Designer aminoglycosides: the race to develop improved antibiotics and compounds for the treatment of human genetic diseases

Mariana Hainrichson, Igor Nudelman and Timor Baasov*

Received 16th August 2007
First published as an Advance Article on the web 9th October 2007
DOI: 10.1039/b712690p

Aminoglycosides are highly potent, broad-spectrum antibiotics that exert their bactericidal therapeutic effect by selectively binding to the decoding aminoacyl site (A-site) of the bacterial 16 S rRNA, thereby interfering with translational fidelity during protein synthesis. The appearance of bacterial strains resistant to these drugs, as well as their relative toxicity, have inspired extensive searches towards the goal of obtaining novel molecular designs with improved antibacterial activity and reduced toxicity. In the last few years, a new, aminoglycoside dependent therapeutic approach for the treatment of certain human genetic diseases has been identified. These treatments rely on the ability of certain aminoglycosides to induce mammalian ribosomes to readthrough premature stop codon mutations. This new and challenging task has introduced fresh research avenues in the field of aminoglycoside research. Recent observations and current challenges in the design of aminoglycosides with improved antibacterial activity and the treatment of human genetic diseases are discussed.

Introduction

Protein synthesis is one of the fundamental processes in all living cells, and therefore, it is not surprising that the RNA and protein machinery of the prokaryotic ribosomes are the target of about half of the antibiotics characterized thus far.[1] Among the different classes of clinically important antibiotics that interfere with protein synthesis *via* this target (*e.g.* aminoglycosides, macrolides and oxazolidinones), aminoglycosides (Scheme 1) represent gold standard drugs for the treatment of serious Gram-negative pathogens. Streptomycin, the first representative of this class of antibiotics, was discovered by Waksman *et al.* in 1944 and was the first effective antibiotic against *Mycobacterium tuberculosis*.[2] In the following decades several milestone drugs, such as neomycin, kanamycin, tobramycin and others, were isolated from soil bacteria by intense search for natural products with antibacterial activity.[3–5] However, the prolonged clinical and veterinary use of aminoglycosides has resulted in the rapid spread of antibiotic resistance to this family of antibacterial agents in pathogenic bacteria.[6] The relative toxicity to mammals is another critical problem of these drugs that largely limits their intensive clinical use.[7,8] Systematic studies on direct chemical modification of existing aminoglycoside drugs, with the aim of circumventing the resistance mechanisms, without either diminishing their activity or increasing their toxicity, has opened up a new era in the history of aminoglycosides. Earlier investigations in this direction have yielded several semi-synthetic drugs such as amikacin, dibekacin, netilmicin and isepamicin that were introduced into clinical use in the 1970s and 1980s.[9,10] The latest semi-synthetic aminoglycoside introduced into human antibacterial therapy was arbekacin, a kanamycin B derivative used in Japan since 1990.[9]

The Edith and Joseph Fischer Enzyme Inhibitors Laboratory, Schulich Faculty of Chemistry, Technion-Israel Institute of Technology, Haifa 32000, Israel. E-mail: chtimor@tx.technion.ac.il; Fax: +972-4-829-5703; Tel: +972-4-829-2590

Recent advances in biochemical and structural studies on resistance mechanisms,[11,12] along with high-resolution structures of aminoglycosides in complex with their ribosomal targets,[13,14] have brought considerable molecular insights into mechanisms of biological action that stimulated the development of innovative approaches towards improved aminoglycoside derivatives and mimetics.[11,15–18] However, although the prokaryotic selectivity of action is critical to the therapeutic utility of aminoglycosides as antibiotics, they are not entirely selective to bacterial ribosome; they also bind to the eukaryotic A site,[19] and promote mistranslation.[20,21] The use of this disadvantage of aminoglycoside antibiotics for the possible treatment of human genetic diseases caused by premature nonsense mutations is extremely challenging.[22] In this perspective, we will briefly review some aspects of aminoglycosides including their molecular mechanism of action and main resistance mechanisms, development of new designs with improved activity against resistant bacteria, structure–toxicity relationship, and potential application to the emerging therapeutic field of the treatment of genetic disorders.

2-Deoxystreptamine-containing aminoglycosides and their molecular mechanism of action

The majority of natural aminoglycosides consist of a common non-sugar ring, named 2-deoxystreptamine (2-DOS) that carries sugar substituents at the 4-, 5- and 6-positions (Scheme 1). According to the current nomenclature, the 2-DOS ring is numbered as ring II, which in most structures also represents the central ring. The sugar ring bound at position 4 of 2-DOS is ring I, and the sugar ring bound either at position 5 or at position 6 of 2-DOS is ring III. The 4,5- and 4,6-disubstituted 2-DOS derivatives are the two most important classes of clinically useful aminoglycoside antibiotics. Neomycin B, a representative of the 4,5-disubstituted 2-DOS sub-class, is used topically in the form of creams and lotions for the treatment of bacterial

Mariana Hainrichson

*Mariana Hainrichson was born in 1979 in Ukraine and immigrated to Israel in 1990. After finishing high school in 1998, she spent two years in the Israeli Army. After receiving her BSc (*Cum Laude*) in 2003 in Biotechnology and Food Engineering from the Technion, she joined the research group of Prof. Timor Baasov at the Schulich Faculty of Chemistry first as an MSc degree student and currently as a PhD student in the frame of the Interdepartmental Program in Biotechnology. Her research work is concerned with the design and biochemical evaluations of semi-synthetic aminoglycosides as potential antibiotics as well as potential new drugs for the treatment of human genetic diseases.*

Igor Nudelman

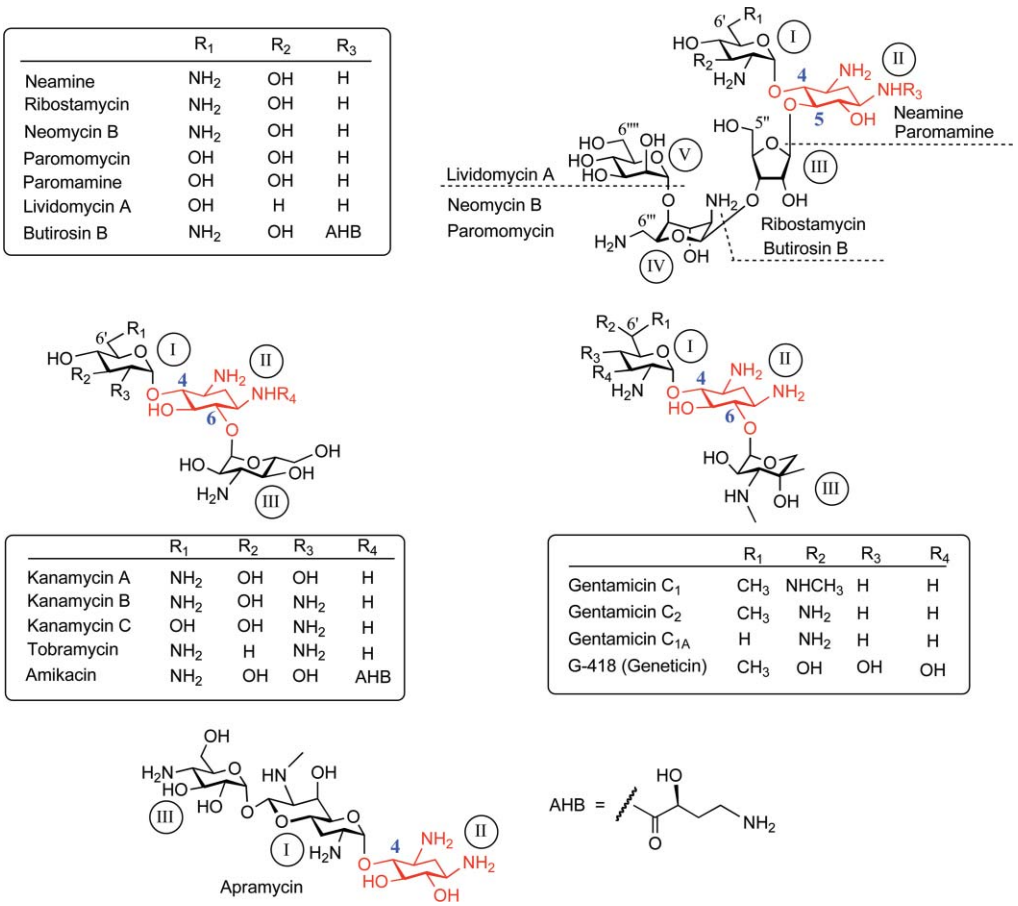
Igor Nudelman was born in 1980 in Ukraine and immigrated to Israel in 1992. After three years of army service during 1998–2001 he joined the Faculty of Biology in Technion as an undergraduate student and received his BA in Molecular Biochemistry in 2005. He then joined the research group of Prof. Timor Baasov at the Schulich Faculty of Chemistry first as an MSc degree student and currently as a PhD student. His thesis work is concerned with the design and synthesis of aminoglycoside-based structures as potential new drugs for the treatment of human genetic diseases.

