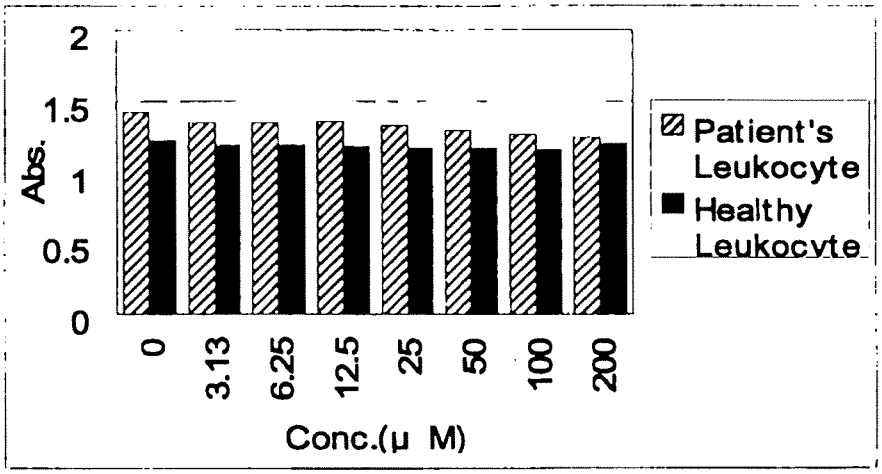

Figure 2. MTT *in vitro* evaluation results for patient's and healthy leukocyte by dibromide

Figure 1 shows that among the four samples epoxide **3a** and dibromide **5** killed effectively K562 leukemia cell, however, the other samples **2** and **3b** did not kill the leukemia cell at all just as the control (DMSO). Figure 2 shows that even the active sample **5** did not kill the healthy leukocyte (blast 0 %) at all but partially kill the patient's leukocyte (blast 40 %) selectively. Figure 3 shows that the separated four diastereomeric dibromide (retention times for each diastereomer are shown in Figure 3) have the higher activity for K562 leukemia cell. Unfortunately all 2-amino-3-hydroxy-1-phenylphospholane 1-oxide derivatives **4a** and **4b** in Table 1 did not kill the leukemia cell shown by *in vivo* evaluation of MTT method. The results of *in vivo* evaluation experiments shown in Figures

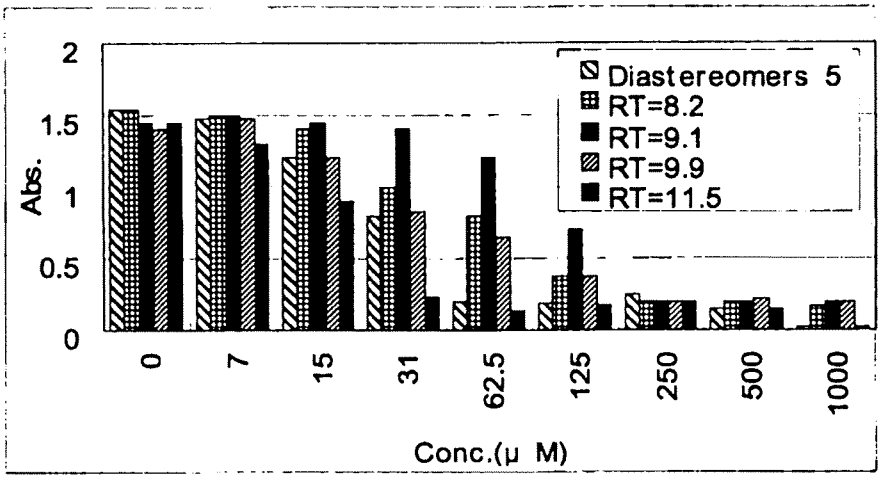

Figure 3. MTT *in vitro* evaluation results for K652 leukemia cell by the four separated diastereomers of 2,3-dibromide **5**. (RT means the retention time (min.) of the diastereomers recorded by HPLC on silica gel column with eluent ($CHCl_3$: MeOH = 20 : 1))

1-3 show that the prepared phospholanes or phospha sugars of epoxide and dibromide have quite efficient anti-cancer activities for leukemia cells in manners of (i) wide spectra, (ii) high activities, and (iii) high specificities. The further research on the structure-activity and the mechanism of the phospha sugars as the anti-tumor agents will be disclosed separately.

Experimental Section:

General Procedures and Methods:

NMR spectra were collected on JEOL EX300 (300MHz) and HITACHI H-90 (90MHz) instruments. TLC plates of "Chromato Sheet" of Wako Pure Chemical Industries were used. HPLC instruments of JASCO Corporation equipped with silica gel column (Wakopak; 4.6 mm in diameter × 250 mm in length) and UV detector were used. Silica gel of "Wakogel C-200" (75 – 150 μm) was used for column chromatography. The eluents used for TLC, HPLC, and column chromatography are shown in parenthesis.

2,3-Epoxy-1-phenylphospholane 1-Oxide 3:

2-Bromo-3-hydroxyphospholane **2** (2.5 g, 9.7 mmol) was dissolved in 50 ml of potassium hydroxide solution (0.5 *N*) and the solution was stirred at 40 □ for 1 hr. After completion of the reaction the solvent was evaporated and the product was extracted with $CHCl_3$ (30 ml × 3 times). The $CHCl_3$ layer was separated and evaporated under diminished pressure. The residue was separated by column chromatography on silica gel (EtOAc : MeOH = 20 : 1) to afford 0.83 g (44 % yield) of **3a** and 0.49 g (26 % yield) of **3b**.

Compound **3a**; ^{1}H NMR ($CDCl_3$), δ (ppm):1.94-2.17 (m, 2H, H-4),1.94-2.68 (m, 2H, H-5), 3.51 (dd, 1H, J_{HCP}=28.7Hz J=3.0Hz, H-2), 3.93-3.96 (m, 1H, H-3), and 7.51-8.00 (m, 5H, Ph-H); TLC (EtOAc : MeOH = 20 : 1), Rf: 0.55.

Compound **3b**; ^{1}H NMR ($CDCl_3$), δ (ppm): 1.94-2.17 (m, 2H, H-4), 1.94-2.68 (m, 2H, H-5), 3.45 (dd, 1H, J_{HPC}=29.1 Hz, J=3.0Hz, H-2), 3.82-3.86 (m, 1H, H-3), and 7.76-7.51 (m, 5H, Ph-H); TLC (EtOAc : MeOH = 20 : 1), Rf: 0.39.

Typical Procedure for 2-Amino-3-hydroxy-1-phenylphospholane 1-Oxide 4 (2-Diethylamino Derivative 4b$_1$):

A mixture of 2,3-epoxy-1-phenylphospholane 1-oxide **3b** (0.050 g, 0.26 mmol) with 20 ml of aqueous ammonia was stirred at room temperature for 2 days. After evaporation of volatile materials reaction products were separated by silica gel column chromatography ($CHCl_3$: CH_3OH = 10:1) to give 0.065 g (0.26 mmol; in aquantitative yield) of **4b$_1$**.

Compound **4b**$_1$; ^{1}H NMR ($CDCl_3$), δ (ppm): 1.63-2.43 (m, 4H, H-4,4',5,5'), 2.95 (dd, 1H, *J*=2.2 Hz, *J*=8.0 Hz, H-2), 4.01-4.11 (m, 1H, H-3), 7.33-7.75 (m, 5H, Ph-H), TLC (EtOAc : MeOH = 20 : 1), Rf: 0.18.

Synthesis of 2,3-Dibromo-3-methyl-1-phenylphospholane 1-Oxide 5 (R=Me)

To a mixture of 3-methyl-1-phenyl-2-phospholene 1-oxide **1** (R=Me) 0.266 g (1.38 mmol) and manganese dioxide 0.239 g (2.38 mmol; 2.0 eq.) in dichloromethane (5 ml) was added dichloromethane (5 ml) solution of bromine 0.400 ml (7.81 mmol; 5.7 eq.), and then stirred for 12 h under Ar atmosphere. Reduction of excess bromine with sodium sulfite solution, and then the reaction mixture was extracted by chloroform (10 ml x 3 times). The organic layer was neutralized with saturated sodium hydrogencarbonate, washed with saturated sodium chloride solution, and then dried over sodium sulfate. Removal of the solvent under reduced pressure followed by separation from the residue gave 0.376 g (yield 78%) of **5** (R=Me). RT value of each diastereomer is shown in Figure 3.

5 (R=Me); m.p. 189.20 °C; b.p. 280.24 °C; TLC ($CHCl_3$: MeOH = 20 : 1), Rf = 0.42; MS (m/z), 353.20 (MH^+); IR (KBr) 1126 cm^{-1} (P=O), 748 cm^{-1}, 1396 cm^{-1} (C-Br); ^{1}H-NMR ($CDCl_3$, 300 MHz), δ(ppm); 1.67 (s, 3H, CH_3), 2.36-2.46 (m, 2H, H-4), 2.97-3.02 (m, 2H, H-5) 4.28-4.31 (m, 1H, C-2), 7.51-7.70 (m, 5H, Ph-H).

Conclusion:

The stereospecific epoxidation of 1-phenyl-2-phospholene 1-oxide **1** to form *erythro* epoxides **3a** was performed by using sodium peroxide. Epoxidation of 2-phospholene derivatives **1** with hydrogen peroxide as well as epoxide formation via bromohydrin route from 2-phospholene **1** afforded a mixture of the *erythro* and *threo* diastereomers **3**. The nucleophilic substitution reaction of 2,3-epoxyphospholane 1-oxides **3a** and **3b** occurred at C-1 position with amines to give the *N*-glycoside derivative **4** of phospha sugars. 2,3-Dibromophospholane derivatives **5** (R=Me) were prepared as a mixture of four diastereoisomers. MTT *in vitro* bio-assay method revealed that the prepared phospholanes or phospha sugars have quite efficient anti-cancer activities for leukemia cells in manners of (i) wide spectra, (ii) high activities, and (iii) high specificities and selectivities.

