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Specific anticancer activity of a new bisabolane sesquiterpene against human leukemia cells inducing differentiation *in vitro*

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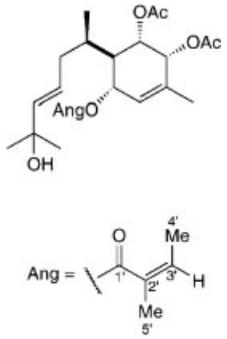
A bisabolane sesquiterpene, *rel*-(1*S*,4*R*,5*S*,6*R*)-4,5-diacetoxy-6-[(*R*)-5-hydroxy-1,5-dimethylhex-3-enyl]-3-methylcyclohex-2-enyl (*Z*)-2-methylbut-2 -enoate, which was newly isolated from the roots of *Leontopodium longifolium*, presented specific anticancer activity against human leukemia HL-60 cells, but did not inhibit proliferation of human hepatoma SMMC-7721 cells and human normal hepatocytes L02 cells. Nitroblue tetrazolium (NBT) reduction, phagocytosis of latex beads, and cell electrophoresis all demonstrated that this bisabolane sesquiterpene presented its anticancer activity against human leukemia HL-60 cells *in vitro* via inducing cell differentiation. Our results may have implications for treatment of human leukemia with the sesquiterpene.

1. Introduction

It was believed for a long time that once carcinogenesis had occurred, cancer cells could not be reversed into normal cells, leading to the incorrect idea that one could only cure cancer through killing cancer cells, until Pierce et al. (1960) found that teratocarcinoma cells in mouse could spontaneously differentiate into normal cells. Then attention began to be paid to cancer differentiation and to possibilities for curing cancer through inducing cell differentiation. From the pioneer work of Friend et al. (1971) who found that dimethyl sulfoxide (DMSO) stimulated erythroleukemia cells to differentiate along the erythroid pathway, to recent work in which all-*trans* retinoic acid (ATRA) is used to cure acute promyelocytic leukemia (APL), differentiation therapy has been widely applied. However, most differentiation inducers at present have severe toxic side-effects. For example, ATRA induces complete remission in a high proportion of patients with APL (Leung et al. 2005; Kizaki et al. 1996), but most of these patients develop ATRA resistance and relapse, resulting in a severe ATRA syndrome (Garcia-Suarez et al. 2004). Therefore, new differentiation inducers with high efficiency and low toxicity should be explored.

Recently, a new bisabolane sesquiterpene, *rel*-(1*S*,4*R*,5*S*,6*R*)-4,5-diacetoxy-6-[(*R*)-5-hydroxy-1,5-dimethylhex-3-enyl]-3-methylcyclohex-2-enyl (*Z*)-2-methylbut-2-enoate, was isolated from the roots of *Leontopodium longifolium*, a plant widely found throughout northwest China (Li et al. 2006). As a traditional Chinese herbal medicine, *L. longifolium* has long been used for releasing cough, as an expectorant, and for the treatment of bronchitis (Zhao 2004). Details of its isolation and structure elucidation have been reported by the laboratory of Prof. Zhongjian Jia (Li et al. 2006). Here, we report that the sesquiterpene presents anticancer activity via inducing differentiation of human leukemia HL-60 cells.



rel-(1*S*, 4*R*, 5*S*, 6*R*)-4,5-diacetoxy-6-[(*R*)-5-hydroxy-1,5-dimethylhex-3-enyl]-3-methylcyclohex-2-enyl (*Z*)-2-methylbut-2-enoate.

2. Investigations and results

2.1. Specific anticancer activity of the bisabolane sesquiterpene against human leukemia cells in vitro

Treating cells with the sesquiterpene resulted in a concentration- and time-dependent arrest of cell proliferation in human leukemia HL-60 cells (Fig. 1A and 1B). However, the sesquiterpene at the same concentration range did not affect the proliferation of human hepatoma SMMC-7721 cells (Fig. 1C) and human normal hepatocyte L02 cells (Fig. 1D). At the same time, the trypan blue stain exclusion assay showed that the sesquiterpene did not influence the viability of HL-60 cells at the concentrations indicated (Fig. 2).

