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Effects of three diterpenoids on tumour cell proliferation and telomerase activity

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Xerophilusin B (XB), macrocalin B (MB) and eriocalyxin B (EB) belong to *ent*-kaurene diterpenoids. The inhibition effects of three *ent*-kaurane compounds on the proliferation of six tumour lines (K562, HL-60, A549, MKN, HCT and CA) were estimated by methyl-thiazol-tetrazolium, and the telomerase activity in K562 cells was measured by the telomeric repeat amplification protocol-enzyme-linked immunosorbent assay. We found that XB, MB and EB could significantly inhibit the proliferation of tumour cells. Within the concentration range of 10^{-4}–10^{-8} mol L^{-1}, three *ent*-kaurane compounds could markedly inhibit the telomerase activity in K562 cells, and their effects on telomerase were exhibited in a dose-dependent manner. Our results demonstrate that XB, MB and EB can inhibit the proliferation of tumour cells and the telomerase activity, which provides data for developing *ent*-compounds as antitumour drugs.

Keywords: XB; MB; EB; cell proliferation; telomerase activity

1. Introduction

Xerophilusin B (XB), macrocalin B (MB) and eriocalyxin B (EB) are natural compounds, which belong to *ent*-kaurene diterpenoids. XB and MB had been isolated from *Isodon xerophilus* (Chen, Lin, & Xu, 1984; Hou et al., 2000), and EB had been isolated from *Isodon eriocalyx* var. *laxiflora* (Niu et al., 2002). Both *I. xerophilus and I. eriocalyx* var. *laxiflora* are native plants from Yunnan Province, China. The structures of XB, MB and EB have been elucidated on the basis of their spectral properties and X-ray crystallographic analysis (Chen et al., 1984; Hou et al., 2000; Niu et al., 2002). Our present research demonstrated that XB, MB and EB could significantly inhibit growth of K562, HL-60, A549, MKN, HCT and CA tumour cells *in vitro*.

Telomerase, a ribonucleoprotein enzyme, utilises its own RNA as a template to add the hexanucleotide to the ends of replicating chromosomes, endowing the cell with the ability of unlimited proliferation. Telomerase is active in the majority of tumour cells but not in normal somatic cells (Franco et al., 2004; Kim et al., 1994; Morin, 1989). Therefore, telomerase is a new target for cancer therapy. Our experiments further showed that XB, MB and EB could markedly inhibit telomerase activity in K562 tumour cells *in vitro*.

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2. Materials and methods

2.1. *Cells and reagents*

K562 (human chronic myelogenous leukaemia cells), HL-60 (human acute promyelocytic leukaemia cells), A549 (human lung carcinoma cells) and HCT (human colorectal carcinoma cells) were purchased from ATCC. MKN (human gastric adenocarcinoma cells) and CA (human live carcinoma cells) were kindly provided by Shanghai Institute of Materia Medica, Chinese Academy of Science. All tumour cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT), penicillin ($100\,\text{units}\,\text{mL}^{-1}$) and streptomycin ($100\,\text{mg}\,\text{mL}^{-1}$). Cisplatin was kindly provided by Kunming Institute of Precious Metals, China. XB, MB and EB were kindly provided by Professor Handong Sun from Kunming Institute of Botany, Chinese Academy of Science. DMSO (dimethyl sulphoxide) and MTT (methyl-thiazol-tetrazolium) were purchased from Sigma Company. The telomeric repeat amplification protocol-enzyme-linked immunosorbent assay (TRAP-ELISA) detection kit was purchased from Boehringer Mannheim, Germany.

2.2. *Cytotoxicity against tumour cells*

Xerophilusin, macrocalin and eriocalyxin were dissolved with 0.1% DMSO. Cytotoxic activity of three compounds was determined using MTT assay. Briefly, K562, HL-60, A549, MKN, HCT and CA cells ($4 \times 10^4\,\text{mL}^{-1}$) were added to 96-well plates, 9 μL per well. After treating at various concentrations of the compounds (10^{-4}–$10^{-8}\,\text{mol}\,\text{L}^{-1}$) for 48 h at 37°C, 5% CO_2, 10 μL of MTT solution ($5\,\text{mg}\,\text{mL}^{-1}$) per well was added to each culture medium, which were incubated for a further 4 h. Then, 10% SDS-5% isobutanol–$0.012\,\text{mL}\,\text{L}^{-1}$ HCl was added to each well (100 μL per well). After 12 h at room temperature, the OD of each well was measured on an ELISA reader (Bioteck EL-340) at wavelengths of 570 and 630 nm. The growth inhibition effects from exposure of cells to each compound were analysed by concentration effect curve.

