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DNA Alkylation by Leinamycin Can Be Triggered by Cyanide and Phosphines

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Abstract—Previous work has shown that alkylation of DNA by the antitumor agent leinamycin (**1**) is potentiated by reaction of the antibiotic with thiols. Here, it is shown that other soft nucleophiles such as cyanide and phosphines can also trigger DNA alkylation by leinamycin. Overall, the results suggest that reactions of cyanide and phosphines with leinamycin produce the oxathiolanone intermediate (**2**), which is known to undergo rearrangement to the DNA-alkylating episulfonium ion **4**.

Leinamycin (**1**) is a potent antitumor antibiotic that contains a unique 1,2-dithiolan-3-one 1-oxide heterocycle.[1,2] Reaction of thiols with this sulfur heterocycle in leinamycin initiates chemistry that leads to oxidative DNA damage *and* DNA alkylation.[3–6] Thiol-triggered DNA alkylation by leinamycin involves initial conversion of the parent antibiotic to the oxathiolanone form **2**,[5,7] followed by rearrangement to the episulfonium ion **4** that efficiently modifies double-stranded DNA at the N7-position of guanine residues (Scheme 1).[5] The same DNA-alkylating episulfonium ion (**4**) is also produced by a slower, thiol-independent pathway initiated by hydrolysis of leinamycin's sulfur heterocycle (Scheme 1).[8,9] Although hydrolytic and thiolytic activation of leinamycin occur by different chemical pathways, both processes are believed to proceed via the intermediacy of the same crucial oxathiolanone species (**2**).

In order to increase our overall understanding of leinamycin's fundamental chemical properties, we investigated the ability of nucleophiles other than thiol to trigger DNA alkylation by this antibiotic. So-called 'soft' nucleophiles such as thiols, phosphines, cyanide, and iodide typically display high 'thiophilicity'. In other words, these nucleophiles react readily with electrophilic sulfur centers.[10] Therefore, we anticipated that various soft nucleophiles might react with leinamycin's electrophilic sulfur heterocycle in a manner similar to thiols—that is, in a manner that potentiates the DNA-alkylating properties of the antibiotic. Here we report that phosphines and cyanide are able to trigger DNA alkylation by the antitumor antibiotic leinamycin. Results of our chemical model reactions and DNA-damage experiments suggest that reaction of cyanide and phosphines with leinamycin affords the critical oxathiolanone intermediate (**2**), which subsequently undergoes rearrangement to the DNA-alkylating episulfonium ion **4**.

The results of previous chemical model reactions suggested that phosphines might be capable of triggering DNA alkylation by leinamycin. Specifically, we found that the reaction of triphenylphosphine with 3*H*-1,2-benzodithiol-3-one 1-oxide (**6**) provides a good yield of the cleft-shaped dimer **8**.[11,12] In the context of the current work, it is important to note that this reaction is most easily rationalized[11] by invoking the involvement of the 3*H*-2,1-benzoxathiol-3-one intermediate **7** (Scheme 2), which corresponds to the 'activated form' of leinamycin (**2**). Importantly, past work has shown that **6** provides an accurate model for predicting the chemical reactivity of leinamycin's sulfur heterocycle.[5,7]

As part of the current work chemical model reactions were performed and the results suggest that cyanide can trigger DNA alkylation by leinamycin. Treatment of **6** with sodium cyanide (10 equiv) in dry tetrahydrofuran containing 18-crown-6 (0.07 equiv) affords 2-thiocyanatobenzoic acid (**10**) as the only detectable product (by TLC). The compound was isolated in 75% yield as its methyl ester derivative after treatment of the reaction with diazomethane.[13,14] We envision that **10** is formed

