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Enediyne-Mediated Cleavage of RNA

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Abstract—RNA cleavage by enediyne anticancer antibiotics was shown to occur with no apparent sequence selectivity, but RNA structure appears to be important in those substrates where cleavage was observed. Neocarzinostatin (NCS) cleaved a wider variety of RNA substrates than either esperamicin (ESP) or calicheamicin (CAL), and dynemicin (DYN) has yet to cleave any RNA substrate tried. NCS, ESP, and CAL were all observed to cleave RNA substrates near the 5'-end, and all three compounds exhibited cleavage in single-stranded loop regions of the RNA substrates. NCS required no thiol for activation and subsequent cleavage, but ESP and CAL required addition of thiol, as expected, for cleavage to occur. An RNA hairpin substrate containing a UCCU sequence, equivalent to the TCCT sequence preferred by CAL in double-stranded DNA substrates, was cleaved by CAL, but no retention of selectivity for the UCCU site was retained by CAL in this RNA substrate. This study confirms an earlier observation that RNA is a substrate for enediyne cleavage, and indicates that nucleic acid cleaving compounds such as the enediynes could be useful probes of RNA three-dimensional structure.

Introduction

The extremely potent enediyne antitumor antibiotics, which include the neocarzinostatin chromophore,[1] the esperamicins,[2] the calicheamicins,[3] the dynemicins[4] (see Fig. 1) and more recently the kedarcidin chromophore[5] and the C1027 chromophore[6] are believed to exhibit their biological activity by degradation of double-stranded DNA. The observed biological activity *in vivo* requires reductive bioactivation followed by oxidative cleavage of (preferentially) double-stranded DNA molecules resulting in single-strand breaks and/or double strand breaks. The chemical cleavage mechanism for the enediynes involves activation by a thiol for all enediynes except C1027, which needs no thiol for activation,[7] followed by a Bergman-type rearrangement to generate a diradical species. After binding in the minor groove, the diradical then goes on to abstract one of several hydrogens at either the C4', C5' or C1' position on the deoxyribose ring of a DNA molecule.[8] Abstraction of a hydrogen leads to collapse of the deoxyribose ring and ultimately cleavage of the phosphodiester bond, liberating DNA fragments with 3'-phosphates or phosphoglycolates and 5'-aldehydes, base propenals, free base, and the production of abasic sites.[9]

NCS is capable of abstracting hydrogens at all three sites on the deoxyribose ring of DNA. In the trinucleotide sequence AGT/ACT, double-stranded lesions are produced predominantly by C4'-hydrogen abstraction of the T of AGT, and C5'-hydrogen abstraction at the T of ACT.[10] Activation by different thiols affects the partitioning of the breakdown products arising from C4'- or C5'-hydrogen abstraction. Hydrogen abstraction at the C1'-position leads to production of abasic sites, which can be identified by subsequent alkaline treatment of the DNA.

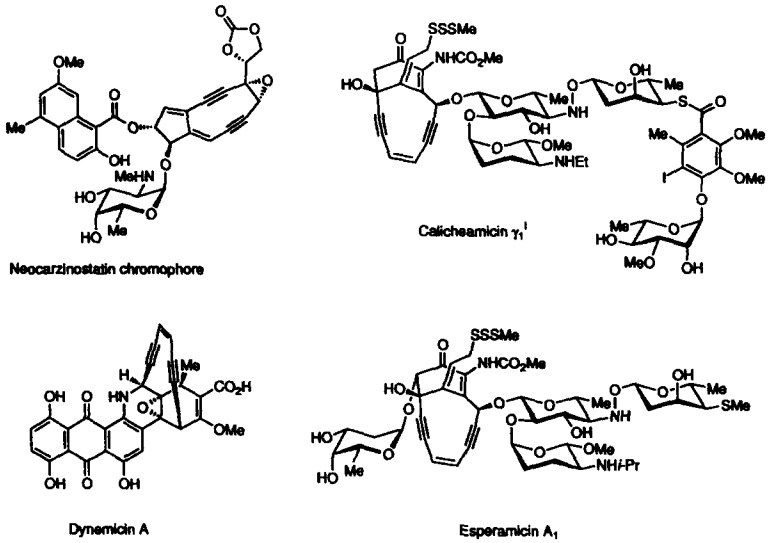


Figure 1. Structures of the enediynes neocarzinostatin (NCS), calicheamicin (CAL), esperamicin (ESP), and dynemicin (DYN).

Analysis of double-stranded cleavage sites in DNA by CAL showed C5'-hydrogen abstraction on one strand,[11] and C4'-hydrogen abstraction was observed on the complementary DNA strand.[12] On the other hand, ESP cleavage of DNA was believed to be predominantly single-stranded,[2] resulting from almost exclusive C5'-hydrogen abstraction.[13] Recent studies, however, indicate that ESP also generates double-strand lesions, with C4'-H abstraction on one DNA strand leading to cleavage of that strand, and C1'-H abstraction on the other strand leading to production of abasic sites.[14] In contrast, DYN was found to preferentially abstract the C1'-hydrogen from the deoxyribose ring, leading to strand breaks with formation of 5'- and 3'-phosphate termini.[15] Dynemicin's preferential C1'-hydrogen abstraction is distinct from the C5'-hydrogen abstraction seen with NCS, ESP, CAL, and C1027.

Sequence selectivity for these drugs is less well understood. Esperamicin and dynemicin exhibit little sequence selectivity in the DNA cleavage observed; however, calicheamicin, neocarzinostatin, kedarcidin and C1027 show varying degrees of sequence selectivity within a given DNA sequence.[1,5,7,10,16] The sequence selectivity for CAL seems to be due to a combination of the complementarity of the enediyne portion of CAL γ1 with DNA secondary structure, and stabilization of the thiobenzoate carbohydrate moiety within the DNA minor groove.[17] Other researchers have pointed out that sequence preferences observed for DNA cleavage by neocarzinostatin and calicheamicin are due to microstructural variations in the DNA geometry, rather than because of actual sequence selectivity.[18] Recent experiments by Kappen and Goldberg[18] have shown that neocarzinostatin will selectively cleave single-stranded bulged DNA bases in small, double-stranded, bent DNA structures containing bulged nucleotides, which supports structure rather than sequence as the determinant for cleavage selectivity.

