DNA cleavage induced by antitumor antibiotic leinamycin and its biological consequences

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The natural product leinamycin has been found to produce abasic sites in duplex DNA through the hydrolysis of the glycosidic bond of guanine residues modified by this drug. In the present study, using a synthetic oligonucleotide duplex, we demonstrate spontaneous DNA strand cleavage at leinamycin-induced abasic sites through a β-elimination reaction. However, methoxyamine modification of leinamycin-induced abasic sites was found to be refractory to the spontaneous β-elimination reaction. Furthermore, this complex was even resistant to the β-elimination reaction with hot piperidine treatment. Bleomycin and methyl methanesulfonate also induced strand cleavage in a synthetic oligonucleotide duplex even without thermal treatment. However, methoxyamine has a negligible effect on DNA strand cleavage induced by both drugs, suggesting that the mechanism of DNA cleavage induced by leinamycin might be different from those induced by bleomycin or methyl methanesulfonate. In this study, we also assessed the cytotoxicity of leinamycin against a collection of mammalian cell lines defective in various repair pathways. The mammalian cell line defective in the nucleotide excision repair (NER) or base excision repair (BER) pathways was about 3 to 5 times more sensitive to leinamycin as compared to the parental cell line. In contrast, the radioresistant mutant xrs-5 cell line deficient in V(D)J recombination showed similar sensitivity towards leinamycin compared to the parental cell line. Collectively, our findings suggest that both NER and BER pathways play an important role in the repair of DNA damage caused by leinamycin.

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1. Introduction

Leinamycin appears to be structurally distinct from any known class of antibiotics, consisting of an unusual 1,3-dioxo-1,2-dithiolane moiety connected to an 18-membered lactam ring through a spiro linkage. Early studies by the discoverers of this natural product compound have shown that leinamycin is a potent DNA-damaging agent with promising antitumor activity against various tumor models and human cancer cell lines. Follow-up studies further demonstrated that the reaction of leinamycin with cellular thiol leads to the generation of a DNA-alkylating episulfonium ion via a novel rearrangement reaction (Fig. 1). The episulfonium ion then associates non-covalently with duplex DNA and alkylates the N7-position of guanine residues with very high efficiency. Recent in vitro studies by the Gates group revealed that the leinamycin–guanine adduct in double-stranded DNA undergoes an unusually rapid depurination (t1/2 = 3 h), generating an AP site in the DNA duplex (Fig. 1). Furthermore, our recent study confirmed abasic site formation by leinamycin in cultured human cancer cells by utilizing the aldehyde-reactive probe (ARP) assay. In previous studies, leinamycin was also shown to produce DNA strand breaks in both naked DNA and genomic DNA isolated from leinamycin-treated cancer cells. In mammalian cells, enzymatic lyase and phosphohydrolase activities are known to convert AP sites to strand breaks. Alternatively, spontaneous cleavage of AP sites can occur via β-elimination, producing 3′- and 5′-blocked ends with an unsaturated aldehydic (β-4-hydroxy-2-pentenal) phosphate and 5′-phosphate groups, respectively although AP sites are known to be relatively stable under physiological conditions. In addition, leinamycin has the potential to cause strand breaks by an oxidative mechanism like ionic radiation and other agents including bleomycin. Therefore, the aim of this study was to follow up on earlier reports of DNA strand breaks by leinamycin under physiological conditions and further clarify the chemical mechanism by which leinamycin produces DNA strand breaks through a detailed study of products resulting from the reaction of double-stranded oligomers with leinamycin.

Although leinamycin is now well known to cause various types of DNA damage, including DNA adducts, AP sites, and DNA strand breaks under physiological conditions, very little information is available regarding the cellular repair mechanisms of DNA...
damage caused by leinamycin. The nucleotide excision repair (NER) pathway is a typical pathway for the removal of bulky, helix-distorting lesions caused by chemical mutagens, while the base excision repair (BER) pathway is responsible for removing alkylated and oxidized nucleobases and repairing AP sites and single strand breaks generated from AP sites. Thus, in the present study, we explored the DNA repair systems involved in the repair of DNA damage produced by leinamycin by examining the extent to which NER, BER, and other repair pathways could contribute to the survival of mammalian cells after exposure to leinamycin.