Timor Baasov

Dr Timor Baasov is Professor and Dr Irving and Jeanette Benveniste Chair in Life Sciences at the Technion. His research interests are in the areas of bioorganic and carbohydrate chemistry and biocatalysis, including development of new chemical and enzymatic strategies for the synthesis of biologically active compounds and designed molecules as mechanistic probes for enzymatic reactions, carbohydrate-mediated biological recognition and drug design. He received his BSc and MSc degrees in chemistry from Tel-Aviv University and PhD from the Weizmann Institute. He was Chaim Weizmann Postdoctoral Fellow at Harvard University during 1986–1988 in the group of Prof. Jeremy R. Knowles.

infections occurring from skin burns, wounds and dermatitis.[23] Paromomycin, another representative of 4,5-disubstituted 2-DOS that differs from neomycin B in that it has a 6′-OH instead of a 6′-NH_2 in ring I, is used therapeutically against intestinal parasites.[23] On the other hand, the 4,6-disubstituted 2-DOS subclass contains several antibiotics, such as gentamicin, amikacin and tobramycin that have important clinical applications in the treatment of serious Gram-negative bacterial infections, especially in cases of opportunistic bacteria accompanying cystic fibrosis (CF), AIDS and cancer.[23] One such bacterium, *Pseudomonas aeruginosa*, is a major cause of mortality among CF patients, and respiratory system infections caused by this pathogen are often treated by aerosol inhalation therapy of gentamicin.[24] The only known representative of the natural 4-monosubstituted 2-DOS aminoglycoside antibiotics is apramycin, which has an unusual bicyclic moiety as a ring I connected to the 2-DOS *via* a glycosidic linkage. Because of its relative toxicity, apramycin is only used in veterinary medicine and never in humans.[25]

All of these three sub-classes of 2-DOS-containing aminoglycosides (4,5- and 4,6-disubstituted and apramycin), target a phylogenetically conserved decoding site (A-site) of bacterial 16S rRNA in the 30S ribosomal subunit[26] (Fig. 1). Upon binding to the 16S rRNA A site, 2-DOS aminoglycosides decrease the fidelity of translation. As a consequence, erroneous proteins that are truncated or incorrectly folded accumulate, which then leads to bacterial cell death. During the last decade, several achievements in bacterial ribosome structure determination,[27,28] along with crystal and NMR structures of bacterial A-site oligonucleotide models,[29–31] have provided fascinating insights into our understanding of the decoding mechanism in prokaryote cells and of how 2-DOS aminoglycosides induce the deleterious misreading of the genetic code. During decoding, a critical step in



Scheme 1 Structures of the 4-, 4,5- and 4,6-substituted 2-DOS derivatives that target the prokaryotic A-site. The 2-DOS scaffold is shown in red and its substituted carbon numbers are in blue.

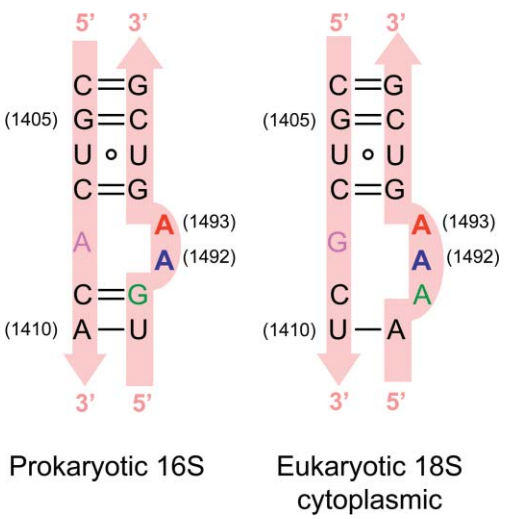


Fig. 1 Secondary structures of the prokaryotic 16S and eukaryotic 18S ribosomal RNA A sites. The *E. coli* numbering is used for both systems and only two relevant base changes are highlighted in pink and green. The conserved adenines A1492 and A1493 are in blue and red, respectively.

aminoacyl-tRNA selection is based on the formation of a mini-helix between the codon of the mRNA and the anti-codon of the cognate aminoacyl-tRNA. In this process, the conformation of the A-site is changed from an "off" state, where the two conserved adenines A1492 and A1493 are folded back within the helix, to an "on" state, where A1492 and A1493 are flipped out from the A-site and interact with the cognate codon–anticodon mini-helix.[32,33] This conformational change is a molecular switch that irreversibly determines on the continuation of translation. The binding of aminoglycosides such as paromomycin to the bacterial A-site changes the conformation equilibrium of the conserved adenines A1492 and A1493 by stabilizing the "on" state conformation even in the absence of cognate tRNA–mRNA complex (Fig. 2). Thus, the affinity of the A-site for a non-cognate mRNA–tRNA complex is increased upon aminoglycoside binding, preventing the ribosome from efficiently discriminating between non-cognate and cognate complexes and leading to the assembly of proteins of incorrect sequence.[34]

While this mechanism of action is now well accepted for the majority of the 4,5- and 4,6-disubstituted 2-DOS aminoglycosides, the primary effect of apramycin on bacterial protein synthesis is inhibition of the elongation step by blocking ribosome translocation.[35] It was suggested that because of its unusual structure and distinct mode of binding to the decoding site, apramycin may form additional interactions with the ribosomal protein S12, which is involved in the translocation process.[36] However, the 3D crystal structure of apramycin complexed to the bacterial A site RNA construct demonstrated that apramycin penetrates the

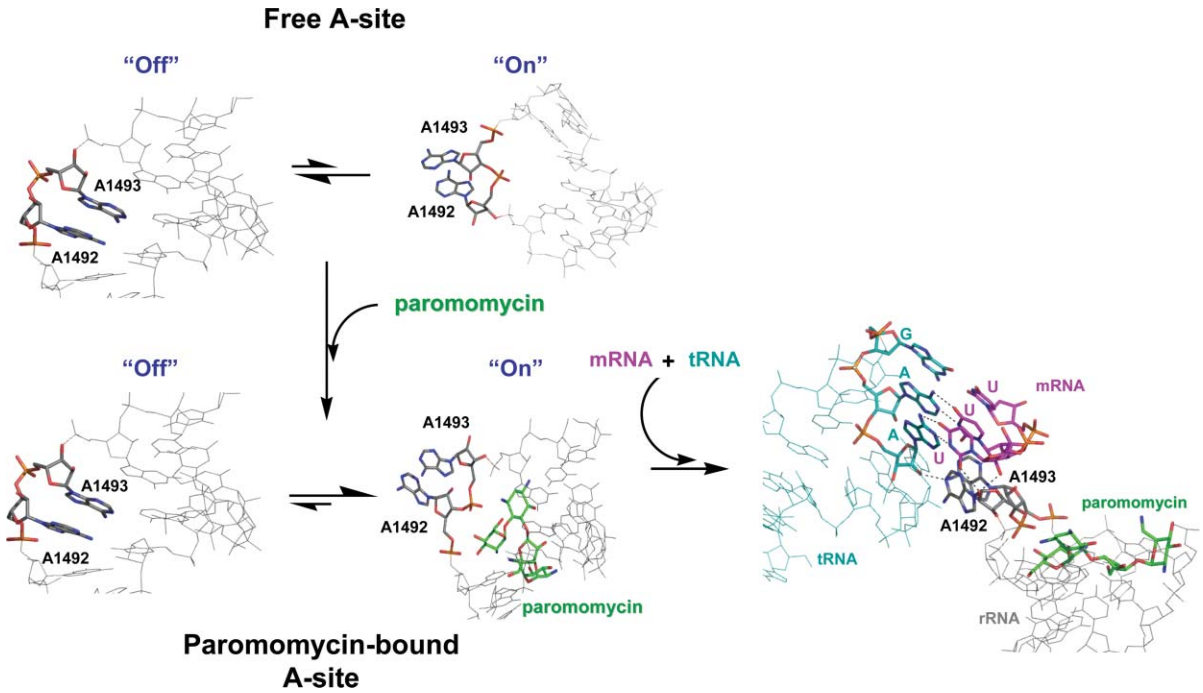


Fig. 2 The molecular basis of the aminoglycoside-induced miscoding as resolved by X-ray crystal structures. At the bacterial decoding site (A-site), two flexible adenines A1492 and A1493 are in conformational equilibrium with a predominance of an intrahelical "off state" conformation. The binding of 2-DOS aminoglycoside paromomycin (green) shifts the equilibrium by stabilizing the "on state" conformation even in the absence of mRNA or tRNA. In the "on state" conformation the A1492 and A1493 are able to create hydrogen bonds with the bases of the mini-helix formed by the near-cognate tRNA anticodon (cyan) and the mRNA codon (magenta) leading to miscoding. All structures are from PDB accession number 1IBL[32] except for the ligand-free "off state" that was taken from PDB accession number 1J5E.[31]

internal loop of the bacterial A site and stabilizes the decoding "on" state conformation with the two critical adenine residues A1492 and A1493 bulged out.[36] This observation, along with the recent crystallographic investigation of a series of aminoglycosides bound to the A site oligonucleotide model[13] suggest that the actual molecular mechanism of this "molecular switch" system is more complex and that additional thermodynamic and kinetic factors are likely to govern the impact of aminoglycosides on prokaryotic translation. The most recent investigation to characterize the energetics and dynamics associated with the aminoglycoside–rRNA interaction demonstrated that the aminoglycoside-induced reduction in the mobility of the A1492 residue is an important determinant of antibacterial activity.[37]

Major mechanisms of bacterial resistance to aminoglycosides

There are several general mechanisms of bacterial resistance to antibiotics, including enzymatic modification of the drugs, chemical alteration or point mutations of the drugs' targets, and reduction of the antibiotic concentration inside the bacterial cells *via* efflux pumps.[6] The primary resistance mechanism to aminoglycosides is the bacterial acquisition of enzymes, collectively called aminoglycoside-modifying enzymes (AMEs), which modify the antibiotics by *N*-acetyltransferase (AAC; acetyl CoA-dependent acetylation of an amino group), *O*-nucleotidyltransferase (ANT; ATP-dependent transfer of AMP on a hydroxyl group), or *O*-phosphotransferase (APH: ATP-dependent phosphorylation of a hydroxyl group) activities[12,23] (Fig. 3). Each class of these enzymes performs a specific reaction and the turnover products of these reactions lack antibacterial activity. Furthermore, each class of these enzymes is comprised of many distinct members that show regio- and substrate specificity. For example, AAC(3)-Ib is an aminoglycoside acetyltransferase that acetylates the antibiotics at the 3-amino group, and the Ib designation indicates that gentamicin and fortimicin A serve as substrates.[38]

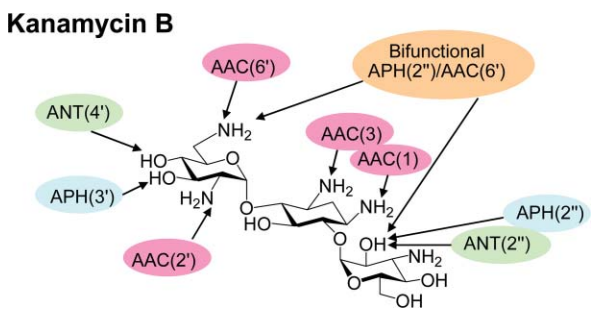


Fig. 3 Target sites of aminoglycosides modifying enzymes as exemplified on the structure of kanamycin B. Three classes of aminoglycoside-modifying enzymes include AAC (aminoglycoside *N*-acetyltransferase), ANT (aminoglycoside *O*-nucleotidyltransferase) and APH (aminoglycoside *O*-phosphotransferase). The numbers in parentheses indicate the location of the functional group (amine or hydroxyl) that undergoes enzymatic modification. Bifunctional APH(2″)/AAC(6′) denotes a single enzyme that modifies aminoglycoside at two different positions (2″-OH and 6′-NH_2) by phosphorylation of the 2″-OH group (APH activity) and acetylation of the 6′-NH_2 group (AAC activity).