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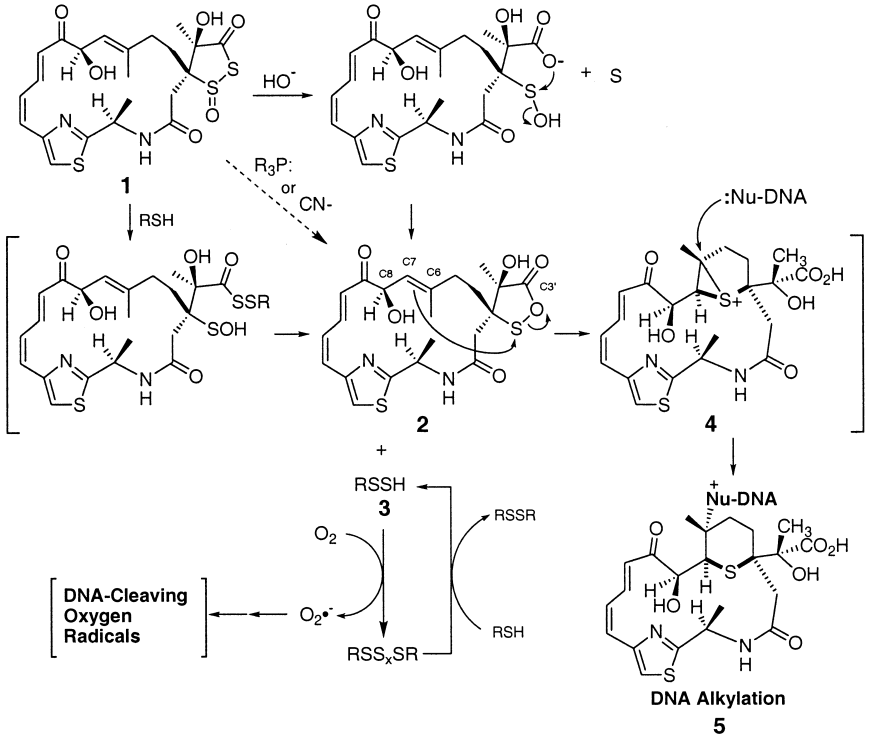
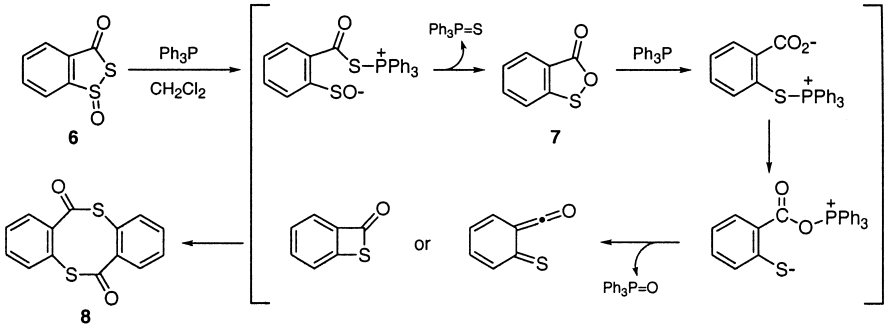
by a mechanism involving initial attack of the soft cyanide nucleophile on the soft central sulfur atom of **6** to afford the sulfenic acid derivative **9**, followed by cyclization to yield 3*H*-2,1-benzoxathiol-3-one (**7**) and one equivalent of thiocyanate ($^{-}$SCN) (Scheme 3). Attack of a second equivalent of cyanide on **7** is expected to yield 2-thiocyanatobenzoic acid (**10**). Consistent with the proposed mechanism, the reaction does not proceed to completion when less than two equivalents of cyanide are employed. In addition, we find that one equivalent of thiocyanate is produced in the reaction (quantitatively detected by the characteristic absorbance of Fe^{3+}–thiocyanate complexes at 450 nm).[15] The results of this model reaction suggest that attack of cyanide ion on leinamycin will produce the 'activated' oxathiolanone form of the antibiotic (**2**).

