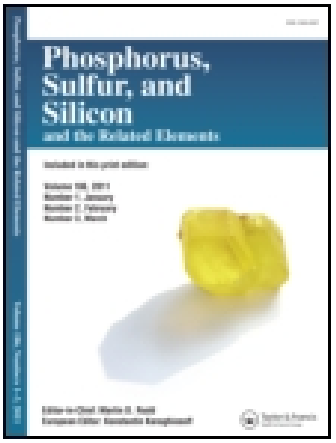

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Research on Phospha Sugar Analogues to Develop Novel Multiple Type Molecular Targeted Antitumor Drugs Against Various Types of Tumor Cells

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RESEARCH ON PHOSPHA SUGAR ANALOGUES TO DEVELOP NOVEL MULTIPLE TYPE MOLECULAR TARGETED ANTITUMOR DRUGS AGAINST VARIOUS TYPES OF TUMOR CELLS

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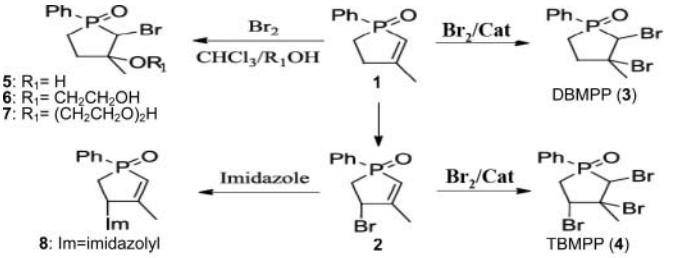
GRAPHICAL ABSTRACT



Preparation of phospha sugar derivatives **2**–**8** from 2-phospholene 1.

Abstract *The synthesis and antitumor activity evaluation of new branched phospha sugars, especially deoxybromophospha sugar derivatives or bromophospholanes of 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP: **3**) and 2,3,4-tribromo-3-methyl-1-phenylphospholane 1-oxide (TBMPP: **4**), against various types of leukemia cell lines as well*

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*as the results of the mechanistic studies for characterizing and developing the novel multiple type molecular targeted antitumor agents are reported in this paper. DBMPP and TBMPP were prepared from 1-phenyl-3-methyl-2-phospholene 1-oxide (**1**). The isomer mixture of phospha sugars prepared were evaluated as novel antitumor agents by MTT in vitro method. DBMPP and TBMPP were characterized by flow cytometry and Western blot analysis and were revealed to be potential antitumor agents against leukemia cell lines of K562 (one type of leukemia cell lines of CML) and U937 (one type of leukemia cell lines of AML) as well as against the various types of leukemia cell lines and also against solid tumor cell lines of stomach, skin, and lung cancers by MTT evaluation and observation by a handstand phase-contrast microscope. The results of the flow cytometry indicated that the mechanism of apoptosis induced by phospha sugar derivatives not only to tumor cells of leukemia cell lines of U937 but also to tumor cells of various kinds of leukemia cell lines selectively to decrease the tumor cell viability of various kinds of leukemia cell lines. The Western blot analyses for phospha sugar DBMPP against U937 leukemia cell lines showed that the phospha sugar affected on the expressions of the factors of cell cycles in the manners of suppressing the expression of the accelerator factors of cell cycles of tumor cells and enhancing the expression of suppressor factors of cell cycles of tumor cells by the medications of phospha sugars. TBMPP enhanced the expression of IER5 and then suppressed the expression of Cdc25B, which is the common factor to accelerate the cell cycles of various kinds of tumor cells. Therefore, suppression of the expression of Cdc25B by TBMPP implies that the branched deoxybromophospha sugar derivatives might be novel and potential multiple type molecular targeted antitumor agents against various kinds of tumor cell lines.*

Keywords Phosphorus heterocycles; branched deoxybromophospha sugars; molecular targeted antitumor agents; MTT *in vitro* evaluation; flow cytmetry; Western blot analysis