The discovery that RNA is sometimes a terrific substrate for cleavage by Fe(II)·bleomycin[19,20] led us to question whether the enediynes might also cleave RNA substrates under the appropriate conditions. Published literature accounts do not include any mention of RNA cleavage by enediynes, even though nowhere in the cleavage mechanism is the 2'-position of the DNA sugar involved. However, Fe(II)·bleomycin was assumed not to cleave RNA molecules until 1989, based on several experiments with RNA substrates and BLM attempted in the early 1970s,[21,22] so it is perhaps not surprising that until recently,[23] no one had shown cleavage of RNA by the enediynes. Since RNA molecules adopt a different double-helical structure when base-paired than do double-stranded DNA molecules,[24] it is easy to rationalize that the enediynes cannot bind RNA molecules and thus no cleavage has been observed. In fact, there are substantial differences between the two double-helical conformations adopted by RNA and DNA. However, there is no chemical reason RNA cannot be a substrate for enediyne cleavage reactions, and so we decided to investigate dynemicin (DYN), esperamicin (ESP), calicheamicin (CAL), and neocarzinostatin (NCS) for their ability to cleave several structurally distinct RNA substrates.

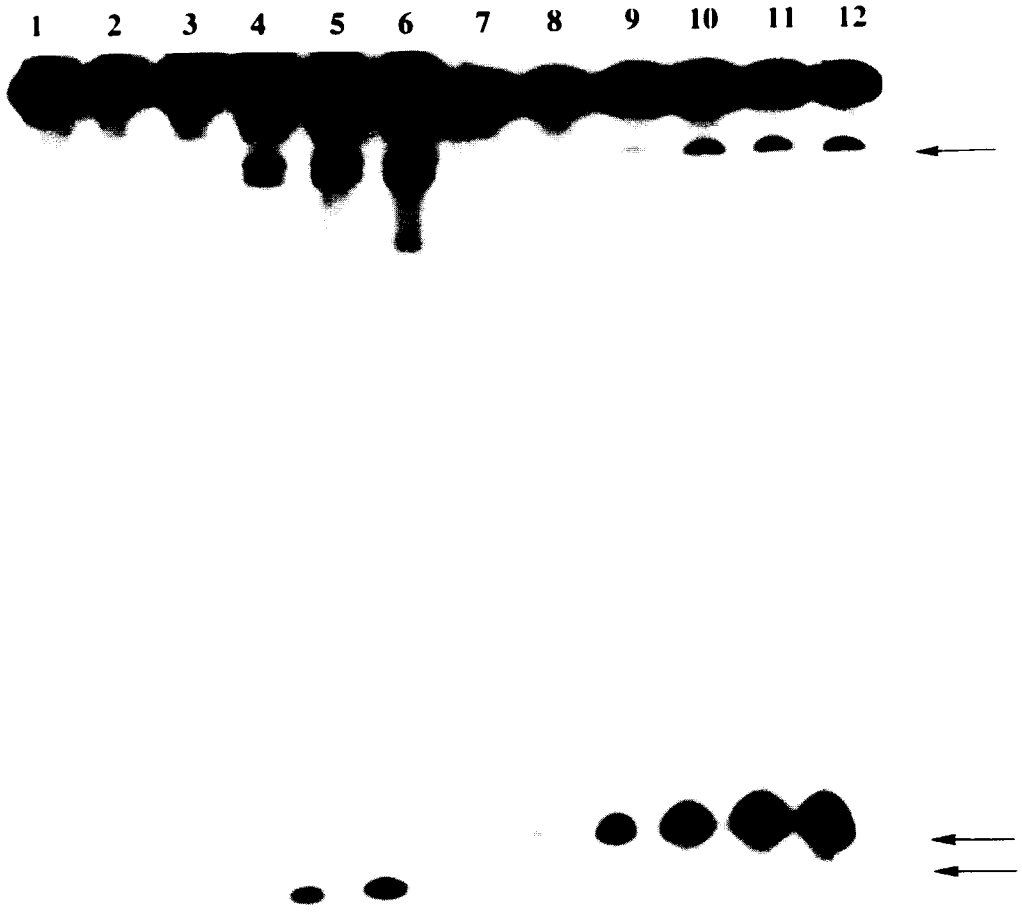
Two RNA hairpin molecules, one $tRNA^{His}$ precursor, and one proposed RNA pseudoknot were used as substrates for enediyne cleavage. We wish to report that the enediynes CAL, ESP, and NCS can cleave some RNA molecules, with all three exhibiting cleavage of two RNA hairpin structures, but little evidence for any sequence-dependence in the cleavage sites was observed. In addition, the $tRNA^{His}$ precursor and the proposed RNA pseudoknot were cleaved by NCS. Evidence for C4'-hydrogen abstraction by NCS at one site in the $tRNA^{His}$ precursor is also presented.

Results and Discussion

The enediynes, like bleomycin, cleave double-stranded DNA substrates by first abstracting a hydrogen atom from the deoxyribose ring of DNA, followed by ultimate scission of the phosphodiester bond to give multiple products including 5'-aldehydes, 3'-phosphates and phosphoglycolates, free base, and base propenals. As with BLM, there is no chemical reason the enediynes cannot cleave RNA molecules. Based on the initial success using a precursor $tRNA^{His}$ transcript as a substrate for NCS cleavage, we decided to further investigate enediyne cleavage of RNA using additional RNA substrates having distinct secondary and tertiary structures. We chose as substrates two RNA hairpins, one taken from the $tRNA^{His}$ anticodon stem/loop sequence, and another designed to contain a UCCU sequence that corresponds to the TCCT sequence preferred by CAL in cleavage of double-stranded DNA substrates. The other RNA substrate investigated was a proposed RNA pseudoknot taken from the sequence of an HIV-1 mRNA for a selenium-binding protein.[25] In addition to double- and single-stranded regions, pseudoknots are believed to contain a triple-strand region, making them potentially interesting substrates for the enediyne molecules.