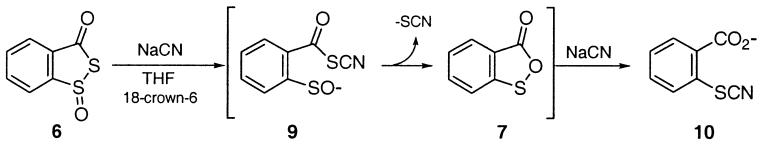
Our first evidence that phosphines and cyanide can, in fact, trigger DNA damage by leinamycin was obtained using a plasmid-based assay that can detect DNA alkylation. In this assay, supercoiled plasmid DNA that ha been exposed to an alkylating reagent is subjected to a 'plasmid-compatible' Maxam–Gilbert-type workup involving warming the alkylated DNA in the presence of *N*,*N*′-dimethylethylenediamine (DMEDA, 100 mM, 1.5 h, 37 °C) or putrescine (100 mM, 6 h, 37 °C). Thermal treatment induces loss of certain alkylated bases from the DNA (e.g., purines alkylated at N7) to yield



Scheme 1.



Scheme 2.



Scheme 3.

abasic sites,[16] which are subsequently converted to strand cleavage sites by the reaction with the polyamine (DMEDA or putrescine).[17,18] The number of resulting DNA-cleavage events can be quantitatively assessed using agarose gel electrophoresis to monitor the conversion of supercoiled plasmid (form I) into the open circular form (form II) that occurs when the supercoiled plasmid sustains a single-strand break.

Using this plasmid-based alkylation assay, we find that treatment of supercoiled plasmid DNA with leinamycin (500 nM) and Ph_3P (10–250 μM) or potassium cyanide (0.5–5 mM) for 6 h at 24 °C, followed by the DMEDA or putrescine workup, leads to significantly more strand breaks than does treatment of the DNA with leinamycin alone (Figs. 1 and 2). Cleavage efficiency increases with increasing concentration of the phosphine or cyanide triggering agent. Relatively high concentrations of sodium cyanide are required to obtain efficient cleavage, presumably because relatively low concentrations of the nucleophilic cyanide ion exist in neutral aqueous solution (pK_a of HCN ~9.4). Consistent with the lower thiophilicity of phosphines and cyanide relative to thiols,[10] higher concentrations of these agents are required to achieve DNA-alkylation yields comparable to the thiol-triggered process under these conditions (compare thiol-triggered DNA cleavage shown in Fig. 3 to the cleavage shown in Figs. 1 and 2). It is noteworthy that water soluble phosphines such as tris(2-hydroxyethyl)phosphine are comparable to triphenylphosphine in their ability to trigger DNA alkylation in this system (data not shown). The notion that DNA cleavage observed in these assays results from alkylation rather than an oxidative, radical-mediated process is supported by the finding that addition of oxygen radical scavengers (e.g., EtOH, 100 mM) or catalase (which destroys H_2O_2) has no significant effect on the cleavage yields in our assays (data not shown).

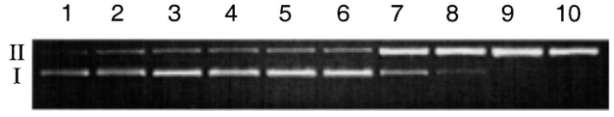


Figure 1. DNA cleavage by leinamycin (**1**) with varying concentrations of triphenylphosphine. Supercoiled plasmid DNA (pBR322, 38 μM in bp) was incubated for 6 h at 24 °C with **1** (0.5 μM) and various concentrations of Ph_3P in sodium phosphate buffer (50 mM, pH 7) followed by treatment with putrescine (100 mM) for 6 h at 37 °C. The resulting DNA cleavage was analyzed using agarose gel electrophoresis. The numbers in parentheses following the description of each lane indicate the mean number of strand breaks per plasmid molecule calculated using the equation $S = -\ln f_I$ where f_I is the fraction of plasmid present as form I. The values are corrected for strand breaks present in the untreated DNA. Lane 1, DNA alone; lane 2, **1** (0.5 μM) (0.1); lane 3, Ph_3P alone (10 μM) (0.0); lane 4, Ph_3P alone (50 μM) (0.0); lane 5, Ph_3P alone (100 μM) (0.0); lane 6, Ph_3P (250 μM) (0.0); lane 7, **1** (0.5 μM) + Ph_3P (10 μM) (1.0); lane 8, **1** (0.5 μM) + Ph_3P (50 μM) (2.1); lane 9, **1** (0.5 μM) + Ph_3P (100 μM) (4.4); lane 10, **1** (0.5 μM) + Ph_3P (250 μM) (4.4).