INTRODUCTION

Well known typical pseudo sugars are *carba*-, *aza*-, and *thi*a-sugars,[1–4] which have a carbon–carbon–carbon, carbon–nitrogen–carbon, or carbon–sulfur–carbon linkage, respectively, instead of the carbon–oxygen–carbon linkage in the hemiacetal ring of the normal sugars. These pseudo sugars are known to exist in nature and are also chemically prepared by applying methodologies of synthetic sugar chemistry, where common sugars are generally used as the starting materials, for synthesizing pseudo sugars via a series of reactions of protection of specific hydroxyl groups, carbon-hetero atom bond formation, deprotection, and finally formation of the heterocycles by the reconstruction of the hemiacetal ring of sugars.[5] Pseudo sugars are known to exert important biological activities, therefore, many studies on not only the isolation and synthesis of the pseudo sugars but also on the characterization and evaluation of the biological activities are actively performed and reported.[6–9] On the other hand, phospha sugars, being classified into one new category of the pseudo sugars having the carbon–phosphorus–carbon linkage in the hemiacetal ring of sugars, are not yet found in nature and the syntheses of them are rather difficult compared with the typical pseudo sugars such as *aza*- and *thia*-sugars.[4,5,10–12]

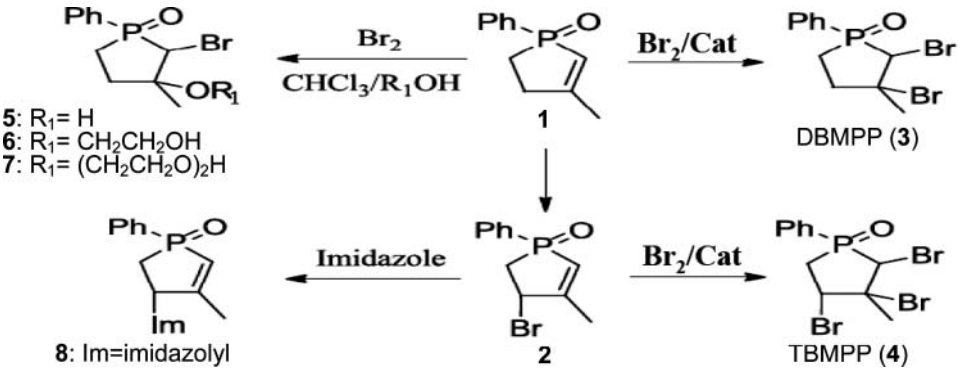
We have been searching biologically active phospha sugars and we have first found new phospha sugar derivatives which provided good antitumor activities against leukemia cell lines by *in vitro* evaluation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) methods.[13] In this paper we will deal with the successful preparation of branched phospha sugars including deoxybromophospha sugars of

2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP: **3**) and 2,3,4-tribromo-3-methyl-1-phenylphospholene 1-oxide (TBMPP: **4**). The evaluations and characterizations of the DBMPP and TBMPP by the MTT method and the Western blot analysis revealed that phospha sugars have excellent characters as antitumor agents with high activities, wide spectra, good selectivity, and specificity against various kinds of leukemia cell lines. Western blot analysis for DBMPP and TBMPP against leukemia cell lines showed that DBMPP suppressed the expression of several tumor cell cycle accelerators and enhanced the expression of tumor cell cycle suppressors. TBMPP enhanced the expression of IER5 and then suppressed the expression of Cdc25B.

RESULTS AND DISCUSSION

Synthesis of Phospha Sugar Derivatives

Branched deoxypbromophospha sugar derivatives of 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP: **3**) and 2,3,4-tribromo-3-methyl-1-phenylphospholane 1-oxide (TBMPP: **4**) were prepared from the starting material of 3-methyl-1-phenyl-2-phospholene 1-oxide (**1**) and 4-bromo-3-methyl-1-phenyl-2-phopholene 1-oxide (**2**) as shown in Scheme 1.[12] Here, DBMPP (**3**) and TBMPP (**4**) were prepared by simple and efficient synthetic methods by addition of bromine to the C=C double bond of the 2-phospholenes **1** and **2** with or without catalyst in yields of 90% and 69%, respectively. Deoxybromophospha sugar derivatives with oxygen functionalities OR_1 (**5** (R_1 = H), **6** ($R_1 = CH_2CH_2OH$), and **7** ($R_1 = (CH_2CH_2O)_2H$)) on the 3-position were prepared by the reaction of **1** with bromine in the presence of water, ethylene glycol, and diethylene glycol, respectively, in yields of 55%, 88%, and 58%. 4-Imidazolyl derivative **8** was prepared from 4-bromo derivative **2** by the substitution reaction of the 4-bromo group by imidazole in a yield of 93% (Scheme 1).



