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Interaction of aminoglycosides and their copper(II) complexes with nucleic acids: implication to the toxicity of these drugs

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Cupric complexes of eight aminoglycosidic antibiotics were screened for their specific behavior towards $tRNA^{Phe}$, both in oxidative and neutral surrounding. Without H_2O_2, the cleavage efficiency was dependent on the resultant charge of the molecule. A comparative assay using $tRNA^{Phe}$ devoid of the natural hypermodification in the anticodon loop proved that hypermodification is indispensable for site recognition and subsequent cleavage. The intensity of single and double strand scissions in plasmid DNA also proceeded in a charge-dependent manner. Unlike free antibiotics, their cupric complexes in the presence of H_2O_2, facilitated plasmid linearisation and degradation. The participation of ROS in those processes was confirmed using NDMA as a reporter molecule, whose consumption was influenced by the protonation state of the complex.

Introduction

Aminoglycosides are still problem antibiotics since any therapy based on these drugs requires constant monitoring of their concentrations in blood plasma. The reason for this caution is the coexistence of various toxic effects such as high ototoxicity and nephrotoxicity, irreversible uptake and the possibility of the cellular membrane damage.[1] Misreading of the genetic code and inhibition of translocation after binding to the ribosome, which is a part of their bactericidal action, may also have a negative impact on the host tissues. Moreover, this group of antibiotics became suspected of another set of deleterious side effects, *i.e.* haemato-,[2] hepato-[3] and neurotoxicity.[4] Therefore, there is a pressing need to design improved, modified aminoglycosides, but also to gain a thorough insight into the chemical basis of their action. Many new representatives of this class have been introduced as therapeutic agents since the discovery of streptomycin, in 1943. Isolation of gentamicin from *Micromonospora purpurea* appeared to be a spectacular breakthrough in clinical treatment. Introduction of subsequent new and modified drugs helped either to decrease the toxicity or combat bacterial resistance. The latter occurs as a result of covalent modification of an antibiotic or the RNA,[5] which is the main target of the drug. Aminoglycosides interfere with the majority of molecules of this group, including ribosomal RNA,[6,7] HIV-1 viral Rev responsible element,[8] its different constructs,[9] TAR RNA,[10] group I introns,[11,12] yeast $tRNA^{Phe}$,[13] hammerhead ribozyme[14] and RNase P RNA.[15] This allows for the conclusion that RNA recognition is shape-specific rather than sequence-specific. The presence of metal ions is the underlying reason for many of those effects. It was proved that aminoglycosides might displace divalent metal ions from RNA surroundings. Due to the fact that antibiotics of this group possess 3–6 amino functions, they carry a positive charge at physiological pH. This charge allows them to compete with metal ions, especially Mg^{2+}, to a variety of binding sites.[16] Neomycin B with its six amines was demonstrated to displace essential metal ions from major regions in intron RNA, to inhibit splicing, and to protect the catalytic core from Fe^{2+} induced hydroxyl radical cleavage.[17] The crystal structure of the $tRNA^{Phe}$ complex with neomycin showed the overlap of the neomycin binding site with known divalent metal ion binding sites in this nucleic acid. The same authors also provided evidence for protection of yeast $tRNA^{Phe}$ from Pb^{2+} induced cleavage by neomycin B, which may be regarded along with other aminoglycosides as a "metal mimic".[18]

Apart from amino functions, aminoglycosides also possess several hydroxyl groups, which together constitute preferable donor sets for metal ions. Combination of such binding sites was found to be very attractive, especially for copper(II) ions (*e.g.* refs. 19–23). The resulting complexes were capable of hydrogen peroxide conversion and activation through radical intermediates. These reactive oxygen species were believed, as in the case of Fe–gentamicin[24,25] and Fe–neomycin[26] systems, to play important roles in the molecular basis of aminoglycoside-induced ototoxicity. Copper complexes of aminoglycosides were previously examined for their ability to cleave both RNA[7,27–29] and DNA[28–31] and the presence of H_2O_2 was proved to enhance their destructive abilities, mainly through hydroxyl radical induction and Cu(II) to Cu(III) oxidation.[23,32]

In this paper we present the results of DNA and RNA cleavage, based on the comparison of the *in vitro* action of the main representatives of aminoglycosides, and provide evidence for specific hypermodification requirement in $tRNA^{Phe}$ for the cleavage to occur.

Experimental

Materials

Geneticin, kasugamycin, sisomicin, amikacin, kanamycins A and B, tobramycin, neomycin B, $CuCl_2$, H_2O_2, NDMA, Chelex-100 resin, sodium and potassium phosphates and all other simple chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Low EEO agarose was purchased from AppliChem (Darmstadt, Germany). Bromophenol blue and glycerol were obtained from POCH (Gliwice, Poland). *Escherichia coli* DH5α strain (Gibco BRL, Gaithersburg, MD) was used for amplification of pBluescriptSK+ plasmid (Stratagene, La Jolla, CA, cat. no. 212205; GeneBank accession number X52325). LB medium and bactotryptone were purchased from Difco (Detroit, MI). Bacterial alkaline phosphatase, Taq polymerase, T7 RNA polymerase and T4 polynucleotide kinase were from MBI Fermentas (Vilnius,

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Lithuania), (γ-^{32}P)ATP was purchased from ICN (Costa Mesa, CA). Electrophoretic reagents: boric acid, acrylamide, bis-acrylamide and urea were obtained from Serva (Heidelberg, Germany).