2.2. Effects of the bisabolane sesquiterpene on differentiation of human leukemia cells

2.2.1. Nitroblue tetrazolium reduction

Exposure of HL-60 cells to the sesquiterpene (12.5 ~ 200 μM) resulted in a significant increase in the percentage of cells

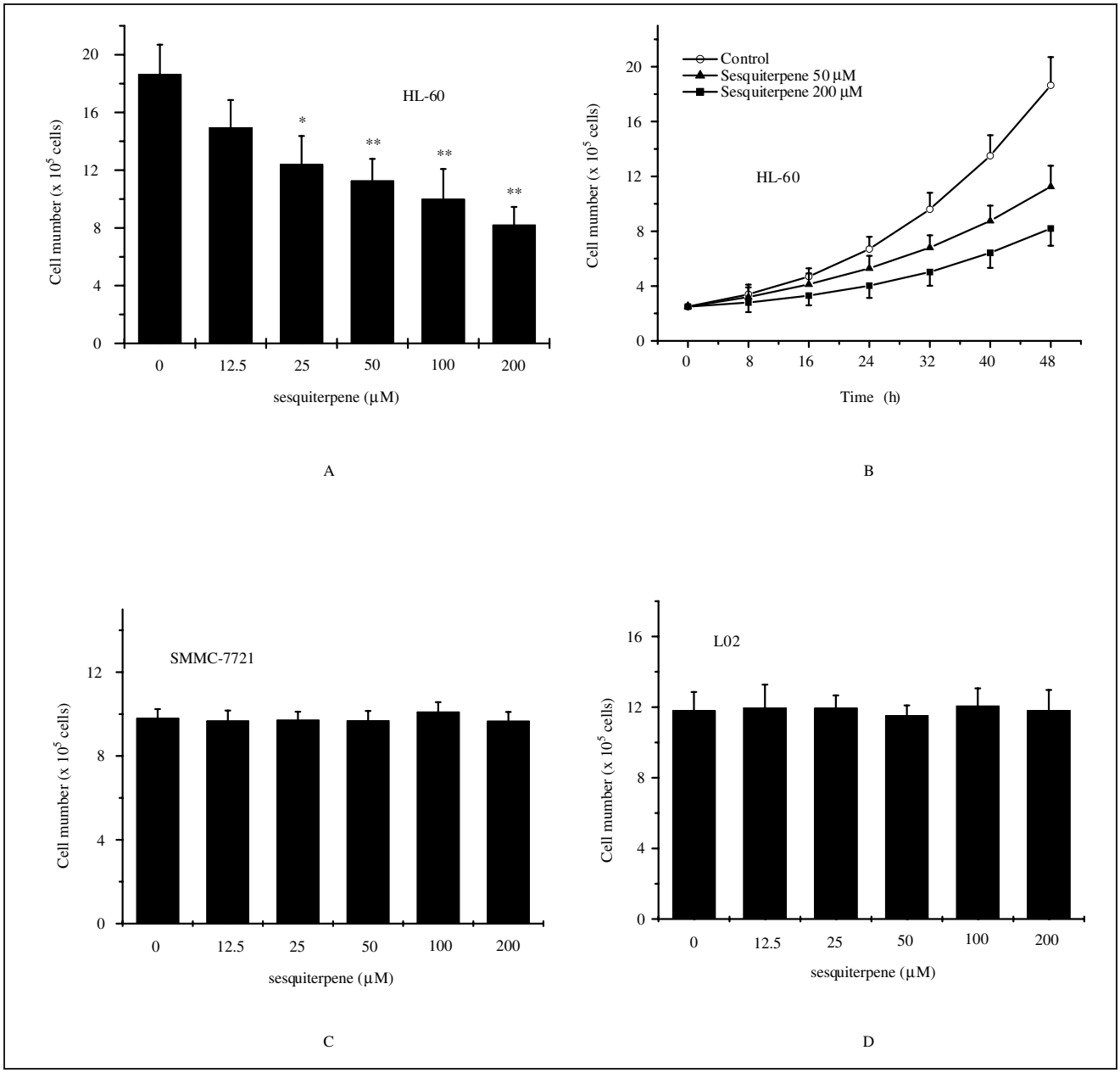


Fig. 1: The sesquiterpene inhibited proliferation of human leukemia HL-60 cells in concentration- (A) and time-dependent (B) manners, but did not inhibit proliferation of human hepatoma SMMC-7721 cells (C) and human normal hepatocytes L02 cells (D) at the indicated concentrations. All data represent mean values of triplicate measurements ± SD. * $p < 0.05$, ** $p < 0.01$ vs. the control group in A

with nitroblue tetrazolium (NBT) reduction activity, compared with control cells (Fig. 3A). In a correlation analysis, treatment with the sesquiterpene at different concentrations showed a negative relationship between cell proliferation and NBT reduction ($R = -0.87518$, Fig. 3B).

2.2.2. *Phagocytosis of latex beads*

After treatment with 200 μM sesquiterpene, HL-60 cells became obviously larger and phagocytosed a number of 1-μm latex beads (Fig. 4B), compared with control cells (Fig. 4A). Dead cells were stained red by eosin Y, which excluded the interference of dead cells