Directed cleavage of RNA within an imperfect complementary complex by oligonucleotide—bleomycin A_5 conjugates

P. E. Vorobjev,[a] I. A. Pyshnaya,[a] E. Wickstrom,[b] and V. F. Zarytova[a]★

[a]Novosibirsk Institute of Bioorganic Chemistry, Siberian Branch of the Russian Academy of Sciences, 8 prosp. Akad. Lavrent´eva, 630090 Novosibirsk, Russian Federation. Fax: +7 (383 2) 33 3677. E-mail: zarytova@niboch.nsc.ru
[b]Thomas Jefferson University, USA, Philadelphia PA 19107, Walnut Street 1025, Suite 425. Fax: +10 (215) 955 4580. E-mail: eric@tesla.jci.tju.edu

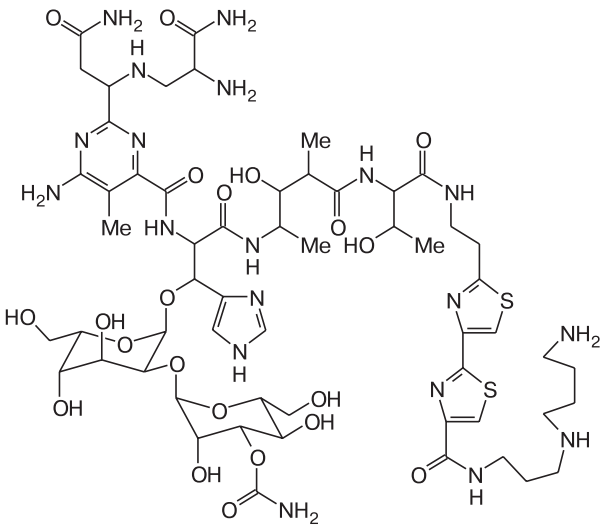
It was demonstarted for the first time that RNA can be subjected to site-specific oxidative cleavage induced by the glycopeptide antibiotic bleomycin A_5 (Blm) covalently linked to the 3´-terminus of an oligodeoxyribonucleotide through two, three, or four residues of hexaethylene glycol phosphate $(p\text{-}heg)_n$. The oligonucleotide conjugate with bleomycin forms an imperfect complementary complex with the RNA to be cleaved (5´-prCGGAGUUGGAAAACAAUGAAAAGGCCCCCA/Blm-$(p\text{-}heg)_n$-3´-pdGCCTCACCTTTTGTTA). The cleavage occurs at the only nucleotide residue (U) preceding a one-nucleotide bulge in the RNA chain, which is formed due to imperfect complementarity between the oligodeoxyribonucleotide and the RNA to be cleaved.

Key words: bleomycin, oxidative cleavage of RNA, antisense oligonucleotides.

Reactive derivatives of oligonucleotides serve as a powerful tool in studies of the functions of nucleic acids (NA) and proteins, they are widely used in the modern molecular biology. Since these derivatives can bind site-specifically to NA thus influencing their functions, they can also find use in medicine as promising selective antitumor and antiviral drugs. Antisense oligonucleotides are able to "turn off" expression of a particular gene in the stage of translation through the formation of a complementary complex with mRNA.

Oligonucleotide conjugates with the glycopeptide antibiotic bleomycin (**1** (Blm)) occupy a special place among reactive oligonucleotide derivatives due to the ability of bleomycin containing the metal-binding center to perform oxidative cleavage of several NA molecules.[1] The iron-containing complex of bleomycin is activated by molecular oxygen. The activated bleomycin complex induces selective oxidation of a deoxyribose (ribose) residue. In the presence of molecular oxygen, this leads to DNA (RNA) chain cleavage. In the presence of a reducing agent, repeated activation of the bleomycin molecule and repeated NA cleavage can take place. When involved in conjugates with oligonucleotides, the bleomycin residue retains the above-described properties.