Isolation of yeast tRNA^{Phe}

Yeast tRNA^{Phe} of specific phenylalanine acceptance 1200–1400 pmol/A_{260} unit was prepared from crude baker's yeast tRNA by standard column chromatography procedures including benzoylated DEAE-cellulose and Sepharose-4B (Pharmacia, Sweden). Final purification was done by HPLC on TSK-gel DEAE 2SW column (Toyo Soda, Japan).

DNA template construct and *in vitro* transcription of tRNA^{Phe}

The DNA template for *in vitro* transcription of tRNA^{Phe} synthesis was prepared as follows:[33] two DNA oligomers were synthesized, 5′-*TAATACGACTCACTATA*GCGGATTTAGCUCAGTTGG-3′ and 5′-TGGTGCGAATTCTGTGGAT CGAACACAGGACCTCCAGATTTCAGTCTGGCGCT CTCCCAACTGAGC TAAATCCGC-3′ (letters in italics mark the T7 RNA polymerase promoter; complementary sequences are underlined). Equimolar amounts of both oligomers were annealed and a double-stranded DNA template was generated by PCR. The reaction mixture contained: 1 μM both DNA oligomers, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM each dNTP and 25U ml^{-1} Taq polymerase. The reaction was performed on a Biometra UNO II thermocycler for seven cycles of 30 s at 94 °C, 30 s at 55 °C and 2 min at 72 °C. The mixture was extracted with phenol–chloroform (1 : 1), the reaction product precipitated with ethanol, dissolved in TE buffer and used in transcription reactions.

The *in vitro* transcription reaction contained 0.4 μM DNA template, 40 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM DTT, 1 mM each NTP, 2.5 mM guanosine and 3000 U ml^{-1} T7 RNA polymerase. Following incubation of the mixture at 37 °C for 4 h, the RNA transcript was purified on a denaturing 8% polyacrylamide gel, excised, eluted with 0.3 M sodium acetate, pH 5.3, precipitated with ethanol, recovered by centrifugation, dissolved in sterile water, and stored at −20 °C.

The DNA anticodon stem oligomer

The DNA oligomer 5′d(GAGCGCCAGACTGAAGATCTGG)3′, which corresponds to nucleotides 22–43 of the anticodon stem of yeast tRNA^{Phe}, acDNA, was synthesized chemically at the 0.2 μmol scale. The oligomer was deprotected after synthesis and purified on denaturing 8% (w/v) polyacrylamide gels. After localization by UV shadowing the DNA band was excised, eluted with 0.3 M sodium acetate, pH 5.3, 1 mM EDTA and precipitated with three volumes of ethanol. The DNA was recovered by centrifugation, dissolved in TE buffer and stored at −20 °C.

Labeling of RNA and DNA

Yeast tRNA^{Phe} was dephosphorylated with bacterial alkaline phosphatase and subsequently 5′-end-labeled with (γ-^{32}P)ATP and polynucleotide kinase. In the case of ^{32}P- labeling of tRNA^{Phe} transcript and acDNA oligomer the dephosphorylation step was omitted. The oligomers were purified on denaturing 12.5% polyacrylamide gels, located by autoradiography, excised and eluted with the 0.3 M sodium acetate buffer, pH 5.1, 1 mM EDTA. The eluted RNA and DNA were precipitated with ethanol, dissolved in water and stored at −20 °C before use.

Cleavage reaction

Prior to the reaction, the ^{32}P labeled tRNA^{Phe} was supplemented with carrier tRNA^{Phe} to the final RNA concentration of 1 μM and subjected to the denaturation/renaturation procedure by heating the samples at 65 °C for 2 min and slow cooling to 25 °C. The ^{32}P labeled acDNA oligomer was treated before the reaction in the same way. Cleavage reactions induced by antibiotics (50 μM) as well as their copper(II) complexes (50 μM) were performed in 50 mM sodium phosphate buffer at pH 7.4, in the absence or presence of H_2O_2 (50 μM) for 1 h. Further details of the reaction conditions are specified in the Fig. legends. All reactions were stopped by mixing with equal volume of 8 M urea/dyes/20 mM EDTA solution and loaded on a 15% polyacrylamide, 7 M urea gel. Electrophoresis was done at 60 W for 3 h, followed by autoradiography at −80 °C with an intensifying screen. For quantitative analysis gels were exposed to phosphorimaging screens and quantified using a Typhoon 8600 Imager with Image-Quant software (Molecular Dynamics).

In order to assign the cleavage sites, products of cleavage reaction were run along with the products of alkaline degradation and limited T_1 nuclease digestion of the same tRNA^{Phe}. The alkaline hydrolysis ladder was generated by incubation of ^{32}P labeled tRNA^{Phe} with five volumes of formamide and 2 mM $MgCl_2$ in boiling water for 15 min. Partial T_1 nuclease digestion was performed in denaturing conditions (50 mM sodium citrate, pH 4.5, 7 M urea) with 0.1 unit of the enzyme. The reaction mixture was incubated for 10 min at 55 °C.

