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Aminoglycoside Interactions with RNAs and Nucleases

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Abstract One of the major challenges in medicine today is the development of new antibiotics as well as effective antiviral agents. The well-known aminoglycosides interact and interfere with the function of several noncoding RNAs, among which ribosomal RNAs (rRNAs) are the best studied. Aminoglycosides are also known to interact with proteins such as ribonucleases. Here we review our current understanding of the interaction between aminoglycosides and RNA. Moreover, we discuss briefly mechanisms behind the inactivation of aminoglycosides, a major concern due to the increasing appearance of multiresistant bacterial strains. Taken together, the general knowledge about aminoglycoside and RNA interaction is of utmost importance in the process of identifying/developing the next generation or new classes of antibiotics. In this perspective, previously unrecognized as well as known noncoding RNAs, apart from rRNA, are promising targets to explore.

Keywords RNA · Aminoglycosides · Metal ions · Small ligands · Antibiotics

1 Introduction

Until relatively recently it was believed that the long struggle for control over infectious disease was almost over. Smallpox was eradicated 1980, vaccine programs were in place to protect the world's children against major killer diseases, and a variety of antibiotic drugs were effectively suppressing countless microbial infections. However, cautious optimism has been overtaken by a fatal complacency that is costing millions of lives, and threatening global socioeconomic development. According to the World Health Report 1996 by the WHO, infectious diseases are still the leading cause of death in the world, killing at least 17 million people every year (http://www.who.int/whr/1996/en/). Diseases such as tuberculosis and malaria once believed to be under control are re-emerging with renewed ferocity. Another major challenge today is the developing resistance to antibiotics, and some infections are virtually untreatable due to the occurrence of multiresistant bacteria (see, for example, Davies 1994; Davies and Wright 1997). Other important aspects are the appearance of "new" infectious agents such as the severe acute respiratory syndrome (SARS) virus, the geographical allocation of infectious agents due to, e.g., increased traveling, and the use of medications that suppress the immune system. Thus, far from being over, the struggle to control infectious diseases has become increasingly difficult, and this situation has resulted in increased costs for healthcare for the society worldwide. Consequently, one of the major challenges in medicine today is the development of new antibiotics as well as effective antiviral agents.

The plethora of properties and functions associated with RNA molecules, i.e., non-coding RNA (ncRNA), has led to the realization that RNA molecules frequently are associated with the development and progression of diseases: genetic disorders, tumor progression, autoimmune diseases (Sullenger and Gilboa 2002). Numerous RNA molecules are also essential for the growth of microbial pathogens (e.g., bacteria, virus, parasites, etc.) and thereby are essential for the progression of infectious diseases (Gottesman 2004). Thus, RNA is indeed a potential drug target and this is witnessed by the fact aminoglycosides interact with RNA and interfere with its function (e.g., von Ahsen et al. 1991 reviewed in Davies et al. 1993).

In this chapter we will review what is currently known about the interaction between aminoglycosides and RNA. However, first we will briefly give an overview about aminoglycosides, resistance against aminoglycosides, and the interaction between RNA and metal ions and other small ligands. Finally, we will discuss the interaction between aminoglycosides and protein enzymes that depend on metal ions for activity followed by a brief outline of future perspectives.

1.1 Aminoglycosides

Aminoglycosides are secondary metabolites that are produced and secreted by the producer to ensure a growth advantage in relation to its neighbors (Davies 1994; Davies and Wright, 1997; Zembower et al. 1998; for further information about classification and biosynthesis of aminoglycosides and other antibiotics, we refer the reader to a recent and excellent book: Walsh 2003). Streptomycin was identified and isolated as early as 1944, and since then many other aminoglycosides have been identified. Also, semisynthetic aminoglycosides such as amikacin and tobramycin have been generated. Aminoglycosides show predictable pharmacokinetics and have been used over the years in the clinic for the treatment of infections caused by both Gram negatives and positives including *Mycobacterium tuberculosis*. In many cases, aminoglycosides work in synergy with other antibiotics. Although many of them are very potent drugs, they are also associated with high toxicity as exemplified by neomycin B. It is well established that aminoglycoside treatment is associated with nephro- and ototoxicity, where the latter is irreversible (Mingeot-Leclercq and Tulkens 1999; Hutchin and Cortopassi 1994; Begg and Barclay 1995). Noteworthy is that chemical approaches have had little effect addressing these toxicity-associated problems. The aminoglycosides are divided into two main classes, the 2-deoxystreptamine-containing and streptomycin antibiotics. The former class includes neomycin B and kanamycin A and B, while the latter is exemplified by streptomycin (Fig. 1). The 2-deoxystreptamine class is further divided into 4,6-disubstituted deoxystreptamine (e.g., kanamycin A and B) and 4,5-disubstituted deoxystreptamine (e.g., neomycin B and paromomycin).

Antibiotics such as aminoglycosides are mainly produced by bacteria, and in the case of aminoglycosides, the main producers are found among the actinomycetes. These carbohydrate antibiotics are also referred to as aminocyclitol, and for their synthesis the activity of a large number of gene products are required. In the case of streptomycin, approximately 30 genes in *Streptomyces griseus* are turned on in response to changes in the environment such as changes in nutrient supply or stationary growth and the outcome is secretion of streptomycin. Thus, antibiotic-producing bacteria have put a large investment into their production.

