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Antibiotics and the search for new principles*

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The title of this paper was suggested by comments written by Ernest Gale some years ago [14] as an introduction to a symposium on antimicrobial drug action. "Antibiotics are selective agents provided by Nature for our enlightenment and we can hope, from a study of their actions, not only to improve our knowledge of the application of known principles, but also to find new principles". He then went on to elucidate one such principle: inactivation of a functional macromolecule via distortion of its structure, as exemplified by the intercalation of actinomycin D or proflavine into DNA with consequent unwinding of the helix. The conceptual framework for those comments, and for work being carried out in Gale's laboratory at that time, was derived ultimately from Paul Ehrlich who first enunciated the principle that drugs don't act unless they bind to something and that selective toxicity involves the ability of drugs to seek out (like 'magic bullets') receptors or targets specific to parasites and absent or unavailable in host cells.

In the mid 1960s it was becoming clear that ribosomes are the targets for many of the antibiotics that inhibit protein synthesis and it had already been shown by D. Vazquez [36] in a simple but seminal analysis, that the selective action of chloramphenicol depends upon the ability of the drug to bind to the prokaryotic-type ("70S") ribosomes of bacteria, chloroplasts and mitochondria but not to the "80S" particles present in eukaryotic cytoplasm. It was therefore reasonable at that time to suppose that inhibitors of protein synthesis could be used to unravel the details of ribosomal structure-function relationships, in much the same way that other inhibitors had been used when the pathways of intermediary metabolism were first elucidated. Moreover, a knowledge of antibiotic target sites within ribosomes, coupled with some understanding of the modes of action of such drugs (e.g. inhibition of peptide bond formation; prevention of the binding of aminoacyl-tRNA to the ribosome-mRNA complex; stimulation of translational inaccuracy, etc.) should eventually allow defined functions to be attributed to specific ribosomal components. In other words, ribosomes are enzymes, enzymes have active sites, and antibiotics that bind into those active sites can be used to characterize them, both physically and functionally. Much of what is known about the modes of action of ribosome-inhibitors (and other antibiotics) has been reviewed at length elsewhere [15]. This paper deals mainly with progress in characterizing the target macromolecule(s) for ribosome-inhibitors.

ANTIBIOTIC BINDING SITES IN RIBOSOMES

Over the years, considerations of antibiotic target sites within ribosomes have usually been predicated upon an assumption for which there is remarkably little evidence, that is, that antibiotics bind to ribosomal proteins. In all probability this idea had several origins, but familiarity with enzyme-substrate complexes was undoubtedly most persuasive. In contrast, RNA was (and is) not renowned for binding small molecules non-covalently. Nevertheless, recent evidence (both direct and indirect) now points irresistibly to the conclusion that some, and probably many, inhibitors of protein synthesis act by binding primarily or even exclusively to ribosomal RNA, calling to mind Luigi Gorini's repeated assertion that streptomycin acts in that way [16].

Although antibiotics typically bind to ribosomes with gratifyingly low stoichiometries (for many drugs single "tight" attachment sites have been reported per ribosome) it has proven extraordinarily difficult to locate the target sites of small molecules (M_r usually < 1000) within such

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a complex macromolecular aggregate. To emphasize the nature of the problem, the *E. coli* ribosome with a mass of about 2.3×10^6 Da contains 3 species of RNA and over 50 proteins. Moreover, "tight" binding sites for antibiotics are not usually very tight; binding is non-covalent with dissociation constants typically in the range 10^{-5} M to 10^{-7} M, although there are exceptions at either end of that range. Not surprisingly, therefore, binding of antibiotics to isolated ribosomal components has not usually been detectable, although thiostrepton does bind readily to 23S rRNA and ribosomal protein L15 has been reported to bind erythromycin [32]. The latter is a unique observation: no other native antibiotic molecule has ever been shown to bind to any ribosomal protein. Of course, some specifically modified antibiotic molecules have been shown to do so but, with due respect to those chemists who designed them, there is no guarantee that the covalent reactions of such affinity probes necessarily label the same target macromolecules that participate in the non-covalent binding of native drug molecules. What is really needed, in order to locate the attachment sites precisely, is a means of chemically modifying the ribosome with something small at single selected sites so as to abolish the binding of specific antibiotics. This is not a fanciful objective: it can be achieved using enzymes obtained from those organisms that produce inhibitors of protein synthesis and defend themselves against their products via specific modification of their ribosomes. Significantly, in all of the several cases yet documented, such ribosomal modification involves the RNA and not the proteins.

