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Antibiotic bacitracin induces hydrolytic degradation of nucleic acids ☆

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ABSTRACT

Background: Bacitracin is a polypeptide antibiotic active against Gram-positive bacterial strains. Its mechanism of action postulates disturbing the cell wall synthesis by inhibiting dephosphorylation of the lipid carrier. We have discovered that bacitracin induces degradation of nucleic acids, being particularly active against RNA.
Methods: In the examination of the nucleolytic activity of bacitracin several model RNA and DNA oligomers were used. The oligomers were labeled at their 5′ ends with ^{32}P radioisotope and following treatment with bacitracin the cleavage sites and efficiency were determined.
Results and conclusions: Bacitracin induces degradation of RNA at guanosine residues, preferentially in single-stranded RNA regions. Bacitracin is also able to degrade DNA to some extent but comparable effects to those observed with RNA require its 10-fold higher concentration. The sites of degradation in DNA are very infrequent and preferentially occur near cytidine residues. Free radicals are not involved in the reaction, and which probably proceeds *via* a hydrolytic mechanism. The phosphate groups at the cleavage sites are present at the 3' ends of RNA products and at the 5' ends of DNA fragments. Importantly, the presence of EDTA does not influence RNA degradation but completely inhibits the degradation of DNA. For DNA degradation divalent metal ions like Mg^{2+}, Mn^{2+} or Zn^{2+} are absolutely necessary.
General significance: The ability of bacitracin to degrade nucleic acids *via* a hydrolytic mechanism was a surprising observation, and it is of interest whether these properties can contribute to its mechanisms of action during antibiotic treatment.

1. Introduction

Bacitracin is a polypeptide antibiotic active against Gram-positive bacterial strains. It is produced as a mixture of closely related compounds by *Bacillus subtilis* var *Tracy*. Since its discovery in the 1940s, bacitracin has been extensively applied against numerous bacterial infections [1,2]. It is used only intramuscularly and topically because its absorption from the alimentary canal is very poor. The intramuscular application is limited to infants only due to high nephrotoxicity of bacitracin [3] while the topical ointment is very effective, especially when the antibiotic is applied as a zinc salt accompanied by neomycin and polymyxin B [4]. Oral administration is applied only if gastrointestinal sterilization is required [5]. The drug is also used in ophthalmic medicine as a solution and in veterinary medicine as food supplement preventing breeding animals from infections. Bacitracin is advisable to be used, like other antibiotics, only against bacterial infections, especially the methicillin-resistant *Staphylococcus aureus* (MRSA) [6]. Its mechanism of action postulates disturbing the cell wall synthesis by inhibiting dephosphorylation of the lipid carrier [2].

The involvement of bacitracin in viral infections therapy has been discussed in literature. The recent work of Lara et al. [7] shows that inhibition of HIV infectivity by bacitracin can arise as the drug can inhibit the protein disulfide isomerase (PDI). Besides, it can also be considered an antiviral agent against T-tropic HIV-1 infection making such viruses like HIV or RSV exhibit limited ability towards disulfide bond reduction in their capsule [7]. This obviously would reduce their capability of penetrating the cell membrane, however, no direct evidence for the antiviral activity of bacitracin is provided. Inhibition of the PDI interferes only with the life cycle of the virus. Recently, however, the specificity of PDI inhibitory abilities of the antibiotic has been questioned [8]. In the studies on herpes simplex virus (HSV) bacitracin has been proven to be inactive when used alone [9], however, when accompanied by

Abbreviations: PDI, protein disulfide isomerase; HIV, human immunodeficiency virus; RSV, respiratory syncytial virus; HDV, hepatitis delta virus; tRNA, transfer RNA; RNase, RNA specific nuclease; DNase, DNA specific nuclease

☆ Summary statement: A polypeptide antibiotic bacitracin induces degradation of nucleic acids, being particularly active against RNA. Free radicals are not involved in the reaction, and which probably proceeds *via* a hydrolytic mechanism.

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antiviral peptides it acts as a protease inhibitor enhancing the activity of the peptides [10,11].

It has recently been reported that bacitracin may induce degradation of double-stranded DNA [12]. The described process results from the binding of transition metal ions and its mechanism includes generation of free radicals. Similar nucleic acid scissions with oxidative background were observed for a number of other antibiotics belonging to the aminoglycoside group [13–16] or actinomycin D [17]. Metal ions coordinated within these antibiotics sometimes play a crucial role in maintaining their proper structure or function [18]. Moreover, in the case of bacitracin it has been suggested that divalent metal ions may also act as a bridge between the antibiotic and C_{55}-isoprenyl pyrophosphate, disabling dephosphorylation of the latter and thus inhibiting cell wall synthesis [19].

Recently, we have discovered that bacitracin induces degradation of nucleic acids in the absence of metal ions capable of generating free radical species [20]. Such an ability of bacitracin was a surprising observation because a number of antibiotics derived from different therapeutic groups tested in our laboratory did not show similar capabilities. Based on this observation, we performed studies on the nucleolytic properties of bacitracin, whose goal was to obtain answers to the following questions: (i) What is the specificity of bacitracin towards various types of phosphodiester bonds, i.e. whether it is active against RNA or DNA? (ii) Does the degradation occur preferentially at particular nucleotides or sequence stretches, and is the reaction affected by the secondary structure folding of the substrates? (iii) *Via* which mechanism does the degradation of nucleic acids proceed?

2. Results and discussion

2.1. Bacitracin induces degradation of RNA molecules; it also induces degradation of DNA molecules, but with a much lower efficiency

In the examination of the nucleolytic activity of bacitracin three model RNA oligomers were used: 76 nt yeast phenylalanine tRNA (tRNA^{Phe}-RNA), 72 nt antigenomic HDV ribozyme (ribHDV-RNA), and 20 nt oligoribonucleotide (R20-RNA) (Fig. 1). The first two RNAs are similar in length but they differ in the primary sequence and secondary structure folding. Moreover, these RNAs have well-characterized tertiary structures which have been determined by crystallography. These molecules have been used as model RNAs in several studies, also those on specific properties of RNA degraders. The third substrate, R20-RNA, is a single-stranded RNA unable to form any higher-order structure. Thus, the chosen RNAs had various primary sequences and contained selected higher-order structural motifs that occur in large RNA molecules.