Acknowledgment:

Financial supports by Shizuoka Prefectural Government, the Ministry of Health, Labor and Welfare, and the Ministry of Education, Culture, Sports, Science and

Technology, Japan, as well as by some collaborated companies and Shizuoka University are deeply acknowledged.

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Received on October 4,2008.

ISSN: 0254-8704
CODEN: JEBIDP

Reprint from

Journal of Environmental Biology

Founder
Editor-in-chief:
Dr. R.C. Dalela

© Triveni Enterprises, Lucknow (India)
ISSN : 0254-8704
http : //www.jeb.co.in

J. Environ. Biol.
30(6), 945-950 (2009)
info@jeb.co.in

Synthesis and *in vitro evaluation* of 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide for potential anti-proliferative effects

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(Received: May 09, 2008; Revised received: October 20, 2008; Accepted: November 21, 2008)

Abstract: *A novel phospha sugar analogue, 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP), was prepared from 1-phenyl-3-methyl-2- phospholene 1-oxide and evaluated by in vitro MTT method for leukemia cells and microscopic observations for solid tumor cells, e.g., stomach cancer cells. The evaluation revealed clearly that the synthesized phospha sugar analogue DBMPP has competent potentials and excellent anti-cancer activities that killed selectively and specifically the leukemia cells of cell lines of K562 and U937 but did not give any damages on healthy leukocyte. Moreover, it was revealed that DBMPP killed solid cancer cells such as stomach cancer cells and melanoma of cell lines of MKN45 and G361. Therefore, DBMPP should exert anti-proliferative effects for different kinds of tumor cells based on the in vitro evaluations. The cell cycle analyses by flow cytometry for K562 and U937 cells clearly demonstrated that the mechanism of the anti-proliferative effect on the human tumor cells is apoptosis induced by DBMPP.*

Key words: *Human tumor cell lines, 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Apoptosis, DBMPP, Phospha sugars, Carcinostatic drug*
PDF of full length paper is available online

Introduction

Phosphorus compounds and carbohydrates are well investigated from every aspects of sustaining and perishing lives of living things. Therefore, researches on anti-tumour agents are very much active to cure cancer and to improve quality of life of patients. A large number of national products are tested to find out new potential anti-cancer drugs (Sahu *et al.*, 2007a,b). Natural products, enzymes, phosphates, *etc.* are also well studied in the field of medical, life, environmental sciences *etc.* (Leeja and Thoppiril, 2007; Singh and Shelley, 2007; Williams *et al.*, 2007; Estiarte *et al.*, 2008). Among the naturally occurring products cellulose is the most popular material and is consisted of glucose. And then, glucose is one of the typical carbohydrates which contains an oxygen atom in the hemiacetal ring of the Haworth equation. When the oxygen atom of the carbohydrate or sugar structure is replaced by a group or an atom other than the oxygen, *e.g.*, CH_2 or S, the carbohydrate is called as a pseudo sugar.

Phospha sugars have a phosphorus atom in the hemiacetal ring of sugars and they are classified into a category of pseudo sugars. Well known pseudo sugars are *carba-*, *aza-* and *thia-*sugars, with a carbon, nitrogen, and sulphur atom, respectively, instead of the oxygen in the hemiacetal ring of the sugars, which are known to exist in nature and are also prepared by synthetic sugar chemistry. Some of them exert important biological activities, therefore, a lot of studies on them are actively being performed. On the other hand phospha sugars are not yet known and found in nature and the synthesis is rather difficult compared with the other pseudo sugars, *i.e.*, *carba-*, *aza-*, and *thia-*sugars (Yamashita *et al.*, 1987; Reddy *et al.*, 2004), when the synthetic route includes bond formation of a carbon-phosphorus on a secondary carbon atom of the carbohydrate, because of the different reactivity of phosphorus compounds bearing a 3d-orbital (Yamamoto *et al.*, 1982, 1985) from the elements of the period 2, *e.g.* nitrogen.

As these phospha sugars have been expected to show biologically important character we have first tried to find anti-tumor activity by using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) methods (Nakamura *et al.*, 2004) for leukemia cells and found that some of these phospha sugars possess anti-proliferative effects for cultured human leukemia cells. During the way to search the optimized structure of phospha sugars for anti-tumor agents a five-membered novel phosphorus heterocyclic compound, 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide

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(DBMPP) is synthosized, which may be said as a new class of deoxyhalo phospha sugars. In this paper we will deal on the synthesis and *in vitro* evaluation of DBMPP and with the new findings of DBMPP which showed the potential anti-proliferative effects for leukemia cells and human solution and solid tumors. The results of mechanistic studies of the anti-tumor activity on leukemia cells being analyzed by the flow cytometry for cell lines of K562 and U937 are also dealed. We may say that the present new findings may lead to develop a new type of carcinostatic drugs being useful in oncostasis.

Materials and Methods

Synthesis of 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP): To CH_2Cl_2 (10 ml) solution of 3-methyl-1-phenyl-2-phospholene 1-oxide (0.27 g, 1.4 mmol) and manganese(IV) dioxide (0.24 g, 2.4 mmol) was added dropwise CH_2Cl_2 (10 ml) solution of bromine (0.40 ml) and the reaction mixture was stirred for 8 hr at room temperature. The reaction was quenched by added saturated sodium sulfite aqueous solution.The aqueous mixture was extracted with chloroform (10 ml x 3). The organic layer was neutralized with saturated $NaHCO_3$ aqueous solution, washed with saturated NaCl aqueous solution and dried over with anhydrous sodium sulfate; and then the solvent of the filtrate was evaporated under a reduced pressure to give an oily mixture of the products. The mixture was purified by column chromatography on silica gel by using chloroform and methanol (30 : 1) as the eluent to give 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP; 0.37 g, 78% yield) (Fig. 1); m.p. (Shimadu Simultaneous DTA-TG Apparatus (DTG-60A50AH) 189.20°C; b.p. 280.24°C; TLC (Silica gel: Eluent: $CHCl_3$: MeOH = 20 : 1), Rf = 0.42; MS (MALDI-TOF-MS: GL Science (Voyager-DE Porimerix); Matrix: α-Cyano-4-hydroxycinnamic acid (m/z)), 349.29 ($M-H^+$; (Molecular peak-1) isotope peaks: 349.29, 351.29, 353.28, and 355.29); IR (JASCO FT/IR 410 (KBr)): 1126 cm^{-1} (P=O), 748 cm^{-1}, 1396 cm^{-1} (C-Br); ^{1}H-NMR (JEOL JNM-AL300 (300 MHz) and Hitach R90H (90 MHz); Solvent: $CDCl_3$, δ (ppm)); 1.67 (s, 3H, CH_3), 2.36-2.46 (m, 2H, H-4), 2.97-3.02 (m, 2H, H-5) 4.28-4.31 (m, 1H, C-2), 7.51-7.70 (m, 5H, Ph-H) (Fig. 1). HPLC (Apparatus: JASCO HPLC Set (JASCO 860-CO, 880-PU, 875-UV, RI-930, and 807-IT; Column: Silica gel (Wakopac φ 4.6 mm × 250 mm); Eluent: $CHCl_3$: MeOH = 20 : 1), RT (retention time: min) values of diastereo isomers were 8.2, 9.1, 9.9 and 11.5.

2,3-Dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP) was dissolved in dimethylsulfoxide (DMSO) (Sigma Chemical Company, St. Louis, MO, USA), and was diluted into appropriate concentration with DMSO in culture medium immediately before use. The final concentrations of DBMPP in DMSO in all experiments were less than 0.010%, and all the treatment conditions were compared with vehicle controls.

Human tumor cell lines and culture: Chronic myeloid leukemia (K562), promyeloid leukemia (U937), adenocarcinoma (MKN45), and melanoma (G361) cells were purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) 292 mg l^{-1} (or 2 mmol l^{-1}) L-glutamine, 100 μg ml^{-1} streptomycin and 200 U ml^{-1} penicillin (GIBCO-BRL,