Preparation of DNA

pBluescriptSK+ plasmid DNA was isolated from bacterial cells using Nucleobond AX100 cartridges and buffers (Macherey-Nagel, Duren, Germany, cat. no. 740 573). Bacterial strains were cultivated with vigorous shaking (197 rpm) at 37 °C in 100 ml of LB medium (1% yeast extract), 1% bactotryptone, 0.5% sodium chloride and 100 μg ml^{-1} ampicillin until the mid-logarithmic growth phase. Cells were harvested by centrifugation (5000*g*, 10 min, 4 °C) and resuspended in 4 ml of S1 buffer (50 mM Tris–HCl, pH 8.0; 10 mM EDTA; 100 mg ml^{-1} RNase A). Then 4 ml of S2 buffer (200 mM NaOH; 1% SDS) were added, the cell suspension gently mixed, and incubated for 5 min at room temperature. Cell lysate was then mixed with 4 ml of S3 buffer (2.8 M sodium acetate, pH 5.1) and, after 5 min incubation on ice, clarified by filtration. Finally, the cell lysate was loaded on a Nucleobond AX100 column equilibrated with N2 buffer (100 mM Tris–H_3PO_4, pH 6.3; 15% ethanol, 900 mM KCl). After washing with 8 ml of N3 buffer (100 mM Tris–H_3PO_4, pH 6.3; 15% ethanol, 1150 mM KCl) plasmid DNA was eluted from the column using 4 ml of N5 buffer (100 mM Tris–H_3PO_4, pH 8.5; 15% ethanol, 1 M KCl). Finally, DNA was recovered from buffer N5 by isopropanol precipitation and redissolved in 100 μl of 10 mM Tris–HCl, pH 8.0.

DNA strand break analysis

The ability of Cu(II) complexes of aminoglycoside antibiotics to induce single- and double-strand breaks in the absence and presence of H_2O_2 was tested with the pBluescriptSK+ plasmid. The molar ratios of reagents were 1 : 1 : 1 at concentrations of 50 μM. The samples, dissolved in 50 mM phosphate buffer of pH 7.4, contained combinations of DNA (25 μg/ml), $CuCl_2$ and/or antibiotic and H_2O_2. After 1 h incubation at 37 °C, reaction mixtures (20 μl) were mixed with 4 μl of loading buffer (bromophenol blue in 30% glycerol) and loaded on 1% agarose gels, containing ethidium bromide, in TBE buffer (90 mM Tris–borate, pH 8.0; 20 mM EDTA). Gel electrophoresis was done at constant voltage of 4 V cm^{-1} for 60 min. As control for double-strand breaks, reference plasmid samples were linearized with *Eco*RI endonuclease. The gels were photographed and processed with a Digital Imaging System (Syngen Biotech, Wroclaw, Poland).

ROS generation measurements

NDMA, a scavenger molecule, used commonly in studies of hydroxyl radicals and similarly reactive species was used in

spectroscopic absorption experiments. The measurements were followed at 37 °C on a Beckman DU-650 (Beckman, Palo Alto, CA) spectrophotometer, in 1 cm cuvettes, at 440 nm, which is a characteristic wavelength of NDMA absorption. The reaction mixtures contained NDMA (25 μM), aminoglycoside antibiotics and Cu(II) (each 50 μM) and H_2O_2 (0.5 mM), all in sodium phosphate buffer (50 mM).

Results and discussion

tRNA cleavage by aminoglycosides and their copper(II) complexes

The possibility of $tRNA^{Phe}$ cleavage by aminoglycosidic antibiotics was studied previously.[13,28] It is known that these antibiotics bind copper(II) ions effectively[19–23] and much more strongly than any other metal ions, available under natural physiological conditions or as a result of environmental pollution.[34] Recently, we have shown that cupric complexes of amikacin cleave $tRNA^{Phe}$ specifically in a non-oxidative manner.[28] Amikacin, however, has a different structure than the other representatives of this class of antibiotics, owing to an aglycon chain, which takes part in Cu(II) ion chelation.[19] Unsubstituted aminoglycosides bind copper(II) ions with different patterns, generally by terminal aminosugar rings.[20–23] Various coordination modes may potentially lead to differences in the interactions of aminoglycosides with nucleic acids. In this paper we present the results of the cleavage reaction of yeast $tRNA^{Phe}$ in the presence of eight popular aminoglycosides as well as their cupric complexes. Fig. 1 shows the comparison of the strand-break assay performed in the presence of four antibiotics which belong to the kanamycin group: kanamycin A, the product of its acylation, amikacin, kanamycin B and its deoxy-derivative tobramycin. In the parallel reactions the antibiotics were used as their Cu(II) complexes both in the presence and absence of hydrogen peroxide. The antibiotics alone (lanes: 5, 9, 13, 17) and accompanied by H_2O_2 (lanes: 6, 10, 14, 18) or complexed with Cu(II) ions (lanes: 7, 11, 15, 19) were effective $tRNA^{Phe}$ damaging agents. Highly specific cleavage occurred in the anticodon loop (Fig. 2), at hypermodified base Y37 (wybutine), similarly as was previously reported for amikacin[13,28]. Since these antibiotics are redox inactive,[23,32] as one could expect, the addition of H_2O_2 did not influence the cleavage patterns (compare *e.g.* lane 5 with 6, Fig. 1). However, in the reactions performed with the antibiotic cupric complexes additional cleavages at A36 appeared (lanes 7, 11, 15 and 19). This was likely to be a consequence of a slightly different arrangement of the cupric complex binding, in comparison with the antibiotic binding in the vicinity of the hypermodified guanine moiety. Such an effect was not reported previously for the amikacin complex.[28]

