Down-regulation of the nuclear factor-κB by lidamycin in association with inducing apoptosis in human pancreatic cancer cells and inhibiting xenograft growth

JING CHEN[1,2], ZHI-GANG OUYANG[1], SHENG-HUA ZHANG[1] and YONG-SU ZHEN[1]

[1]Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050; [2]North China Coal Medical College, Tangshan, Hebei 063000, China

Abstract. Pancreatic cancer is now one of the most common causes of cancer death worldwide. K-ras mutations are present in up to 90% of pancreatic cancer cases. The expression of mutant K-ras activates the Akt/protein kinase B pathway, resulting in the activation of the nuclear factor-κB (NF-κB) transcriptional factor. Constitutive NF-κB activity plays a key role in pancreatic carcinoma. NF-κB has been shown to inhibit apoptosis in response to chemotherapeutic agents. In the present study, the effects of lidamycin (LDM), a member of the enediyne antibiotic family, were investigated on two established pancreatic cell lines, PANC-1 and SW1990. A dose-dependent inhibition of phospho-Akt and NF-κB activation was found in the cells treated with LDM as determined by Western blot analysis. Moreover, a down-regulation of K-ras mRNA and a protein expression by LDM were observed in both cell lines as determined by reverse transcription-PCR and Western blot analysis. By MTT assay, a remarkable difference in chemosensitivity to LDM, mitomycin, adriamycin, taxol, and gemcitabine was found in both cell lines. The IC_{50} values of LDM for the PANC-1 or SW1990 cells were 0.955±0.414 or 0.426±0.212 nM, respectively, lower than those of the other drugs. Growth inhibition, apoptosis induction and cell cycle arrest were observed in the LDM-treated cells. LDM decreased the invasive potential of pancreatic cancer cells by reducing matrix metalloproteinase-9 activity. Furthermore, LDM was found to suppress the growth of SW1990 xenografts in nude mice. Treatment with an i.v. injection of LDM at the dose of 0.02 and 0.04 mg/kg (once a week for two weeks) inhibited the growth of xenografts by 66 and 72%, respectively. By contrast, an i.p. injection of gemcitabine at the dose of 80 mg/kg inhibited the growth of xenografts by 38%. Our findings suggest that LDM is active in the down-regulation of NF-κB and could play a positive role in relevant targeted chemotherapy for pancreatic carcinoma.

Introduction

Pancreatic cancer is a highly malignant neoplasm characterized by locally advanced unresectable disease or metastasis at the time of diagnosis (1). Current therapies are largely ineffective in this disease and the 5-year survival rate remains dismal (2). Pancreatic cancer is now one of the most common causes of cancer death worldwide. In the United States nearly all patients diagnosed with pancreatic adenocarcinoma die a cancer-related death (3,4). Complete surgical resection remains the only potentially curative treatment modality for patients with pancreatic cancer. Unfortunately, the vast majority of patients present with unresectable or metastatic disease. Even among patients undergoing a potentially curative resection, many will develop metastatic disease. Gemcitabine (2', 2'-difluorodeoxycytidine) is currently considered the optimal treatment for patients with newly diagnosed metastatic pancreatic cancer (5). However, the impact of gemcitabine-based chemotherapy regimens in pancreatic cancer has been at best, modest (6). Therefore, the development of novel approaches for pancreatic cancer treatment is essential as tumor cells are highly resistant to most conventional chemotherapy drugs. As reported, the constitutive activation of the nuclear factor-κB (NF-κB) is a frequent molecular alteration in pancreatic carcinoma and is also found in human pancreatic carcinoma cell lines but not in immortalized, nontumorigenic pancreatic epithelial cells (7). Several studies have shown that the constitutive NF-κB activation plays a key role in pancreatic carcinoma (8). NF-κB is becoming an attractive target for chemoprevention and its modulation for pancreatic cancer prevention is promising (9,10).

