Antitumor efficacy of lidamycin on hepatoma and active moiety of its molecule

Yun-Hong Huang, Bo-Yang Shang, Yong-Su Zhen

Yun-Hong Huang, Bo-Yang Shang, Yong-Su Zhen, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China. Supported by the National High Technology Research and Development Program of China (863 program), No. 2002AA2Z3107. Correspondence to: Professor Yong-Su Zhen, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, 1 Tiantan Xili, Beijing 100050, China. zhenys@public.bta.net.cn

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Abstract
Aim: To study the in vitro and in vivo antitumor effect of lidamycin (LDM) on hepatoma and the active moiety of its molecule.

Methods: MTT assay was used to determine the growth inhibition of human hepatoma BEL-7402 cells, SMMC-7721 cells and mouse hepatoma H22 cells. The in vivo therapeutic effects of lidamycin and mitomycin C were determined by transplantable hepatoma 22 (H22) in mice and human hepatoma BEL-7402 xenografts in athymic mice.

Results: In terms of IC₅₀ values, the cytotoxicity of LDM was 10 000-fold more potent than that of mitomycin C (MMC) and adriamycin (ADM) in human hepatoma BEL-7402 cells and SMMC-7721 cells. LDM molecule consists of two moieties, an apoprotein (LDP) and an enediyne chromophore (LDC) of 843 ku. These two parts of the molecule, connecting each other through non-covalent binding, can be dissociated. LDM can induce DNA damages including double-strand breaks, single-strand breaks and formation of abasic sites which is proved to be the major mechanism of the cytotoxicity of LDM. It is also reported that LDC can interact in DNA minor grooves and cleave double-helical DNA with a remarkable sequence-selectivity. The double-strand cleavage sites occurring predominantly at CTITTA/AAAG, ATATATTAT, CTITTA/AAAG, CTCC/AAAG, and especially GTTAT/ATAAAG, consist of nucleotide sequences with a two-nucleotide 3'-stagger of the cleaved residues.

Conclusion: Both LDM and its chromophore LDC display extremely potent cytotoxicity to hepatoma cells. LDM shows a remarkable therapeutic efficacy against murine and human hepatomas in vivo.

Key words: Lidamycin; Hepatoma; Mitomycin

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Introduction
Lidamycin (LDM, also called C-1027), a macromolecular antitumor antibiotic produced by Streptomyces globisporus C-1027, can markedly inhibit the growth of transplantable tumors in mice including leukemia L1210, P388, sarcoma 180 and melanoma Harding-Passey. LDM consists of an apoprotein (LDP) of 10.5 ku and an enediyne chromophore (LDC) of 843 ku. These two parts of the molecule, connecting each other through non-covalent binding, can be dissociated. LDM can induce DNA damages including double-strand breaks, single-strand breaks and formation of abasic sites which is proved to be the major mechanism of the cytotoxicity of LDM. It is also reported that LDC can interact in DNA minor grooves and cleave double-helical DNA with a remarkable sequence-selectivity. The double-strand cleavage sites occurring predominantly at CTITTA/AAAG, ATATATTAT, CTITTA/AAAG, CTCC/AAAG, and especially GTTAT/ATAAAG, consist of nucleotide sequences with a two-nucleotide 3'-stagger of the cleaved residues.

Recently, LDM has entered phase I clinical trials. In this study, we report the growth inhibition of various carcinoma cell lines by LDM, and the therapeutic efficacy of LDM against hepatoma in mice and human hepatoma xenografts in athymic mice.

Materials and Methods
Materials
Highly purified LDM and LDP were prepared in our institute. MTT was obtained from Sigma Chemical Co. (St.
in diameter, one for each mouse. LDM and MMC in PBS were administrated \( iv \) on d 3 and 10 after tumor implantation. PBS was used as control. Tumor volume (V) was measured twice a week using a caliper and calculated using the formula: 
\[
V (\text{mm}^3) = \frac{1}{2}ab^2
\]
where a and b represent the long diameter and perpendicular short diameter (mm) of the tumor, respectively.

**Statistical analysis**
Significant difference between two values was determined with Student’s \( t \)-test. \( P<0.05 \) was considered statistically significant.

**RESULTS**

**Cytotoxicity of LDM, MMC and ADM to hepatoma cells**

As shown in Figures 1A and B, LDM displayed extremely potent cytotoxicity to hepatoma BEL-7402 and SMMC-7721 cells. In comparison, the cytotoxicity of LDM was much more potent than that of MMC and ADM. The IC\(_{50}\) value of LDM, MMC and ADM was 0.0030, 152, and 51.7 nmol/L for BEL-7402 cells respectively, and 0.064, 1136.6, and 378 nmol/L for SMMC-7721 cells respectively. In terms of IC\(_{50}\) values, the cytotoxicity of LDM to hepatoma cells was 10 000-fold more potent than that of MMC and ADM (Table 1). In addition, the growth inhibitory IC\(_{50}\) value of LDM for mouse hepatoma 22 was 0.025 nmol/L by MTT assay (curve not shown).