HOW ANTIBIOTIC-PRODUCING ORGANISMS AVOID SUICIDE

In order to resist the potentially toxic effects of their products, antibiotic-producing organisms must adopt survival strategies (for review, [5]). The available options include; inactivation or sequestration of intracellular drug molecules and any biologically active precursors thereof, the erection of membrane permeability barriers coupled with efficient efflux mechanisms for removing drug molecules from the cells, and (most relevant to the present exercise) modification or replacement of the target site(s) at which given drugs normally act.

Various enzymes involved in intermediary metabolism or in the synthesis of macromolecules are normally the targets for specific antibiotics – except in the respective producing organisms (Table 1; [5] and text). In most cases involving non-ribosomal target sites, it is not known whether such resistance results from post-translational modification of an otherwise sensitive target enzyme or whether a resistant version of the target is produced de novo. However, multiple genes encoding DNA gyrase B protein (the normal target for novobiocin) are known to be present in *Streptomyces sphaeroides* [33], the producer of novobiocin. One *gyrB* gene, encoding a drug-sensitive DNA gyrase B protein, is expressed constitutively whereas another gene copy, encoding a novobiocin-resistant protein, is induced by exogenous treatment with the drug. Presumably, such induction also occurs during (or prior to) the novobiocin production phase. Conceptually, similar events also occur in *Streptomyces arenae* which produces pentalenolactone, an inhibitor of glyceraldehyde phosphate dehydrogenase [12]. Two GPDH gene copies are present and the drug-sensitive target enzyme present during primary metabolism is replaced by a resistant one as drug-production begins. Little more can be added to the information given in Table 1 (which is not meant to represent a comprehensive list) so far as detailed resistance mechanisms are concerned. However, moving back to inhibitors of protein synthesis, resistance in the producing organisms has been studied in some depth.

Not all organisms that produce inhibitors of protein synthesis are capable of rendering their own ribosomes resistant to antibiotics, autogenous or otherwise. For example, ribosome-based resistance mechanisms have not been detected, despite detailed examination, in the *Streptomyces* species that produce chloramphenicol, tetra-

TABLE 1

Antibiotic resistance resulting from modification of non-ribosomal target sites in producing organisms

Organism	Antibiotic produced	Target
Streptomyces sphaeroides	Novobiocin	DNA gyrase
Streptomyces arenae	Pentalenolactone	GPDH
Amycolatopsis mediterranei[a]	Rifamycin	RNA polymerase
Streptomyces cinnamomeus	Kirrothricin	EF-TU
Pseudomonas fluorescens	Pseudomonic acid	Ile-tRNA synthase
Cephalosporium caerulens	Cerulenin	Fatty acyl synthase

[a] Formerly *Nocardia mediterranei.*

Abbreviations: GPDH, glyceraldehyde-3-phosphate dehydrogenase; EFTu, protein synthesis elongations factor Tu.

cycline, neomycin, streptomycin or various other antibiotics (for further details, [2]). On the other hand, ribosome modification leading to antibiotic resistance has been observed and characterized in organisms that produce thiostrepton, macrolides (such as erythromycin, tylosin, carbomycin, spiramycin, etc.), celesticetin, pactamycin, and aminoglycosides of the kanamycin and gentamicin families. Some examples are listed in Table 2 (for references, [5]). Two significant observations have emerged from these studies: resistance is due to methylation of ribosomal RNA at single sites, each specific for a given resistance phenotype, and such resistance is specific for the autogenous drug or its close relatives. Moreover, whenever binding studies have been carried out with the modified ribosomes, drug resistance has been shown to result from the abolition or severe impairment of drug-target recognition. Evidently, there is the most direct of relationships between antibiotic target sites in ribosomes and the sites at which methylation confers resistance.

