Research and Development of Phospha Sugar Anti-cancer Agents with Anti-leukemic Activity

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ABSTRACT:

We have synthesized three deoxybromophospha sugar analogues, 4-bromo-3-methyl-1-phenyl-2-phospholene 1-oxide (MBMPP (**2**)), 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP (**3**)), and 2,3,4-tribromo-3-methyl-1-phenylphospholane 1-oxide (TBMPP (**4**)), by the reaction of 3-methyl-1-phenyl-2-phospholene 1-oxide (**1b**) and/or **2** with bromine, and investigated their potentials as anti-leukemic agents against human leukemia cell lines of K562 and U937. Cells' growth inhibition was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) *in vitro* assay. All agents showed inhibitory effects on leukemia cell proliferation, indicating that inhibition appeared to be dependent on number of bromine substituent in the heterocyclic structure. Further, the phospha sugar derivatives did not show any inhibitory effects on normal cell proliferation. These agents may facilitate the development of new strategies in molecular targeting anti-leukemic therapy.

Keywords: Phospha sugar, Phospholanes, Anti-cancer agent, Tumor, Leukemia cell lines, MTT *in vitro* evaluation.

INTRODUCTION

Since the finding that 3'-azido-3'-deoxythymidine (AZT) is a chemotherapeutically effective nucleoside for the treatment of acquired immunodeficiency syndrome (AIDS) /1/, many 2,3-dideoxyribonucleosides have been synthesized and tested with the aim to develop more selective agents against human immunodeficiency virus (HIV) /2/. Most of the nucleoside analogues so far synthesized in this context are modified either at the nucleobase moiety or the 5'-position of AZT. The present aim is synthesis of novel phospha sugar analogues to search potentially bioactive and useful phospha sugars or heterocycles, having a phosphorus moiety in place of the ring oxygen of normal sugars.

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

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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 have previously synthesized pentofuranose analogues of phospha sugars from phospholenes as potentially bioactive agents which structurally resemble AZT or ribavirin /3/. Phospha sugars are analogues of normal sugars in which the central oxygen atom of the hemiacetal ring has been replaced by a phosphorus atom moiety. Replacement of the oxygen atom in the hemiacetal ring of normal sugars by a carbon or a heteroatom leads to the formation of pseudo sugars, several of which have been heavily investigated in the fields of synthetic, biological, and medical chemistry /4/. Well known typical pseudo sugars are *carba*-, *aza*-, and *thia*-sugars, with a carbon, nitrogen, and sulphur atom, respectively, instead of the oxygen atom in the hemiacetal ring of the normal sugars /4/. These pseudo sugars are known to exist in nature and are also prepared by synthetic sugar chemistry. Pseudo sugars exert important biological activities, therefore, many studies on the isolation, synthesis, characterization, etc., are actively performed. On the other hand, phospha sugars, one new category of the pseudo sugars which have a phosphorus atom in the hemiacetal ring of sugars, are not yet found in nature and the synthesis of them are rather difficult compared with the typical pseudo sugars /1-5/.

Novel nucleoside derivatives of pseudo or hetero sugars reported to date include *aza*-sugars (or amino sugars: nitrogen instead of an oxygen atom) /6,7/, *thia*-sugars (or *thio*-sugar: sulphur instead of an oxygen atom) /8/, and *carba*-sugar (oxygen atom replaced by a methylene group) /9/. Further, the potential bioactivities of hetero-sugar nucleosides and glycosides have also been reported /10/.