Precursor $tRNA^{His}$ transcript

As observed previously,[23] the precursor $tRNA^{His}$ transcript is cleaved by NCS in the anticodon loop of the tRNA molecule. Figure 2A shows an autoradiogram of NCS cleavage of generally-labeled $tRNA^{His}$ precursor in lanes 1–6 (labeled at every cytosine with α-^{32}P-CTP), and NCS cleavage of 5'-end labeled $tRNA^{His}$ precursor in lanes 7–12. The previously observed cleavage site in the anticodon loop is indicated by the top arrow, and a new site near the 5'-end of the $tRNA^{His}$ substrate is indicated near the bottom with two arrows. The cleavage sites are also shown by arrows in Figure 2B, which is a depiction of the sequence and secondary structure of the $tRNA^{His}$ precursor juxtaposed near the gel. Because the generally labeled tRNA contains a 5'-triphosphate versus just a single 5'-phosphate for the 5'-end labeled tRNA, the two substrates do not co-migrate on the gel, nor do the cleavage products.



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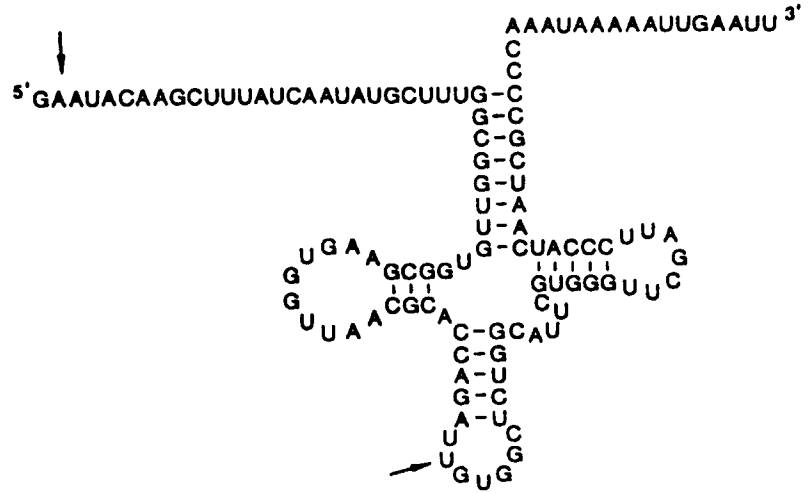

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Figure 2. Cleavage of $tRNA^{His}$ by the neocarzinostatin chromophore. (A) Lanes 1–6 contain ~20,000 cpm of high-specific activity $tRNA^{His}$ precursor; lanes 7–12 contain ~20,000 cpm of 5'- end labeled $tRNA^{His}$ precursor. All lanes contain 5 mM Mg^{2+} and 5 mM NaH_2PO_4, (pH 7.0). Lanes 2–6 and lanes 8–12 contain 20, 40, 60, 80, or 100 μM NCS chromophore, respectively. No thiol was added for activation, and all reactions were incubated 1 h at 25 °C; (B) secondary structure of precursor $tRNA^{His}$ showing sites of NCS cleavage.

The cleavage observed is remarkably selective, and, at least for cleavage at A2 near the 5'-end, relatively efficient. For the generally labeled substrate, cleavage at the anticodon U60 site generates two products, one 59 bases long (the 5'-half of the tRNA), and the other 58 bases long (the 3'-half of the tRNA) while the U60 at the cleavage site itself is invisible on the gel. Lanes 4–6 clearly show the two expected bands. For cleavage at the A2 near the 5'-end, two bands are also expected, one near the bottom (a triphosphate G1 product) and the other just below the intact tRNA band (corresponding to the tRNA missing the 5'-terminal triphosphate GA). Again, both bands are visible in lanes 4–6, with the single triphosphate Gl product near the bottom, and the other band running just below the starting material.

Lanes 7–12 show the same reaction for a 5'-end labeled substrate, where cleavage at each site generates only one band because the other product does not contain a radioactive label and so is thus invisible in the autoradiogram. As expected, cleavage at U60 and A2 are observed as single bands. Interestingly, CAL, ESP, and DYN were unable to cleave this tRNA substrate. In addition, using mature $tRNA^{Phe}$ from *E. coli* as a substrate, none of the four enediynes tested produced

Figure 3. Identification of the terminal 5'-pGp NCS cleavage product of $tRNA^{His}$. Lanes 1 and 2 contain authentic 5'-^{32}P-labeled pGp with and without 80 μM NCS. Lanes 3–7 contain ~20,000 cpm of 5'-end labeled $tRNA^{His}$ precursor. All lanes contain 5 mM Mg^{2+} and 5 mM NaH_2PO_4, (pH 7.0). Lanes 3–6 contain 160, 120, 80, or 40 μM NCS chromophore, respectively. Lane 7 is the control RNA lane without NCS. No thiol was added for activation, and all reactions were incubated 1 h at 25 °C.

cleavage. This is reminiscent of previous results with Fe(II)·BLM indicating that cleavage is observed with some tRNA substrates and not others.[19,20]

Using 5'-^{32}P-labeled pGp as a standard, the cleavage product resulting from NCS cleavage at A2 was identified as 5'-pGp, as indicated by co-migration of the cleaved product and the authentic standard (see Fig. 3, lanes 1 and 2 compared to lanes 3–6). This indicates C4'-hydrogen abstraction is occurring at this site, although with this substrate there is no evidence for production of any 3'-phosphoglycolate product. Also, contrary to literature reports for NCS cleavage of DNA,[8,9,10,26] no thiol is required for the observed RNA cleavage. Addition of thiols such as DTT does not increase NCS cleavage of RNA. Thus, as suggested by Kappen and Goldberg,[18] and supported by Lamothe and Fuchs using model studies with α-hydroxynaphthoate esters,[27] the self-activation of NCS under these conditions appears to be occurring spontaneously.