1.2 Resistance Toward Aminoglycosides

Resistance toward naturally occurring aminoglycosides is an essential property for the bacteria that synthesizes a particular aminoglycoside. This simple fact is probably a key reason why antibiotic resistance has become such a growing medical problem. Here we will briefly review some aspects of the mechanisms behind aminoglycoside resistance in bacteria. For a more extensive discussion

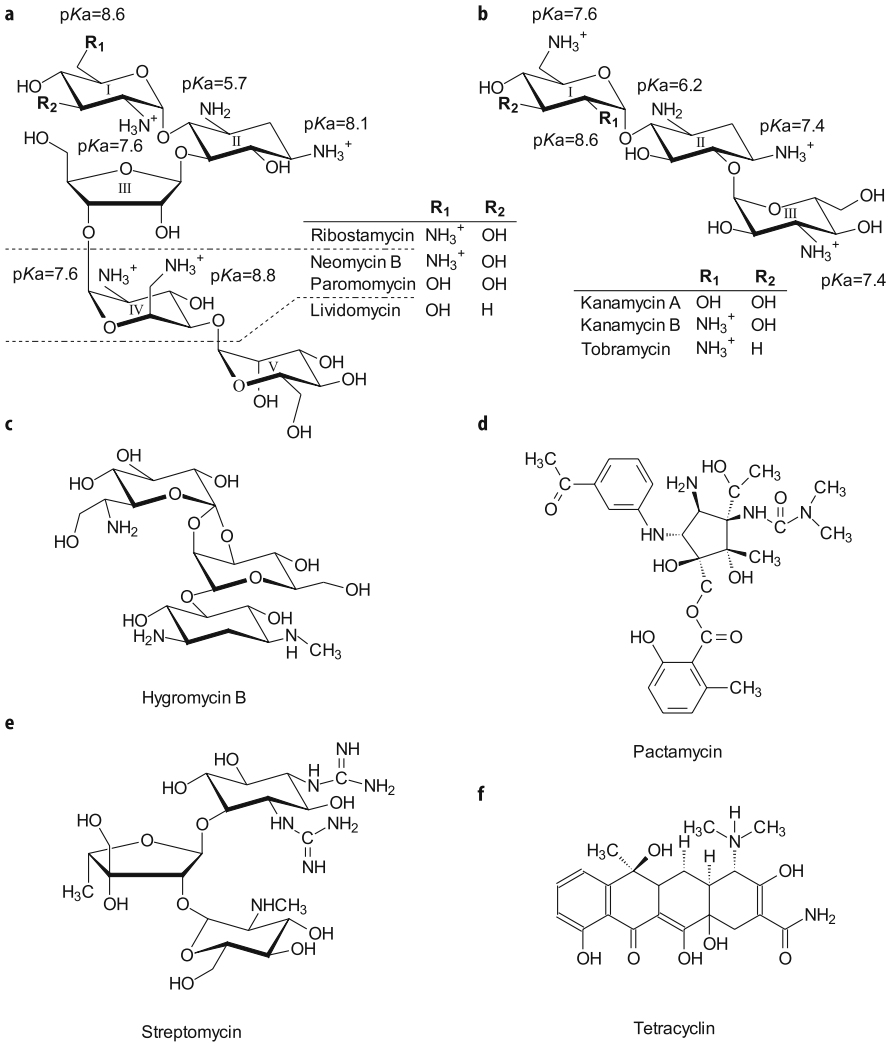

Fig. 1a–f Molecular structures of antibiotics. **a** The neomycin family, **b** kanamycin family, **c** hygromycin B, and **e** streptomycin are all representatives of the aminoglycoside family. Positions where they differ are indicated with R_1 and R_2 and pK_a values for the ammonium groups are indicated. The structures of two other RNA-binding antibiotics that are discussed here are also illustrated: **d** pactamycin and **f** tetracyclin

of the topic, we refer to several recent and excellent reviews (see for example Walsh 2003; Mingeot-Leclercq et al. 1999; Kotra et al. 2000; Dessen et al. 2001; Stewart and Costerton 2001).

Streptomycin is active as a free substance, and in order to ensure that the producer, i.e., *S. griseus*, does not kill itself during synthesis, strepto-

mycin does not become an active substance until the secretion process, during which it is activated via two chemical reactions. Thus, natural systems exist that modify aminoglycosides, as well as other naturally occurring antibiotics, which lead to either activation or inactivation of the antibiotic. With respect to the resistance problem, three main classes of enzymes are involved in chemical and covalent modification of the amino and hydroxyl groups of aminoglycosides: *O*-phosphotransferases, O-nucleotidyltransferases and *N*-acetyltransferases. *O*-Phosphotransferases, also referred to as APH, use ATP as phosphate donor and modify specific hydroxyl groups on the aminoglycoside. *O*-Nucleotidyltransferases (ANT) also use ATP as a donor, resulting in adenylation of hydroxyl groups. The *N*-acetyltransferases (AAC) use acetyl-CoA as donor and modify the amino groups. The genes encoding these enzymes are often carried by transposable genetic elements or plasmids. Within each class, several aminoglycoside-modifying enzymes with different regiospecificities have been identified: Seven APHs, four ANTs, and four AACs are currently known. The crystal structures of some enzymes have been solved, and this has given mechanistic information and generated strategies of how to circumvent the resistance problem. The binding pocket encompassing kanamycin A and the active site of kanamycin nucleotidyltransferase is illustrated in Fig. 2.

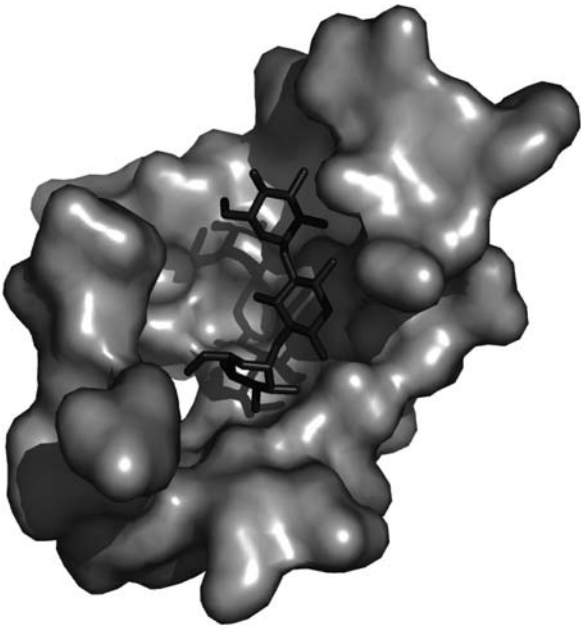

Fig. 2 Aminoglycoside binding to the enzyme kanamycin nucleotidyltransferase. The surface representation shows the interacting region within a radius of 10 Å surrounding the bound kanamycin A (as *stick model*). The figure was made using the molecular graphics program Pymol (DeLano 2002). The structure is according to Pedersen et al. (1995). PDB code 1KNY