Interestingly, the relative efficiency of cleavage induced in the presence of Cu(II)–antibiotic complexes at Y37 strongly depended on the number of amino functions in the aminoglycoside molecules. Stronger cleavage occurred in the presence of kanamycin B and tobramycin, each of them containing five amino groups, than in the presence of amikacin and kanamycin A, containing four amino functions (Fig. 1). In order to confirm this observation we also studied some other antibiotics, such as kasugamycin, geneticin, sisomicin and neomycin B. Although the cleavage patterns were very similar to the pattern presented in Fig. 1, the cleavage intensities at Y37 differed (data not shown). The two-ring kasugamycin, containing one primary and two secondary amines, carried the lowest positive charge at pH 7.4 and it caused the least cleavage of tRNA. On the other hand, the strongest cleavage occurred in the presence of neomycin B, which contains six amino functions. The intensities of cleavages induced by geneticin and sisomicin place them between kasugamycin and neomycin, with respect to their positive charge.

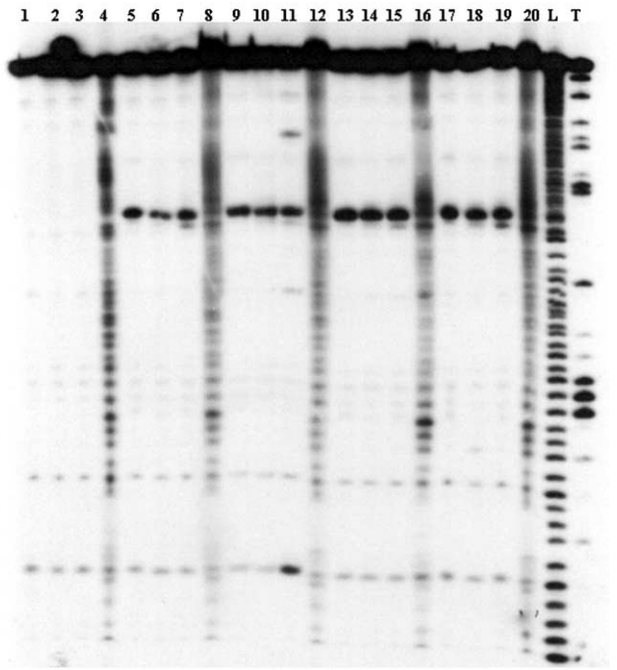

Fig. 1 Specificity of cleavages in yeast $tRNA^{Phe}$, induced by aminoglycosides and their Cu(II) complexes. The samples, incubated for 1 h at 37 °C, were run on a 15% polyacrylamide for 3 h at 60 W in TBE buffer. 5′-end-labeled $tRNA^{Phe}$ was mixed with unlabeled $tRNA^{Phe}$ to obtain final RNA concentration of 1 μM. **Lane 1**, untreated $tRNA^{Phe}$; **lane 2**, + 50 μM H_2O_2; **lane 3**, + 50 μM $CuCl_2$; **lane 4**, + 50 μM $CuCl_2$ + 50 μM H_2O_2; **lane 5**, + 50 μM AK; **lane 6**, + 50 μM AK + 50 μM H_2O_2; **lane 7**, + 50 μM AK + 50 μM $CuCl_2$; **lane 8**, + 50 μM AK + 50 μM $CuCl_2$ + 50 μM H_2O_2; **lane 9**, + 50 μM KMA; **lane 10**, + 50 μM KMA + 50 μM H_2O_2; **lane 11**, + 50 μM KMA + 50 μM $CuCl_2$; **lane 12**, + 50 μM KMA + 50 μM $CuCl_2$ + 50 μM H_2O_2; **lane 13**, + 50 μM KMB; **lane 14**, + 50 μM KMB + 50 μM H_2O_2; **lane 15**, + 50 μM KMB + 50 μM $CuCl_2$; **lane 16**, + 50 μM KMB + 50 μM $CuCl_2$ + 50 μM H_2O_2; **lane 17**, + 50 μM TM; **lane 18**, + 50 μM TM + 50 μM H_2O_2; **lane 19**, + 50 μM TM + 50 μM $CuCl_2$; **lane 20**, + 50 μM TM + 50 μM $CuCl_2$ + 50 μM H_2O_2; **L**, formamide ladder; **T**, limited hydrolysis by RNase T1.

Each of the studied antibiotics forms a few complexes with Cu(II) ion in the whole pH range, which differ by their charge. At pH 7.4 there is usually a mixture of complexes at different fractions and of various proton content.[19–23] The resultant charge is the sum of the charge and fraction products. Fig. 3 presents the dependence of the cleavage extent at Y37 on the resultant charge of the Cu(II)–antibiotic complexes. This correlation appeared to be linear. A possible explanation of this phenomenon is that the cleavage efficiency is a function of the complexes binding strength in the vicinity of Y37, which rises with the increase of the positive charge of the complexes studied.

