# Cellular response and molecular mechanism of antitumor activity by leinamycin in MiaPaCa human pancreatic cancer cells

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**Previous *in vitro* biochemical studies have revealed that the antitumor drug leinamycin causes oxidative DNA damage and DNA alkylation. However, it is still not clear whether the same mechanism(s) of action operate in cultured human tumor cells. Here, we evaluated the effects of leinamycin in the human pancreatic carcinoma cell line MiaPaCa. Leinamycin was highly toxic to MiaPaCa cells *in vitro*, with an $IC_{50}$ value of 50 nM, and extensive DNA fragmentation was observed in leinamycin-treated MiaPaCa cells. Flow cytometric experiments showed that leinamycin was able to disrupt normal cell cycle progression, resulting in an initial arrest of the cells in S phase. With increased time or at higher concentrations of leinamycin, the population of cells in the sub-$G_1$ phase gradually increased, indicative of apoptotic cell death due to DNA damage. Mammalian Chk2, but not Chk1 kinase, was found to be activated in MiaPaCa cells treated with leinamycin, indicating that cellular responses to leinamycin could be attributed to DNA strand break formation rather than DNA adduct formation. Like other DNA-damaging anticancer drugs, the downregulation of telomerase activity was also observed in MiaPaCa cells at cytotoxic concentrations. However, leinamycin failed to induce DNA ligase I expression in MiaPaCa cells, unlike other DNA-damaging agents, which are known to inhibit DNA replication by arresting DNA replication forks. Taken together, the results from our study indicate that the DNA strand breakage caused by the oxidative DNA-damaging property of leinamycin is directly related to the cellular responses of this drug in MiaPaCa cells over the DNA alkylation property in a dose-responsive manner. *Anti-Cancer Drugs* 15:689–696 © 2004 Lippincott Williams & Wilkins.**

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## Introduction

The natural product leinamycin was first identified from the culture broth of a strain of *Streptomyces* during a screening for anticancer drug antibiotics in Japan in 1989 and subsequent studies have demonstrated a high level of antitumor activity of leinamycin in preclinical settings [1,2]. Leinamycin is believed to be a unique thiol-triggered DNA-damaging agent and does not belong to any other known class of DNA-damaging agents [2–5]. As described in Scheme 1, Gates' group proposed that leinamycin damages DNA by at least two chemical mechanisms involving (i) a thiol-triggered release of both hydrodisulfide and polysulfide species that cause oxidative DNA damage, and (ii) the thiol-triggered generation of a DNA-alkylating episulfonium ion [5]. The initial attack of the thiol on leinamycin results in the 1,3-dioxo-1,2-dithiolane ring opening to form sulfenic acid, which then undergoes subsequent cyclization into the 1,2-oxathiolan-5-one moiety with the concomitant release of hydrodisulfide intermediates. Reaction of these polysulfides in the presence of molecular oxygen results in the formation of either hydrogen peroxide or oxygen radicals that cause DNA strand cleavage (Scheme 1). The 1,2-oxathiolan-5-one serves as a source of the episulfonium cation that is able to alkylate DNA bases (Scheme 1). Hara's group succeeded to isolate a leinamycin–$N^7$-guanine adduct, thus directly supporting the proposed mechanism provided in Scheme 1 [2]. Leinamycin, however, did not react with either guanoside nucleotide or with single-stranded DNA, thus prompting a unique interaction of this molecule with double-stranded DNA [2].

Since leinamycin showed a high level of antitumor activity, but did not belong to any known class of DNA-damaging agents, the novel mechanism of action by this compound draws significant attention to the further development of thiol-triggered DNA-cleaving agents as clinically useful anticancer drugs [6–11]. Although DNA cleavage and DNA base alkylation are believed to be the primary mechanisms of action responsible for the antitumor effect of leinamycin through *in vitro*

