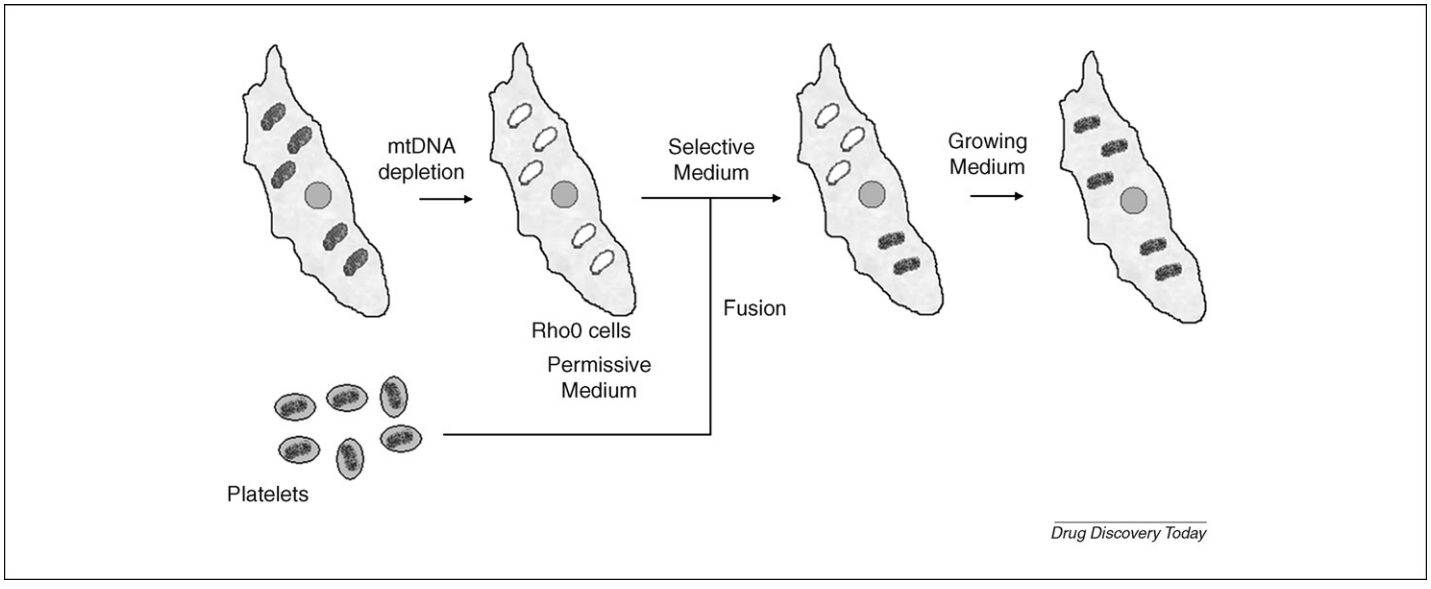

FIGURE 4

Transmitochondrial cell line construction. After removing the mtDNA from the cell by using drugs or mitochondrially targeted restriction endonucleases, the rho0 cells (containing mitochondria but no mtDNA) are fused with platelets and grown in selective medium. In these conditions, only cells repopulated with external mitochondria will be able to survive.

Interestingly, there is evidence that the nuclear genetic background influences the phenotypic expression of this mutation [56]. A polymorphism on chromosome 6 near the TFB1M gene but located outside of the coding region was implicated as a nuclear modifying locus of this mutation in several pedigrees of mitochondrial deafness [57]. Several clues suggest that altered TFB1M expression affects the penetrance of the m.1555G>A mutation by modifying the rRNA methylation. Therefore, nuclear genes involved in the modification or interaction with the mt-rRNAs should be considered when analyzing the phenotypic effects of particular ribosomal antibiotics on the mitochondrial translation.