Much focus has been placed on the the potent antitumor activity of enediyne antibiotics due to their unique ability to damage the DNA of tumor cells by inducing single strand and/or double strand breaks through free radical attacks on the deoxyribose moieties in DNA (11). Lidamycin (LDM, also named as C-1027) is a member of the enediyne antibiotic family, which was produced by a *Streptomyces*

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Correspondence to: Professor Yong-su Zhen, Department of Oncology, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, No. 1 Tiantan Xili, Beijing 100050, China
E-mail: zhenys@public.bta.net.cn

globisporus C-1027 strain isolated in China (12,13). LDM shows extremely potent cytotoxicity, anti-angiogenic activity and a marked growth inhibition of transplantable tumors in mice (14-17). The LDM molecule contains an enediyne chromophore (MW 843 Da) responsible for the extremely potent bioactivity and a noncovalently bound apoprotein (MW 10.5 kDa), which forms a hydrophobic pocket for protecting the chromophore (18,19). The apoprotein and chromophore can be dissociated and reconstituted, and the biological activity of the rebuilt molecule is comparable to that of natural LDM (20).

In order to determine the action of LDM on pancreatic cancer cells and reveal its possible mechanism, we investigated the effects of LDM on two established pancreatic carcinoma cell lines, PANC-1 and SW1990. In the present study, we found that lidamycin was highly active in targeting the Akt/NF-κB signal pathway. Moreover, LDM induced apoptosis and cell cycle arrest in human pancreatic cancer cells and suppressed the growth of xenografts in athymic nude mice.

Materials and methods

Chemicals. LDM was provided by Professor Lian-fang Jin of our institute. Mitomycin (MMC) was the product of Kyowa Hakko Kogyo Co., Ltd., Japan. Adriamycin (ADM) was obtained from Shenzhenwanle Pharmaceutical Co., Ltd, China. Taxol was purchased from Zhongmeishiguibao Pharmaceutical Co., Ltd., China. Gemcitabine was the product of Lilly France S.A., France. All chemicals were of standard analytical grade.

Cell culture. The human pancreatic cancer cell lines, PANC-1 and SW1990 (CRL-1469 and CRL-2172, American Type Culture Collection), were maintained in culture with DMEM (Gibco BRL, Grand Island, New York, USA) supplemented with 10% heat-inactivated FBS (Sigma, St Louis, Missouri, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Western blot analysis. A total protein extract of cells was prepared by incubation for 15 min on ice with an ice-cold hypotonic lysed buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 1% Nonidet P-40, 0.1% SDS, protease inhibitors (1 mM PMSF, 5 mg/ml aprotinin, 5 mg/ml leupeptin and 5 mg/ml pepstatin) and phosphatase inhibitors (20 mM ß-glycerophosphate, 50 mM NaF and 1 mM Na_3VO_4). Lysates were centrifuged at 12,000 g for 12 min. Protein samples of an equal amount were denatured with 1 volume of 6X SDS sample buffer and loaded on SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, Massachusetts, USA). Blots were blocked for 60 min at room temperature with 5% nonfat milk powder and 0.1% Tween-20 in PBS and exposed overnight at 4°C to a primary antibody against matrix metalloproteinase (MMP-9) (Santa Cruz Biotechnology, California, USA), K-ras (Santa Cruz Biotechnology), Akt (Cell Signaling Technology, USA), p-Akt (Cell Signaling Technology), and NF-κB (Santa Cruz Biotechnology), respectively. Blots were washed with TBST [20 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Tween-20] for 5 min

(three times) and exposed for 60 min at room temperature to an appropriate horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology). Complexes of the primary and secondary antibodies were visualized using enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Reverse transcription-PCR (RT-PCR). Total cellular RNA was extracted from the PANC-1 and SW1990 cells using TRIzol reagent (Invitrogen, Carlsbad, California, USA) and the protocol recommended by the manufacturer. RT-PCR amplification was performed on the extracted RNA using the Superscript One-Step RT-PCR kit (Invitrogen) according to the manufacturer's instructions. The primer pair for K-ras was sense 5'-ACTTGTGGTAGTTGGAGCTG, and antisense 5'-CTAACAGTCTGCATGGAGC. RT was performed at 55°C for 15 min. The denaturation and amplification conditions were 95°C for 30 sec followed by up to 25 cycles of PCR. Each cycle of PCR included denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec and extension at 72°C for 40 sec. After PCR amplification, the fragments were analyzed by 2% agarose gel electrophoresis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the positive control. The sizes were estimated by comparison with molecular weight markers.