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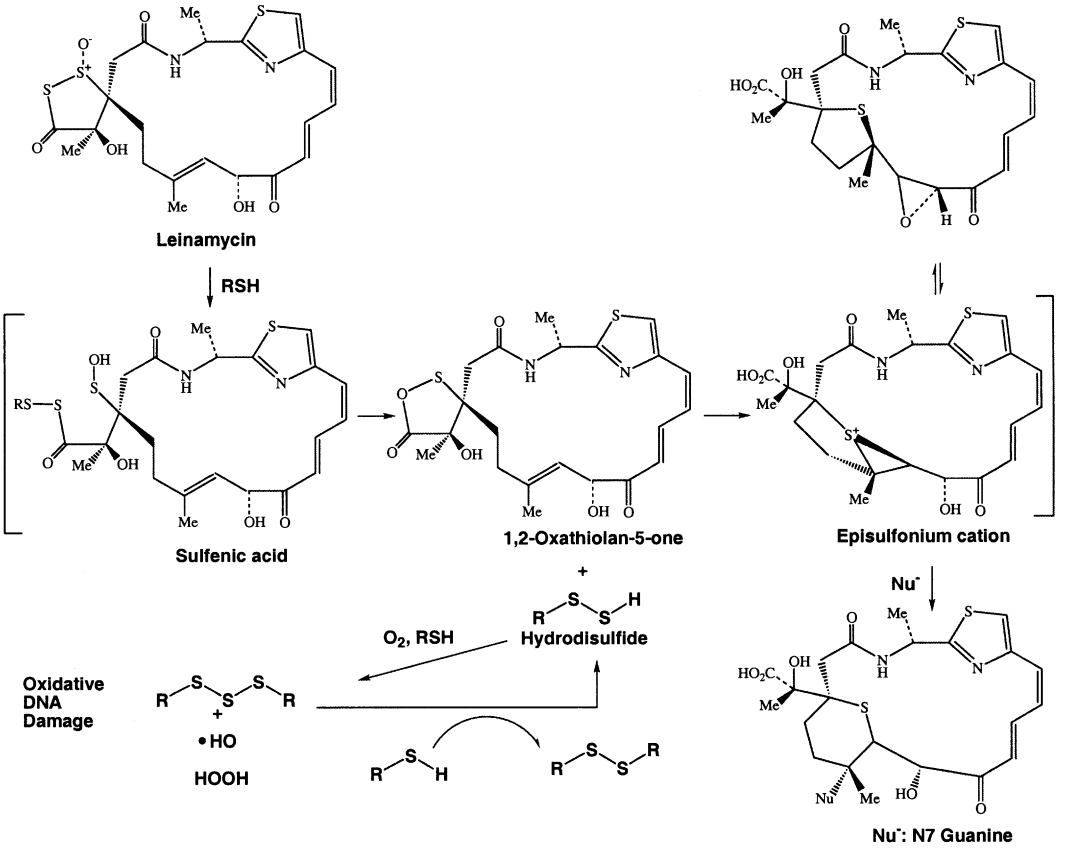
Scheme 1




Thiol-triggered DNA damage by leinamycin.

biochemical studies, it is not yet known whether the same mechanisms of DNA damage operated in cultured human tumor cells. In this report, we explored whether leinamycin exerts its therapeutic effects on human tumor cells by causing DNA damage in treated cells. Leinamycin caused DNA strand breakage in MiaPaCa cells, resulting in cell cycle arrest at S phase, and a gradual increase of apoptotic cells with increased time and at higher concentrations of this drug. We also found that Chk2 kinase, but not Chk1 kinase, was activated in MiaPaCa cells, indicating that DNA damage checkpoint pathways involving Chk2 are activated as a result of DNA damage caused by leinamycin. The results from this study support the idea that the DNA-damaging properties would contribute to the therapeutic effects of leinamycin against human tumor cells.

## Materials and methods

### Chemicals and cell lines

Leinamycin was kindly provided by Dr Kanda (Kyowa Hakko Kogyo, Japan). Cell culture media and supplements were purchased from Cellgro (Herndon, VA). The human pancreatic carcinoma cell line, MiaPaCa, was obtained from ATCC (Rockville, MD).

### Cell culture, drug treatment and MTT cellular growth assays

The MiaPaCa cells were cultured in monolayers at 37°C and 5% $CO_2$ using Dulbecco's modification of Eagle's medium (DMEM) containing 10% fetal bovine serum, L-glutamine and 4.5 g/l glucose without sodium pyruvate. For the drug treatment of cell lines, exponentially growing cells were plated at approximately $10^5$ cells/ml in T-175 $cm^2$ flasks, and leinamycin was added to the media and incubated for the desired times. After drug treatment, the medium in the culture flask was aspirated and the remaining monolayer cells were thoroughly washed once with PBS. The washed cells were then trypsinized and harvested for counting the cell number, and subsequently for the preparation of cell lysates. The cell number was counted using either a Coulter counter or hematocytometer. The cell viability was determined by the Trypan blue dye exclusion test. Cellular growth was assessed using the MTT (Sigma, St Louis, MO) colorimetric dye reduction method as described previously [12].