ANTs, enzymes causing AMP transfer are the smallest class among the AMEs. They modify frequently used aminoglycosides such as gentamicin and tobramycin and only ten of them have been reported to date.[11,12] APHs include a large number of aminoglycoside modifying enzymes and are the most relevant in clinical resistance to aminoglycosides by Gram-negative organisms[39] (Fig. 3). Among these, aminoglycoside 3′-phosphotransferases [APH(3′)s], of which seven isozymes are known, are widely represented. These enzymes catalyze transfer of the γ-phosphoryl group of ATP to the 3′-hydroxyl of many aminoglycosides, rendering the latter inactive since the resulting phosphorylated antibiotics no longer bind to the bacterial ribosome with high affinity. Due to the unusually broad spectrum of aminoglycosides that can be deactivated by APH(3′) enzymes, much effort has been put into understanding the structural basis of their substrate recognition and catalysis.[40–42] These studies revealed several intriguing parallels in the modes of drug recognition with both the resistance enzymes and the ribosomal RNA target. While the AMEs are often plasmid encoded or associated with transposable elements, in several instances they may also be chromosomally encoded. For example, the chromosomal gene *aph*(*3′*)-*IIb*, of *P. aeruginosa* has been identified[43] and held largely responsible for the "uniform resistance" of *P. aeruginosa* to kanamycin. Recently, this gene was cloned and overexpressed in *Escherichia coli* and the recombinant APH(3′)-IIb enzyme was shown to catalyze a highly regiospecific phosphorylation at the 3′-hydroxyl group of various aminoglycosides.[44]

The AACs are the second most abundant of AMEs; over 50 unique members of this class have been identified so far,[11,12] which testifies to their versatility as AMEs. Although the AMEs of all three classes are typically monofunctional enzymes, the recent emergence of genes encoding bifunctional AMEs is another level of sophistication relevant to the clinical use of aminoglycosides. Four genes encoding the following bifunctionioal enzymes have been identified so far: AAC(6′)/APH(2″), ANT(3″)-Ii/AAC(6′)-IId, AAC(3)-Ib/AAC(6′)-Ib′, and AAC(6′)-30/AAC(6′)-Ib′.[38] Among them, the bifunctional AAC(6′)/APH(2″) enzyme has been the most extensively investigated, due to the large number of clinically important aminoglycosides that are susceptible for modification with this enzyme.[45,46]

In spite of the emergence of a large number of diverse AMEs, aminoglycosides are still used as a first choice for the treatment of multi-drug-resistant tuberculosis infection as well as against many other serious Gram-negative pathogens. This is ascribed to the high regio- and substrate specificity of AMEs. For example, several AMEs acting on gentamicin and tobramycin either modify amikacin poorly or do not use it as a substrate. Nevertheless, it is highly noteworthy that despite recent progress in the isolation and characterization of numerous AMEs, almost no progress[47] has been made in the development of potent inhibitors of AMEs. Such inhibitors could in principle provide an important tool for overcoming aminoglycoside resistance. In fact, aminoglycosides that lack the 3′-OH of ring I, like gentamicin, tobramycin, dibekacin and arbekacin are low micromolar inhibitors of APH(3′)s.[39] Furthermore, rational design of such inhibitors capable in overcoming the activities of a large number of AMEs is likely to be more valuable than that of the bifunctional aminoglycosides that target both bacterial rRNA and inhibit resistance-causing enzymes. This is further supported by the fact that the structure-based rational design of novel aminoglycosides is restricted due to their own nature, and the nature of their target, the rRNA. There is severe promiscuity in RNA-aminoglycoside binding, resulting from several factors such as the dominancy of electrostatic interactions in the binding process, the fact that aminoglycosides are "remodeled" according to the RNA topography, and the abundance of water-mediated contacts.[48]

Toxicity of aminoglycosides

One of the major limitations in using aminoglycosides as drugs is their high toxicity to mammals through kidney (nephrotoxicity) and ear-associated (ototoxicity) illnesses. The origin of this toxicity probably results from a combination of different factors/mechanisms such as interactions with phospholipids, inhibition of phospholipases and formation of free radicals.[49,50] Although considered selective to bacterial ribosomes, most aminoglycosides also bind to the eukaryotic A-site but with lower affinities than to the bacterial A-site.[51] The inhibition of translation in mammalian cells is also one of the possible causes for the high toxicity of these agents. Another factor adding to their cytotoxicity is their binding to the mitochondrial 12S rRNA A-site, whose sequence is very close to the bacterial A-site.[1]

Many avenues of research have been pursued in an attempt to alleviate the toxicity associated with aminoglycosides,[52] including the use of antioxidants to reduce free radical levels[53,54] and the use of poly-L-aspartate[55,56] and daptomycin[57,58] to reduce the ability of aminoglycosides to interact with phospholipids. The role of megalin, a multiligand endocytic receptor that is especially numerous in the kidney proximal tubules and the inner ear, in the uptake of aminoglycosides has recently been demonstrated.[50] The administration of agonists that compete for aminoglycoside binding to megalin also resulted in a reduction in aminoglycoside uptake and toxicity.[59] In addition, altering the administration schedule and/or the manner in which aminoglycosides are administered has been investigated as means to reduce toxicity.[60,61]

Structure–toxicity relationship of aminoglycosides

Several studies reported that it may be possible to separate elements of the aminoglycoside structure that induce toxicity from those that are required for an antibiotic effect. First, from the available acute toxicity data on clinically used aminoglycosides[62] along with the toxicities of some designed structures,[63,64] it turns out that two factors that significantly influence the toxicity of aminoglycoside are deamination and/or deoxygenation. Generally, a decrease in the number of amino groups results in reduced toxicity while a decrease in the number of hydroxyl groups results in increased toxicity. For example, paromomycin (Scheme 1), which differs from neomycin in that it has one less amino group, is much less toxic than neomycin (LD_{50} values in mg kg^{-1} of neomycin = 24, paromomycin = 160). Thus, this difference of one charge makes a significant difference in the toxicity of the two compounds. A similar one charge difference between kanamycin B (LD_{50} = 132) and kanamycin A (LD_{50} = 280) and kanamycin C (LD_{50} = 225) make the latter two drugs less toxic than kanamycin B. Further deletion of charged amino groups in ribostamycin makes it less toxic (LD_{50} of ribostamycin = 260) than either neomycin or paromomycin. Such reduction in the toxicity of

aminoglycosides upon the decrease in the number of charged amino groups could be explained by a decrease in nonspecific interactions with other cell components, and by the reduced production of free radicals.

In contrast to deamination, deoxygenation of aminoglycoside results in increased toxicity of the resulted deoxy derivative. For example, removal of 3′-OH in kanamycin B (LD_{50} = 132) gives the significantly more toxic tobramycin (LD_{50} = 79). Further deletion of 4′-OH in tobramycin results in dibekacin (3′,4′-dideoxykanamycin B, LD_{50} = 71) with only a marginal increase in toxicity. This phenomenon was explained by a reduction in the basicity of the amino group; removal of the hydroxyl group (3′-OH) adjacent to the amino group (2′-NH_2) has much more influence than removal of the 4′-OH, which is more distant from the amine. Similar results have been obtained by displacement of the 5-OH with 5-fluorine in kanamycin B and its several clinical derivatives.[63] The toxicities of the resulting fluoro analogs were much lower than the parent compounds and this again was attributed to basicity reduction of the 3-NH_2 group induced by the strongly electron-withdrawing 5-F. Thus, significantly high toxicity of the clinical drugs such as tobramycin (3′-deoxy), gentamicin (3′,4′-dideoxy), dibekacin (3′,4′-dideoxy) and arbekacin (3′,4′-dideoxy) could be ascribed to the increased basicity of the 2′-NH_2 group (ring I) in these drugs caused mainly because of the lack of 3′-hydroxyl or 3′,4′-hydroxyl groups.

An additional factor that was shown to affect the toxicity of aminoglycosides is acylation of the N-1-amine of the 2-deoxystreptamine ring with an AHB ((*S*)-4-amino-2-hydroxybutanoyl) group, although the extent of this effect depends on the aminoglycoside structure (for example, neamine LD_{50} = 125 *vs.* N-1-AHB-neamine LD_{50} = 260; and kanamycin A LD_{50} = 280 *vs.* amikacin LD_{50} = 300) (Scheme 1). Interestingly, a very recent study demonstrated that changing the configuration at single chiral carbon atoms of the aminoglycoside structure can convert the toxic compound to almost non-toxic, while its bactericidal efficacy is not affected.[65] In this study, four components of the native gentamicin, C_1, C_2, C_{2a}, and C_{1A} were separated and their nephro- and cytotoxicity in cell and animal models, along with antibacterial activity, were examined. The component C_2, which is a 6′-diastereomer of C_{2a} (Scheme 1), exhibited little cellular toxicity and no nephrotoxicity while maintaining bactericidal activity. Therefore, this purified gentamicin C_2 component was also suggested as a substitute of the native gentamicin mixture in treating genetic diseases because it could be much more efficient for long-term daily use, although the readthrough efficiency of this component in comparison to that of other components was not determined.

