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Selective Cleavages of tRNA^{Phe} with Secondary and Tertiary Structures by Enediyne Antitumor Antibiotics

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Abstract—Some enediyne antitumor antibiotics induce site-selective cleavages for yeast tRNA^{Phe} with three-dimensional structure. Of special interest is the fact that tRNA^{Phe} is specifically cleaved at the anticodon arm regions by C-1027 and esperamicin A_1 in the presence of Mg^{2+} ions. Although neocarzinostatin strongly breaks tRNA^{Phe} at 5′-GPu steps in the absence of magnesium ions, its cleavage ability is completely lost in the presence of 100 μM Mg^{2+} ions. Dynemicin A, which favors an intercalative binding, causes no strand scissions for the RNA with secondary and tertiary structures. This cutting of tRNA^{Phe} may reveal that RNA as well as DNA constitutes a therapeutically relevant target for certain enediyne antitumor antibiotics. © 1997 Elsevier Science Ltd.

Introduction

The so-called 'enediyne antitumor antibiotics' have attracted increasing attention because of their chemistry, biology and medical application.[1] The enediyne antibiotics such as calicheamicin,[2] esperamicin,[3] dynemicin,[4] neocarzinostatin,[5] C-1027,[6] kedarcidin[7] and maduropeptin[8] possess unprecedented chemical structure, potent anticancer activity, and a fascinating mode of biological action (Fig. 1). They are believed to exert their physiological activity by cleaving DNA duplex and by blocking transcription and replication of DNA. Indeed, the antibiotics show sequence-specific,[1,9] conformation-selective,[10,11] and double-stranded DNA cuttings.[2,5,12,13] Recently, DNA interaction modes between the enediyne antibiotics and oligodeoxynucleotides