Transmitochondrial cell lines for studying the effects of antibiotics

Ideally, to analyze the phenotypic effect of a particular mt-rRNA Single Nucleotide Polymorphism (SNP), we should use a system in which the rest of the variables remain homogeneous. Despite some limitations, such a system actually exists: the transmitochondrial cell lines. In this system, the mtDNA of a parental cell line is removed and the final product, the rho0 cell, is fused with cytoplasts (enucleated cells) or platelets (cell fragments without nucleus), originating cell lines known as 'cytoplasmic hybrids', or 'cybrids' (Fig. 4). By using the same rho0 cell line and different mitochondria sources, we will obtain cell lines sharing the same nuclear background and growing in the same environmental conditions. Thus, the only difference between them will be the mtDNA genotype, and their phenotypic differences will necessarily be due to the particular mtDNAs that they contain.

In fact, this cybrid system was used to demonstrate the mitochondrial inheritance of human chloramphenicol resistance. This resistance was due to the m.2939C>A/MT-RNR2 and m.2991T>C/MT-RNR2 mutations [58,59]. It has been shown that one of the oxygens of the p-NO_2 group of chloramphenicol seems to form hydrogen bonds with N4 of Ec.2452C (m.2939C), a nucleotide involved in tRNA-binding site P. The other p-NO_2 oxygen interacts with O4 of Ec.2504U (m.2991U) through an Mg ion. Both nucleotides are involved in chloramphenicol resistance in bacteria [2]. Furthermore, this system has been shown to be very useful for studying susceptibility to ribosomal antibiotics, such as erythromycin [20,21] and aminoglycosides [52,53,56,60].

An important disadvantage of the cybrid cell lines derives from their origin. The parental cell lines are highly proliferative tumor cells and they generate almost all ATP via glycolysis, despite abundant oxygen and functional mitochondria. These cells can be resistant to xenobiotics that impair mitochondrial function, such as ribosomal antibiotics. However, there is a way to solve this problem. The metabolism of galactose is very slow in these cells and sometimes is not sufficient to synthesize the ATP requirements by glycolysis when OXPHOS is impaired [61]. Then, to increase the detection of the mitochondrial effects induced by drugs, cells can be forced to rely on OXPHOS rather than glycolysis by substituting galactose for glucose in the growth media [62]. Thus, the susceptibility to mitochondrial ribosomal antibiotics would increase.

Because mtDNA only encodes for 13 polypeptides, it is not difficult to analyze the mtDNA-encoded protein levels by using gel electrophoresis [63]. To perform these studies, radioactive methionine and cysteine and an inhibitor of the cytosolic protein synthesis, such as cycloheximide or emetine, are added to the cell culture, meaning only the mtDNA-encoded polypeptides will be marked and the thousands of nuclear-encoded mitochondrial proteins will not appear in the gel. By comparing cybrids harboring particular mtDNA genotypes and growing in the presence of different antibiotics, we can analyze the relationship between an mtDNA genetic background and its susceptibility to an antibiotic.

Nowadays, many other OXPHOS-related functions – such as growth curves in galactose, oxygen consumption, mitochondrial inner membrane potential, ROS production, respiratory complex activities and levels, calcium levels and ATP production – can be analyzed on a large scale, facilitating the screening of many antibiotics and mtDNA genetic backgrounds.

Concluding remarks

There are now thousands of published human mt-rRNA sequences. The sequence of many cytoplasmic eukaryotic, archaebacteria and eubacteria rRNAs showing resistance or susceptibility to different antibiotics is known, and the crystal structure of some ribosomes is also known. We also have laboratory models to analyze specific mtDNA variants. Therefore, it would be useful to correlate different human mt-rRNA polymorphisms and the sensitivity or resistance of mitochondrial protein synthesis to inhibitors of bacterial translation. Such a pharmacogenomic approach might help to optimize antibiotic regimens on the basis of the patient's genetic background [4] and to increase the number of available antibiotics.

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