The impact of bacitracin on DNA was studied applying 39 nt (M39-DNA) and 72 nt (M72-DNA) DNA oligomers (Fig. 1). The DNAs differ in their primary sequences and moreover, M72-DNA might potentially fold into a defined secondary structure since this oligomer is complementary to a fragment of the HDV ribozyme sequence. The model DNA as well as RNA substrates was relatively short allowing precise analysis of the specificity of bacitracin-induced reactions and the nature of products arising after the cleavage of phosphodiester bonds.

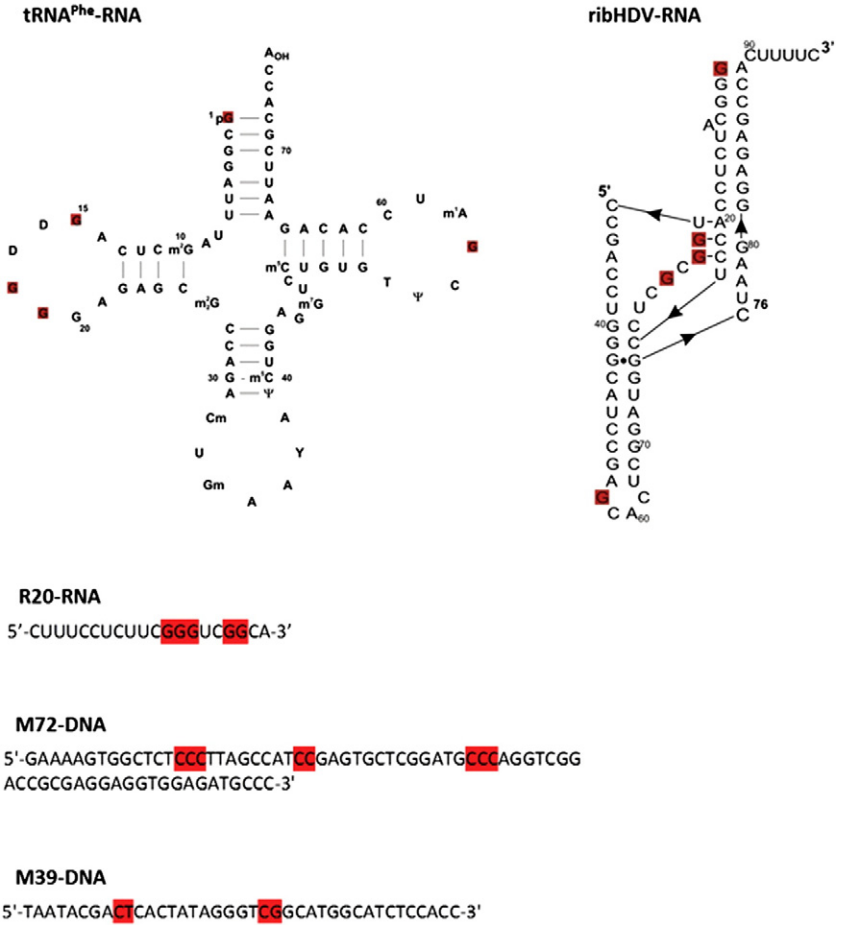

Fig. 1. Oligonucleotide substrates used in the evaluation of the nucleolytic properties of bacitracin. tRNA^{Phe}-RNA, secondary structure of phenylalanine-specific tRNA^{Phe} isolated from baker's yeast; rybHDV-RNA, secondary structure of antigenomic HDV ribozyme; R20-RNA, 20-mer oligoribonucleotide; M72-DNA 72-mer DNA oligomer; M39-DNA 39-mer DNA oligomer. The major sites of degradation in the presence of bacitracin are marked.

Following incubation of tRNA^{Phe}-RNA with 5 μM bacitracin for 60 min at 37 °C approximately 40% of the starting amount of RNA was degraded (Fig. 2A,D). At a higher concentration of the antibiotic, the degradation level increased and at 100 μM it achieved 95%. The degradation of ribHDV-RNA was more efficient than that of tRNA^{Phe}-RNA (Fig. 2B,E). Already in the presence of 5 and 10 μM bacitracin it reached 60 and 80%, respectively, while at 50 μM bacitracin almost complete disappearance of the RNA substrate was observed. Under the same time and temperature conditions, unstructured R20-RNA was almost completely degraded into shorter products in the presence of 20 μM bacitracin (Fig. 2C,F). Thus, independently of the different lengths of these three RNA molecules it turned out that in the presence of 20 μM bacitracin after 60 min at 37 °C the degradation level reached 80–95% of the starting amount of RNA. The kinetics of the degradation of tRNA^{Phe}-RNA and R20-RNA in the presence of 22 μM bacitracin was also examined (Fig. 3). For both RNAs about 50% degradation was observed already after 1 min of incubation. After 20 min the fraction cleaved reached a *plateau* at more than 90% for both tRNA^{Phe}-RNA and R20-RNA. The calculated rates of substrate disappearance k_{obs} were $0.80 \pm 0.10\ min^{-1}$ for tRNA^{Phe}-RNA and $1.01 \pm 0.16\ min^{-1}$ for R20-RNA.

Bacitracin was also able to degrade single-stranded DNA to some extent (Fig. 4). However, the degradation of M39-DNA and M72-DNA required significantly higher concentrations of the antibiotic than in the case of RNA molecules. An effect comparable to that observed for RNA with 20 μM bacitracin was achieved for DNA with 250 μM antibiotic, which indicates that in order to degrade DNA, the concentration of bacitracin has to be at least 10-fold higher.