Another strategy used by microorganisms that results in resistance (or increased tolerance) is by modification of the targets. In the context of RNA, this means modification of the base or the ribose as well as replacing the entire nucleotide with another nucleotide, i.e., introducing a mutation. For example, *Micromonospora purpurea*, which produces gentamicin, protects itself by methylation of its 16 S ribosomal (r)RNA (Thompson et al. 1985). Also, eukaryotic ribosomes show a decreased affinity ($\geq$tenfold; Table 1) toward aminoglycosides due to substitution of A1408, with G resulting in a G1408/A1493 base pair not present in the bacterial rRNA. In addition, the eukaryotic ribosome lacks the C1409/G1491 base pair (Recht et al. 1998; Vincens and Westhof 2001, 2002, 2003; see also Walter et al. 1999).

Table 1 Summary of apparent inhibition constants for a selected number of aminoglycosides inhibiting different RNA activities (concentrations are given in micromolars). Given K_i values are defined as the concentration resulting in 50% inhibition, with the exception of values that are appK_i[#] values and calculated K_i values[##]

RNA	Aminoglycosides				
	Neomycin B	Paromomycin	Kanamycin A	Tobramycin	Reference
sunY td group I intron	1.3[#]	100	$\gg$1,000	nd	von Ahsen et al. 1992
Hammerhead	13.5				Stage et al. 1995
Hairpin	190	600	nd	nd	Earnshaw and Gait 1998
E. coli RNase P RNA −C5 protein	35	190	nd	nd	Mikkelsen et al. 1999
E. coli RNase P RNA +C5 protein	60	nd	nd	nd	Mikkelsen et al. 1999
Human RNase P	$\geq$600	nd	nd	nd	Eubank et al. 2002
Charging of *E. coli* $tRNA^{Phe}$	300	nd	nd	nd	Mikkelsen et al. 2001
Charging of yeast $tRNA^{Asp}$				0.036[#]	Walter et al. 2002
E. coli tmRNA	70	225	1,400	1,600	Corvaisier et al. 2002
Genomic HDV RNA	28[#]	1,000	1,000	nd	Rogers et al. 1996
A-site (bacterial) 16S rRNA	nd	0.11	nd	2	Griffey et al. 1999
A-site (eukaryotic) 18S rRNA	nd	>20	nd	1.4	Griffey et al. 1999
Affinity to[1] RRE RNA[##]	0.9	nd	100	10	Luedtke et al. 2003

nd, not determined. [1]Measured as Rev peptide displacement from the HIV-1 Rev response element (RRE)

Antibiotic resistance can also be achieved by active transport or efflux of the antibiotic out of the bacteria, resulting in a concentration that is too low to cause harm to the bacteria. This strategy to achieve resistance is very common among bacteria, and for further discussion on this topic we refer to Walsh (2003). Finally, we would like to mention that antibiotic resistance is also manifested as a result of the formation of bacterial biofilms. Here the mechanisms of resistance are different in relation to the discussion above, and we refer to a recent review covering this topic (Stewart and Costerton 2001).

1.3 Properties and Functions of RNA

It has become evident during the past few years that RNA plays a much more vital role in all living organisms than initially anticipated, when it was believed that the only role of RNA was to physically convey genetic information stored in DNA to functionally acting proteins. Today it is clear that RNA, besides being the physical link between DNA and protein, plays several other key roles, i.e., structural, functional, regulatory, and informational. A large number of ncRNAs have recently been identified, and today we know that such ncRNA molecules have several fundamental functions essential for cell growth, survival, and development. The functions that RNA carries out or participates in include RNA processing and protein translation, acting as structural scaffolds, transporters, gene regulators, and biocatalysts. In fact, most likely RNA and not protein constitutes the active center where peptide bond formation takes place (Ban et al. 2000; Nissen et al. 2000). Moreover, the rapid increase in available genome sequences has permitted researchers to search for and analyze regulatory RNAs, which used to be impossible. In the last 2 years, several novel and biologically important small RNAs have been discovered in a variety of organisms from bacteria to mammalian cells. In bacteria, these small RNAs are sometimes referred to as sRNA, while the novel small RNAs in eukaryotes include, for example, micro (mi)RNA and small interfering (si)RNA. In eukaryotes the large collection of novel small and ncRNAs have been demonstrated to be involved in gene silencing via RNA and to play essential roles in controlling all steps of gene expression, including transcription, chromatin modification, epigenetic memory, and alternative splicing (Mattick 2003). The recognition that many of the recently discovered ncRNAs in both bacteria and eukaryotes possibly act as regulators of gene expression has led to the initiation of vigorously pursued research efforts worldwide. Moreover, we note that the role of small RNAs in immunity was recently discussed by McManus (2004).

2
Small Ligands and RNA

2.1
RNA, Metal Ions and Small Molecules

Our increased knowledge of RNA function/structure has lead to the realization that divalent metal ions such as Mg^{2+} play crucial roles for RNA function, being both structurally and/or catalytically important. On average, there is one Mg^{2+}-ion bound per 3–4 nucleotides of the negatively charged RNA. It has been demonstrated that binding of Mg^{2+} to RNA is important for RNA folding, RNA–RNA interactions, RNA–protein interactions, and various catalytic processes such as cleavage of RNA, transfer (t)RNA charging, and codon–anticodon interaction (Gesteland et al. 1999). Other biologically relevant divalent metal ions, such as Ca^{2+} and Mn^{2+}, also bind to RNA. The former binds with approximately the same affinity to RNA as Mg^{2+}, while the latter binds 3–4 times stronger (Brännvall et al. 2001). Addition of, for example, Ca^{2+} to RNase P RNA cleavage and $tRNA^{Ala}$ alanyl-tRNA synthetase charging reactions result in reduced activities (Brännvall and Kirsebom 2001 and references therein). This raises the interesting possibility that biocatalysts that depend on RNA for activity are up- or down-regulated depending on the intracellular concentrations of Mg^{2+} and Ca^{2+}. For example, the flux of Ca^{2+} is perturbed in tumor cells (Berridge et al. 1998) and in bacterially infected cells (Uhlen et al. 2000). Another possibility is that binding of different metal ions influences the interaction between RNA and other cellular factors such as proteins (Brännvall et al. 2004).