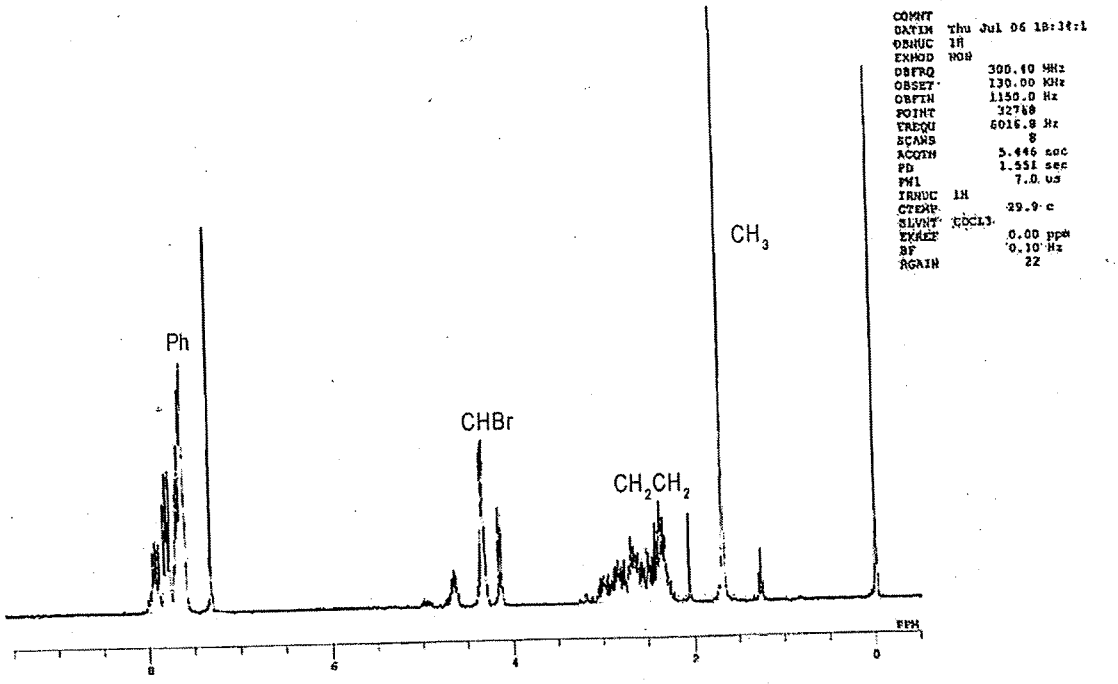

Fig. 1: ^{1}H-NMR spectrum (300 MHz, $CDCl_3$) of DBMPP

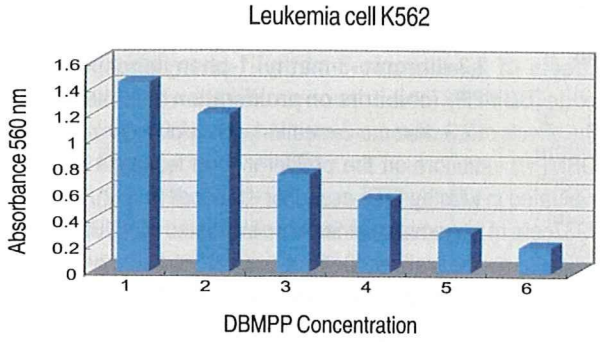

Fig. 2: Inhibition from cell proliferation of K562 cell by treatment with 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP). The cells were treated at concentrations (1) 0 μmol l^{-1} (non-treatment), (2) 12.5 μmol l^{-1}, (3) 25 μmol l^{-1}, (4) 50 μmol l^{-1}, (5) 100 μmol l^{-1} and (6) 200 μmol l^{-1} at 37°C for 48 hr. The cell proliferation or inhibition was measured as the function of the absorbance at 560 nm visible light and evaluated *in vitro* by MTT assay

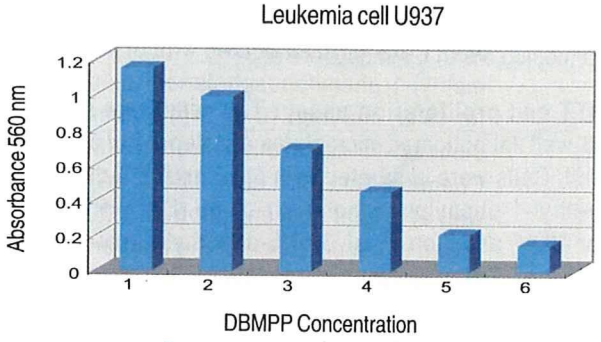

Fig. 3: Inhibition from cell proliferation of U937 cell by treatment with 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP). The cells were treated at concentrations (1) 0 μmol l^{-1} (non-treatment), (2) 12.5 μmol l^{-1}, (3) 25 μmol l^{-1}, (4) 50 μmol l^{-1}, (5) 100 μmol l^{-1}, and (6) 200 μmol l^{-1} at 37°C for 48 hr. The cell proliferation or inhibition was measured as the function of the absorbance at 560 nm visible light and evaluated *in vitro* by MTT assay

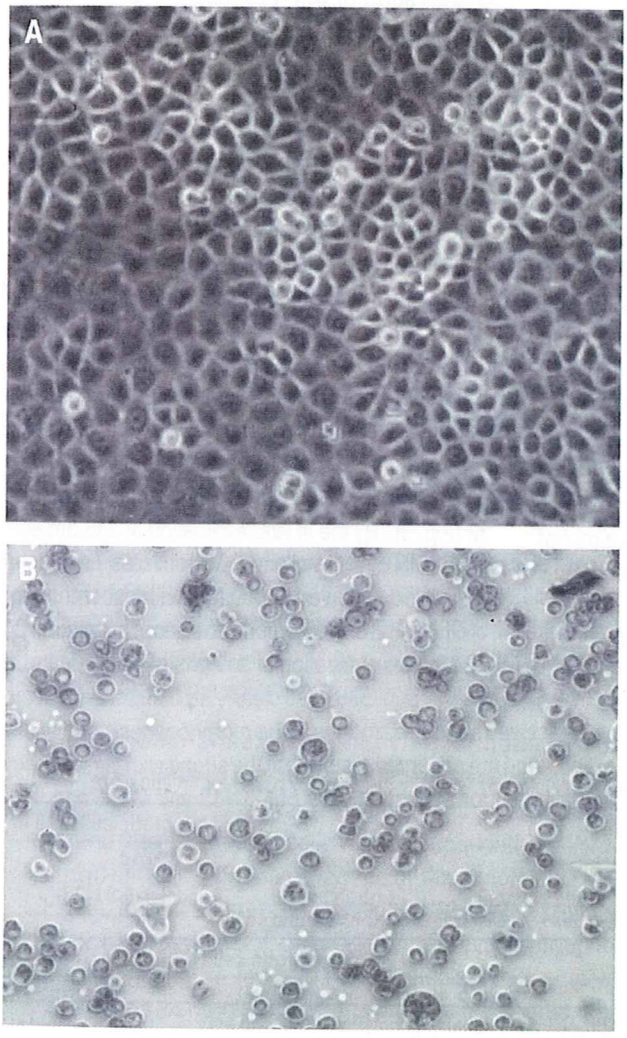

Fig. 4: Handstand phase-contrast microscopic observations (x 100) for adenocarcinoma stomach cells (MKN45): (A) Untreated and (B) treated with 100 μmol l^{-1} of 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP) at 37°C for 24 hr

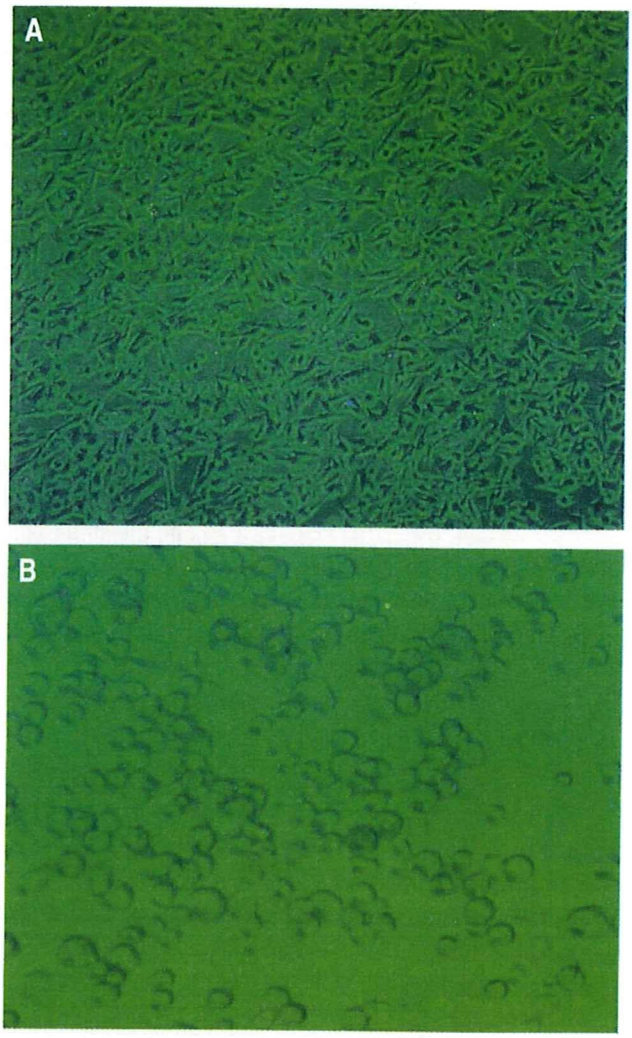

Fig. 5: Handstand phase-contrast microscopic observations (x 100) for melanoma skin cells (G361): (A) Untreated and (B) treated with 100 μmol l^{-1} of 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP) at 37°C for 24 hr

Gaithersburg, MD, USA). All cells were maintained in a humidified 5% CO_2 atmosphere at 37°C.

MTT cell proliferation assay: The cells were seeded in 96-well flat bottomed microplates at a density of 5 × 10^4 per well. Cells were incubated with or without 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP), at 37°C for 48 hr, and then 10 µl 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) solution (Sigma Chemical Co., St. Louis, MO, USA) was added to each well at a final concentration of 1.0 µg ml^{-1} $well^{-1}$. Cells grown in the presence of the medium alone were used as the controls. After incubation at 37°C for 4 hr, absorbance was measured at a wavelength of 560 nm by using a microplate reader for *in vitro* evaluation (Nakamura *et al.*, 2004; Mimori *et al.*, 2004).

Handstand phase-contrast microscopy: Cultured cancer cells in flasks were photographed with a handstand phase-contrast microscope. Adenocarcinoma (MKN45) and melanoma (G361) cells were cultured in 1 ml complete medium containing 5 x 10^5 cells in the presence of 50, 100 and 200 µmol l^{-1} of 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP), whose cells were maintained in a humidified 5% CO_2 atmosphere at 37°C. The cultured cells at 37°C for 12, 24 and 48 hr of incubation were observed by handstand phase-contrast microscope for *in vitro* evaluation (Moriwaki *et al.*, 1996, 2005).