### Preparation of cell lysates (S-100)

Cultured human cells were washed once in phosphate buffered saline (PBS) and resuspended in ice-cold lysis

buffer containing 10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM β-mercaptoethanol, 1 mM dithiothreitol, 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), along with 10% glycerol ($10^6$ cells/20 µl of buffer). The cell suspension was incubated for 30 min on ice and was centrifuged for 30 min in a microcentrifuge at 13 000 r.p.m. at 4°C. The resulting supernatant (cell lysate) was carefully transferred into a new tube. The protein concentrations of the cell lysates were determined using a commercially available protein assay kit (Bio-Rad, Hercules, CA) and were normalized by adjusting the volume of cell lysate with cold lysis buffer. The cell lysates were stored in a −80°C freezer until use.

#### Western immunoblot analysis

Total cellular proteins were separated on a 10% SDS–polyacrylamide gel. The proteins were then electroblotted to a polyvinylidene difluoride membrane, and the membrane was blocked with 10 mM Tris and 150 mM NaCl (TBS) plus 0.1% Tween containing 5% defatted milk powder. The membrane was incubated for 1 h at room temperature with monoclonal antibodies against either phospho-Chk1, phospho-Chk2 (Upstate Biochemicals, Lake Placid, NY), DNA ligase I (Genetex, Houston, TX) or actin as a control. They were then washed and probed with either anti-rabbit or anti-mouse IgG secondary antibody conjugated with horseradish peroxidase (Amersham Life Science, Arlington Heights, IL). The signal was detected using an enhanced chemiluminenescence Western blotting system (Amersham Life Science).

#### Genomic DNA analysis

To isolate high-molecular-weight chromosomal DNA from MiaPaCa cells, the harvested cells ($1 \times 10^6$) were lysed in 10 µl of lysis buffer containing 10 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.5% Triton X-100 and 0.1 mg/ml RNase A. After incubation for 1 h at 37°C, the lysate was further incubated for 30 min at 50°C in the presence of 0.2 mg/ml Proteinase K. DNA was precipitated by adding 20 ml of 5 M NaCl and 120 ml of isopropanol, dissolved in TE buffer, and separated by 0.7% agarose gel electrophoresis under neutral conditions. The gels were stained with ethidium bromide, destained in water and photographed for documentation. The negative films were scanned and analyzed using ImageQuant Program (Molecular Dynanics, Sunnyvale, CA).

#### Telomerase assay

A telomerase assay was performed as described previously [13]. Reaction mixtures (20 µl) containing 4 µl of cell extracts (60 µg of total cell protein), 50 mM Tris-OAc (pH 8.5), 50 mM K-OAc, 1 mM $MgCl_2$, 5 mM BME, 1 mM spermidine, 1 µM 5′-biotinylated telomere primer ($TTAGGG_3$), 1.2 µM [α-$^{32}$P]dGTP (800 Ci/mmol), 1 mM dATP and 1 mM dTTP were incubated at 37°C for 30 min. Reactions were terminated by adding 20 µl of streptavidin–Dynabeads. Streptavidin–Dynabeads bind selectively to their desired targets (5-biotinylated primer), forming a magnetic bead–target complex. This complex was separated from the suspension using a magnet (Dynal MPC) and was washed several times with washing buffer (2 M NaCl) to eliminate [α-$^{32}$P]dGTP background. The telomerase reaction products were separated from the magnetic beads by protein denaturation with 5.0 M guanidine–HCl at 90°C for 30 min. After recovery of these products, analysis was performed by PAGE. Gels were dried on filter paper and developed by autoradiography on a sensitive film (Kodak; Biomax-MS).

#### Flow cytometry

MiaPaCa cells were treated with leinamycin, and the whole cells were harvested after 3, 6, 24 and 48 h of drug treatment. Cell pellets were fixed by incubation in 0.5% paraformaldehyde (EM Grade) in PBS. For flow cytometric analysis, cell pellets were gently resuspended in 1 ml hypotonic propidium iodide (PI) solution (50 µg/ml PI in a hypotonic sodium citrate solution with 0.3% NP-40 and 1.0 mg/ml RNase A) at $1.0 \times 10^6$ cells/ml and vortexed and stained for 30 min at room temperature in the dark. Prior to flow cytometric measurements, samples were filtered through a 37-µm nylon mesh into 12 × 75 mm tubes and stored at 4°C. All samples were analyzed with an Epics Elite flow cytometer (Coulter Cytometry, Miami, FL) using a 15 mW argon ion laser operated at 6 A of power at 488 nm. Photomultiplier tube voltage was adjusted for each control sample to position the $G_0/G_1$ peak to channel 240 on a 1024 channel presentation. Histograms were analyzed for cell cycle compartments using MultiCycle-Plus Version 4.0.