Cell proliferation assay. PANC-1 and SW1990 cells were plated in triplicate in 96-well plates with 3,000 cells/well and 4,000 cells/well, respectively. After overnight incubation, triplicate wells were treated with varying concentrations of LDM, MMC, ADM, taxol, and gemcitabine for 48 h. The effects on cell growth were examined by MTT (methylthiazolyltetrazolium) assay. In brief, 20 μl MTT solution (5 mg/ml in PBS) (Sigma) were added to each well and incubated for 4 h at 37°C. The MTT, formazan, was dissolved in 150 μl DMSO and absorbance was measured by a Microplate Reader (Thermo Labsystem, USA, Multiskan MK3) at a wavelength of 570 nm.

Flow cytometry
Cell cycle analysis. Cells were harvested and resuspended in PBS, incubated with 200 μg/ml RNase A (Sigma) for 30 min at 37°C, and stained with 50 μg/ml propidium iodide (Sigma) in the dark for 30 min. The samples were analyzed on a fluorescence-activated cell sorter (EPICS XL, Coulter).

FITC-Annexin V/PI apoptosis assay. Cells were harvested and resuspended in 200 μl Binding Buffer. Then 10 μl FITC-labeled enhanced Annexin V (Baosai Biotechnology Ltd., Beijing) and 100 ng propidium iodide, were added. Upon incubation in the dark (15 min, room temperature or 30 min at 4°C), the samples were diluted with 300 μl Binding Buffer. Flow cytometry was carried out on a FACScan instrument (Becton-Dickinson) and data were processed by WinMDI/PC-software.

Staining with Hoechst 33342. PANC-1 (3.5×10^4) and SW1990 (4.5×10^4) cells per well were seeded into 12-well plates and exposed to LDM for 48 h. Then 0.1 ml Hoechst 33342 (Sigma) at a concentration of 2 μg/ml was added to each well

and incubated at room temperature for 15 min. The cells were fixed and observed using a fluorescence microscope (BH2 system, Olympus).

Cell migration/invasion assays. Cell migration was examined using transwell cell migration chamber plates (12-well inserts, 8 μm pore size; Corning, USA). Panc-1 cells (20,000) or SW1990 cells (30,000) were suspended in 100 μl DMEM containing 1% serum with varying concentrations of LDM and placed in the upper compartment of the chamber. The lower compartment of the chamber was filled with 600 μl DMEM containing 20% FBS. In order to determine cell invasion, 50 μl matrigel were dispersed on the upper side of the transwell cell migration chamber and dried at 37°C for 2 h. The transwell plates were incubated at 37°C and 5% CO_2 for 48 h. The nonmigratory or noninvasive cells were removed with a cotton-tipped applicator, and the cells that penetrated through to the bottom of the chamber were stained with hematoxylin.

In vivo antitumor activity. Twenty-four athymic female BALB/c (*nu/nu*) mice (20±2 g, obtained from the Institute for Experimental Animals, Chinese Academy of Medical Sciences, China) at the age of 4-6 weeks were used for SW1990 human pancreatic tumor xenografts. SW1990 tumors for implantation were initially grown from injections of SW1990 cells at a dose of 5x10^6 cells/mouse. A tumor piece of 2 to 3 mm in diameter was implanted subcutaneously into each experimental animal. After 10 days of tumor growth, the animals were randomly divided into groups (n=6) in a manner that minimized the difference in tumor size between the groups. Each animal received 200 μl of either PBS (vehicle control) or LDM by i.v. injection once a week for 2 weeks. The doses of LDM were 0.02 and 0.04 mg/kg, respectively. The animals received <300 μl of gemcitabine at a dose of 80 mg/kg by i.p. injection as the positive control. The mice were weighed and tumor sizes were measured with a vernier caliper and recorded every other day. Tumor volume was calculated using the formula: Length x width^2 x 0.5.