in the experimental results. Phagocytotic ability of the treated cells depended on concentration of the sesquiterpene in the range of $12.5 \sim 200$ μM (Fig. 4C). The correlation between cell proliferation and phagocytotic ability was also negative ($R = -0.97444$, Fig. 4D).

2.2.3. *Cell electrophoresis*

For HL-60 cells treated with the sesquiterpene, the electrophoresis time was prolonged in a dose-dependent manner, and showed significant difference at concentrations of $50 \sim 200$ μM (Fig. 5A). In the same way, the correlation between cell proliferation and electrophoresis time was obviously negative ($R = -0.84597$, Fig. 5B).

3. Discussion

HL-60 cells are one kind of human acute promyelocytic leukemia distinguished by Collins et al. (1977), which can differentiate into diverse terminals, induced by different agents. HL-60 cells are useful models for research to understand the characteristics of human leukemia and search for drugs to cure it. Presently, the main differentiation inducers in clinical use are ATRA (Avvisati and Tallman

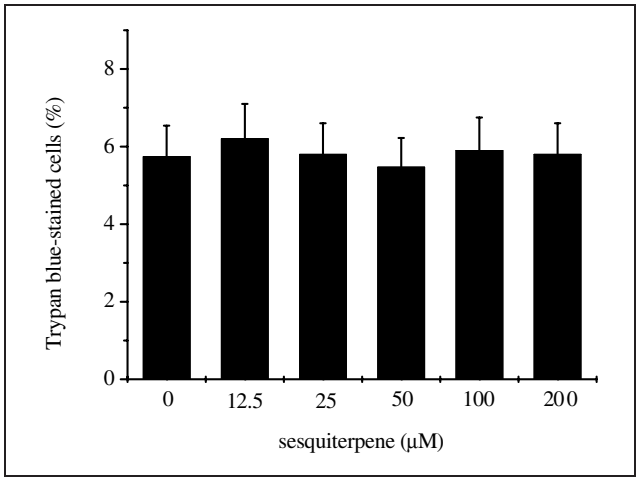


Fig. 2: Effect of the sesquiterpene on viability of human leukemia HL-60 cells at indicated concentrations. All data represent mean values of triplicate measurements ± S.D.