We have been aware that commercially available bacitracin is isolated from bacterial cultures and preparations are composed of a series of derivatives of a similar structure and with similar – but not identical – characteristics. It has also been suggested that sometimes the preparations may be contaminated with proteases [21]. In most experiments we examined the properties of bacitracin, which was purchased from Sigma-Aldrich Co. Therefore, we decided to test whether the formulation of bacitracin derived from another source, Calbiochem, exhibits similar nucleolytic properties. A comparison of the degradation patterns of two oligonucleotides, R20-RNA and M39-DNA, in the presence of two different preparations of bacitracin showed that these preparations have nearly identical ability to degrade RNA and DNA molecules (Fig. 5). Moreover, mass spectra using the Autoflex MALDI-ToF spectrometer confirmed that those samples did not contain detectable amounts of compounds with a molecular mass much higher than that of bacitracin which would suggest their contamination with proteins (Supplementary data, Figs. S1, S2).

2.2. Degradation of RNA occurs preferentially at guanosine residues in single-stranded stretches while DNA is cleaved at certain cytosine nucleotides

Cleavages induced by bacitracin occurred in single-stranded RNA regions, for tRNA^{Phe}-RNA primarily at G15, G18, G19 and at G57 in the D and TΨC loops, for ribHDV-RNA at G28, G30, G31 and at G58 in the looped regions L3/P3 and L4, and for the single-stranded R20-RNA at G12, G13, G14, G17 and G18 (Figs. 1, 2). It is interesting that a similar specificity of RNA degradation is exhibited by T1 ribonuclease, an enzyme which has a molecular mass ca. 10-times larger [22–25]. Furthermore, it was noted that bacitracin removes the phosphate group from the 5′ end of RNA molecules, or, alternatively, from the 5′-terminal nucleotide that had been earlier cut off. This effect was observed both for tRNA^{Phe}-RNA and ribHDV-RNA but not in the case of R20-RNA (Fig. 2). In the first two RNAs the 5′-terminal residue is guanosine while cytidine is present at the 5′ end of the third molecule.

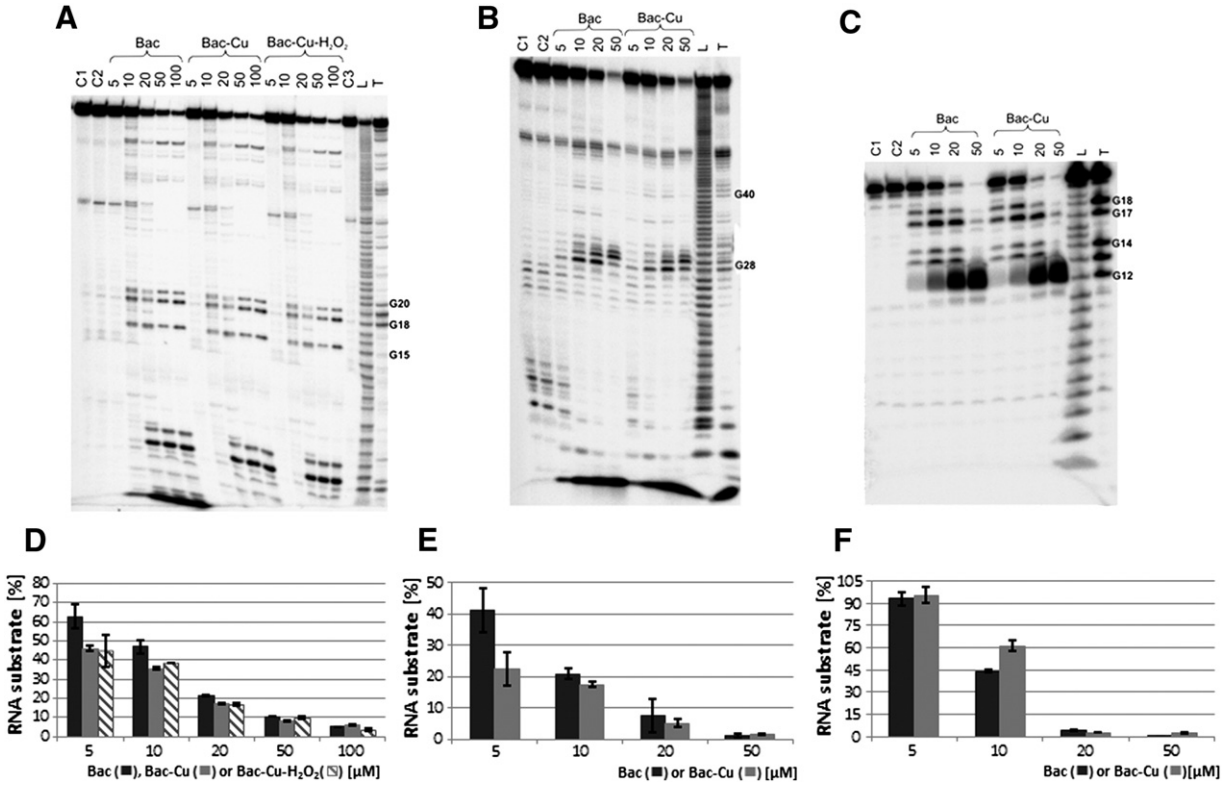

Fig. 2. Nucleolytic degradation of RNA substrates in the presence of bacitracin (Bac), its complex with Cu^{2+} ions (A, B and C), as well as Cu^{2+}-complexes in the presence of H_2O_2: (A) — tRNA^{Phe}-RNA, (B) — ribHDV-RNA, and (C) — R20-RNA. Concentrations of bacitracin and its complex are expressed in μM. Reaction conditions: 50 mM Tris–HCl pH 7.5, 37 °C, 60 min, total concentration of RNA 80 μg/ml. C1, control lane; C2, 50 μM Cu^{2+}; L, alkaline hydrolysis ladder; T, digestion with RNase T1. The gels were quantified and the data were expressed as the fraction of the RNA substrate remaining after treatment with bacitracin, its complex with Cu^{2+} ions as well as the complex in the presence of H_2O_2: (D) — tRNA^{Phe}-RNA, (E) — ribHDV-RNA, and (F) — R20-RNA. The bar graphs show average values and standard deviations for three independent experiments.