Apoptosis analysis: DNA content analysis at the each stage of the cell cycles was performed by using propidium iodide (PI) staining. Cells (K562 and U937) were cultured in 2 ml complete medium containing 1 x 10^6 cells in the presence of 50, 100, and 200 µmol l^{-1} of 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP), at the indicated concentrations in the figure and incubated at 37°C. After 24 and 48 hr of incubation, the cells were washed twice with cold PBS, fixed with 70% ethanol for overnight before treatment with 100 µg ml^{-1} RNase A and then stained with 50 µg ml^{-1} of PI. The relative DNA content per cell was measured by flow cytometry by using an Epics Elite flow cytometer (Coulter Immunotech, Marseille, France) (Nishizawa *et al.*, 2000; Nakamura *et al.*, 2004).

Results and Discussion

Effects of 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP) inhibitors on proliferation of leukemia cells: The effects of 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP) inhibitors on the proliferation of leukemia cells were evaluated *in vitro* by MTT assay for K562 cell (Fig. 2). K562 and U937cells of leukemia cell lines were incubated with DBMPP at the indicated concentrations (0~1000 µmol l^{-1}) of the phospha sugar for 24 hr at 37°C. DBMPP strongly suppressed the cell proliferation of K562 cell in a dose-dependent manner and the intensity of absorbance at 560 nm decreased. The decrease of the absorbance means the death of the cell and clearly indicates that DBMPP possesses the growth inhibitory effect on K562 cell and that half of the absorbance intensity was achieved by 50 µmol l^{-1} of DBMPP. Similar growth inhibition by DBMPP was also shown for U937 cell (Fig. 3). The anti-proliferative effect of DBMPP on U937 cell was much more efficient than that of Gleevec (inatinib mesylate, also known as STI-571), a promising new oral treatment for patients with myeloid leukemia (CML) (Druker *et al.*, 2000).

Observation of anti-proliferative effect of DBMPP on solid cancer by a handstand phase-contrast microscope: Adenocarcinoma stomach cancer cell (MKN 45) was observed by a handstand phase-contrast microscopic measurement to have been received the aggression and killed by 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP) at the lower concentration than 100 µmol l^{-1} and became finally extinct (Fig. 4). On the process of the cancer cell MKN45 being first received the damage and finally become extinct was observed under the microscopic observation at about 12 hr after the dose of DBMPP to the cells. And then, the MKN45 cells damaged had disappeared at 24 hr after the dose via cellulae processus peculiar to a cancer cell (the detailed data will be shown elsewhere) (Kobayashi *et al.*, 2000; Suzuki *et al.*, 2002). Moreover, similarly to MKN45 cells, melanoma skin cells (G361) also received the aggression at the lower concentration of DBMPP than 100 µmol l^{-1} and became extinct in 24 hr (Fig. 5). By the *in vitro* evaluation of anti-proliferative effects on both cells of MKN45 and G361 cell clearly demonstrated that the cells received serious damages in the morphogenesis and finally extinct. And the observed results that the strong sticking tendency of malignant for almost all cells of MKN45 and G361 and then

Table - 1: Cell cycle analysis for K562 and U937 cells treated with 50 and 100 µmol l^{-1} of DBMPP at 37°C for 24 and 48 hr

Human tumor cell lines	% of apoptotic cells (24 hr)			% of apoptotic cells (48 hr)		
	DBMPP untreated	DBMPP (50 µmol l^{-1})	DBMPP (100 µmol l^{-1})	DBMPP untreated	DBMPP (50 µmol l^{-1})	DBMPP (100 µmol l^{-1})
K562	2.3± 0.4	64.8 ± 1.5	80.4 ± 3.2	2.5± 0.4	84.3 ± 4.1	92.4 ± 4.1
U937	2.5± 0.4	65.6 ± 2.3	79.2 ± 3.9	2.2± 0.4	80.2 ± 3.5	89.6 ± 3.6

Data represent the mean value with standard deviation (± SD) of three independent experiments

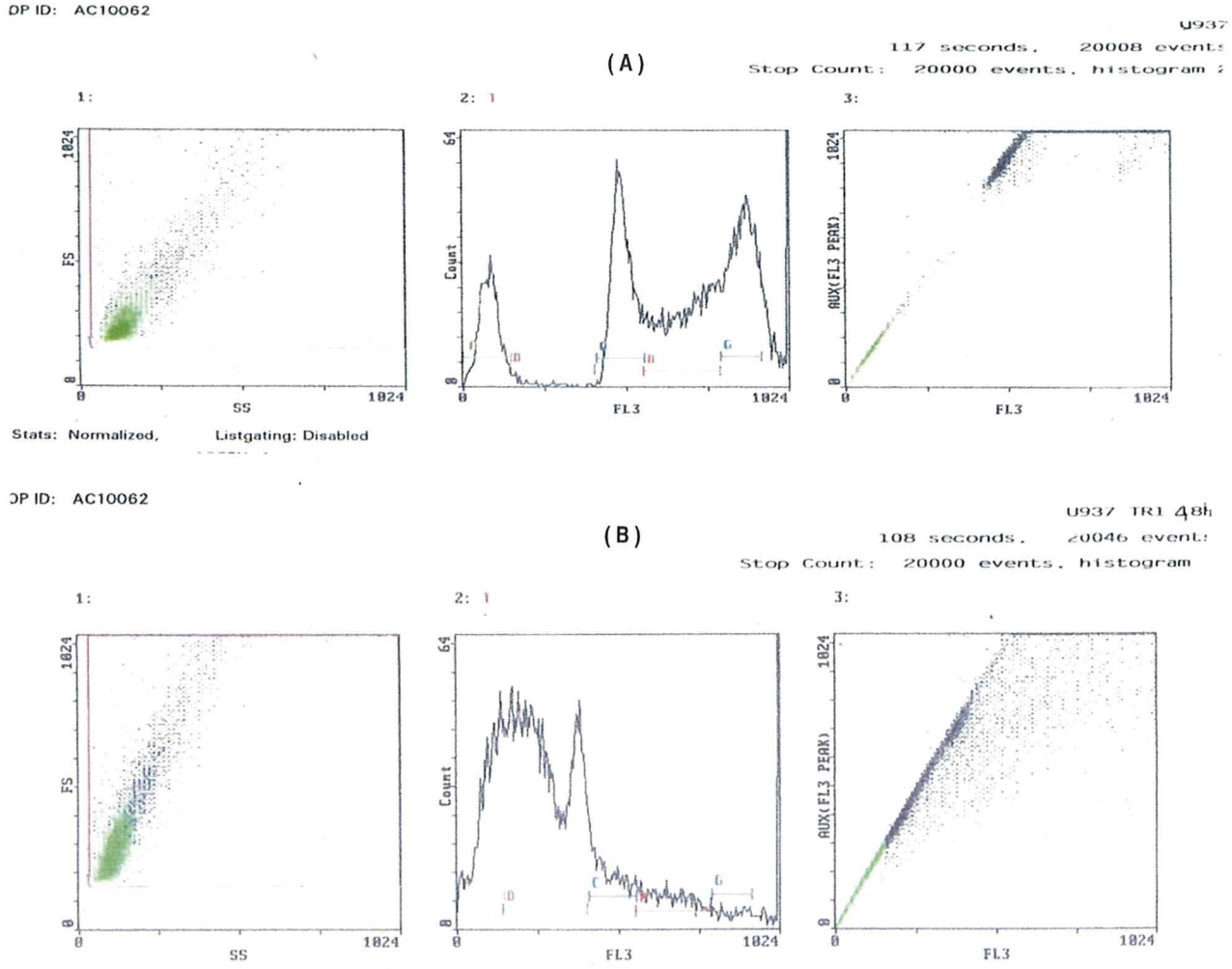

Fig. 6: Results of cell cycle analysis for U937 cells by using an Epics Elite flow cytometer. (A) Un-treated (B) treated with 100 μmol l^{-1} of DBMPP at 37°C for 48 hr

became extinct by DBMPP at the lower concentration within 24 hr have a significant meanings for developing phospha sugars as a novel class of anti-tumor drugs hereafter. These observations are quite rare and the present new findings by DBMPP are considered to be an epoch-making event by chemically synthesized materials (Fig. 4, 5).

2,3-Dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP) induced apoptosis for leukemia cells: All leukemia cells (K562 and U937) were treated at 37°C for 24 hr and/or 48 hr, and then the treated cells were subsequently stained with propidium iodide (PI) and analyzed by flow cytometry (Table 1, Fig. 6). In contrast to the observation for 12 hr, after treatment of leukemia cells with 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP), induction of apoptosis (Nakamura *et al.*, 2004) was certainly observed and the observations were reproduced. Treatment of all leukemia cells (K562 and U937), of stomach cells (MKN45) and melanoma skin cells (G361) with DBMPP led to a time- and dose-dependent induction of the apoptosis (Nakamura *et al.*, 2004). Doses of 50 and 100 μmol l^{-1} were sufficient to induce apoptotic changes of the cells (Tahir *et al.*, 2001).

From the cell sorter or flow cytometer analyses of the cell cycles shown in Fig. 6, the important conclusions can be said as follows: (i) It is acquired from the flow cytometryic data that the cell cycle is stoped at the process by induction of DBMPP; (ii) DBMPP induces an apoptosis and annihilates the canner cells at the Gap 2 period (Nakamura *et al.*, 2004) and (iii) it is also suggested that DBMPP is acting on the gene of a mitosis (Tahir *et al.*, 2001). It may be mostly plausible from the result obtained by the *in vitro* evaluation that DBMPP can be a novel carcinostatic agent or drug which acts on the gene at the new mitosis stage by controlling the mitosis at the lower concentration. And by further research and development on phospha sugars' anti-tumor agents based on the present results it may be possible that the medicinal chemistry will lead to find new chemicals as molecular targeting therapeutic drugs for the human cancers.