2.3. *Extraction of proteins containing telomerase*

After treating at various concentrations of the compounds (10^{-4}–$10^{-8}\,\text{mol}\,\text{L}^{-1}$) for 48 h at 37°C, 5% CO_2, K562 cells were transferred to a tube of RNase-free Eppendorf and centrifuged at 3000 *g*, 4°C for 10 min. Then, the supernatant was removed and cells were centrifuged at 300 *g*, 4°C for 15 min, after which 200 μL of CHAPS lysis buffer (0.5% CHAPS, 10 mM Tris-HC1, 1 mM $MgCI_2$, 1 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol, and 0.1 mM benzamidine; Boehringer Mannheim, Germany) was added and inoculated for 30 min at 4°C. The protein concentration was measured by Bradford assay (Pierce Biotechnology, Rockford, USA).

2.4. *Detection of telomerase activity in K562 cells*

An amount of 0.6 μg extracts were added to PCR tube. After 30 min of primer extension at 25°C, PCR was subjected to denaturation (94°C, 30 s), annealing (50°C, 30 s), and extension (72°C, 90 s) for 30 cycles. The amplification product was denatured and

hybridised to a digoxigenin-labelled, telomeric repeat-specific detection probe. Finally, the PCR product was detected with an antibody against digoxigenin that was conjugated to peroxidase. OD values represented telomerase activity and were measured at the wave length of 450 nm. The inhibitory rate of telomerase was calculated according to the formula = (1 − sample OD/negative control OD) × 100%. In this experiment, positive control was cisplatin (10^{-4} mol L^{-1}).

2.5. *Statistical analysis*

The telomerase activity in K562 cells was represented as mean ± standard deviation (SD). Statistical analysis was performed using student's *t*-test. The statistical significance was determined at $p < 0.05$.

3. Results

3.1. *The chemical structure of XB, MB and EB*

Xerophilusin and macrocalin had been isolated from *I. xerophilus*, and EB had been isolated from *I. eriocalyx* var. *laxi flora*. Their chemical structures were elucidated according to their spectral properties and X-ray crystallographic analysis (Figure. 1).

3.2. *The effects of XB, MB and EB on tumour cells proliferation*

The cell proliferation was detected by MTT assay. The result illustrated in Table 1 showed that XB and MB could significantly inhibit proliferation of K562, HL-60, A549, MKN and CA tumour cells, and EB could significantly inhibit proliferation of K562, HL-60, A549, MKN, HCT and CA tumour cells. XB and MB slightly inhibited growth of HCT cells, having IC_{50} values between 17.00 and 171.0 μmol L^{-1}.

3.3. *The effects of XB, MB and EB on telomerase activity in K562 cells*

The effects of XB, MB and EB on telomerase in K562 cells were measured by TRAP-ELISA. As shown in Table 2, within the concentration range of 10^{-4}–10^{-8} mol L^{-1}, XB, MB and EB could significantly inhibit telomerase activity as compared to PBS. At the concentration of 10^{-4} mol L^{-1}, inhibition effect of XB, MB and EB on telomerase in K562 cells was equal to that of cisplatin. These results showed that XB, MB and EB could

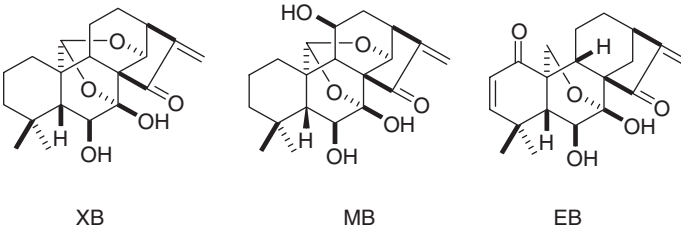

Figure. 1. The structure of three compounds. XB, MB and EB the structure of the three compounds were elucidated on the basis of their spectral properties and X-ray crystallographic analysis.

Table 1. The effects of XB, MB and EB on tumour cells proliferation.

Compound	Chemical formula	IC_{50} (μmol L^{-1})					
		K562	HL-60	MKN	HCT	A549	CA
XB	$C_{20}H_{26}O_5$	2.11	0.80	0.48	17.00	1.09	4.70
MB	$C_{20}H_{26}O_6$	22.30	5.67	4.60	171.00	5.87	2.81
EB	$C_{20}H_{24}O_5$	0.45	0.46	0.22	34.40	0.24	21.50

Notes: Six tumour cells proliferation were analysed by MTT assay. The result showed XB and MB had significant inhibition effects on K562, HL-60, A549, MKN and CA tumour cells. EB had significant inhibitory effects on K562, HL-60, A549, MKN, HCT and CA tumour cells.