The best example of this concerns resistance to thiostrepton in *Streptomyces azureus* from which a resistance gene (*tsr*) has been isolated and shown to encode a rRNA methylase enzyme [34]. That enzyme has also been isolated from *S. azureus* directly and has been shown to monomethylate 23S rRNA, generating 2′-*O*-methyladenosine at position 1067 of the polymer sequence [35]. Cause and effect linking resistance to RNA methylation was established by in vitro reconstitution analysis in which *E. coli* ribosomes were dissociated into RNA and protein components, and then re-assembled after exposure of the RNA to purified *tsr* protein in the presence and absence of the methyl transfer cofactor, *S*-adenosylmethionine. Reconstituted particles containing methylated 23S RNA were totally resistant to thiostrepton in functional assays and failed to bind the drug as assayed by equilibrium dialysis. Since thiostrepton normally binds to ribosomes with a dissociation constant of less than 10^{-9} M, this implies a change in binding affinity of at least 5–6 orders of magnitude consequent upon the introduction of a single methyl group into the intact ribosome. Thiostrepton also binds directly in vitro to purified 23S rRNA [31] with single site specificity and a respectable affinity (K_{diss} approx. 10^{-7} M), which is again abolished by specific methylation. More recently, a 61-mer oligonucleotide fragment of 23S rRNA, embracing residue A-1067 and containing about 2% of the intact RNA chain, has been shown to bind the drug indistinguishably from 23S RNA (J. Thompson, personal communication). Studies are now in progress to define the tertiary structure of this RNA fragment and the manner in which thiostrepton interacts with it [9]. This is important. The binding of thiostrepton to 23S rRNA represents the first characterized example of non-covalent binding of any small molecule to any nucleic acid with single-site specificity.

TABLE 2

Antibiotic resistance resulting from methylation of ribosomal RNA in producing organisms

Organism	Antibiotic produced
Streptomyces azureus	Thiostrepton
Saccharopolyspora erythraea [a]	Erythromycin
Streptomyces fradiae	Tylosin
Streptomyces caelestis	Celesticetin
Streptomyces kanamyceticus	Kanamycin
Micromonospora purpurea	Gentamicin
Streptomyces tenjimariensis	Istamycin
Streptomyces pactum	Pactamycin

[a] Formerly *Streptomyces erythraeus*.

ANTIBIOTICS AND RIBOSOMAL RNA

Only in the case of thiostrepton has direct binding of a drug to isolated rRNA been demonstrated. However, in addition to the methylation data referred to above, there are other lines of evidence that point irresistibly to the conclusion that an assortment of ribosome-inhibitors recognise RNA in the particle. Thus, in 'footprinting' studies [8,22,23], the binding of various drugs to ribosomes has been shown to protect specific rRNA residues from chemical or nuclease attack (or, in some cases, to enhance such attack). There are also impressive correlations with other data derived from genetic studies. When the *rrnH* ribosomal RNA operon of *E. coli* was isolated, subjected to mutagenesis and then reintroduced into *E. coli* on a multicopy plasmid, transformants resistant to various antibiotics could be selected. The mutations were then mapped within the plasmid-located rRNA genes and characterized by sequencing [10,26]. Mutations leading to antibiotic resistance have also been studied in the mitochondria of yeast [7,21,28,29] and mammalian cell lines [1,20,27], in chloroplasts of *Euglena* [24], *Chlamydomonas* [17] and tobacco [4,11,13], in archaebacteria of the genus *Halobacterium* [18,19] and in the nuclei of *Tetrahymena thermophila* [30]. These organelles and organisms each possess only single sets of rRNA genes (*T. thermophila* has multiple copies of a single active *rrn* operon) so that mutations occurring therein are not recessive, as they would be in other organisms (such as *E. coli*) with multiple *rrn* operons. In these various genetic systems, antibiotic resistance mutations have been mapped within rRNA genes and characterized by DNA or rRNA sequencing.