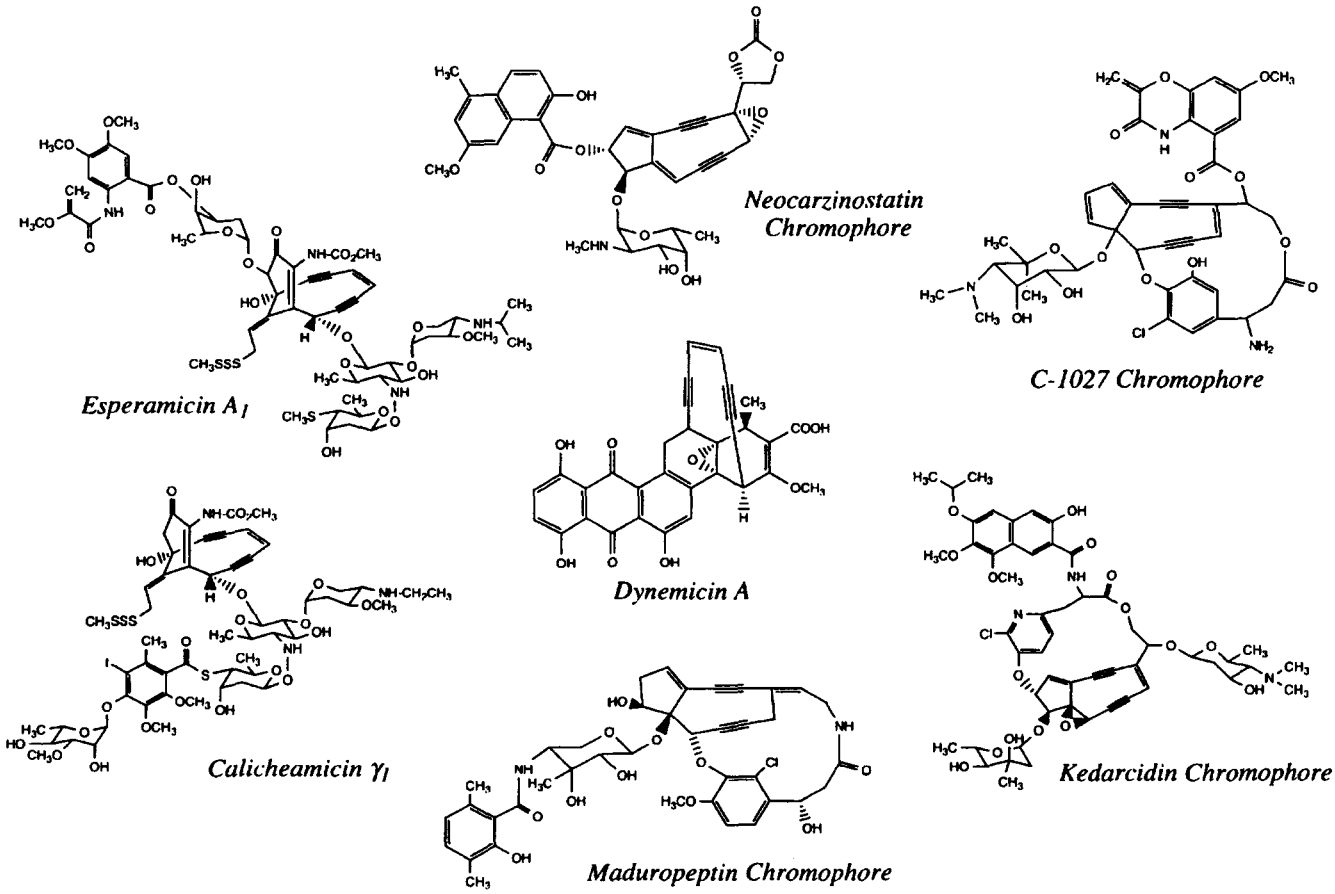

Figure 1. Chemical structures of the enediyne antitumor antibiotics.

were also demonstrated by high-resolution ^{1}H NMR.[14–16] The compounds containing the bicyclic enediyne structure are themselves converted into diradical species through the Bergman cyclization. The diradical species can abstract hydrogen atoms from the deoxyribose backbone of DNA duplex, and the novel mechanism of DNA damage reveals valuable implications for their application as potent cancer chemotherapeutic agents.

Although the enediyne-meditated RNA damage has been little investigated,[17,18] one obvious potential therapeutic locus for these enediyne antibiotics may be RNA. In recent years it was established that RNA is sometimes a terrific substrate for cleavage by metallobleomycin, a famous DNA-cutting drug.[19–21] For the cleavage event by the enediyne antibiotics, the RNA structure appears to be very important: RNA molecules specifically require folded tertiary structures for their biological and chemical functions. Yeast tRNA^{Phe} is an attractive candidate for initial studies, because its three-dimensional structure has been well characterized by X-ray crystallography[22] and high-resolution ^{1}H NMR spectroscopy.[23] Therefore, we chose yeast tRNA^{Phe} in this work and investigated selective cleavages of tRNA^{Phe} with secondary and tertiary structures by some enediyne antitumor antibiotics.

Results and Discussion

Figure 2 shows cleavage patterns of the 5′-end ^{32}P-labeled yeast tRNA^{Phe} by esperamicin A_1. In the absence of Mg^{2+} ions, where the tertiary structure of RNA^{Phe} is disrupted, the RNA break was strong but considerably random, indicating less selectivity of the drug to the secondary structure of the RNA. Indeed, the treatment of a shorter fragment of the tRNA (residues 47–46) with esperamicin A_1 also gave a ladder of cutting pattern similar to that of the full-length tRNA^{Phe}. In the presence of Mg^{2+} ions, where tRNA^{Phe} attains correct three-dimensional structure and is biologically active, by contrast, site-selective cleavage was detected in spite of reduced cutting intensity. Inspection of the autoradiogram reveals that the cleavage sites (O2′-methyl G34, A35, A36, and A38) are restricted to the anticodon arm of the tRNA. Since magnesium ions have no effects on the aromatization of esperamicin A_1,[24] the site specificity is due to the conformational folding of the tRNA induced by Mg^{2+} ions. As evidently demonstrated by the crystal structure of tRNA^{Phe},[22] there are tertiary base interactions between D and T4C arms but not in the anticodon arm. Therefore, the most likely explanation for the stereoselectivity is that the tight D-T4C arm interactions displace esperamicin A_1 from these arms, allowing the moderately unfolded anticodon arm to be accessible to the drug. It is well known that the esperamicin A_1-induced DNA strand scission is limited to double-stranded substrates and occurs with the greatest efficiency near oligo (purine)/oligo (pyrimidine) tracts.[3,9,24] These recognition characteristics are not