## Results

#### Growth inhibitory effect and DNA-damaging properties of leinamycin on MiaPaCa cells

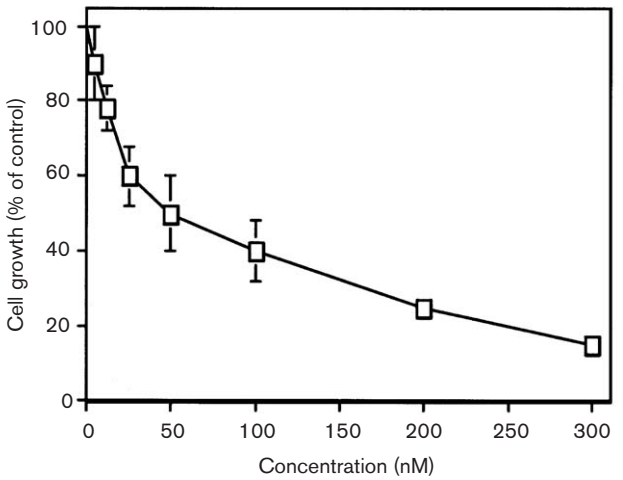
The ability of leinamycin to inhibit the growth of MiaPaCa cells was determined by the MTT assay and Trypan blue exclusion after incubating cells with this drug in the range of 0–300 nM for 96 h. As described in Figure 1, leinamycin was highly toxic to MiaPaCa cells, with an $IC_{50}$ value of about 50 nM. To test whether leinamycin induced DNA strand breaks in cultured MiaPaCa cells, the cells were exposed to 100 and 200 nM leinamycin, and harvested at 3, 6, 24 and 30 h. The genomic DNA was then isolated from the harvested cells and analyzed by 0.7% agarose gel electrophoresis under neutral conditions. As shown in Figure 2, leinamycin was able to induce significant DNA strand break formation in MiaPaCa cells. DNA strand breaks appeared as early as 6 h after treatment with 200 nM leinamycin, and gradually increased in both dose- and time-dependent manners. However, oligonucleosomal DNA fragmentation that is associated with apoptosis

was not observed in MiaPaCa cells, even at 200 nM concentrations, indicating that the DNA strand breakage that appeared on the agarose gel would be a result of the direct DNA strand cleavage caused by leinamycin, rather than apoptotic processes. These results support the previous reports that DNA strand breaks could be caused by leinamycin through the oxidative damage of genomic DNA.

### Effect of leinamycin on the cell cycle progression of MiaPaCa cells

To further evaluate the DNA-damaging properties of leinamycin, we examined the cell cycle progression of MiaPaCa cells after exposure to leinamycin at 100 and 200 nM concentrations. As shown in Figure 3, there was a significant increase in the S phase population of MiaPaCa cells treated with 100 nM leinamycin within 3 h and this pattern continued until 24 h after drug treatment. During the same time period, there was a gradual increase of a sub-$G_1$ fraction of MiaPaCa cells after leinamycin exposure, which could be attributed to apoptosis or strand break formation [14]. After 48 h treatment, an increase in S phase cells was not observed, but the cells in the sub-$G_1$ fraction were significantly increased. After exposure to 200 nM leinamycin, cells in the sub-$G_1$ fraction were gradually increased as time progressed, while the arrest of cells in the S phase was not as significant as the cells treated with 100 nM (Fig. 3). Furthermore, progression into the $G_2$ phase from the cells arrested in the S phase was much slower at 200 nM than at 100 nM leinamycin, indicating the majority of S phase arrested cells would rather undergo apoptotic death than progress into the $G_2$ phase and complete the cell cycle. In general, DNA alkylating drugs are known to initially arrest the cell cycle at S phase followed by the arrest at $G_2$ at lower levels of drug, while at high levels of the drug, the cell cycle predominantly arrests at the $G_1$ phase with an accumulation of sub-$G_1$ phase cells. Therefore, the predominant accumulation of cells in the S and sub-$G_1$ phases and lack of cells progressing into the $G_2$ phase could be attributable to the DNA cleaving properties of leinamycin.