Interestingly, it was recently shown that aminoglycosides stabilize DNA and RNA triplexes. A clear correlation between the toxicity (LD_{50} values) of these antibiotics and their ability to stabilize DNA triple helixes was demonstrated and it was suggested that aminoglycosides may be able to aid H-DNA formation *in vivo*, which might be one of the reasons for their toxicity.[66]

Aminoglycoside derivatives with improved activity against resistant bacteria

To tackle the problem of bacterial resistance caused by enzymatic modification, many semi-synthetic analogs of natural aminoglycosides have been synthesized during recent years.[9,15] One of the most successful approaches is either *N*-acylation or *O*-alkylation of one the amino or hydroxyl groups on the aminoglycoside scaffold, or a combination of both. At the early stages, this approach has led to the development of amikacin by *N*-1-acylation of kanamycin A with an AHB group (Scheme 1).[67] This antibiotic has been in clinical use since 1977. Using this strategy, Chang and coworkers recently synthesized a series of analogs of kanamycin and neomycin sub-classes by introducing an AHB group at the N-1 position.[68,69] The most potent compound from this series was the kanamycin B derivative, compound **1**, which was also named JLN027 (Fig. 4), that showed even better activity than the clinically used amikacin against resistant bacteria harboring APH(3′)-I.

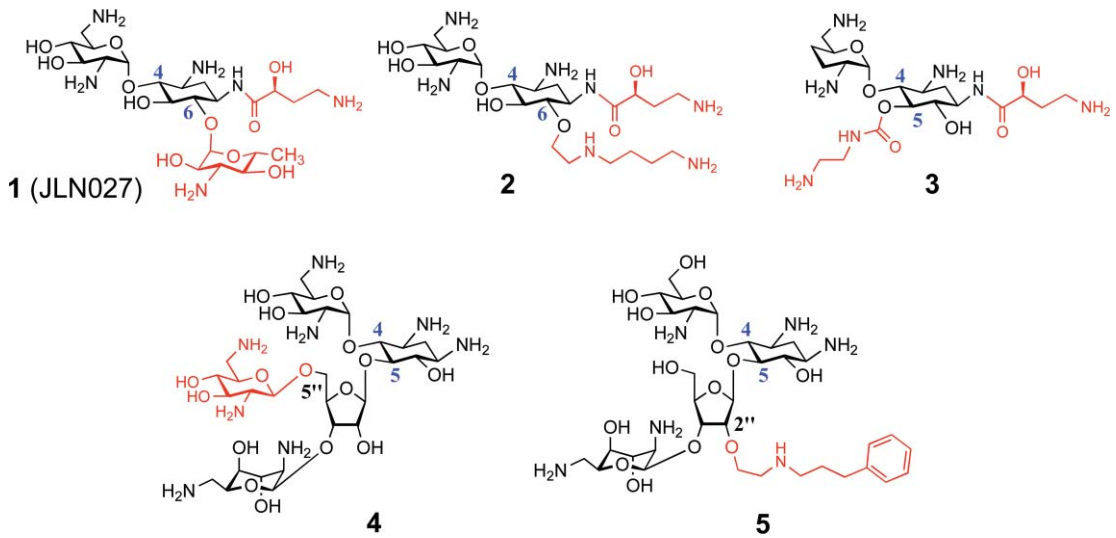


Fig. 4 Representative structures of the semi-synthetic aminoglycoside derivatives that were designed for improved activity against resistant bacterial strains.

Another example of a semi-synthetic derivative that was designed by a more rational strategy, is the neamine derivative **2** (Fig. 4), reported by Mobashery and colleagues.[70] Unlike the majority of the natural and semi-synthetic aminoglycosides that contain at least three rings (Scheme 1), compound **2** consists of a unique pseudo-disaccharide core that is substituted at position N-1 by AHB and at position O-6 by aliphatic diamine. This derivative was shown to be highly active against various resistant and pathogenic bacteria, including *P. aeruginosa* and *Staphylococcus aureus*. The crystal structure of compound **2** complexed to the rRNA A site construct exhibited the same extrahelical conformation of the critical A1492 and A1493 residues as in complexes with natural aminoglycosides.[71] This observation confirmed that the molecular basis of action of compound **2** is the same as that of natural aminoglycosides.

Compound **3** (Fig. 4), recently reported by Minowa *et al.*,[72] in addition to consisting of the pseudo-disaccharide core structure also has a different advantage; it lacks both 3′- and 4′-hydroxyl groups and therefore is intrinsically insusceptible to the AMEs such as APH(3′) and ANT(4′). This derivative showed excellent activity against *S. aureus* expressing AAC(6′)/APH(2″) and against *P. aeruginosa* strains expressing AAC(6′) and AAC(3). Molecular modeling studies suggested that the O5-linked arm interacts significantly with the A-site while the terminal amino group of this arm forms two hydrogen bonds with O6 and N7 of the G1491 residue.

In contrast to the studies described above, we have recently hypothesized that since aminoglycosides exert their antibacterial activity by selectively recognizing and binding to rRNA, it is likely that by adding additional recognition/binding elements to an intact aminoglycoside molecule, improved binding to rRNA and better antibacterial performance against resistant strains are expected to result. Using this strategy, we have generated a new class of pseudo-pentasaccharide derivatives of neomycin B by linking a variety of sugars at the C5″-OH group of neomycin B[73,74] *via* glycosidic linkage. The new derivatives, such as compound **4** (Fig. 4), exhibited similar or better antibacterial activities to that of the parent neomycin B against selected bacterial strains, and especially good activities were observed against *P. aeruginosa*.[73,74] In a complementary study, these compounds were also shown to be potential anti-anthrax drugs, having a dual effect by inhibiting the anthrax lethal factor toxin and at the same time also displaying anti *Bacillus anthracis* activity.[75]

Using a similar strategy, Hanessian and colleagues[76,77] have more recently reported the synthesis of a series of paromomycin derivatives in which the backbone of the parent drug is maintained intact and various *N*-aminoalkyl or *N*-arylalkyl ether appendages are attached at the 2″ position. Some of the lead structures obtained in this study were also investigated by X-ray crystallography, which revealed a new mode of binding in the A-site rRNA. The new derivatives such as compound **5** (Fig. 4) show potent inhibitory activity against a sensitive strain of *S. aureus* and excellent survival rate in a mouse septicemia protection assay.

Potential of aminoglycosides to treat genetic diseases

In the last few years, it was shown that besides their use as antibiotics, aminoglycosides could have therapeutic value in the treatment of human genetic disorders caused by premature stop codons (nonsense mutations).[78–80] In these genetic disorders one of the three stop codons (UAA, UAG or UGA) replaces an amino acid-coding codon, leading to premature termination of the translation and resulting in truncated proteins. Currently, hundreds of such nonsense mutations are known, and several were shown to account for certain cases of fatal diseases, including cystic fibrosis (CF), Duchenne muscular dystrophy (DMD), ataxia-telangiectasia, Hurler syndrome, hemophilia A, hemophilia B, Tay-Sachs, and more.[81] For many of those diseases there is presently no effective treatment, and although gene therapy seems like a potential possible solution for genetic disorders, there are still many critical difficulties to be solved before this technique can be used in humans.

The potential of aminoglycosides in the treatment of these diseases results from their ability to suppress the nonsense mutations by inducing the ribosomes to "readthrough" the premature stop codons, *via* insertion of a random amino acid by a near-cognate tRNA, generating full-length proteins from part of the mRNA molecules (Fig. 5). The termination of protein synthesis is signaled by the presence of a stop codon in the mRNA, and is mediated by release factor proteins. The efficiency of translation termination is usually very high, and in intact cells the misincorporation of an amino acid at a stop codon (suppression) normally occurs at a frequency of around 10^{-4}. The enhancement of termination suppression by aminoglycosides in eukaryotes is thought to occur in a similar mechanism to the aminoglycosides′ activity in interfering with translational fidelity during protein synthesis (Fig. 2): the binding of certain aminoglycosides to the ribosomal A-site probably induces conformational changes that stabilize near-cognate mRNA–tRNA complexes, instead of inserting the release factor. Aminoglycosides suppress the various stop codons with dramatically different efficiencies (UGA > UAG > UAA), and the suppression effectiveness is further dependent upon the identity of the fourth nucleotide immediately downstream from the stop codon (C > U > A ≥ G) as well as the local sequence context around the stop codon.[79,82]

The fact that aminoglycosides could suppress premature nonsense mutations in mammalian cells was first demonstrated by Burke and Mogg in 1985, who also pointed out the therapeutic potential of these drugs in the treatment of genetic disorders.[83] The first genetic disease examined was CF, the most prevalent autosomal recessive disorder in the Caucasian population, affecting 1 in 2,500 newborns. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein. The first experiments of aminoglycoside-mediated suppression of CFTR stop mutations demonstrated that premature stop mutations found in the *CFTR* gene could be suppressed by G-418 and gentamicin, as measured by the appearance of full-length, functional CFTR in bronchial epithelial cell lines.[84,85] Most importantly, clinical studies showed that gentamicin can suppress stop mutations in affected patients: in a double-blind, placebo-controlled, crossover trial, it was reported that gentamicin treatment improved transmembrane conductance across the nasal mucosa in a group of 19 patients carrying CFTR stop mutations.[86] Other genetic disorders for which the therapeutic potential of aminoglycosides were tested in *in vitro* systems, cultured cell lines, or animal models include DMD,[87] Hurler syndrome,[88] nephrogenic diabetes insipidus,[89] nephropathic cystinosis[90] retinitis pigmentosa,[91] and ataxia-telangiectasia.[92] In most of these studies the production of