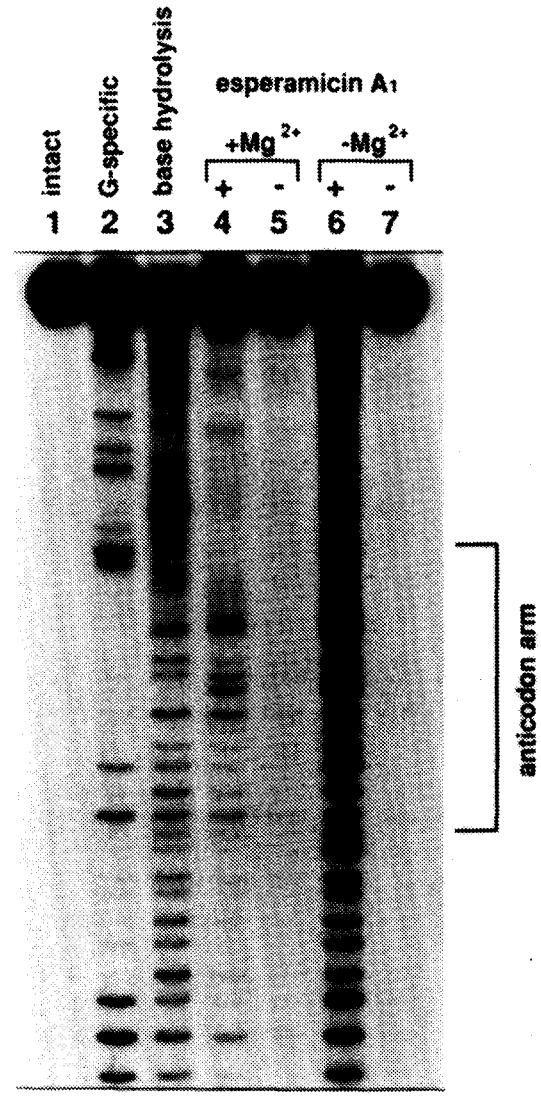

Figure 2. Cleavage of yeast tRNA^{Phe} by esperamicins A_1. The 5′-end-labeled tRNA was treated as follows: lane 4, 10 μM esperamicin A_1, 1 mM dithiothreitol, and 5 mM Mg^{2+}; lane 5, 1 mM dithiothreitol and 5 mM Mg^{2+}; lane 6, 10 μM esperamicin A_1 and 1 mM dithiothreitol; and lane 7, 1 mM dithiothreitol. Lanes 1–3 indicate intact tRNA, tRNA digestion with ribonuclease T1, and alkaline hydrolysis of tRNA, respectively.

applicable to RNA–esperamicin interaction; that is, esperamicin A_1 binds and cleaves RNA and DNA in different manners. Esperamicin C (lacking of deoxyfucose–anthranilate moiety from esperamicin A_1) and esperamicin D (lacking of thiomethylhexapyranose moiety from esperamicin C)[24] showed a cleavage preference similar to that of esperamicin A_1 in the absence of Mg^{2+} ions, but its cleavage activity was significantly lower than that of esperamicin A_1. In the presence of Mg^{2+} ions, on the other hand, no strong cuttings induced by esperamicins C and D were detected even in the anticodon arm region. The results suggest that the deoxyfucose–anthranilate moiety presumably plays an important role in tRNA^{Phe} binding of esperamicin antibiotics. It is also interesting that the RNA cleavage activity of calicheamicin γ_1 was evidently