**Fig. 1**

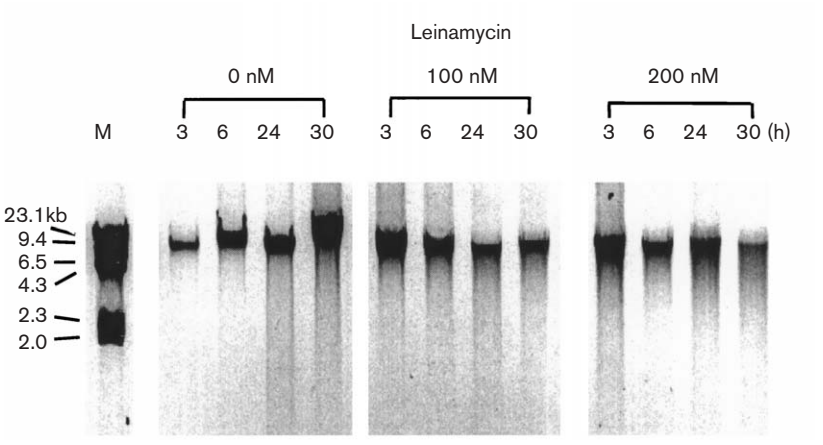



Cytotoxicity of leinamycin against MiaPaCa cells. For the cytotoxicity assay, MiaPaCa cells were incubated with the indicated concentrations of leinamycin for 96 h. At the end of incubation, cytotoxicity was measured using the MTT assay.

### Downregulation of telomerase activity by leinamycin in MiaPaCa cells

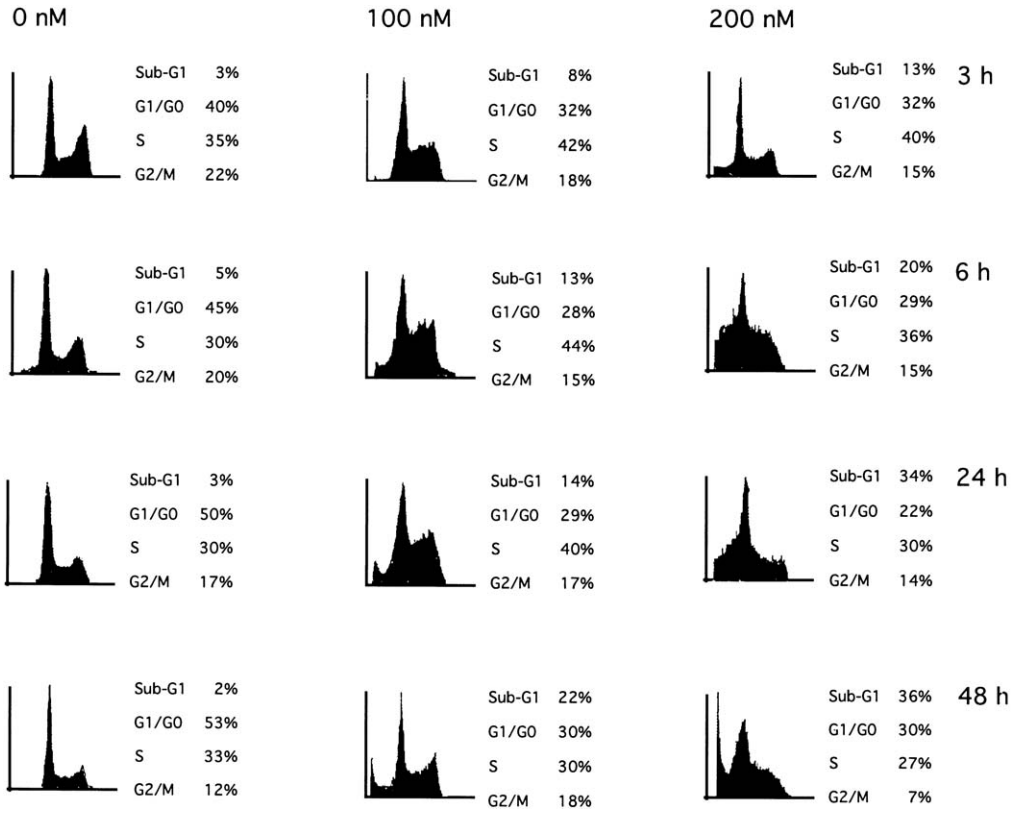
Telomerase activity is frequently associated with malignant phenotypes and can be considered an ubiquitous tumor marker. Many DNA-damaging cytotoxic agents, including cisplatin, are known to downregulate

**Fig. 2**




DNA cleavage in MiaPaCa cells treated with leinamycin. MiaPaCa cells were incubated with the indicated concentrations of leinamycin for 3, 6, 24 and 30 h. Chromosomal DNA was isolated from the harvested cells and analyzed by 0.7% agarose gel electrophoresis to monitor an increase in DNA cleavage in MiaPaCa cells treated with leinamycin.