RNA hairpin transcripts

Because tRNA molecules are known for their three-dimensional structure, we were interested in whether an RNA molecule with only secondary structure would be a substrate for cleavage by enediynes. Thus, we synthesized a small 14-mer RNA hairpin, based on the first 12 nucleotides in the anticodon stem/loop region of the tRNA^{His} substrate. We then treated this RNA hairpin with NCS, ESP, CAL, and DYN. As shown in Figure 4, in addition to NCS cleavage (lanes 2 and 3), both ESP and CAL also cleave this RNA substrate (see lanes 4 and 5 and 6 and 7, respectively), but DYN does not. The positions of cleavage are indicated to the right of the gel in the sequence and secondary structure shown for this substrate. Notice that between the three drugs, every position in the loop is cleaved, and NCS, CAL, and ESP all exhibit cleavage in both single- and double-stranded regions of the RNA. These results demonstrate that tertiary structure is not necessary for

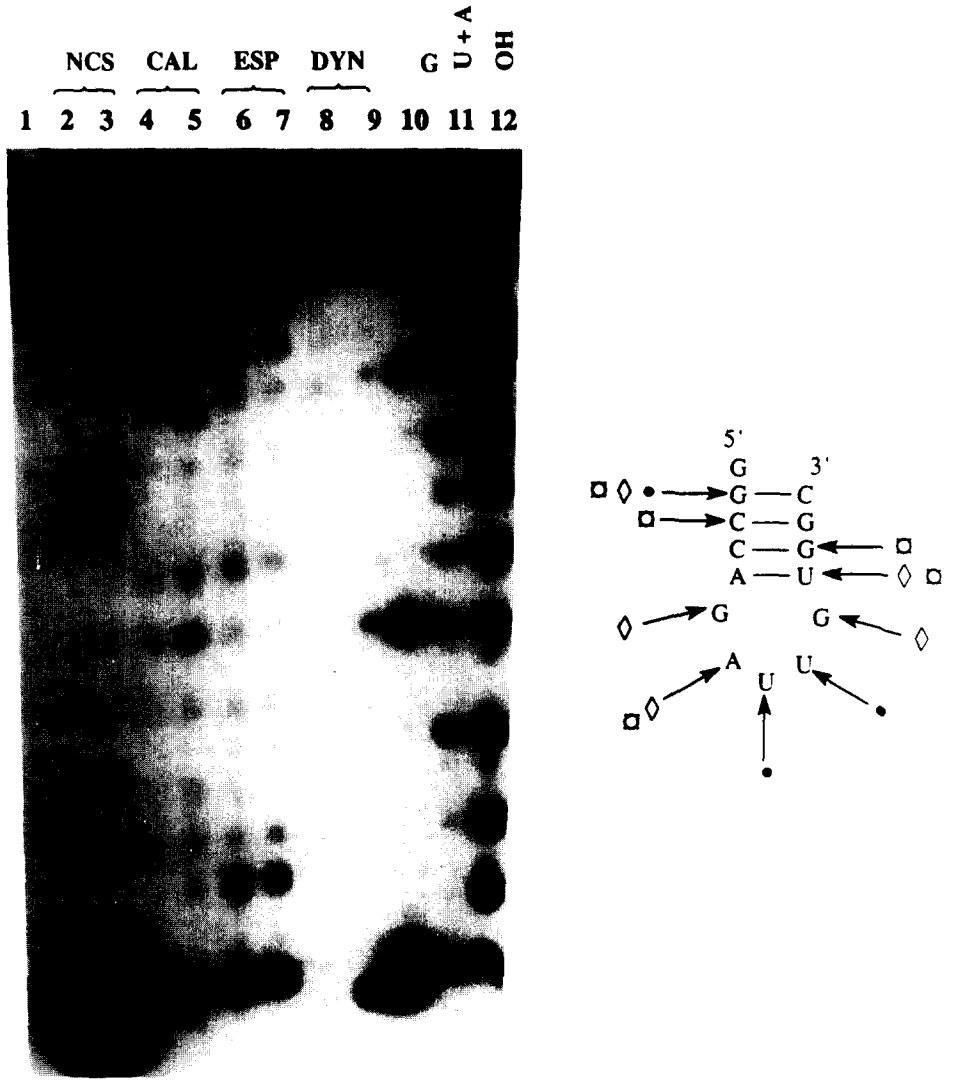

Figure 4. Cleavage of a 14-mer RNA hairpin by NCS, CAL, ESP, and DYN. All lanes contain ~20,000 cpm of 5'-end labeled 14-mer RNA hairpin, 5 mM Mg^{2+}, and 5 mM NaH_2PO_4, (pH 7.0). Lanes 2 and 3, 4 and 5, 6 and 7, and 8 and 9 contain 40 or 80 μM NCS, CAL, ESP, and DYN respectively. Lanes 4–9 also contain 5 mM DTT. All reactions were incubated 1 h at 25 °C. ◊ CAL, □ ESP, ● NCS.

efficient cleavage by NCS. In fact, the smaller 14-base RNA hairpin substrate appears to be a better substrate in general for cleavage by NCS, ESP, and CAL.

Although the cleavage requires 40 and 80 μM enediyne concentration and thus is not as efficient as that seen with DNA substrates, there are nonetheless several interesting aspects to the RNA cleavage observed. Namely, NCS, CAL, and ESP exhibit strong cleavage at G2 near the 5'-end of the RNA. Using 5'-^{32}P-labeled GMP (pGp) as a marker, the strong cleavage band generated at this site by NCS was identified as pGp, making the band running just ahead of it the corresponding 3'-phosphoglycolate product. As seen with the $tRNA^{His}$ substrate (*vide supra*), this indicates the chemistry for NCS is predominantly C4'-hydrogen abstraction at this position. Note, however, that for this substrate, 3'-phosphoglycolate product is readily observed, unlike the NCS cleavage observed with the $tRNA^{His}$ precursor.