Here, we report that the formation of DNA strand breaks by leinamycin can be attributed to the sequence of reactions involving DNA alkylation, depurination, and spontaneous strand cleavage at the resulting AP sites. The results are consistent with our hypothesis that the rapid generation of AP sites is central to the biological activity of leinamycin, establishing a strong link between the DNA-damaging properties of leinamycin and its bioactivity. Our data also suggests that both NER and BER pathways contribute to the repair of DNA damage caused by leinamycin, indicating that both leinamycin–guanine adducts and AP sites may play a role in the potent activity of leinamycin against human cancer cell lines.

2. Materials and methods

2.1. Materials

All oligonucleotides were manufactured by Sigma Genosys (Woodlands, Texas). T4 polynucleotide kinase was purchased from New England Biolabs, and all radioactive nucleotides were from Amersham Life Sciences. Bleomycin and methyl methanesulfonate (MMS) were purchased from Sigma–Aldrich, and leinamycin was a kind gift from Dr. Yutaka Kanda of Kyowa Hakko Kogyo Ltd.

2.2. Cell lines

MiaPaCa, a parent Chinese hamster ovary cell line (AA8), NER-defective cell lines (UV41, UV135, UV5), BER-defective cell line (EM-9), and a recombination repair-defective cell line (Xrs-5) were obtained from American Type Culture Collection (Rockville, MD) and cultured according to their instructions. All cell lines were cultured in RPMI1640 supplemented with 10% FCS.

2.3. Purification and radiolabeling of oligonucleotides

5’-End labeled duplex oligonucleotides was prepared as described below. Each strand was labeled at the 5’ termini with [γ-32P] ATP and T4 polynucleotide kinase, heat-treated to inactivate the enzyme, and annealed with the unlabeled complementary strand. The resulting duplex oligonucleotides were run through a Sephadex G-10 column to remove unincorporated γ-32P-ATP. The annealed duplex oligonucleotides were further purified with a 16% nondenaturing PAGE gel. The DNA was visualized by exposure to X-ray film and purified from the gel by using the crush and soak method.

2.4. DNA cleavage reaction

5’-End labeled duplex oligonucleotides with 32P (100 fmol) were incubated with various concentrations of leinamycin, activated bleomycin [Fe(II)-BLM] (50 mM), or MMS (1 mM) in a 20 µL reaction mix containing 20 mM Tris–HCl, pH 7.6, 20 mM NaCl, and 1 mM DTT. The reaction was terminated by passing through a Sephadex G-10 column to remove unreacted drug molecules. To map the cleavage sites, each DNA solution was subjected to thermal treatment at 90 °C for 30 min in the presence of 1 M piperidine. The DNA solution was dried in a Speed Vac (Savant) and redissolved in 15 µL of neutral formamide dye and electrophoresed in a 16% urea–acrylamide gel for 2 h at 2000 V. To determine frank DNA strand breakage sites, the DNA solution was mixed with an equal volume of 20 µL of a neutral formamide dye and electrophoresed in a 16% urea–acylamide gel for 2 h at 2000 V. To determine damage caused by leinamycin, the dried gel was exposed to a PhosphorImager screen and quantified using ImageQuant software.

2.5. MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, which is based on the ability of viable cells to metabolize the MTT dye to a purple formazan, was used to
measure the growth inhibitory effect of leinamycin on in vitro cell cultures. After solubilizing the formazan, the absorbance at 595 nm is measured using a Synergy HT Multi-Mode microplate reader (BioTek). The number of surviving cells is directly proportional to the level of the formazan product created.

2.6. Clonogenic survival assay

The long-term cytotoxic effect of leinamycin on the survival of the cells was measured by the clonogenic survival assay. For this assay, cells were plated in triplicate at 400 cells per well in a 6-well plate. After 24 h, cells were treated with various concentrations of leinamycin. After 14 days, colonies were stained with 1% methylene blue in 50% ethanol. After washing with distilled water, relative frequencies of viable cells were calculated by solubilizing the methylene blue from the colonies with isopropyl alcohol containing 0.4 M HCl, and the resulting blue color was quantified by measuring absorbance at 595 nm using a Synergy HT Multi-Mode microplate reader.

