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tRNA^{Phe} cleavage by aminoglycosides is triggered off by formation of an abasic site

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Abstract

This communication reports the characteristics of the mechanism of highly specific tRNA^{Phe} cleavage, which occurs in the anticodon loop in the presence of aminoglycoside antibiotic—neomycin B. The data prove that the cleavage requires previous depurination of the polynucleotide chain at position 37, which is occupied by a hypermodified guanine base—wybutine. The results suggest that the phenomenon, previously considered as selective with respect to the presence of tRNA hypermodification, may concern far more RNA molecules, namely the ones carrying abasic sites.

Keywords: Depurination; Abasic site; RNA cleavage; tRNA^{Phe}; Aminoglycoside antibiotics; Neomycin B

The three-dimensional structure of yeast phenylalanine transfer RNA is well established [1] and thus this molecule has often been employed in the studies of interactions of xenobiotics or metal-based compounds with RNA. Interactions of divalent metal cations have been proved to be essential for stabilization of tRNA^{Phe} tertiary structure [2]. In particular, charge neutralization by a polyvalent cation (i.e., Mn(II) or $[Co(NH_3)_6]^{3+}$) promotes the formation of U-turn fold of tRNA^{Phe} [3]. On the other hand, some metal ions, for example, Mg(II), Pb(II), Eu(II) or Mn(II), exhibit high cleavage efficacy towards the D-loop of this molecule [4,5].

The tRNA^{Phe} has also been proven as a target for aminoglycoside antibiotics [6]. In our recent studies, we have obtained the data concerning its specific cleavage induced by several aminoglycosides as well as their copper(II) complexes [7–9]. This process occurred specifically at a hypermodified nucleoside present in the anticodon loop, at Y37, and was claimed to be dependent on its presence [9]. However, specific pretreatment of tRNA^{Phe} may lead to the Y-base (wybutine) excision [10,11]. The removal of wybutine is responsible for the conformational changes, which take place in the tRNA structure [11,12]. The newly formed abasic site may also favor interactions with antibiotics or other agents, what was not taken into account in the previous studies on aminoglycoside-induced tRNA cleavage [6–9].

Depurination is a common DNA lesion that arises spontaneously or by repair of oxidatively modified bases in order to avoid promutagenic alterations. The amount of abasic sites increases with age and is strongly dependent on base excision repair activity [13]. The role of metal ions and their complexes in the initiation of depurination processes is well substantiated. Among several DNA cleavage products, free bases were present in the case of iron, copper or ruthenium complexes, as well as antibiotics like neocarzinostatin or bleomycin [14,15]. Action of the latter was subjected to in-depth

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analysis and reviewed, drawing attention also to depurination mechanisms [16,17]. Formation of the abasic sites has been recognized as a source of errors in DNA synthesis [18], as a trigger for strand scission under alkaline conditions or in the presence of lyases [19] and even was implied in mechanisms of spontaneous human cancers [20]. Apurinic/apyrimidinic sites in DNA are primarily important targets for apurinic/apyrimidinic endonucleases (AP-endonucleases) [21].

Contrary to DNA, RNA has rarely been examined with respect to abasic site formation within its molecules. Some ribosome-inactivating proteins show activity towards depurination of yeast rRNA [22] or synthetic oligoribonucleotides [23]. Similar behavior has been observed during the studies of HIV RNA, yeast ribosomal RNA or luciferase mRNA depurination by selected antiviral proteins [24–26]. Another protein, ricin, exhibits cytotoxic activity by interaction with the ribosome to catalyze depurination reaction [27]. $tRNA^{Phe}$ as well as its chemically modified derivatives or $tRNA^{Phe}$ mutants have also been the objects of investigations in this field. Their structure–function mapping by rhodium complex reveals that specific chemical modification of $tRNA^{Phe}$ promotes selective depurination [28].

A multitude of stimuli that are able to trigger off nucleic base loss make this issue significant and worth considering, especially from a pharmacological point of view, since an abasic site may attract antibiotics to induce polynucleotide strand breakage.