**Fig. 3**



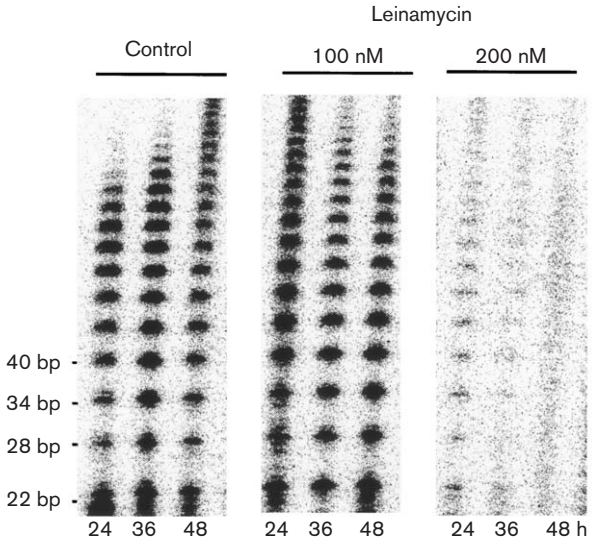

Flow cytometric analysis of leinamycin-treated MiaPaCa cells. MiaPaCa cells were treated with leinamycin at various concentrations for the indicated times. The cells were harvested, fixed and DNA was stained with propidium iodide. The DNA content was analyzed using a flow cytometer.

telomerase activity in human tumor cells at concentrations that cause significant growth inhibition [15,16]. Thus, the assessment of telomerase activity in tumor cells treated with anticancer drugs was used as a marker of tumor cell death by anticancer drugs [17–19]. In this study we determined whether leinamycin could down-regulate telomerase activity in MiaPaCa cells, like other DNA-damaging anticancer drugs. As shown in Figure 4, an apparent increase was observed in the telomerase activity of control cells over time, since increased telomerase activity is related to increased cell proliferation. However, leinamycin significantly reduced telomerase activity in MiaPaCa cells at more than cytostatic concentrations, while telomerase activity remained unchanged in the cells treated with cytostatic concentrations of leinamycin. Since the level of telomerase activity correlates with the cells' proliferating potential or cell viability after exposure to the anticancer drugs, our results suggest that leinamycin is an effective therapeutic agent limiting the proliferating capability of tumor cells after exposure [19].

### Activation of Chk2 kinase by leinamycin in MiaPaCa cells

Previous studies revealed that most DNA-damaging agents activate DNA checkpoint mechanisms irrespective of their mode of actions [reviewed in 20]. Chk2 is known to be activated in response to direct DNA cleaving agents such as ionizing radiation (IR), while replication arresting agents, such as the alkylating agents, aphidicolin and hydroxyurea, activate Chk1 [20,21]. Therefore, we tested whether DNA checkpoint mechanisms are activated in MiaPaCa cells by leinamycin. As shown in Figure 5, we found that Chk2 kinase, but not Chk1 kinase, was activated in MiaPaCa cells, indicating that DNA strand cleavage rather than replication inhibition by leinamycin–base adducts might be responsible for activating DNA-damaging checkpoints in MiaPaCa cells. In our previous study, we observed an increase of DNA ligase I levels in MiaPaCa cells after exposure to DNA replication arresting agents, such as cisplatin and Ara-C [22,23]. As shown in Figure 5, however, there was a slight decrease of DNA ligase I levels in MiaPaCa cells after exposure to 100 and 200 nM leinamycin, indicating that leinamycin might cause a type of DNA damage different from other DNA replication arresting agents in the cultured cells. Thus, these results also support the more critical role of the DNA cleaving property over the guanine alkylating property of leinamycin in mediating cytotoxic effects in cultured pancreatic human tumor cells.

**Fig. 4**




Effect of leinamycin on telomerase activity in MiaPaCa cells. MiaPaCa cells were incubated with the indicated concentrations of leinamycin, and harvested at 24, 36 and 48 h. The cell lysates were prepared from harvested cells and telomerase activity was analyzed.