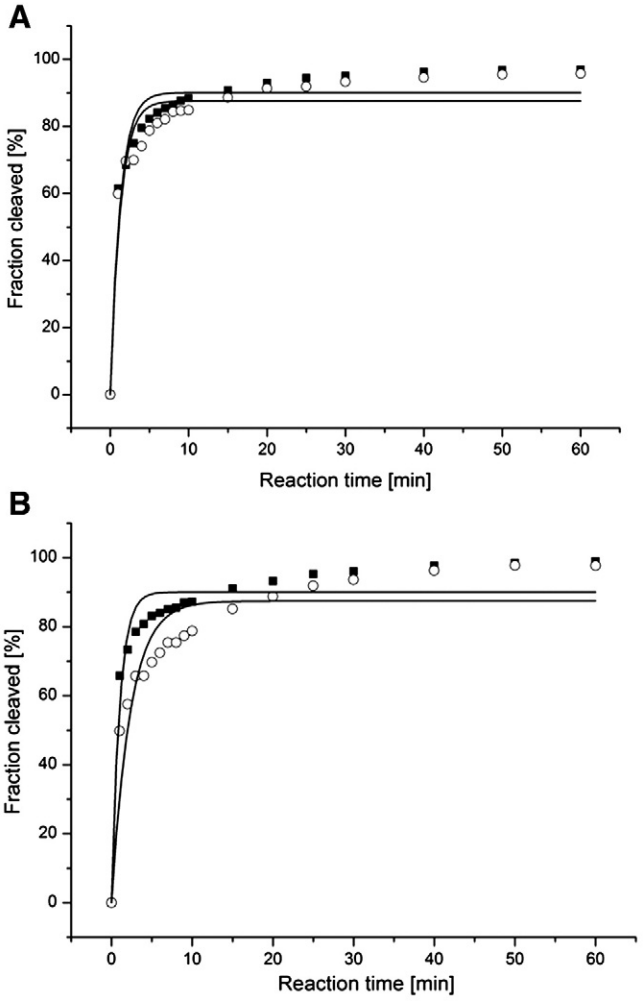

Fig. 3. Time course of the degradation of tRNA^{Phe}-RNA (A) and R20-RNA (B) in the presence of 22 μM bacitracin (filled squares) and Cu^{2+}–bacitracin complex (circles). The following reaction conditions were applied: 50 mM Tris–HCl pH 7.5, 37 °C; total concentration of RNA 80 μg/ml.

The patterns of the specific degradation of DNA by bacitracin were significantly different than those for RNA molecules. The cleavages in M72-DNA and M39-DNA were very infrequent, and preferentially they occurred in the sequences close to some cytidine residues (Fig. 4). In combination with the information that the degradation of DNA requires the presence of some divalent metal ions (see the next chapter), it may be suggested that the sites of DNA degradation are localized near the sites of strong metal ion binding. Since such sites are likely not too numerous in short DNA molecules thus cleavages within their sequences occur rarely.

The activity of bacitracin against double-stranded DNA has recently been studied by Ming's group (Fig. 10 in reference [12]). They have shown that free bacitracin used at a concentration as high as 23 mM does not change the migration of plasmid DNA in an agarose gel suggesting that no interaction of the antibiotic with DNA takes place. The complex of bacitracin with Cu^{2+} ions has caused strong retardation of DNA in the gel but no short DNA degradation products have been observed. As expected, under oxidative conditions the complex has rapidly cleaved plasmid DNA *via* a free radical cleavage mechanism [12].

In order to check whether bacitracin may act as a phosphatase or pyrophosphatase [^{32}P] radiolabeled nucleoside triphosphates: [γ-^{32}P] ATP and [α-^{32}P]UTP were incubated with 25 and 250 μM bacitracin for 60 min at 37 °C, also in the presence of Mg^{2+}, Mn^{2+} or EDTA. Since there were no degradation products observed, those nucleoside triphosphates were not substrates for bacitracin cleavage (data not shown).

2.3. A nucleic acid degradation reaction induced by bacitracin proceeds by hydrolytic mechanism

The application in the reaction with tRNA^{Phe}–RNA of not only bacitracin but also of its complex with Cu^{2+} ions, and additionally of the complex in the presence of H_2O_2, had almost no effect on the reaction efficiency or cleavage pattern (Fig. 2). Similarly, almost no differences in the degradation level by the bacitracin–Cu^{2+} complex relative to the free antibiotic were noted for the ribHDV-RNA and R20-RNA. Moreover, the time course of the degradation reaction of tRNA^{Phe}-RNA and R20-RNA in the presence of 22 μM bacitracin or of the Cu^{2+}–bacitracin complex revealed that the reaction proceeded with the same rate or even 2 times slower with the complex (Fig. 3). The calculated rates of substrate disappearance k_{obs} were 0.80 ± 0.10 min^{-1} and 0.79 ± 0.12 min^{-1} for tRNA^{Phe}-RNA, and 1.01 ± 0.16 min^{-1} and 0.43 ± 0.07 min^{-1} for R20-RNA. All these observations strongly argue against the free-radical mechanism of the reaction and suggest a hydrolytic degradation process.