Since Cu(II) complexes of aminoglycosides possess specific redox activity[23,32] we performed the cleavage experiments described above also in the presence of H_2O_2. As expected, the addition of H_2O_2 had no impact on the antibiotic reactivity in the absence of metal ion. However, in the presence of Cu(II) ions, several new cleavages were observed. They were mainly located near Y37 in the anticodon loop of $tRNA^{Phe}$ (lanes: 8, 12, 16, 20, Fig. 1). Some weaker cleavages also appeared in the D-stem. In this region, the most noticeable damage site was localized at D17, asbin the case of amikacin.[28] The cleavages caused by the Cu(II)–H_2O_2 system were also numerous but weaker (lane 4, Fig. 1) and they occurred at different sites. Although the strand breakages in the D and anticodon loops occurred to some extent already in the absence of antibiotics or their cupric complexes, no cleavage was observed at Y37. This suggested that guanine hypermodification was essential for aminoglycoside binding and the polynucleotide chain scission

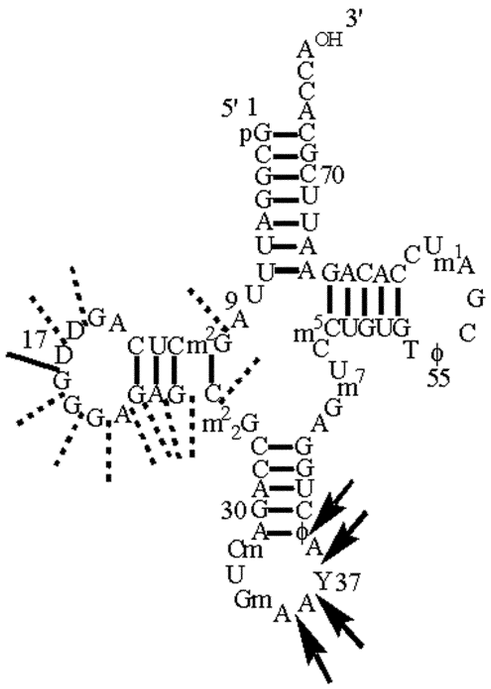

Fig. 2 The secondary structure of the yeast tRNA^{Phe} with cleavage sites induced by aminoglycosides and their Cu(II) complexes marked as follows: arrows for antibiotics and Cu(II)–antibiotics; lines (higher intensity cleavage) and dotted lines (lower intensity cleavage) for Cu(II) aqua ions and Cu(II)–aminoglycoside complexes in the presence of H_2O_2.

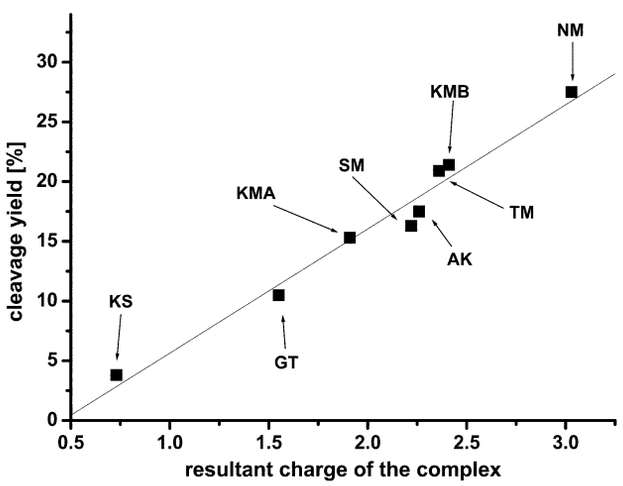

Fig. 3 Dependence of the extent of cleavage at Y37 on the resultant charge of the Cu(II)–antibiotic complexes at pH 7.4.

resulting from this interaction. Furthermore, no cleavage was observed after treatment of tRNA^{Phe} with some other antibiotics such as lincomycin, which belongs to lincosamides or vancomycin, a glycopeptide (data not shown), indicating that this might be a characteristic feature of aminoglycosides.

Interestingly, specific fragmentation of yeast tRNA^{Phe} in the anticodon loop also occurs after the excision of Y37 base at acidic conditions and subsequent breakage of the polynucleotide chain with aniline.[35,36] The reaction requires pH below 3 and incubation for 3 h at room temperature followed by treatment with 0.5 M aniline at pH 4.5 for the next several hours. Although these conditions significantly differ from those used for fragmentation of tRNA^{Phe} with antibiotics, further investigations are necessary to find out whether the exceptional lability of the N-glycosidic bond of the Y base contributes to the mechanism by which antibiotics cleave tRNA^{Phe} in the anticodon loop.