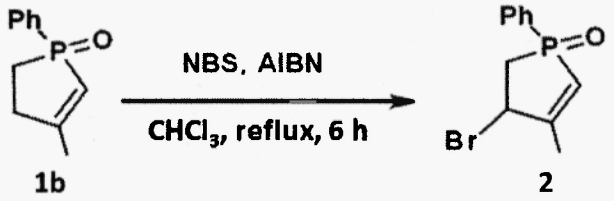
Given this potential for bioactivity, phospha sugar chemistry is one of the most rapidly developing areas of research /11/. One report has suggested that acetyl derivatives of the glucopyranose pattern of phospha sugar have potential as anti-cancer agents /12/. *Carba*-sugar derivatives are known to be effective in hampering some enzyme activity /9/. 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/. Based on this background, we have synthesized 4-bromo-3-methyl-1-phenyl-2-phospholene 1-oxide (MBMPP (**2**)), 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP (**3**)), and 2,3,4-tribromo-3-methyl-1-phenylphospholane 1-oxide (TBMPP (**4**)) in this paper.

Acute myeloid leukemia (AML) is the most common type of blood cancer in adults. If untreated, this form of leukemia usually progresses quickly. In a healthy person, bone marrow makes the blood stem cells that mature into infection-fighting white blood cells, oxygen-carrying red blood cells, and blood-clotting platelets. When a person has AML, cells called myeloid stem cells usually develop instead into a type of immature white blood cell called myeloblasts, which never go on to become healthy, infection-fighting white blood cells. 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 /15/. 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 remains to be defined.

We have been searching biologically active phospha sugars and/or phosphorus heterocycles, and we have first found new anti-tumor phospha sugar analogues by using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) *in vitro* evaluation methods for some of these phospha sugars or phosphorus heterocycles against leukemia cells /6/. In the present study, we have investigated not only the syntheses but also the anti-leukemic effect of the deoxybromophospha sugar derivatives of MBMPP (**2**), DBMPP (**3**), and TBMPP (**4**) in regulating proliferation against human leukemia cell lines of K562 and U937. And we will deal with the synthesis and the *in vitro* evaluation of these phosphorus heterocycles or phospha sugars. From the results of the research we may say that dibromide DBMPP (**3**) and/or tribromide TBMPP (**4**) may lead to develop a new type of carcinostatic agents being useful in tumor oncostasis.

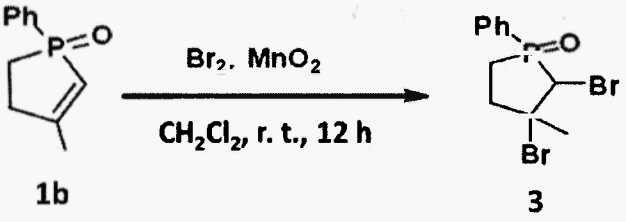
RESULTS AND DISCUSSION

Deoxybromophospha sugars or bromophospholene and bromophospholane derivatives MBMPP (**2**), DBMPP (**3**), and TBMPP (**4**) were prepared from 3-methyl-1-phenyl-2-phospholene 1-oxide (**1b**) by substitution and/or addition reaction of bromo radical and/or bromine in the presence of catalyst (**Schemes 1-3**).



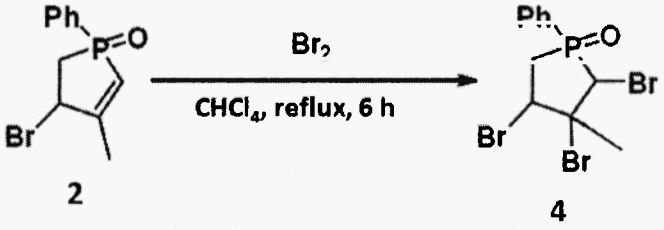
Scheme 1

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



Scheme 2

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



Scheme 3

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

Little is known about the stereoselectivity of addition reaction to the 2,3-double bond and allylic substitution reaction of 2-phospholene derivatives. *Cis*-geminal dihydroxylation of 1-phenyl-2-phospholene 1-oxide with OsO_4 affords *anti*-configuration of C(2)OH against P=O caused by electronic repulsion of P=O oxygen with OsO_4 oxygen /16/. The stereochemistry of addition reaction of OH^- group to the formed bromonium intermediate proceeds from less hindered side to give mainly *erythro* bromohydrin of phospholane /17-19/. However, the stereoselectivity of addition of bromine to the 2,3-double bond and bromo radical replacement of allylic hydrogen of $C(4)H_2$ of the 2-phospholenes are less known. Therefore, the stereochemical research on the introduced bromo group is still under way, nevertheless, to search higher anti-cancer activity and to optimize the structure as the novel anti-tumor drug by evaluating phospha sugar analogues are required.

