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Identification of ribosomal mechanisms mediating drug resistance

Objectives The ribosome is the target of many important antiinfective agents. In spite of decades of use of ribosomal drugs, we still do not understand the principles governing selectivity and toxicity of these agents. Detailed understanding of the mechanisms governing resistance towards drugs targeting protein synthesis has long been hampered by the lack of a suitable model organism (*rrn* resistance mutations are in general recessive, and bacteria harbour several rRNA operons in their chromosome). To learn more about ribosomal mutations conferring resistance to antiinfective agents and to learn how these agents work, a derivative of *M. smegmatis* was rendered single rRNA operon allelic by means of gene inactivation techniques. In this system, genetic manipulation of the single rRNA operon results in cells carrying homogeneous populations of mutant ribosomes. This model allows the isolation of recessive drug resistance mutations within *rrn*. The goal is to characterise the mechanisms of action of these drugs and to understand the determinants involved in drug selectivity and toxicity. In addition, the fitness cost of defined resistance determinants was studied, as this is the single most important parameter that determines the spread of resistance.

Conclusions The results demonstrated that the genetic background influences the fitness cost of resistance determinants and falsified the current perception that drug resistance determinants inevitably carry a fitness burden. The studies on drug-target interactions of antiinfective agents targeting the ribosome identified the mechanisms involved in the development of resistance at a molecular level and defined the biological importance of the different structural interactions observed by crystallographic studies. The elucidation of these mechanisms identifies the molecular basis for the large differences in drug susceptibility observed in phylogenetically distinct ribosomal systems, e.g. bacterial, protozoal, cytoplasmatic, mitochondrial ribosomes. These findings provide the rational basis for understanding the mechanisms that determine the selectivity and toxicity of ribosomal antibiotics. This should help in developing future drugs with activity against prevailing resistance determinants and in synthesis of antibiotics endowed with less toxicity and defined specificity.

Main results and findings

The role of G2447 in peptidyl transfer and as determinant of resistance to oxazolidinones
The isolation and characterisation of linezolid-resistant bacteria revealed two classes of mutants:

- Ribosomes from class I mutants are resistant to oxazolidinones in an in vitro peptidyl transferase assay, indicating that resistance maps to the ribosome component. Introduction of a wild-type ribosomal RNA operon into linezolid-resistant strains restored linezolid sensitivity in class I mutants, indicating that resistance (i) maps to the rRNA and (ii) is recessive. Sequencing of the entire *rrn* operon identified a single nucleotide alteration in 23S rRNA of class I mutant strains, 2447G → T (*Escherichia coli* numbering), locating the drug binding site to the catalytic centre of the peptidyltransferase region. The finding that nucleotide identity at position G2447 of the 23S rRNA is dispensable for ribosome function *in vivo* argues against its critical role in peptidyltransfer. In line with these observations, mutation of the conserved base 2451A →U of 23S rRNA alters the structure of the peptidyl transferase centre, changes the pattern of pH-dependent rearrangements, as probed by chemical modification of 23S rRNA and was found to result in resistance to linezolid. Therefore, A2451 seems to function as a pivot point in ordering the structure of the peptidyl transferase centre rather than taking part in chemical catalysis. Introduction of mutation *rrl*2447T into *M. smegmatis* rrn- resulted in a linezolid-resistant phenotype, demonstrating a cause–effect relationship of the 2447G →T alteration. The 2447G →T mutation, which renders *M. smegmatis* linezolid resistant, confers lethality in *E. coli*. This finding is strong evidence of structural and functional differences between the ribosomes of Gram-positive and Gram-negative bacteria.
- Ribosomes from class II mutants show wild-type susceptibility to linezolid in vitro, pointing to a non-ribosomal mechanism of resistance. In agreement with the results of the in vitro assay, class II mutants show a wild-type sequence of the complete rRNA operon. The lack of cross-resistance of the class II mutants to other antibiotics suggests a resistance mechanism other than activation of a broad-spectrum multidrug transporter.

Molecular basis for toxicity of hygromycin B, a universal inhibitor of translation Hygromycin B inhibits protein synthesis in archaebacteria, eubacteria, fungi and in higher eukaryotes. Hygromycin B binds largely in a sequence-specific manner close to the very top of conserved helix 44 of 16S rRNA, between the A (aminoacyl) and the P(peptidyl) sites. The localization of hygromycin B binding to this region is consistent with the recently suggested involvement of helix 44 in movements during the translocation step. Because of its universal activity, hygromycin B cannot be used for systemic application in clinical medicine.

The isolated resistance mutations were found to localize exclusively in 16S rRNA. The mutations observed are in close proximity to the hygromycin B binding site. The 16S rRNA positions involved in hygromycin B resistance are highly conserved in all three domains of life. The observation that ribosomal alterations mediating resistance to hygromycin B exclusively involve universally conserved nucleotides within rRNA provides an explanation for the lack of specificity of hygromycin B, i.e. the observation that hygromycin B is toxic for almost all organisms.

The molecular basis of aminoglycoside binding Nucleotide A1408, the shifted U1495-U1406 pair and the Watson-Crick base pair C1409-G1491 closing the binding pocket are critical for the molecular interactions of aminoglycosides with the ribosome:

- Pseudo base-pair formation between ring I of the aminoglyosides and A1408 is the key molecular interaction for specific binding.
- Mutagenesis of the wobble base pair U1495-U1406 discriminates between 4,5- and 4,6-aminoglycosides. In addition, the sensitivity of aminoglycosides to various subtle alterations of the U1406-U1495 pair is correlated with differences in their substituents. However, major displacement of 1406 or 1495 towards the deep groove makes the ribosome highly resistant to all aminoglycosides.
- Mutagenesis of C1409-G1491 discriminates poorly between 4,5- and 4,6-aminoglycosides, but differentially affects aminoglycosides with an OH- versus a NH3+-group at position 6' of ring I (presumably, this is due to the difference in energy of H-bonding between OH- versus NH3+ and N1 of A1408).

These results define the biological importance of the different structural interactions observed by crystallographic studies and provide the rational basis for understanding the mechanisms that determine the selectivity and toxicity of ribosomal antibiotics.

Drug resistance and fitness To study the cost of chromosomal drug resistance mutations in bacteria, the fitness cost of mutations that confer resistance to different classes of antibiotics affecting bacterial protein synthesis (aminocyclitols, 2-deoxystreptamines, macrolides) was investigated. A model system based on an in vitro competition assay with defined laboratory mutants was used. Selected mutations were introduced by genetic techniques to address the possibility that compensatory mutations ameliorate the resistance cost. The results can be summarised as follows:

- The chromosomal drug resistance mutations studied often had only a small fitness cost.
- Compensatory mutations were not involved in low-cost or no-cost resistance mutations.
- Natural sequence polymorphism within the drug binding site is a major denominator of the fitness cost associated with a resistance determinant.
- When drug resistance mutations found in clinical isolates were considered, selection of mutations that have little or no fitness cost in the in vitro competition assay seems to occur.

These results argue against expectations that link decreased levels of antibiotic consumption with a decline in the level of resistance.

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