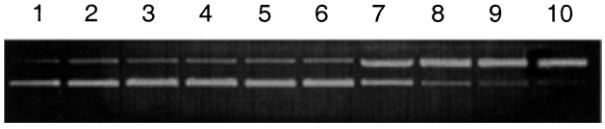


Figure 2. DNA cleavage by leinamycin (**1**) with varying concentrations of cyanide. Supercoiled plasmid DNA (pBR322, 38 μM in bp) was incubated for 6 h at 24 °C with **1** (0.5 μM) and various concentrations of KCN in sodium phosphate buffer (50 mM, pH 7) followed by treatment with putrescine (100 mM) for 6 h at 37 °C. The resulting DNA cleavage was analyzed using agarose gel electrophoresis. The numbers in parentheses following the description of each lane indicate the mean number of strand breaks per plasmid molecule calculated using the equation $S = -\ln f_I$ where f_I is the fraction of plasmid present as form I. The values are corrected for strand breaks present in the untreated DNA. Lane 1, DNA alone; lane 2, **1** (0.5 μM) (0.1); lane 3, KCN alone (250 μM) (0.0); lane 4, KCN alone (1 mM) (0.0); lane 5, KCN alone (5 mM) (0.0); lane 6, KCN alone (10 mM) (0.0); lane 7, **1** (0.5 μM) + KCN (250 μM) (0.8); lane 8, **1** (0.5 μM) + KCN (1 mM) (1.5); lane 9, **1** (0.5 μM) + KCN (5 mM) (1.8); lane 10, **1** (0.5 μM) + KCN (10 mM) (2.6).

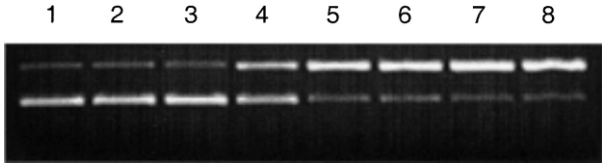


Figure 3. DNA cleavage by leinamycin (**1**) with varying concentrations of 2-mercaptoethanol. Supercoiled plasmid DNA (pBR322, 38 μM in bp) was incubated for 7 h at 24 °C with **1** (0.5 μM) and various concentrations of of 2-mercaptoethanol in sodium phosphate buffer (50 mM, pH 7) followed by treatment with DMEDA (100 mM) for 1.5 h at 37 °C. The resulting DNA cleavage was analyzed using agarose gel electrophoresis. The numbers in parentheses following the description of each lane indicate the mean number of strand breaks per plasmid molecule calculated using the equation $S = -\ln f_I$ where f_I is the fraction of plasmid present as form I. The values are corrected for strand breaks present in the untreated DNA. Lane 1, DNA alone; lane 2, **1** (0.5 μM) (0.1); lane 3, thiol alone (100 μM) (0.0); lane 4, **1** (0.5 μM) + thiol (1 μM) (0.5); lane 5, **1** (0.5 μM) + thiol (5 μM) (1.2); lane 6, **1** (0.5 μM) + thiol (10 μM) (1.3); lane 7, **1** (0.5 μM) + thiol (50 μM) (1.6); lane 8, **1** (0.5 μM) + thiol (100 μM) (1.7).