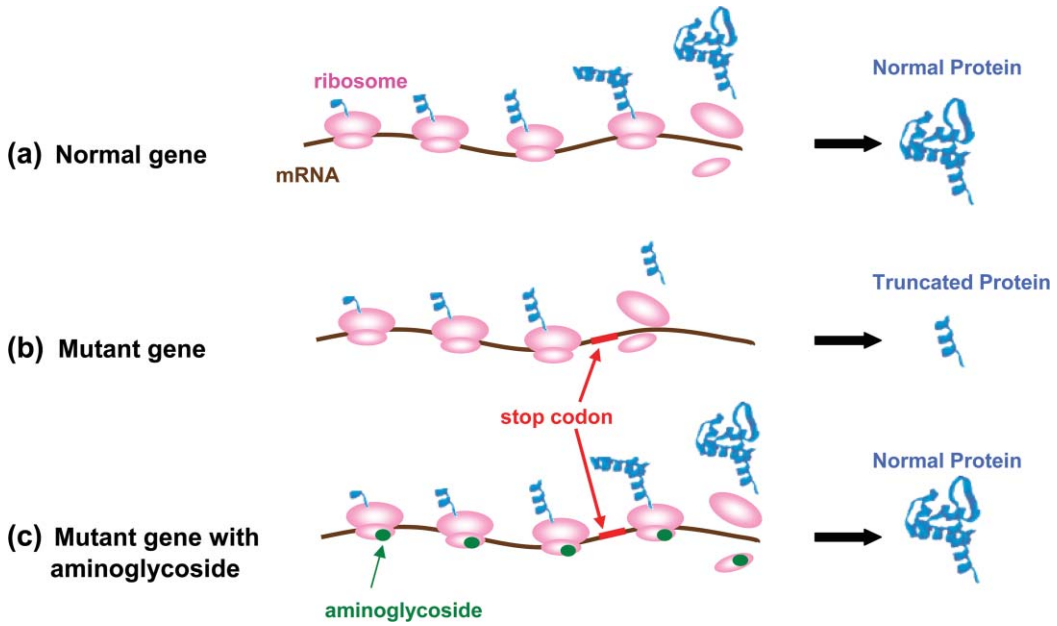


Fig. 5 Aminoglycoside-mediated readthrough of a premature stop codon in a eukaryotic system is represented schematically. (a) In wild type cells, normal mRNA encodes the complete proteins. (b) In mutant cells, the mRNA contains a premature stop codon, and truncated proteins are translated. (c) Aminoglycosides occasionally allow the incorporation of a random amino acid at the internal stop codon of the mutant mRNA. The truncated and miscoded proteins are still made, but in some frequencies, full-length proteins result from the aminoglycoside-induced readthrough.

full-size functional proteins were demonstrated with efficiencies varying from 1% to 25% depending on the aminoglycoside used, the stop codon and the sequence context surrounding it. It is important to note that in cases of recessive disorders, where protein expression is essentially absent, like CF, DMD, Hurler syndrome, ataxia-telangiectasia and others, the production of even 1 percent of normal protein function may restore a near-normal or clinically less severe phenotype.[22] Therefore, it is primarily in recessive disorders that aminoglycosides have provided the greatest promise in both cell culture experiments and clinical trials.[22]

Structure–readthrough relationship of aminoglycosides

To date, there is still no clear answer to the question why some aminoglycosides induce termination suppression, while others do not. To make things even more complicated, the identity of the stop codon and the sequence context surrounding it influence the readthrough activity differently among the various aminoglycosides that do have this activity.[79] Comparison of the *in vitro* suppression activity of several commercial aminoglycosides in mammalian system have generally shown that aminoglycosides with a 6′-OH group on ring I (such as G-418 and paromomycin, Scheme 1) are more effective than those with an amine at the same position.[79,93] One of the key differences between the prokaryotic and eukaryotic A-site is the nucleotide in the 1408 position: an adenine in the prokaryotes and a guanine in the eukaryotes (Fig. 1). An A1408G mutation in various engineered bacteria leads to enhanced resistance towards aminoglycosides, but much higher levels of resistance are observed towards aminoglycosides with 6′-NH_2 than towards those with 6′-OH,[94,95] in agreement with the suppression results obtained in the mammalian system. The crystal structures of the bacterial A-site in complex with different aminoglycosides show that indeed the 6′ functional groups form key H-bonds with the A1408 base, and models of the A1408G mutations based on these structures suggest that this mutation would prevent such interaction completely in 6′-NH_2 molecules.[30,94] However, this is not universal for all the aminoglycosides since gentamicin that contains a 6′-NH_2 group is among the most powerful readthrough inducers, and neomycin B can induce readthrough in certain constructs containing different stop codon mutations.

Challenges in the design of novel nonsense readthrough inducers

Over the past decade, the main challenge to organic synthesis with respect to aminoglycosides has been directed towards improving their antibacterial activity, and almost no efforts were made to optimize their activity as stop codon readthrough inducers. To date, nearly all suppression experiments for the potential use of these drugs for the treatment of human genetic diseases have been performed with commercially available aminoglycosides.[79] Recently, a set of neamine derivatives, such as the compound **6**, also named TC007 (Fig. 6), was shown to promote readthrough of the survival motor neuron-1 (SMN) protein in fibroblasts derived from spinal muscular atrophy (SPA) patients; however, these compounds were originally designed as antibiotics and no conclusions were derived for further improvement of the readthrough activity of these derivatives.[96]

One of the factors that probably largely hampered the inspiration towards the development of new readthrough inducers was the lack of detailed information on the molecular mechanism of aminoglycoside-induced misreading and nonsense mutation suppression in mammalian cells. Fortunately, in a recent seminal work of Westhof and coworkers, the X-ray structures of human

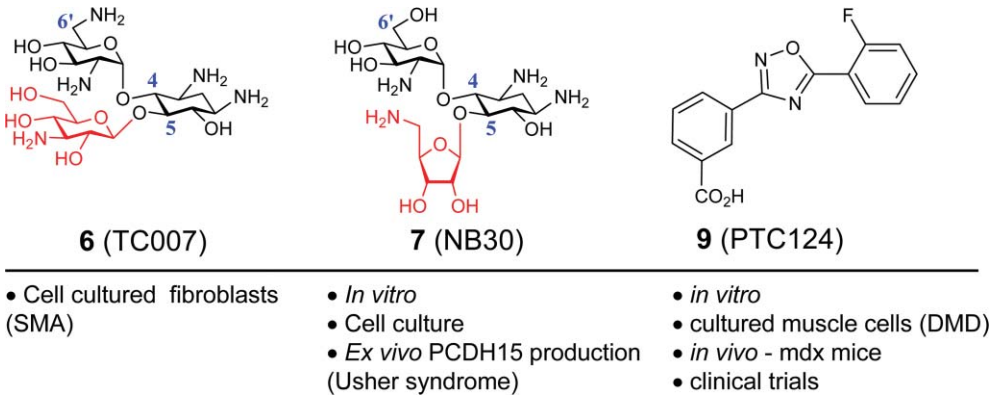


Fig. 6 Structures of semi-synthetic aminoglycosides (compounds **6** and **7**) and non-aminoglycoside PTC124 (compound **9**) that induce translational readthrough at premature stop codons.

cytoplasmic rRNA A-site models in both native and in complex with the aminoglycoside apramycin, were determined.[97,98] Two different conformations of the free cytoplasmic A-site were obtained and were suggested to correspond to its "on" state, with the two adenine residues A1492 and A1493 fully bulged-out, and its "off" state, with A1491 fully bulged-out and A1493 halfway bulged-out.[97]

These structures suggest that the molecular decoding mechanism is likely to be conserved in prokaryotes and eukaryotes but that the translation inhibition mechanism appears to be different; the aminoglycoside apramycin specifically binds and stabilizes the non-decoding "off" state of the cytoplasmic A site thereby probably inhibiting translocation of the eukaryotic ribosome instead of disturbing decoding fidelity.[98,99] More recently, very similar structures of an oligonucleotide containing the human ribosomal decoding site sequence free of the bound ligand and in complex with the apramycin[100] was also reported by Hermann and coworkers. However, to date there are still no structures of the human A-site in complex with any of the aminoglycosides that induce readthrough. In addition, a recent comparative study of the rRNA binding properties demonstrated that both paromomycin and G-418, the two powerful readthrough inducers, bind to the human A site oligonucleotide model with markedly lower affinities than those they exhibit for the *E. coli* rRNA A site.[101] Also in these studies, by combining the fluorescence quantum yield and lifetime data, the extent of drug-induced base destacking of the base at position 1492 (by *E. coli* numbering) was quantified, which revealed that the binding of G-418, but not that of the paromomycin, induces the destacking of base 1492 in the human rRNA A site sequence. Thus, whether the aminoglycoside-induced base destacking, or other factor(s) that govern the energetics and dynamics associated with aminoglycoside-induced stop codon readthrough at the human rRNA A-site, is not clear yet and requires further investigation.

Nevertheless, the most critical factor that largely limits the potential of aminoglycosides for suppression therapy is their high human toxicity. Even though various approaches to reduce aminoglycoside antibiotic toxicity have been investigated (see above), few have been implemented into standard clinical use other than changes in the administration schedule.[102] Unique protocols, however, must be designed for the administration of aminoglycosides to suppress stop mutations. For example, the use of subtoxic doses of gentamicin in the clinical trials probably caused the reduced readthrough efficiency obtained in the *in vivo* experiments compared to the *in vitro* systems.[103] The aminoglycoside G-418 (Scheme 1) shows the best termination suppression activity in *in vitro* translation–transcription systems,[79] however, its use as a therapeutic agent is not possible since it is lethal even at very low concentrations. For example, the LC_{50} of G-418 against human fibroblast cells is 0.04 mg ml^{-1}, compared to 2.5–5.0 mg ml^{-1} for gentamicin, neomycin and kanamycin.[104]

Currently, only a limited number of aminoglycosides, including gentamicin, amikacin, and tobramycin, are in clinical use as antibiotics for internal administration in humans. Among these, tobramycin does not have suppression activity, and gentamicin is the only aminoglycoside tested in animal models and clinical trials. Although some studies have shown that due to their relatively lower toxicity in cultured cells, amikacin[105] and paromomycin[93] can represent alternatives to gentamicin for suppression therapy, no clinical trials with these aminoglycosides have been reported yet.