Statistical analysis. Results are expressed as the means ± SD. Treatment effects were compared using the Student's t-test and differences between the means were considered to be significant when P≤0.05.

Results

Effects of LDM on activation of proteins in Akt/protein kinase B pathway. K-ras mutations are present in up to 90% of pancreatic cancer cases (21). The expression of mutant K-ras activates the Akt/PKB pathway (22), resulting in the activation of the NF-κB transcriptional factor. We measured the levels of mRNA encoding K-ras in PANC-1 and SW1990 cells treated with different concentrations of LDM for 48 h by RT-PCR analysis. The protein expression of K-ras, Akt, p-Akt and NF-κB was determined by Western blot analysis. The result of RT-PCR showed that the levels of K-ras mRNA were decreased after treatment with LDM at 2 nM in the PANC-1 cells and 1 nM in the SW1990 cells. Meanwhile, LDM reduced the expression of the K-ras protein in either
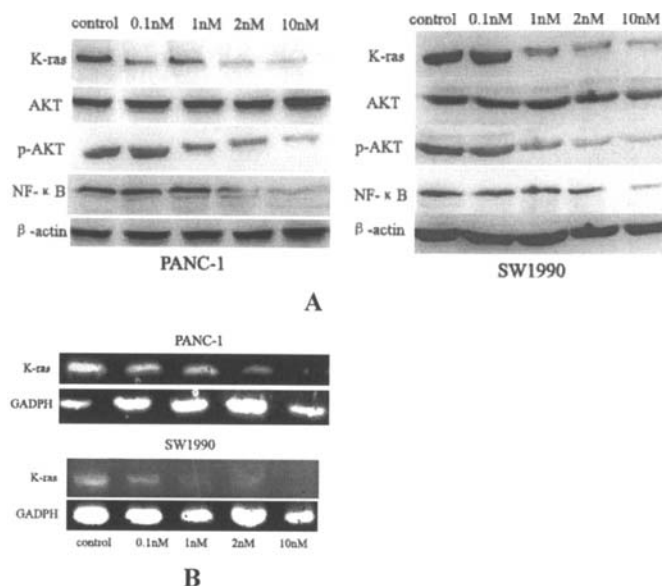

Figure 1. The levels of K-ras, AKT, p-AKT, NF-κB, and ß-actin in the PANC-1 and SW1990 cells treated with various concentrations of lidamycin (LDM) for 48 h were determined by Western blot analysis (A). The level of mRNA encoding K-ras in the PANC-1 and SW1990 cells treated with the indicated concentrations of LDM was determined by reverse transcription-PCR analysis. The level of glyceraldehyde-3-phosphate dehydrogenase mRNA was used as the loading control (B).

cell line. A dose-dependent decrease in the K-ras protein expression was observed after the exposure of the cells to increasing concentrations of LDM (1, 2, or 10 nM). Moreover, the levels of phospho-Akt and NF-κB also decreased in a dose-dependent manner. Additionally, the maximal inhibition of NF-κB protein levels was observed in the PANC-1 and SW1990 cells after exposure to 10 nM LDM for 48 h, whereas the levels of Akt protein were unaffected by LDM (Fig. 1).

Induction of apoptosis by LDM in PANC-1 and SW1990 cells. The Hoechst 33342 staining that is sensitive for DNA detection was used to assess the changes in nuclear morphology following the treatment of LDM. The nuclei of untreated cells were normal and exhibited diffused staining of the chromatin. After exposure to LDM for 48 h, most cells presented typical morphological changes of apoptosis such as chromatin condensation or a shrunken nucleus (Fig. 2). Some condensed nuclei were observed when cells were exposed to higher concentrations of LDM.

Flow cytometry combined with FITC-Annexin V/PI staining showed that LDM at 1 nM induced earlier apoptosis in both cell lines. The ratio of apoptosis was significantly enhanced when cells were incubated with 2 or 10 nM LDM for 48 h (Fig. 3).