Addition of Mn^{2+} influences the accuracy of, for example, RNA-mediated cleavage of RNA and protein-mediated cleavage of DNA (Brännvall and Kirsebom 2001; Hsu and Berg 1978). In this perspective, it is interesting to note that certain bacteria, for example *Borrelia burgdorferi*, an intracellular parasite causing Lyme disease (Posey and Gherardini 2000) and certain *Lactobacillus* spp. (Archibald and Duong 1984) have elevated intracellular concentration of Mn^{2+}. Clearly this emphasizes the importance of understanding the interaction between RNA and metal ions from a biological perspective, as well as investigating in detail the biological consequences as a result of the interaction between RNA and different metal ions. Noteworthy in this context is the recent finding that other ligands, such as vitamin B12 and thiamine, interact with specific structural motifs of RNA and thereby influence the expression of specific genes. These structural motifs are referred to as riboswitches, i.e., structural domains in the non-coding regions of mRNAs, acting as metabolite-responsive genetic switches (see for example, Nahvi et al. 2002; Winkler et al. 2002; Mandal et al. 2003; Vitreschak et al. 2004). Moreover, tRNA has been demonstrated to be involved in regulation of many "amino-acid-related" genes (e.g., amino

acyl tRNA synthetase genes) in gram-positive bacteria (for a recent review see Grundy and Henkin 2004).

An interesting aspect regarding the function of small RNAs is their potential role for virulence. In fact, recently, an RNA involved in regulation of the expression of an *E. coli* toxin gene was identified (Vogel et al. 2004), and we foresee many others yet to be identified. For the interaction between RNA and other small ligands, we refer to Hermann (2003). Thus, small ligands of various classes, from simple metal ions to more complex organic compounds, interfere with and regulate RNA function.

Taken together, an increased knowledge of the way metal ions and other small ligands such as aminoglycosides (see the following sections) interact with RNAs and carry out their functions is fundamental and necessary in order to understand the mechanism of action of the different RNA molecules that exist in a cell. This knowledge can be exploited to identify small ligands that bind a given RNA specifically and interfere with its function. Needless to say, these ligands can subsequently be used as leads to develop novel drugs/antibiotics.

2.2
Aminoglycoside Binding to RNA and Displacement of Divalent Metal Ions

The bacterial ribosome is a primary target for various antibiotics such as the aminoglycosides, which bind to 16 S rRNA in the A-site and interfere with the decoding process. Aminoglycosides also bind to other RNAs and interfere with their functions, for example: inhibition of several ribozymes including RNase P RNA; inhibition of tRNA charging and inhibition of splicing (Table 2; see Sect. 2.3). Currently, the understanding of how aminoglycosides interact with RNA and interfere with its function has become an increasingly important research field that is exploited worldwide to identify novel aminoglycoside-based antibiotics.

Structural studies and biochemical probing analysis have been used to obtain information regarding the interaction between RNA and various aminoglycosides (for reviews see, for example, Kotra et al. 2000; Walter et al. 1999; Vicens and Westhof 2003; Yonath and Bashan 2004). Here we will only highlight some aspects of the RNA-aminoglycoside interaction based primarily on the high-resolution RNA-aminoglycoside structure complexes that recently have been reported (e.g., Vicens and Westhof 2001, 2002, 2003b; Mikkelsen et al. 2001; Brodersen et al. 2000; Carter et al. 2000; Ogle et al. 2000; Schlünzen et al. 2000; Piolleti et al. 2001 for binding of several other antibiotics to the ribosome).

Crystal studies and nuclear magnetic resonance (NMR) spectroscopy of different complexes containing either 4,5- (e.g., tobramycin and geneticin) or 4,6-disubstituted (e.g., paromomycin and neomycin B) (see, for example, Fourmy et al. 1996, 1998a,b) have revealed that aminoglycosides often bind in the deep groove: in the A-site of the ribosomal 16S rRNA (Fig. 3) and below

Table 2 A compilation of novel RNA drug targets as indicated

RNA	Function	Bacteria	Essential	Human	Structural differences	Potential drug target
Group I intron	Gene regulation expression	In certain bacteria		No	Not relevant	Possibly
RNase P RNA	tRNA biosynthesis	Yes	Yes	Yes	Yes	Yes
tRNA charging	Translation	Yes	Yes	Yes	Yes	Yes
tmRNA	Scavenging	Yes	No[##]	No	Not Relevant	Yes
4.5S RNA	Protein export secretion	Yes	Yes	Yes	Yes[#]	Possibly
Spot 42 RNA	Gene regulation	Yes	No	No	Not relevant	Not known[###]
6S rRNA	Inhibitor of RNA polymerase	Yes	No	No	Not relevant	Not known

[#]Not including low GC gram-positive bacteria [##]Has been demonstrated to be essential in *Neisseria* spp. (see text) but not in *E. coli* under laboratory conditions [###]Might be a model system to use to exploit antisense RNA as a drug target

the D-loop in yeast $tRNA^{Phe}$ (Fig. 4). Based on these and biochemical studies (see Sect. 2.3), it is clear that (1) electrostatic interactions and (2) hydrogen bond formation directly between RNA residues/backbone and amino/hydroxyl groups on the aminoglycoside and water-mediated interactions are crucial to achieve high affinity. In the case of binding to the 16S rRNA A-site, the dissociation constant has been determined to be as low as 1.5 μM for both 4,5- and 4,6-disubstituted aminoglycosides (Hyun Ryu and Rando 2001) while for binding to yeast $tRNA^{Phe}$ the affinity is higher (Table 1; Mikkelsen et al. 2001). However, increasing pH above the physiological level results in reduced binding and no inhibition due to deprotonation of amino groups on the aminoglycoside (Fig. 5). The latter observation emphasizes experimentally the importance of electrostatic interactions between the positively charged aminoglycoside and the negatively charged RNA. Moreover, it is the functional groups of the neamine domain (Fig. 1), which is shared between these aminoglycosides, that play a decisive role in the interaction with RNA.