In the hydrolytic cleavage mechanisms of phosphodiester bonds, it is important to know whether the phosphate group remains at the 3′ or 5′ end of the ribose residue at the cleavage site [26,27]. We thus decided to determine the localization of these phosphate groups in the RNA and DNA fragments generated in the presence of bacitracin. To this end, the electrophoretic migration of the degradation products of R20-RNA was compared with the migration of products of alkaline hydrolysis as well as limited digestion with nuclease S1 and RNase T1 (Fig. 6). It is known that alkaline hydrolysis and RNase T1 digestion products possess phosphate groups at their 3′ ends, whereas nuclease S1 digestion products — at their 5′ ends. The migration of RNA fragments generated with bacitracin corresponded to those after alkaline hydrolysis and RNase T1 digestion, and it was clearly different than in the case of the products of nuclease S1 digestion. This is convincing evidence that phosphate groups are found at the 3′ end of the RNA fragments arising in the reaction with bacitracin. An analogous experiment was performed for the reaction products of M39-DNA (Fig. 6). In that case the migration of the products generated with bacitracin corresponded to the migration of products after digestion with nuclease S1. Thus, the phosphate groups were found at the 5′ ends of the arising DNA fragments.

In order to elucidate the mechanisms of RNA and DNA degradation by bacitracin, it was important to establish whether the degradation with bacitracin required divalent metal ions. It turned out that the reaction of ribHDV-RNA was not affected by the presence of 2 mM EDTA (Fig. S3, Supplementary data). A similar observation was also made for tRNA^{Phe}-RNA (data not shown). On the contrary, the degradation of DNA was completely inhibited by EDTA, both in the case of M39-DNA and M72-DNA (Fig. 4A,C; 0.2 and 2 mM EDTA was used, respectively) Thus, the degradation of DNA requires the presence of divalent metal ions. It was determined that these include Mg^{2+}, Mn^{2+} or Zn^{2+} ions (Fig. 4). The degradation of DNA-M39 and DNA-M72, which was observed without the addition of divalent metal ions (and with no EDTA; Fig. 4A,C) could be explained by the fact that the commercially available bacitracin was supplied as a complex with Zn^{2+} ions.

2.4. The impact of selected degradation conditions on the effectiveness of bacitracin

The observed level of RNA degradation was dependent on its total concentration. The degradation effectiveness of three different RNA molecules, used at concentrations of 80 μg/ml incubated with 20 μM bacitracin for 60 min at 37 °C was 80–95% of the initial amount of RNA (Fig. 2). Decreasing the concentration of R20-RNA to 8, 0.8, 0.08 and about 0.01 μg/ml, with 25 μM bacitracin for 2 min, led to an increased degradation effect (Fig. S3, Supplementary data). In the case of isotope [^{32}P]-

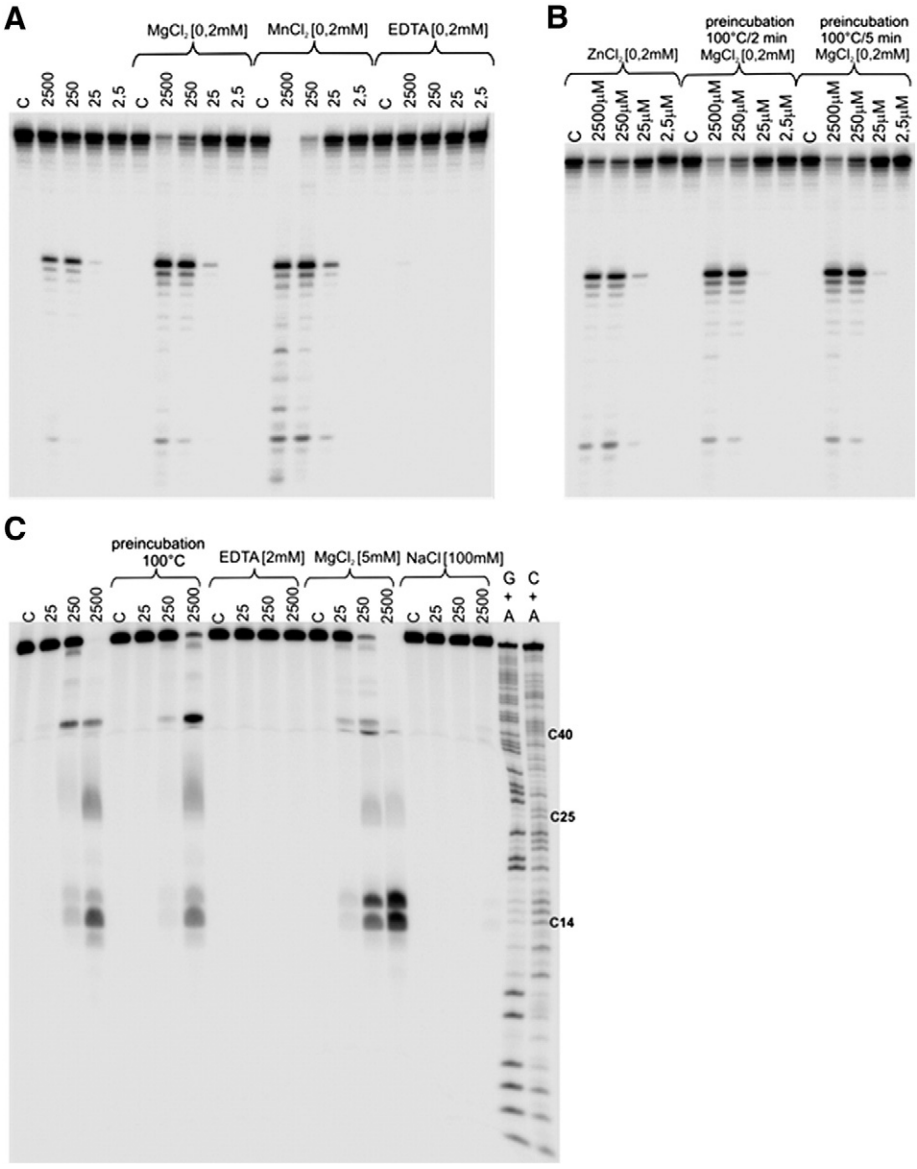

Fig. 4. Autoradiograms of the degradation products of M39-DNA (A and B) and M72-DNA (C) with bacitracin (Bac) at the concentration in the range of 2.5–2500 μM in the presence of factors potentially affecting the reaction. The reactions were performed in the following conditions: 50 mM Tris–HCl pH 7.5, 37 °C, 60 min., total concentration of DNA 30 μg/ml. C, control lanes; G + A, C + A, sequencing lanes.

tagged R20-RNA at a concentration of approx. 0.01 μg/ml and 2 min of incubation, with 10 μM bacitracin, we observed a complete degradation of the initial RNA, while 15% degradation occurred with 1 μM bacitracin (Fig. 6).