Changes of cell cycle progression induced by LDM. Flow cytometric cell cycle analysis showed that LDM at 1 nM induced G2/M arrest in PANC-1 cells. The accumulation of PANC-1 cells in the S phase was induced by 2 and 10 nM LDM. About 50% of the SW1990 cells were arrested in the S phase after treatment with 1 or 2 nM LDM (data not shown).

Effects of LDM on the proliferation of PANC-1 and SW1990 cells in culture. The inhibition of cell proliferation was
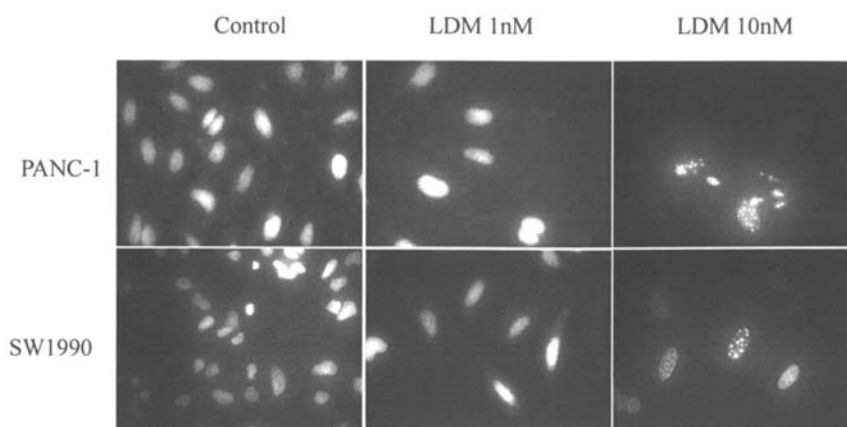
Figure 2. PANC-1 and SW1990 cells were treated with the indicated concentrations of lidamycin for 48 h and then stained with the DNA-binding dye, Hoechst 33342 (x200).
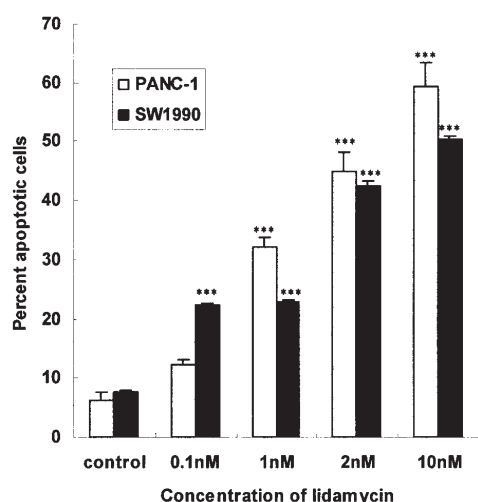

Figure 3. Induction of cell apoptosis by lidamycin. Apoptosis was determined by the FITC-Annexin V/PI apoptosis assay. Data represent the means ± SD of three independent experiments. ***P<0.001 vs the control.
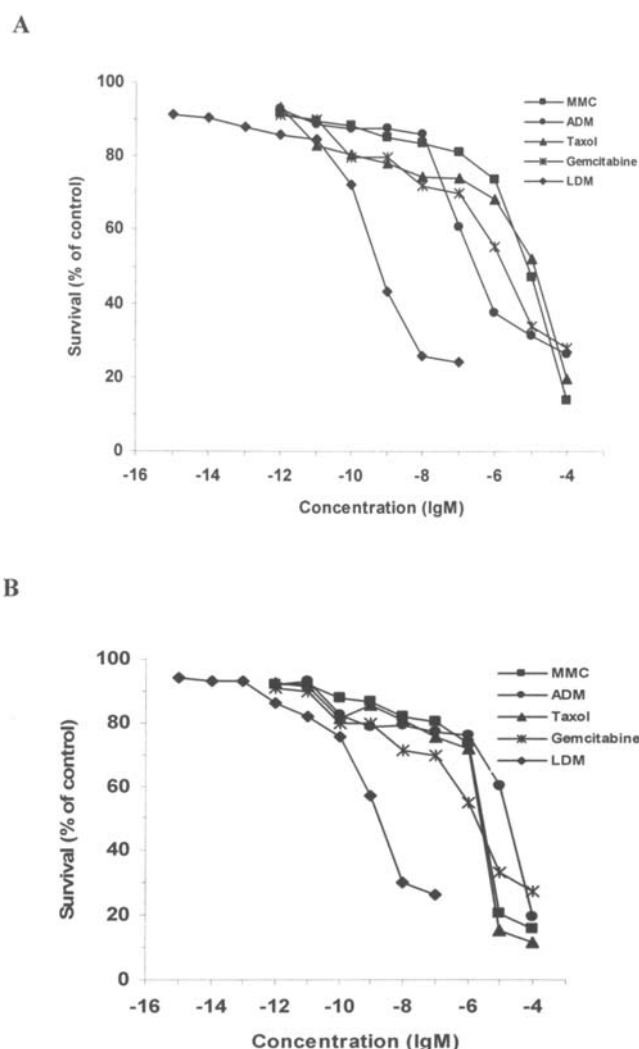