Scheme 1 Preparation of phospha sugar derivatives **2**–**8** from 2-phospholene 1.

Antitumor Activity and Cell Cycle Analysis[13,14]

Antitumor activity for the prepared phospha sugar derivatives **2**–**8** was evaluated by MTT *in vitro* method against K562 and U937 cell lines, whose results of antitumor activities are summarized in Table 1. Among these branched phospha sugar derivatives, bromo derivatives were active, especially DBMPP (**3**) and TBMPP (**4**) were quite active,

Table 1 Antitumor activities (IC_{50}) of phospha sugar derivatives against leukemia cell lines of K562 and U937 for 48 h at 37°C)

Phospha sugars	K562 IC_{50} (μM)	U937 IC_{50} (μM)
1	>200	>900
2	48	83
3 (DBMPP)	23	24
4 (TBMPP)	3.2	2.3
5	34	ND[a]
6	>100	ND[a]
7	63	ND[a]
8	>100	ND[a]
(Imatinib mesylate)	0.48	>500

[a]ND = not yet determined.

against leukemia cell lines. DBMPP and TBMPP were more active against U937 cell lines than imatinib mesylate (Glivec, Gleevec), however, they did not give any damages against healthy or normal leukocytes (Table 1). Therefore, here we have focused on DBMPP and TBMPP for further investigation of phospha sugar derivatives to develop novel antitumor agents.

DBMPP and TBMPP have wide spectral antitumor activities against various kinds of leukema cell lines (Table 2). The cell cycle analysis revealed that DBMPP induced apoptosis to stop the progress of the cell cycle of K562 and U937 cell lines likewise the manner of imatinib mesylate at the Sub G1 stage (Figure 1: against K562 cell lines). Figure 1 shows that the apoptosis of 78% for the cell cycles was induced by DBMPP (20 μM) against K562 cell lines. TBMPP (20 μM) also induced the apoptosis against various leukemia cell lines about 80% (Figure 2). Branched deoxybromophospha sugars DBMPP and TBMPP have good to excellent antitumor activities against wide spectral leukemia cell lines.

Western Blot Analysis[13]

Western blot analysis for phospha sugars was performed against leukema cell lines. DBMPP enhanced the expression of tumor accelerator factors of FoxM1, KIS, Skp2,

Table 2 Antitumor activities (IC_{50}) of phospha sugar derivatives DBMPP and TBMPP against various kinds of leukemia cell lines (for 48 h at 37°C)

Cell lines	TBMPP (**4**) IC_{50} (μM)	DBMPP (**3**) IC_{50} (μM)
HL60	4.8 ± 1.0	18 ± 1.5
NB4	3.2 ± 0.9	15 ± 1.4
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
MEG-01	8.6 ± 1.4	27 ± 1.9
SHG3	5.4 ± 0.6	26 ± 2.1
(Healthy or normal leukocyte)[a]	>>200	>>200

[a]Blast 0% (no leukemia cells).

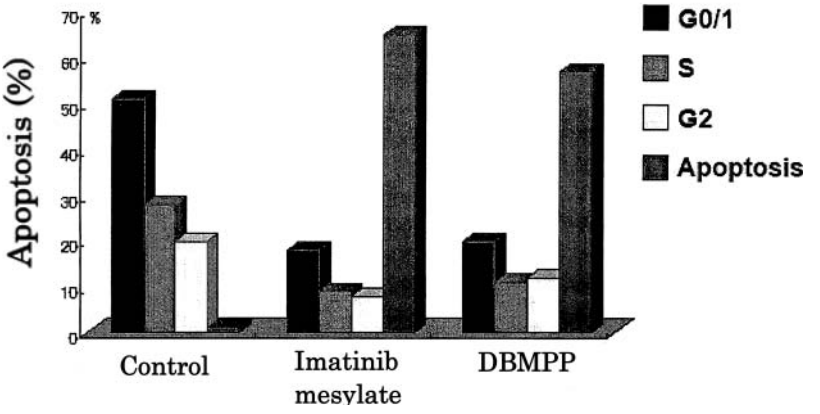


Figure 1 Results of the flow cytometry observed by DBMPP and Imatinib mesylate (Glivec, Gleevec) against K562 cell lines for 24 h at 37°C showing apoptosis (%) induced.