Materials and methods

Procedure for Y-base removal from $tRNA^{Phe}$. Yeast $tRNA^{Phe}$ (Sigma Chemical) was labeled at its 5′ end using [γ-^{32}P]ATP and polynucleotide kinase in standard conditions and purified by polyacrylamide gel electrophoresis. Subsequently, the ^{32}P-labeled RNA (2.5×10^6 c.p.m.) was supplemented with tRNA carrier to a final RNA concentration of 4.5 A_{260} U/ml, incubated in 50 mM ammonium formate, pH 2.85, for 4 h at 37 °C, and precipitated with ethanol. The pellet was dissolved in buffer A: 0.3 M NaCl, 10 mM $MgCl_2$, and 10 mM sodium acetate, pH 4.5. The RNA was loaded onto a small column with BD-cellulose, which had been equilibrated with 10 column volumes of buffer A. $tRNA^{Phe}(-Y)$ was eluted from the column with buffer B: 1.0 M NaCl, 10 mM $MgCl_2$, and 10 mM sodium acetate, pH 4.5 [10–12]. Finally, $tRNA^{Phe}(-Y)$ was ethanol precipitated in the presence of 1 μl of glycogen (10 mg/ml) and dissolved in sterile water.

Analysis of $tRNA^{Phe}(-Y)$ cleavage reactions. Cleavage reactions of $tRNA^{Phe}(-Y)$ (ca. 60,000 c.p.m., 0.1 μM RNA in 40 μl reaction volume) induced by neomycin B (Sigma Chemical) and its copper(II) complexes (0.1–100 μM) were performed in 50 mM sodium phosphate or Tris buffers at pH 7.4, at 37 °C. The reactions were terminated by mixing their aliquots with equal volumes of 8 M urea/dyes/20 mM EDTA solution and freezing the samples on dry ice. Subsequently, the cleavage products were analyzed on 15% polyacrylamide, 7 M urea gels. Electrophoresis was performed at 1500 V for 3 h, followed by autoradiography at −80 °C with an intensifying screen. For quantitative analysis the 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 ^{32}P-labeled $tRNA^{Phe}$. The alkaline hydrolysis ladder was generated by incubation of $tRNA^{Phe}$ with 5 volumes of formamide/2 mM $MgCl_2$ in boiling water for 10 min. Partial T_1 nuclease digestion was performed in denaturing conditions (50 mM sodium citrate, pH 4.5, 7 M urea) with 0.1 U of the enzyme. The reaction mixture was incubated for 10 min at 55 °C.

To determine the cleavage rate reaction aliquots were taken at specified time points, quantified after electrophoretic separation, and subsequently the logarithm of the percent RNA remaining was plotted as a function of time. The negative slope of the least-squares plot yielded the cleavage rate.

Results and discussion

It has been observed that several aminoglycoside antibiotics induce efficient cleavage of yeast $tRNA^{Phe}$ in the anticodon loop [6–9]. This process seemed to depend on the presence of a hypermodified guanine residue, called wybutine, in position 37 of the tRNA polynucleotide chain. Such conclusion has also been supported by the results of our recent studies, in which we compared susceptibility to aminoglycoside attack the native $tRNA^{Phe}$ and its unmodified in vitro transcript [9]. Exceptional lability of the N-glycosidic bond of the hypermodified Y nucleoside was not, however, taken into account in the previous studies. Hence, we have hypothesized that the cleavage at position 37 may occur not as triggered off by wybutine, but in the aftermath of its excision. In order to verify this assumption we exposed the $tRNA^{Phe}$ to conditions of wybutine removal from the anticodon loop [10,11], and subsequently, the $tRNA^{Phe}(-Y)$ was examined for its susceptibility to degradation induced by neomycin B (Fig. 1). This antibiotic

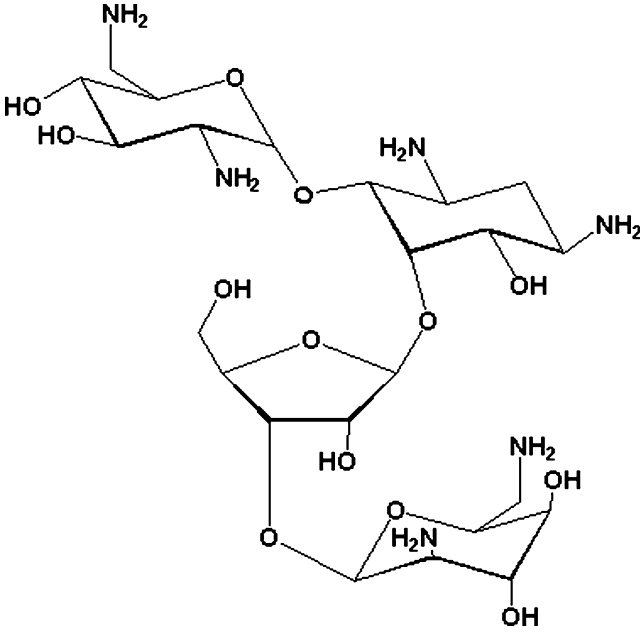

Fig. 1. The molecule of neomycin B in its fully deprotonated form.