Table 2. The effects of XB, MB and EB on the telomerase activity.

	XB		MB		EB	
(mol/L)	Telomerase activity	Inhibitory rate (%)	Telomerase activity	Inhibitory rate (%)	Telomerase activity	Inhibitory rate (%)
10^{-4}	0.042 ± 0.002*	85.9	0.039 ± 0.002*	86.9	0.039 ± 0.003*	86.9
10^{-5}	0.043 ± 0.001*	85.5	0.046 ± 0.004*	84.5	0.035 ± 0.003*	88.2
10^{-6}	0.053 ± 0.017*	82.2	0.063 ± 0.001*	78.8	0.039 ± 0.004*	86.9
10^{-7}	0.057 ± 0.007*	80.8	0.096 ± 0.008*	67.7	0.070 ± 0.027*	76.4
10^{-8}	0.069 ± 0.022*	76.8	0.108 ± 0.046*	63.6	0.110 ± 0.017*	63.0
PBS	0.297 ± 0.004		0.297 ± 0.004		0.297 ± 0.004	
Cisplatin	0.040 ± 0.003**	86.5	0.040 ± 0.003**	86.5	0.040 ± 0.003**	86.5

Notes: The effects of XB, MB and EB on telomerase activity in the K562 cells were assayed by TRAP-ELISA. The results were expressed as mean ± SD.
*$p < 0.01$ for telomerase activity in K562 cells treated with XB, MB and EB when compared to PBS.
**$p < 0.01$ for telomerase activity in K562 cells treated with cisplatin as compared to PBS.

significantly inhibit the telomerase activity in K562 cells, and inhibition effects were exhibited in a dose-dependent manner.

4. Discussion

It has been demonstrated that *ent*-kaurene diterpenoids have biological activity such as antibacterial and potent antitumour activities (Fuji et al., 1985, 1989; Fujita et al., 1976; Osawa et al., 1994). Our continuing research demonstrates that *ent*-kaurene diterpenoids can significantly inhibit growth of tumour cells *in vitro* (Sun et al., 1994, 1995a, b). Therefore, it is worthy to further study their mechanism.

K562 cells express high levels of telomerase activity, which provide a good model to explore inhibitory activity on telomerase (Faraoni et al., 2005). Cisplatin is a common antitumour drug that can inhibit telomerase activity (Burger & Double, 1997; Ishibashi & Lippard, 1998; Kondo et al., 1995), therefore it is used as positive control.

Oridonin, a diterpenoid isolated from *Rabdosia rubescens*, has antitumour effects (Zhou et al., 2007). Recent research demonstrated that oridonin could supress

telomerase activity. Li and Wang (2004) found that both 3.43 and 6.86 μmol L^{-1} oridonin could inhibit telomerase activity in K562 cells. However, the inhibition effect of 3.43 μmol L^{-1} oridonin on telomerase was more significant. The mechanism by which oridonin inhibits telomerase activity may involve cell-cycle arrest in G_2–M phase (Li & Wang, 2004). In this study we investigated the effects of XB, MB and EB on telomerase in K562 cells. We found that XB, MB and EB could significantly inhibit telomerase activity in K562 cells. Unlike oridonin, the higher the concentrations of XB, MB and EB, the lower the telomerase activity in K562 cells. At concentrations ranging from 10^{-4} to 10^{-8} mol L^{-1}, inhibition effects of XB, MB and EB on the telomerase in K562 cells were exhibited in a dose-dependent manner.

As to the mechanisms of how three *ent*-kaurene diterpenoids inhibit telomerase activity, we propose the following reasons. First, three compounds can inhibit growth of tumour cells, therefore the telomerase activity measured in K562 cells may represent the telomerase activity from survived cells. Second, it has been demonstrated that telomerase activity during S phase is maximal, and is minimal in G_1–M phase (Zhu et al., 1996). Cytocytic drugs can arrest tumour cells in G_1–S phase. XB, MB and EB exhibit significant cytocyticity on K562 cells, which causes cells to be in a non-proliferating state, reducing telomerase activity. Therefore, the effects of XB, MB and EB on telomerase may correlate with medicating cell-cycle arrest. Third, in terms of the effects of three compounds on K562 cells, IC_{50} of telomerase activity is far lower than IC_{50} of cell proliferation, which implies that reduction of telomerase activity is a progressive process. According to our present results, we cannot conclude whether the effects of XB, MB and EB on telomerase in K562 cells are irrevisible. It is interesting to further study direct molecular interactions between the three compounds and the telomerase activity.

In summary, XB, MB and EB can significantly inhibit tumour cells proliferation and telomerase activity. However, a defined mechanism, such as relations of structure and effect, should be further studied.

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