Studying the interaction between yeast $tRNA^{Phe}$ and aminoglycoside demonstrated that aminoglycoside binding to the RNA resulted in displacement of a divalent metal ion, and hence it was suggested that aminoglycosides could be considered as "metal mimics" (Fig. 4). That aminoglycoside binding could

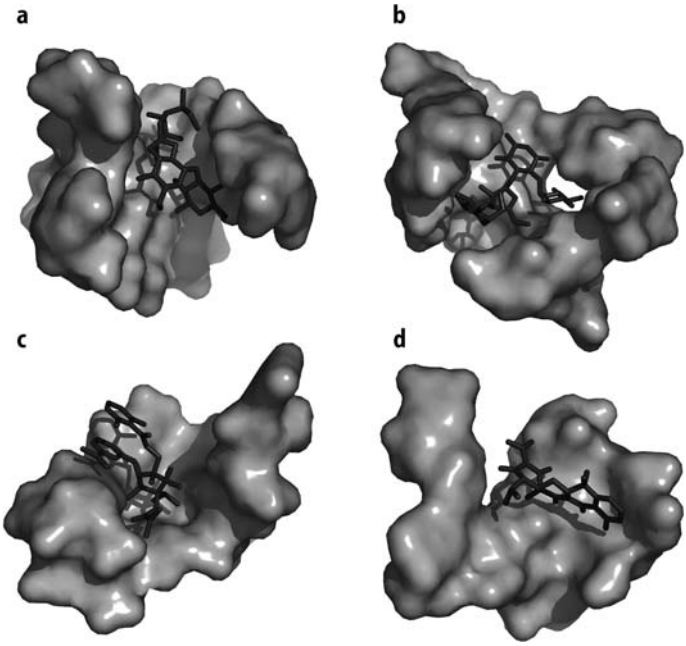

Fig. 3a–d Specific binding pockets for different antibiotics in *Thermus thermophilus* 30S ribosomal subunit. All surface representations show the interacting region within a radius of 10 Å surrounding the bound antibiotic. **a** Hygromycin B (Brodersen et al. 2000—PDB file 1HNZ). **b** Paromomycin (Ogle et al. 2001—PDB file 1IBK). **c** Tetracyclin (Brodersen et al. 2000—PDB file 1HNW). **d** Pactamycin (Brodersen et al. 2000—PDB file 1HNX). The figures where made using the molecular graphics program Pymol (DeLano 2002)

result in displacement of functionally important metal ions was originally suggested by Hermann and Westhof (1998), and the structural analysis as well as biochemical studies of aminoglycoside binding to various RNA is in keeping with this (see the following section). In fact, displacement of Mg^{2+} also occurs when paromomycin binds to the 30S ribosomal subunit (Fig. 4), providing further evidence that divalent metal ion and aminoglycoside binding sites in RNA overlap (Carter et al. 2000). This is in keeping with recent studies using small RNA model molecules representing the 16S rRNA A-site (Mikkelsen et al. 2001; Summers et al. 2002). Whether Mg^{2+} plays a direct functional role in the decoding process is not clear, but Porschke and coworkers have discussed the role of Mg^{2+} in the decoding process (e.g., Labuda et al. 1984). Nonetheless, from the structural studies of the 16S rRNA A-site, it is evident that addition of aminoglycoside stabilizes the bulging conformation of two functionally important adenines, A1492 and A1493. These adenines play an essential role in the decoding process during translation. Thus, this gives a structural reason as to why addition of aminoglycosides results in increased errors during translation, as has been shown be Ehrenberg and coworkers, among others (for a review see