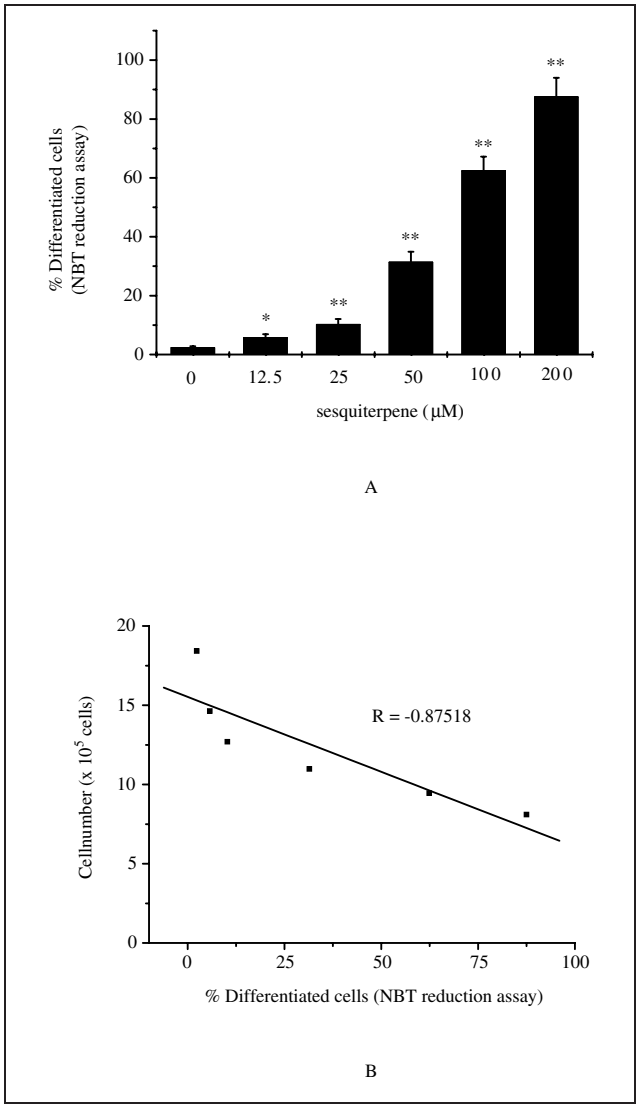


Fig. 3: Effect of the sesquiterpene on NBT reduction activity of human leukemia HL-60 cells at indicated concentrations (A) and negative correlation between cell proliferation and NBT reduction (B). All data represent mean values of triplicate measurements ± S.D. * $p < 0.05$, * $p < 0.01$ vs. the control group in A

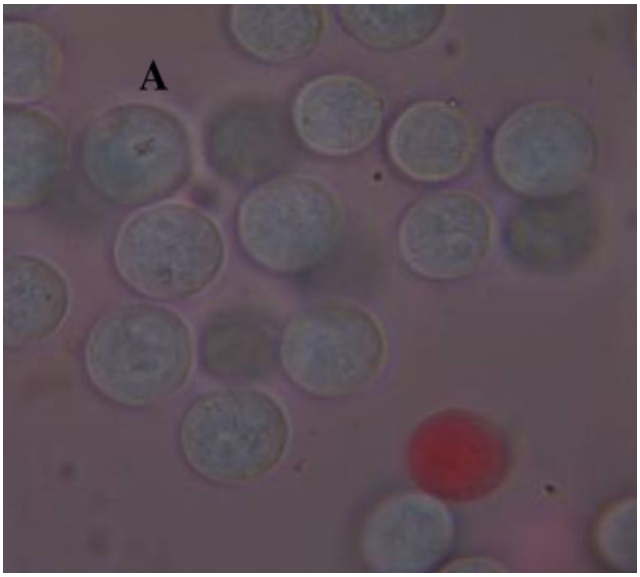
2003) and arsenic trioxide (As_2O_3) (Liu and Han 2003), both of which are restricted in use due to their severe toxic side-effects, so that new differentiation inducers are in great demand. Here, we report that a newly-isolated bisabolane sesquiterpene, *rel*-(1*S*,4*R*,5*S*,6*R*)-4,5-diacetoxy-6-[(*R*)-5-hydroxy-1,5-dimethylhex-3-enyl]-3-methylcyclohex-2-enyl(*Z*)-2-methylbut-2-enoate, induced differentiation of HL-60 cells.

