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Review Article

RNA-acting antibiotics: in-vitro selection of RNA aptamers for the design of new bioactive molecules less susceptible to bacterial resistance

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Abstract

During the last few years, antibiotic multiresistance has been increasing, not only in hospitals, but also, more worryingly, in general medicine. Different ways are being explored to bypass this problem. RNA-acting antibiotics such as aminosides (aminoglycosides) bind to bacterial RNA causing premature termination of proteins and mistranslation in bacteria. It is now possible to study the interactions of such antibiotics with their target by in-vitro selection of RNA molecules that recognize these antibiotics (RNA aptamers, SELEX method). The knowledge of the antibiotic–RNA interactions represents a promising way for the rational design of new bioactive compounds less susceptible to bacterial resistance.

Introduction

Advanced molecular biology has led to the discovery of a new generation of molecules called aptamers (Ellington & Szostak 1990). These molecules, nucleic acids (DNA, RNA) or polypeptides, are able to recognize different molecular targets. Some of them, called ribozymes, can catalyse chemical or biochemical reactions (Doherty & Doudna 2001).

This review focuses on RNA aptamers and their use in therapy, especially as tools in antibiotic therapy, although they can be used as therapeutic agents in, for example, antiviral therapy.

The first signs of penicillin resistance were observed in 1947, only two years after the first use of this antibiotic. Resistance was only found in hospitals. Today, multiresistance is found outside of hospitals and is getting out of control. A mathematical model has shown that changes in the prevalence of resistance after a successful intervention will occur on a time scale of one week to a month, considerably faster than in community-acquired infections (Lipsitch et al 2000). It is therefore necessary to find a new generation of antibiotics to bypass multiresistance. To this end, it is important to have a better understanding of the mechanism of action of classic antibiotics in order to find a way to avoid multiresistance.

DNA or RNA aptamers are molecules selected for their ability to bind strongly and selectively different molecules (for a review see Famulok 1999). They can be selected in order to bind small molecules (see Meli et al 2002) or to inhibit or activate protein functions. Diagnostic (Charlton et al 1997) and therapeutic applications have been developed against, for example, thrombin and HIV-Rev protein (Burke & Berzal-Herrantz 1993; Ellington & Conrad 1995; Schneider et al 1995; Toulmé & Giégé 1998; Boiziau et al 1999; Watson et al 2000). Structures of aptamer complexes reveal the molecular key interactions conferring specificity to the aptamer–ligand association (Patel et al 1997; Hermann & Patel 1999, 2000). Such RNA molecules allow the study of the binding properties of RNA-acting antibiotics to their target site(s) and may therefore offer the possibility of modifying antibiotics to avoid resistance. They are

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endowed with a nanomolar affinity and stringent specificity, which permits discrimination of different antibiotic family members.

This review introduces the concept of bacterial resistance against antibiotics and then focuses on aptamer–antibiotic complexes.

RNA-acting antibiotics, antibiotics and resistance

Mechanism of action of antibiotics

There are 17 classes of antibiotics, among which the mechanisms of action involved to fight bacterial infections are different. The mechanism of action of each class of antibiotics is specific because it takes place at different stages in bacterial metabolism (Asselineau & Zalta 1986), for instance on the bacterial wall, on the membrane, or on nucleic acids.

Here, we focus our attention on RNA-acting antibiotics and especially on antibiotics that perturb and/or disrupt protein synthesis. In this field, a completely new class of the oxazolidinone antibiotics was recently discovered, the only one in 35 years. These antibiotics are indicated for infections by Gram-positive bacteria including vancomycin-resistant strains of *Enterococcus* (bacteraemia and nocosomial pneumonia) (Shinabarger et al 1997; Swaney et al 1998). Oxazolidinones are the only initiation inhibitors that prevent the formation of a functional 70S initiation complex.

RNA-acting antibiotics and perturbation of protein synthesis

Different types of antibiotics bind to RNA and perturb protein synthesis, such as aminosides (Figure 1), the macrolides (e.g. erythromycin), tetracyclines, viomycin and

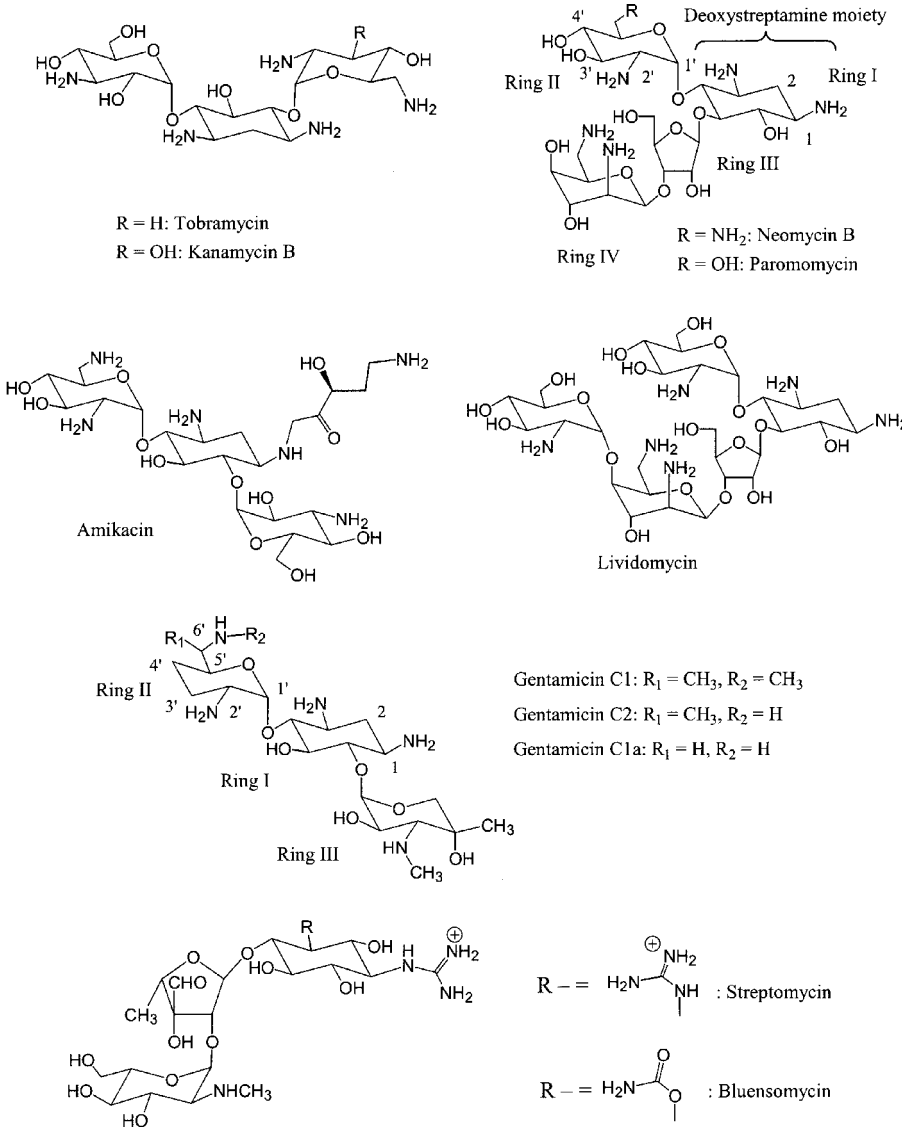

Figure 1 Structure of aminosides (aminoglycosides).