These deoxybromophospha sugars or bromosubstituted phosphorus heterocycles are evaluated by MTT *in vitro* method for anti-cancer agents against human leukemia cell lines. As shown in **Figure 1**, human leukemia K562 cells

were inhibited the proliferation by MBMPP (**2**), DBMPP (**3**), TBMPP (**4**), and Gleevec® (imatinib mesylate) in dose dependent manner. Results showed that the effect of MBMPP (**2**) was > 30 μM, DBMPP (**3**) was 28.0 ± 1.7 μM, and TBMPP (**4**) was 9.1 ± 0.8 μM with regard to 50% cell growth inhibition (IC_{50}) value. Data are shown as mean ± S.D. in triplicate culture and are representative of three independent experiments. Gleevec® is a unique treatment for certain forms of cancer. It works by targeting, and turning off, specific proteins in cancer cells that cause the cancer cells to grow and multiply. Gleevec® of low dose showed inhibition of 40% cell viability. But Gleevec® of high dose has no effect on the cell viability any more.

Interestingly, the strength of the effect depended on the number of bromine substituent introduced into the phospha sugar molecules was first observed for phospha sugars. The effect of the number of the bromine substituent in isatin derivatives, which induce apoptosis, on anti-cancer activity is reported /20/.

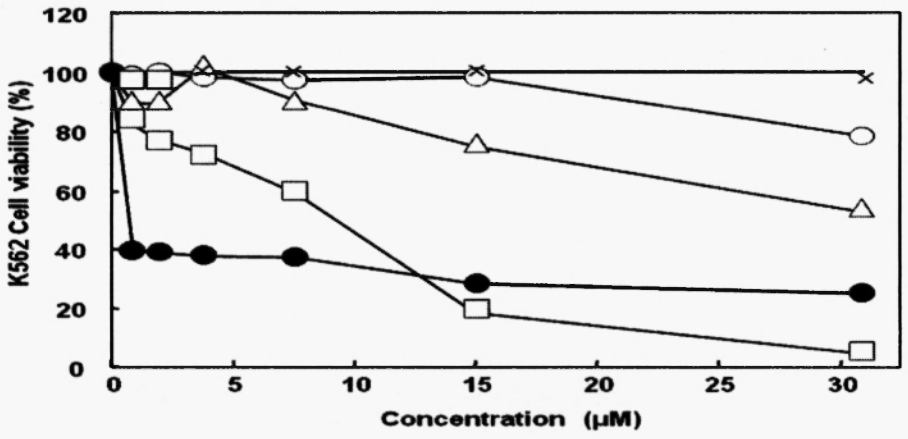


Fig. 1: Effect of MBMPP (**2**), DBMPP (**3**), TBMPP (**4**), and Gleevec® on inhibition of human leukemia cells (K562) proliferation measured by MTT assay: K562 cells were shown being untreated (×), treated with **2** (○), treated with **3** (△), treated with **4** (□), and treated with Gleevec® (●) at the indicated concentration.

As shown in **Figure 2**, human leukemia U937 cells were inhibited the proliferation by MBMPP (**2**), DBMPP (**3**), and TBMPP (**4**), but were not so effectively inhibited by Gleevec® in dose dependent manner. The decrease of cell viability for U937 cells was observed for MBMPP (**2**), DBMPP (**3**), and TBMPP (**4**) at the low concentration of 62.5, 7.5, and 1.85 μM, respectively. They are much more active against the U937 cell lines than Gleevec®. Results showed that the effect of MBMPP (**2**) was 91.5 ± 3.0 μM, DBMPP (**3**) was 22.0 ± 1.8 μM, and TBMPP (**4**) was 6.2 ± 1.1 μM with regard to IC_{50} value. Data are shown as mean ± S.D. in triplicate culture and are representative of three independent experiments. Gleevec® has almost no effect against U937 cells viability because of its narrow spectral character. Interestingly, these findings strongly indicate that the phospha sugars must be quite active anti-tumor agents against wide spectra of tumor cell lines, and the strength of the effect is depended on the number of bromine substituent introduced into the molecules.