Cyclin D1, Survivin, Aurora-B, Actin against U937 cell lines. On the other hand, DBMPP suppressed the expression of tumor suppressor factors of $p27^{Kip1}$ and $p21^{Cip1}$ against U937 cell lines, and then the phospha sugar induced apoptosis and stops the cell cycle progress (Figure 3). Similarly, DBMPP enhanced the expression of tumor accelerator factors of Aurora-A, Aurora-B, Survivin, FoxM1, Skp2, hKIS, KPC1, and Pirh1 against K562 cell lines, and then affected on the cell cycle progression (Figure 4).[13]

The Western blot analysis for TBMPP against leukemia cell lines showed the enhanced expression of IER5, and then suppressed the expression of Cdc25B (Figure 5).[14] Cdc25B is known to be a common factor of cell cycle progression for many kinds of different tumor cell lines, therefore, phospha sugars TBMPP and DBMPP may be expected

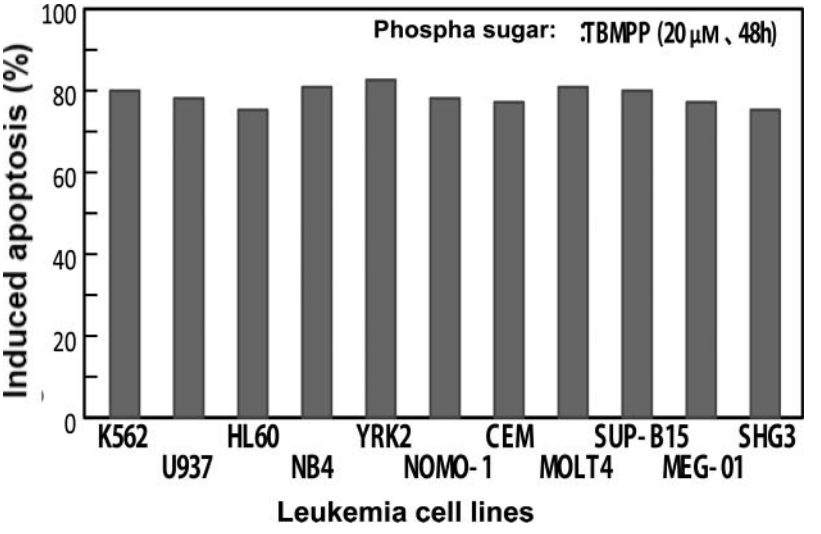


Figure 2 Apoptosis (%) induced by TBMPP (20 μM) for 48 h at 37°C.

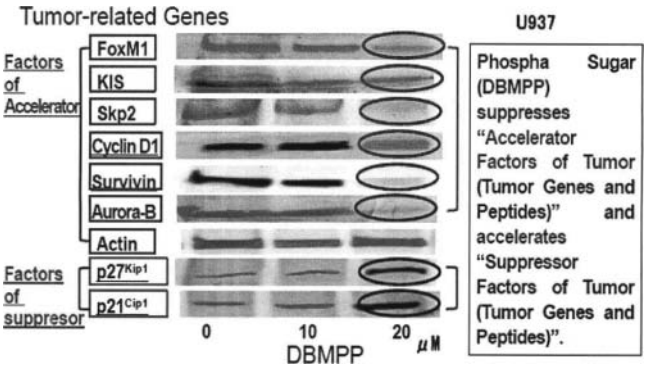


Figure 3 Western blot analysis by DBMPP against U937 cell lines.

to be quite efficient antitumor agents against many kinds of leukemia cell lines and to cure effectively tumor patients suffered from different types of leukemia cell lines.

EXPERIMENTAL

Instruments

TLC (Silica gel: Wako Chromato sheet and/or Merk Kieselgel 60; Eluent : $CHCl_3$: MeOH = 20 : 1, in R*f* value); melting point apparatus (Gallenkamp, in °C) and thermal analysis instrument (Shimazu DTG-60A50AH, TGA and DSC, in °C); HPLC (GL Science: GL-7410 HPLC Pump and GL-7450 UV Detector); MS (MALDI-TOF-MS: GL Science, Voyager-DE Porimerix; Matrix: α-Cyano-4-hydroxycinnamic acid, in *m*/*z*); IR