The data described above, and the challenge of identifying novel life-saving drugs, prompted us to attempt to design new aminoglycosides with improved termination suppression activity and lower toxicity. As an initial effort towards this goal, we recently reported the synthesis of a series of new derivatives of paromomycin that were examined for their ability to readthrough stop codon mutations both *in vitro* and *ex vivo* in mammalian cultured cells.[106] Notably higher readthrough activity in cultured cells, compared to paromomycin and gentamicin was observed for the pseudo-trisaccharide derivative, compound **7**, also named NB30 (Fig. 6). However the other new structure, compound **8** (also named NB33, Fig. 7), had no significant readthrough activity, while it strongly inhibited translation. Antibacterial tests indicated that both NB30 and NB33 have increased selectivity in their action towards eukaryotic cells than towards prokaryotic cells. Cell toxicity tests using three kidney-derived cell lines confirmed that NB30 is 6–15 fold less toxic than the clinically used aminoglycosides gentamicin and paromomycin in all three cell lines tested.[107] Encouraged by these observations, we further examined the impact of NB30 on an actual genetic disorder caused by nonsense mutation. The *ex vivo* tests of the *PCDH15* gene nonsense mutations, the underlying cause of type 1 Usher syndrome (USH1), demonstrated the production of full-length protein and

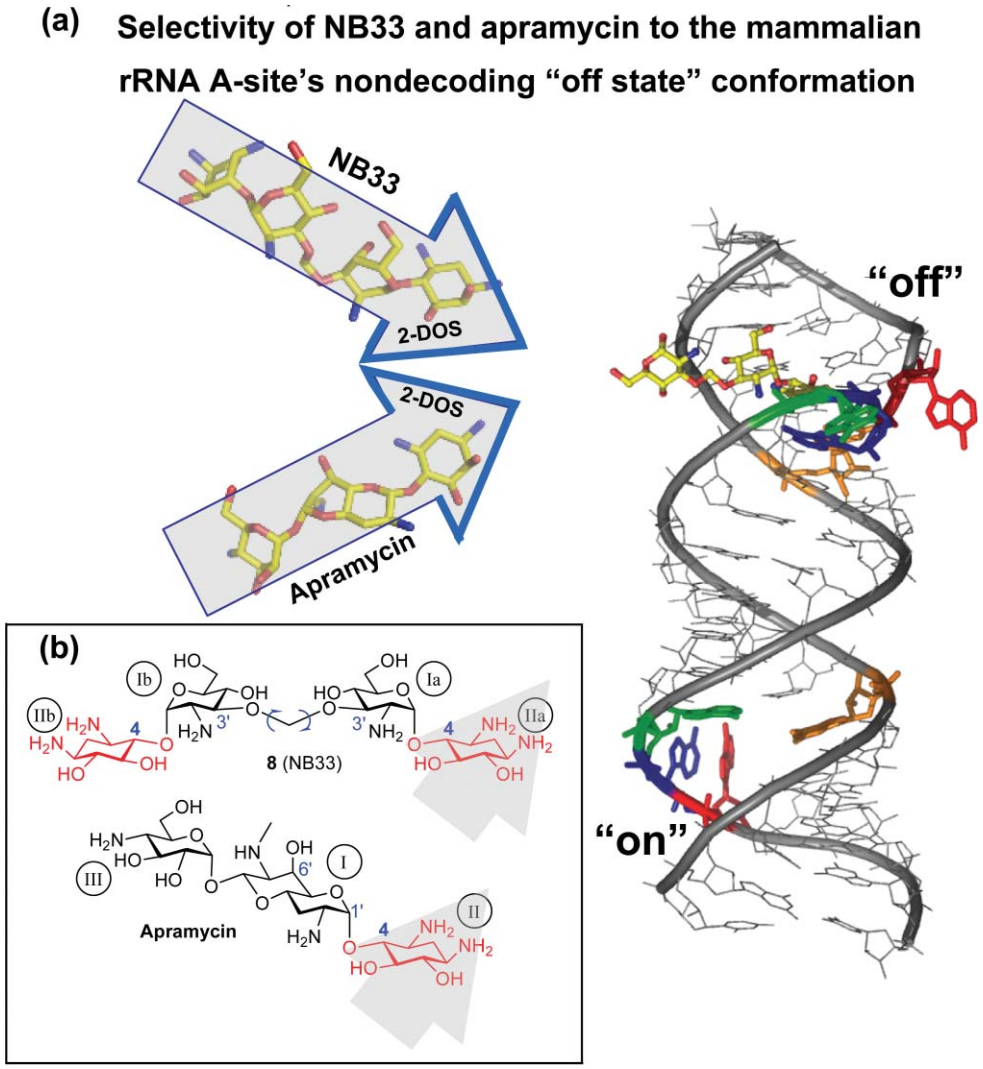


Fig. 7 Molecular basis for the selectivity of NB33 (compound **8**) and of apramycin to the eukaryotic A-site "off state" conformation as determined by a recent comparative study.[99] (a) Both NB33 and apramycin bind to the *H. sapiens* 18S cytoplasmic A site RNA construct (only the NB33–rRNA complex is shown on the right) and selectively stabilize its "off state" conformation, in which A1491 (green) and A1493 (red) are bulging out in their extrahelical conformation and A1492 (blue) stays inside the A site helix. The *E. coli* numbering is used for the RNA atoms and the X-ray structure was taken from PDB accession number 2O3V.[99] The arrows (left panel) symbolize the direction of the ligands (NB33 and apramycin) to the "off state" conformation with "arrowheads" highlighting the 2-DOS moiety. (b) Chemical structures highlighting the 2-DOS moiety and the comparative flexibility of NB33 to that of apramycin.

that the readthrough inducing activity of NB30 was shown to be higher than paromomycin, but similar to gentamicin.[107] Due to its reduced toxicity, however, the use of NB30 for suppression of nonsense mutations may be more beneficial, since it is expected to be accompanied by less negative side effects. These data, in addition to providing the proof of concept, pave the way for the development of novel aminoglycoside-based small molecules that selectively target mammalian cells by means of optimizing the efficiency of aminoglycoside-induced suppression of premature stop mutations; this progress may offer promise for the treatment of many genetic diseases.

The observed increased selectivity of action of NB30 and NB33 towards eukaryotic *versus* prokaryotic ribosome drew our attention and prompted us to ask several fundamental questions: what structural and mechanistic features are responsible for the observed selectivity increase of these synthetic derivatives? Can a general molecular principle for their structure–activity relationship be devised? To address these questions, we performed comparative biochemical and structural analysis of NB30 and NB33, together with a series of different 4-, 4,5- and 4,6-substituted 2-DOS aminoglycosides, in both eukaryotic and prokaryotic systems.[99] These studies revealed that NB33 is the strongest inhibitor of eukaryotic protein translation and the weakest inhibitor of prokaryotic protein translation of all of the aminoglycosides tested. NB33 was also found to bind the eukaryotic (human cytoplasmic) decoding-site RNA with a greater affinity than to the prokaryotic decoding-site RNA.

The molecular basis for the observed eukaryotic-target selectivity increase of NB33 was revealed by solving the 3D structure of NB33 complexed to the *H. sapiens* 18S cytoplasmic A site RNA

construct (Fig. 7). This structural study demonstrated that unlike the majority of 4,5- and 4,6-disubstituted 2-DOS aminoglycosides that selectively bind to the prokaryotic A-site and stabilize its "on state" conformation, NB33 seems to stabilize a pre-existing "off state" conformation of the free eukaryotic decoding-site RNA.[99] This drug-bound "off" conformational state does not interfere with the decoding process and consequently explained the lack of readthrough activity (<1%) of NB33. Interestingly, the observed unique action of NB33 on the eukaryotic ribosome was found to be virtually identical to that of the natural drug apramycin,[98–100] and it was suggested that these similar properties are largely determined by both being mono-substituted 2-DOS derivatives (Fig. 7); the unique unbranched pattern of the 2-DOS ring serves as an "arrowhead" allowing both NB33 and apramycin to easily penetrate into the eukaryotic A-site and grab and stabilize its "off" state conformation

Unlike NB33, apramycin, however, also binds to the prokaryotic A-site in the decoding "on state"[36] and has antibacterial activity, indicating that the unbranched pattern of the 2-DOS ring may be important, but it alone is not enough for sufficient discrimination between prokaryotic and eukaryotic A-sites. The observed increase of NB33's selectivity towards eukaryotic *versus* prokaryotic A-site was therefore rationalized by an exquisitely balanced interplay involving its Ib-spacer-Ia-IIa ring pattern. Such a selective three-dimensional structural–electrostatic complementarity of NB33 to the eukaryotic A-site "off" state conformation is a very unique precedent and serves as an inspiration for the design of other, more potent, aminoglycoside-based structures that will selectively target the eukaryotic ribosome. It will be more beneficial if this challenging task is directed towards the discovery of potent readthrough inducers with high eukaryotic specificity. The increased specificity and selectivity for the cytoplasmic rRNA A site can decrease the useful dosing ranges and subsequently decrease the anticipated toxicity of such structures.

Trying to avoid the limitations of rational design, the biopharmaceutical company PTC Therapeutics (NY, USA) is trying to discover new suppression drugs by screening large chemical libraries for nonsense readthrough activity. Using this approach a new non-aminoglycoside compound, structure **9** (also called PTC124, Fig. 6),[108,109] was discovered. The fact that it has no antibacterial activity and no reported toxicity suggest that its mechanism of action on the ribosome is different than that of the aminoglycosides. The FDA has granted fast track and orphan drug designations to PTC124 for the treatment of both CF and DMD caused by nonsense mutations, and the preliminary results of phase II clinical trials in CF and DMD patients seems very promising.

Perspectives

This abbreviated overview illustrates that combined efforts over the past few years between organic and biological chemistry have significantly advanced our understanding of how aminoglycosides might induce deleterious misreading of the genetic code in prokaryotic cells and how chemical redesign of the existing drugs can evade the resistance mechanisms that have evolved in pathogenic bacteria. Little progress, however, has been made towards the discovery of new aminoglycoside derivatives with diminished toxicity, which indeed is one of the remaining and perhaps the most challenging task. The latest pioneering structural studies on the eukaryotic decoding site provide, for the first time, a clear visual selectivity window between the prokaryotic and eukaryotic decoding sites, which can be exploited for the rational design of highly prokaryotic-specific aminoglycosides exhibiting diminished human toxicity. Separation of the elements of the aminoglycoside structure that cause toxicity from those that are required for highly specific binding to the prokaryotic decoding site can guide the rationale for the development of such designs.

Compelling evidence is now available that certain aminoglycoside structures can induce mammalian ribosomes to readthrough premature stop codon mutations and generate full-length functional proteins. Unfortunately, however, more than ten years elapsed before the idea of treating genetic diseases with aminoglycosides (1985) was tested in an animal model (1999), and almost 20 years went by until gentamicin was tested for the first time in CF patients (2003). Nevertheless, the high toxicity of gentamicin along with the reduced readthrough activity of its subtoxic doses indicates that a systematic search for new designs is required to extrapolate the approach to the point where it can actually help patients. Although the discovery of an "ideal readthrough inducer" is still a challenging task, the recent observations discussed here illustrate that this may be an achievable goal. In this avenue of research, as is true for the discovery of new aminoglycoside-based antibiotics, the human toxicity of aminoglycosides should be placed as a central problem. As a possible solution to this drawback, the therapeutic window discussed above between the prokaryotic and eukaryotic decoding sites can be further exploited for the development of new structures that selectively target the eukaryotic cytoplasmic rRNA A site. Such structures exhibiting extensive specificity and selectivity for the cytoplasmic rRNA A site can decrease the functional dosing ranges and subsequently decrease the anticipated toxicity, making them potential drugs for the treatment of human genetic disorders.