## Discussion

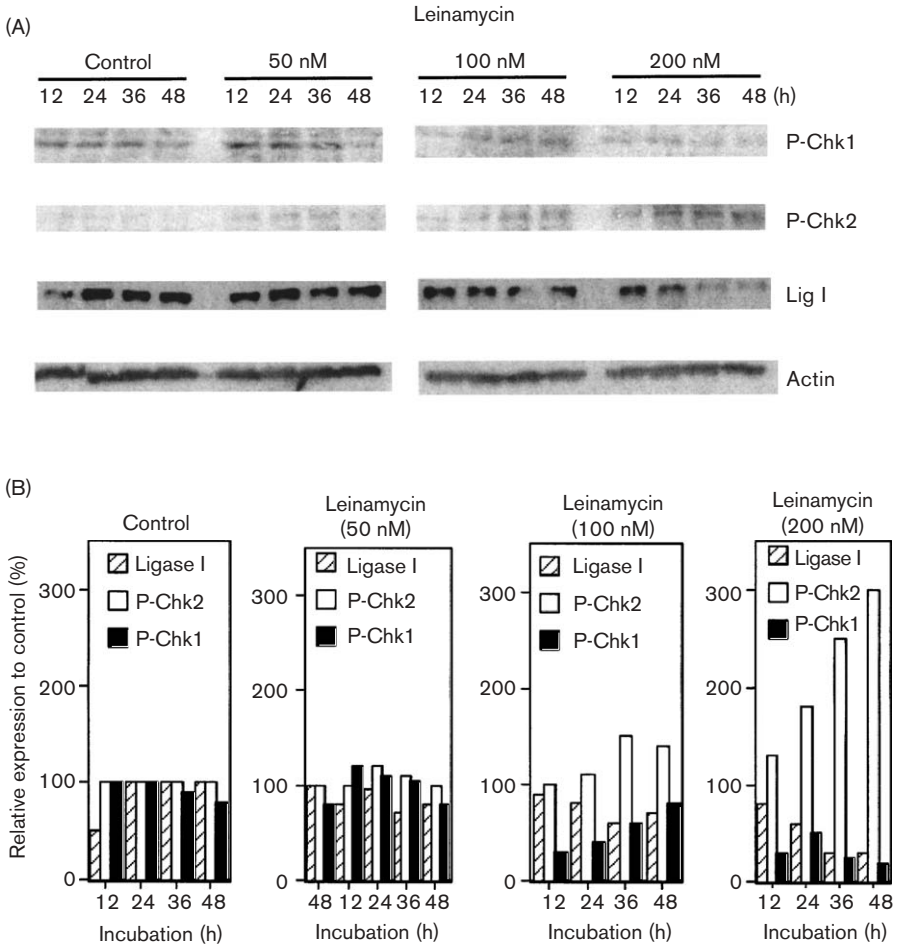
Leinamycin is one of a new class of anticancer agents that contains a 1,3 dioxo-1,2-dithiolane moiety [2]. This dithiolane moiety is responsible for causing oxidative DNA damage as well as the alkylation of guanine residues based on previous *in vitro* biochemical studies [2–6]. In this present study we address the biological relevance of the DNA-damaging properties of leinamycin using a human pancreatic carcinoma MiaPaCa cell line as a model system.

The results from this study demonstrated that leinamycin is highly cytotoxic to MiaPaCa cells, with an $IC_{50}$ value of 50 nM, which is comparable to the potency of this drug against other tumor cell lines in previous studies [7,8]. We also found that the exposure of MiaPaCa cells to leinamycin effectively caused DNA strand breaks in both time- and dose-dependent manners, and DNA strand breaks appeared as early as 3 h after MiaPaCa cells were exposed to high concentrations of this drug. DNA strand breaks on the chromosomal DNA were previously observed in human tumor cells treated with several DNA-damaging agents, such as IR, bleomycin or enediyne compounds [24–26]. Thus, the strand breakage formation by leinamycin was analogous to these DNA-cleaving agents.

Apoptosis is known as a major mode of cell death induced by several DNA-damaging agents, so we evaluated the induction of apoptosis after the exposure of MiaPaCa cells to leinamycin by using flow cytometric analysis. At cytostatic concentrations (100 nM), leinamycin caused an initial arrest of the cell cycle at S phase and a subsequent increase in the population of cells at the sub-$G_1$ phase, which is a characteristic of apoptosis. After exposure to a cytotoxic concentration (200 nM) of leinamycin, the percentage of apoptotic cells was significantly higher than at the cytostatic concentration (100 nM) initially and throughout the exposed times. The generation of apoptotic cells immediately after the treatment with leinamycin indicates that oxidative DNA cleavage by this drug might be more prevalent inside cells rather than DNA alkylation. Some DNA alkylating drugs, such as cisplatin, are known to induce apoptosis only after a prolonged arrest of cells in the S phase [27–29]. Thus, the appearance of apoptotic cells as early as 3 h after leinamycin treatment and the quick nature of oxidative stress supports the possibility that the DNA-cleaving property of leinamycin is more responsible for inducing apoptotic cell death.