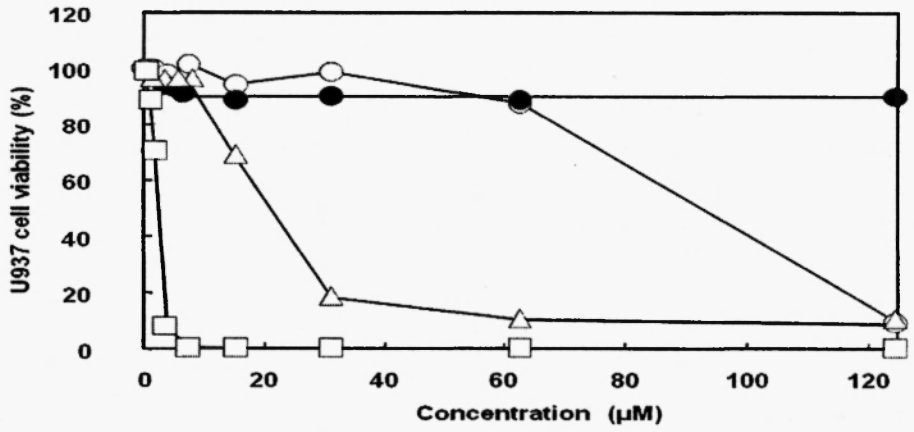


Fig. 2: Effect of MBMPP (**2**), DBMPP (**3**), TBMPP (**4**), and Gleevec® on inhibition of human leukemia cells (U937) proliferation measured by MTT assay: U937 cells were shown being untreated (×), treated with **2** (○), treated with **3** (△), treated with **4** (□), and treated with Gleevec® (●) at the indicated concentration.

Because of no reports being appeared about the toxicity of halogen derivatives of phospha sugars, we have investigated the toxicology effect of the phospha sugar derivatives of DBMPP (**3**) in regulating proliferation by human normal cell only (Blast 0%) and mixture of 60% of human normal cell and 40% K562 cells (Blast 40%) (**Figure 3**). Against the human normal cells (blast 0%) of the leukocytes, there seems to have no effect with DBMPP (**3**) at all on the cell viability by the evaluation with MTT *in vitro* assay method. While in mixed cells (Blast 40%), there was the effect of anti-leukemic activity with DBMPP on the cell viability by 40%, which corresponded with the percentage of K562 cells. The data clearly shows that the phospha sugar derivatives will not give serious damages against normal cell growth and will not kill normal cells, nevertheless, the phospha sugars seem to be quite effective against leukemic cells selectively and specifically. The important results may indicate that the phospha sugar will not have any toxicology against normal cells.