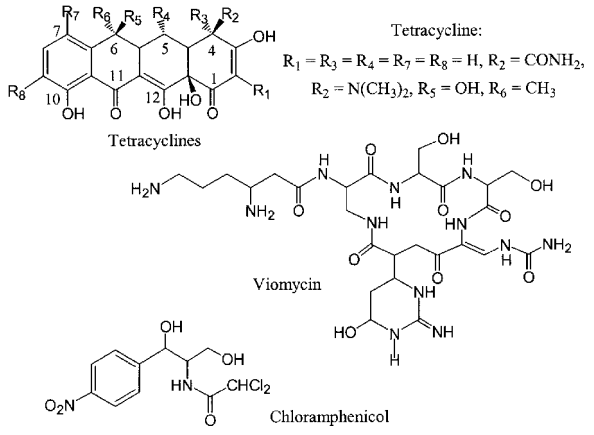

Figure 2 Structure of tetracyclines, viomycin and chloramphenicol.

chloramphenicol (Figure 2). Erythromycin belongs to the group of macrolides produced by the *Streptomyces*. They display a wide spectrum of activity and are effective against Gram-positive cocci, *Haemophilus influenzae*, *Legionella*, *Bordetella*, *Corynebacterium*, Gram-negative cocci, *Mycoplasma*, *Chlamydia* and both Gram-positive and Gram-negative anaerobes. Erythromycin is a small macrolide that binds at the level of the 50S ribosome subunit, inhibiting translocation and therefore protein synthesis. The large macrolides (spiramicin, jasamicin) inhibit the first stages of protein synthesis (Sugimoto et al 1983).

Aminoside antibiotics (aminoglycosides), possessing a deoxystreptamine moiety, constitute a clinically important class of antibiotics that are effective against a broad range of microorganisms (Edson & Terrell 1999). Aminoglycosides are multiple-positive-charged molecules of high flexibility. They bind to the A-site of the decoding region of 16S RNA in the bacterial ribosome and act during amino acid tRNA binding. Aminoglycosides are responsible for the inaccurate transcription that leads to the synthesis of modified proteins (for a review see Wong et al 1998).

Tetracyclines inhibit the process of synthesis by acting at the level of the 30S subunit. Thus, they prevent the binding of amino acid tRNA at the level of the ribosome A-site (Sandar et al 2001).

Chloramphenicol, a small molecule, is another RNA-binding antibiotic that acts on a part of the 50S subunit acceptor site, corresponding to the region receiving the 3′ extremity of the amino acid tRNA. It inhibits the peptide synthetase and thus the formation of peptidic bonds (Leviton 1999).

Bacterial resistance acquisition

The hospital is a very special environment where selection pressure is very high because of the use of many antibiotics to cure and prevent infections. The spreading of resistance is facilitated by the high density and diversity of infected patients, which act as vectors. In France, the prevalence of nosocomial infection is 8%. This prevalence is higher in reanimation units (30%). Three microorganisms are predominately involved: *Escherichia coli* (20%), *Staphylococcus aureus* (16%), and *Pseudomonas* (11%) (Lipsitch et al 2000). Resistant organisms are escaping from hospitals and are now frequently observed in general practice.

With the use of anti-infection drugs, microorganism resistance (bacteria, fungi) to these drugs is incessantly increasing. The first strain of *Staphylococcus* was identified in 1947 and by 1997, *Staphylococcus* was resistant to seven main classes of antibiotics. This is a spectacular evolution (Acar & Courvalin 1998). For each development of new antibiotics, bacteria appear to be able to adapt very quickly. Usually, 2 to 4 years are necessary to develop new mechanisms to acquire resistance. Most synthetic antibiotics are derived from natural substances produced by fungi or by soil bacteria, before being refined by chemists. Thus, resistance could have been expected, since these molecules are not toxic to the microorganisms that produce them, as they have their own arsenal to defend themselves, and in 80% of cases, resistance comes from gene transfer, even between different species.

Natural resistance against some antibiotics has a chromosomal origin, which depends on the genetics of bacteria and is characteristic for a given species (Olsen 1999; Hashimoto-Gotoh et al 2000; Von Freiesleben et al 2000). For instance, some bacteria possess elements in their bacterial wall that can prevent antibiotics from reaching their target, or the target might not exist in a particular type of bacteria. This resistance is not owing to the adaptation of one or more bacteria to antibiotics, but depends on either a bacterial chromosome mutation or acquisition of one or more extra-chromosomal DNA fragments. This phenomenon appears in one or more bacteria in a strain, but not in the bacterial species as a whole. Resistance mutation concerns a low percentage (10–20%) of the pathogenic strains isolated in clinical trials. In contrast to extra-chromosomal resistance, this resistance is not transmittable from one bacteria species to another.

Extra-chromosomal resistance represents most of the cases of bacterial resistance isolated in clinical trials (about 80%). It occurs as a result of bacterial acquisition of DNA particles by conjugation and/or transduction. These extra-chromosomal DNA elements are plasmids and transposons that carry resistance properties.

Transformation is the rarest resistance transfer mechanism. A DNA fragment, present in the extracellular environment, is just picked up by the bacteria. For instance, it may be picked up from other dead bacteria whose membranes have been more or less destroyed.