We also tested the effect of some other factors which might potentially alter the effectiveness of RNA degradation. Comparing the reaction of ribHDV-RNA with 5 and 25 μM bacitracin for 60 min, with and without 5 mM Mg^{2+} ions, some differences in the distribution of the products were observed, with little changes in the total level of RNA degradation (Fig. S3, Supplementary data). Likewise, the degradation was little affected by the presence of 100 mM NaCl. The reactions were also performed in two different buffers, 50 mM Tris–HCl pH 7.5 and 50 mM HEPES–NaOH pH 7.0, without any significant differences in the RNA and DNA degradation (data not shown).

In order to exclude a possibility that the bacitracin nucleolytic properties are due to the contamination of the commercial product with nucleases, some additional experiments were performed. The degradation of ribHDV-RNA with 5 and 25 μM bacitracin was performed with the addition of the protein ribonuclease inhibitor RNasin (Promega), without any significant differences in the reaction effectiveness (Fig. S3, Supplementary data). Bacitracin was pre-incubated at 100 °C for 5 min prior to the reaction of ribHDV-RNA at 5 μM bacitracin concentration for 60 min at 37 °C. It caused only a slight inhibition of the antibiotic's ability to degrade the RNA and the amount of non-degraded RNA increased from about 70 to 80% (Fig. S3, Supplementary data). Likewise, pre-incubation of the bacitracin solution for 5 min at 100 °C, i.e. in the conditions under which most DNases are deactivated, did not alter the degradation of M72-DNA (Fig. 4).

2.5. Bacitracin promoted RNA and DNA degradation: a comparison with nucleases

Bacitracin, like some other nucleases, shows dual specificity: metal ion-independent RNase and magnesium-dependent DNase. It is known that most of the single-strand specific nucleases are either metalloenzymes or metal-requiring enzymes. S1, P1 and mung bean nucleases are Zn^{2+} metalloprotein while the nuclease from *Neurospora crassa* is a cobalt metalloenzyme [27]. On the other hand, *Aspergillus*

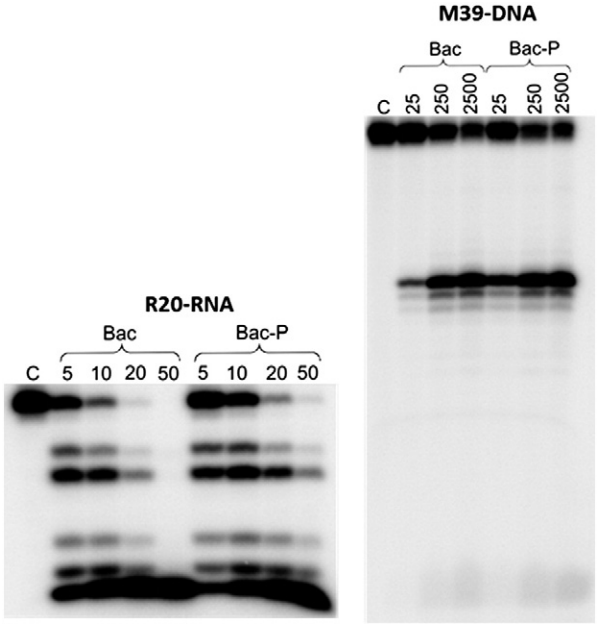

Fig. 5. Comparison of the degradation reactions of RNA-R20 and DNA-M39 induced by two different bacitracin samples purchased from independent suppliers Sigma-Aldrich Co (Bac) and Calbiochem (Bac-P). Reaction conditions: 50 mM Tris–HCl pH 7.5, 37 °C, RNA-R20 — incubation time 2 min, RNA concentration 80 μg/ml, DNA-M39 — incubation time 60 min, DNA concentration 8 μg/ml. C, control lane.

sydowii nuclease is active in a broader spectrum of divalent metal ions: 20 mM Mg^{2+}, 0.4 mM Mn^{2+} or 2 mM Co^{2+}. Bacitracin absolutely requires metal ions for DNA degradation and it is active in the presence of Mg^{2+} at a low, 0.2 mM concentration. Besides, Mg^{2+} ions can be replaced by other metal ions like Mn^{2+} and Zn^{2+} with no changes in activity.

In nucleases, metal ions play a dual role, ensuring proper folding of their catalytic centers and being directly involved in the cleavage mechanisms. An analysis of the resolved crystal structures of P1 and S1 nucleases has revealed a possible role of Zn^{2+} ion which activates a water molecule bound to the enzyme lowering its pK_a value [28]. Such a hydroxide molecule acts as a nucleophile attacking the phosphate P – O3′ bond while another metal ion stabilizes the leaving O3′-group. The final products are DNA fragments containing 5′ phosphate and 3′ hydroxyl groups.

In contrast to bacitracin-induced DNA degradation, the RNA cleavage pattern is not affected by the presence of 2 mM EDTA thus the antibiotic seems to be a metal-independent RNase. Moreover, the specificity of the RNA cleavage resembles that found for RNase T1 [25]. In non-denaturing conditions, bacitracin shows preference to single-stranded regions of highly structured RNAs, yeast $tRNA^{Phe}$ and antigenomic HDV ribozyme and the RNA chain is cleaved at GpN sequences. Very similar degradation patterns have been obtained when RNase T1 has been used to probe these RNA structures [22,24]. Also in the case of unstructured R20-RNA all GpN sites are cleaved by bacitracin yielding a 3′ phosphate and 5′-hydroxyl termini.