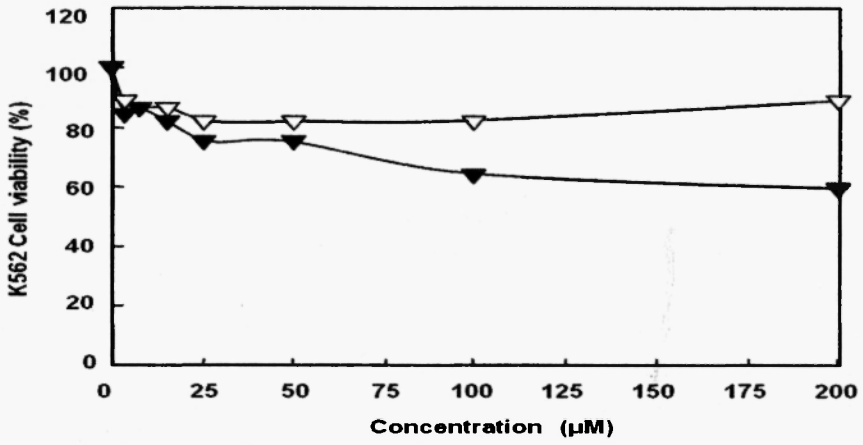


Fig. 3: Effect of DBMPP (**3**) on inhibition of human normal cells and leukemia cells (K562) proliferation measured by MTT assay: Human normal cells (Blast 0%) being treated with **3** (▽) and the mixture of 60% of human normal cells and 40% of K562 (Blast 40%) treated with **3** (▼) were shown at the indicated concentration.

We have been searching biological activity of phospha sugar and/or phosphorus heterocycles, and we have first found new anti-tumor phospha sugar analogues by using MTT *in vitro* evaluation methods for some of these phospha sugars or phosphorus heterocycles (MBMPP (**2**), DBMPP (**3**), and TBMPP (**4**)) against human leukemia cell lines, whose detailed mechanism is now under the way of research and will be published separately. Advanced research on the preparation and optimization of the novel anti-cancer agents as well as the studies on separation of the stereoisomers and stereoselective and stereospecific preparation are now actively in progress.

EXPERIMENTAL SECTION

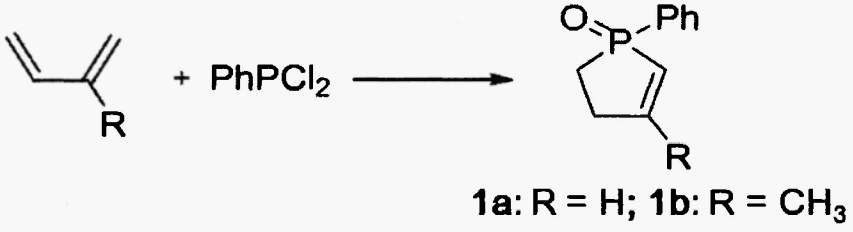
General Procedures and Methods

TLC (Silica gel Wako Chromato Sheet and/or Merk Kieselgel 60; Eluent: $CHCl_3$: MeOH= 20:1); Column chromatography (Silica gel (Wakogel C-200 (75-150 μm)) and/or Activated alumina (Wako 45-150 μm); 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 (*m/z*)); IR (JASCO FT/IR: 410 (KBr), cm^{-1}); ^{1}H-NMR (JEOL JNM-AL300 (300 MHz) ; Solvent: $CDCl_3$, δ (ppm)) were used for separation and analyses of the products.

Chemicals and Chemical Synthesis

*1-Phenyl-2-phospholene 1-oxide (**1a**; R= H) and 3-methyl-1-phenyl-2-phospholene 1-oxide (**1b**; R= CH_3)*:

McCormac reaction prepares 2- or 3-phospholene derivatives from 1,3-dienes and phosphorus(III) halides. 1-Phenyl-2-phospholene 1-oxide (**1a**) and 3-methyl-1-phenyl-2-phospholene 1-oxide (**1b**) were prepared by the McCormac reaction /21/ of 1,3-butadiene and 2-methyl-1,3-butadiene with phenylphosphonous dichloride (**Scheme 4**) in 16% and 68% yield, respectively, and the prepared **1b** was used for the synthesis of **2**, **3**, and **4**.



Scheme 4
Synthesis of 2-phospholene 1-oxides **1** (McCormac Reaction).