Acknowledgements

We are grateful to Dr Dalia Shezifi and our collaborators Dr Sima Yaron and Dr Tamar Ben-Yosef (Technion), Prof. Eric Westhof (Université Louis Pasteur, CNRS, Strasbourg, France) and Prof. Daniel S. Pilch (Robert Wood Johnson Medical School, New Jersey, USA) for their valuable contribution. We thank the Israel Science Foundation founded by the Israel Academy of Sciences and Humanities (grant no. 766/04), the U.S.–Israel Binational Science Foundation (grant no. 2002–126), the Mizutani Foundation for Glycoscience (grant no. 060012), and the Technion V.P.R. fund for their financial support.

References

1 Q. Vicens and E. Westhof, *ChemBioChem*, 2003, **4**, 1018–1023.
2 A. Schatz, E. Bugie and S. A. Waksman, *Proc. Soc. Exp. Biol. Med.*, 1944, **55**, 66–69.
3 S. A. Waksman, H. A. Lechevalier and D. A. Harris, *J. Clin. Invest.*, 1949, **28**, 934–939.
4 H. Umezawa, *Ann. N. Y. Acad. Sci.*, 1958, **76**, 20–26.
5 M. J. Weinstein, G. M. Luedemann, E. M. Oden, G. H. Wagman, J. P. Rosselet, J. A. Marquez, C. T. Coniglio, W. Charney, H. L. Herzog and J. Black, *J. Med. Chem.*, 1963, **122**, 463–464.
6 G. D. Wright, A. M. Berghuis and S. Mobashery, *Adv. Exp. Med. Biol.*, 1998, **456**, 27–69.

7 M. P. Mingeot-Leclercq and P. M. Tulkens, *Antimicrob. Agents Chemother.*, 1999, **43**, 1003–1012.
8 E. Selimoglu, *Curr. Pharm. Des.*, 2007, **13**, 119–126.
9 S. Kondo and K. Hotta, *J. Infect. Chemother.*, 1999, **5**, 1–9.
10 *Aminoglycoside Antibiotics*, ed. H. Umezawa and I. R. Hooper, Springer-Verlag, New York, Heidelberg, 1982.
11 S. B. Vakulenko and S. Mobashery, *Clin. Microbiol. Rev.*, 2003, **16**, 430–450.
12 S. Magnet and J. S. Blanchard, *Chem. Rev.*, 2005, **105**, 477–498.
13 B. Francois, R. J. Russell, J. B. Murray, F. Aboul-ela, B. Masquida, Q. Vicens and E. Westhof, *Nucleic Acids Res.*, 2005, **33**, 5677–5690.
14 T. Hermann, *Curr. Opin. Struct. Biol.*, 2005, **15**, 355–366.
15 X.-S. Ye and L.-H. Zhang, *Curr. Med. Chem.*, 2002, **9**, 929–939.
16 T. Hermann and Y. Tor, *Expert Opin. Ther. Pat.*, 2005, **15**, 49–62.
17 J. Li and C. W. Chang, *Anti-infective Agents in Medicinal Chemistry*, 2006, **5**, 255–271.
18 J. G. Silva and I. Carvalho, *Curr. Med. Chem.*, 2007, **14**, 1101–1119.
19 E. C. Bottger, B. Springer, T. Prammananan, Y. Kidan and P. Sander, *EMBO Rep.*, 2001, **2**, 318–323.
20 D. C. Eustice and J. M. Wilhelm, *Biochemistry*, 1984, **23**, 1462–1467.
21 J. M. Wilhelm, S. E. Pettitt and J. J. Jessop, *Biochemistry*, 1978, **17**, 1143–1149.
22 L. V. Zingman, S. Park, T. M. Olson, A. E. Alekseev and A. Terzic, *Clin. Pharmacol. Ther.*, 2007, **81**, 99–103.
23 S. Jana and J. K. Deb, *Appl. Microbiol. Biotechnol.*, 2006, **70**, 140–150.
24 D. Banerjee and D. Stableforth, *Drugs*, 2000, **60**, 1053–1064.
25 D. Pashov, S. Vangelov and L. Diakov, *Vet. Med. Nauki*, 1987, **24**, 41–46.
26 D. Moazed and H. F. Noller, *Nature*, 1987, **327**, 389–394.
27 A. P. Carter, W. M. Clemons, D. E. Brodersen, R. J. Morgan-Warren, B. T. Wimberly and V. Ramakrishnan, *Nature*, 2000, **407**, 340–348.
28 F. Schluenzen, A. Tocilj, R. Zarivach, J. Harms, M. Gluehmann, D. Janell, A. Bashan, H. Bartels, I. Agmon, F. Franceschi and A. Yonath, *Cell*, 2000, **102**, 615–623.
29 D. Fourmy, S. Yoshizawa and J. D. Puglisi, *J. Mol. Biol.*, 1998, **277**, 333–345.
30 Q. Vicens and E. Westhof, *Biopolymers*, 2003, **70**, 42–57.
31 B. T. Wimberly, D. E. Brodersen, W. M. Clemons, Jr., R. J. Morgan-Warren, A. P. Carter, C. Vonrhein, T. Hartsch and V. Ramakrishnan, *Nature*, 2000, **407**, 327–339.
32 J. M. Ogle, D. E. Brodersen, W. M. Clemons, Jr., M. J. Tarry, A. P. Carter and V. Ramakrishnan, *Science*, 2001, **292**, 897–902.
33 J. M. Ogle, F. V. Murphy, M. J. Tarry and V. Ramakrishnan, *Cell*, 2002, **111**, 721–732.
34 J. M. Ogle and V. Ramakrishnan, *Annu. Rev. Biochem.*, 2005, **74**, 129–177.
35 S. Perzynski, M. Cannon, E. Cundliffe, S. B. Chahwala and J. Davies, *Eur. J. Biochem.*, 1979, **99**, 623–628.
36 Q. Han, Q. Zhao, S. Fish, K. B. Simonsen, D. Vourloumis, J. M. Froelich, D. Wall and T. Hermann, *Angew. Chem., Int. Ed.*, 2005, **44**, 2694–2700.
37 M. Kaul, C. M. Barbieri and D. S. Pilch, *J. Am. Chem. Soc.*, 2006, **128**, 1261–1271.
38 C. Kim, A. Villegas-Estrada, D. Hesek and S. Mobashery, *Biochemistry*, 2007, **46**, 5270–5282.
39 C. Kim and S. Mobashery, *Bioorg. Chem.*, 2005, **33**, 149–158.
40 D. L. Burk, W. C. Hon, A. K. Leung and A. M. Berghuis, *Biochemistry*, 2001, **40**, 8756–8764.
41 D. H. Fong and A. M. Berghuis, *EMBO J.*, 2002, **21**, 2323–2331.
42 D. Nurizzo, S. C. Shewry, M. H. Perlin, S. A. Brown, J. N. Dholakia, R. L. Fuchs, T. Deva, E. N. Baker and C. A. Smith, *J. Mol. Biol.*, 2003, **327**, 491–506.
43 H. Hachler, P. Santanam and F. H. Kayser, *Antimicrob. Agents Chemother.*, 1996, **40**, 1254–1256.
44 M. Hainrichson, O. Yaniv, M. Cherniavsky, I. Nudelman, D. Shallom-Shezifi, S. Yaron and T. Baasov, *Antimicrob. Agents Chemother.*, 2007, **51**, 774–776.
45 D. M. Daigle, D. W. Hughes and G. D. Wright, *Chem. Biol.*, 1999, **6**, 99–110.
46 D. D. Boehr, D. M. Daigle and G. D. Wright, *Biochemistry*, 2004, **43**, 9846–9855.
47 J. Roestamadji, I. Grapsas and S. Mobashery, *J. Am. Chem. Soc.*, 1995, **117**, 80–84.
48 H. Weizman and Y. Tor, in *Carbohydrate-based drug discovery*, ed. C. H. Wong, Wiley-VCH, Weinheim, 2003, vol. 2, pp. 661–683.
49 A. Forge and J. Schacht, *Audiol. Neurootol.*, 2000, **5**, 3–22.
50 J. Nagai and M. Takano, *Drug Metab. Pharmacokinet.*, 2004, **19**, 159–170.
51 M. Kaul, C. M. Barbieri and D. S. Pilch, *J. Mol. Biol.*, 2005, **346**, 119–134.
52 K. M. Keeling and D. M. Bedwell, *Curr. Pharmacogenomics*, 2005, **3**, 259–269.
53 G. Sener, A. O. Sehirli, H. Z. Altunbas, Y. Ersoy, K. Paskaloglu, S. Arbak and G. Ayanoglu-Dulger, *J. Pineal Res.*, 2002, **32**, 231–236.
54 K. Kawamoto, S. H. Sha, R. Minoda, M. Izumikawa, H. Kuriyama, J. Schacht and Y. Raphael, *Mol. Ther.*, 2004, **9**, 173–181.
55 D. N. Gilbert, C. A. Wood, S. J. Kohlhepp, P. W. Kohnen, D. C. Houghton, H. C. Finkbeiner, J. Lindsley and W. M. Bennett, *J. Infect. Dis.*, 1989, **159**, 945–953.
56 D. Beauchamp, G. Laurent, P. Maldague, S. Abid, B. K. Kishore and P. M. Tulkens, *J. Pharmacol. Exp. Ther.*, 1990, **255**, 858–866.
57 N. Thibault, L. Grenier, M. Simard, M. G. Bergeron and D. Beauchamp, *Antimicrob. Agents Chemother.*, 1994, **38**, 1027–1035.
58 N. Thibault, L. Grenier, M. Simard, M. G. Bergeron and D. Beauchamp, *Life Sci.*, 1995, **56**, 1877–1887.
59 A. Watanabe, J. Nagai, Y. Adachi, T. Katsube, Y. Kitahara, T. Murakami and M. Takano, *J. Controlled Release*, 2004, **95**, 423–433.
60 D. Beauchamp and G. Labrecque, *Curr. Opin. Crit. Care*, 2001, **7**, 401–408.
61 C. Bartal, A. Danon, F. Schlaeffer, K. Reisenberg, M. Alkan, R. Smoliakov, A. Sidi and Y. Almog, *Am. J. Med.*, 2003, **114**, 194–198.
62 K. Fujisawa, T. Hoshiya and H. Kawaguchi, *J. Antibiot.*, 1974, **27**, 677–681.
63 T. Shitara, E. Umemura, T. Tsuchiya and T. Matsuno, *Carbohydr. Res.*, 1995, **276**, 75–89.
64 T. Tsuchiya, T. Shitara, S. Umezawa, T. Takeuchi, M. Hamada, N. Tomono and E. Umemura, *Carbohydr. Res.*, 1993, **240**, 307–312.
65 R. M. Sandoval, J. P. Reilly, W. Running, S. B. Campos, J. R. Santos, C. L. Phillips and B. A. Molitoris, *J. Am. Soc. Nephrol.*, 2006, **17**, 2697–2705.
66 D. P. Arya, R. L. Coffee, Jr., B. Willis and A. I. Abramovitch, *J. Am. Chem. Soc.*, 2001, **123**, 5385–5395.
67 H. Kawaguchi, T. Naito, S. Nakagawa and K. I. Fujisawa, *J. Antibiot.*, 1972, **25**, 695–708.
68 J. Li, H. N. Chen, H. Chang, J. Wang and C. W. Chang, *Org. Lett.*, 2005, **7**, 3061–3064.
69 J. Li, F. I. Chiang, H. N. Chen and C. W. Chang, *J. Org. Chem.*, 2007, **72**, 4055–4066.
70 J. Haddad, L. P. Kotra, B. Llano-Sotelo, C. Kim, E. F. Azucena, Jr., M. Liu, S. B. Vakulenko, C. S. Chow and S. Mobashery, *J. Am. Chem. Soc.*, 2002, **124**, 3229–3237.
71 R. J. Russell, J. B. Murray, G. Lentzen, J. Haddad and S. Mobashery, *J. Am. Chem. Soc.*, 2003, **125**, 3410–3411.
72 N. Minowa, Y. Akiyama, Y. Hiraiwa, K. Maebashi, T. Usui and D. Ikeda, *Bioorg. Med. Chem. Lett.*, 2006, **16**, 6351–6354.
73 M. Fridman, V. Belakhov, S. Yaron and T. Baasov, *Org. Lett.*, 2003, **5**, 3575–3578.
74 M. Hainrichson, V. Pokrovskaya, D. Shallom-Shezifi, M. Fridman, V. Belakhov, D. Shachar, S. Yaron and T. Baasov, *Bioorg. Med. Chem.*, 2005, **13**, 5797–5807.
75 M. Fridman, V. Belakhov, L. V. Lee, F. S. Liang, C. H. Wong and T. Baasov, *Angew. Chem., Int. Ed.*, 2005, **44**, 447–452.
76 B. Francois, J. Szychowski, S. S. Adhikari, K. Pachamuthu, E. E. Swayze, R. H. Griffey, M. T. Migawa, E. Westhof and S. Hanessian, *Angew. Chem., Int. Ed.*, 2004, **43**, 6735–6738.
77 S. Hanessian, J. Szychowski, S. S. Adhikari, G. Vasquez, P. Kandasamy, E. E. Swayze, M. T. Migawa, R. Ranken, B. Francois, J. Wirmer-Bartoschek, J. Kondo and E. Westhof, *J. Med. Chem.*, 2007, **50**, 2352–2369.
78 R. J. Kaufman, *J. Clin. Invest.*, 1999, **104**, 367–368.
79 M. Manuvakhova, K. Keeling and D. M. Bedwell, *RNA*, 2000, **6**, 1044–1055.
80 E. Kerem, *Curr. Opin. Pulm. Med.*, 2004, **10**, 547–552.
81 J. Atkinson and R. Martin, *Nucleic Acids Res.*, 1994, **22**, 1327–1334.
82 M. T. Howard, B. H. Shirts, L. M. Petros, K. M. Flanigan, R. F. Gesteland and J. F. Atkins, *Ann. Neurol.*, 2000, **48**, 164–169.
83 J. F. Burke and A. E. Mogg, *Nucleic Acids Res.*, 1985, **13**, 6265–6272.
84 D. M. Bedwell, A. Kaenjak, D. J. Benos, Z. Bebok, J. K. Bubien, J. Hong, A. Tousson, J. P. Clancy and E. J. Sorscher, *Nat. Med.*, 1997, **3**, 1280–1284.

