Tetramethylpyrazine inhibits the proliferation of acute lymphocytic leukemia cell lines via decrease in GSK-3β

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Abstract. Tetramethylpyrazine (TMP) has been proven to be an anticancer agent in many studies. However, its effectiveness in acute lymphoblastic leukemia (ALL) and its molecular mechanisms are still unclear. The present study aimed to evaluate the effect of TMP against Jurkat and SUP-B15 ALL cell lines and to investigate the possible detailed mechanism of action of TMP. A Cell Counting Kit-8 (CCK-8) assay was employed to examine the proliferation of Jurkat and SUP-B15 cells. Flow cytometric analysis was conducted to detect the cell cycle distribution and apoptotic rate. The expression of total glycogen synthase kinase-3β (GSK-3β), cox-2, survivin, bcl-2 and p27 RNA and protein levels was detected by quantitative real-time PCR and western blot assay, respectively. Additionally, western blot analysis was used to determine the whole-cell and nuclear protein levels of GSK-3β downstream transcription factors, NF-κB (p65) and c-myc. TMP inhibited the proliferation of Jurkat and SUP-B15 cells in a dose- and time-dependent manner, with IC50 values of 120 and 200 µg/ml, respectively at 48 h. TMP induced the apoptosis of Jurkat and SUP-B15 cells and synergistically blocked cell cycle progression at the G0/G1 phase. Cells treated with TMP exhibited significantly attenuated GSK-3β, NF-κB (p65) and c-myc expression, followed by downregulation of bcl-2, cox-2 and survivin and an upregulation of p27. The results showed that TMP induced apoptosis and caused cell cycle arrest in Jurkat and SUP-B15 cells through the downregulation of GSK-3β, which may have further prevented the induced translocation of NF-κB and c-myc from the cytoplasm to the nucleus.

Introduction

Glycogen synthase kinase-3β (GSK-3β) is a multifunctional serine/threonine protein kinase that functions as a transducer in the Wnt/β-catenin, Notch, Hedgehog and other signaling pathways to regulate multiple physiological processes, including glycogen metabolism, cell proliferation, differentiation and apoptosis (1,2). GSK-3β is one of the essential cell signal transducers and is recognized for its diverse functions in cell survival (3).

Although its functions in normal cell physiology have been thoroughly investigated, the role of GSK-3β in cancer is still unclear and is the subject of much debate (4,5). In solid tumors, Thiel et al (6) found that treating gastric cancer cells with RNAi targeting GSK-3β or GSK-3β inhibitors induced an increase in cox-2 and promoted the progression of gastric cancer. However, Tsuchiya et al (7) showed that GSK-3β functions as a tumor suppressor in colon cancer by promoting the degradation of β-catenin. In a previous study of leukemia by our group (8), we demonstrated that GSK-3β is unusually highly expressed in the cytoplasm and nuclei of leukemic cells in children with acute lymphoblastic leukemia (ALL), suggesting that GSK-3β may play a role as an oncogene in ALL. There is a great deal of interest in finding effective drugs directed against GSK-3β for the treatment of various types of cancers (9).

Tetramethylpyrazine (TMP) (2,3,5,6-tetramethylpyrazine; C9H12N2) is one of the bioactive ingredients extracted from the rhizome of the Chinese herb *Ligusticum*. Studies have demonstrated that TMP has potent inhibitory effects on a variety of tumors through affecting the proliferation and migration of tumor cells (10-12). In our previous study, TMP was found to inhibit the growth of HL-60 cells by inducing their differentiation (13). There have been no studies concerning the effect of TMP on ALL cells.

In the present study, we treated Jurkat and SUP-B15 ALL cell lines with TMP and evaluated the effects of TMP on proliferation, apoptosis and the cell cycle. We also analyzed the expression of GSK-3β and its downstream transcription factors and apoptosis-related molecules in ALL cells in hopes of providing new insight into the anti-leukemia mechanism of TMP.