3. Results

3.1. Leinamycin can produce single-strand breaks (SSBs) at leinamycin–guanine adduct sites

Previous studies suggested that leinamycin induces a significant amount of DNA adducts that spontaneously depurinate to form AP sites in DNA. In order to investigate the stability of leinamycin-induced AP sites, a 5′-end 32P-labeled 25-bp oligomer duplex (Sp25 in Fig. 2A) was used in this study, containing only one leinamycin-reactive site on one strand of DNA. As shown in Figure 2A, DNA strand breakage was observed in Sp25 even without hot piperidine treatment and strand breakage products increased gradually in a time-dependent manner. When these DNA cleavage products were compared with the products produced by Maxam–Gilbert sequencing reactions, which generate fragments terminating in a 3′ phosphate group, a majority of the product bands produced by leinamycin were found to migrate consistently slower than the 3′ phosphate products of Maxam and Gilbert DNA cleavage (Fig. 2A). However, the major band derived from leinamycin cleavage conigrated with the bands derived from Maxam and Gilbert sequencing when oligomer duplex DNA treated with leinamycin was subsequently heat treated at 95°C for 30 min in 1 M piperidine (Fig. 2B). The DNA fragments containing a 4-hydroxy-2-pentenal-5-phosphate residue that is generated following β-elimination of the 3′-phosphoryl group from the AP site (Fig. 4B). The pentenal oxime product that is likely to result from this reaction is refractory to the base-catalyzed δ-elimination that would yield the 3′-phosphoryl end product (band c in Fig. 4A).

3.2. Protective effect of methoxyamine on leinamycin-induced strand breaks

Methoxyamine is known to block strand cleavage at AP sites by reacting with the aldehyde group at the same sites in DNA and preventing β-elimination and hydrolysis of the 3′-phosphodiester of an abasic site. To obtain clear evidence that DNA strand breaks could spontaneously occur via β-elimination reactions at aldehyde abasic sites, we tested whether methoxyamine decreased the yield of DNA strand breaks induced by leinamycin. As shown in Figure 4A, treatment of oligomer duplexes with leinamycin in the presence of methoxyamine significantly decreased the number of direct DNA strand breaks (band a in Fig. 4A) produced by leinamycin in the absence of hot piperidine treatment. This presumably results from trapping of the AP site by methoxyamine to yield the stable oxime derivative. Methoxyamine also alters the fate of leinamycin–guanine adducts that are subjected to thermal workup in the presence of 10% piperidine (bands b and c in Fig. 4A). Under these conditions, methoxyamine primarily traps the 3′-4-hydroxy-2-pentenal-5-phosphate residue that is generated following β-elimination of the 3′-phosphoryl group from the AP site (Fig. 4B). The pentenal oxime product that is likely to result from this reaction is refractory to the base-catalyzed δ-elimination that would yield the 3′-phosphoryl end product (band c in Fig. 4A).

3.3. Protective effect of methoxyamine on DNA strand breaks induced by bleomycin and methyl methanesulfonate

Bleomycin and methyl methanesulfonate are well known to induce abasic sites, which undergo spontaneous DNA strand cleavage. To investigate whether these agents produce DNA strand breaks in the same way we observed in leinamycin-treated DNA, we examined the effect of methoxyamine on DNA strand breaks induced by bleomycin and methyl methanesulfonate. As shown in Figure 5A, methoxyamine has little effect on the number of direct DNA strand breaks in oligomer duplexes produced by these agents. Methoxyamine also did not alter the amount of DNA cleavage after thermal workup in the presence of 10% piperidine. The oxime derivatives produced by reacting abasic sites with methoxyamine was not observed in both bleomycin and MMS treated DNA. This observation clearly suggests that DNA cleavage induced by leinamycin might be different from those induced by bleomycin or methyl methanesulfonate.