Mechanisms of bacterial resistance

Resistance of certain bacterial species to antibiotics can be linked to several resistance mechanisms (Davies 1994; Robert-Dernuet 1995), such as a reduction in wall permeability, which prevents antibiotic penetration. Bacteria use various means in order to prevent the access of antibiotics to their targets. They use membrane transporters, which expulse the antibiotic (multidrug resistance: *Bacillus subtilis*, *Staphylococcus aureus*, *Mycoplasma genitalium*) (Nikaido 1994), so that its intracellular concentration will

remain insufficient to be toxic (e.g. tetracycline). Another possibility is the modification of porins or a decrease in their number following a mutation, which limits the passage of molecules and reduces the sensitivity of bacteria to some classes of antibiotics. Other types of mechanisms developed by bacteria against antibiotics involve enzyme synthesis, or modification or substitution of the target by another non-vulnerable molecule.

Bacterial enzyme synthesis (Cocito et al 1997; Bernhardt et al 2001) Antibiotics may be inactivated by bacterial enzyme synthesis (β-lactamase, peptidyltransferase and acetyltransferase for chloramphenicol, pyruvyltranferase for fosfomycin). This is the most widespread mechanism for antibiotic inactivation in nature. Bacteria synthesize an enzyme that modifies antibiotics and makes them harmless. Inactivation may occur at different stages. It may be intracellular in the case of antibiotics whose targets are cytoplasmic (aminosides, chloramphenicol). On the other hand, β-lactams aim at extracellular targets, and have to be inactivated before contact with the cell. β-Lactamases are excreted into the culture medium (Gram-positive bacteria) or in the periplasmic space (Gram-negative bacteria) and intercept the antibiotic even before it reaches the target.

High-level aminoglycoside resistance involves enzymatic acetylation, phosphorylation and ribosylation as primary causes in most clinical isolates (Wright et al 1998; Kondo & Hotta 1999; Mingeot-Leclerc et al 1999).

The study of these mechanisms of antibiotic inactivation and determination of the recognition sites necessary for RNA binding are promising ways for progressing the development of antibiotics less susceptible to bacterial resistance.

Modification or substitution of the target (Sedgwick et al 1975; De et al 2001) Modification of the site of action or antibiotic target (ribosome, DNA gyrase, RNA polymerase) inhibits binding of an antibiotic to its target (β-lactams, erythromycin, lincomycin). For instance, resistance to macrolides results from the methylation of the 23S ribosomal RNA, which causes a decrease in the affinity of these antibiotics for the ribosome. Resistance to quinolones results in the modification of the DNA gyrase; therefore antibiotics will no longer bind to the enzyme and will no longer affect DNA replication.

Bacteria can develop a metabolic pathway replacing the reactions blocked by antibiotics. This is called target substitution (synthesis of enzyme insensitive to multibacterial agent). The target may be substituted for another non-vulnerable molecule. Thus, two different molecules (one sensitive, the other not sensitive) possessing the same function coexist in the same bacteria (e.g. sulfamide resistance).

Overproduction of antibiotic target (sulforamid, trimethoprim) and sequestration of antibiotic by binding to a protein (β-lactams, fusidic acid, bleomycin) are two other mechanisms of resistance.

These modifications of antibiotic sensibility are particularly well studied in *Staphylococcus aureus* (penicillin G and meticillin resistance), *Streptococcus pneumoniae* and *Neisseria gonorrhooe* (penicillin G resistance), *Neisseira meningitidis* (sulfamide resistance), *Haemophilus influenza* (ampicillin and chloramphenicol resistance) and enterobacteria (extremely variable).

In-vitro selection of RNA aptamers for the design of new antibiotics

RNA molecules are the functional targets for different antibiotics such as aminoglycosides. In order to study and understand antibiotic–RNA recognition, high-affinity aptamers have been prepared by in-vitro selection (SELEX method; Tuerk & Gold 1990).

SELEX method

The systematic evolution of ligands by an exponential enrichment procedure (SELEX) was developed by Tuerk & Gold (1990). This method allows the selection from an infinite number of nucleic acid molecules (starting from a population of 10^{15} to only 10 molecules) according to their affinity for a ligand or their capacity to catalyse a reaction. The first step in this procedure is to create a pool of DNA with an internal degenerate sequence flanked with defined sequences that are necessary for the next steps of reverse transcription, polymerase chain reaction (PCR) and in-vitro transcription. This pool is transcribed and selected according to its ability to bind to a target or to catalyse a reaction.

Selected RNAs are reverse transcribed and amplified by PCR in double-stranded DNA, which is competent for subsequent in-vitro transcription. This transcribed RNA is selected again. Many rounds of enrichment result in the exponential increase of the best sequenced aptamers to determine a consensus sequence and a structure.

The creation of a DNA pool is a critical stage because it determines the multiplicity of molecules for selection. The choice of molecule selected is a function of the type of study being carried out. For instance, for a molecular evolution study, to modify the specificity of a ribozyme, the sequence of the ribozyme is broken down and selected against another target. To study interaction between an antibiotic and 16S rRNA, a pool of RNA is used.

Different methods of selection can be used. By affinity chromatography, an aptamer is selected according to its affinity for a small part of the molecule. Thus, this aptamer will be able to interact selectively with this antibiotic. If the selection is made against a bigger target such as a protein, a gel shift assay is used. For example, an aptamer that potentiates the inhibitory effect of valyldiphenylester phosphonate, a neutrophil elastase inhibitor, has been selected through this method. SELEX is also used to study the interactions of antibiotics with their nucleic acid targets.

Aminoglycosides

Aminoglycosides bind strongly to RNA and this property has been used to control gene expression (Werstuck &

Green 1998). Aminoside antibiotics are thought to function for the most part by binding to the decoding region of bacterial 16S RNA (for a review see Wong et al 1998). Binding of aminoglycosides to this region can cause premature termination of proteins and mistranslation, and consequently bacterial death. They have also been found to inhibit group I intron self-splicing by binding at specific RNA sites (von Ashen et al 1992), and to act on a large group of RNA targets. For example, the neomycin family has been shown to bind to the A-site on 16S ribosomal RNA and cause inhibition of bacterial protein biosynthesis (Moazed & Noller 1987; von Ashen et al 1992; Purohit & Stern 1994; Wallis et al 1995, 1997; Wong et al 1998). Neomycin B inhibits self-splicing at the same low micromolar concentration that is found to disrupt ribosome function (Jiang et al 1999). It also blocks HIV-Rev and HIV-Tat protein bindings to its viral RNA recognition element, the Rev responsive element (RRE; Zapp et al 1993; Werstuck et al 1996; Wang et al 1997; Tok et al 2001) and the trans-acting responsive sequence (TAR; Mei et al 1995), respectively. Neomycin is the best inhibitor for the hammerhead ribozyme cleavage reaction (Clouet d'Orval et al 1995; Stage et al 1995) and inhibits the self-cleavage reaction of the hepatitis delta virus ribozyme (Rogers et al 1996). Hairpin ribozyme cleavage is also catalysed by aminoglycosides such as neomycin B in the presence of spermine and in the absence of metal ions (Earnshaw & Gait 1998). Neomycin B binds the site 1 mRNA of thymidylate synthase (Tok et al 1999) and the RNA stem-loop that regulates alternative splicing of tau exon 10 within the gene coding for human tau protein (Varani et al 2000). Copper derivatives of aminoglycosides such as neomycin B are also efficient cleavage agents for cognate RNA motifs (Sreedhara et al 1999).