Having identified an RNA substrate cleaved by CAL, we decided to investigate the sequence selectivity observed for CAL and double-stranded DNA substrates, and determine whether CAL retains this sequence selectivity when it cleaves RNA. Thus, we designed and synthesized an RNA hairpin containing UCCU in the stem region of the hairpin, the RNA equivalent to TCCT. As before, of the four enediynes tested, NCS, CAL, and ESP all exhibited cleavage, but DYN did not (see Fig. 5). Both CAL and ESP showed cleavage at a number of sites, including sites in both single- and double-stranded regions of the RNA hairpin, but CAL did not retain any apparent selectivity for the 'UCCU' site. In DNA, the sequence selectivity of CAL for TCCT sequences arises from the complementarity of the enediyne region of CALγ1 and the DNA secondary structure, as well as from stabilization of the thiobenzoate carbohydrate moiety within the minor groove of DNA.[17] Because RNA forms an A-form helix, the minor groove of RNA is quite different from that of

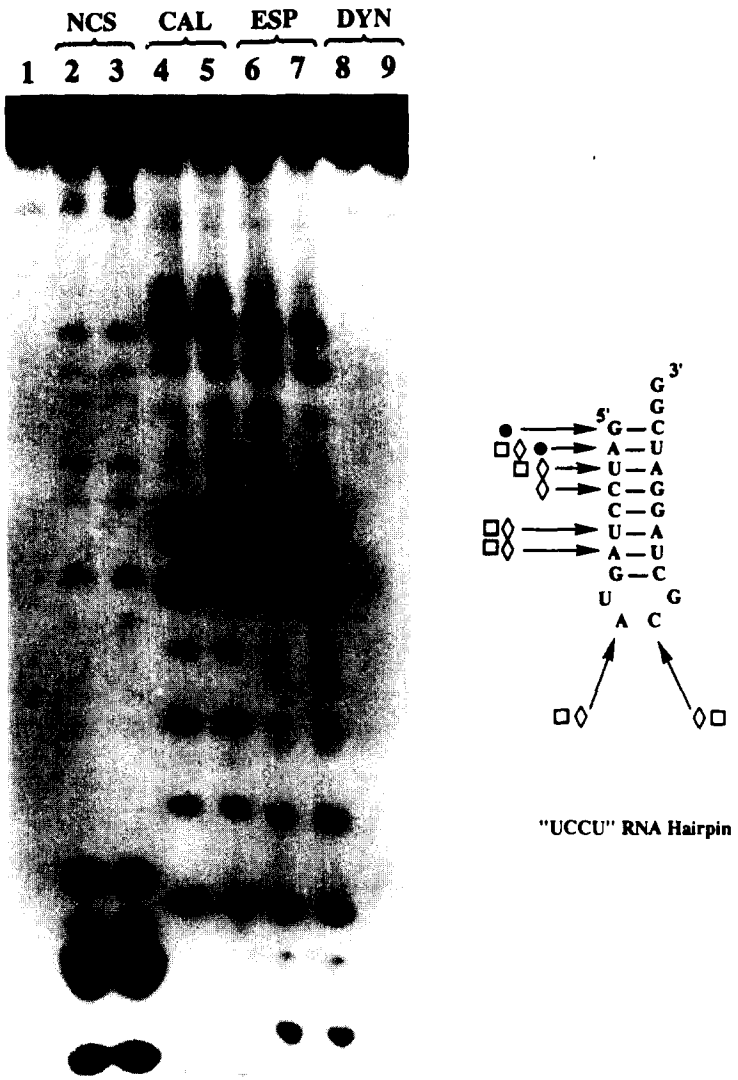

Figure 5. Cleavage of a 22-mer 'UCCU' RNA hairpin by NCS, CAL, ESP, and DYN. All lanes contain ~20,000 cpm of 5'-end labeled 22-mer 'UCCU' RNA hairpin, 5 mM Mg^{2+}, and 5 mM NaH_2PO_4, (pH 7.0). Lanes 2 and 3, 4 and 5, 6 and 7, and 8 and 9 contain 40 or 80 μM NCS, CAL, ESP, and DYN respectively. Lanes 4–9 also contain 5 mM DTT. All reactions were incubated 1 h at 25 °C. ◊ CAL, ¤ ESP, ● NCS.

DNA. Thus, the same complementarity and stabilization is apparently not occurring with this UCCU sequence as no selectivity by CAL for the UCCU site in this substrate was observed. Surprisingly, NCS cleaved almost exclusively near the 5'-end of the RNA, and not a single site was observed in the single-stranded loop region. It is also noteworthy that all the cleavage observed by the three enediynes is essentially on one side of the RNA hairpin. This suggests that no double-stranded cleavage is taking place. Analysis of the observed cleavage does not provide much insight into the chemistry of cleavage. However, based only on the bands observed, one can see that NCS is generating different chemical products from either CAL or ESP, as evidenced by the four bands near the bottom of the gel (lanes 2 and 3) which do not co-migrate with any of the cleavage products generated by CAL or ESP (lanes 4 and 5 and 6 and 7, respectively). Also, while many of the cleavage products generated by CAL and ESP do co-migrate, careful examination of the bands indicates that several products are generated by CAL and ESP which do not co-migrate. Treatment of NCS-generated RNA cleavage products from this substrate with piperidine indicates the presence of abasic sites, again indicating C4'- (and C1'-?) chemistry (unpublished results), but we have not done this experiment for the other enediynes so we cannot say the same for CAL and ESP.

