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Antibiotic susceptibility of mammalian mitochondrial translation

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Abstract **All medically useful antibiotics should have the potential to distinguish between target microbes (bacteria) and host cells. Although many antibiotics that target bacterial protein synthesis show little effect on the translation machinery of the eukaryotic cytoplasm, it is unclear whether these antibiotics target or not the mitochondrial translation machinery. We employed an in vitro translation system from bovine mitochondria, which consists of mitochondrial ribosomes and mitochondrial elongation factors, to estimate the effect of antibiotics on mitichondrial protein synthesis. Tetracycline and thiostrepton showed similar inhibitory effects on both *Escherichia coli* and mitochondrial protein synthesis. The mitochondrial system was more resistant to tiamulin, macrolides, virginiamycin, fusidic acid and kirromycin than the *E. coli* system. The present results, taken together with atomic structure of the ribosome, may provide useful information for the rational design of new antibiotics having less adverse effects in humans and animals.**

Keywords: Protein synthesis; Antibiotics; Ribosome; Mitochondria; *E. coli*

1. Introduction

Protein synthesis is a multi-step process that includes initiation, elongation, termination and recycling. Each process requires ribosomes, tRNAs and several translation factors. The ribosome is a universally conserved ribonucleoprotein complex that catalyzes the ordered polymerization of amino acids directed by the genetic information on mRNA. Various clinically useful antibiotics prevent protein synthesis by interacting with the bacterial ribosome or translation factors. Good antibiotics do not target the cytoplasmic ribosomes of host cells because their structures are adapted to fit the structures of bacterial ribosomes and not those of eukaryotic ribosomes. Mitochondrial (mt) ribosomes are categorized as a bacteria-type ribosome on the basis of shared antibiotic susceptibilities and sequence similarities of ribosomal proteins and RNAs [7,11,22,31–33]. If antibiotics could penetrate or be transported into mitochondria, the mt ribosome would be exposed to the antibiotics, resulting in the inhibition of mt translation. Aminoglycosides, which target bacterial ribosomes to induce misreading, are the most commonly used antibiotics. Autotoxicity is a major irreversible adverse effect of aminoglycosides and occurs in a dose-dependent fashion [13]. The A1555G mutation in mt 12S rRNA gene confers susceptibility to aminoglycosides and can cause non-syndromic deafness [13,28]. Although the molecular pathogenesis of aminoglycoside-induced deafness is not fully understood, it is possible that aminoglycosides directly bind to the mt rRNA and induce translation disorders in protein synthesis [15].

Using a modified 'fragment reaction' (puromycin reaction) for measuring the peptidyl-transferase activity of the ribosomal large subunit, the antibiotic susceptibility of the bovine mt 39S large subunit was compared to those of *Escherichia coli* and bovine cytoplasmic ribosomal subunits many years ago [11]. The mt large subunit showed low susceptibility to lincosamines and macrolides, indicating that the binding sites for certain of these antibiotics had been altered.

With the advent of an in vitro mt translation system established by our group from bovine mitochondria [16,23,34], it is now possible to characterize several antibiotics with respect to their abilities to target the small subunit as well as the large subunit of the mt ribosome. In addition, kirromycin and fusidic acid, which target elongation factors, can also be characterized in this system. The present study describes the antibiotic susceptibility of the mammalian mt translation system.

2. Materials and methods

2.1. Materials

E. coli total tRNA was purchased from Boehringer Mannheim. Tetracycline, fusidic acid, thiostrepton, kirromycin and virginiamycin M1 were purchased from Sigma–Aldrich. Josamycin, spiramycin, midecamycin acetate were gifts from Meiji seika Co. Ltd. Tiamulin was a gift from Novartis Co. Ltd, Shanghai, PR China. [^{14}C] phenylalanine was purchased from Amersham-Pharmacia.