Materials and methods

Chemicals and reagents. Both the human acute T lymphoblastic leukemia Jurkat and Ph⁺ ALL SUP-B15 cells were...
obtained from the American Type Culture Collection (ATCC; USA), and preserved in our laboratory. RPMI-1640 and fetal bovine serum (FBS) were obtained from Gibco and HyClone, USA, respectively. A nuclear and total protein extraction kit and Annexin V-FITC/PI apoptosis detection kit were purchased from KeyGen Biotechnology, China. Western blotting detection reagents and Cell Counting Kit-8 (CCK-8) were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). TMP was purchased in 4 mg/2 ml injection ampules from Rongsheng Pharmaceutical Co., Ltd., China. TRIzol reagent was supplied by Life Technologies, USA. Real-time polymerase chain reaction (PCR) primers were synthesized by Huada Biotechnology Co. Ltd., Shanghai, China, and its mixture was obtained from Takara, Japan. Antibodies against GSK-3β, NF-κB, c-myc, bcl-2, p27KIP1, β-actin and lamin B1 were purchased from Abcam Co., USA. Antibody against survivin was purchased from GeneTex Co., USA.

Cell culture. Both Jurkat and SUP-B15 cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 µg/ml streptomycin, 100 U/ml penicillin and 1 mM L-glutamine. They were maintained at 37˚C in a humidified atmosphere of 5% CO₂.

Cell proliferation assay. Cell proliferation was detected with the CCK-8 assay following the manufacturer's instructions. Both Jurkat and SUP-B15 cells were seeded at a density of 1x10⁴ cells/well in 96-well microtiter plates and cultured for 24, 48 and 72 h with different concentrations of TMP (20, 40, 80, 160 and 320 µg/ml). Control cells were not treated with TMP. At each indicated time point, the cells were combined with 10 µl CCK-8 kit solution and incubated for an additional 3 h. Absorbance was measured at 450 nm (A value). Three replicate wells were used for each analysis. The cell growth inhibition rate (IR) (IR = 1 - A of treated wells/A of control wells) and the half maximal inhibitory concentration (IC₅₀) value were calculated.

Cell apoptosis by Annexin V/PI assay. The cell apoptosis rate was measured by flow cytometry (FACSCalibur; BD Biosciences, USA) with Annexin V/PI staining assay. After being synchronized, Jurkat cells were incubated with TMP (0, 60, 120 and 180 µg/ml) for 48 h, while SUP-B15 cells were incubated with TMP (0, 100, 200 and 300 µg/ml) for 48 h. Cells (>1x10⁶) were collected and washed twice with cold 0.01 M phosphate-buffered saline (PBS). According to the manufacturer's instructions, cells were resuspended in binding buffer and stained with Annexin V-FITC (5 µl) and PI (5 µl), respectively, and then analyzed by flow cytometry. Early apoptosis was defined as being Annexin V-positive and PI-negative, while late apoptosis and necrosis were defined as being both Annexin V-positive and PI-positive.

Cell cycle assay. The flow cytometric analysis was employed to determine the cell cycle distribution of the TMP treated Jurkat and SUP-B15 cells. Briefly, ~1x10⁶ cells were collected. After fixation with 70% cold ethanol at -20˚C overnight, the cells were washed twice with PBS, and labeled with 50 mg/ml PI containing 100 µg/ml DNase-free RNase A. The cells were cultured for 30 min at 37˚C in the dark and analyzed for DNA content by flow cytometry.

Preparation of RNA extraction and quantitative real-time PCR analysis. The expression of GSK-3β, c-myc, cox-2, survivin, bcl-2 and p27 mRNA in the TMP-treated Jurkat and SUP-B15 cells was analyzed by quantitative real-time PCR analysis. After the co-culture of cells with TMP at different concentrations for 48 h, total RNAs were isolated using TRIzol reagent according to the manufacturer's protocol. Reverse transcription was used to synthesize complementary DNAs. The primer sequences and annealing temperature used for PCR are listed in Table I. cDNA (0.8 µl) was added to 10 µl of the PCR mixture containing 3.4 µl of H₂O, 0.4 µl each of 5’ and 3’ primers (10 µmol·l⁻¹), and 5 µl of 2X SYBR Premix Ex Tag II.
The reaction conditions were as follows: 39 cycles for 30 sec at 95°C, 5 sec at 95°C, 30 sec at annealing temperature, and 1 min at 65°C. The results were normalized against β-actin and appeared as a target mRNA:β-actin ratio.