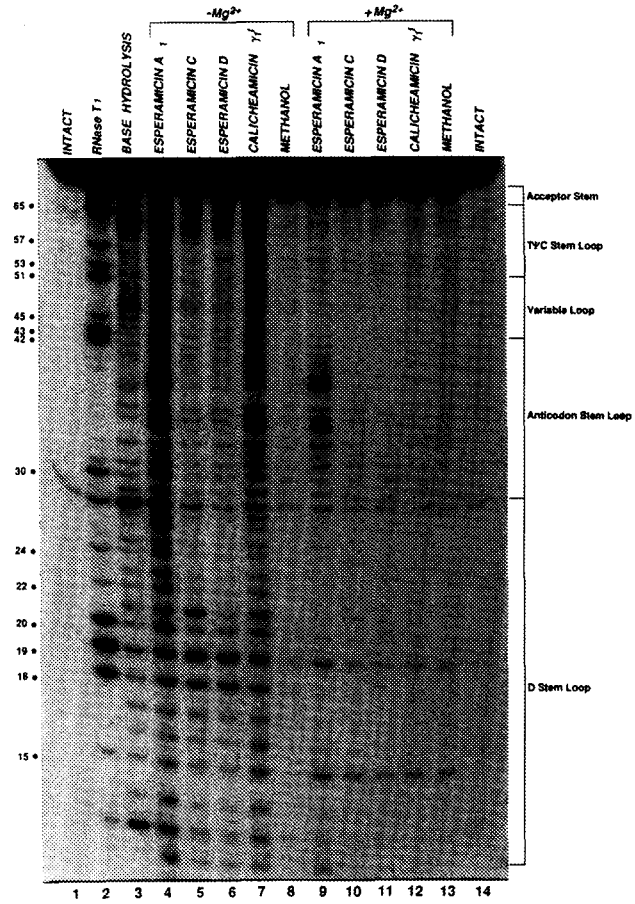

Figure 3. Cleavage of yeast tRNA^{Phe} by esperamicins A_1, C and D, and calicheamicin γ_1 in the absence and presence of Mg^{2+} ions. The 5′-end-labeled tRNA was treated as follows: lane 4, 10 μM esperamicin A_1 and 1 mM dithiothreitol; lane 5, 10 μM esperamicin C and 1 mM dithiothreitol; lane 6, 10 μM esperamicin D and 1 mM dithiothreitol; lane 7, 10 μM calicheamicin γ_1 and 1 mM dithiothreitol; lane 8, 1 mM dithiothreitol; lane 9, 10 μM esperamicin C, 1 mM dithiothreitol, and 5 mM Mg^{2+}; lane 11, 10 μM esperamicin D, 1 mM dithiothreitol, and 5 mM Mg^{2+}; lane 12, 10 μM calicheamicin γ_1, 1 mM dithiothreitol, and 5 mM Mg^{2+}; and lane 13, 1 mM dithiothreitol and 5 mM Mg^{2+}. Lanes 1–3 and 14 indicate intact tRNA, tRNA digestion with ribonuclease T_1, alkaline hydrolysis of tRNA, and intact tRNA, respectively.

lower than that of esperamicin A_1 both in the absence and presence of Mg^{2+} ions (Fig. 3).

Figures 4 and 5 present typical autoradiographic results for strand breaks of the 5′-end ^{32}P-labeled tRNA^{Phe} by C-1027 or neocarzinostatin. In neocarzinostatin and C-1027, each enediyne chromophore showed the same sequence preference as its holo drug. Here, some control experiments showed that the cleavage is not due to the apoprotein. Similar RNA break is also known to be unaffected by thiol.[17] In the absence of Mg^{2+} ions, C-1027 induced strong cleavage at the residues of A14, A29, U33, U52, and G57. The addition of magnesium ions suppressed the cleavages detected above, but caused the new breaks at G30, A31, A36 and Y37 in the anticodon loop. C-1027 is known to involve 5′-AGA and 5′-TAT as DNA recognition sites.[6] However, 5′-AGA sequence of tRNA^{Phe} was not cleaved by this antibiotic. The anticodon loop in RNA molecules forms a single-stranded region of the molecule that does not contain any tertiary interactions with other regions of the tRNA molecule. The results strongly indicate that Mg^{2+}-dependent conformational change and stabilization of the host tRNA have significant effects on the location and efficiency of the RNA cleavage by C-1027. On the other hand, DNA break of C-1027 is insensitive to the presence of Mg^{2+} ions. As demonstrated by a fluorescence quenching study,[18] C-1027-RNA^{Phe} interaction is clearly different in the absence and presence of Mg^{2+} ions. The result suggests that the tRNA binding of C-1027 is stronger in the presence of Mg^{2+} ions than in the absence of magnesium ions. The estimated binding constant (9.9×10^5 M^{-1}) of C-1027 chromophore-tRNA^{Phe} complex in the presence of Mg^{2+} ions is smaller than that (2.7×10^6 M^{-1}) of the corresponding DNA complex.[25] As to the binding constant, the tightly bound class of Mg^{2+} ions to tRNA has been reported to be in the order of 0.9×10^5 M^{-1}.[26] Accordingly, the binding constant of the C-1027 chromophore–tRNA^{Phe} complex is approximately 10 times that of the tRNA^{Phe}–Mg^{2+} complex. In the 5′-end-labeled tRNA, the bands of the cleaved product migrated close to those of the marker fragments or a little faster than the markers generated by the alkaline hydrolysis. The marker fragments are known to have 2′,3′-cyclic phosphate at their 3′-termini. On the other hand, the 3′-end-labeled tRNA gave the cleaving bands that migrated ahead of the marker-bearing 5′-hydroxyl group. Bacterial alkaline phosphatase quantitatively converted each band into the band comigrating with the marker (data not shown). This observation is consistent with the formation of the fragments having 5′-phosphate termini. It is demonstrated that C-1027 oxidizes DNA through predominant hydrogen abstraction at the C-4′ carbon of deoxyribose.[6] We cannot decide if this RNA cleavage proceeds by C1′- or C4′-hydrogen abstraction, because there is no direct evidence for production of any 3′-phosphoglycolate product. The related observation with neocarzinostatin and RNA·DNA hybrids provided direct evidence for C1′-hydrogen abstraction.[27] The relative cutting efficiencies of DNA and RNA by C-1027 clearly differ. Cleavage of DNA by C-1027 is about 20-fold more efficient than that of RNA under similar experimental conditions. Taken together, these results show that there are significant differences in the modes of recognition and cleavage between DNA and RNA by C-1027. Recently, our NMR study revealed interaction mode of the benzoxazolinate and aminosugar moieties of C-1027 with DNA, in particular the intercalation of benzoxazolinate group.[16] On the other hand, neocarzinostatin strongly cleaved tRNA^{Phe} at 5-G<u>Pu</u> steps such as G19, G20, A21, G43, A44, and A66 in the absence of Mg^{2+} ions. It is noteworthy that all the strong cutting sites are located on double-strand and single-strand junctions. This observation suggests that neocarzinostatin preferentially binds to flexible regions in the RNA. The weak cleavage occurred at 5′-Pu<u>N</u> steps including A5, 2-methyl C11, D16, G22, A23, C<u>25</u>, G30, A31, A35, 7-methyl G46, U52, T54, and 1-methyl A58. When the tRNA was treated with neocarzinostatin in the presence of Mg^{2+} ions, the cleavage efficiency of neocarzinostatin was considerably reduced by 10 μM