The NMR structure of an A-site RNA model complexed with aminoglycosides provides the structural basis for the functioning of the decoding site and for understanding of aminoglycoside–RNA interactions. Fourmy et al (1996, 1998a, b) suggested that the decoding site is an irregular helix that binds antibiotics via its major groove, and might contact the codon–anticodon complex via its minor groove. Understanding RNA–aminoglycoside recognition will be extremely useful for the design of potent and selective antagonists of RNA function (Schroeder et al 2000).

RNA aptamers to some characteristic antibiotic molecules such as tobramycin, lividomycin, neomycins, gentamicin and streptomycin (Figure 3) have been extensively studied (e.g. see Wang et al 1996; Hamasaki et al 1998; for review see Schroeder et al 2000). RNAs capable of binding tetracycline, viomycin or chloramphenicol have also been isolated. We present here the characteristics of these aptamers to antibiotics.

Tobramycin (Wang & Rando 1995; Wang & Tor 1997; Cho et al 1998; Hamasaki et al 1998; Jiang & Patel 1998) Specific RNA aptamers that bind the aminoglycoside antibiotic tobramycin can readily be selected from a randomized pool. The average K_d for the pool selected under low stringent conditions was in the micromolar range (Wang et al 1996). The RNA, synthesized from an individual chosen clone, bound tobramycin with a 1:1 stoichiometry and with about the same affinity as observed for the binding of tobramycin to ribosomes and introns. Selection at greater stringent conditions led to the discovery of the highest binding affinity recorded for any small molecule to an RNA or DNA aptamer. Here clear consensus sequences, located in predicted stem-loop structures, were synthesized and were able to bind tobramycin with high affinity.

The approaches reported here should be generally applicable to the study of the binding of other aminoglycosides to RNA. Fluorescence anisotropy measurements by CRT (5-carboxytetramethylrhodamin labelled tobramycin) were used for the determination of the dissociation constant between the RNA and the aminoglycosides.

By selecting under stringent conditions, tobramycin aptamers that bind tobramycin with subnanomolar dissociation constants were found, and differentiated from similar aminoglycosides with respect to binding. One of these aptamers, J6 (Figure 3), bound stoichiometrically to tobramycin with $K_d = 0.77 \pm 0.03$ nM, while binding to similar aminoglycosides with K_d values 10^3 to 10^4 times higher. An important question is which part of the full J6 construct is important for both high-affinity and specific aminoglycoside binding.

Obtaining high-affinity minimum-sized constructs is also important for possible structural determination. The 109-nucleotide J6 aptamer can be simplified to 39-nucleotide aptamers without substantial loss of affinity and specificity with respect to tobramycin binding.

Elimination of the trinucleotide bulge and the single A bulge decreases binding affinities markedly, and the 1:1 stoichiometry of binding is lost. The trinucleotide bulge and the single A bulge could be important with respect to governing the overall structure of the construct and/or they could be part of the tobramycin binding site. Structural studies on J6f1 (40-mer) and similar constructs should reveal the way in which the trinucleotide bulge, the single A bulge, and 6-base loop are important for high-affinity tobramycin binding (Hamasaki et al 1998).

Chemical modification interference assays were used to investigate which nucleotides are important for tobramycin binding to J6f1 RNA. The 5′ end labelled J6f1 RNA was modified under denaturing conditions using diethyl pyrocarbonate (DEPC) and hydrazine. These reagents modify the base moieties and may interfere with base-specific recognition by tobramycin. Modification of five consecutive bases (G14–U18) by DEPC, and modification of U18, U19, and U20 by hydrazine, abolished tobramycin binding. In contrast, modification of the three-nucleotide bulge did not interfere with tobramycin binding. Consequently, it appears most likely that tobramycin binds to the stem-loop region, cantered at the single base bulge of J6f1 RNA. Deletion of either a three-nucleotide bulge (A9 U10 A11) or a one-nucleotide bulge (C23) from J6f1 RNA abolished high-affinity and stoichiometric tobramycin binding. These results suggest that these two bulges have significant effects on the specific conformation of duplex regions of J6f1 (Wang & Rando 1995).

One plausible mode of aminoglycoside–RNA recognition is through electrostatic interactions between the

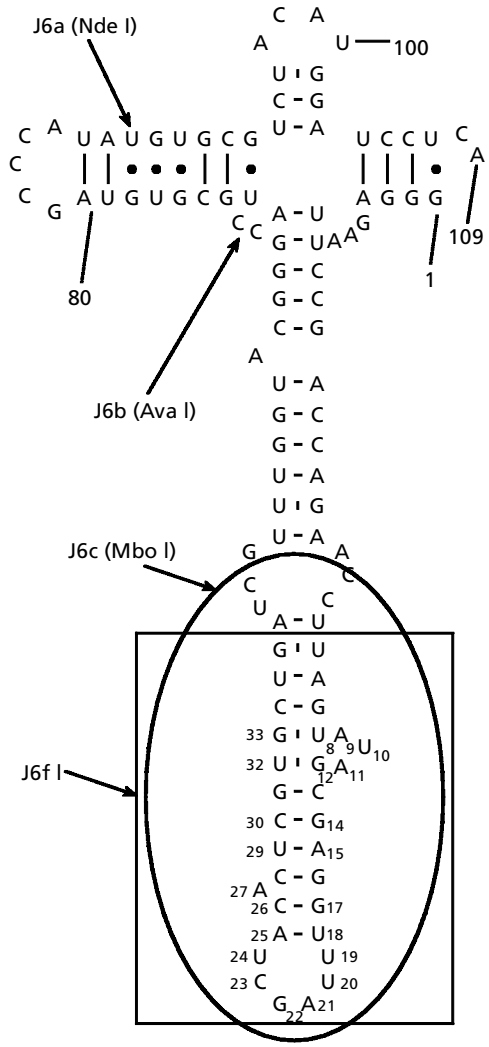

Figure 3 Secondary structure plot of J6 RNA (Hamasaki et al 1998). Reproduced with permission from Biochemistry 37, 2, 658 (1998) © 1996 American Chemical Society.

positively charged amino groups of the aminoglycoside and the phosphate backbone of the RNA. To probe this possibility, ethylation interference experiments were performed with ethyl nitrosourea. No strong interference patterns were observed, suggesting that the phosphate backbone is not involved in recognition. The Ce (III)-ion-mediated cleavage pattern was not disturbed by tobramycin, suggesting that phosphate moieties are not part of the aminoglycoside-binding domain. This is in contrast with other studies showing that the neomycin inhibition of a hammerhead ribozyme is strongly dependent on ionic interaction between the aminoglycoside and the RNA phosphate moieties (Wang & Tor 1997).