has previously shown the highest efficacy in promoting tRNA cleavage [9] and thus was chosen for further detailed studies.

The direct comparison of neomycin-induced cleavage of $tRNA^{Phe}(-Y)$ and $tRNA^{Phe}$ revealed that only the $tRNA^{Phe}(-Y)$ was susceptible to the scission in the presence of this aminoglycoside (Fig. 2). Fig. 3 shows the time-dependent changes in distribution of $tRNA^{Phe}(-Y)$ cleavage products with neomycin B at concentration: 0.1 μM (lanes 4–10), 1 μM (lanes 11–17), and 10 μM (lanes 18–24). The kinetic parameters of this process are included in Table 1. Clearly, the cleavage takes place much more effectively at higher antibiotic concentrations. In the control experiment (lanes 1–3), slow breakage of the abasic linkage also occurs with the rate constant, which amounts to $16 \times 10^{-6}\ min^{-1}$. For the spontaneous break-up of a phosphodiester bond, the rate constant of ca. $10^{-7}\ min^{-1}$ has been found [29]. Interestingly, after better separation of $tRNA^{Phe}(-Y)$ cleavage products, with neomycin B at concentration 0.1, 1, and 10 μM for 24 h, doublet bands are observed on the gel (Fig. 4, lanes 2–4). The upper band is seen already after 10 min of incubation, while the lower one appears not earlier than after 2–4 h (Fig. 3). Most likely, these are the products of ribose moiety break-up at the position of RNA cleavage. It seems that the decay of ribose residue occurs in the long-term aftermath of the cleavage at the abasic site. Similarly, the presence of heterogeneous products has been reported as a result of chemical modification of RNA, when the phosphodiester bond was cleaved by aniline or piperidine following the base excision [30].

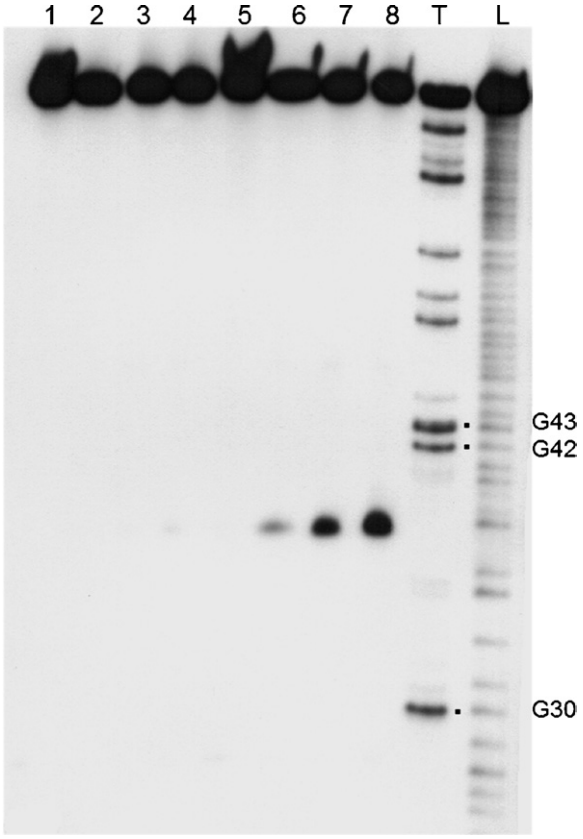

Fig. 2. Autoradiogram of a polyacrylamide gel for the comparison of $tRNA^{Phe}$ (lanes 1–4) and $tRNA^{Phe}(-Y)$ (lanes 5–8) susceptibility to the cleavage by neomycin B (10 μM) following incubation at: 0, 5, 15, and 45 min, respectively. T, T_1 ladder; L, formamide ladder.