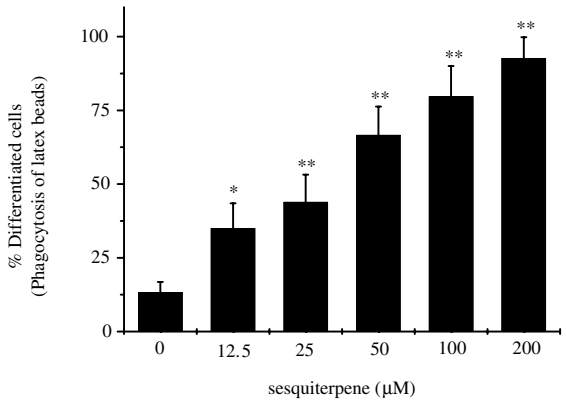
In our experiments, the sesquiterpene particularly inhibited the growth of human leukemia HL-60 cells, but had little effect on another kind of tumor cells (human hepatoma SMMC-7721 cells) or normal cells (human normal hepatocytes L02 cells) (Fig. 1). These results were also confirmed by a previous report using the sulforhodamine B (SRB) method (Li et al. 2006). Recently, three new bisabolane sesquiterpenes were isolated from the roots of *Leontopotium longifolium*. Besides the sesquiterpene reported here, *rel*-(1*S*,4*R*,5*S*,6*R*)-4,5-diacetoxy-6-[(*R*)-1,5-dimethylhexa-3,5-dienyl]-3-methylcyclohex-2-enyl (*Z*)-2-methylbut-2-enoate (compound **1**) and *rel*-(1*R*,2*S*,4*R*,5*S*)-4-acetoxy-2-[(*R*)-5-hydroxy-1,5-dimethylhex-3-enyl]-5-methylcyclohexyl (*Z*)-2-methylbut-2-enoate (compound **2**) were also isolated. Compound **1** has a similar structure to the sesquiterpene tested, and also inhibited the growth of HL-60 cells with no effects on proliferation of SMMC-7721 and L02 cells, but compound **2** did not influence proliferation of any of the three cell lines (Li et al. 2006). Compound **2** lacks 5-acetoxy in its structure compared with the other two compounds, indicating that 5-acetoxy might play an important role in the biological activity of the sesquiterpene tested. More details of the structure and proliferation-inhibitory activity of these three compounds were elaborated in a report by Li et al. (2006).

Interestingly, we found that the growth-inhibitory effect of the sesquiterpene tested could not be attributed directly to its cytocidal activity, since HL-60 cells treated with the same concentrations of the sesquiterpene did not exhibit obvious cell death, compared with the control cells (Fig. 2). A great deal of evidence has shown that cell proliferation arrest and withdrawal from the cell cycle are preconditions for cell differentiation (Studzinski and Harrison 1999). Thus, the differentiation of HL-60 cells was investigated.

NBT reduction is a universal method to measure the differentiation of HL-60 cells (Meyer and Kleinschnitz 1990; Kizaki et al. 1996; Zhao et al. 2006). When HL-60 cells differentiate into normal human phagocytes, they can recover the ability of generating superoxide anion (O_2^-), an oxygen free radical, in order to kill phagocytized bacteria (Johnston et al. 1975), and the radical can be detected by its capacity to reduce NBT (Beauchamp and Fridovich 1971). Furthermore, HL-60 cells were examined by the latex bead ingestion assay, which is a routine method to analyze the action of phagocytes (Thompson et al. 2005; Craig et al. 2005) and also to detect differentiation of HL-60 cells (Sokoloski and Sartorelli 1987; Vassiliadis et al. 1992; Kramer et al. 2002). Undifferentiated HL-60 cells could not reduce NBT and exhibited only a minimal ability to ingest latex beads. After treatment with the sesquiterpene, HL-60 cells not only showed increased NBT reduction ability (Fig. 3A), but also recovered the capacity for phagocytosis (Fig. 4C), demonstrating the differentiation-inducing effect of the sesquiterpene in HL-60 cells.