Figure 4. Growth inhibition of PANC-1 (A) and SW1990 (B) cells by lidamycin and other drugs. Cells were exposed to the drugs for 48 h and determined by MTT assay. Results were derived from three independent experiments.

measured by MTT assays. PANC-1 or SW1990 cells were treated with LDM of different concentrations. Meanwhile, certain traditional antitumor drugs including MMC, ADM and taxol were involved in the assays. Both cell lines showed a decreased cell proliferation after treatment with the drugs, especially the cells exposed to LDM (Fig. 4A and B). The IC_{50} values of LDM for the PANC-1 or SW1990 cells were 0.955±0.414 or 0.426±0.212 nM, respectively, lower than those of the other drugs (Table I).

Inhibition of cell migration and invasion by LDM. The effect of LDM on the migration or invasion of the PANC-1 and SW1990 cells through an extracellular matrix was examined using a transwell cell migration chamber assay. A dose-dependent reduction in cell migration or invasion in both cell lines was found after exposure to LDM (Fig. 5). The expression of MMP-9 was determined by Western blot analysis. PANC-1 and SW1990 cells treated with LDM showed a dose-dependent decrease in the expression of MMP-9. The level of MMP-9 was reduced by 1 nM LDM (Fig. 6).

Effect of LDM on growth of SW1990 tumor xenografts. The growth of the established s.c. tumors in the nude mice was decreased significantly when an i.v. injection of LDM was

Table I. Comparison of the chemosensitivity to various anti-cancer drugs in the PANC-1 and SW1990 cells.

Drugs	IC$_{50}$ (μM)	
	PANC-1	SW1990
MMC	4.240±0.162	5.082±0.148
ADM	0.451±0.014	2.017±0.028
Taxol	0.578±0.085	0.603±0.042
Gemcitabine	1.874±0.168	3.724±0.143
LDM	0.0009±0.0004	0.0004±0.0002

MMC, mitomycin; ADM, adriamycin; LDM, lidamycin. MTT assay was performed after the cells were treated with different drugs for 48 h. Results were derived from three independent experiments.

Table II. Inhibitory effects of gemcitabine and LDM on the growth of human pancreatic cancer SW1990 xenografts in nude mice.

Groups	Dose (mg/kg)	BWC (g)	Tumor weight (g)	Inhibition rate (%)
Control	-	-1.16	1.27±0.18	-
Gemcitabine	80	-2.65	0.77±0.34	38.87[a]
LDM	0.02	-1.54	0.43±0.13	65.71[b]
	0.04	-1.86	0.27±0.08	78.27[b]

Results were recorded on day 31 after tumor implantation, with all athymic mice alive (n=6). LDM, lidamycin; BWC, average body weight change; [a]P<0.01; [b]P<0.001 compared with the control group.

Figure 6. The level of matrix metalloproteinase-9 and ß-actin in PANC-1 and SW1990 cells treated with various concentrations of lidamycin was determined by Western blot analysis.