Previously, we have demonstrated that oligonucleotide conjugates with bleomycin A_5 efficiently and site-specifically cleave single-stranded DNA,[2] whereas cleavage of RNA is inefficient.[3] It is known that some RNAs with different biological functions are efficiently cleaved by free bleomycin.[4] In particular, the cleavage of 5S yeast ribosomal RNA occurs at the nucleotide residues U in the 5´-GUA-3´ sequences preceding one-nucleotide bulges.[4] Apparently, these regions of the RNA structure serve as sites of the preferential binding of bleomycin in the conformation favorable for selective oxidation of the ribose residue.



1

In the present study, we demonstrated that the structure of an RNA target can be subjected to local changes with the use of the oligonucleotide component of conju-

Published in Russian in *Izvestiya Akademii Nauk. Seriya Khimicheskaya,* No. 7, pp. 1097—1099, July, 2002.

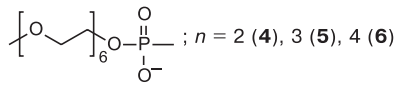
gates by forming complexes which contain regions of preferential cleavage by bleomycin. This approach was used for the cleavage of a 30-mer fragment of the oncogene *c-myc* mRNA (**2**) by free bleomycin and conjugates of the latter with a 16-mer oligodeoxyribonucleotide.

5′ ^{32}prCGGAGUUGGAAAACAAUGAAAAGGCCCCCA
2

3′ pdGCCTCACCTTTTGTTA
3

U
5′ ^{32}prCGGAGUGGAAAACAAUGAAAAGGCCCCCA
3′ pdGCCTCACCTTTTGTTA
Duplex **2**—**3**

pdGCCTCACCTTTTGTTA
(heg-p)$_n$Blm
4—**6**

(heg-p) =  ; n = 2 (**4**), 3 (**5**), 4 (**6**)

Experimental

Bleomycin A_5 hydrochloride was purchased from the Institute of Organic Synthesis (Latvia) and 30-mer oligoribonucleotide **2** was purchased from CyberSyn (USA).

Oligodeoxyribonucleotide **3** was prepared by the phosphoramidite method on an automatic ASM-700 synthesizer (Biosset, Russia). Oligodeoxyribonucleotides with linkers were synthesized starting from the monomers which were prepared according to procedures described previously.[5] The relative sizes of the linkers and the hybrid duplex were estimated using the HyperChem 5.0 program package. Conjugates **4**, **5**, and **6** were synthesized according to a known procedure.[6] (The synthesis will be described in detail elsewhere.) The conjugates were isolated by high-performance ion-exchange chromatography on a Resource Q, 1 mL column (Pharmacia Biotech, Sweden) and high-performance reversed-phase chromatography on a Polysil RP-18 sorbent (Teoreticheskaya praktika [Theoretical Practice], Russia). The homogeneity of the conjugates was confirmed by gel electrophoresis in a denaturing polyacrylamide gel followed by visualization using the Stains-all dye (Acros Organics, USA). The electronic spectra of the conjugates have a characteristic absorption band of the bleomycin residue in the region of 300—330 nm. The ratio $\varepsilon_{260}/\varepsilon_{310} = 12.4$ for compounds **4**, **5**, and **6** is close to the calculated value $\varepsilon_{260}/\varepsilon_{310} = 12.1$ for the 1 : 1 stoichiometry of the addition of the oligonucleotide to the antibiotic.

The ^{32}P-labeled RNA target was prepared according to a known procedure[7] with the use of [γ-^{32}P] ATP (Biosan, Russia) and polynucleotide kinase (EC 2.7.1.78) (SibEnzim, Russia).

The concentrations of oligonucleotides and their derivatives were determined by spectrophotometry. The molar absorption coefficients of oligonucleotides at $\lambda = 260$ nm were calculated according to a procedure described previously.[8] The contribution of the antibiotic residue was taken equal to 16000 L mol^{-1} cm^{-1}.[9]

The modification of the ^{32}P-labeled RNA target was carried out in a buffer solution (10 μL) containing 0.2 *M* LiCl, 0.01 *M* Tris-HCl (pH 7.5), and 0.05 *M* 2-mercaptoethanol. The reaction was initiated by adding a solution of $Fe(NH_4)_2(SO_4)_2$ prepared in argon-saturated twice-distilled water to the concentration of $1 \cdot 10^{-4}$ mol L^{-1}.* The concentration of the RNA target was $1 \cdot 10^{-6}$ mol L^{-1}. The concentrations of the conjugates and free bleomycin were $1 \cdot 10^{-5}$ mol L^{-1}. The reactions were carried out at 20 °C for 4 h. The reaction mixtures were analyzed by electrophoresis in 20% polyacrylamide gel under denaturing conditions (0.09 *M* Tris-H_3BO_3, pH 8.3, 8 *M* urea, 40 °C).