To investigate further the importance of specific bases, a series of mutations were introduced into J6f1 RNA. All the mutations of the base pairs, except for substitution of G14–C30 with a G–U pair, had significant effects on CRT binding. Most mutations abolished specific CRT binding altogether, whereas mutations of the A15–U29, G17–C26 and U18–A25 base pairs (to U–A/A–C, G–U, and U–G pairs) showed non-stoichiometric binding with CRT. A series of mutations in the loop region (J6f1 series) were also tested to examine the local sequence requirements for binding. U19, U20, and U24 bases are critical for the CRT binding. In contrast, A21, G22, and C23 could be mutated to other bases with little effect on binding.

Mutations of the single-base bulge A27 to G or U also abolished specific CRT binding. It appears that identity of the single-base bulge A27 may be critical for tobramycin binding. The single-base bulge and stem regions appear to be critical for tobramycin binding. Elimination of the three-nucleotide bulge (A9 U10 A11) eliminated specific CRT binding.

To confirm the role of this bulge in tobramycin binding, the sequence A9 U10 A11 was replaced by a UUU sequence. This mutant sequence showed weak, non-stoichiometric binding with CRT. In addition, mutation of two wobble base pairs (G12–U32 and U8–G33) to canonical Watson–Crick base pairs (G–C and C–G) showed similar non-stoichiometric binding with CRT. This result is consistent with the idea that the three-nucleotide bulge sequence and the adjacent wobble base pairs are important structurally for specific interactions with tobramycin.

On the basis of these results, tobramycin-J6f1 RNA interactions may be different from other aminoglycoside–RNA interactions. It appears that specific interactions do occur between tobramycin and certain nucleotides in the stem-loop region. The trinucleotide bulge appears to be essential for maintaining the correct overall structure of the tobramycin binding site without actually being part of the binding site (Cho et al 1998).

RNA aptamers I (Figure 4A) and II (Figure 4B) directed against tobramycin contain a common U10–A17 loop segment, except at position 15, which is a C in aptamer I and a G in aptamer II. This RNA segment adopts close conformations, as do the bound tobramycin in the structures of the RNA aptamer I and the RNA aptamer II complexes (Wang & Rando 1995; Jiang & Patel 1998).

The structure of tobramycin that binds RNA aptamers I and II has been characterized by multidimentional NMR spectroscopy on the complex containing uniformly labelled ^{13}C, ^{15}N RNA aptamer (Jiang & Patel 1998).

For aptamer RNA I, tobramycin adopts a defined alignment and binds to the RNA major groove cantered about a stem-loop junction site. A portion of the bound tobramycin is encapsulated between the floor of the major groove and a looped-out cytosine residue that forms a flap over the binding site in the complex.

For aptamer RNA II, tobramycin inserts into the deep groove cantered about mismatch pairs and is partially encapsulated between its floor and a looped out guanine base that flaps over the bound antibiotic. Several potential intermolecular hydrogen bonds between the charged NH_3 groups of tobramycin and acceptor atoms on base pair edges and backbone phosphates anchor the aminoglycoside antibiotic within its sequence/structure specific RNA binding pocket.

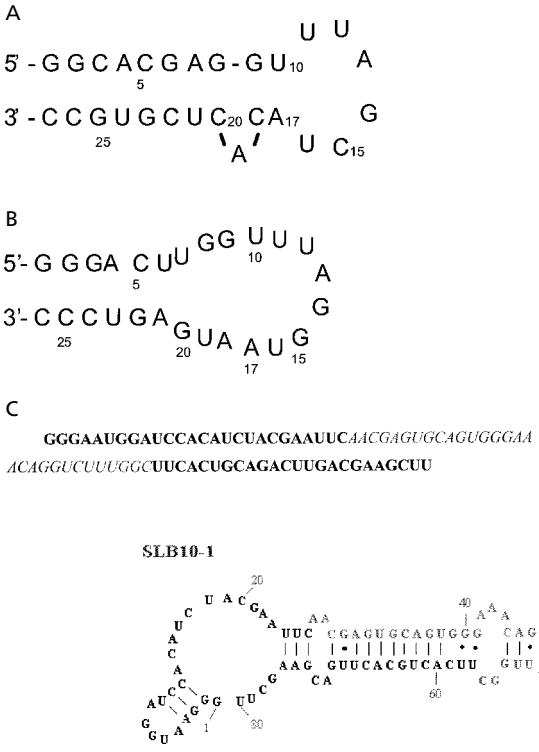

Figure 4 A. RNA aptamer I to tobramycin (Jiang & Patel 1998). B. RNA aptamer II to tobramycin (Jiang & Patel 1998). C. Structure of SLB10-1 aptamer to lividomycin (Lato & Ellington 1996). Reproduced with permission (D. J. Patel).

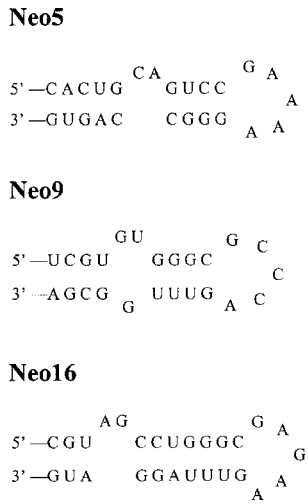

Figure 5 Secondary structure representation of three clones that specifically recognize neomycin (Wallis et al 1995). Reproduced with permission from Chemistry & Biology, Vol. 2 (1995) pp 543–552. © 1995 Elsevier Science.

Lividomycin (Lato et al 1995 (also RNA aptamers to kanamycin A); Lato & Ellington 1996) Aptamers that can bind the aminoglycoside lividomycin have been selected (Lato & Ellington 1996). After four rounds of selection and amplification, the selected pool was found to bind tightly and specifically to lividomycin with a K_d of approximately 300 nM. Thus, although there are a large number of different sequences that can be captured by lividomycin, only a few of these sequences are likely to be found in the context of a given bacterial or eukaryotic genome. After six extra rounds of selection and amplification, two aptamer species remained.