![Figure 1](image)

**Cytotoxicity of LDM and its constituents (LDC and LDP) to hepatoma cells**

The inhibitory effects of LDM and its constituents, LDC
LDC displayed extremely potent cytotoxicity to human hepatoma BEL-7402 and SMMC-7721 cells. However, another constituent LDP was much less active to hepatoma cells. The \( IC_{50} \) value of LDC on BEL-7402 and SMMC-7721 cells was 0.0032 and 0.0597 nmol/L, respectively. However, the growth inhibitory \( IC_{50} \) value of LDP on BEL-7402 and SMMC-7721 cells was 2.896 and 1.896 nmol/L respectively. In terms of \( IC_{50} \) values, the potency of LDC was similar to LDM, but LDP was 10-fold less potent than LDM and LDC (Table 1).

### Inhibitory effect of LDM and MMC on hepatoma 22 in mice

The in vivo therapeutic efficacy of LDM and MMC on the growth of murine hepatoma 22 (H22) in Kunming mice was investigated. As shown in Table 2, LDM and MMC inhibited the growth of hepatoma 22 tumors in a dose dependent manner. In experiment I, LDM with single i.v.

| Table 2 Inhibition of the growth of mouse hepatoma 22 by LDM and MMC |
|------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Experiment | Groups | Dose (mg/kg) | \( \times \) times | Number of mice | Body weight (g) | Tumor weight (g) | Inhibition rate(%) | \( P \) |
| I | Control | 0.1 | 10 10 | 19.6 35.0 | 4.19±1.36 | -- | -- | <0.01 |
| | LDM | 0.05 | 10 10 | 19.6 30.6 | 1.19±0.50 | 71.5 | <0.01 | <0.01 |
| | | 0.025 | 10 10 | 19.6 27.3 | 1.60±0.61 | 61.8 | <0.01 | <0.01 |
| | | 0.0125 | 10 10 | 19.6 27.3 | 1.65±0.50 | 60.6 | <0.01 | <0.01 |
| | MMC | 0.00625 | 10 10 | 19.6 27.3 | 2.48±0.78 | 40.8 | <0.01 | <0.01 |
| II | Control | 0.1 | 10 10 | 20.2 35.2 | 3.83±1.22 | -- | -- | <0.01 |
| | LDM | 0.05 | 10 10 | 20.2 32.3 | 0.43±0.16 | 88.8 | <0.01 | <0.01 |
| | | 0.025 | 10 10 | 20.2 29.6 | 0.67±0.20 | 82.5 | <0.01 | <0.01 |
| | | 0.0125 | 10 10 | 20.2 32.1 | 0.96±0.30 | 74.9 | <0.01 | <0.01 |
| | | 0.00625 | 10 10 | 20.2 31.9 | 1.55±0.63 | 59.5 | <0.01 | <0.01 |
| | MMC | 5 | 10 10 | 19.6 23.9 | 1.98±0.77 | 48.3 | <0.01 | <0.01 |
| | 2.5 | 10 10 | 20.2 32.1 | 1.01±0.29 | 73.6 | <0.01 | <0.01 |
| | 1.25 | 10 10 | 20.2 33.1 | 1.76±0.76 | 54.0 | <0.01 | <0.01 |
| III | Control | 0.1 | 10 10 | 20.3 33.2 | 3.83±1.48 | -- | -- | <0.01 |
| | LDM | 0.05 | 10 10 | 20.3 33.2 | 3.3±1.04 | 53.2 | <0.01 | <0.01 |
| | | 0.025 | 10 10 | 20.3 33.2 | 0.35±0.06 | 89.5 | <0.01 | <0.01 |
| | | 0.0125 | 10 10 | 20.3 33.2 | 0.63±0.23 | 81.1 | <0.01 | <0.01 |
| | | 0.00625 | 10 10 | 20.3 33.2 | 0.96±0.45 | 71.2 | <0.01 | <0.01 |
| | MMC | 5 | 10 10 | 19.8 25.1 | 1.08±0.59 | 67.6 | <0.01 | <0.01 |
| | 2.5 | 10 10 | 19.8 25.1 | 1.45±0.88 | 56.5 | <0.01 | <0.01 |
| | 1.25 | 10 10 | 19.8 25.1 | 2.28±0.96 | 31.5 | <0.01 | <0.01 |

**Table 1 Inhibition of proliferation by LDM, MMC, ADM and the LDM constituents LDC and LDP in hepatoma cells (mean±SD)**

| Drugs | IC\(_{50}\) (nmol/L) |
|-------|-----------------|
| LDM | 0.003±0.0006 | 0.064±0.013 |
| MMC | 152.0±27.3 | 113.6±72.2 |
| ADM | 51.7±6.5 | 378.0±39.6 |
| LDC | 0.0032±0.0005 | 0.0597±0.0064 |
| LDP | 2.896.0±345.5 | 1.896.0±354.0 |

LDM: lidamycin; LDC: LDM enediyne chromophore; LDP: LDM apoprotein; ADM: adriamycin, MMC: mitomycin C.