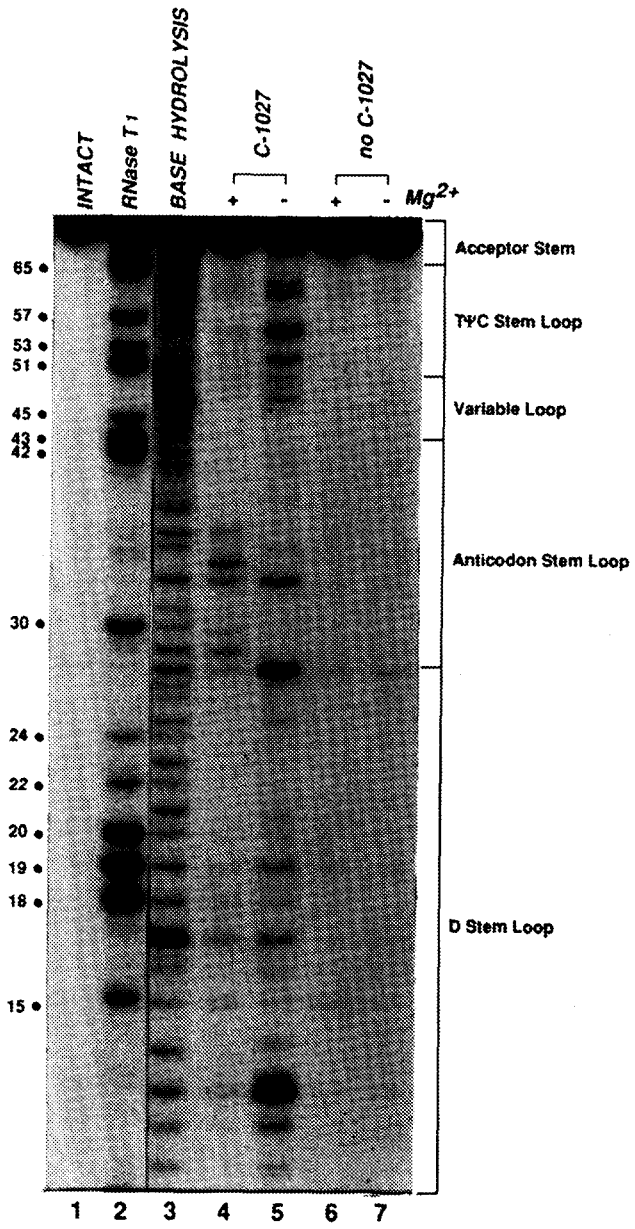

Figure 4. Cleavage of yeast $tRNA^{Phe}$ by C-1027. The 5′-end-labeled tRNA was treated as follows: lane 4, 100 μM C-1027 amd 5 mM Mg^{2+}; lane 5, 100 μM C-1027; lane 6, 5 mM Mg^{2+}; and lane 7, none. Lanes 1–3 show intact tRNA, tRNA digestion with ribonuclease T_1, and alkaline hydrolysis of tRNA, respectively.