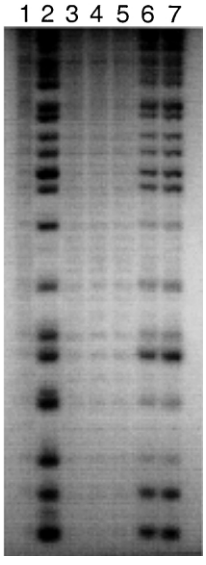


Figure 4. Triphenylphosphine-triggered alkylation of DNA by leinamycin. In the alkylation reactions, a 5′-^{32}P-labeled 145-base pair DNA fragment was incubated at 37 °C with leinamycin (13 μM) and triphenylphosphine (1 mM) or 2-mercaptoethanol (1 mM) for 2 h in sodium phosphate buffer (50 mM, pH 7.0), followed by Maxam–Gilbert workup, denaturing 20% polyacrylamide gel electrophoresis, and imaging of the gel by exposure to Fuji X-ray film at 4 °C for 60 h. Lane 1, DNA alone; lane 2, Maxam–Gilbert G rxn; lane 3, leinamycin (13 μM) alone; lane 4, Ph_3P alone (1 mM); lane 5, 2-mercaptoethanol alone (1 mM); lane 6, leinamycin (13 μM) + Ph_3P (1 mM); lane 7, leinamycin (13 μM) + 2-mercaptoethanol (1 mM).

To further characterize phosphine and cyanide-triggered DNA damage by leinamycin, we examined the resulting DNA cleavage using denaturing polyacrylamide gel electrophoresis (PAGE). We find that incubation of a 5′-^{32}P-labeled 145-base pair restriction fragment of duplex DNA with leinamycin (13 μM) and Ph_3P (1 mM), followed by Maxam–Gilbert workup (0.1 M piperidine, 90 °C, 30 min) affords cleavage at every guanine residue in the fragment (Fig. 4). Under these conditions (2 h incubation), phosphine-triggered DNA alkylation (lane 6, Fig. 4) is significantly more efficient than background alkylation by leinamycin alone (lane 3) and is comparable to thiol triggered DNA alkylation (lane 7).

Similarly, treatment of a 5′-^{32}P-labeled 19-base pair oligonucleotide duplex with leinamycin (50 μM) and sodium cyanide (10 mM), followed by Maxam–Gilbert workup, produces cleavage specifically at guanine residues. Phosphorimage analysis of the gel reveals that the yield of cyanide-triggered DNA alkylation is nearly equivalent (~90%) to that afforded by the thiol-triggered alkylation process and the sequence-specificity of guanine alkylation resulting from the cyanide-promoted activation of leinamycin is nearly identical to that of the thiol-mediated process (Fig. 5). In this experiment, DNA alkylation by leinamycin in the absence of any activating agent (lane 2, Fig. 5) is approximately 15% of that obtained in the thiol-activated case (lane 4, Fig. 5).

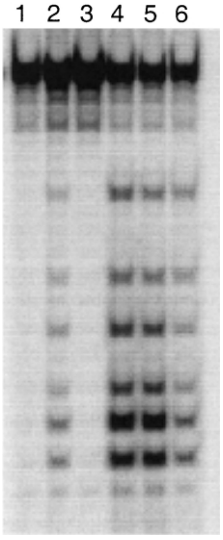


Figure 5. Cyanide-triggered DNA alkylation by leinamycin. In the alkylation reaction, a DNA duplex consisting of a radiolabeled 30-mer (5′-^{32}P-ATA ATT TGT ATA GGG AGA GAA AGT TAA TAA-3′) hybridized to a complementary 19-mer (5′-TTA TTA ACT TTC TCT CCC T-3′) was incubated at 37 °C with leinamycin (50 μM) and potassium cyanide (10 mM) for 22 h in Hepes buffer (20 mM, pH 7.0) containing herring sperm DNA (80 μM bp), followed by Maxam–Gilbert workup, denaturing 16% polyacrylamide gel electrophoresis, and documentation of the gel by phosphorimager analysis. The clearly separated bands in the lower and middle portion of the gel result from alkylation and cleavage at the six guanines in the duplex region of the labeled oligonucleotide. Lane 1, DNA alone; lane 2, leinamycin alone; lane 3, β-ME alone (0.5 mM); lane 4, leinamycin + β-ME (0.5 mM); lane 5, leinamycin + KCN (10 mM); lane 6, Maxam–Gilbert G reaction.

Our results clearly show that phosphines and cyanide can trigger DNA alkylation by the antibiotic leinamycin. Leinamycin has recently been shown to undergo slow conversion to the DNA-alkylating episulfonium ion (**4**) in aqueous buffered solution;[9] however, under the conditions of the experiments described here, DNA alkylation by this pathway is relatively inefficient (lane 2, Fig. 1; lane 2, Fig. 2, lane 3, Fig. 4) though it can clearly be seen in the experiments where leinamycin is incubated alone with DNA for longer times at 37 °C (e.g., lane 2, Fig. 5). In all experiments, the efficiency of phosphine- and cyanide-triggered DNA alkylation is well above the background levels of alkylation resulting from the incubation of DNA with leinamycin alone.