Data for 1-phenyl-2-phospholene 1-oxide (**1a**; R = H; Registry number: 703-03-7): Formula: $C_{10}H_{11}OP$; Mol. Wt.: 178.17; Yield 16%; bp 145-152 °C (0.08 mmHg); R_f = 0.38 ($CHCl_3$: MeOH = 20 : 1); HPLC (Wakosil 5SIL, $CHCl_3$/MeOH = 20:1, flow rate 0.5 mL/min, λ = 254 nm): t_R = 10.88 min; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ = 2.12-2.21 (m, 2H, C(5)H_2), 2.78-2.94 (dd, 2H, C(4)H_2), 6.25-6.36 (dt, 1H, C(3)H), 7.05-7.23 (dt, 1H, C(2)H), 7.47-7.50 (m, 3H, *m,p*-Ph), 7.64-7.71 (m, 2H, *o*-Ph); MALDI-TOF-MS (*m/z*): 179.6 (MH^+, 100).

Data for 3-methyl-1-phenyl-2-phospholene 1-oxide (**1b**; R = Me; Registry number: 707-61-9): Formula: $C_{11}H_{13}OP$; Mol. Wt.: 192.19; Yield 68%; bp 148-161 °C (0.10 mmHg); R_f = 0.32 ($CHCl_3$: MeOH = 20 : 1); HPLC (Wakosil 5SIL, $CHCl_3$/MeOH = 20:1, flow rate 0.5 mL/min, λ = 254 nm): t_R = 10.06 min; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ = 2.08 (s, 3H, CH_3), 2.17-2.29 (m, 2H, C(5)H_2), 2.59-2.83 (dd, 2H, C(4)H_2), 5.90-5.99 (dd, 1H, C(2)H), 7.43-7.71 (m, 5H, Ph); MALDI-TOF-MS (*m/z*): 193.7 (MH^+, 100).

4-Bromo-3-methyl-1-phenyl-2-phospholene 1-oxide (MBMPP (2)):

Under an Ar atmosphere, **1b** (192 mg, 1.00 mmol, 1.0 eq) was dissolved in chloroform ($CHCl_3$, 3.0 mL), and then *N*-bromosuccinimide (NBS; 214 mg, 1.20 mmol, 1.2 eq) was added to the substrate solution and heated at 50 °C. To the mixture was added azobisisobutyronitrile (AIBN; 24.6 mg, 0.15 mmol, 0.15 eq) and the reaction mixture was refluxed for 6 h. After the reaction, the reaction mixture was cooled, diluted with $CHCl_3$, and vacuum filtered. The filterate was collected, washed by 10% Na_2SO_3 solution, water, and brine, and then dried over anhydrous Na_2SO_4. Evaporation of the solvent followed by purification through silica gel column with an eluent ($CHCl_3$: MeOH = 20:1) afforded MBMPP (**2**; 177 mg, 0.65 mmol) in 65% yield.

Data for MBMPP (**2**): Formula: $C_{11}H_{12}BrOP$; Mol. Wt.: 271.09; TLC: R_f = 0.42 ($CHCl_3$: MeOH = 20 : 1); HPLC (Wakosil 5SIL, $CHCl_3$: MeOH = 20:1, flow rate 0.5 mL/min, λ = 254 nm): t_R = 8.23 min; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ = 2.22 (s, 3H, CH_3), 2.65-3.10 (m, 2H, C(5)H_2), 4.96-5.17 (dd, 1H, C(4)H), 5.91-6.38 (dd, 1H, C(2)H), 7.49-7.87 (m, 5H, Ph); MALDI-TOF-MS (*m/z*): 271.4 (MH^+, 100), 273.3 (MH^+, 75).

2,3-Dibromo-3-methyl-1-phenylphospholane 1-oxide (DBMPP (3)) :

2-Phospholene 1-oxide **1b** (192 mg, 1.00 mmol, 1.0 eq) was dissolved in CH_2Cl_2 (10 mL), and then MnO_2 (174 mg, 2.00 mmol) was added to the substrate solution, which was followed by slow addition of CH_2Cl_2 solution of bromine (50.2 μL; 2.00 mmol, 2.0 eq) and kept stirring at room temperature for 12 h. The reaction mixture was diluted with CH_2Cl_2 and vacuum filtered. The filterate was collected, washed with 10% Na_2SO_3 solution, water, and brine, and then dried over anhydrous Na_2SO_4. Evaporation of the solvent followed by purification through silica gel column with eluents ($CHCl_3$: MeOH = 30:1 – 15:1) afforded DBMPP (**3**; 195 mg, 0.558 mmol) in 56% yield.