Figure 7. Effect of lidamycin on the growth of SW1990 xenografts in nude mice. Treatment started on day 12 with a once per week i.v., x2 regimen.

given over a period of 3 weeks compared with the control PBS-treated animals (Fig. 7). Treatment with LDM at the dose of 0.02 and 0.04 mg/kg inhibited the growth of pancreatic carcinoma SW1990 xenografts by 66 and 72%, respectively. T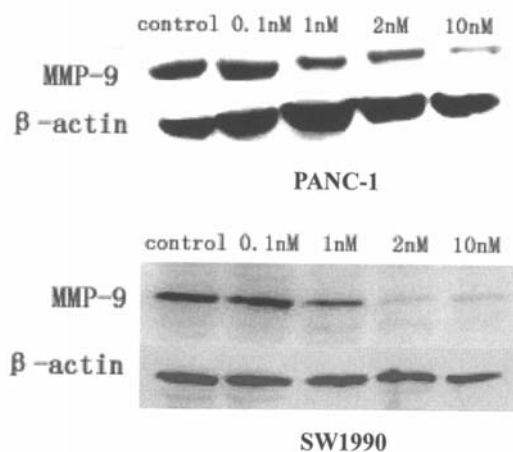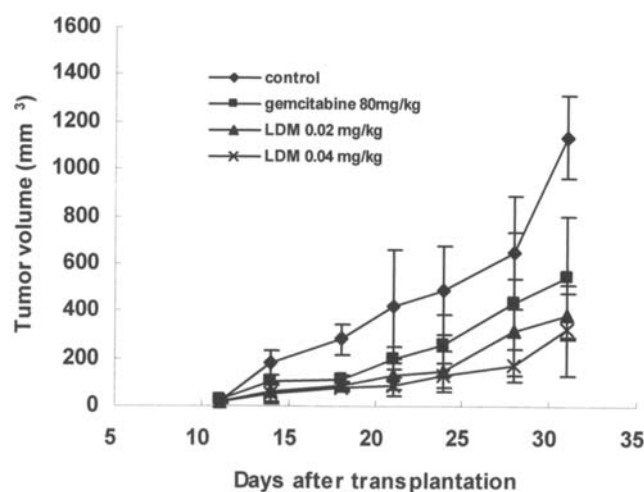he antitumor activity of LDM is more remarkable than that of gemcitabine. The body weights of the animals showed no significant differences between the control and treated groups (Table II).
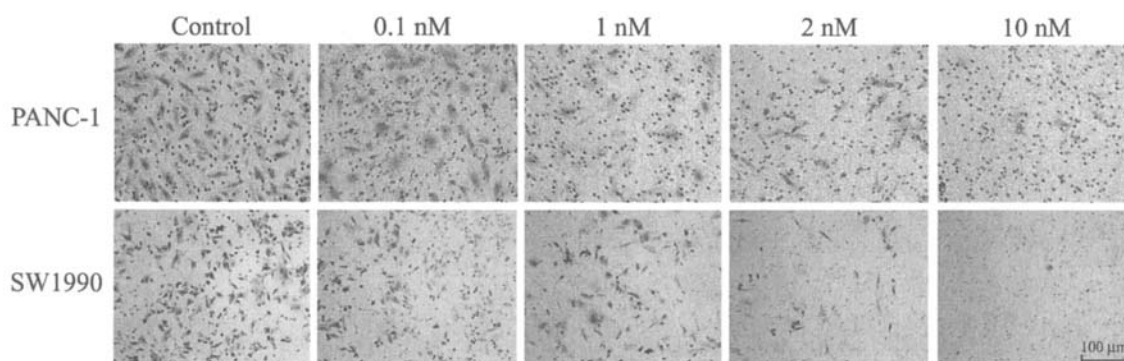

Figure 5. Reduction of pancreatic cell invasion by lidamycin (LDM). PANC-1 and SW1990 cells were treated with the indicated concentrations of LDM. The cells were placed in the upper chamber of a transwell insert coated with matrigel and incubated for 48 h. Cells that invaded through the transwell membrane were stained with hematoxylin (x100).