The similarities in the nature of the DNA damage resulting from activation of leinamycin by phosphine, cyanide and thiol suggest that all three reactions generate the same DNA-alkylating intermediate (**4**, Scheme 1). Studies employing the leinamycin model compound 3*H*-1,2-benzodithiol-3-one 1-oxide (**6**, Schemes 2 and 3) further suggest that attack of cyanide or phosphine on the sulfur heterocycle of leinamycin converts the antibiotic to its 'activated' oxathiolanone form **2** which undergoes rearrangement to the DNA-alkylating episulfonium ion **4**. Consistent with this mechanism, LC/MS analysis reveals that treatment of leinamycin with phosphines in aqueous solution affords the characteristic rearrangment product[5] resulting from hydrolysis of the episulfonium ion (**4**). Overall, these studies reveal novel, chemically interesting routes by which the antitumor antibiotic leinamycin can be converted to its DNA-alkylating form.

Acknowledgements

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13. To a mixture of **7** (400 mg, 2.17 mmol), 18-crown-6 (40 mg, 0.15 mmol) and NaCN (1.07 g, 21.8 mmol) was added THF (30 mL) to produce a light yellow suspension. After stirring for 12 h at 24 °C under dry nitrogen, the reaction was complete as monitored by TLC. Then HCl (2 M, 15 mL) was added to the reaction mixture, and the resulting clear solution was extracted with brine (20 mL). CAUTION: All steps of this procedure should be performed in a well ventilated fume hood because highly toxic HCN gas may be produced. The aqueous layer was extracted with CH_2Cl_2 (2×30 mL) and to the combined organic layers was added diazomethane (25 mL of a 0.3 M solution in ether; CAUTION: explosion hazard!). The resulting solution was dried over Na_2SO_4 and the solvent removed under reduced pressure to yield a yellow oil which was purified by column chromatography on silica gel (6:1 hexane/EtOAc) to produce the methyl ester of compound **10** (315 mg, 75%) as a white solid: mp 67–69 °C, R_f 0.61 (3:1 hexane/EtOAc); ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.12 (dd, J=8, 1.5 Hz, 1H), 7.92 (dd, J=8, 1 Hz, 1H), 7.65 (ddd, J=8 Hz, 8 Hz, and 1.5 Hz, 1H), 7.42 (ddd, J=8 Hz, 8 Hz, and 1 Hz, 1H), 3.97 (s, 3H); ^{13}C NMR (62.5 MHz, $CDCl_3$) δ 166.4, 134.1, 131.6, 130.8, 127.8, 127.5, 126.1, 111.5, 52.8; HRMS (EI) m/z calcd for $C_9H_7NO_2S$ 193.020, found 193.020. Identity of this compound was further confirmed by comparison (NMR, TLC) to a sample prepared from anthranilic acid by the route of Simchen and Wenzelburger.[14]
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15. Vogel, A. *Vogel's Textbook of Quantitative Inorganic Analysis*, 4th ed.; Longman: London, 1978. To a mixture of **7** (51 mg, 0.275 mmol) and KCN (179 mg, 2.75 mmol) was added DMF (5.5 mL). Solution immediately turned yellow, and after stirring for 15 min at room temperature the reaction was judged to be complete by TLC. In order to detect the presence of thiocyanate, 5 μL aliquots of the reaction mixture were added to 1 mL of an $FeCl_3$ solution (1 mM, in water) containing 20% DMSO and 60 mM HCl. Resulting iron–thiocyanate complexes were detected using UV–vis spectroscopy by their absorbance at 450 nm. The yield of thiocyanate ion was determined by comparing the absorbance to a calibration curve prepared by measuring the absorbance of iron thiocyanate complexes prepared from known concentrations of thiocyanate.
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