In order to confirm the importance of guanine hypermodification for the strand breakage process, an additional experiment was performed. We compared susceptibility to the cleavage reaction of natural tRNA^{Phe}, its analogue synthesized by *in vitro* transcription (devoid of modified nucleotides) and a DNA oligomer of the tRNA anticodon stem sequence, acDNA. Both tRNA molecules were used to find out whether the cleavage required a specific sequence of nucleic bases only or the presence of natural modifications, particularly the Y base. The acDNA was used to see if the sugar moiety, ribose or 2′-deoxyribose, might have any impact on the cleavage process. Fig. 4 presents the result of the strand break assay performed in the presence of kanamycin A alone, with copper(II) ions and/or H_2O_2. Clearly, the cleavage occurred only with natural tRNA^{Phe} while both unmodified tRNA^{Phe} and acDNA remained unchanged. Thus, the tRNA anticodon stem neither in its RNA or DNA form was able to undergo highly specific breakage. In native tRNA^{Phe} the cleavage occurred at position 37 of the polynucleotide chain where the hypermodified guanine residue was present. Since similar modifications occur in the anticodon loops of several tRNA^{Phe} molecules, including the human one, it is conceivable that also those molecules may undergo specific fragmentation in the presence of aminoglycoside antibiotics or their metal-ion complexes. Fig. 4 shows that only the complex with H_2O_2 affected the structures of both unmodified tRNA and acDNA in a similar manner as native tRNA^{Phe}. As had been previously observed,[28,29] in an oxidative surrounding, metal ion complexes produce multiple cleavages, which probably result from the reactive oxygen species attack on the ribonucleic acid. We have recently provided evidence that these species are hydroxyl radicals, which are by-products of H_2O_2 conversion.[23,32] The breakages they cause within nucleic acid are random and cannot be considered as a characteristic feature of the complexes used. These radical forms however, are crucial in DNA cleavage processes.

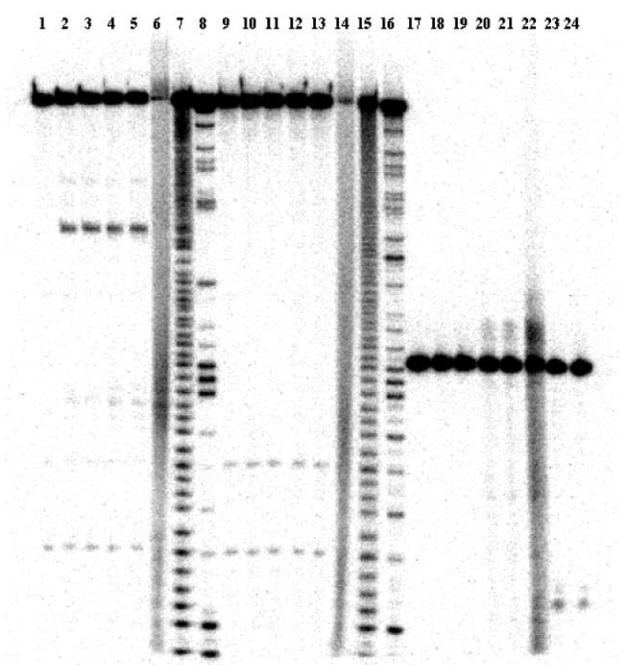

Fig. 4 Specificity of cleavages induced by aminoglycosides and their Cu(II) complexes within yeast tRNA^{Phe} in comparison with unmodified tRNA^{Phe} and the DNA oligomer of anticodon loop sequence. Conditions as on Fig. 1. **Lanes 1–8**, tRNA^{Phe}, lanes 9–17, unmodified tRNA^{Phe}, **lanes 18–24**, DNA with: lanes 1, 9, 17, untreated; **lanes 2, 10, 18**, + 50 μM KMA; **lanes 3, 11, 19**, + 100 μM KMA; **lanes 4, 12, 20**, + 50 μM KMA + 50 μM H_2O_2; **lanes 5, 13, 21**, + 50 μM KMA + 50 μM $CuCl_2$; **lanes 6, 14, 22**, + 50 μM KMA + 50 μM $CuCl_2$ + 50 μM H_2O_2; **lanes 7, 15, 23**, formamide ladder; **lanes 8, 16, 24**, limited hydrolysis by RNase T1.

BluescriptSK$^+$ plasmid damage by Cu(II)–aminoglycoside complexes

DNA differs from RNA by forming a right-handed double helix consisting of two antiparallel strands. RNA is mainly single-stranded with local regions of short complementary base pairing. Considering the interactions of aminoglycosides and their complexes with tRNA we cannot predict their effect on DNA molecules.

Plasmid DNA, which we used in our experiments on aminoglycoside–DNA interactions, differs from the linear one in the shape of its molecule, which forms a closed circular form. This was of great advantage for the monitoring of the interactions described herein using the electrophoretic separation method.

In our previous work, on the oxidative action of copper(II)–amikacin towards oxidation susceptible molecules, we provided evidence that in the presence of hydrogen peroxide the complexes exhibited nicking activity in the interaction with plasmid DNA.[28] Accumulation of the random breakages resulted in double strand scission, which led to the plasmid linearisation. Such action is typical for cupric complexes in oxidative surroundings, but the character of the ligand may greatly influence this process.[37,38] Some ligands may decrease the damaging activity of Cu(II) ions but others, mainly drugs, usually enhance it. In our recent studies, aminoglycosides have been shown to promote Cu(II) oxidation to Cu(III) species.[23,32] These antibiotics, as well as their complexes, are positively charged in physiological pH, which allows them to interact with the negatively charged sugar–phosphate backbone of DNA. As the complexes studied by us had a different resultant charge, we performed a comparative, electrophoretic experiment focused on the damage yield brought by the systems studied.