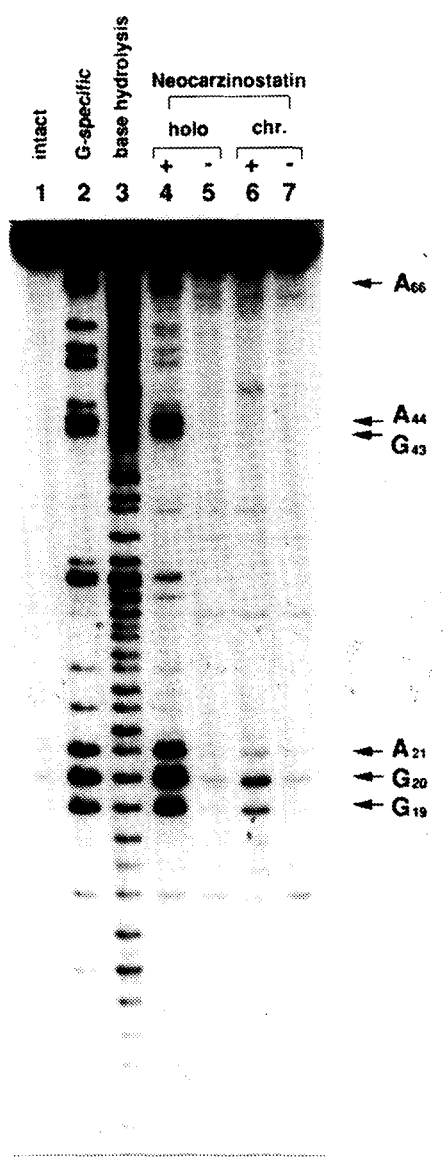

Figure 5. Cleavage of yeast $tRNA^{Phe}$ by neocarzinostatin. The 5′-end-labeled tRNA was treated as follows: lane 4, 10 μM holo-neocarzinostatin and 1 mM dithiothreitol; lane 5, 1 mM dithiothreitol; lane 6, 10 μM neocarzinostatin chromophore and 1 mM dithiothreitol; and lane 7, 1 mM dithiothreitol. Lanes 1–3 indicate intact tRNA, tRNA digestion with ribonuclease T_1, and alkaline hydrolysis of tRNA, respectively

magnesium ions and completely abolished by 100 μM Mg^{2+} ions. Under the physiological condition, therefore, $tRNA^{Phe}$ is not a suitable target substrate for neocarzinostatin. Similar cutting loss for $tRNA^{Phe}$ with tertiary structure has also been observed in studies of bleomycin-iron complex.[20,28] In the secondary and tertiary structures of RNA^{Phe}, the cleavage sites by esperamicin γ_1, C-1027, and neocarzinostatin are summarized in Figures 6 and 7.

Dynemicin A, which is a hybrid molecule containing anthraquinone and enediyne cores, effectively cleaves DNA duplex. In particular, the antibiotic preferentially breaks conformationally flexible regions of DNA such as the B–Z junction, and bulge and nick sites.[29] However, the present careful experiments demonstrated that dynemicin A is unable to cut this RNA substrate both in the absence and presence of magnesium ions. Presumably, this result is due to the fact that DNA can provide intercalative binding sites favorable for dynemicin A, but RNA cannot.

Of interest is the fact that RNA is employed as one target for the cleavage by certain enediyne antibiotics. The drugs are believed to exert their antitumor effects by damaging DNA. This study clearly shows the ability of enediyne antibiotics to cleave RNA. In particular, C-1027 and esperamicin A_1 specifically attack the anticodon arm of $tRNA^{Phe}$ with tertiary structure. When $tRNA^{His}$ was used as RNA substrate, it has also been

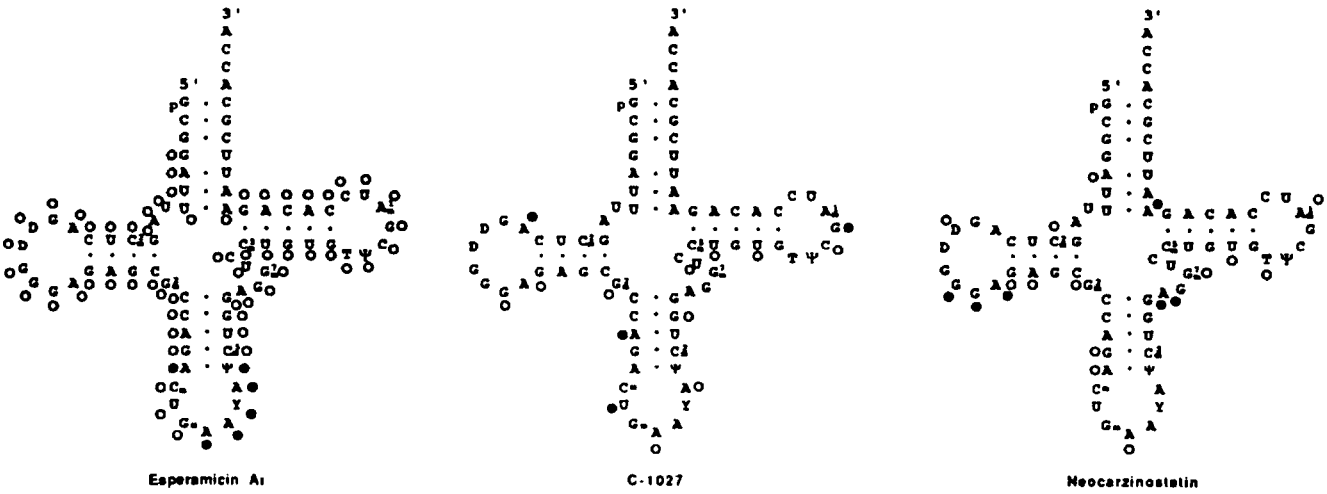

Figure 6. Cleavage sites of yeast tRNA^{Phe} induced by esperamicin A1, C-1027, and neocarzinostatin in the absence of Mg^{2+} ions. Closed and open circles show major and minor sites of cleavage by the enediyne antibiotics.

observed that esperamicin, calicheamicin, and neocarzinostatin exhibit apparent cleavage in the single-stranded loop regions.[17] In larger RNAs, the fully unfolded regions may be important for targeting of the enediyne antibiotics. Certainly, the RNA cleavage by the enediyne drugs significantly depends on higher-order structures of RNAs that are required for their biological and chemical functions. Considering the paucity of RNA repair mechanisms, RNA as well as DNA is probably an important biological target for certain enediyne antibiotics such as C-1027 and esperamicin A_1.