As human promyelocytes, HL-60 cells can differentiate into granulocytes or macrophages/monocytes (Collins et al. 1977; Koeffler 1983), depending on the various differentiation inducers. For example, HL-60 cells were induced





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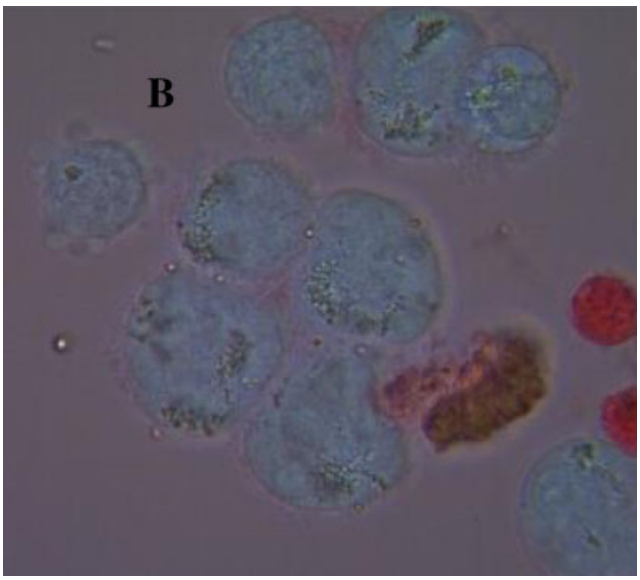

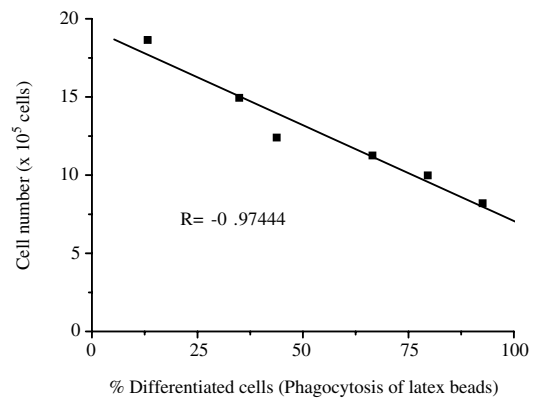

D

Fig. 4: Effect of the sesquiterpene on phagocytotic ability of HL-60 cells, measured by phagocytosis of latex beads. Cell phagocytotic ability and morphological changes without (A) or with (B) treatment of 200 μM sesquiterpene. Dead cells dyed red by eosin Y. Percentage of cells with phagocytotic ability after treatment with different concentrations of sesquiterpene (C) and negative correlation between cell proliferation and phagocytotic ability (D). All data represent mean values of triplicate measurements ± SD. * $p < 0.05$, ** $p < 0.01$ vs. the control group in C

to differentiate along the granulocytic pathway by exposure to 6-methylmercaptopurine ribonucleoside (Sokoloski and Sartorelli 1987), but into monocyte-like cells by exposure to 1,25-dihydroxyvitamin D3 (Watanabe et al. 1996). After treatment with the sesquiterpene, HL-60 cells became obviously larger than control cells, indicating that the HL-60 cells were more likely to differentiate into macrophages, a finding which needs further investigation.