The predicted secondary structure of the dominant anti-lividomycin aptamer is shown in Figure 4C. The dominant aptamer (SLB10-1) uses sequences in the third constant region to form a long stem. It is unclear whether this stem is important for function or whether it as been sequestered to inhibit the formation of alternate inactive conformers.

Individual aptamers were cloned and sequenced. Matching sequences were identified using the BLAST program. RNA secondary structures were predicted using the MUFOLD program with a standard parameter set. The sequence of SLB10-1 is "similar" to several human cDNA clones of unknown function and miscellaneous genes in the mouse, goat and chicken.

Neomycins Neomycins constitute a family of aminoglycoside antibiotics characterized by Waksman & Lechevalier (1949). They inhibit protein synthesis by three mechanisms. First, by blocking synthesis initiation, then by blocking translation, inducing a premature termination and, finally, by the incorporation of defective amino acids. The neomycin family is divided into different classes: neomycins A, B, C, LP-B and LP-C and paromomycin I and II (Figure 1).

Amino groups make them highly polar. They cross membranes poorly and are not absorbed by the intestine. They have been used in two therapy forms: an oral administration to eliminate the intestinal bacteria (before general intestine surgery) or a topic application in a variety of skin infections. They are rarely used by intravenous route because of their kidney and auditory toxicity.

There is great diversity in the secondary structure of the neomycin targets, but it recognizes all of them. Wallis et al (1995) have used SELEX to select 21 clones of RNA aptamers that specifically recognize neomycin. Neomycin binds with high affinity (K_d = 100 nM) and high specificity (affinity for neomycin is 100-fold higher than for paromomycin) to selected aptamers.

Sequence comparison, footprinting of the sites of interaction of neomycin with selected RNAs and partial alkaline hydrolysis experiments identified a hairpin stem-loop structural motif as the neomycin-binding site (Figure 5). The consensus sequence of this motif is: 5′-GGGCGN-NRAGUUU-3′, where N = nucleotide and R = purine.

This motif includes a GNNRA loop, the stem of which might feature a widened major groove owing to the presence of G–U base pairs and an asymmetric bulge. Such a motif has been identified in the central part of RRE and equally in the 16S rRNA. Indeed, an in-vitro selection for neomycin B binding was carried out with an RNA pool containing a 47-nucleotide domain of the decoding region of the 16S rRNA, mutated at 30% per base position (Famulok & Hüttenhofer 1996). The degenerate region was composed

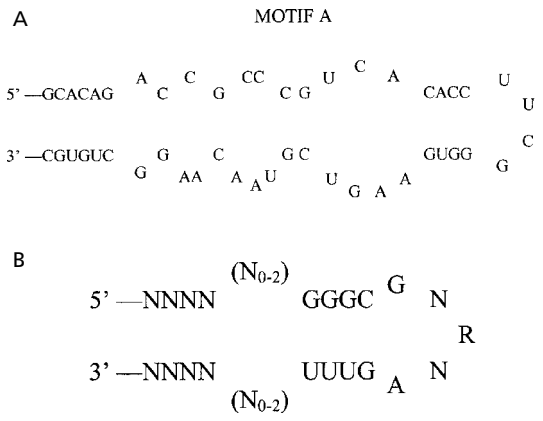

Figure 6 A. Secondary structure of the 16S rRNA and the A motif (Famulok & Hüttenhofer 1996). B. Secondary structure of the B motif (Famulok & Hüttenhofer 1996). Parts A & B reproduced with permission from Biochemistry 35, 14, 4265–4270 (1996) © 1996 American Chemical Society.

of an oligonucleotide analogue (motif A) (Figure 6A) of the decoding region in 30S subunit, which has previously been shown to interact with neomycin B and tRNA ligands. After five cycles of selection/amplification, RNA sequences were isolated and were able to bind specifically to neomycin B. Cloning and sequencing showed that none of the isolated clones shared primary sequence or secondary structure homology with the decoding region of 16S RNA. Instead, a new set of sequences was isolated, which could be folded into a defined hairpin structure termed motif B (Figure 6B).

Motif B binds to neomycin B at Mg^{2+} concentrations at which binding of the other RNAs tested was significantly lower or not detectable. This is consistent with motif B exhibiting a higher affinity for neomycin B than motif A under these conditions. Thus, the A-site motif in 16S rRNA (motif A) might not be an optimal target for neomycin B recognition.

Finally, in-vitro selection allowed the determination of neomycin conformation in its binding with an RNA aptamer. Recognition of a cognate RNA aptamer by neomycin B was recently studied (Cowan et al 2000). The data reported are consistent with the dominance of H-bonding in comparison with the ionic contacts as demonstrated previously for tobramycin (Cho et al 1998).

It was demonstrated that the antibiotic paromomycin binds specifically to an RNA oligonucleotide that contains the 30S subunit A-site (Fourmy et al 1996). The solution structure of the RNA–paromomycin complex determined by NMR spectrometry revealed that the antibiotic binds in the major groove of the model A-site RNA within a pocket created by an A–A base pair and a single bulged adenine. Another NMR study showed that two universally conserved residues of the A-site of 16S rRNA are displaced towards the minor groove of the RNA helix in the presence of paromomycin (Fourmy et al 1998b).

Detailed comparative NMR studies and biochemical experiments show that ring I and ring II of neomycin class aminoglycosides (Figure 1; neamine molecule) are sufficient to confer specificity for the binding to a model A-site RNA (Fourmy et al 1998a). Another NMR study of an RNA aptamer to neomycin B with 100 nM affinity showed that the neamine moiety is sandwiched between the major groove floor of a ''zipper-up'' G–U mismatch and a looped-out purine base that flaps over the bound antibiotic (Jiang et al 1997). Specific intermolecular hydrogen bonds were also observed between the charged amines of neomycin B and bases mismatch edges and backbone phosphate.

Gentamicin (Yoshizawa et al 1998) One of the most important aminoglycoside used clinically is gentamicin (a mixture of three components, gentamicin C1, C2 and C1a; Figure 1). Binding of each gentamicin component to the ribosome and to a model RNA oligonucleotide (27 nucleotides) was studied biochemically. On binding of each component, the same bases of the A-site RNA were protected against chemical modification by DMS. Gentamicin C1 binds with the lower affinity compared with gentamicin C1a and C2 (K_d = 0.5, 0.01, and 0.025 μM, respectively). Molecular modelling showed that for gentamicin C1, in which the 6′ amino group of gentamicin C2 is methylated, rotation around the 6′ carbon and the 6′ nitrogen is restricted in the binding pocket. The limited rotation may disrupt some of the possible hydrogen bonds and electrostatic interactions.