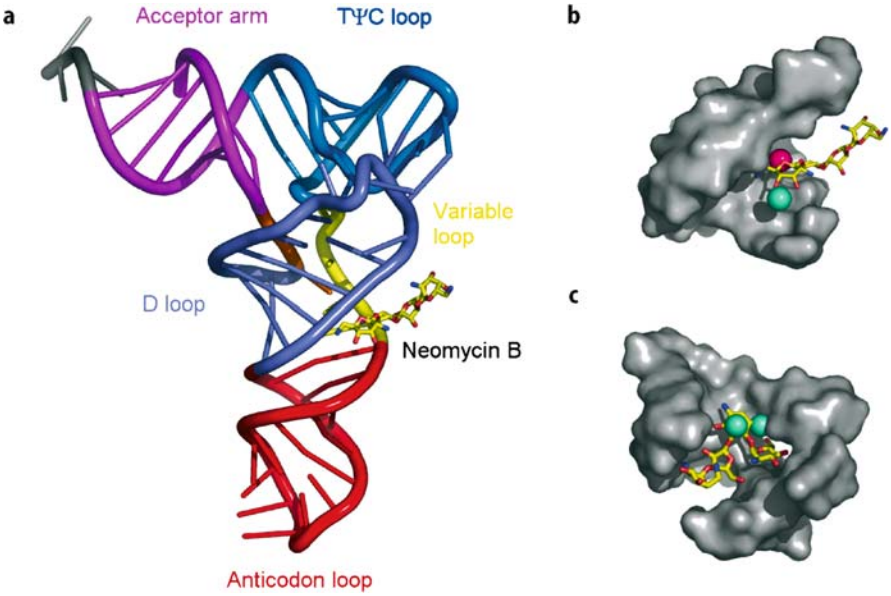

Fig. 4a–c Displacement of divalent metal-ions by aminoglycosides. **a** 3D ribbon-and-stick model of neomycin B overlapping the metal ion-binding pocket in the deep groove below the D loop in yeast $tRNA^{Phe}$ (Mikkelsen et al. 2001—PDB file 1I9 V). Individual regions of the molecule colored according to Jovine et al. (2000). **b** Divalent metal ions from yeast $tRNA^{Phe}$-Pb^{2+} (and Mg^{2+}) structures superimposed on the yeast $tRNA^{Phe}$-neomycin B structure (Brown et al. 1983—PDB file 1TN1; Brown et al. 1985—PDB file 1TN2; Jovine et al. 2000—PDB file 1EVV; Shi and Moore 2000—PDB file 1EHZ; Mikkelsen et al. 2001). Yeast $tRNA^{Phe}$ represented as a surface model surrounding the bound neomycin B at a 10 Å radius. Magnesium and lead ions are colored in *cyan* and *magenta*, respectively. **c** *Thermus thermophilus* 30S-Paromomycin structure superimposed with overlapping divalent metal ion binding sites (Ogle et al. 2000—PDB codes 1IBK and 1IBM). Surface representation of *Thermus thermophilus* 30S surrounding the bound paromomycin and magnesium ions 433 and 437 at a 10 Å radius. The figures where made using the molecular graphics program Pymol (DeLano 2002) and in generating the tRNA stick model, the program Nuccyl (Jovine 2003; www.mssm.edu/students/jovinl02/research/nuccyl.html) was used

Schroeder et al. 2000). Moreover, both crystal and NMR studies have revealed structural information about resistance against various aminoglycosides. This information is crucial for the work aiming at identifying the next generation of aminoglycosides.

In this context, we would like to mention that studies where aminoglycoside RNA aptamers have been studied have also provided valuable information and we refer to various articles covering this aspect of RNA-aminoglycoside interaction (apart from the references already mentioned, see Patel et al. 1997; Herrmann and Patel 2000).

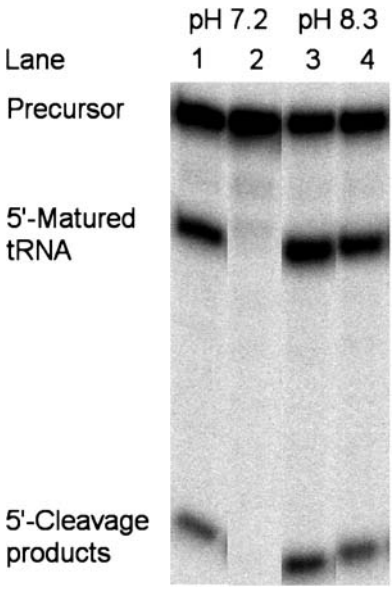

Fig. 5 The electrostatic interaction of aminoglycosides with RNA is pH dependent. Monitoring M1 RNA cleavage activity of a tRNA precursor, pSu3, at two different pH conditions at 37 °C. *Lanes 1* and *3* show M1 RNA cleavage activity in the absence of Neomycin B whereas *lanes 2* and *4* show cleavage in the presence of 1 mM neomycin B. The time of cleavage in all cases was 2 min. Concentration of M1 RNA and pSu3 were 82 nM and 5.2 nM, respectively (for details see Mikkelsen et al. (1999))

2.3 RNA as a Drug Target

Given that RNA is essential for bacterial growth, and the possible importance of RNA for virulence makes RNA a suitable and promising drug target. This potential is witnessed by the fact that many antibiotics targeting the ribosome have been and are still used in the clinic today. As discussed above, high resolution structural studies of the bacterial ribosome in complex with a number of different antibiotics have revealed that drugs bind with high specificity to bacterial rRNA (16S and 23S) and tRNA. For example, there are drugs that interact with or close to the decoding region on the 30S subunit as well as the peptidyl transfer center on the 50S subunit (Fig. 3). Although the ribosome is the primary RNA target known today, other RNA molecules, both naturally occurring RNA and in vitro-selected RNA aptamers, have been demonstrated to interact with antibiotics such as aminoglycosides as well as other classes of drugs (see above). In this section, we will discuss other RNAs, primarily bacterial RNAs that have been demonstrated to interact with aminoglycosides and as such are potential drug targets when searching for novel antibiotics. In addition, we will mention some other functional RNAs that potentially could

be targeted and/or used in the process to identify novel lead compounds. These RNA molecules/targets are summarized in Table 2.

To function as a suitable drug target the overall criteria is that the targeted RNA has to be unique for the infectious agent (e.g., bacteria, virus, or fungi). Alternatively, the structural differences comparing the targeted RNA in the infectious agent with that of the host homolog are large enough to result in higher tolerance toward the drug for the host RNA.

2.3.1 tRNA

tRNA plays a central role in protein synthesis by bringing the amino acid to the ribosome. Prior to this, the various tRNAs have to be charged with the correct amino acid, and the tRNA structure is essential in this process. During the charging process, different aminoacyl-tRNA synthetases recognize and charge the respective cognate tRNA. Thus, tRNA aminoacylation is a cellular process that can be targeted when searching for novel bacterial drugs. Actually, one anti-infective drug based on pseudomonic acid is directed against and inhibits isoleucyl-tRNA synthetase (IleRS) derived from several gram-negative and gram-positive bacteria without affecting human IleRS (Sutherland et al. 1985; Ward and Campoli-Richards 1986). Besides pseudomonic acid, it has also been demonstrated that various aminoglycosides interact with $tRNA^{Phe}$ and $tRNA^{Asp}$ and inhibit aminoacylation. In the case of *E. coli*-$tRNA^{Phe}$, the inhibition is most likely due to an overlap between the aminoglycoside binding site and the positive identity elements that are essential for aminoacylation by phenylalanyl-tRNA synthetase (Mikkelsen et al. 2001) while inhibition of $tRNA^{Asp}$ is a consequence of an aminoglycoside-induced destabilization of the L-shape tRNA structure (Walter et al. 2002).