Western blot analysis. Western blot analysis was conducted for GSK-3β, c-myc, Cox-2, survivin, bel-2, NF-κB and p27 expression. Jurkat and SUP-B15 cells of the treated and control group were lysed in lysis buffer containing protease inhibitor. According to the nuclear and total protein extraction kit manufacturer's protocol, the following procedures were followed. Nuclear and total protein samples (100 µg) were separated on 10% polyacrylamide resolving gels and 5% stacking gels and then transferred at 100 V and 250 mA for 90 min. The membranes were incubated with primary antibodies overnight at 4°C. Loading controls of total and nuclear protein samples were confirmed with β-actin and lamin B1 antibodies, respectively.

Statistical analysis. The results are presented as the mean ± SEM of three independent experiments. Treated groups were compared with the control group by one-way analysis of variance (ANOVA). p<0.05 was considered to indicate a statistically significant result.

Results

Effect of TMP on the proliferation of Jurkat and SUP-B15 cells. To investigate the antiproliferative effect of TMP on ALL cells, Jurkat and SUP-B15 cells were exposed to different concentrations of TMP for 24, 48 and 72 h. As shown in Fig. 1, TMP notably inhibited the growth of both cell lines in a dose- and time-dependent manner, although Jurkat cells were more sensitive to TMP. The IC50 values of TMP in the Jurkat and SUP-B15 cells at 48 h were 120 and 200 µg/ml, respectively. Concentrations of 60, 120 and 180 µg/ml of TMP were used to treat the Jurkat cells, and concentrations of 100, 200 and 300 µg/ml were used to treat the SUP-B15 cells in subsequent experiments. The results suggested that TMP is an antitumor agent for human ALL cells.

TMP induces apoptosis in Jurkat and SUP-B15 cells. To confirm whether TMP exerts its inhibitory effect through inducing apoptosis, Jurkat and SUP-B15 cells were incubated with concentration gradients of TMP from 20 to 320 µg/ml for 48 h and apoptosis was detected by the Annexin V/PI double staining assay using flow cytometry. In Fig. 2, the apoptotic rates of Jurkat cells in the control, 60, 120 and 180 µg/ml TMP groups were 3.05±0.05, 4.03±0.2, 6.15±0.3 and 16.64±0.65%, respectively, whereas the rates of SUP-B15 cells in the control, 100, 200 and 300 µg/ml were used to treat the SUP-B15 cells in subsequent experiments. The results suggested that TMP is an antitumor agent for human ALL cells.

GSK-3β expression in the TMP-treated Jurkat and SUP-B15 cells. To determine whether GSK-3β participates in the apoptotic induction by TMP, we evaluated the expression of GSK-3β with quantitative real-time PCR and western blot analysis. As shown in Fig. 3, GSK-3β expression decreased significantly both at the protein and RNA levels compared with the control group in the Jurkat and SUP-B15 cells treated with TMP. These results imply a potential inhibitory effect of TMP on GSK-3β expression in ALL.