The mechanism of RNA digestion by RNase T1 involves base–acid catalysis that basically includes two processes, i.e. the abstraction of a proton from 2′ OH group of ribose moiety and then stabilization of the pentacovalent bipyramidal transition state by protonation. In fact, an analysis of the crystal structure data has revealed that two residues His40 and His92 of RNase T1 act as acid catalysts, while Glu58 performs the base catalysis [25]. RNase A, another well studied ribonuclease with pyrimidine residues specificity, requires two His residues for its catalytic activity, namely His12 and His119, in order to form a 2′,3′-cyclic phosphate intermediate that is stabilized by the nearby Lys41 [29].

In endonucleolytic activity of several peptides the necessity of an imidazole ring of the His residue has been reported [30]. A 30-amino acid-long peptide being a part of the N-terminal end of zinc-finger protein (ZFY) cleaved single-stranded RNA at pyrimidine residues utilizing the transesterification reaction, however without DNase specificity. Similarly to bacitracin, divalent metal ions did not affect the peptide activity. However, the determined k_{obs} values differed significantly. While for bacitracin k_{obs} was in the range of 0.6–0.7 min^{-1}, at least 20-fold lower k_{obs} was observed for the peptide [30]. An even lower cleavage activity has been observed for a series of tripeptide-acridine conjugates mimicking RNase A [31]. In that case, however, mutational studies have suggested that the imidazole ring of His residue at position three does not play a significant role in the cleavage mechanism. In contrast,

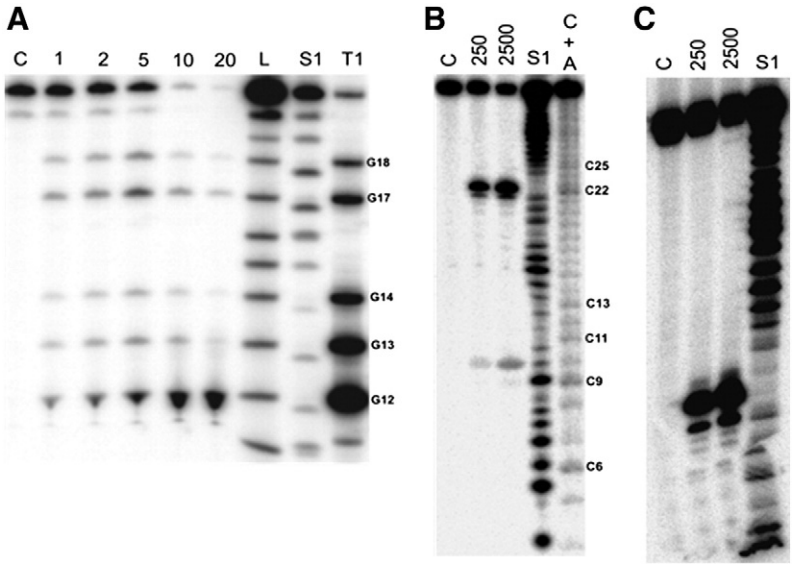

Fig. 6. (A) High resolution separation of degradation products of R20-RNA with varying concentrations of bacitracin (in μM), under the following reaction conditions: RNA concentration 0.01 μg/ml, 50 mM HEPES–NaOH pH 7.0, 37 °C, 2 min. C, control lane; L, alkaline hydrolysis; S1, T1, digestion with nuclease S1 and RNase T1. (B and C) Autoradiograms showing the degradation products of M39-DNA as an effect of varying concentrations of bacitracin (250 and 2500 μM), under the following reaction conditions: 50 mM HEPES–NaOH buffer pH 7.0; temp. 37 °C; time 60 min; total concentration of DNA 8 μg/ml. C, control lane; S1, digestion with nuclease S1, C + A, sequencing lane. The short (B) and long electrophoresis runs (C) of the gel are shown.

elimination of the primary amino group of residue one which likely was involved in the stabilization of the pentacoordinated cyclic transition state resulted in a dramatic loss of activity [31]. Finally, it has to be noted that bacitracin contains a His residue in position 10 which may participate in the RNA degradation mechanism. However, all the bacitracin congeners have this residue and the synthesis of bacitracin mutants would be required to identify the amino acids involved in the mechanism of RNA degradation.

Several other antibiotics besides bacitracin can induce degradation of RNA acting *via* a hydrolytic mechanism or in reactions involving free-radical species. In particular, some aminoglycoside antibiotics have been shown to induce hydrolytic cleavages in RNA molecules. Neomycin B cleaves the HIV TAR RNA construct with moderate efficiency, with k_{obs} of 3.7×10^{-3} min^{-1} at the 25 μM concentration of the antibiotic and neutral pH [32]. Moreover, the application of the $tRNA^{Phe}$ substrate with an abasic site in the anticodon loop increases k_{obs} of the neomycin B-induced cleavage to 10.1 min^{-1} [24]. Interestingly, paromomycin, which differs from neomycin B in the replacement of one of its amino group with a hydroxyl group, is inactive during the RNA degradation *via* the hydrolytic mechanism [32]. However, antibiotics have more frequently been used in the form of their stable complexes with redox metal ions, like Fe^{2+} or Cu^{2+} [18]. In the presence of H_2O_2 these complexes are a source of free radicals which break sugar moieties of RNA or DNA chains. Such polynucleotide chain scissions occur most efficiently at places which are close to the binding sites of the metal ion- antibiotic complexes to the targeted molecules. For example, the bleomycin–Fe^{2+} complex generates cleavages in the bacterial $tRNA^{His}$ precursor predominantly in the anticodon loop at U35 [13]. Also, the amikacin–Cu^{2+} complex splits yeast $tRNA^{Phe}$ in the anticodon loop [14]. These complexes are also able to degrade DNA plasmids *via* the free radical mechanism.