2.2. Preparation of ribosome, elongation factors and aminoacyl-tRNA

The 55S mt ribosome and 70S *E. coli* ribosome were prepared as described previously [16]. Mt EF-G was partially purified by DEAE–Sepharose and Superdex 200 chomotography (Amersham-Pharmacia) from bovine liver [9]. The concentration of mt EF-G was determined by quantifying the intensity of the CBB-stained band on a SDS–PAGE gel by using Fluor-S MAX (Bio-rad). Recombinant *E. coli* EF-Tu,

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E. coli EF-G, and bovine mt EF-Tu were expressed in *E. coli* and purified as described [30,40]. The aminoacylation of *E. coli* tRNA was carried out with an *E. coli* S100 fraction under the conditions described previously [23], and protein synthesis was quantified by measuring the amount of incorporated [^{14}C] phenylalanine using a scintillation counter (ALOKA, Liquid Scintillation Counter, LSC-6100).

2.3. Measurement of the inhibition of poly(U)-directed poly(Phe) synthesis by antibiotics

The in vitro translation was carried out as described in the literature [16,34] with slight modifications. Appropriate amounts of antibiotics and 0.08 μM *E. coli* ribosomes were pre-incubated for 25 min on ice, and then at 37 °C for 5 min in a buffer containing 50 mM Tris–HCl (pH 7.5), 6.5 mM $MgCl_2$ and 60 mM KCl. Poly(U)-directed polyphenylalanine synthesis by *E. coli* ribosomes was performed in 20 μL of the same buffer containing 0.08 μM *E. coli* ribosomes (pre-incubated with each antibiotic), 0.5 mM GTP, 2 mM DTT, 0.1 mM spermine, 1 mg/ml poly U, 25 mM phosphoenolpyruvate, 2.5 unit/ml pyruvate kinase, 0.3 μM [^{14}C] Phe-tRNA, 0.6 μM EF-G, 0.6 μM EF-Tu, and each antibiotic at 37 °C for 20 min. Then, 10 μL of the reaction mixture was spotted onto a filter paper (Whattman 3MM), followed by deacylation of aminoacyl-tRNA through boiling. The TCA-insoluble products were then quantified by liquid scintillation counting (ALOKA, LSC-6100).

For the mt translation system, the pH of the Tris–HCl buffer was changed to 8.5, and the concentrations of $MgCl_2$, KCl, DTT and Spermine were changed to 7.5, 5, 1, and 0.5 mM, respectively. All the other procedures, except for ribosomes, EF-G and EF-Tu, which were all mitochondrial, were the same as those used in *E. coli* translation system. The initial experiments were performed over a wide range of antibiotic concentrations (0.1 μM to 10 mM) to obtain a rough estimation of the IC_{50} value for each antibiotic. Based on the results, we determined the IC_{50} value for each antibiotic within a narrow rage of concentrations both in *E. coli* and mt systems. Each experiment was repeated at least three times, and the average was taken to be the final IC_{50} value.

2.4. Site-directed mutagenesis of E. coli ribosomal 23S rRNA

We used *E. coli* strain NT101 (derived from TA542 [2] which was kindly provided by Dr. Cathy Squires of Tuft University) which is deleted for all chromosomal rRNA operons and, instead, contains a rescue plasmid, pRB101, carrying the *rrnB* and *sacB* genes. Using a Quick change site-directed mutagenesis kit (Stratagene), the A2058G point mutation was introduced into the 23S rRNA gene on another plasmid, pRB102, which shares the same replication origin (pSC101) as pRB101 but has a different antibiotic marker (Km). NT101 cells were transformed with the A2058G mutant plasmid, and colonies resistant to kanamycin and sucrose were selected to obtain a strain having a ribosome with the A2058G mutation.

3. Results and discussion

The poly(U)-directed polyphenylalanine synthesis system of mitochondria was constructed with bovine mt ribosomes, recombinant mt EF-Tu and partially purified mt EF-G from bovine liver mitochondria. The conditions were optimized as described previously [16,23,34]. As a control system, we employed the *E. coli* system which consists of *E. coli* ribosomes, recombinant *E. coli* EF-Tu, and EF-G. Polyphenylalanine incorporation for 20 min. was plotted against the concentration of each antibiotic and the IC_{50} value of each antibiotic was then added to Table 1.