Fig. 5(A) and (B) present the extent of the pBluescriptSK+ plasmid damage caused by cupric ions alone, antibiotics and their complexes, all in the presence of H_2O_2. As we have reported before, neither the drug nor hydrogen peroxide, nor Cu(II) ions have any impact on the plasmid structure.[28,29] The studied aminoglycosides accompanied by H_2O_2 unwound plasmid DNA, probably by nicking, which led to the formation of an open form (depicted as form II, Fig. 5(A)) but measurable amounts of superhelical form (form I) were still present (lanes: 4, 6, 8, 10). Only traces of linear DNA (form III) were observed in the absence of metal ions. A different phenomenon could be observed when the reaction mixtures additionally contained Cu(II) ions. These ions, together with H_2O_2, produced single strand breakages whose accumulation led to linearisation, but only in small percentage (lane 3, Fig. 5(A) and (B)). Enhancement of the destructive effects was observed on lines 5, 7, 9 and 11, illustrating plasmid damage after the addition of the complex. It is of note that copper(II)–aminoglycoside complexes in the absence of H_2O_2 or ascorbate do not cleave DNA in an oxidative manner.[28–30] Rather, they induce single strand nicks converting superhelical plasmid to its opened form but without further linearisation and degradation to short fragments. This mechanism has been recognized as hydrolytic and not leading to the linear form III.[30] We have previously postulated that the damage mechanism is dependent on the generation of reactive oxygen species.[23,32] The yields of the plasmid decomposition processes, as was the case of RNA cleavage (see Fig. 2), seem to depend on the resultant charge of the complexes at physiological pH. Unlike DNA destruction, the RNA hydrolysis occurred already in the absence of H_2O_2, so both reactions proceeded in different conditions. Nevertheless, the impact of the charge value seems also to play an important role in this process. In the case of the cupric complex of kasugamycin, which carries the lowest positive charge, some superhelical DNA remained unaffected (lane 7, Fig. 5(B)) and only insignificantly less than in the case of the Cu(II)–H_2O_2 system. The majority of plasmid molecules exist in nicked form. The number of random, single strand breakages is insufficient to convert this open form to the linear one so the latter form occurs here only occasionally to a small extent. Its amount is better measurable when the system contains any other antibiotic and prevails over the nicked plasmid DNA in the presence of highly charged complexes of sisomicin (lane 9, Fig. 5(B)) or tobramycin (lane 11, Fig. 5(A)). Cu(II)–kanamycin B (lane 9, Fig. 5(A)), being more positively charged than the complexes mentioned, converts both superhelical and open forms to the linear one, whose contribution is relatively high. The most efficient degradation within a plasmid molecule is observed when the reaction mixture contains Cu(II)–neomycin B in the presence of H_2O_2. The resultant positive charge of this complex is the highest of all the complexes studied and such species not only decompose the superhelical and open forms but also decrease the amount of the linear plasmid (lane 11, Fig. 5(B)), which results from the enhanced activity of this particular complex towards DNA degradation. Similar observations have been made in the case of Cu(II)–bleomycin interaction with plasmid DNA, where the bleomycin derivative, lacking a positively charged group, has appeared to be a less effective DNA unwinding agent.[39]

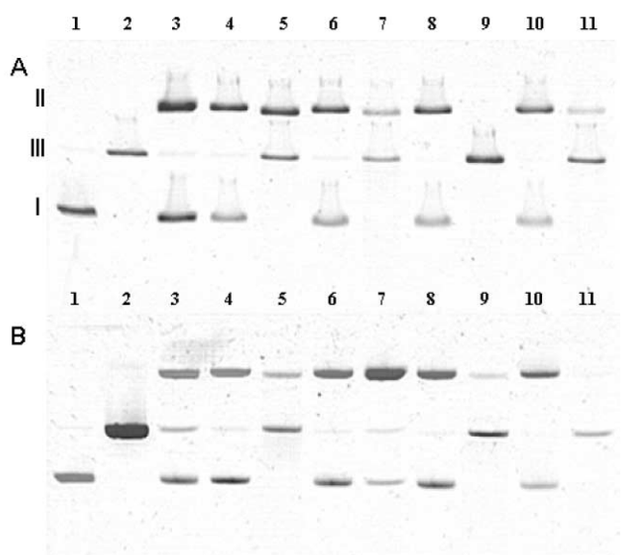

Fig. 5 Agarose gel electrophoresis of pBluescriptSK+ plasmid cleavage by Cu(II)–aminoglycoside complexes in the presence of H_2O_2. The samples, incubated for 1 h at 37 °C, were run on a 1% agarose gel, containing ethidium bromide for 1 h at 4 V cm^{-1} in TBE buffer. (A) **Lane 1**, untreated plasmid; **lane 2**, plasmid linearized with *Eco*RI endonuclease; **lane 3**, plasmid + 50 μM $CuCl_2$; **lane 4**, + 50 μM AK; **lane 5**, + 50 μM AK + 50 μM $CuCl_2$; **lane 6**, + 50 μM KMA; **lane 7**, + 50 μM KMA + 50 μM $CuCl_2$; **lane 8**, + 50 μM KMB; **lane 9**, + 50 μM KMB + 50 μM $CuCl_2$; **lane 10**, + 50 μM TM; **lane 11**, + 50 μM TM + 50 μM $CuCl_2$, all with H_2O_2. (B) **Lanes 1–3**, the same as above; **lane 4**, plasmid + 50 μM GT; **lane 5**, + 50 μM GT + 50 μM $CuCl_2$; **lane 6**, + 50 μM KS; **lane 7**, + 50 μM KS + 50 μM $CuCl_2$; **lane 8**, + 50 μM SM; **lane 9**, + 50 μM SM + 50 μM $CuCl_2$; **lane 10**, + 50 μM NM; **lane 11**, + 50 μM NM + 50 μM $CuCl_2$, all with H_2O_2.