The results of the present study revealed that the phospha sugar derivative of 1,2-dibromo-1,2-dideoxy-2-methyl-P-phenyl *phospha*-tetrofuranose or 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP) has a potential anti-cancer activity for human leukemia cells like naturally occurring materials of L-asparaginase emzyme (Sahu *et al.*, 2007) and solid tumor cells as the first and valuable findings. The mechanism of the activity of the synthesized DBMPP is based on the induction of apoptosis for the cells, and then the mechanism of the agents resembles "Imatinib Mesylate (Gleevec)".

Acknowledgments

The authors greatly acknowledge financial support by the Satellite Shizuoka of Japan Science and Technology Agency (JST) as well as by the Headquarters of JST and Shizuoka Prefecture Industry Creating Agency. Mrs. Kiyoshi Sibata, Professor Reiji Aoshima and Ms. Masako Suzuki are also acknowledges to flow cytometry, experimental supports and mass spectrometry, respectively.

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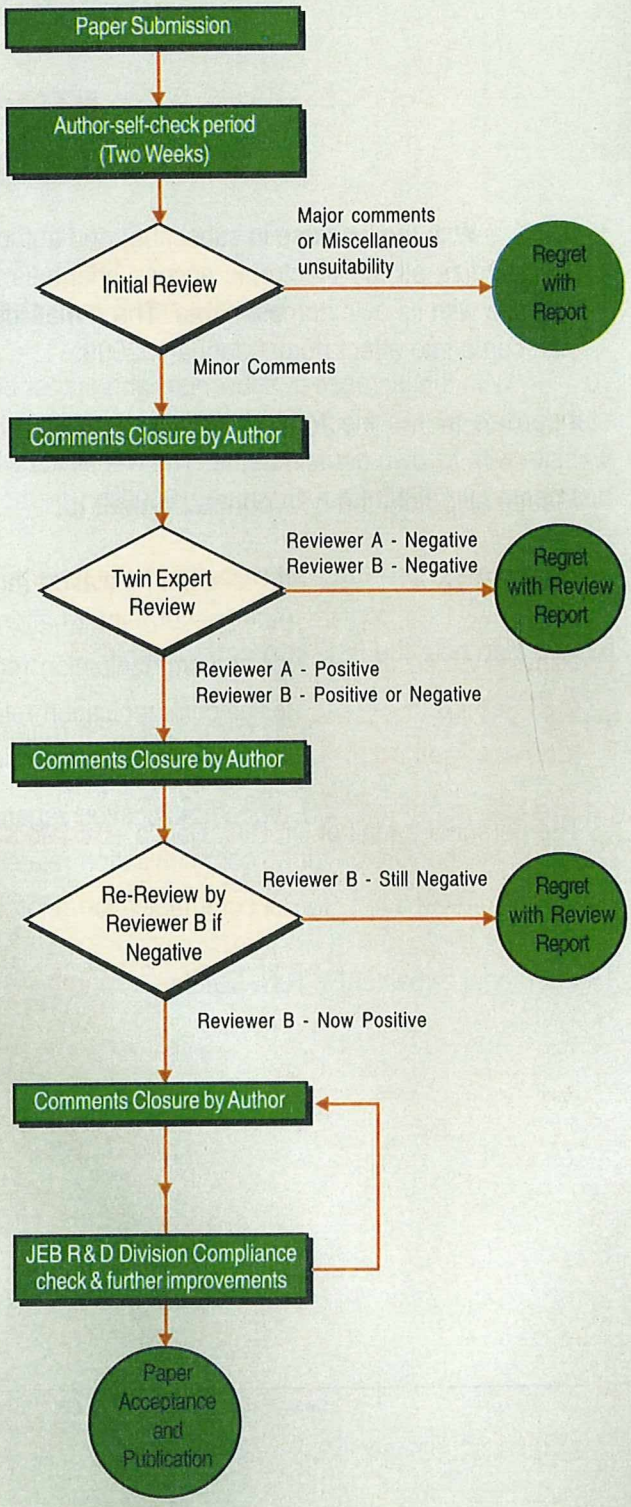

Regd. No.: 35574/79 Coden : JEBIDP ISSN : 0254-8704

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With the increase in subscriber and author base, Journal is growing in stature and outreach. In order to standardize all the electronic access channels on a single platform, we have decided to host the Journal website with its own domain name. The e-mail ids will also be aligned to the new journal website. This change has come into effect from 1 January, 2009.

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Invest New Drugs
DOI 10.1007/s10637-009-9255-3

PRECLINICAL STUDIES

Development and pharmacologic characterization of deoxybromophospha sugar derivatives with antileukemic activity

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Received: 22 March 2009 / Accepted: 31 March 2009

Summary Here, we synthesized two phospha sugar derivatives, 2,3,4-tribromo-3-methyl-1-phenylphospholane 1-oxide (TMPP) and 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DMPP) by reacting 3-methyl-1-phenyl-2-phospholene 1-oxide with bromine, and investigated their potential as antileukemic agents in cell lines. Both agents showed inhibitory effects on leukemia cell proliferation, with mean IC_{50} values of 6.25 μmol/L for TMPP and 23.7 μmol/L for DMPP, indicating that inhibition appeared to be dependent on the number of bromine atoms in the structure. Further, TMPP at 10 μmol/L and DMPP at 20 μmol/L induced G2/M cell cycle block in leukemia cells, and TMPP at 20 μmol/L induced apoptosis in these cells. TMPP treatment effected a reduction in both cell cycle progression signals (FoxM1, KIS, Cdc25B, Cyclin D1, Cyclin A, and Aurora-B) and tumor cell survival ($p27^{Kip1}$ and $p21^{Cip1}$), as well as induced the activation of caspase-3 and -9. Further, treatment with TMPP significantly reduced the viability of AML specimens derived from AML patients, but only slightly reduced the viability of normal $ALDH^{hi}$ progenitor cells. We also observed that FoxM1 mRNA was overexpressed in AML cells, and treatment with TMPP reduced FoxM1 mRNA expression in AML cells. Here, we report on the synthesis of TMPP and DMPP and demonstrate that these agents hinder proliferation of leukemia cells by FoxM1 suppression, which leads to G2/M cell cycle block and subsequent caspase-3-dependent apoptosis in acute leukemia cells. These agents may facilitate the development of new strategies in targeted antileukemic therapy.

Keywords Leukemia · Deoxybromophospha sugar · Cell cycle · Apoptosis

Abbreviations

TMPP	2,3,4-tribromo-3-methyl-1-phenylphospholane 1-oxide
DMPP	2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide
AML	acute myeloid leukemia
ALDH	Aldehyde Dehydrogenase
FoxM1	The forkhead box M1
CFU-GEMM	colony forming unit-granulocyte, erythroid, macrophage, megakaryocyte

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Published online: 14 May 2009

CFU-GM	colony forming unit-granulocyte, macrophage
BFU-E	burst forming unit-erythroid

Introduction

Phosphorus compounds perform vital functions in the growth, sustenance, and reproductive processes of all living organisms. Organophosphorus compounds in particular have been found to possess potential applications in both life sustaining and life extinguishing processes. These characteristics suggest the possible clinical use of these compounds as drugs with lower toxicity yet higher efficacy than existing drugs.

We previously synthesized pentofuranose analogs of phospha sugars from phospholenes as potentially bioactive agents which structurally resemble AZT or ribavirin [1, 2]. Phospha sugars are analogs of normal sugars in which the central oxygen atom of the hemiacetal ring has been replaced by a phosphorus atom. Replacement of the oxygen atom in the hemiacetal ring of normal sugars by a carbon or heteroatom leads to the formation of pseudo-sugars, several of which have been heavily investigated in the fields of synthetic, biological, and medical chemistry [3]. Novel nucleoside derivatives of pseudo- or hetero-sugars reported to date include *aza*-sugar (nitrogen instead of an oxygen atom; amino sugar) [4–7], *thio*-sugar (or *thia*-sugar; sulfur instead of an oxygen atom) [8, 9], and *carba*-sugar (oxygen atom replaced by a methylene group) [10, 11]. Further, the potential bioactivity of hetero-sugar nucleosides and glycosides (e.g., glycosidase and nojirimycin) has also been reported [12].

Given this potential for bioactivity, phospha sugar chemistry is one of the most rapidly developing areas of research [15–18]. One report has suggested that acetyl derivatives of the glucopyranose pattern of phospha sugar have potential as anticancer agents [19]. *Carba*-sugar derivatives are known to be effective in hampering some enzyme activity [10, 11]. Given that *aza*-sugar compounds are known to influence carbohydrate processing in the human body, extensive, on-going research and development using the compound has been employed to combat virus infection, cancer, and tuberculosis [13, 14]. Against this background, we synthesized 2,3-dibromo-3-methyl-1-phenylphosholane 1-oxide (DMPP) and 2,3,4-tribromo-3-methyl-1-phenylphospholane 1-oxide (TMPP).

Acute myeloid leukemia (AML) is characterized by the excess production of leukemic blasts arrested at various stages of granulocytic and monocytic differentiation, and it is this stage which determines the AML subtype (French-American-British [FAB] classification, AML: M1 to M5) [20]. To effectively cure a patient with AML, this proliferation of leukemic cells must be halted. Given that chemotherapy rarely eradicates the leukemic clones, efforts are now being made to find innovative new therapies which inhibit the proliferation of AML cells [21]. However, the effect of cell cycle progression and apoptosis resistance on the pathogenesis of AML remains to be defined.