2.3.2 Small Stable RNAs

In bacteria, several small stable RNAs, apart from tRNA, have been identified; for example, RNase P RNA, transfer messenger (tm)RNA, 6S RNA, 4.5S RNA, and Spot 42 RNA [see Table 2; for a recent review regarding small RNAs in *E. coli* see Gottesman (2004)]. Among these, only RNase P RNA and tmRNA are known to be inhibited by aminoglycosides. Beside these two stable RNAs, it is known that aminoglycosides both bind and inhibit the function of a large variety of different small RNA molecules or structures, including group I introns and viral RNAs. Thus, it is conceivable that also other stable RNAs interact with aminoglycosides or similar compounds. Therefore, it will be of interest to elucidate if aminoglycosides inhibit the function of, for example, small novel regulatory RNAs that recently have been identified in various bacteria.

2.3.2.1 RNase P

RNase P is ubiquitous and responsible for generating the 5′ end of almost all tRNA in bacteria, archaea, and eukarya. In bacteria, RNase P consists of the catalytic RNA subunit and a basic protein, the C5 protein, in a 1:1 ratio (Guerrier-Takada et al. 1983; Vioque et al. 1988; see also Fang et al. 2001). This endoribonuclease plays an essential role in the processing of tRNA as well as other RNA transcripts, e.g., mRNA (Altman and Kirsebom 1999). Based on secondary structure comparison, bacterial and RNase P RNA of mammalian origin show distinct structural differences (Kirsebom and Virtanen 2001 and references therein). Moreover, human RNase P consists of at least nine protein subunits and is therefore more complex in its protein composition relative to bacterial RNase P, which only has one protein component (Kovrigina et al. 2003). These differences make bacterial RNase P with its catalytic RNA subunit a suitable target. So far bacterial RNase P is not known to be targeted by any of the antibiotics used in the clinic today. However, as discussed above, aminoglycosides do indeed interact with and inhibit RNase P RNA from various bacterial origins and, in the case of *E. coli* RNase P, even in the presence of the C5 protein (Table 1; Mikkelsen et al. 1999; Eubank et al. 2002). Based on Pb^{2+}-induced cleavage studies, it was suggested that addition of aminoglycosides interferes with the binding of functionally important metal ions. Gopalan and coworkers showed that aminoglycosides do not inhibit the action of human RNase P to the same extent as in the case of bacterial RNase P (Eubank et al. 2002). Taken together, this clearly indicates that RNase P is a suitable drug target.

2.3.2.2 Transfer Messenger RNA

tmRNA is of approximately the same size as RNase P RNA. Apirion and coworkers observed tmRNA as early as 1978 and they referred to it as 10S A RNA (*E. coli* RNase P RNA was referred to as 10S B RNA; Gegenheimer and Apirion 1981). Its function was not apparent until 1996 when it was shown that it rescued stalled ribosomes by acting as a transfer-messenger (tm)RNA. This results in the synthesis of a tag at the C-terminus on the growing polypeptide that subsequently is recognized by proteases resulting in degradation of the tagged polypeptide. Thus, tmRNA plays a role in degradation of unwanted and incorrect polypeptides (Keiler et al. 1996; Withey and Friedman 2003). tmRNA is apparently unique to bacteria since no homolog in mammalians has been identified. Its presence in *E. coli* is not essential, since strains lacking tmRNA are viable under various laboratory conditions, although it cannot be excluded that tmRNA is essential under more natural conditions (or its presence gives a growth advantage). It should be noted that in *Neisseria gonorrhoeae* tm-

RNA is essential for growth (Huang et al. 2000). This raises the possibility that tmRNA may also be essential for growth of other bacteria. Felden and coworkers demonstrated that aminoglycosides bind to tmRNA and interfere with aminoacylation of tmRNA. The inhibition of aminoacylation is not a result of binding of the aminoglycoside to the aminoacyl-acceptor stem where the major determinant for alanyl-tRNA-synthetase, rather the data, suggested that aminoglycoside binding perturbed the conformation of the aminoacyl-acceptor stem of tmRNA (Corvaisier et al. 2002). These data clearly suggest that tmRNA is a potential and suitable drug target, if not in the case of all bacteria, at least to some.

2.3.2.3 Spot 42 RNA, 6S RNA, and 4.5S RNA

Beside RNase P RNA and tmRNA, no other "classical" stable non-coding RNAs, i.e., 4.5S RNA, 6S RNA, and Spot 42 RNA, have so far, to our knowledge, been demonstrated to interact with aminoglycosides. However, given the generality for the RNA/aminoglycoside interaction, it is expected that these RNAs should also interact with aminoglycosides. Thus, it would definitely be of interest to investigate whether aminoglycosides indeed bind to these and inhibit their cellular function. Noteworthy is that the 4.5S RNA is involved in secretion and is essential for viability. Moreover, comparing the structures of bacterial (excluding low GC content gram-positive bacteria e.g., *Mycoplasma* spp.) 4.5S RNA and the corresponding human signal recognition particle (SRP) RNA reveals structural differences that clearly would be possible to explore in the quest for novel drug targets/candidates. Likewise, the 6S RNA is an interesting candidate as a drug target, since a homolog of 6S RNA has not been identified in mammals. The bacterial 6S RNA functions as an RNA regulator that inhibits RNA polymerase, and it is up-regulated during the stationary phase of bacterial growth (Wassarman and Storz 2000).

The Spot 42 RNA (Ikemura and Dahlberg 1973) functions in sugar metabolism where it is involved in translational regulation of *galK* (Møller et al. 2002). Spot 42 RNA represent a class of RNA involved in translational regulation by an antisense RNA mechanism, and as such might be of interest as a model system to identify inhibitors that interfere with anti-sense RNA. For a recent review of other small RNAs that have been identified in bacteria, we recommend Gottesman (2004).