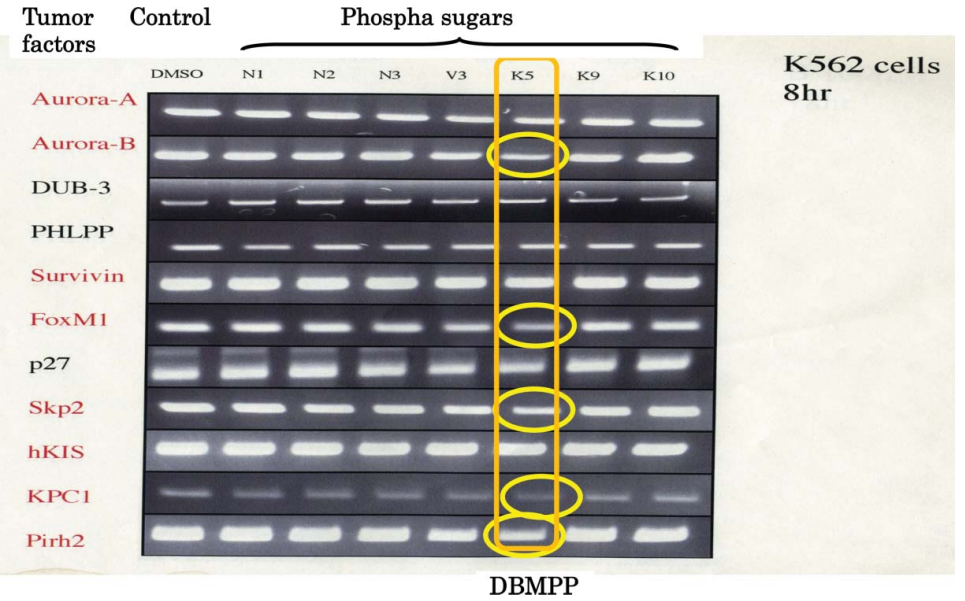


Figure 4 Western blot analysis by DBMPP against K562 cell lines. (Color figure available online).

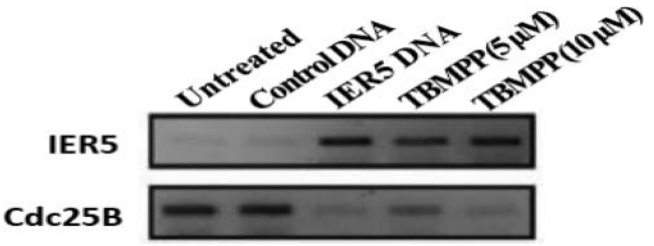


Figure 5 Western blot analysis; Acceleration of IER5 expression and suppression of Cdc25B expression by TBMPP.

(JASCO FT/IR 410 (KBr), in cm^{-1}); ^{1}H-NMR (JEOL JNM-AL300 (300 MHz); Solvent: $CDCl_3$, in δ (ppm) from TMS) were used to record and collect the data for analyzing the products.

Materials

3-Methyl-1-phenyl-2-phospholene 1-oxide (**1**),[15] was prepared and used as the starting material.

Synthesis of 4-bromo-3-methyl-1-phenyl-2-phospholene 1-oxide (2)

3-Methyl-1-phenyl-2-phospholene 1-oxide (**1**: 0.96 g, 5.0 mmol; 1.0 eq.) was dissolved in CCl_4 (5 mL), and to the solution NBS (1.33 g, 1.5 eq.) was added and stirred to make clear solution at 70 °C. To the solution was then added AIBN (0.12 g, 0.75 mmol; 0.15 eq.) and the solution of the reaction mixture was refluxed for 1 day. The reaction mixture was treated with saturated aqueous solution of sodium hydrogensulfite, and then the solution was washed with saturated sodium hydrogencarbonate solution, brine, and water followed by drying over anhydrous Na_2SO_4, which was followed by removal of the solvent afforded crude product. Silica gel column chromatograpy of the product with the mixed eluent of chloroform and methanol (20 : 1) afforded product **2** (0.88 g, 3.3 mmol) in a yield of 65%; Molecular equation: $C_{11}H_{12}BrOP$; M.W.: 271.01; TLC: $R_f = 0.42$ ($CHCl_3$:MeOH = 20 : 1); MS (*m/z*), 271.5(MH^+, 100), 273.5(MH^+, isptope, 90); ^{1}H-NMR ($CDCl_3$, 300 MHz), $\delta = 2.23$ (s, 3H, CH_3), 2.70–2.89 (m, 1H, C5), 2.96–3.15 (m, 1H, C5), 5.14–5.20 (m, 1H, C4), 6.17 (t, $J = 20.1$ Hz, 1H, C2), 7.48–7.65(m, 3H, *o,p*-Ph), 7.80–7.87(m, 2H, *m*-Ph).