In the present study, we investigated the antileukemic effect of the phospha sugar derivatives TMPP and DMPP in regulating proliferation and apoptosis in a series of leukemic cell lines, and in AML patient samples.

Materials and methods

Chemical synthesis

2,3-Dibromo-3-methyl-1-phenylphospholane 1-oxide (DMPP) was prepared by reacting 3-methyl-1-phenyl-2-phospholene 1-oxide with bromine in the presence of manganese dioxide. 2,3,4-Tribromo-3-methyl-1-phenyl-phospholane 1-oxide (TMPP) was prepared similarly by reacting 4-bromo-3-methyl-1-phenyl-2-phospholene 1-oxide with bromine. The reaction mixtures were extracted with chloroform, washed with saturated NaCl solution, and dried with anhydrous sodium sulfate. These reaction agents were then dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Company, St Louis, MO) and diluted in culture medium immediately before use. The final concentration of DMSO in all experiments was less than 0.01%, and all treatment conditions were compared with vehicle controls.

Cells and cell cultures

Human leukemia cell lines HL60, NB4, U937, NOMO-1, CEM, MOLT4, SUP-B15, K562, and Meg-01 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). YRK2 and SHG3 cells were harvested in our laboratory from bone marrow samples of AML (M5a) and CML patients. Samples of these cells, except for SUP-B15 cells, were cultured in RPMI 1640 media containing 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 μg/ml streptomycin, and 200 U/ml penicillin (GIBCO-BRL, Gaithersburg, MD). SUP-B15 cells were cultured in RPMI 1640 containing 20% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 μg/ml streptomycin, and 200 U/ml penicillin (GIBCO-BRL).

Primary samples

This study analyzed six adult patients (age range, 34 to 78 years) with M1 (n=21), M2 (n=56), M4 (n=32) and M5 (n=18) leukemia (FAB classification). Primary leukemia

cell specimens were obtained from patients before the start of any treatment, and normal hematopoietic cells (including bone marrow and peripheral blood mononuclear cells) were extracted from healthy donors after obtaining informed consent. Mononuclear cells (MNCs) were purified by Ficoll-Hypaque density-gradient centrifugation, and any remaining erythrocytes in the neutrophil pellet were removed by hydrolysis. The MNCs were then counted, and viability was determined by trypan blue exclusion.

Cell proliferation assay

Cells were seeded in 24-well flat-bottomed microplates at a density of 3×10^4 per well and incubated at various concentrations of TMPP or DMPP for 5 days. The cells were then washed with phosphate-buffered saline (PBS), harvested, and suspended in a 0.4% trypan blue solution for trypan blue exclusion assay, in which viable cells were counted with a hemocytometer at the indicated incubation day. For the MTT assay, the cells were seeded in 96-well flat-bottomed microplates at a density of 5×10^5 per well. The cells were incubated at various concentration of TMPP or DMPP for 24 h. After incubation, 10 μl 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma) was added to each well at a final concentration of 1 mg/ml. After incubation at 37°C for 4 h, absorbance was measured at a wavelength of 560 nm using a microplate reader.

Cytotoxic assay

Cytotoxic activity of TMPP and DMPP against various leukemia cells (IC_{50} value) was determined. Cells were seeded in triplicate at 5,000 to 10,000 per well in 24-well plates. The day after plating, TMPP or DMPP were added to the wells by titrating over six or more dilutions from 10 to 1,000 μmol/L. Control cells without treatment were seeded as well. The cells were then grown for five days, and viable cells were counted. After washing with PBS, cells were placed in isotonic solution and counted immediately. IC_{50} was determined by a sigmoidal dose-response calculation (XLFit 3, IDBS, Inc., Emeryville) and represented the concentration of TMPP or DMPP that produced 50% of maximum response.

Cell cycle analysis

Propidium iodide (PI) (Sigma Chemical Company, St. Louis, MI) staining was used to analyze DNA content. TMPP- or DMPP-treated cells were cultured at 37°C in 2 ml of complete medium containing 1×10^6 cells. After incubation for 48 h, the cells were washed twice with cold PBS, fixed with 70% ethanol overnight, treated with 100 μg/ml RNase A, and then stained with 50 μg/ml PI. For apoptosis analysis, the relative DNA content per cell was measured by flow cytometry using an Epics Elite flow cytometer (Coulter Immunotech, Marseille, France). The percentage of cells in the apoptotic sub-G1 phase, as well as G1, S, and G2/M phases, was calculated using the ModFit program (Becton, Dickinson and Company, San Jose, CA).

Western blotting

The TMPP-treated leukemia cells were harvested, washed with cold PBS, and resuspended in lysis buffer containing 0.5% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 150 mM NaCl, 1 mM sodium orthovanadate, and 1 mM dithiothreitol supplemented with one Complete Mini protease inhibitor tablet (Boehringer Mannheim GmBH, Indianapolis, IN) per 20 ml lysis buffer immediately before use. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were then blocked with 0.5% milk in PBS for 1 h at room temperature. After being washed in Tris-buffered saline Tween (TBS-T), the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Amersham Biosciences Inc., Arlington Heights, IL) for 1 h and exposed to X-ray film at room temperature. The signal was detected by chemiluminescence using an ECL detection kit (Amersham Bioscience Inc.). The following commercially available antibodies and dilutions were used for western blotting: rabbit polyclonal anti-FoxM1 antibody (MPP2 K-19, 1:500) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal anti-$p27^{Kip1}$ antibody (1:1000) (Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-$p21^{Cip1}$ antibody (1:1000) (Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-Cdc25B2 antibody (1:500) (Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-Cyclin D1 antibody (1:500) (Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-Cyclin A antibody (1:500) (Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-KIS antibody (1:500) (ABGENT, Inc., San Diego, CA), rabbit polyclonal anti-Aurora-B antibody (1:500) (ABGENT, Inc.), mouse monoclonal anti-bcl-2 antibody (BD Biosciences Pharmingen, San Diego, CA), mouse monoclonal anti-caspase-9 antibody (BD Biosciences Pharmingen), mouse monoclonal anti-caspase-3 (CPP32) antibody (BD Biosciences Pharmingen), and mouse monoclonal anti-PARP antibody (BD Biosciences Pharmingen). To ensure equal protein volume loading, similar experiments were performed using a mouse monoclonal anti-actin antibody (C-4; ICN Biomedicals, Inc., Aurora, OH) as an internal control.

Immunofluorescent staining

U937 cells were cytocentrifuged onto glass slides, fixed in 4% paraformaldehyde, premeabilized in 0.5% Triton X-100, stained with anti-FoxM1 rabbit polyclonal antibody (Santa Cruz, CA), and incubated with fluorescence-linked anti-rabbit immunoglobulin G (Santa Cruz, CA). The cells were viewed by phase-contrast or fluorescence microscopy (IMT-2; OLYMPUS, Tokyo, Japan).

Isolation of primitive hematopoietic cells by Aldehyde Dehydrogenase (ALDH) activity

Following the purification process, the MNCs were further fractionated according to ALDH activity by staining with Aldefluor reagent (StemCo Biomedical, Inc., Durham, NC) according to the manufacturer's specifications. Aldefluor substrate (0.625 μg/mL) was added to between 2 and 7×10^6 cells/mL suspended in proprietary Aldefluor assay buffer, then incubated for 20 to 30 min at 37°C to allow the conversion of Aldefluor substrate to a fluorescent product. The amount of intracellular fluorescence was measured by flow cytometry. $ALDH^{hi}$ cells were selected by FACS (Becton, Dickinson and Company).

Viability of AML and $ALDH^{hi}$ cells with regard to TMPP administration, and expression of FoxM1 mRNA

AML cells were obtained from PB or BM of AML patients, and normal $ALDH^{hi}$ progenitor cells were obtained from BM of healthy volunteers. The AML and normal $ALDH^{hi}$ cells were untreated or treated with TMPP at concentrations ranging from 0.25 to 16 μmol/L. To determine cell number, AML cells and $ALDH^{hi}$ progenitor cells were treated with TMPP, and any nonviable cells were identified after 3 min of staining with 0.4% trypan blue (Sigma). The number of unstained (nonviable) cells was counted using a hemocytometer.

Quantification of FoxM1 mRNA in AML and $ALDH^{hi}$ cells

AML cells were obtained from PB or BM of AML patients, and normal $ALDH^{hi}$ progenitor cells were obtained from BM of healthy volunteers. The AML and normal $ALDH^{hi}$ cells were untreated or treated with TMPP at 5 μmol/L for 48 h. Total RNA was extracted from these cells using an RNeasy system (Quiagen, Tokyo, Japan), and 2 μg RNA was reverse transcribed using a 1st strand cDNA synthesis kit (Roche, Indianapolis, IN). Oligonucleotide sequences for each primer were as follows: FoxM1, sense 5'-GGGCGCACGGCGGAAGATGAA-3', antisense 5'-CCACTCTTCCAAGGGAGGGCTC-3'; and G3PDH; sense 5'-GAACGGGAAGCTCACTGGCATGGC-3', antisense 5'-TGAGGTCCACCACCCTGTTGCTG-3'. In each experiment, RT-PCR was performed in duplicate. The real-time RT-PCR was performed using SYBER-Green dye on an ABI PRISM 7700 Sequence detector (Perkin-Elmer/Applied Biosystems, Foster City, CA). For real time using SYBER-Green, dissociation curve was obtained for melting curve analysis to confirm PCR product specificity.

Statistical analysis

Experiments were repeated at least three times and results were combined and represented graphically as the mean values ± the standard deviation (SD). The significance of the *in vitro* results was determined using Student's *t* test.