When exposed to DNA-damaging anticancer drugs, cells often activate specific signal transduction pathways called the DNA damage checkpoints, which are responsible for sensing any major changes in the basic structure and function of the DNA duplex that may need repair [30,31]. Human phosphatidylinositol 3-kinase-related kinases (PIKKs), including ATM, ATR and DNA-PK, play a key role in DNA damage checkpoint pathways as the most proximal signal sensors and transducers [20].

**Fig. 5**




Chk2 activation in response to leinamycin. MiaPaCa cells were treated with 0, 50, 100 or 200 nM leinamycin for the indicated times. The levels of phospho-Chk1, phospho-Chk2, DNA ligase I and actin proteins in cell lysates were examined by Western immunoblot.

The downstream kinases (Chk1 and Chk2) are known to regulate cell cycle progression and DNA repair perhaps through transcription [21–32]. In response to DNA strand breaks, such as IR and UV, ATM is known to phosphorylate Chk2, while ATR mainly phosphorylates Chk1 in response to DNA replication arresting agents, such as the alkylating agents cisplatin, hydroxyurea and aphidicolin [20,21,32]. Our study demonstrated that Chk2 kinase rather than Chk1 kinase was activated in MiaPaCa cells after exposure to leinamycin, indicating that DNA damage checkpoints are activated as a result of DNA strand breaks directly caused by leinamycin. Our previous studies suggested that DNA ligase I levels elevated in MiaPaCa cells after exposure to the DNA alkylating agent cisplatin or other anticancer agents that are known to arrest DNA replication [22,23]. Induction of DNA ligase I expression is believed to be required for a specific repair process of DNA strand breaks generated from the arrest of DNA replication by these agents. Interestingly, an increase in DNA ligase I levels was not observed in MiaPaCa cells treated with leinamycin, suggesting that the predominant type of DNA damage that the cells face and respond to after exposure to leinamycin would be oxidative DNA cleavage rather than base alkylation.

Telomeres are specialized nucleoproteins at the ends of eukaryotic chromosomes, which consist of a tandem array of TTAGGG repeats complexed with specific telomeric-repeat binding proteins [33]. They protect the chromosomal ends from degradation or aberrant recombination. Without proper telomere maintenance mechanisms, eukaryotic cells progressively lose telomeres after each cell cycle because of the end replication problem, eventually resulting in replicative senescence [34]. Telomeres of all cancer cells are primarily maintained by telomerase, a specialized reverse transcriptase that adds telomeric repeats to the ends of chromosomes [35].

Previously, the correlation between telomerase activity and antitumor effects of cytotoxic anticancer drugs was investigated in various human cancer cell lines [17–19]. These studies revealed that the activity of telomerase correlated significantly with the number of viable cells after exposure to cytotoxic anticancer drugs, raising the possibility of utilizing this enzyme to predict tumor response to chemotherapy. In this study, leinamycin was found to significantly downregulate telomerase activity in MiaPaCa cells at more than cytotostatic concentrations, like other DNA-damaging cytotoxic agents. The down-regulation of telomerase activity in drug-treated cells is regarded as an index of chemosensitivity to that drug, so leinamycin could be as effective as other DNA-damaging anticancer drugs in suppressing the proliferation of human tumor cells. The downregulation of telomerase activity by leinamycin is expected to accelerate the erosion of telomere DNA, limiting the long-term survival of tumor cells treated with this drug.

In conclusion, this present study clearly demonstrates the biological relevance of the DNA-damaging properties of leinamycin using a human pancreatic carcinoma MiaPaCa cell line as a model system. Furthermore, our results indicate that the DNA-cleaving properties of leinamycin could be more important for exerting the drug's biological activities over its alkylating property inside cells, although it is known to cause both DNA cleavage and DNA base alkylation by *in vitro* biochemical assays. This information regarding the mechanisms of action by leinamycin in a cell-based system will provide useful information for the further development of this class of compounds as clinically useful anticancer drugs.

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