3.4. Methoxyamine potentiates the cytotoxic effect of leinamycin in cancer cells

Methoxyamine is also known to potentiate the cytotoxic properties of AP sites in cells by capping the AP site and preventing processing.
In this study, we have verified the formation of AP sites in human cancer cells treated with leinamycin through experiments showing that methoxyamine potentiates the cytotoxic effect of leinamycin in MiaPaCa cells. As shown in Figure 6A, the MTT assay revealed that methoxyamine alone has little effect (less than 20% growth inhibition up to 25 mM methoxyamine) in MiaPaCa cells, but methoxyamine enhances the cytotoxic effect of leinamycin in MiaPaCa cells, suggesting that leinamycin generates significant quantities of AP sites. Methoxyamine enhanced the cytotoxic effect of leinamycin by 2.5-fold in MiaPaCa cells (IC₅₀ values were reduced from 40 to 15 ng/mL, Fig. 6B).

3.5. DNA repair mechanisms are involved in modulating leinamycin cytotoxicity in mammalian cells

Our work has shown that leinamycin can generate DNA adducts, AP sites, and DNA strand breaks under physiological conditions. It is interesting to determine which of these lesions contribute to the potent cancer-cell killing properties of leinamycin. Accordingly, we set out to shed light on this issue by identifying repair systems that mitigate the cytotoxic effects of leinamycin in mammalian cells. In this study, a panel of Chinese hamster ovary (CHO) cell lines were used, each of which are deficient in one of the following repair pathways: base excision repair (BER), nucleotide excision repair (NER), and DNA double-strand break repair and V(D)J recombination (xrs-5). The cytotoxicity of leinamycin was measured by the clonogenic survival assay, which is widely used to determine the long-term drug effect on the survival of cells by measuring the ability of the cells to continue proliferating and to form colonies on plates following drug treatment. As shown in Figure 7A, leinamycin showed more cytotoxic effects against NER mutant cell lines (UV5/ERCC2-deficient, UV41/XPF-deficient, and UV135/XPG-deficient) compared to the wild type with IC₅₀ values of 10 ng/mL for AA8 (wild), 3 ng/mL for UV5, 3 ng/mL for UV41, and 2 ng/mL for UV135. Thus, wild-type AA8 proved the most resistant to leinamycin-induced cytotoxicity. These results suggest that the NER system is involved in the repair of leinamycin–guanine adducts in DNA. The effect of XRCC1 on leinamycin cytotoxicity in mammalian cells was evaluated using the EM9 cell line, which has mutated XRCC1 and exhibits defective repair of single strand DNA breaks as well as AP sites. As shown in Figure 7B, EM9 cells were >3-times more sensitive to leinamycin cytotoxicity as compared to the parental cell line AA8, suggesting that a genetic inhibition of BER results in enhanced leinamycin cytotoxicity in mammalian cells. Taken together, our

Figure 2. Time-dependent cleavage of ³²P-labeled Sp25 treated with leinamycin. After incubation at 37 °C for the indicated durations, without piperidine treatment (A) or followed by piperidine treatment (B), the treated DNA fragments were analyzed by 16% denaturing polyacrylamide gel. (C) Graphic representation of data presented in panels A and B. The cleavage products, without piperidine treatment (open circle) or followed by piperidine treatment (closed circle) were measured by densitometric scanning of autoradiograms. (D) Proposed mechanism of DNA strand breakage at an abasic site through the β-elimination reaction and the subsequent δ-elimination reaction by hot piperidine treatment.
results indicate that NER and BER are most likely capable of modu-
ling the cytotoxicity of leinamycin and that lesions induced by
leinamycin are repaired by BER or NER. To further clarify a clear cor-
relation between NER/BER deficiency and cytotoxicity to leinamy-
cin, additional experiments might be needed in our future studies
to demonstrate that leinamycin can produce more DNA strand
breaks in NER- or BER-defective mutant cells compared to the
parental AA8 cells.

In contrast, the radiosensitive mutant xrs-5 cell line, which is
defective in DNA double-strand break repair and V(D)J recombi-
nation due to the defective Ku antigen (86 kDa subunit)37, had a sim-
ilar sensitivity towards leinamycin like the parental cell line
(Fig. 7B). It remains possible that leinamycin yields double-strand
breaks through the generation of closely-spaced, opposing abasic
sites, and that these lesions contribute to the biological activity
of leinamycin, but that these lesions are not efficiently repaired
by the mammalian repair machinery employing the Ku antigen.