Data for DBMPP (**3**): Formula: $C_{11}H_{13}Br_2OP$; Mol. Wt.: 352.00; TLC: R_f = 0.23 ($CHCl_3$: MeOH = 20 : 1); HPLC (Wakosil 5SIL, $CHCl_3$: MeOH = 20:1, flow rate 0.5 mL/min, λ = 254 nm): t_R = 6.558 min; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ = 1.24-1.31 (m, 2H, P-C(5)H_2), 1.73-2.17 (m, 2H, C(4)H_2), 2.17 (s, 3H, CH_3), 4.15-4.23 (m, 1H, C(2)H), 7.51-7.75 (m, 5H, Ph); MALDI-TOF-MS (*m/z*): 350, 352, and 354 (MH^+, isotope peaks (1 : 2: 1), 100).

2,3,4-Tribromo-3-methyl-1-phenylphospholane 1-oxide (TBMPP (4)):

To the carbon tetrachloride (CCl_4, 15 mL) solution of MBMPP (**2**; 2.0 mmol) heated to over 75 °C was added bromine (0.40 mL, 8.0 mmol, 4.0 eq) in CCl_4. After stirring the reaction mixture at 80 °C for 8 h to compete the reaction, the reaction mixture was cooled, diluted with $CHCl_3$ (30 mL), washed with 10% Na_2SO_3 solution, water, and brine, and then dried over anhydrous Na_2SO_4, filtered, evapolated, and chromatographed on silica gel to give TBMPP (**4**; 1.0 mmol) in 50% yield.

Data for TBMPP (**4**): Formula: $C_{11}H_{12}Br_3OP$; Mol. Wt.: 430.90; MALDI-TOF-MS (*m/z*): 351.5 and 353.5 (MH^+ - Br, isotope peaks (1 : 2: 1), 100); 429.4, 431.4, 433.4, and 435.4 (MH^+, isotope peaks (1 : 3 : 3: 1), 30).

Cell lines and Cell Culture

Human chronic myeloid leukemia cell lines (K562), human monocytic cell lines (U937), human gastric cancer cell lines (MKN45) were purchased from American Type Culture Collection (ATCC) (Rockville, MD, USA). These Cells were maintained in plastic tissue culture flasks containing RPMI1640 medium (Sigma Co.,) supplemented with 10% heat-inactivated fetal calf serum (FCS) 2 mmol/L L-glutamine, 100 mg/L streptomycin and 200 U/mL penicillin (GIBCO BRL. Rockville, MD, USA). Cells were incubated in a humidified atmosphere of 95% air / 5% CO_2 at 37 °C.

MTT cell proliferation assay

For the MTT assay, the cells were seeded in 96-well flat-bottomed microtiter-plate at a density of 5 × 10^4 cells per well. Cells were incubated with or without MBMPP (**2**), DBMPP (**3**), or TBMPP (**4**) for 72 h (including 24 h pre-incubation) at 37 °C. Bromide **2**, **3**, or **4** was dissolved in dimethylsulfoxide (DMSO) (Sigma Co., St. Louis, Mo., USA),

and was diluted into appropriate concentration with DMSO in culture medium immediately before use. The final concentrations of **2**, **3**, and **4** in DMSO in all experiments were less than 0.01%, and all the treatment conditions were compared with vehicle control. After incubation for 72 h including pre-incubation at 37 °C, 10 μL of 3-(4,5-dimethylthiaxol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) solution (Sigma Co., Ltd.) was added to each well at a final concentration of 1 mg/mL. After incubation with MTT at 37 °C for 4 h, absorbance was measured at a wavelength of 560 nm using microtiter-plate reader. Cells' grown in complete medium alone were used as controls.

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