Results

In vitro antiproliferative effects of TMPP/DMPP in leukemia cells

Antiproliferative effects of TMPP and DMPP (Fig. 1) were tested in U937 and YRK2 cells (Fig. 2). Both cell lines showed a significant reduction in cell proliferation over the time course examined at 10 μmol/L TMPP or 20 μmol/L DMPP. U937 cells treated with DMPP and TMPP showed a growth inhibition of 14.8% and 5.6%, respectively, at 3 days' treatment (Fig. 2(a)). YRK2 cells treated with DMPP and TMPP showed a growth inhibition of 19.7% and 2.8%, respectively, at the same time point (Fig. 2(b)). Cytotoxic effects of TMPP and DMPP were tested in several human

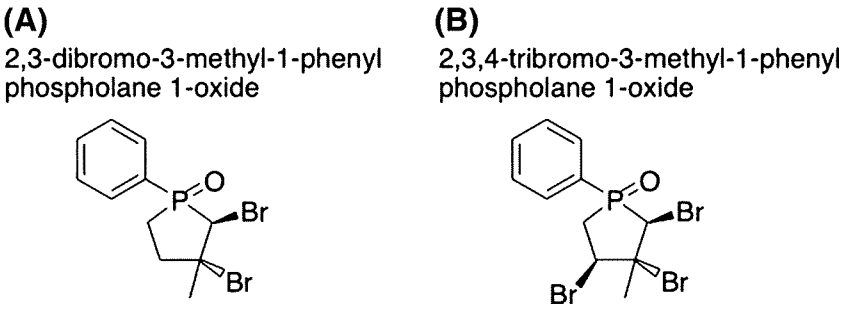


Fig. 1 Chemical structure. Chemical structure of DMPP (**a**) and TMPP (**b**)

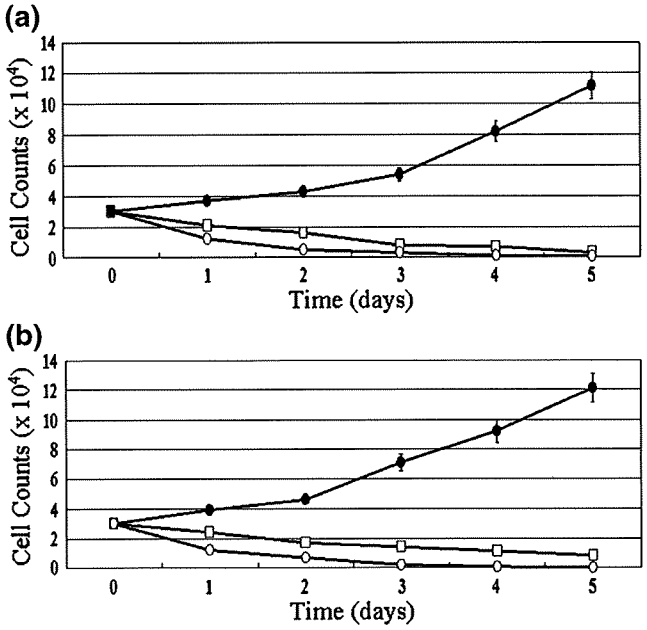


Fig. 2 Effect of TMPP/DMPP on leukemia cell proliferation. U937 (**a**) and YRK2 (**b**) leukemia cells were either untreated (•), treated with 10 μmol/L TMPP (○), or treated with 20 μmol/L DMPP (□) for 5 days. After incubation, the cells were harvested and counted. Data are shown as mean±S.D. in triplicate culture and are representative of three independent experiments

leukemia cell lines (HL60, NB4, U937, YRK2, NOMO-1, CEM, MOLT4, SUP-B15, K562, Meg-01, and SHG3) (Table 1). Results showed that the effects of TMPP were three to five times stronger than DMPP with regard to IC_{50} value. Interestingly, the strength of the effect depended on the number of bromine atoms introduced into the molecule.

Cell cycle analysis in leukemia cells treated with TMPP/DMPP

As shown in Fig. 3(a), treatment of U937 cells with 10 μmol/L TMPP resulted in an increase in the number of cells in the G2/M and S phases, and a slight decrease in the number in the G1 phase, in a time-dependent manner as measured by flow cytometry. Treatment of U937 cells with 20 μmol/L DMPP showed similar results. However, TMPP more strongly induced cell cycle arrest than DMPP, which is consistent with the inhibition pattern observed by MTT assay. Further, treatment of the U937 cells with 10 μmol/L TMPP for 40 h resulted in a marked accumulation of cells in the G2/M phase, and a concentration of 20 μmol/L TMPP significantly increased the number of apoptotic cells (Fig. 3(b)). The number of cells in the G2/M phase increased to 1.5 to 2 times that of the control level in all leukemia cell lines following treatment with 10 μmol/L TMPP (Fig. 3(c)), and the apoptotic fraction increased from 72±3% to 83±2% following treatment with 20 μmol/L TMPP compared to control levels (Fig. 3(d)). These findings indicate that exposure of leukemia cells to TMPP leads to G2/M block of cell cycle progression at a concentration of 10 μmol/L and to apoptosis at 20 μmol/L.

TMPP-induced cell cycle arrest and apoptosis in leukemia cells

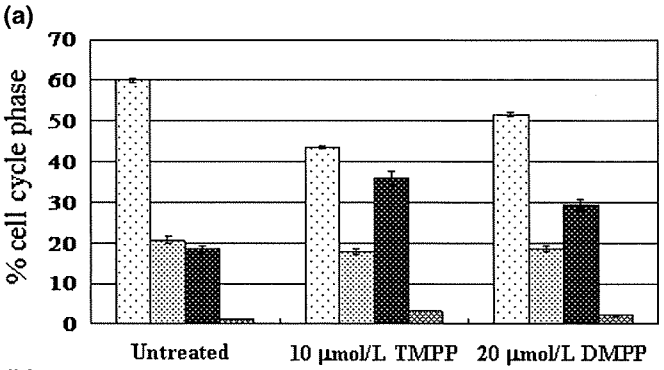
Given our previous finding (in press) that FoxM1 regulates transcription of essential cell cycle regulatory genes, we examined whether exposure of leukemia cells to TMPP affected expression of cell cycle regulators such as FoxM1, KIS, Cdc25B, $p27^{Kip1}$, $p21^{Cip1}$, Cyclin D1, Cyclin A, and Aurora-B kinase. Protein extracts were isolated from U937 and YRK2 cells treated with TMPP, and western blot analysis was performed to measure protein levels of these cell cycle regulators (Fig. 4(a)). Results showed that TMPP treatment reduced FoxM1, KIS, Cdc25B, Cyclin D1, Cyclin A and Aurora-B protein levels in a dose-dependent manner in U937 and YRK2 cells compared to untreated leukemia cells. However, exposure to TMPP actually increased levels of $p27^{Kip1}$ and $p21^{Cip1}$ protein. These results indicate that TMPP regulates the expression of cell cycle regulatory proteins.

We also examined the effect of 48-h treatment with TMPP in U937 and YRK2 cells in relation to expression of several apoptotic regulatory proteins. As shown in Fig. 4(b), the treatment of leukemia cells with TMPP resulted in reduction of BCL-2 protein expression in a dose-dependent manner. Further, with regard to caspase-9 and -3, treatment with TMPP induced significant cleavage of procaspase-9 and -3 in a dose-dependent manner. Moreover, TMPP treatment increased the levels of the cleaved form of PARP in a dose-

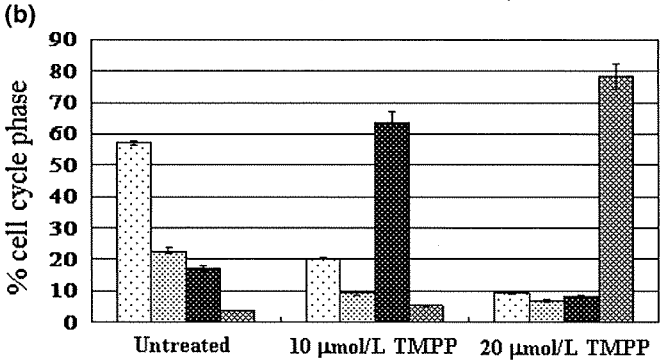
Table 1 TMPP/DMPP inhibition of AML cell proliferation

Cell lines	TMPP IC_{50}(μmol/L)	DMPP IC_{50}(μmol/L)
HL60	4.8±0.7	18±2.3
NB4	3.2±0.9	15±1.4
U937	6.2±1.1	22±1.8
YRK2	5.3±1.3	28±2.6
NOMO-1	5.5±0.8	18±2.1
CEM	6.9±0.3	29±2.4
MOLT4	6.7±1.2	26±1.8
SUP-B15	7.1±1.0	24±2.8
K562	9.1±0.8	28±1.7
Meg-01	8.6±1.4	27±1.9
SHG3	5.4±0.6	26±2.1

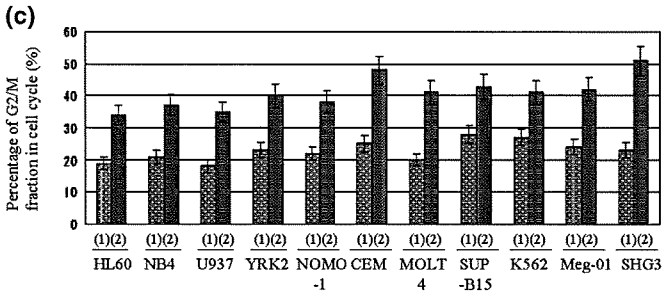
The cytotoxic activity of TMPP and DMPP. IC50 values were determined for each cell line. Data are shown as mean ± S.D. in triplicate culture and are representative of three independent experiments