The structure of the RNA complexed to gentamicin C1a was solved by magnetic resonance nuclear spectroscopy. Gentamicin C1a binds in the major groove of the RNA. The 3′ and 4′ hydroxyl groups in paromomycin are substituted by hydrogen atoms in gentamicin C1a. These hydroxyl groups make backbone contacts (Fourmy et al 1996) in the paromomycin complex that may be compensated by hydrophobic interactions between a guanine and ring II. The comparison of the positions of ring I and II in both structures clearly revealed how similar hydrogen bond donor groups specifically contact the RNA. The gentamicin–RNA structure suggests why aminoglycosides with a 4,6-substituted ring I are clinically preferred. The ring III of these aminoglycosides makes specific contact with the A-site; in contrast, aminoglycosides with a 4,5-substituted ring I do not make additional base-specific contact beyond rings I and II. Gentamicin–RNA contact is apparently highly cooperative, as disruption of ring III guanine hydrogen bonds leads to a severe loss of affinity. The structure leads to a general model for specific ribosome recognition by aminoglycoside antibiotics and a possible mechanism for translational inhibition and miscoding. The results obtained provided a basis for the understanding of the resistance mechanisms to aminoglycosides. Modification enzymes target rings I and II (acetylation or phosphorylation). These modifications lead to steric hindrance and interaction penalties that prevent binding. In the neomycin class, 3′ and 4′-hydroxyl groups of ring II are phosphorylation and adenylation targets, respectively. These two hydroxyl groups are absent in gentamicin components, with little consequence for RNA binding, and they are not a target for these classes of aminoglycoside modification enzymes.

Streptomycin (Wallace & Schroeder 1998) The amino cyclitol antibiotic streptomycin (Figure 1) interacts with the 16S ribosomal RNA and, in addition, inhibits group I intron splicing. Two kinds of aptamers have been isolated by in-vitro selection: aptamers from the normal selection that bind streptomycin and bluensomycin, and RNA from a counter selection that discriminates between streptomycin and bluensomycin.

Bluensomycin (Figure 1) is a streptomycin analogue, differing from streptomycin by containing a carbamido group instead of a guanidino group. Bluensomycin does not inhibit group I intron splicing of the T4 thymidylate synthase gene.

Two motifs were found in different locations within different clones and were chosen for further investigations. The minimum sequence necessary to bind streptomycin was determined by subjecting RNAs from clones containing motifs 1 and 2 to partial alkaline hydrolysis followed by affinity chromatography.

By testing motifs 1 and 2 for binding characteristics on streptomycin Sepharose using equilibration buffer containing 250 mM NaCl and different concentrations of Mg^{2+}, it was shown that Mg^{2+} does not compete with streptomycin for binding to the RNA aptamer. This suggests that the binding is specific and that Mg^{2+} is not an essential cofactor for the interaction of streptomycin with these RNAs.

By performing Pb^{2+}-induced cleavage in the presence or absence of streptomycin and Mg^{2+}, conformational changes were detected in the RNA on addition of both ligands.

Chemical probing using DMS and ketoxal was performed to propose a secondary structure model, to locate the region of RNA–antibiotic interaction and to ascertain whether the motifs can discriminate between bluensomycin and streptomycin.

Two natural streptomycin binding sites are known, one in the core of the 16S rRNA and one in the core of self-splicing group I introns. This suggests that the binding site is probably composed of several secondary structure elements that converge. It seems to be a general rule for RNA aptamers that bind their ligands with high affinity and that discriminate between analogous compounds, that the RNA does not present a preformed pocket to the ligand. The RNA is rather unstructured in the absence of the ligand. The aptamers adopt a complex structure only in the presence of the ligand, using it as an essential building block for structure formation.

Tetracycline (Berens et al 2001) Tetracycline is an antibiotic that inhibits prokaryotic translation by interfering with binding of the aminoacyl-tRNA to the ribosomal A-site (Sandar et al 2001). Recently, the structure of the 30S ribosomal subunit, the target of tetracycline, complexed with the antibiotic was described (Brodersen et al 2000). Aptamers with high affinity to tetracycline have been isolated by in-vitro selection. One of them, cb28, which has a comparable affinity to tetracycline, was characterized as the small ribosomal subunit. The cb28 aptamer binds only to typical tetracyclines, while atypical tetracyclines were recognized. The hydroxyl group at position 6 is an essential determinant for recognition and binding is magnesium dependent. The secondary structure of cb28 was determined by lead cleavage and DMS modification. Protection from lead cleavage indicated a conformation change in the RNA, which was confirmed in the DMS modification pattern. Photo-induced affinity incorporation of tetracycline into cb28 resulted in a crosslink. The mode of binding of tetracycline to the cb28 aptamer resembles its interaction with the primary binding site on the small ribosomal subunit.

Viomycin (Wallis et al 1997) Viomycin (Figure 2) is a small cyclic peptide antibiotic containing the amino acids arginine, serine and lysine, which are known to occur in functional domains of RNA binding proteins. Indeed, viomycin inhibits translation and splicing of group I introns and it induces RNA–RNA interactions. It is a member of the tuberactinomycin family, which is widely used against tuberculosis.

To achieve a better understanding of the principles underlying binding and recognition of viomycin by RNA, viomycin-specific RNA aptamers with high affinity (K_d = 11–16 μM) have been selected. These RNA molecules share one commonly recurring motif, a stem-loop important for binding, although it is not sufficient to achieve full viomycin binding activity. Indeed, the loop is engaged in a long-range interaction with sequences near the 3′ end of RNA molecules, resulting in the formation of a pseudoknot (Figure 7). The formation of this pseudoknot was tested by mutational analysis in the presence or absence of viomycin and results were in good agreement with the proposed structure. In particular, these studies show that addition of viomycin to the RNA results in protection of bases within the consensus motif from chemical modifications and protection of backbone positions from Pb^{2+} cleavage, confirming that this region is involved in viomycin binding. By comparing the natural viomycin binding site with the novel pseudoknot motif, it was noticed that they all fold into pseudoknot structures. Thus, the result of the selection, and the fact that natural target sites for viomycin are composed of RNA pseudoknots, suggests that peptide antibiotic has a specificity for and is able to recognize particular pseudoknots.