Synthesis of 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP (3))

3-Methyl-1-phenyl-2-phospholene 1-oxide (**1**: 0.21 g, 1.1 mmol; 1.0 eq.) was dissolved in $CHCl_3$ (5 mL), and to the solution Cu(II)Br_2 (0.12 g, 0.53 mmol; 0.49 eq.) was added $CHCl_3$ (10 mL) solution of bromine (0.5 mL; 2.7 g, 17 mmol; 16 eq.) dropwisely and stirred for 1 h at room temperature. The reaction mixture was treated with saturated sodium hydrogensulfite solution and was extracted with $CHCl_3$. The $CHCl_3$ extract was washed with saturated sodium hydrogencarbonate, brine, and water, and then dried over anhydrous Na_2SO_4. Evaporation of the solvent from the solution of the product followed by silica gel column chromatography with the mixed eluent of chloroform and methanol (20

: 1) afforded 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (**3**; 0.35 g, 0.99 mmol) in 90% yield as a colorless crystal; Molecular equation: $C_{11}H_{13}Br_2OP$; M.W.: 352; TLC: $R_f = 0.23$ ($CHCl_3$:MeOH = 20 : 1); MS (*m/z*), 351.5 (MH^+, 60), 353.5 (MH^+, isptope peak, 100), 355.5 (MH^+, isptope peak, 40); ^{1}H-NMR ($CDCl_3$, 300 MHz), $\delta = 2.17$ (s, 3H, C3-CH_3), 2.44–2.58 (m, 2H, C5), 2.60–2.83 (m, 2H, C4), 4.67 (dd, $J = 1.5$ Hz, $J = 7.2$ Hz, 1H, C2), 7.32–7.56 (m, 5H, Ph).

Synthesis of 2,3,4-tribromo-3-methyl-1-phenylphospholane 1-oxide (TBMPP (4))

Similarly, the reaction of 4-bromo-3-methyl-1-phenyl-2-phospholene 1-oxide (**2**: 0.16 g, 6.0 mmol; 1.0 eq.) with bromine (1.2 mL; 6.6 g, 41 mmol; 6.8 eq.) in CCl_4 under reflux temperature for 24 h and work-up procedure afforded TBMPP (1.7 g, 4.0 mmol) in 69% yield; Molecular equation: $C_{11}H_{12}Br_3OP$; M.W.: 431; TLC: $R_f = 0.62$ ($CHCl_3$: MeOH = 20 : 1); Mp: 142–144 °C; MS (*m/z*): 429.0 (MH^+, 45), 431.1 (MH^+, isptope, 100), 433.1 (MH^+, isptope, 95), 435.1 (MH^+, isptope, 30); ^{1}H-NMR ($CDCl_3$, 300 MHz), $\delta = 2.14$ (s, 3H, C3-CH_3), 2.73–2.90 (m, 1H, C5), 3.04–3.14 (m, 1H C5), 4.26 (d, $J =$ 1.8 Hz,1H, 4C), 4.68–4.86 (m, 1H, C2), 7.52–7.79 (m, 5H, Ph).

Synthesis of 2-bromo-3-hydroxy-3-methyl-1-phenylphospholane 1-oxide (5)

The reaction of 3-methyl-1-phenyl-2-phospholene 1-oxide (**1**: 0.24 g, 1.3 mmol; 1.0 eq.) with bromine (0.5 mL; 2.7 g, 17 mmol; 13 eq.) in the mixed solvent of $CHCl_3$ (2 mL) and water (8 mL (340 eq.) of diluted sodium hydroxide aqueous solution) under stirring for 3 d at room temperature was progressed. The reaction mixture was treated with saturated sodium hydrogensulfite solution and the product was extracted with $CHCl_3$. The extract was washed with saturated sodium hydrogencarbonate solution, brine, and water, and then the solution of the product was dried over anhydrous Na_2SO_4. Evaporation of the solvent followed by silica gel column chromatography with the mixed eluent of chloroform and methanol (20 : 1) afforded product **5** (0.20 g, 0.69 mmol) in 55% yield; Molecular equation $C_{11}H_{14}BrO_2P$; M.W.: 289; TLC: $R_f = 0.45$ ($CHCl_3$: MeOH = 20 : 1); MS (*m/z*): 289.5 (MH^+), 291.5 (MH^+, isptope); ^{1}H-NMR ($CDCl_3$, 300 MHz): $\delta = 1.69$ (s, 3H, C3-CH_3), 2.01–2.51 (m, 2H, C4, C5), 4.32 (m, 1H, C2), 7.48–7.71 (m, 5H, Ph).