4. Discussion

The various types of DNA damage caused by leinamycin,
including DNA alkylation, depurination, and DNA strand breaks,
have been implicated from previous studies from several
laboratories.3,8,15–19 However, the exact mechanism by which this
compound produces DNA strand breaks in DNA was not character-
ized. We hypothesized that the rapid evolution of DNA strand
breaks by leinamycin can be attributed to the sequence of reactions
involving DNA alkylation, depurination, and strand cleavage at the
resulting AP sites. In the present study, the type of DNA strand
breaks produced by leinamycin in DNA were further characterized
by using a denaturing polyacrylamide gel electrophoresis analysis
of a duplex DNA oligomer (Sp25) treated with leinamycin. This
analysis revealed that even without hot piperidine treatment,
DNA treated with leinamycin was cleaved to provide fragments
possessing a modified sugar moiety, which is readily decomposed
to a 3'-phosphate terminus by subsequent thermal treatment in
1 M piperidine. It was found that heating the DNA containing an
abasic site induces β-elimination to provide 3’-termini possessing
a 4-hydroxy-2-pentenal-5-phosphate group.30,31 This 3’-terminus
is readily decomposed to a 3’-phosphate terminus via α-elimination
by subsequent thermal treatment with piperidine (1 M). Interest-
ingly, methoxyamine effectively decreases the yield of DNA strand
breaks produced by leinamycin in duplex DNA oligomers in a dose-
dependent manner. Because methoxyamine is known to block
strand cleavage at abasic sites by reacting with the aldehyde group

Figure 3. Dose-dependent cleavage of 32P-labeled Sp25 treated with leinamycin. After incubation at 37 °C for 24 h at the indicated durations, without piperidine treatment
(A) or followed by piperidine treatment (B), the treated DNA fragments were analyzed by 16% denaturing polyacrylamide gel. (C) Graphic representation of data presented in
panels A and B. The cleavage products, without piperidine treatment (open circle) or followed by piperidine treatment (closed circle) were measured by densitometric
scanning of autoradiograms.
at AP sites in DNA and preventing β-elimination and the hydrolysis of the 3'-phosphodiester of an abasic site,\textsuperscript{30,31} our results suggest that true DNA strand breaks induced by leinamycin are a result of spontaneous cleavage via β-elimination and the hydrolysis of the 3'-phosphodiester of an abasic site to generate a 3'-hydroxy-2-pentenal-5-phosphate group and a 5'-phosphate group. The AP sites are known to easily convert into DNA strand nicks under physiological conditions as the β-elimination reaction breaks the C3-OP bond 3' to an AP site.\textsuperscript{21,27} Thus, our study provides strong evidence that leinamycin-mediated strand breaks could arise primarily through leinamycin’s DNA alkylating properties and its ability to subsequently generate labile AP sites.