The net charges at the surface of tumor cells are generally greater than those of the corresponding normal cells (Capo et al. 1983; Sato et al. 1979), thus, the electrophoresis time of tumor cells should be less than that of normal cells with the same intensity of electric field, and the increase in electrophoresis time has been taken as an indicator of tumor cell differentiation, as has been reported in tumor cells including human gastric adenocarcinoma MGc80-3 cells (Li et al. 1997), human hepatoma BEL-7402 cells (Zheng and Zheng 2002), human hepatoma SMMC-7721 cells (Zhang et al. 2005; Pan et al. 2004; Kang et al. 2001) and even murine macrophage-like P388D1 tumor cells (Capo et al. 1983). In our results, the higher the concentration of the sesquiterpene with which were treated HL-60 cells, the more time they needed to move the same distance, demonstrating that the surface charges of human leukemia HL-60 cells are apparently also greater than those of the corresponding normal cells, which has not been reported before as far as we are aware, just like the other kinds of tumor cells mentioned above. The results thus indicated that treatment with the sesquiterpene decreased the surface charge of HL-60 cells and induced cell differentiation. It has been suggested that changes of some enzyme activities within the cell membranes might accompany the change of charge state and affect the charge at the cell surface (Pan et al. 2004), so that the alteration of cell surface charges could exert an important influence on the malignant phenotypic reversion of tumor cells, including HL-60 cells.

In brief, the sesquiterpene induced differentiation in human leukemia HL-60 cells, as demonstrated by NBT reduction, phagocytosis of latex beads, and cell electrophoresis.

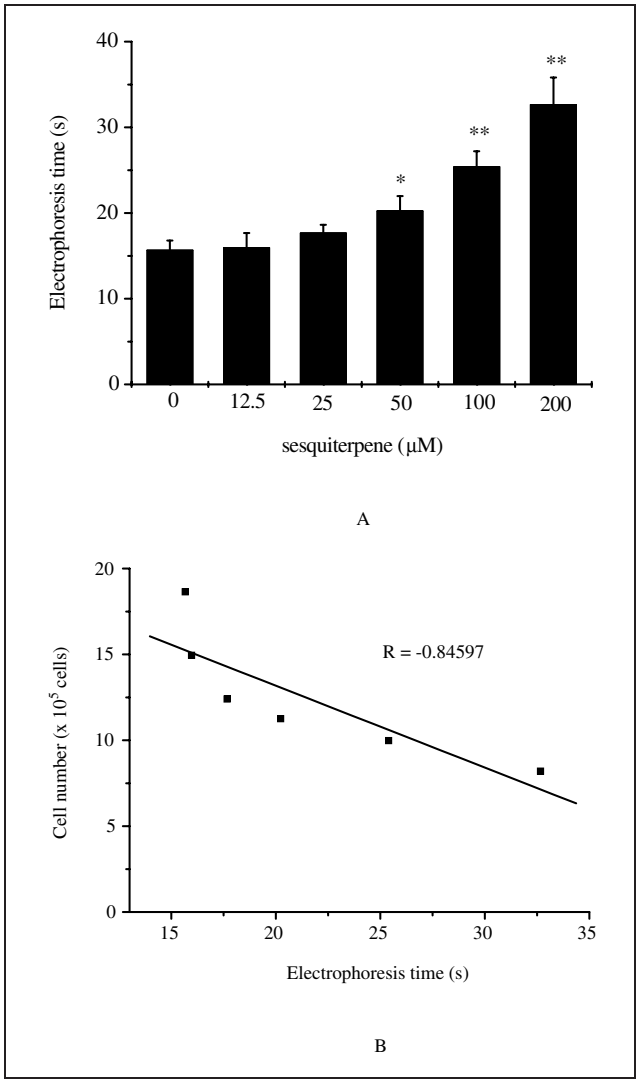

Fig. 5: Cell electrophoresis time change after treatment with different concentrations of sesquiterpene (A) and negative correlation between cell proliferation and electrophoresis time (B). All data represent mean values of triplicate measurements ± S.D. * $p < 0.05$, ** $p < 0.01$ vs. the control group in A

Furthermore, according to correlation analysis, treatment with the sesquiterpene showed a negative relationship between cell proliferation and differentiation (Fig. 3B, Fig. 4D and Fig. 5B), that is, the sesquiterpene inhibited HL-60 cell proliferation due to its differentiation-inducing effect, which was measured by three different methods.

In conclusion, the sesquiterpene presented its specific anticancer activity against human leukemia HL-60 cells *in vitro* by inducing cell differentiation, but had little effect on cell death, which reflected its low toxicity, or on proliferation of other kinds of cells. Hence, our results suggest that the sesquiterpene could be considered as a candidate drug to cure human leukemia, though the differentiation mechanism needs to be investigated.