The gel-electrophoresis autoradiographs were obtained by exposure of a CP-BU NIF 100 film (AGFA, Belgium) at −20 °C.

The extent of cleavage of the RNA target was determined as the ratio between the intensity of the band corresponding to the cleavage product and the overall intensity of the autoradiograph lane. The relative intensities of the bands were determined with the use of the GelPro program package.

Results and Discussion

The 30-mer fragment of the oncogene *c-myc* mRNA (nucleotides 7041—7070; hereinafter, RNA target **2**) was used as the target. It was demonstrated that this fragment was not cleaved by free bleomycin in the concentration range of 1—10 μmol L^{-1}. No cleavage by bleomycin was also observed within the perfect hybrid duplex formed by RNA target **2** and the 17-mer oligodeoxyribonucleotide 3′-pdGCCTCA**A**CCTTTTGTTA-5′ (the data are not presented). The efficient cleavage of this RNA target by free bleomycin was achieved within the hybrid duplex with oligodeoxyribonucleotide **3** (Fig. 1). In this case, an imperfect duplex with a one-nucleotide bulge in the RNA chain is apparently formed due to imperfect complementarity between 16-mer oligonucleotide **3** and RNA target **2**. The cleavage occurred at the nucleotide residue U6 adjacent to the bulge containing the residue U7. The degree of cleavage was 30%. Hereinafter, only direct cleavages of the RNA chain, which were observed without additional treatment, were registered.

Then, we attempted to form an analogous imperfect duplex with the use of bleomycin derivatives of oligonucleotides. Conjugates **4**, **5**, and **6**, whose sequences are identical with the sequence of oligonucleotide **3**, were synthesized. For the bleomycin residue that is attached to the 3′-terminus of the oligonucleotide to reach the desired cleavage site, flexible linkers consisting of two, three, or four hexaethylene glycol phosphate residues, respectively, were inserted into the conjugates.

* Since the amount of oxygen dissolved in water under normal atmospheric pressure is sufficient for the activation of the iron complex with bleomycin, no oxygen was specially introduced into the reaction mixture.

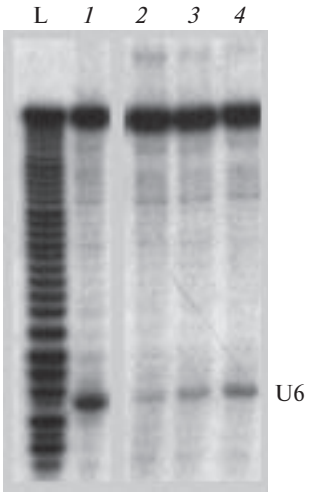


Fig. 1. Gel-electrophoresis autoradiograph of the cleavage products of the ^{32}P-labeled RNA target in the presence of oligonucleotide **3** and free bleomycin (*1*), of conjugate **4** (*2*), of conjugate **5** (*3*), and of conjugate **6** (*4*). L is statistical hydrolysis of the RNA target in 0.2 *M* $NaHCO_3$, pH 8.9. The reaction conditions are given in the Experimental section.