Experimental

Materials

Yeast tRNA^{Phe} obtained from Boehringer Mannheim was purified by HPLC. Esperamicins A_1, C, and D were a kind gift from Dr T. W. Doyle (Bristol–Myers Squibb), and calicheamicin γ_1 was generously provided by Dr G. A. Ellestad (Lederle Laboratories). Purified chromoprotein C-1027 was kindly supplied by Dr T. Otani (Taiho, Tokushima) and nonprotein C-1027 chromophore was extracted from lyophilized C-1027 with distilled methanol at 0 °C. Neocarzinostatin was obtained from Yamanouchi Pharmaceutical Comp. (Tokyo). T4 polynucleotide kinase and T4 RNA ligase were purchased from Takara Shuzo (Kyoto) and New England Biolabs, respectively. Ribonuclease T_1 was supplied from Boehringer Mannheim. Distilled water was purified through a Sybron Nanopure II system, and all other chemicals used were of commercial reagent grade.

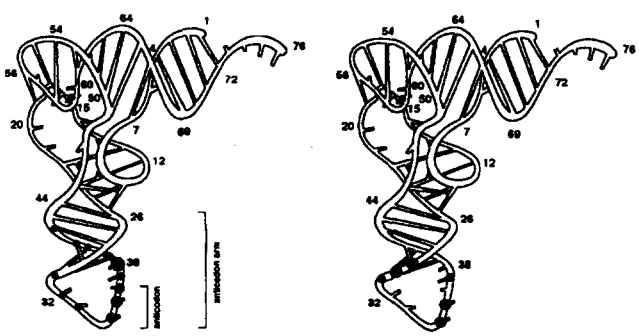

Figure 7. Three-dimensional structure of tRNA^{Phe} and cleavage sites induced by esperamicin A_1 (left) and C-1027 (right) in the presence of Mg^{2+} ions. Large and small closed circles indicate major and minor sites of cleavage by the enediyne antibiotics.

Purified tRNA^{Phe} was dephosphorylated by using calf intestinal alkaline phosphatase and then 5′-^{32}P end-labeled with T4 polynucleotide kinase and [γ-^{32}P] ATP. The RNA was also 3′-end-labeled by ligation with [5′-^{32}P]pCp, as described by England et al.[30] After the end-labeling, tRNA^{Phe} was purified by electrophoresis in a 15% polyacrylamide/7 M urea gel and eluted from the gel in buffer solution. The eluted tRNA^{Phe} was precipitated with ethanol and stored in 10 mM Tris-HCl buffer (pH 7.4).

Methods

The ^{32}P end-labeled tRNA^{Phe} was renatured by heating to 90 °C or 1 min in 10 mM Tris–HCl buffer (pH 7.4) and the solution was slowly cooled to room temperature prior to use. A standard reaction mixture (20 μL) contained the end-labeled tRNA^{Phe} (3 pmol) and the enediyne drug (10–100 μM) in 10 mM Tris–HCl buffer (pH 7.4) including 5 mM $MgCl_2$ and 85 mM NaCl. When required, dithiothreitol (1 mM) was added to activate the enediyne antibiotics. The cleavage reaction was allowed to proceed at 37 °C for 30 min. After ethanol precipitation, the each sample was analyzed on a 15% polyacrylamide/7 M urea slab gel. For calibration of the gel band positions, the ^{32}P end-labeled tRNA^{Phe} was cleaved at G residues by digestion with ribonuclease T_1 or subjected to alkaline hydrolysis (non-specific) reaction. Autoradiography of the gels was

carried out on Fuji medical X-ray film at −80 °C overnight, and the autoradiograms were scanned with a laser densitometer (LKB Model 2222 Ultro-Scan XL).