Chloramphenicol (Burke et al 1997) Many antibiotics, including chloramphenicol (Figure 2), inhibit the peptidyl transferase activity of ribosomes. Chloramphenicol interacts with ribosomes through the central loop of domain V in 23S RNA, also known as the peptidyl transferase loop (Leviton 1999). Chloramphenicol binding aptamers have been selected from two independent initially random RNA populations. These aptamers share a structural motif: a pseudosymmetric motif, which consists of a highly conserved central helix of 5 to 6 base pairs flanked by A-rich bulges and additional helices. A 1:1 RNA–chloramphenicol stoichiometry has been established. Chloramphenicol aptamers have a well-defined secondary structure with a binding pocket that appears to be stabilized by chloramphenicol.

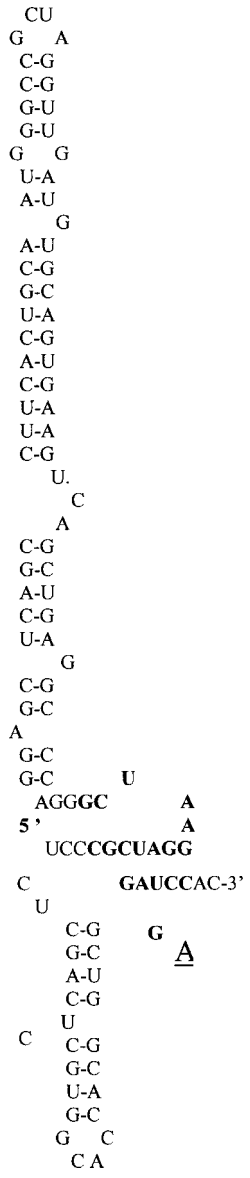

Figure 7 Proposed secondary structure of one selected aptamer to viomycin (Wallis et al 1997). Reproduced with permission from Chemistry & Biology, Vol. 4 (1997) pp 357–366. © 1997 Elsevier Science.

Similarities have been observed between the RNA motif and the chloramphenicol binding site in 23S r RNA.

Conclusion

Development of novel aminoglycoside drugs and antibiotics that target RNA

Because small molecules inhibit RNA functions involved in prokaryotic and eukaryotic translation and viral replication, RNA macromolecules are now considered as major targets for therapeutic intervention (Chow & Bogdan 1997; Pearson & Prescott 1997; Borman 2000; Hermann 2000; Schroeder et al 2000). Davies (1990) suggested that antibiotics were not invented for the purpose of killing bacteria, but rather to act as effector molecules in the regulation of many biological activities, for example aminoglycosides, which act on RNA during protein synthesis.

The architecture of the RNA folds is beginning to be understood (for reviews see Hermann & Patel 1999, 2000) and the first crystallographic structure of the ribosome (Muth et al 2000; Nissen et al 2000) has provided the structural basis for understanding ribosome activity in peptide bond synthesis. NMR structures of an A-site RNA model complexed with aminoglycosides give interesting structural information on aminoglycoside–RNA interactions (Fourmy et al 1996, 1998a, b). In order to progress the rational design of new antibiotics, in-vitro selection and studies of RNA aptamers to the different antibiotics is an interesting way to circumvent the problem of studying large natural RNA–target complexes. The in-silico strategy for the docking of cationic drugs to RNA targets using molecular dynamic simulations should contribute to this progress (Hermann & Westhof 1999, 2002).

At the same time, a series of novel techniques have been developed to measure specificity and affinity of small molecules for RNA (Hendrix et al 1997; Hamasaki & Rando 1998; Wong et al 1998). Combinatorial chemistry was used to generate libraries of neomycin B ''mimetics'' (e.g. Park et al 1996; Greenberg et al 1999) and chemical modifications of aminoglycosides resulted in an increased affinity for their RNA targets (e.g. Wang & Tor 1997; Tok et al 1999a, b, 2001; Litovchick et al 2000; Hamasaki & Ueno 2001). New aminoglycosides were obtained that can resist or inhibit aminoglycoside-modifying enzymes (for reviews see Kotra et al 2000; Kotra & Mobashery 2001). Using such a strategy, dimers of neamine that target rRNA and inhibit resistance-causing enzymes were synthesized (Sucheck et al 2000). Aminoglycosides were also modified in order to decrease the strength of their electrostatic interactions with aminoglycoside 3′-phosphotransferases types Ia and IIa, responsible for resistance (Roestamadjli et al 1995a, b; Roestamadjli & Mobashery 1998; Kotra et al 2000).

In less than 10 years, these approaches have led to new RNA-acting drugs and antibiotics. Thus, the use of aptamers to understand antibiotic mechanisms of action and bacterial resistance is very promising. In-vitro selection and NMR studies have allowed the nucleic acid sequences essential for antibiotic binding to the natural targets to be determined. Similarities in the aptamer motif selected and the ribosomal RNA binding site were observed. It also makes easier the research of mutated sites when resistance appears, finding molecules with high affinity and high specificity to kill bacteria, and discovering other potential targets for antibiotics.

Necessity of control in antibiotic use

In 20 years, antibiotics use per year and per person has multiplied by two. In France, this use increases by about 4% per year. The following rules should be observed:

antibiotic prescription is not always an obligation, optimal posology should be respected, and there should be better follow-up of treatment. The progression of bacterial multiresistance is monitored by coordination structures (committee of drugs, CCLIN, Onerba), which allows updating of the activity spectrum of antibiotics. It also allows coordinated strategies to be created to prevent the spread of bacterial resistance and to intervene when necessary. For instance, CCLIN has been present in every public hospital since 1998. It takes an active interest in staff training, with respect to hygiene and in the monitoring of resistance.

The development of totally new classes of antibacterial agents should allow bypassing of the resistance problem. For instance, by decreasing the pathogenic potential of bacteria by targeting virulence factors or by working on intercellular communication molecules. To further this, two strategies have been developed: the development of new molecules derived from old ones, or the development of new molecules that can act on original bacterial targets or that block bacterial resistance mechanisms (Bergogne-Berezin 1999).

Genomic approaches, characterization and total sequencing of the genome and its exploitation, and proteomics (systematic analyses of all bacterial proteins) are also under development. Monitoring systems for resistance are being developed and are currently used to control antibiotic use. Thus, while pharmaceutical industries are fighting resistance by creating new antibiotics, clinicians, microbiologists and hygienists are protecting the future of these products by increasing the control of their use in hospitals and in general practice, and by defining better protocols for the use of existing antibiotics.

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