The supporting spectroscopic experiment, focused on reactive oxygen species formation, confirmed the influence of the reagent's charge on its oxidative activity. Fig. 6 presents the curves of ROS formation for the cupric complexes of the studied antibiotics. In our recent studies we have characterized these forms as hydroxyl radicals.[23,32] Only Cu(II)–kasugamycin failed to produce $^{\bullet}OH$ species, yielding relatively high amounts of superoxide radicals. The reason for the different behavior of Cu(II)–kasugamycin complexes, despite a similar set of donors, is probably the formation of mutually perpendicular nitrogen-containing chelate rings.[40] In the presence of both Cu(II) ions and H_2O_2 the remainder of the antibiotics exhibited high efficacy towards NDMA consumption with respect to the charge of the complex at pH 7.4. The mechanism of interaction between NDMA and copper-bound radicals may be quite complex. On the basis of cyclic voltammetry, gasometry and

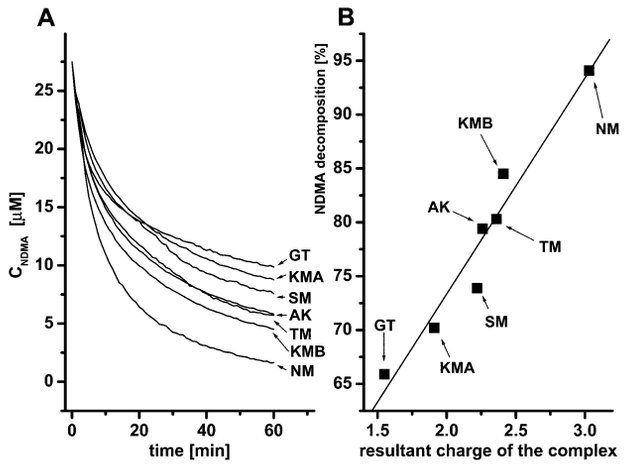

Fig. 6 (A) The experimental curves of kinetic measurements of NDMA bleaching by Cu(II)–aminoglycoside–H_2O_2 systems at 37 °C. The reaction mixtures contained NDMA, 25 μM; antibiotic, 50 μM; $CuCl_2$, 50 μM and H_2O_2, 0.5 mM, in 50 mM sodium phosphate buffer. (B) Correlation of NDMA decomposition yield with the resultant charge of the complex at pH 7.4.

ROS generation measurements we have recently proposed an oxidative mechanism suitable for the above interactions.[23,32] The processes yielding hydroxyl radicals described therein proceed *via* a Cu(II)/Cu(III) redox pair. Oxidation of cupric ions to their high-valence species, which is needed for $^{\bullet}OH$ production, happens more easily at pH close to neutral, in which highly protonated species occur. The same complex form, in which a central metal ion undergoes oxidation, is responsible for effective ROS generation.[23] Moreover, on the basis of our current study we postulate that the protonated amino functions of the complexes may act as anchors in the binding of the complex to DNA. The strength of that interaction increases with the number of protonated amino functions. The copper(II)-bound hydroxyl radicals may then get sufficiently close to the sugar–phosphate backbone of the double helix to cause strand scission.

Conclusions

The results of our experiments may contribute to the understanding of yet unknown mechanisms of aminoglycoside induced toxic effects, since the redox active metal ions and ROS are believed to participate in these effects. It was shown that under specific conditions, in which neutrophils and other cells are activated, reactive oxygen intermediates and micromolar quantities of redox active metal ions, including copper(II), could be released.[41] Cupric ions were also found in nuclei in association with chromosomal DNA.[42] Inside the cell, Cu(II) species may cause many non-specific toxic effects such as base oxidation and cleavage of phosphodiester linkages, which may result in *e.g.* carcinogenic processes.[43] It has also been recently shown, that such reaction proceeds *via* Cu(II) oxidation to Cu(III) species, and it is accompanied by the release of radical intermediates.[44] Considering these facts in light of our results, we suggest that Cu(II)–aminoglycoside complexes are potentially dangerous genotoxic agents. These complexes may induce specific cleavage in some RNA molecules, which carry hypermodified bases. The presence of such modification appears to enhance their binding and subsequent hydrolysis of the RNA molecule. Endogenous hydrogen peroxide however, is needed for the complex-induced oxidative DNA damage. The radical intermediates generated in this process seem to be responsible for strand scissions within plasmid DNA. Both RNA and DNA damage, despite distinct chemical basis, proved in our experiments to be charge dependent. The resultant charge of the complexes studied was the primary feature affecting their interactions with the negatively charged sugar–phosphate backbone as well as their oxidative reactivity.

Abbreviations

AK	amikacin
GT	geneticin
KMA	kanamycin A
KMB	kanamycin B
KS	kasugamycin
NM	neomycin
SM	sisomicin
TM	tobramycin
D	dihydrouridine
Y	wybutosine
NDMA	*N*,*N*-dimethyl-*p*-nitrosoaniline
ROS	reactive oxygen species

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