85 M. Howard, R. A. Frizzell and D. M. Bedwell, *Nat. Med.*, 1996, **2**, 467–469.
86 M. Wilschanski, Y. Yahav, Y. Yaacov, H. Blau, L. Bentur, J. Rivlin, M. Aviram, T. Bdolah-Abram, Z. Bebok, L. Shushi, B. Kerem and E. Kerem, *N. Engl. J. Med.*, 2003, **349**, 1433–1441.
87 E. R. Barton-Davis, L. Cordier, D. I. Shoturma, S. E. Leland and H. L. Sweeney, *J. Clin. Invest.*, 1999, **104**, 375–381.
88 K. M. Keeling, D. A. Brooks, J. J. Hopwood, P. Li, J. N. Thompson and D. M. Bedwell, *Hum. Mol. Genet.*, 2001, **10**, 291–299.
89 K. Sangkuhl, A. Schulz, H. Rompler, J. Yun, J. Wess and T. Schoneberg, *Hum. Mol. Genet.*, 2004, **13**, 893–903.
90 A. Helip-Wooley, M. A. Park, R. M. Lemons and J. G. Thoene, *Mol. Genet. Metab.*, 2002, **75**, 128–133.
91 C. Grayson, J. P. Chapple, K. R. Willison, A. R. Webster, A. J. Hardcastle and M. E. Cheetham, *J. Med. Genet.*, 2002, **39**, 62–67.
92 B. Xi, F. Guan and D. S. Lawrence, *J. Am. Chem. Soc.*, 2004, **126**, 5660–5661.
93 M. T. Howard, C. B. Anderson, U. Fass, S. Khatri, R. F. Gesteland, J. F. Atkins and K. M. Flanigan, *Ann. Neurol.*, 2004, **55**, 422–426.
94 P. Pfister, S. Hobbie, Q. Vicens, E. C. Bottger and E. Westhof, *ChemBioChem*, 2003, **4**, 1078–1088.
95 M. I. Recht, S. Douthwaite and J. D. Puglisi, *EMBO J.*, 1999, **18**, 3133–3138.
96 V. B. Mattis, R. Rai, J. Wang, C. W. Chang, T. Coady and C. L. Lorson, *Hum. Genet.*, 2006, **120**, 589–601.
97 J. Kondo, A. Urzhumtsev and E. Westhof, *Nucleic Acids Res.*, 2006, **34**, 676–685.
98 J. Kondo, B. Francois, A. Urzhumtsev and E. Westhof, *Angew. Chem., Int. Ed.*, 2006, **45**, 3310–3314.
99 J. Kondo, M. Hainrichson, I. Nudelman, D. Shallom-Shezifi, C. M. Barbieri, D. S. Pilch, E. Westhof and T. Baasov, *ChemBioChem*, 2007, **8**, 1700–1709.
100 T. Hermann, V. Tereshko, E. Skripkin and D. J. Patel, *Blood Cells, Mol. Dis.*, 2007, **38**, 193–198.
101 M. Kaul, C. M. Barbieri and D. S. Pilch, *J. Am. Chem. Soc.*, 2004, **126**, 3447–3453.
102 R. Schiffelers, G. Storm and I. Bakker-Woudenberg, *J. Antimicrob. Chemother.*, 2001, **48**, 333–344.
103 G. Karpati and H. Lochmuller, *Ann. Neurol.*, 2001, **49**, 693–694.
104 V. G. Chernikov, S. M. Terekhov, T. B. Krokhina, S. S. Shishkin, T. D. Smirnova, E. A. Kalashnikova, N. V. Adnoral, L. B. Rebrov, Y. I. Denisov-Nikol'skii and V. A. Bykov, *Bull. Exp. Biol. Med.*, 2003, **135**, 103–105.
105 M. Du, K. M. Keeling, L. Fan, X. Liu, T. Kovacs, E. Sorscher and D. M. Bedwell, *J. Mol. Med.*, 2006, **84**, 573–582.
106 I. Nudelman, A. Rebibo-Sabbah, D. Shallom-Shezifi, M. Hainrichson, I. Stahl, T. Ben-Yosef and T. Baasov, *Bioorg. Med. Chem. Lett.*, 2006, **16**, 6310–6315.
107 A. Rebibo-Sabbah, I. Nudelman, Z. M. Ahmed, T. Baasov and T. Ben-Yosef, *Hum. Genet.*, 2007, DOI: 10.1007/s00439-007-0410-7.
108 S. Aurino and V. Nigro, *Acta Myol.*, 2006, **25**, 5–12.
109 E. M. Welch, E. R. Barton, J. Zhuo, Y. Tomizawa, W. J. Friesen, P. Trifillis, S. Paushkin, M. Patel, C. R. Trotta, S. Hwang, R. G. Wilde, G. Karp, J. Takasugi, G. Chen, S. Jones, H. Ren, Y. C. Moon, D. Corson, A. A. Turpoff, J. A. Campbell, M. M. Conn, A. Khan, N. G. Almstead, J. Hedrick, A. Mollin, N. Risher, M. Weetall, S. Yeh, A. A. Branstrom, J. M. Colacino, J. Babiak, W. D. Ju, S. Hirawat, V. J. Northcutt, L. L. Miller, P. Spatrick, F. He, M. Kawana, H. Feng, A. Jacobson, S. W. Peltz and H. L. Sweeney, *Nature*, 2007, **447**, 87–91.