TMP decreases the expression of GSK-3β downstream of NF-κB and c-myc in the Jurkat and SUP-B15 cells. GSK-3β has been identified to play a vital role in the NFκB- and c-myc-mediated survival of cancer cells. Since NFκB and c-myc are downstream transcription factors of GSK-3β, we examined the effect of TMP on the levels of NFκB and c-myc in the Jurkat and SUP-B15 cells. The results showed that the expression of NFκB and c-myc in the Jurkat and SUP-B15 cells was decreased in both the whole-cell and nuclear lysates (Fig. 4). These findings suggest that TMP may exert its effect on ALL cell apoptosis
Figure 2. TMP induces apoptosis in Jurkat and SUP-B15 cells. Flow cytometric analysis of TMP-induced apoptosis in Jurkat and SUP-B15 cells using Annexin V-FITC/PI staining. (A) Flow cytometric analysis of Annexin V/PI staining in Jurkat (upper panel) and SUP-B15 (lower panel) cells treated with TMP and control are shown. The concentration of TMP is shown above each plot, and Annexin V/PI staining was performed after a 48-h treatment. The lower right quadrant represents early apoptotic cells, and the upper right quadrant indicates late apoptotic cells undergoing secondary necrosis. (B) TMP induced apoptosis in the ALL cell lines in a dose-dependent manner. *p<0.05 compared with the control cells, which were untreated ‘0’. TMP, tetramethylpyrazine.

Figure 3. GSK-3β expression in the TMP-treated Jurkat and SUP-B15 cells. (A and B) GSK-3β mRNA levels in the Jurkat and SUP-B15 cells, respectively, following treatment with concentration gradients of TMP as detected by real-time PCR. (C and D) Protein levels of GSK-3β in whole lysates detected by western blotting in the Jurkat and SUP-B15 cells following treatment with TMP, respectively. *p<0.05 compared with the control. GSK-3β, glycogen synthase kinase-3β; TMP, tetramethylpyrazine.
by downregulating the transcriptional activity of NF-κB and c-myc through GSK-3β regulation.

**Effect of TMP on the expression of survivin, bcl-2, cox-2 and p27 in the Jurkat and SUP-B15 cells.** Bcl-2, cox-2 and survivin are downstream molecules of NF-κB or c-myc that are relevant for apoptosis, and we evaluated the effect of TMP on their expression levels in the Jurkat and SUP-B15 cells (Fig. 5). The results revealed that bcl-2 and survivin protein expression was decreased significantly in both cell lines after exposure to TMP compared with the control groups, whereas changes in the mRNA levels were not significant. Consistent with the PCR results, the cox-2 protein level was decreased significantly in both the Jurkat and SUP-B15 cells. Considering the fact that TMP resulted in the arrest of Jurkat and SUP-B15 cells in the G0/G1 phase of the cell cycle and p27<sup>kip1</sup> regulates and blocks cell cycle progression through the G1-S transition, real-time RT-PCR and western blotting were conducted to examine the p27 expression in the ALL cells (Fig. 5). The results showed that TMP upregulated the expression of p27 in Jurkat and SUP-B15 cells, suggesting a potential mechanism through which TMP affects the cell cycle.

**Discussion**

ALL is a heterogeneous group of malignant clonal diseases that originate from pluripotent hematopoietic stem cells. Although improved therapeutic strategies have achieved long-term survival rates of more than 80% in children, the survival rate is less than 40% in adults (14). Patients for whom induction chemotherapy fails require intensive chemotherapy and may suffer from severe side-effects of the treatments. Therefore, novel agents are urgently required. The pathogenesis of ALL is the result of multiple factors and genes working in concert. In particular, aberrant cell signal transduction plays a vital role in its occurrence and development. As noted above, GSK-3β acts as an oncogene in ALL and plays an important role in ALL tumorigenesis and progression. In the present study, we adopted GSK-3β as a promising drug target for new, effective and low-toxicity anticancer drugs (15). The small-molecule inhibitors of GSK-3β are mainly divided into two categories: ATP-competitive and non-ATP competitive agents. The former category includes bis-indole and pyrazine, for example, and lithium is a representative of the latter group (16,17). TMP, which is a pyrazine, has been widely used in the clinic for the treatment of cardiovascular and neurovascular diseases (18,19), and it has an excellent safety profile. Recently, beyond its traditional function, TMP has been found to have antitumor effects (20). In the present study, we showed a novel effect of TMP on human Jurkat and SUP-B15 ALL cell lines,