Synthesis of 2-bromo-3-(2-hydroxyethoxy)-3-methyl-1-phenylphospholane 1-oxide (6)

The $CHCl_3$ (2 mL) solution of the mixture of 3-methyl-1-phenyl-2-phospholene 1-oxide (**1**: 0.050 g, 0.26 mmol; 1.0 eq.), ethylene glycol (3 mL, 3.3 g, 53 mmol; 200 eq.), and bromine (0.3 mL; 1.6 g, 10 mmol; 39 eq.) was stirred for 2 d at room temperature. The work-up procedure of the reaction mixture by treating with saturated sodium hydrogensulfite and washing with sodium hydrogencarbonate, brine, and water followed by drying over anhydrous Na_2SO_4 and removal of the solvent, and chromatography on silica gel (eluent: $CHCl_3$: MeOH = 20; 1) afforded product **6** (0.077 g, 0.23 mmol) in a yield of 88%; Molecular equation: $C_{13}H_{18}BrO_3P$; M.W.: 333; TLC: $R_f = 0.15$ ($CHCl_3$: MeOH = 20 : 1);

MS (*m/z*): 333.1 (MH^+, 100), 335.1 (MH^+, isptope, 85); ^{1}H-NMR ($CDCl_3$, 300 MHz), $\delta =$ 1.57 (s, 3H, C3-CH_3), 2.10–2.31 (m, 2H, C5), 2.40–2.44 (m, 2H, C4), 3.63–3.65 (m, 2H, -CH_2OH), 3.82–3.85 (m, 2H, -OCH_2-), 4.19 (s, 1H, -OH), 7.50–7.82 (m, 5H, Ph)

Synthesis of 2-bromo-3-(2-(2-hydroxyethoxy)-ethoxy)-3-methyl-1-phenylphospholane 1-oxide (7)

Similarly, the reaction of 3-methyl-1-phenyl-2-phospholene 1-oxide (**1**: 0.050 g, 0.26 mmol; 1.0 eq.) with bromine (0.5 mL; 1.6 g, 10 mmol; 38 eq.) in diethylene glycole (3 mL; 3.3 g, 31 mmol; 120 eq.) for 2 d at room temperature under stirring and the work-up procedure followed by silica gel column chromatography (eluent: $CHCl_3$: MeOH = 20 : 1) afforded product **7** (0.055 g, 0.15 mmol) in 58% yield; Molecular equation $C_{15}H_{22}BrO_4P$; M.W.: 377; TLC: $R_f = 0.15$ ($CHCl_3$: MeOH = 20 : 1); MS (*m/z*): 377.3 (MH^+, 100), 379.3 (MH^+, isptope, 90); ^{1}H-NMR ($CDCl_3$, 300 MHz), $\delta =$ 1.53 (s, 3H, C3-CH_3), 2.07–2.13 (m, 2H, C5), 2.34–2.53 (m, 2H, C4), 3.84 (s, 1H, OH), 3.59–3.84 (m, 8H, -CH_2CH_2-), 4.15–4.17 (m, 1H, C2), 7.46–7.77 (m, 5H, Ph)

Synthesis of 2-Imidazolyl-3-methyl-1-phenyl-2-phospholane 1-oxide (8)