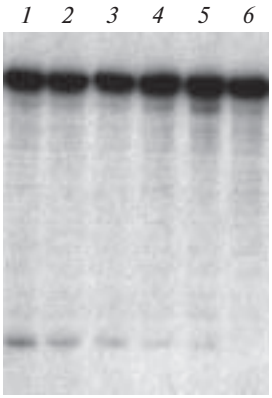


Fig. 2. Gel-electrophoresis autoradiograph of the cleavage products of the ^{32}P-labeled RNA target by conjugate **6** in the presence of oligonucleotide **3** at the concentration of $0.1 \cdot 10^{-6}$ (*1*), $0.5 \cdot 10^{-6}$ (*2*), $1 \cdot 10^{-6}$ (*3*); $5 \cdot 10^{-6}$ (*4*), and $1 \cdot 10^{-5}$ mol L^{-1} (*5*); *6* is the RNA target under the reaction conditions. The reaction conditions are given in the Experimental section.

According to an estimate made with the use of molecular models, the lengths of all three linkers are larger than the distance between the 3′-terminal phosphate group of oligonucleotide **1** in conjugates **4**, **5**, and **6** and the cleaved nucleotide residue in the RNA target involved in the hybrid duplexes.

As can be seen from Fig. 1, the cleavage of the RNA target in the presence of conjugates **4**, **5**, and **6** occurred at the nucleotide residue U6. The efficiency of cleavage in the complex depends on the length of the linker between the bleomycin residue and the oligonucleotide. The extent of cleavage of the RNA target by conjugates **4** and **5** was 8% and 12%, respectively. The maximum efficiency of cleavage (18%) was achieved in the presence of oligonucleotide **6** containing four hexaethylene glycol phosphate residues in the linker. Evidently, duplexes formed by conjugates **4**, **5**, and **6** with the RNA target, like that formed by oligonucleotide **3**, contain the one-nucleotide bulge in the RNA chain.

The enhancement of the efficiency of RNA cleavage with an increase in the length of the linker in the conjugate is apparently associated with the fact that bleomycin must be bound in a definite conformation necessary for the interaction between the activated bleomycin residue and ribose.

The efficiency of the modification of the RNA target by conjugate **6** decreases upon the addition of oligonucleotide **3**, which competes with conjugate **6** for the binding site in the RNA target (Fig. 2). This indicates that the cleavage of the RNA target by conjugates **4**—**6** is complementary addressed.

The above-considered data provide evidence that the use of the oligonucleotide component of bleomycin-containing conjugates for the formation of bulged complexes, which contain regions of the preferential cleavage of RNA by bleomycin, makes it possible to perform efficient complementary addressed RNA cleavage by such conjugates.

This study was financially supported by the Fogarty International Research Collaboration Award (FIRCA, Project TW 01094), by the Russian Foundation for Basic Research (Project Nos. 99-04-49731a and 02-04-49597a), and by the Russian Academy of Sciences (VI Competition of Research Projects of Young Scientists of the Russian Academy of Sciences, Project No. 216).

References

1. R. M. Burger, *Chem. Rev.*, 1998, **98**, 1153.
2. D. S. Sergeyev, P. E. Vorobjev, and V. F. Zarytova, *Nucleosides Nucleotides*, 1997, **16**, 1575.
3. J. B. Smith, P. E. Vorobjev, V. F. Zarytova, and E. Wickstrom, *American Association for Cancer Research Annual Meeting*, Philadelphia, Pennsylvania, April 10—14, 1999, 133.
4. S. E. Holmes, B. J. Carter, and S. M. Hecht, *Biochemistry*, 1993, **32**, 4293.
5. I. A. Pyshnaya, D. V. Pyshnyi, E. M. Ivanova, V. F. Zarytova, G. M. Bonora, C. Scalfi-Happ, and H. Seliger, *Nucleosides Nucleotides*, 1998, **17**, 1289.
6. V. F. Zarytova, D. S. Sergeyev, and T. S. Godovikova, *Bioconjugate Chem.*, 1993, **4**, 189.
7. K. L. Berkner and W. R. Folk, *J. Biol. Chem.*, 1977, **252**, 3176.
8. *Handbook of Biochemistry and Molecular Biology: Nucleic Acids*, Ed. T. E. Fasman, CRC Press, Cleveland, 1975, p. 589.
9. J. Sugiura, K. Ishuzu, and K. Miyoshi, *J. Antibiot.*, 1979, **32**, 453.

Received February 13, 2002;
in revised form March 26, 2002