RNA pseudoknot transcript

The final RNA substrate that we investigated was a proposed RNA pseudoknot structure. RNA pseudoknots, like tRNA molecules, have a high degree of tertiary structure. Besides containing two single-stranded loop regions and two double-stranded stem regions, they are also believed to contain a possible triple-strand region.[28] In addition, most pseudoknot sequences that have been identified serve as translational regulators of some kind.[29–36] Even more importantly, many pseudoknot sequences have been identified in viral mRNA sequences, in particular in retrovirus mRNA sequences.[32] We chose an RNA sequence from the mRNA of a selenium-binding protein in HIV-1, which has been proposed to fold into a pseudoknot,[25] as a substrate for enediyne cleavage.[37] Upon treatment with NCS (and Fe(II)·BLM), three NCS cleavage sites were observed, as seen in Figure 6. As with all RNA substrates utilized to date, NCS cleavage near the 5'-end of the RNA molecule was observed. Two other NCS cleavage sites were also identified, and these are seen in the second double-stranded stem of the pseudoknot, nearer to the 3'-end of the molecule. One NCS site is directly opposite the only BLM cleavage site observed for this RNA. The observed cleavage is Mg^{2+} dependent, and is completely abolished in the absence of Mg^{2+} or spermidine, both molecules known to stabilize the structure of RNA in solution (data not shown). Of particular interest are the sites of cleavage. Fe(II)·BLM- and enediyne-mediated cleavage in the stem 2 region suggests that the 3'-end of the pseudoknot is double-stranded, thus supporting the proposed pseudoknot structure, because in DNA, enediynes require double-stranded substrates,[8] or at least, double-stranded regions in single-stranded DNA,[18] for cleavage to occur. The cleavage sites produced by NCS and Fe(II)·BLM suggest that drugs such as the enediynes and BLM could be used as structural probes to help verify the existence of pseudoknot structures.

Conclusion

We have shown that the enediynes NCS, CAL, and ESP cleave a variety of RNA substrates, including tRNA transcripts, RNA hairpins, and a proposed RNA pseudoknot. NCS cleaved all RNA molecules tested, CAL and ESP cleaved the RNA hairpins, and DYN did not cleave any RNA substrate. This lack of cleavage by DYN could reflect the preference for C1'-hydrogen abstraction, and in RNA substrates, the C1'-position may not be accessible to the diradical. In particular, the sugar pucker in RNA is generally C3'-*endo*, versus C2'-*endo* for DNA. Therefore, the hydrogens available for radical abstraction may not be the same in RNA as in DNA. Also, because the equivalent minor groove in RNA is very shallow, and the equivalent major groove is very deep and narrow, it is not altogether clear that the enediynes are still binding in the 'minor' groove of RNA to produce the observed cleavage. However, the cleavage results with NCS and the tRNA precursor and small hairpin RNA indicating C4'-hydrogen abstraction provide tentative evidence that the equivalent minor groove is occupied by the enediynes in RNA, suggesting that the chemistry of cleavage is also similar to that observed for DNA.

All observed cleavage of RNA by NCS involves a thiol-independent activation, while RNA cleavage by CAL and ESP requires addition of thiol. Cleavage in both single- and double-stranded regions of RNA was observed, but double-stranded cleavage has not been verified. In addition, CAL, ESP, and particularly NCS show strong selectivity in the cleavage observed, although CAL does not retain its selectivity for a UCCU sequence, equivalent to the TCCT sequence preferred with DNA substrates. In addition, strong NCS cleavage sites were observed in all RNA substrates near 5'-terminal bases, and C4'-hydrogen abstraction at these sites leads to the production of 5'-pGp in the $tRNA^{His}$ precursor and the 14-mer RNA substrate.

Although the pseudoknot structure and small RNA hairpin structure have not been rigorously established, experimental data support both structures as drawn. Enzymatic mapping and computer analysis support the pseudoknot conformation, as does the observed cleavage by NCS and Fe(II)·BLM. The small 14-mer RNA hairpin was predicted by computer analysis using the STAR program[38] to be stabilized by –3.2 kcal mol^{-1}, and this RNA is also a substrate for double-strand-specific ribonuclease V1. In addition, it is known that RNA oligonucleotides as small as 10–12 bases form stable hairpin structures.[39] Thus, the results presented