Table 1
The rate constants for the $tRNA^{Phe}(-Y)$ cleavage in the presence of neomycin B both free antibiotic and in complex with Cu(II), as well as the rate enhancements with respect to the control reaction

Neomycin B concentration [μM]	Rate constants $[min^{-1} \times 10^{-3}]$	Rate enhancement
0 (control)	0.016	—
0.1	0.08	5
1	2.23	139
10	7.2	450
10 + Cu(II)	6.8	425
100	10.1	631
100 + Cu(II)	8.9	556

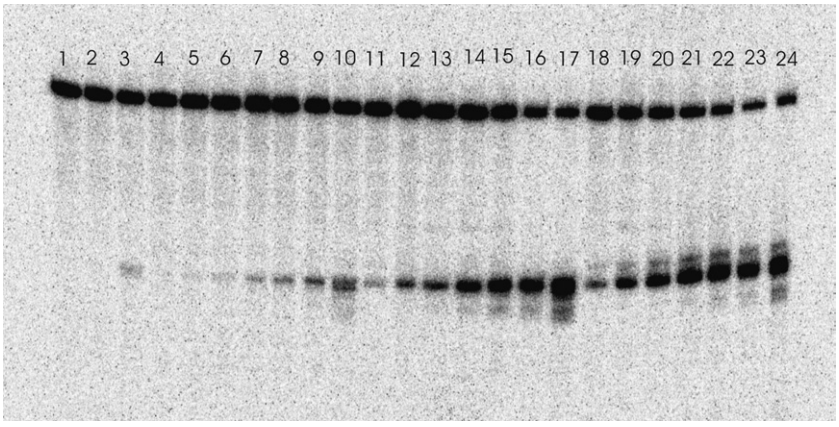

Fig. 3. Autoradiogram of a polyacrylamide gel for the cleavage of $tRNA^{Phe}(-Y)$ by neomycin B. Lanes 1–3, control samples (without neomycin B) incubated for 30 min, 8 and 24 h, respectively; lanes 4–10, $tRNA^{Phe}(-Y)$ + 0.1 μM neomycin B; lanes 11–17, +1 μM neomycin; and lanes 18–24, +10 μM neomycin, incubated for 0, 10, 30, and 60 min, 4, 8, and 24 h, respectively, for each concentration.

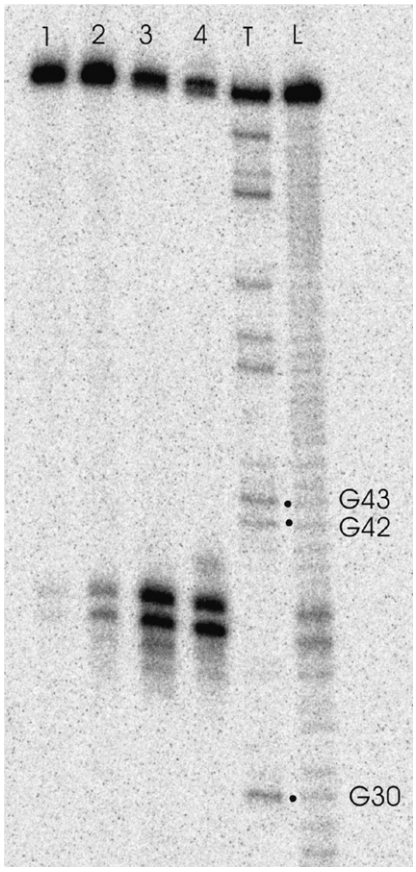

Fig. 4. Autoradiogram of a polyacrylamide gel for the $tRNA^{Phe}(-Y)$ cleavage by neomycin B following 24 h incubation. The long run of the gel is shown. Lane 1, control; lanes 2–4, $tRNA^{Phe}(-Y)$ + antibiotic at conc. 0.1, 1, and 10 μM, respectively; T, T_1 ladder; and L, formamide ladder.

The $tRNA^{Phe}(-Y)$ was also subjected to the action of neomycin B complex with Cu(II) ion. Copper(II) revealed to exert negligible influence on the kinetics of cleavage. The rate constants seem not to differ for the antibiotic and its cupric complex at concentration of 10 μM (Table 1). Nevertheless, at 100 μM concentration, slight but measurable drop in the strand breakage rate in the case of the complex with respect to the free antibiotic is noticed. It is also well reflected in decrease of the rate enhancements (Table 1). Metal ion, coordinating to amino functions of the drug, may thus lower its affinity to bind RNA, what has been observed earlier [9].