4. Experimental

4.1. Reagents and cell lines

The bisabolane sesquiterpene, *rel*-(1*S*,4*R*,5*S*,6*R*)-4,5-diacetoxy-6-[(*R*)-5-hydroxy-1,5-dimethylhex-3-enyl]-3-methylcyclohex-2-enyl (*Z*)-2-methylbut-2-enoate, was generously donated by Prof. Zhongjian Jia (State Key Laboratory of Applied Organic Chemistry, College of Chemistry and Chemical Engineering, Lanzhou University, Lanzhou, China). Trypan blue, trypsin, nitroblue tetrazolium (NBT), 12-*O*-tetradecanoyl phorbo1 13-acetate, latex beads, eosin Y and agar were purchased from Sigma (Sigma, St. Louis, MO). RPMI-1640 was purchased from Gibco Laoratories (Santa Clara, CA). Bovine serum was obtained from Si-Ji-Qing Biotechnology Co. (Hangzhou, China). All other reagents were of analytical reagent grade.

Human leukemia HL-60 cells, human hepatoma SMMC-7721 cells and human normal hapatocytes L02 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

4.2. Cell culture and treatment

HL-60 cells, SMMC-7721 cells and L02 cells were grown in RPMI-1640 medium supplemented with 10% inactivated bovine serum, penicillin 100 units/mL, streptomycin 100 μg/mL, and 2 mM glutamine at 37 °C in 5% CO_2 in a humidified incubator. The sesquiterpene was added to the culture medium until the cells were harvested. Cells were treated at the indicated concentrations for 48 h or for the indicated time.

4.3. Cell proliferation and viability assay

Cells were seeded in 5 mL culture bottles at a density of 5×10^4 cells/mL, harvested every 8 h until 48 h and mixed with an equal volume of 0.4% trypan blue solution for 5 min. The stained and unstained cells were counted under a phase-contrast microscope by one investigator. Mean values were obtained from three separate experiments.

4.4. Nitroblue tetrazolium (NBT) reduction

NBT reduction was performed as previously described (Kizaki et al. 1996). The HL-60 cells were suspended at a density of 2×10^5 cells/mL, and mixed with an equal volume of solution containing 1.25 mg/mL NBT, 17 mg/mL bovine albumin, and 1 mg/mL 12-*O*-tetradecanoyl phorbo1 13-acetate for 30 min at 37 °C. Then the medium was discarded, and formazan deposits were dissolved in 100 μL dimethyl sulfoxide (DMSO) and measured at 580 nm by a microplate spectrophotometer reader (Multiskan Ascent, Labsystems).

4.5. Phagocytosis of latex beads

To examine phagocytotic ability, the HL-60 cells were treated with the sesquiterpene for 48 h at 37 °C and incubated with 1-μm latex beads (Sigma) at a concentration of 1×10^9 beads/mL 4 h prior to the time when cells were harvested. Then the cells were washed five times with PBS, stuck on slide glasses, stained with eosin Y, and observed under a microscope.

4.6. Cell electrophoresis

Cell electrophoresis was performed as previously described (Kang et al. 2001; Zheng and Zheng 2002). The HL-60 cells were collected and washed with D-Hanks' solution twice, then resuspended at a density of 1×10^9 cells/L. The cell electrophoresis determination was performed with a round plastic tube with an electric-bridge filled with NaCl 10%-agar 1% and Ag−AgCl electrodes at a direct current voltage of 40 V, at room temperature of 24 °C, taking sucrose 9% as the electrophoretic medium and using a microcapillary electrophoresis apparatus. The results were expressed as the average time during which a cell moves over a distance of 120 μM and 50 cells in each group were determined.

4.7. Statistical analysis

All data were collected from at least three separate experiments. The statistical analysis was done using one-way ANOVA, and p values less than 0.05 or 0.01 were denoted as * or ** respectively.

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