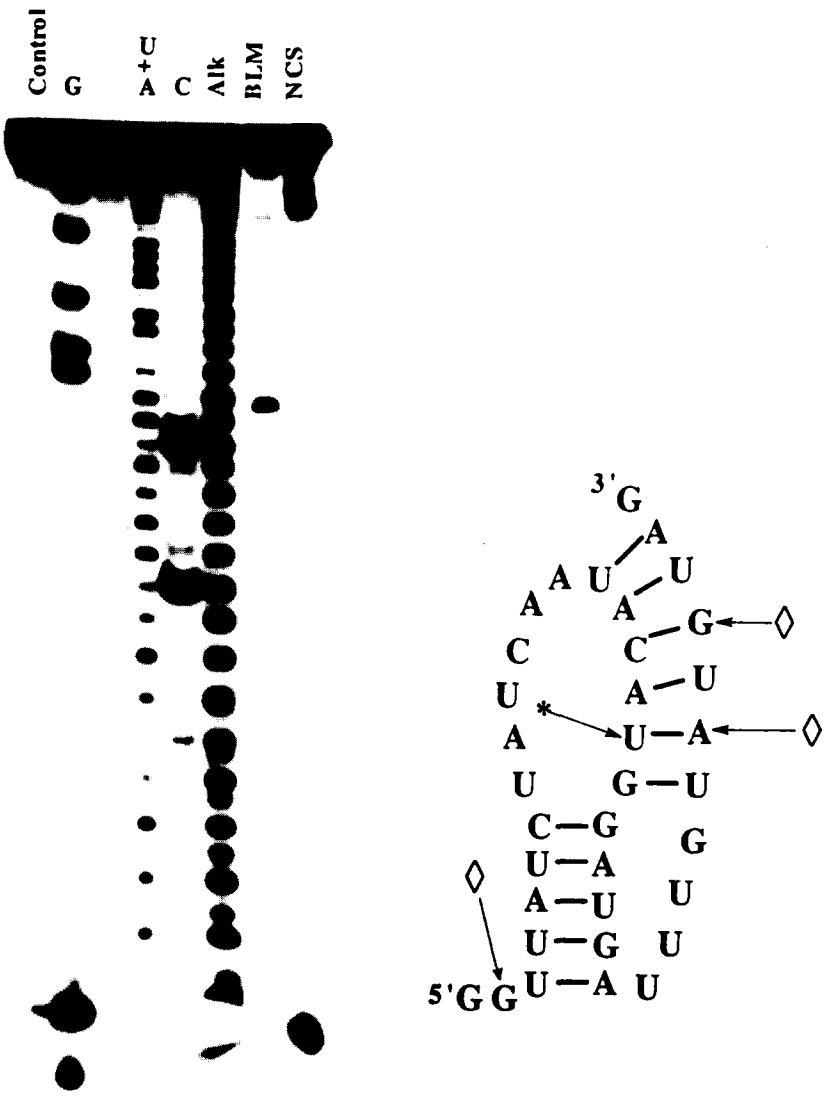

Figure 6. NCS and BLM cleavage of a 35-mer RNA pseudoknot from an HIV selenium-binding protein mRNA sequence. All lanes contain ~10,000 cpm of 5'-end labeled 35-mer RNA pseudoknot, and the BLM and NCS reaction lanes also contained 5 mM Mg^{2+}, 5 mM NaH_2PO_4, (pH 7.1), and 40 μM Fe(II)·BLM or 100 μM NCS, respectively. The BLM and NCS cleavage reactions were incubated 1 h at 25 °C. ◊ NCS, * BLM.

support the idea that enediyne recognition and cleavage of nucleic acids has more to do with secondary and tertiary structure than sequence, particularly for RNA substrates, and that structural variation in RNA molecules is a major factor impacting the presence (or absence) of enediyne cleavage sites in nucleic acid molecules.

The single-strand cleavage of the $tRNA^{His}$ precursor and RNA hairpins by NCS, requiring no added thiol for activation, is very similar to the results obtained by Goldberg and coworkers with a DNA sequence from the *E. coli* $tRNA^{Phe}$ molecule.[18] In addition, for all the single-stranded cleavage seen with NCS, CAL, and ESP, the sites of cleavage are not a 2-base bulge, or a single-stranded base adjacent to a double-stranded region, but rather several of the cleavage sites are situated in the middle of 4, 5, or 7-base single-stranded loop regions of the RNA molecules. This degree of cleavage in single stranded regions has not been reported previously for enediyne cleavage of nucleic acid substrates. The most likely explanation for the observed cleavage in the loops of RNA hairpins is that the enediynes are binding to the double-stranded stem regions of the hairpins, and the diradicals are able to abstract hydrogens from the sugars in the loop regions nearby. The lack of cleavage in any $tRNA^{His}$ loop region except the anticodon loop may reflect the inaccessibility of the sugars and bases in those stem/loop regions due to their involvement in tertiary interactions with other regions of the tRNA molecule. The anticodon loop in tRNA molecules is known to be

a completely single-stranded region of the molecule which does not contain any tertiary interactions with other regions of the tRNA molecule. This is consistent with NCS cleavage being observed only in this stem/loop region and no other stem/loop.

Curiously, with every RNA substrate studied, including several not discussed here, NCS exhibits highly selective cleavage at the 5'-terminal and/or 5'-penultimate base. In fact, with time-dependent reactions, NCS actually removes the 5'-phosphate label, consistent with cleavage at the 5'-terminal base, causing the percentage of observable cleavage to decrease with time. It is possible that the electronic environment of the 5'-end phosphate on the RNA is particularly appealing to the NCS chromophore, but just how this sorts out on a molecular level is unclear at this time.

Enediyne-mediated cleavage of RNA requires further investigation to better understand recognition and cleavage of RNA, elucidate the chemistry of cleavage, and to investigate the possibility for therapeutic relevance. To that end, studies of enediyne cleavage of numerous other RNA molecules, including hairpins, additional pseudoknots, and different tRNAs, both as transcripts or mature molecules, are ongoing.

Experimental

Materials

Eco R1, calf intestine alkaline phosphatase (CIPase) and T4 polynucleotide were from U.S. Biochemicals. Kodak XAR-2 film was from Sigma. Plasmid DNA pBR322, RNasin® ribonuclease inhibitor, and SP6 RNA polymerase were from Promega. Radioisotopes α-^{32}P-CTP (~3,000 Ci mmol^{-1}) and γ-^{32}P-ATP (~7,000 Ci mmol^{-1}) were from ICN Radiochemicals. Dynemicin A, esperamicin A1, and bleomycin were from Bristol-Myers Squibb; calicheamicin was from American Cyanamid; and neocarzinostatin was a gift from the National Cancer Institute. Plasmid pSP64 encoding for the precursor tRNA^{His} was a gift from Christopher J. Green from SRI International.

Oligonucleotides used for the Uhlenbeck method of *in vitro* RNA transcription[40] were synthesized on a Cyclone II DNA synthesizer from Milligen/Biosearch using a 0.2 μmol scale synthesis and standard phosphoramidite chemistry followed by concentrated ammonia deprotection and purification on Milligen Oligo columns. The DNA oligomers were quantitated using UV spectrophotometry at 260 nm and then checked for purity by gel electrophoresis or HPLC analysis. The DNA was annealed by combining equimolar amounts of each strand and then heating at 65 °C for 5 min in 10 mM Tris–HCl, 1 mM EDTA, pH 7, followed by slow cooling to room temperature.