![Figure 4. TMP decreases the expression of NF-κB and c-myc in the Jurkat and SUP-B15 cells. The protein levels of NF-κB (p65) and c-myc in the ALL cell lines detected by western blotting are shown. The expression of NF-κB and c-myc in the Jurkat and SUP-B15 cells was downregulated in both the whole lysates and in the nucleus. TMP, tetramethylpyrazine.](image-url)
i.e., targeting the GSK-3β pathway. Our research found that TMP inhibited the proliferation of Jurkat and SUP-B15 cells in a dose- and time-dependent manner by inducing apoptosis and arresting the cell cycle at the G0/G1 phase. Thus, we report that TMP may be an effective anti-ALL agent. Studies have demonstrated that therapy targeting GSK-3β inhibits the proliferation of several cancer cell lines (21,22). Our experiments showed that GSK-3β expression was significantly decreased in the Jurkat and SUP-B15 cells following treatment with TMP. Therefore, we showed for the first time that TMP exerts antitumor effects on ALL cells by inhibiting GSK-3β signaling.

The substrates of GSK-3β are mainly glycogen synthase and transcription factors such as elf2, HSF-1, c-jun, c-myc and c-myb, among others (23). Here, we found that TMP decreased the total and nuclear expression of transcription factors NF-κB and c-myc in the Jurkat and SUP-B15 cells. NF-κB is a pleiotropic transcription factor that regulates the transcription of its downstream target genes that are critical for the regulation of tumorigenesis, apoptosis and a wide variety of cellular functions (24). In normal cells, NF-κB is sequestered in the cytoplasm in its inactive form through binding with the inhibitor proteins of the IκB family (25). However, NF-κB was found to be persistently activated in leukemic cells and regulated the anti-apoptotic mechanism (26). Kotliarova et al (27) used GSK-3β inhibitors and RNAi technology to reduce the activity of GSK-3β, leading to a decrease in NF-κB activity, which caused the apoptosis of glioma cells. Ougolkov et al (28) found that the inhibition of GSK-3β activity can silence the expression of the downstream anti-apoptotic target genes of NF-κB in chronic lymphocytic leukemia (CLL) B cells, thereby promoting the apoptosis of CLL B cells. All previous studies suggest that NF-κB is affected by GSK-3β inactivity.

c-myc, which belongs to the myc family of transcription factors, is a regulator gene that codes for a nucleoprotein that binds to nuclear DNA. By promoting or inhibiting transcription of target genes, c-myc participates in the start of intracellular signal transduction and in the expression of a wide variety of genes (29). C-myc regulates target gene expression by forming a dimer with MAX proteins. Studies have shown that c-myc...
is constitutively highly expressed in acute leukemia (30). Dysfunction of c-myc led to the unregulated expression of many genes, some of which are involved in cell proliferation and apoptosis.

Further experiments in the present study showed that the expression levels of cox-2, bcl-2 and survivin, the downstream target genes of NF-κB and c-myc, were decreased.

Our previous results showing that the expression of GSK-3β was suppressed by TMP as well as other related literature suggest that GSK-3β may be a potential target for TMP in ALL cells. In our experiments, TMP caused both Jurkat and SUP-B15 cells to be arrested in the G0/G1 phase, which may be associated with an increase in the expression of p27KIP1. p27KIP1 protein plays a pivotal role in the regulation of the cell cycle as an inhibitor of cyclin-dependent kinases (CDKs) through inhibiting the checkpoint kinase CDK2/cyclin E1 complex and blocking cell cycle transition from the G0/G1 to the S phase (31,32). The inhibition of GSK-3β has been reported to induce cell cycle arrest at the G1 phase via the activation of p27KIP1 (8), which is consistent with our results.

In summary, our experimental results showed that TMP can induce apoptosis in ALL cells by downregulating GSK-3β and TMP plays a role as an antimtumor agent. TMP, which is an approved treatment option for vascular diseases, exhibits broad potential for the treatment of leukemia, and our results provide a theoretical basis for its clinical application.

References