The outcome of the studies outlined above is quite

TABLE 3

Antibiotics implicated in interactions with ribosomal RNA

Thiostrepton	Macrolides	Lincosamides
Chloramphenicol	Vernamycin B	
Streptomycin	Spectinomycin	Aminoglycosides
Pactamycin	Hygromycin B	Tetracycline

startling. A list of the antibiotics implicated in RNA-based ribosomal interactions by one or more of the above means would include modified peptides (such as thiostrepton), macrolides, aminoglycosides, lincosamides, chloramphenicol etc., embracing drug molecules with hydrophilic, hydrophobic or amphiphilic character (see Table 3; for structures see [15]). Thus RNA would appear to possess a hitherto unsuspected capacity to recognise small molecules of diverse chemical types.

In considering the proposition that active sites of the ribosomal enzyme contain (or are composed of) RNA and that antibiotics bind to RNA when inhibiting ribosomal function, the following arguments are germane. The original machinery for specific protein synthesis must have been protein free; the structure of rRNA has been conserved to a remarkable extent during 3×10^9 years or so of evolution (which implies the existence of good reasons why this should be so); and RNA is now known to act catalytically in other contexts. Thus, in the 1980s we have seen the emergence of a new principle of fundamental importance to biochemistry, that of catalysis mediated by RNA or enzymes with essential RNA subunits. Examples are RNase P of *E. coli* and other eubacteria that processes the 5′ ends of tRNA transcripts, self-splicing RNA molecules in *Tetrahymena* nuclei and in yeast mitochondria, signal recognition particles, snRNPs, scRNPs, etc. (for review see [3]). Studies with antibiotics and ribosomes have contributed incisively to the realisation that RNA was probably the first catalytic macromolecule in evolutionary terms. Indeed, the ribosome itself (the most abundant object on this planet) is now revealed as the biggest and best of the extant RNA enzymes. To return to where we began, having commented on the potential usefulness of antibiotics as probes in biochemical systems, and having pointed to the principle whereby structural distortion can lead to the inactivation of macromolecules, Gale [14] anticipated the importance of 'studies of the interactions of ribosomes or other forms of active RNA with streptomycin, chloramphenicol, tetracycline etc.'

A NEW PRINCIPLE EMERGING?

Studies with thiostrepton have been central to the realisation that antibiotics can bind to rRNA and that the latter constitutes the 'heart' of the ribosomal enzyme. They have also given rise to a provocative new observation that is presented here by way of conclusion.

The thiostrepton-resistance gene (*tsr*) from *Streptomyces azureus* encoding the rRNA methylase, has been incorporated for selective purposes into most of the cloning vectors developed for deliberate gene transfer among actinomycetes. In the absence of other specialised requirements to influence the choice of recipient, *Streptomyces lividans* is the most commonly used host strain in such manipulations. Startlingly, it has recently been discovered [25] that thiostrepton turns on gene expression in *S. lividans* strains containing *tsr*, under which conditions the organism is immune to the inhibitory effects of the drug. Subsequently, one of the proteins thus produced was purified, the gene (*tipA*) encoding it was isolated via reverse genetics, and the promoter was transplanted into a promoter probe vector where it proved to be inducible by thiostrepton. This occurred in an organism that does not produce thiostrepton and therefore would not normally expect to encounter the drug. Whether or not thiostrepton affects gene expression in *S. azureus* is not yet clear. The mechanism of induction of the *tipA* promoter is currently being investigated. Conceivably, the drug might act in a classical manner by binding to a regulatory protein, thereby relieving negative control or facilitating gene activation in a positive sense. However, the fact that the drug can bind to rRNA also raises the possibility that induction of the *tipA* promoter could involve direct binding of thiostrepton to DNA. In any event, thiostrepton exerts this effect at very low concentrations, certainly as low as those required for inhibition of protein synthesis. All of which makes one wonder whether this kind of effect is restricted to thiostrepton or whether this might represent the 'tip' of an iceberg and that other antibiotics might also have the capacity to influence gene expression, as J. Davies has speculated in a recent stimulating review [6]. If so, we may need to revise our use of the very word 'antibiotic', and that might point us towards other new principles.

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