Discussion

NF-κB is getting more attention as a potential target for the therapy of pancreatic carcinoma. As is known, NF-κB is a transcriptional factor that is involved in a wide spectrum of cellular functions including apoptosis and cell cycle control. The permanent activation of NF-κB in pancreatic cancer cells is associated with a profound resistance towards chemotherapy. NF-κB is retained in the cytoplasm by the inhibitory protein, IκB (23). Several mediators in the signal transduction pathway including Akt are known to activate NF-κB through the phosphorylation of IκB (24). Activated NF-κB translocates to the nucleus, resulting in the transcription of genes that encode key determinants in inflammation, tumorigenesis, and apoptosis. NF-κB has been shown to inhibit apoptosis in response to chemotherapeutic agents (25). Targeting NF-κB in combination with other critical growth-regulating molecules/pathways could overcome the innate chemoresistance of cancer cells and provide additional therapeutic options or improved response. Compounds targeting the NF-κB pathway can sensitize pancreatic tumor cells by counteracting resistance mechanisms and therefore deserve further evaluation as chemotherapy for, and possibly chemoprevention of, pancreatic cancer (26,27). In this study, LDM decreased NF-κB expression through inhibiting the phosphor-Akt level. The total Akt level was not decreased. These results indicate that LDM can inhibit the activation of Akt.

Molecular genetic analysis has identified mutation in the *p53*, *p16*, and *Smad4* tumor suppressor genes and the *ras* proto-oncogene in >80% of pancreatic cancers (28). Sequence analysis identified activating *ras* mutation as the earliest and most common genetic mutation in pancreas cell transformation and tumor progression (29). K-*ras*, H-*ras*, and N-*ras* mutations are involved in ~25 to 30% of all human cancers (30). However, K-*ras* mutations (in codons 12,13, and 16) are present in nearly all pancreatic adenocarcinomas (31,32). Mutant K-*ras* is becoming an attractive target for the treatment of pancreatic cancer. Therefore, K-*ras*-directed therapy is justified (33). In this study, a dose-dependent decrease in K-ras mRNA and protein expression was found in the cells after exposure to increasing concentrations of LDM.

The capacity of pancreatic cells to remodel the extracellular matrix is an important component of tumor invasion (34). There are several enzymes capable of degrading the extracellular matrix of the basement membrane. MMPs play an important role in tumor invasion and metastasis (35). This process is accomplished by the dissolution of the basement membrane extracellular matrix by MMP-2 and MMP-9, the gelatinases for which type IV collagen is a specific substrate. Pancreatic cancer is a highly aggressive tumor that produces metastases in the early course of disease development. This process is mediated, in part, by the activity of MMP-2 and MMP-9 on the extracellular matrix of the basement membrane. Our findings suggest that LDM can inhibit pancreatic cancer cell migration and invasion by blocking the expression of MMP-9 in the cells.

LDM is currently being evaluated in phase II clinical trials as a potential chemotherapeutic agent in China. It can induce apoptosis or mitotic cell death in many cancer cells (36-38). Recent studies have indicated that LDM induces unusual DNA damage responses to double-strand breaks (39). It alters cell cycle progression and induces chromosomal aberrations (40,41). The LDM-induced antitumor effect can be amplified by other molecules (42). The biosynthesis of LDM and another enediyne antibiotic, calicheamicin, has drawn much attention (43,44). In our study, LDM was shown to exhibit extremely potent cytotoxicity to PANC-1 and SW1990 cells. The IC_{50} values of LDM for the PANC-1 and SW1990 cells were 0.955 and 0.426 nM, respectively, lower than those of MMC, ADM and taxol (13,20). LDM-treated cells showed growth arrest and an increasing apoptotic ratio. This could be related to certain molecular changes involving the Akt/NF-κB signal pathway. The results of this study also demonstrate the antitumor activity of LDM in nude mice. The growth of pancreatic xenografts was markedly suppressed by LDM at the dose of 0.02 and 0.04 mg/kg, and its antitumor activity was stronger than gemcitabine (80 mg/kg).

In conclusion, LDM can down-regulate the Akt/NF-κB signal pathway, which in turn can contribute to the induction of apoptosis and tumor growth inhibition. This result indicates that LDM can play a positive role in NF-κB-targeted chemotherapy for pancreatic carcinoma.

Acknowledgements

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