The previous studies of aminoglycosides and their Cu(II) complexes were performed in phosphate buffers [7–9]. The reason for that choice was very low values of the stability constants of copper(II) complexes with phosphates. Herein we tested also the Tris buffer, however, no differences were noticed between the data obtained in these both buffers (data not shown). Thus, different buffering salts do not seem to influence the tRNA cleavage.

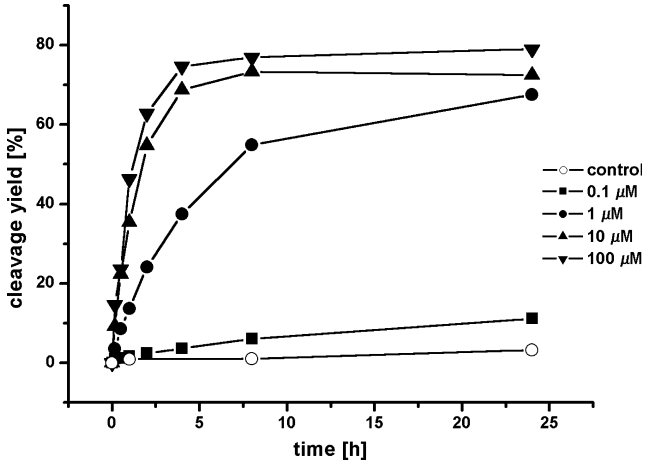

Fig. 5. The kinetics of $tRNA^{Phe}(-Y)$ cleavage induced by neomycin B at concentrations from 0.1 to 100 μM.

Fig. 5 presents the courses of time-dependent $tRNA^{Phe}(-Y)$ cleavage at the abasic site for variable antibiotic concentration. The most striking observation is that neomycin is active at concentration as low as 0.1 μM. The rate constant for this process is low (see Table 1), however, significant. It suggests that randomly depurinated sites within RNA may become a target for even vestigial antibiotic concentration. The reaction rate substantially increases with antibiotic concentration from 0.1 to 1 μM. The latter value is particularly important as it fits in the range of physiological concentrations, which may be reached, i.e., upon the drug injection. Despite distinct kinetics, the final cleavage yields for neomycin B at concentrations 1, 10, and 100 μM are almost equal after 24 h of incubation. The rate constant values are, however, more differentiated (Table 1). The rate enhancement in the presence of 0.1 μM antibiotic is only fivefold with respect to the control reaction, but already in the case of 10 μM antibiotic the reaction is accelerated by a factor of ca. 500. Since the cleavage of $tRNA^{Phe}(-Y)$ at the abasic site is 160-fold faster than the spontaneous breakage of a phosphodiester bond (Table 1 and [29]), in the presence of 10 μM antibiotic the summarized acceleration of cleavage at the abasic site reaches five orders of magnitude.

The precise mechanism of neomycin-induced $tRNA^{Phe}(-Y)$ cleavage at the abasic site is not known. In the crystal structure of $tRNA^{Phe}$–neomycin complex, the antibiotic molecule is positioned in the deep groove below the D-loop [31], in a far distance from the anticodon loop. Thus, such bound neomycin could not be directly involved in the cleavage of $tRNA^{Phe}(-Y)$, and, most likely, antibiotic molecules acting from solution induce the reaction. Strong dependence of the cleavage rate on neomycin concentration seems to support this conclusion although the detailed explanation of the cleavage mechanism requires further experimentation.

Conclusion

The data described in this communication on neomycin-induced cleavage of $tRNA^{Phe}(-Y)$ suggest that aminoglycosides may be regarded as agents of potential destructive effects on RNA. Accidental depurination of RNA might be followed by subsequent strand cleavage at the abasic sites, which is strongly enhanced in the presence of aminoglycosides. Since this phenomenon occurs at very low concentrations of antibiotics, it might be of biological significance. Although these conclusions are drawn from the observations on $tRNA^{Phe}$ with a single abasic site and the studies need to be broadened, they constitute substantially new facts in aminoglycosides–RNA interactions.

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