To an acetonitril (3 mL) solution of 4-bromo-3-methyl-1-phenyl-2-phospholene 1-oxide (**2**: 0.050 g, 0.18 mmol; 1.0 eq.) was added imidazole (0.062 g, 0.91 mmol; 5.1 eq.) and the mixture was stirred for 24 h at 60°C. The reaction mixture was worked-up by addition of $CHCl_3$ followed by washing with saturated sodium hydrogencarbonate solution, brine, and water, and then dried over anhydrous Na_2SO_4 followed by removal of the solvent to afford crude product **8**, whose chromatography on silica gel with an eluent of mixed solvent ($CHCl_3$: MeOH = 20 : 1) afforded product **8** (0.042 g, 0.16 mmol) in a yield of 89%; Molecular equation: $C_{14}H_{15}N_2OP$; M.W.: 258; TLC: $R_f = 0.2$ ($CHCl_3$: MeOH = 20 : 1); MS (*m/z*): 259.5 (MH^+, 100); ^{1}H-NMR ($CDCl_3$, 300 MHz), $\delta = 1.88$ (s, 3H, C3-CH_3), 2.36–2.49 (m, 1H, C5), (2.85–2.95 (m, 1H, C5), 5.16(s, 1H, C2), 6.31 (d, $J = 21.6$ Hz, im-C4-H), 7.13 (d, $J = 20.9$ Hz, im-C5-H), 7.52–7.72 (m, 5H, Ph), 7.63 (s, 1H, im-C2-H)

Evaluations and Analyses

MTT In Vitro Evaluation for Phospha Sugars Against Leukemic Cells[13]

Antitumor activities of phospha sugars **2–8** prepared were evaluated by MTT method against K562 (human chronic myelogenous leukemia) and U937 (human acute myelogenous leukemia) cell lines as well as HL60, NB4, YRK2, NOMO-1, CEM, MOLT4, SUP-B15, MEG-01, and SHG3 cell lines. Cells were seeded in 96-well flat-bottomed microplates at a density of 5×10^4 per well and incubated with various concentrations of phospha sugar derivatives for antitumor activity assay or without any phospha sugars for control experiments, for 48 h at 37°C, and then 10 μL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added to each well at the final concentration of 1.0 μg/mL/well. After incubation for 24 h or 48 h at 37°C, absorbance was measured at a wavelength of 560 nm by using a microplate reader for the *in vitro* evaluation.

Cell Cycle Analyses[13]

Propidium iodide (PI) (Sigma) staining was used to analyze cellular DNA content. Cells treated with Imatinib mesylate (Glivec, Gleevec) (1 μM) or phospha sugars DBMPP or TBMPP were cultured at 37°C in 2 mL of complete medium containing 1×10^6 cells. After incubation for 24 h or 48 h, the cells were washed twice with cold PBS, fixed with 70% ethanol overnight before treatment with 100 μg/mL RNase A, and then stained with 50 μg/mL of PI. For apoptosis analysis, the relative DNA content per cell was measured by flow cytometry using an Epics Elite flow cytometer (Coulter Immunotech). 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).

Western Blot Analyses[13,14]

For Phospha Sugar Treated Leukemia Cells. Leukemia cells treated with phospha sugars (DBMPP or TBMPP) were harvested, and then 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) 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). The membranes were then blocked with 0.5% milk in PBS for 1 h at room temperature. After being washed with 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.) 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 blot analysis: rabbit polyclonal anti-FoxM1 antibody (MPP2 K-19, 1:500) (Santa Cruz Biotechnology, Inc.), 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.), rabbit polyclonal anti-Aurora-B antibody (1:500) (ABGENT, Inc.), mouse monoclonal anti-bcl-2 antibody (BD Biosciences Pharmingen), mouse monoclonal anti-caspase-9 antibody (BD Biosciences Pharmingen), mouse monoclonal anticaspase-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 by using a mouse monoclonal antiactin antibody (C-4; ICN Biomedicals, Inc., Aurora, OH) as an internal control.

For Transfected Cells. Cells transfected with scrambled shRNA or with IER5 shRNA-#1 or -#2 were harvested. After 3 d, Western blot analysis was performed using the following antibodies: goat polyclonal anti-IER5 (Abcam), rabbit polyclonal anti-Cdc25B (Santa Cruz), anti-CHK1 (Santa Cruz), anti-WEE1 (Santa Cruz), anti-Aurora-B (Santa Cruz), mouse monoclonal anti-Cyclin B1 (Santa Cruz), and anti-Survivin (Santa Cruz). To ensure equal protein volume loading, similar experiments were performed by using a mouse monoclonal antiactin antibody (C-4; ICN Biomedicals, Inc., Aurora, OH) as an internal control.

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