Fluorescence spectral measurements were performed on a Hitachi fluorescence spectrophotometer F-3010. To a solution of 10 μM C-1027 chromophore at pH 7.4, $tRNAs^{Phe}$ (0–20 μM) were added. The C-1027 chromophore was exited at 320 nm. Apparent binding constant for the complex of C-1027 chromophore and $tRNA^{Phe}$ was evaluated by the neighbor-exclusion method of McGhee and von Hippel.[31]

Acknowledgement

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References

1. Smith, A. L.; Nicolaou, K. C. *J. Med. Chem.* **1996**, *39*, 2103.

2. Zein, N.; Poncin, M.; Nilakantan, R.; Ellestad, G. A. *Science* **1989**, *244*, 697.

3. Long, B. H.; Golik, J.; Forenza, S.; Ward, B.; Rehfuss, R.; Dabrowiak, J. C.; Matsirad, S. I. T.; Broolshire, K. W.; Doyle, T. W. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 2.

4. Sugiura, Y.; Shiraki, T.; Konishi, M.; Oki, T. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 3831.

5. Goldberg, I. H. *Acc. Chem. Res.* **1991**, *24*, 191.

6. Sugiura, Y.; Matsumoto, T. *Biochemistry* **1993**, *32*, 5548.

7. Zein, N.; Colson, K. L.; Leet, J. E.; Schroeder, D. R.; Solomon, W.; Doyle, T. W. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 2822.

8. Schroeder, D. R.; Colson, K. L.; Klohr, S. E.; Zein, N.; Langley, D. R.; Lee, M. S.; Matson, J. A.; Doyle, T. W. *J. Am. Chem. Soc.* **1994**, *116*, 9351.

9. Uesugi, M.; Sugiura, Y. *Biochemistry* **1993**, *32*, 4622.

10. Ichikawa, A.; Kuboya, T.; Aoyama, T.; Sugiura, Y. *Biochemistry* **1992**, *13*, 6784.

11. Kappen, L. S.; Goldberg, I. H. *Science* **1993**, *261*, 1319.

12. Zein, N; Shinha, A. M.; McGahrem, M. J.; Ellestad, G. A. *Science* **1988**, *240*, 1198.

13. Kusakabe, T.; Uesugi, M.; Sugiura, Y. *Biochemistry* **1995**, *34*, 9944.

14. Walker, S.; Murnick, J.; Kahne, D. *J. Am. Chem. Soc.* **1993**, *115*, 7954.

15. Gao, X; Stassinopoulos, A.; Rice, J. S.; Goldberg, I. H. *Biochemistry* **1995**, *34*, 40.

16. Okuno, Y; Iwashita, T.; Otani, T.; Sugiura, Y. *J. Am. Chem. Soc.* **1996**, *118*, 4729.

17. Battigello, J. M. A.; Cui, M.; Roshong, S.; Carter, B. J. *Bioorg. Med. Chem.* **1995**, *3*, 839.

18. Totsuka, R.; Aizawa, Y.; Uesugi, M.; Okuno, Y.; Matsumoto, T.; Sugiura, Y. *Biochem. Biophys. Res. Commun.* **1995**, *208*, 168.

19. Carter, B. J.; de Broom, E.; Long, E. C. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9373.

20. Hüttenhofer, A.; Hudson, S.; Noller, H. F.; Mascharak, P. K. *J. Biol. Chem.* **1992**, *267*, 24471.

21. Guan, L. L.; Totsuka, R.; Kuwahara, J.; Otsuka, M.; Sugiura, Y. *Biochem. Biophys. Res. Commun.* **1993**, *191*, 1338.

22. Kim, S. H.; Quigley, G. J.; Suddath, F. L.; Mcpherson, A.; Sneden, D.; Kim, J. J.; Weinzierl, J.; Rich, A. *Science* **1973**, *179*, 285.

23. Hüttenhofer, A.; Noller, H. F. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 7851.

24. Sugiura, Y.; Uesawa, Y.; Takahashi, Y.; Kuwahara, J.; Golik, J.; Doyle, T. W. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 7672.

25. Matsumoto, T.; Sugiura, Y. *Biochem. Biophys. Res. Commun.* **1994**, *205*, 1533.

26. Schimmel, P. R.; Redifield, A. G. *Annu. Rev. Biophys. Bioeng.* **1980**, *9*, 181.

27. Zeng, X.; Xi, Z.; Kappen, L. S.; Tan, W.; Goldberg, I. H. *Biochemistry* **1995**, *34*, 12435.

28. Holmes, C. E.; Carter, B. J.; Hecht, S. M. *Biochemistry* **1993**, *32*, 4293.

29. Sugiura, Y.; Kusakabe, T. In *DNA and RNA Cleavers and Chemotherapy of Cancer and Viral Diseases*; Meunier, B., Ed.; Kluwer Academic: Dordecht, 1996; pp 65–73.

30. England, T. E.; Bruce, A. G.; Uhlenbeck, O. C. *Methods Enzymol.* **1980**, *65*, 65.

31. McGhee, J. D.; von Hippel, P. H. *J. Mol. Biol.* **1974**, *86*, 469.

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