Tiamulin, a pleuromulin derivative, is known to strongly inhibit bacterial translation in vitro and the peptidyl transferase (PTase) reaction between formylmethionyl (fMet)-tRNA and puromycin [12,20,27]. The IC_{50} of tiamulin in the *E. coli* system was determined to be 0.48 μM, while the IC_{50} in the mt system was 58.1 μM (Fig. 1A and Table 1), which represents nearly a one hundred-fold difference. Tiamulin occupies the PTase center, and thereby sterically hinders the correct positioning of tRNA [5,27]. The binding sites of tiamulin at the PTase center of *E. coli* 23S rRNA have been assigned as A2058, A2059, U2506, U2584 and U2585 (according to the *E. coli* numbering system) by antibiotic foot-printing [27]. A comparison of the tiamulin binding sites in *E. coli* and mt rRNAs, shows that A2058 is replaced by a G in mitochondria, while all the other binding sites are conserved. Therefore, the resistance of the mt system to tiamulin is probably conferred by the base difference at position 2058 in the mt large subunit rRNA. To confirm this, we constructed an *E. coli* ribosome with the A2058G mutation by site-directed mutagenesis and measured its IC_{50} value with tiamulin. As expected, the IC_{50} of the A2058G *E. coli* ribosome increased to 18.2 μM. This strongly indicates that the resistance of the mt system to tiamulin is due to the presence of a G residue in the mt rRNA binding site.

Macrolide antibiotics are powerful inhibitors of protein synthesis in bacteria [14]. These antibiotics are composed of a large lactone ring (from 14 to 16 carbon atoms) to which several sugars (sometimes amino sugars) are attached. They can be broadly divided structurally and functionally into relatively homogenous groups, as represented by the erythromycin and spiramycin groups [8]. The spiramycin group carries a 16-membered lactone ring, of which only one position, C5, is frequently glycosylated with a disaccharide. Macrolide specifically binds to the PTase center and to the entrance of the peptide tunnel in the 50S subunit. It interferes with the interaction of peptidyl-tRNA with the ribosomal P-site, and/or induces a conformational change in the ribosome [4,17,18,29,35].

In the present study, we investigated the susceptibility of *E. coli* and mt ribosomes to three 16-membered macrolides, spiramycin, josamycin and midecamycin. The results (Fig. 1B and Table 1) clearly indicated that IC_{50} values for these macrolides are much higher in the mt system than in the *E. coli* system. The main binding sites for these macrolides are certain bases located within the central loop of domain V of 23S

Table 1
IC_{50} values (μM) of various antibiotics in *E. coli* and mitochondrial systems

Antibiotics	Mitochondria	*E. coli*	*E. coli* A2058G	Categories	Target
Tiamulin	58.1 ± 2.6	0.48 ± 0.04	18.2 ± 2.3	Pleuromutilin	Large subunit
Josamycin	12.3 ± 1.6	0.35 ± 0.04	59.5 ± 2.5	Macrolide	Large subunit
Spiramycin	24.9 ± 0.5	0.27 ± 0.04	37.2 ± 0.63	Macrolide	Large subunit
Midecamycin	76.6 ± 1.6	0.43 ± 0.03	78.2 ± 4.6	Macrolide	Large subunit
Virginiamycin	3.5 ± 0.2	0.22 ± 0.02	–	Mikamycin	Large subunit
Kirromycin	64.9 ± 2.3	0.30 ± 0.02	–		EF-Tu
Fusidic acid	2020 ± 90	33.0 ± 3.0	–		EF-G
Thiostrepton	0.60 ± 0.05	0.48 ± 0.02	–		Large subunit
Tetracycline	170 ± 10	150 ± 10	–		Small subunit