Finally, molecular tools have been constructed in which an artificial ribonuclease is attached to a target-specific oligonucleotide [33]. Such tools can precisely cleave single- or double-stranded RNA in a similar way as the restriction enzymes cleave DNA. Neamine, a part of the antibiotic neomycin B has been attached to a 16-mer peptide nucleic acid oligomer targeting the HIV TAR element. The construct cleaves the RNA inhibiting virus replication [34]. Likely, also bacitracin can be applied in that manner, acting as an antibiotic-oligonucleotide conjugate which has dual specificity, that of RNase and DNase.

3. Conclusions

It has been shown that bacitracin, one of the polypeptide antibiotics most frequently used in the antimicrobial therapy, has the ability to degrade nucleic acids, being especially active against RNA molecules. Bacitracin also has the capability of degrading single-stranded DNA to some extent. The degradation of DNA requires, however, substantially higher antibiotic concentrations and a comparable effect as for RNA is achieved with at least 10-fold higher concentrations of bacitracin.

No significant changes in RNA degradation are observed when bacitracin is in the complex with Cu^{2+} ions, and additionally, when H_2O_2 is included in the reaction mixture. This observation argues against the involvement of free radicals in the degradation and suggests that it occurs *via* a hydrolytic mechanism. In the degradation products, phosphate groups are located at the 3′ ends of the RNA and at the 5′ ends of the DNA fragments. It is important that RNA degradation is not affected by the presence of 2 mM EDTA, while EDTA completely inhibits DNA degradation. Thus, in contrast to RNA, DNA degradation necessarily requires the presence of divalent metal ions, like Mg^{2+}, Mn^{2+} or Zn^{2+}.

It was found that bacitracin induces the degradation of RNA at guanosine residues, preferentially in single-stranded RNA regions. Strikingly, RNase T1, an enzyme with the molecular mass *ca.* 10-times larger, shows a similar specificity. The specificity of DNA degradation is different and very infrequent cleavages occur preferentially in the vicinity of some cytidine residues. At the same time, the requirement of the presence of divalent metal ions may suggest that the regions of DNA degradation are located in the vicinity of these metal ions strong binding sites.

The high nucleolytic activity of bacitracin in comparison to other synthetic RNase mimics suggests that the RNA cleavage reaction proceeds *via* a base–acid catalysis in which more than one amino acid of this antibiotic are involved. Mutational studies of bacitracin should result in those amino acids identification and possibly in obtaining the antibiotic's derivatives with even better nucleolytic properties.

4. Experimental procedures

4.1. Materials

Bacitracin samples used in this study were purchased from Sigma-Aldrich Co (lot 1323372) and Calbiochem (lot D00103597). The other materials were from the following sources: [γ-^{32}P]ATP with specific activity 5000 Ci/mmol was from Hartmann Analytic, T4 polynucleotide kinase, T1 and S1 nucleases were from Fermentas. All chemicals were purchased from Sigma-Aldrich and Serva.

4.2. RNA and DNA oligomers

Model RNA substrates: baker's yeast tRNA specific for phenylalanine, $tRNA^{Phe}$-RNA, was purchased from Roche Diagnostic, the antigenomic HDV ribozyme, ribHDV-RNA, was prepared by *in vitro* transcription [22,23], and the 20-mer oligoribonucleotide, RNA-R20, was purchased from Future Synthesis, Poznan. DNA oligomers M39 and M72 were synthesized by Oligo Service, Warsaw. The nucleotide sequences of all these RNA and DNA oligomers are shown in Fig. 1.

The RNA and DNA oligomers were labeled at their 5′-ends using [γ-^{32}P]ATP and polynucleotide kinase under standard conditions. The labeled oligomers were purified by electrophoresis and identified in the gel by autoradiography. Radioactive bands were excised, and the oligomers were eluted from the gel with 0.3 M potassium acetate, 1 mM EDTA at pH 5.5. The labeled oligomers were ethanol precipitated, dissolved in water and stored at −20 °C before use.

4.3. Bacitracin degradation reaction

Prior to reactions with bacitracin, [^{32}P]-end-labeled oligonucleotide substrates were supplemented with unlabeled crude tRNA (for RNA) or appropriate DNA oligomers (for DNA) to obtain the specified concentrations. The oligonucleotides were renatured in the buffer: 50 mM Tris–HCl pH 7.5 by incubating at 100 °C for 1 min and at 0 °C for 5 min. Subsequently, bacitracin was added, and the reaction proceeded at 37 °C during the time intervals and at the bacitracin concentration specified in the figure legends. The reactions were quenched by mixing with an equal volume of an 8 M urea/dyes/20 mM EDTA solution, and the samples were loaded on 12% polyacrylamide, 0.75% bisacrylamide, 7 M urea gels. Electrophoresis was performed at 65 W for 3 h, followed by autoradiography at −70 °C with an intensifying screen. For quantitative analysis, gels were exposed to phosphorimaging screens and quantified using the FLA 5100 image analyzer (Fuji) and MultiGauge software (Fuji).

4.4. Determination of cleavage sites

To assign the cleavage sites in RNA, products of the bacitracin degradation reactions were run along with the products of alkaline hydrolysis and limited T1 and S1 nuclease digestion of the same RNA molecule. The alkaline hydrolysis ladder was generated by incubation of [^{32}P]-end-labeled RNA with 5 volumes of formamide/2 mM $MgCl_2$ in boiling water for 15 min. Partial T1 nuclease digestion was performed in denaturing conditions (50 mM sodium citrate, pH 4.5, and 7 M urea), with 0.1 unit of the enzyme. S1 nuclease digestion was carried out in

10 mM Tris–HCl, pH 7.2, 40 mM NaCl, 10 mM $MgCl_2$, and 1 mM $ZnCl_2$ was additionally added. To terminate the enzymatic digestion reactions, a mixture of 8 M urea/dyes/20 mM EDTA was added and the samples were immediately frozen on dry ice.

To assign the cleavage sites in DNA, products of the bacitracin degradation reactions were run along with the products of limited S1 nuclease digestion and G + A/C + A sequencing reactions of the same DNA molecule.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.01.034.

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