(%)	Untreated	10 μM TMPP	20 μM DMPP
G1	60.1 ± 1.7	43.6 ± 1.2	51.5 ± 1.3
S	20.6 ± 2.1	17.7 ± 1.1	18.6 ± 1.2
G2/M	18.5 ± 1.1	35.9 ± 2.5	29.3 ± 1.5
Apoptosis	1.1 ± 0.4	3.2 ± 0.5	2.1 ± 0.4



(%)	Untreated	10 μM TMPP	20 μM TMPP
G1	57.2 ± 2.3	20.4 ± 2.6	9.2 ± 2.4
S	22.7 ± 1.9	9.2 ± 2.9	6.9 ± 0.8
G2/M	17.2 ± 1.8	63.7 ± 3.2	8.1 ± 2.2
Apoptosis	3.7 ± 1.3	5.3 ± 1.7	78.2 ± 6.1



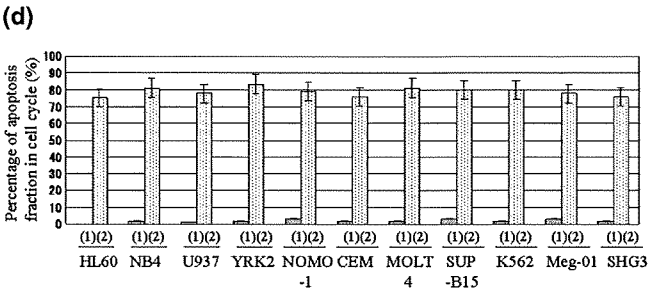


Fig. 3 Effect of TMPP/DMPP on cell cycle and apoptosis of leukemia cells. Cell cycle analysis of U937 cells. FACS analysis was performed on U937 cells treated with 10 μmol/L TMPP or 20 μmol/L DMPP for 16 h (**a**). FACS analysis was then performed on U937 cells treated with 10 and 20 μmol/L TMPP for 40 h (**b**). The G2/M (**c**) and apoptosis (**d**) fractions were analyzed in all leukemia cell lines treated with 10 and 20 μmol/L TMPP for 16 h and 40 h, respectively. Bars represent untreated cells (1) and cells treated with TMPP (2). Data are shown as mean±S.D. in triplicate culture and are representative of three independent experiments

dependent manner. In contrast, TMPP treatment did not affect procaspase-8 levels (data not shown). These results indicate that treatment of leukemia cells with TMPP induced down-regulation of anti-apoptotic proteins and was associated with the activation of caspase cascades.

Localization of FoxM1 in leukemia cells

As shown in Fig. 5(a), U937 cells were inhibited the proliferation by TMPP-dose-dependent manner. Interestingly, immunofluorescent staining in U937 cells revealed that FoxM1 was constitutively present in the nucleus. TMPP treatment significantly attenuated the cytoplasmic signals, and inhibited the transfer of FoxM1 protein from cytoplasm to nucleus (Fig. 5(b)). Moreover, FoxM1 expression in cytoplasm was reduced by TMPP-dose dependent manner (Fig. 5(c)). These findings showed that TMPP regulated the subcellular localization of FoxM1.

TMPP inhibited AML cells derived from AML patients

AML cells from PB or BM donated by AML patients accounted for between 78% and 92% of MNCs, and hematopoietic progenitor cells from bone marrow donated by healthy volunteers were obtained according to ALDH activity using the Aldefluor substrate and FACS. Elevated ALDH expression is an intrinsic property in many types of stem cells. Murine xenotransplantation models have shown that cells with elevated ALDH activity are able to repopulate *in vivo* [22]. $ALDH^{hi}$ hematopoietic progenitor cells, including $CD34^{+}$, $CD133^{+}$, c-kit^{+}, and Lin^{-} cells, were selected according to side scatter (SSC) and FITC

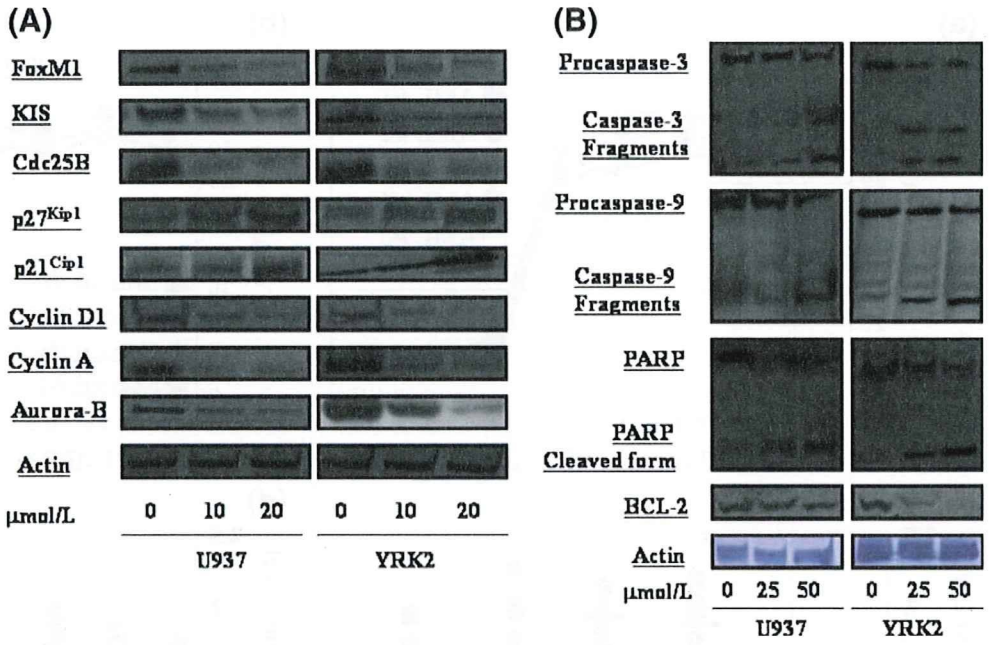


Fig. 4 Effect of TMPP treatment on cell cycle and apoptosis-related proteins. Western blot analysis was performed on TMPP-treated and untreated cells to investigate the expression of cell cycle-related proteins (**a**). Western blot analysis was performed on TMPP-treated and untreated cells to investigate the expression of apoptosis-related proteins (**b**)

properties. The ALDH^{hi} selected populations in healthy volunteers represented 1.24±0.27% of MNCs. With regard to AML cells from AML patients (M2: AML #1, #2, #5; M4: AML #6, #8), cell viability was reduced by TMPP in a dose-dependent manner (Fig. 6(a)), particularly at a molarity of 4 μM. In contrast, the viability of ALDH^{hi} cells obtained from healthy volunteers was only slightly reduced by TMPP (Fig. 6(b)). Moreover, high levels of FoxM1 mRNA were detected in all (127/127) primary leukemia specimens tested, and the mean ratios of FoxM1 to G3PDH in AML specimens were 1.72±0.48 (M1; 1.82, M2; 1.95, M4; 1.62, M5; 1.47). In contrast, the mean ratio of FoxM1 to G3PDH in normal ALDH^{hi} cells was 1.12± 0.15. These results demonstrated that increased levels of FoxM1 mRNA were detected in nearly all of these samples from various leukemia specimens compared to normal ALDH^{hi} cells (Fig. 6(c)). To assess the expression of FoxM1 in clinical specimens treated with TMPP, AML cells from AML patients (M2: AML #1, #2, #5; M4: AML #6, #8) and normal ALDH^{hi} cells were treared with TMPP

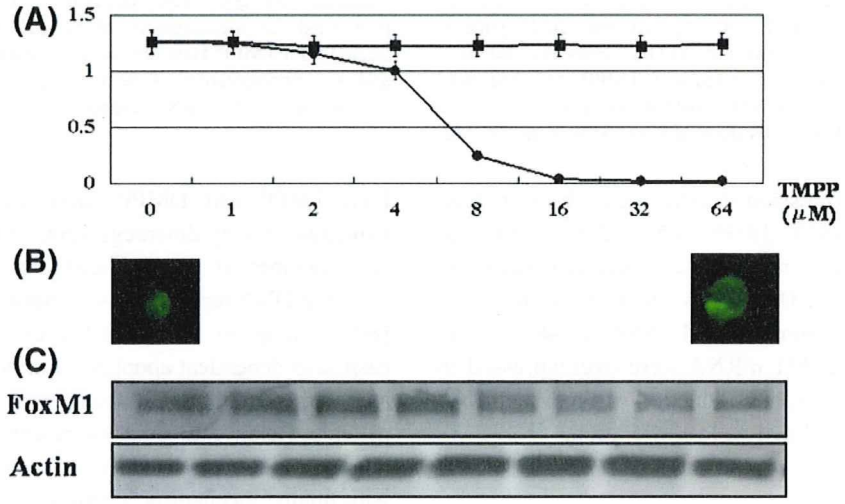


Fig. 5 TMPP inhibited leukemia cell proliferation through the regulation of cytoplasmic localization of FoxM1. (**a**) U937 cells were untreated (■) and treated with TMPP (●) at the indicated concentration for 72 h. The cell proliferation was measured by MTT assay. Data shown as mean ± S.D. in triplicate culture and are representative of three independent experiments. (**b**) U937 cells were treated with TMPP at the indicated concentration for 24 h, cytocentrifuged, and fixed with 4% paraformaldehyde for 10 min. The fixed cells were immunostained using antibody of FoxM1. Control is the left panel (fluorescence image). The treatment with 64 μmol/L TMPP is the right panel (fluorescence image). Original magnification × 400. (**c**) The changes of FoxM1 protein expression was evaluated by TMPP-dose dependent manner in U937 cells