2.3.3 Other RNA Molecules Interacting with Aminoglycosides

Other RNA-based activities inhibited by aminoglycosides include both a number of ribozymes and viral RNAs. Among the ribozymes, the group I intron is one of the best-studied examples. In their pioneering work, Schroeder and

coworkers (von Ahsen et al. 1991) demonstrated that aminoglycosides bind and inhibit group I intron self-splicing (for a review see Schroeder et al. 2000). Given the existence of self-splicing RNA group I introns in bacteria, these are indeed potential drug targets to explore. This is also apparent based on the knowledge that group I introns have not been identified in mammalian cells. Other ribozymes known to be inhibited by aminoglycosides are the self-cleaving hammerhead ribozyme, the hairpin ribozyme (Stage et al. 1995; Earnshaw and Gait 1998), and the self-cleaving hepatitis delta virus HDV RNA (Rogers et al. 1996; Chia et al. 1997). In this context, we note that the aminoglycoside streptomycin is apparently a better inhibitor of nuclear pre-mRNA splicing compared to inhibition of the group I intron splicing (Hertweck et al. 2002). Since no other aminoglycosides have been tested for inhibition of nuclear pre-mRNA splicing, it would be of value to study this in more detail. This is not only important in the evaluation of the group I intron as a potential target, but this information is also of significance in order to be able to ensure that the next generation of aminoglycosides is more specific/efficient against chosen bacterial RNA targets.

Aminoglycosides are known to bind to human immunodeficiency virus (HIV) RNA and, in some cases, even to prevent viral replication. In a classic study by Zapp et al. (1993), it was shown that aminoglycosides, in particular neomycin B, bound to the Rev response element (RRE) of HIV-1 and selectively blocked Rev protein binding to the RRE, resulting in inhibition of viral growth. Later, it was shown that aminoglycosides also bind to the HIV Tat-responsive element (TAR) and prevent binding of Tat protein. In this case, both structural and molecular dynamic simulations suggest that aminoglycoside binding induces conformational changes in the RNA and thereby prevent Tat protein binding (Mei et al. 1995; Hermann and Westhof 1999; Faber et al. 2000). These findings emphasize that aminoglycoside also targets viral RNA and influences viral replication by perturbing essential RNA protein interactions. These studies, therefore, highlight the possibility that RNA protein interactions, which are essential for viral growth, serve as potential drug targets to explore for drug development. Of course, any essential ribonucleoprotein complexes present in any infectious agent (e.g., viral, bacterial, parasitic) would serve as a potential target.

3
Aminoglycoside Inhibition of Metalloenzymes

It has been observed that some aminoglycosides inhibit enzymes involved in cleavage and formation of phosphodiester bonds (Lazarus and Kitron, 1973; McDonald and Mamrack, 1995; Woegerbauer et al. 2000). However, the mechanism of inhibition has not been fully understood. Based on the studies performed on aminoglycoside inhibition of RNA function, it has become clear that

the RNA-binding property of aminoglycosides is a key reason why they inhibit RNA function. Most likely, as discussed above, the binding results in a disturbed structure of the RNA and/or displacement of functionally important divalent metal ions. To investigate if a related mechanism of inhibition was also plausible for enzymes involved in cleavage and formation of phosphodiester bonds, we investigated if aminoglycosides could interact with and inhibit the polymerizing activity of *E. coli* DNA polymerase I and/or the exoribonuclease activity of human poly(A)-specific ribonuclease (PARN) (Ren et al. 2002). These enzymes encompass active sites that form negatively charged binding pockets that resemble aminoglycoside binding sites in RNA. Moreover, both active sites of these two enzymes depend on and coordinate divalent metal ions. Our studies (Ren et al. 2002) showed that aminoglycosides inhibited the activity of both enzymes and suggested that this was caused by the aminoglycoside distorting the active sites and/or displacing functionally important divalent metal ions. Thus, the property of aminoglycosides to bind to negatively charged binding pockets seems to be a generally applicable property of aminoglycosides. As a matter of fact, this property is even relevant for the binding of kanamycin B to the active site of the bacterial kanamycin-modifying enzyme kanamycin nucleotidyltransferase (Fig. 2), as this active site encompasses a negatively binding pocket and binds divalent metal ions (Pedersen et al. 1995; Sakon et al. 1993). Thus, proteins that depend on metal ions for activity are potential targets for aminoglycosides. Therefore, in the process to develop RNA-specific drugs based on aminoglycosides, it is essential to ensure that these do not interact and interfere with protein function, such as various nucleases/polymerases that depend on metal ions for activity. In addition, these findings identify nucleases and polymerases as potential targets in the process of identifying novel drugs/lead compounds.

4 Concluding Remarks and Future Aspects

Over the last decades, very few new classes of antibiotics have been introduced. The recent increase in the appearance of multiresistant bacteria emphasizes the urgent need for new substances that can be used in the process of identifying novel antibiotics. Here we have discussed the potential of aminoglycosides and related molecules or derivatives thereof as potential starting molecules for the search of novel antibiotics. We have primarily focused on the RNA-binding property of aminoglycosides and discussed some potential RNA molecules that could serve as drug targets.

Aminoglycosides were introduced in the early 1950s and have been an important class of antibiotic used in the clinic since then. As discussed here, functional and structural studies of RNA in the last 15 years have demonstrated that RNA is a primary target for aminoglycosides. Importantly, aminoglycoside

binding to RNA results in conformational changes and/or displacement of divalent metal ions. Therefore, the identification of metal ion binding sites can be explored to identify novel aminoglycoside derivatives that bind RNA.

In the process of identifying novel drugs, several laboratories worldwide have used chemistry to modify existing aminoglycosides. For example, such laboratories have (1) used the synthesis of aminoglycoside conjugates with intercalators, such as acridine attached to them, to make dimeric aminoglycosides, (2) replaced existing sugar moieties with different sugar derivatives, or (3) introduced arginine residues at specific positions on the aminoglycoside (Agnelli et al. 2004; Cheng et al. 2001; Luedtke et al. 2003; Litovchick et al. 2001; Sucheck and Shue 2001; Yao et al. 2004). Many of these new aminoglycoside derivatives have been demonstrated to bind various RNAs with improved affinities and to be more resistant toward enzymatic modification. However, a difficult problem to address yet is toxicity. Nonetheless, this demonstrates that it is possible to use aminoglycosides in the search for new and more efficient drugs against a number of various pathogens such as bacteria and viruses.

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