Figure 4. (A) A high-resolution denaturing PAGE analysis showing single-stranded cleavage products of a 5'-end-labeled Sp25 by treatment with leinamycin in the presence of increasing concentrations of methoxyamine. A+G, purine-specific Maxam and Gilbert sequencing; T+C, pyrimidine-specific Maxam and Gilbert sequencing; lanes 1–7, DNA cleaved by leinamycin at 37 °C for 24 h in the presence of increasing concentrations of methoxyamine following hot piperidine treatment; lanes 8–14, DNA cleaved by leinamycin at 37 °C for 24 h in the presence of increasing concentrations of methoxyamine without hot piperidine treatment. (B) Structures at the 3' end of the DNA fragment represent bands a–c of the autoradiogram in (A).
Since unrepaired AP sites are potentially lethal or mutagenic, by blocking DNA replication, stalling RNA polymerase II during transcription, or promoting topoisomerase II-mediated double strand breaks, AP sites are recognized and incised by AP endonuclease (APE). APE incises the DNA backbone immediately 5′ to the AP site via its 5′-endonuclease activity, producing a single strand break with a normal 3′-hydroxyl group and an abnormal 5′-deoxyribose 5-phosphate (dRP) residue. This sugar-phosphate product is removed by 5′-deoxyribophosphodiesterase (5′-dRpase) activities. Removal of the sugar-phosphate products allows the subsequent restoration of nucleotides by DNA polymerase β followed by religation of the DNA strand. These AP sites can undergo spontaneous chemical cleavage via 3′-phosphodiester of an abasic site, leaving a 3′ unsaturated sugar-phosphate product (trans-4-hydroxy-2-pentenal-5-phosphate) and a 5′-phosphate product. The conversion of AP sites into DNA strand breaks is believed to be catalyzed by physiological concentrations of polyamines and histones inside cells. The removal
of trans-4-hydroxy-2-pentenal-5-phosphate from the substrate containing 3'-incised AP sites is mediated by a hydrolytic reaction requiring Drosophila S3-associated dRP activities.\textsuperscript{41} Based on genetic studies, endonucleases Apn1 or Apn2 are the major contributors to removal of 3'-dRP in yeast.\textsuperscript{41–45} APE1 is responsible for hydrolyzing these β-elimination products.\textsuperscript{46} However, when Nei-like (NEIL)1 carries out β-elimination after base excision, polynucleotide kinase (PNK) is known to be required to generate the 3' OH terminus from the 3' unsaturated sugar-phosphate product.\textsuperscript{47} Since leinamycin causes different types of DNA damage, including N7-guanine alkylation, AP site formation, and DNA strand breaks, we assessed the type of DNA repair pathways responsible for repairing DNA damage in cultured mammalian cell lines exposed to leinamycin. We used a panel of cell lines defective in NER, BER, or DNA double-strand break repair.\textsuperscript{35–37} The cell lines defective in NER are typically hypersensitive to chemical agents causing bulky and helix-distorting lesions in duplex DNA.\textsuperscript{35} For example, N7-deoxyguanosine adducts of the carcinogen aflatoxin B1 are repaired by NER. On the other hand, cells defective in BER are generally hypersensitive to agents causing AP sites and single-stranded DNA breaks.\textsuperscript{36} Since NER is the main pathway for the removal of bulky, helix-distorting lesions caused by alkylating agents, NER could, in principle, contribute to the survival of mammalian cells after exposure to leinamycin via removal of the leinamycin–guanine adducts.\textsuperscript{22} The XRCC1 protein and AP endonuclease (APE1) play a central role in the BER pathway, which is involved in the repair of AP sites.\textsuperscript{36} XRCC1 is known to act as both a scaffold and a modulator of the different activities involved in the initial step of the BER repair of AP sites.\textsuperscript{36} The radiosensitive mutant xrs-5 derived from the Chinese hamster ovary (CHO) cell line is defective in DNA double-strand break repair and V(DJ) recombination due to the defective Ku antigen (86 kDa subunit).\textsuperscript{37} Therefore, xrs-5 is known to be hypersensitive to agents causing DNA double-strand breaks.\textsuperscript{37} We observed that a panel of mammalian cell lines defective in either NER or BER pathways was about 3 to 5 times more sensitive to leinamycin compared to the parental wild type cell line after long-term exposure to the drug. We also assessed the role of methoxyamine, a small molecule inhibitor of BER, in the response of MiaPaCa human pancreatic cancer cells to leinamycin cytotoxicity.\textsuperscript{30,31} Methoxyamine was able to increase leinamycin cytotoxicity in MiaPaCa cells, suggesting that a genetic or biochemical impairment of BER results in increased leinamycin-induced cytotoxicity in mammalian cells. In contrast, little difference in the cytotoxic effect of leinamycin was observed between the parental cell line and the xrs-5 cell line defective in DNA double-strand break repair, suggesting that the formation of DNA double-strand breaks is minimal in leinamycin-treated cells. These results suggest that both NER and BER pathways are in part responsible for the repair of DNA damage caused by leinamycin, suggesting that leinamycin–guanine adducts, AP sites, and single-stranded DNA breaks serve as toxic lesions.

5. Conclusion

Our results are consistent with our hypothesis that leinamycin’s unique ability to simultaneously generate a burst of AP sites along with DNA strand breaks represents a new chemical route that explains the potent cell killing activity of leinamycin against human cancer cells. Therefore, we have established a strong link between the DNA-damaging properties of leinamycin and its potent activity against human cancer cell lines. Our data also suggests that both NER and BER pathways are partially responsible for the repair of DNA damage caused by leinamycin and that both leinamycin–guanine adducts and AP sites serve as toxic lesions.

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References and notes