**Figure 2** Growth inhibition of hepatoma BEL-7402 cells (A) and SMMC-7721 cells (B) by LDM, LDC and LDP.
administration at the doses of 0.1, 0.05, 0.025, 0.0125, and 0.00625 mg/kg inhibited tumor growth by 84.7%, 71.6% and 61.8%, 60.6% and 40.8% (P<0.01), respectively. Whereas, the inhibition rate of MMC at the doses of 5, 2.5, and 1.25 mg/kg was 58.9%, 49.9%, and 44.2%, respectively. The therapeutic index (TI) of LDM and MMC was 15 and 2.5, respectively. The therapeutic index of LDM was much higher than that of MMC. In experiments II and III, the drugs were administered on d 3 and 7. The growth inhibition rate by LDM was also higher than that of MMC. At doses used in these experiments, no body weight loss and other severe side-effects were observed, implying that LDM at tolerated doses displayed remarkable therapeutic efficacy.

Inhibition effect of LDM and MMC on the growth of human hepatoma BEL-7402 in athymic mice

For further in vivo evaluation, the athymic mice bearing BEL-7402 hepatoma xenografts were treated by injection of LDM and MMC into the tail vein for the first time 3 d after subcutaneous tumor inoculation. At that time the tumor diameters ranged from 2 to 4 mm. Then the drugs were administered for the second time on d 10. As shown in Figure 3, LDM significantly inhibited the growth of BEL-7402 tumors. According to the tumor volume on d 24 (Table 3), the inhibition rate by LDM at the doses of 0.05 and 0.025 mg/kg was 68.7% (P<0.05) and 27.2%, respectively. MMC at the dose of 1.25 mg/kg inhibited tumor growth by 34.5%. LDM showed much higher efficacy against the growth of hepatoma xenografts.

Figure 3 Inhibitory effect of LDM and MMC on the growth of human hepatoma BEL-7402 xenografts in athymic mice.

Although the cause is not fully understood, there are several known risk factors, including over 40 years of age, male sex, cirrhosis, and exposure to hepatitis viruses (hepatitis B, C, D and G), etc. Although surgical operation could cure some patients with hepatoma, many patients with carcinomas are not good surgical candidates because of large tumor size, diminished liver function, or cirrhosis. Therefore, it is urgent to develop new drugs for the treatment of carcinoma.

It was reported that LDM strongly inhibits DNA synthesis in hepatoma BEL-7402 cells. In terms of effective concentration, LDM is over 1 000-fold potent than MMC or ADM. LDM also inhibits RNA synthesis in hepatoma BEL-7402 cells without affecting protein synthesis, blocks BEL-7402 cells at G2/M phase and induces mitotic cell death of human hepatoma BEL-7402 cells. In the present study, LDM showed extreme cytotoxicity to hepatoma cells, including BEL-7402 SMMC-7721 and H22 cells in vitro. In our study, LDM displayed obvious tumor inhibition effect in vivo. The therapeutic efficacy of LDM was 6-fold higher than that of MMC. In addition, LDM showed potent anti-tumor effect on human hepatoma BEL-7402 xenografts in athymic mice in a dose dependent manner. LDM has drawn much attention of researchers because of its potent anti-tumor effect, unique structure and action mechanism. Its apoprotein gene has been successfully cloned and nucleotide sequencing was determined. Recently, as an example of enediyne anti-tumor antibiotics, the biosynthetic genes of LDM have become a hot spot. In our studies on targeted anti-tumor drugs, LDM could act as a novel “effector moiety” to construct molecule-downsized and highly potent monoclonal antibody drugs. The downsized Fab~2~LDM immunoconjugate is generated by chemical methods, which shows potent anti-tumor effect both in vitro and in vivo. We have demonstrated in this study that LDC has a similar potency as intact LDM molecule. In contrast, its apoprotein LDP displays weak effect on tumor cells. Recently, an energized fusion protein, Fv-LDP-AE, has been prepared. The highly potent fusion protein is composed of Fv fragment of antibody, protein moiety of LDM molecule, and the active enediyne (AE) of lidamycin. The molecular weight of enediyne-energized fusion protein Fv-LDP-AE is only 38.7 ku, much smaller than the reported immunotoxin Fv-PE (67 ku). The method of preparing energized fusion protein Fv-LDP-AE may also provide a useful technical platform to construct scFv-based engineering and enediyne-energized fusion proteins specifically targeted to various cancers expressing different molecular markers.

In summary, both lidamycin and lidamycin chromophore have extremely potent cytotoxicity to liver cancer cells, and lidamycin shows remarkable therapeutic efficacy against murine transplantable hepatoma and human hepatoma xenografts.

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