Methods

Preparation of 5'-end labeled RNA substrates.
Precursor tRNA^{His}. The precursor tRNA^{His} substrate was prepared by first linearizing plasmid pSP64 (pSP64-His) encoding the tRNA^{His} precursor using the restriction enzyme Eco R1. The reaction volume was 500 μL total containing 60 μg of pSP64-His plasmid, 100 units *Eco* Rl, 100 mM NaCl, 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, and 1 mM DTT. The DNA was quantified using UV spectrophotometry and a 1.5 mg mL^{-1} stock solution of the linear plasmid was prepared. The linearized pSP64-His was then used as a substrate for *in vitro* RNA transcription. The reaction volume was 100 μL total containing 40 mM Tris– HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 100 units RNasin® ribonuclease inhibitor, 0.5 mM ATP, GTP, CTP, and UTP, 3 μg of linear pSP64-His plasmid, and 40 units SP6 RNA polymerase. The reaction was incubated overnight at 37 °C. The RNA transcript was then purified using a Nensorb-20 column. The purified pre-tRNA^{His} was then quantified using UV spectrophotometry, and dried under vacuum centrifugation. The purified pre-tRNA^{His} transcript was then dephosphorylated with calf intestinal alkaline phosphatase (CIPase). The reaction volume was 100 μL total containing ~16 μg of tRNA^{His}, 26 units of CIPase, 0.5 M Tris–HCl (pH 8.5), and 0.1 mM EDTA. The reaction was incubated 30 min at 37 °C. Then, another microliter of CIPase (26 units) was added, and the reaction was incubated 30 min more at 37 °C. The reaction was stopped by addition of 10 × STE buffer (1 M NaCl, 100 mM Tris–HCl, 10 mM EDTA, pH 8.0) to a final concentration of 1 ×, and SDS to a final concentration of 1%. The mixture was then heated 15 min at 65 °C. Calf intestinal alkaline phosphatase was removed by phenol extraction followed by several diethyl ether extractions to remove the phenol. The RNA was then precipitated at –80 °C overnight by addition of 0.3 M sodium acetate (final concentration) and 2.5 volumes of absolute ethanol. The precipitated RNA pellet was recovered by centrifugation and dried under vacuum centrifugation.

Next, the pre-tRNA^{His} was 5'-end-labeled witb γ-^{32}P-ATP. The reaction volume was 50 μL total containing ~10 μg of the pre-tRNA^{His}, 60 units of T4 polynucleotide kinase, 50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM DTT, 10 mM 2-mercaptoethanol, and 1 mCi γ-^{32}P-ATP. The reaction was incubated 1 h at 37 °C and quenched by addition of 30 μL of polyacrylamide loading buffer [80% (v/v) deionized formamide, 50 mM Tris–borate, pH 8.3, 1 mM EDTA, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue]. The sample was then loaded onto a 20% polyacrylamide denaturing gel (7 M urea) and run 4 h at 350 V in 1 × TBE (89 mM Tris–borate, 2 mM EDTA, pH 8.3). The band corresponding to the 5'-end labeled tRNA^{His} was visualized by autoradiography, excised, and the tRNA was recovered by overnight elution in crush and soak buffer [0.5 M ammonium acetate, 10

mM magnesium acetate, 1 mM EDTA (pH 8.0), 0.1% SDS], followed by overnight ethanol precipitation at –80 °C and then centrifugation. The RNA pellet was then dried under vacuum centrifugation and a stock solution was prepared to give ~20,000 cpm μL^{-1}.

RNA hairpin and RNA pseudoknot substrates. Following automated synthesis and annealing of the DNA templates, the Uhlenbeck method of *in vitro* transcription[40] was used to synthesize the RNA hairpin and RNA pseudoknot substrates. Reactions were essentially the same as described above for the precursor $tRNA^{His}$ molecule, except T7 RNA polymerase was used instead of SP6 RNA polymerase.

Preparation of high specific activity $tRNA^{His}$ transcript ('Generally-Labeled' $tRNA^{His}$). Transcription reactions were 20 µL total volume containing 40 mM Tris–HCl (pH 7.50), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 13 units RNasin® ribonuclease inhibitor, 0.5 mM ATP, GTP, and UTP, 12.5 µM CTP, 1.5 µg of DNA template, 20 units of T7 RNA polymerase, and 50 µCi of α-^{32}P-CTP. The reaction was incubated for 6–12 h at 37 °C, quenched by the addition of 10 µL of loading buffer and run 2 h at 350 V in 1 × TBE buffer. The results were visualized by autoradiography after a 1 min exposure to Kodak XAR-2 film and the RNA recovered by excision from the gel followed by elution in crush and soak buffer overnight, then ethanol precipitation at –80 °C and centrifugation. The RNA pellet was then dried under vacuum centrifugation and a stock solution prepared containing ~20,000 cpm μL^{-1}.

Cleavage of various RNA molecules by NCS, CAL, ESP, and DYN. Reaction volumes were 5 µL total containing ~20,000 cpm of either 5'-end labeled RNA or high specific activity RNA, 20 mM DTT, 5 mM sodium phosphate (pH 7.0), and enediyne drug at concentrations varying between 20 µM and 200 µM. NCS reactions were also frequently carried out in the absence of DTT. Reactions were incubated 1 h at room temperature and quenched by addition of 3 µL polyacrylamide loading buffer. The samples were loaded onto a 20% polyacrylamide denaturing (7 M urea) sequencing gel (0.4 mm) and run ~5 h at 2,000 V in 1 × TBE buffer. Results were visualized by autoradiography after overnight exposure to Kodak XAR-2 X-ray film.

RNA sequencing reactions. Aliquots of 5'-end labeled RNA containing ~20,000 cpm were sequenced by treatment with ribonucleases T_1, U_2, Cl_3, Ym, and BC, and also by treatment under alkaline conditions using standard protocols described with the U.S. Biochemicals RNA sequencing kit.[41]

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37. Other studies in our laboratory with this RNA substrate using enzymatic and chemical mapping techniques support the pseudoknot structure (see Mei Cui Masters Thesis, *Characterization of RNA Pseudoknots*, University of Toledo 1995).

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41. See United States Biochemicals Corporation RNA Sequencing Kit, Nuclease Method, and references therein.

Note added in proof. During correction of this manuscript an article on a related topic, *Bulge-Specific Cleavage in Transactivation Response Region RNA and Its DNA Analogue by Neocarzinostatin Chromophore* appeared in print by H. Goldberg, *Biochemistry* **1995**, *34*, 5997.

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