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Novel Molecular Targeted and Wide Spectrum Antitumor Agents: Preparation and Preclinical Evaluation of IER5/Cdc25B Targeted Low-molecular-weight Phospha Sugar Derivatives

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Novel broad spectrum low-molecular-weight antitumor agents of phospha sugar derivatives were investigated and developed. Branched deoxybromophospha sugars, such as DBMPP and TBMPP, which target IER5/Cdc25B and innovate in chemo-therapeutic treatments against various type of cancer cells. These successful preclinical *in vitro* and *in vivo* evaluations observed might be expected to afford clinically useful drugs and innovative medical treatments for various kinds of cancers.

1. Introduction

It is important to treat as well as to diagnose cancer more efficiently and safely than the present clinical technologies for both medical reasons and societal needs at the earlier stage of tumors by R and D on novel medical materials. We have been very much interested in chemistry and biological activities of carbohydrates and phosphorus compounds not only on the basis of medicinal chemistry but also as an opportunity for improved treatments for cancer patients as well as diagnosing cancer tissues. In this research we will deal with phospha sugar antitumor agents to provide innovative cancer therapy technologies.

2. Experimental

2.1 General

TLC (Silica gel: Wako Chromato Sheet and/or Merk Kieselgel 60; Eluent: $CHCl_3$: MeOH = 20 : 1, Rf value); 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 (JASCO FT/IR 410 (KBr), in cm^{-1}); and ^{1}H-NMR (JEOL JNM-AL300 (300 MHz); Solvent: $CDCl_3$, in δ (ppm) from TMS) were used for analyzing the products.

2.2 Materials

2.2.1 Starting material:

3-Methyl-1-phenyl-2-phospholene 1-oxide (Registry number: 707-61-9; *Rf* = 0.32 ($CHCl_3$: MeOH = 20 : 1); bp 148-161 °C (0.10 mmHg); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ = 2.08 (s, 1H, C3-CH_3), 2.17-2.29 (m, 2H, C5), 2.59-2.83 (dd, 2H, C4), 5.90-5.99 (dd, 1H, C2), 7.43-7.71 (m, 5H, Ph); MS (*m/z*): 193.7 (MH^+, 100); HPLC (Wakosil 5SIL, $CHCl_3$: MeOH = 20 : 1, flow rate 0.5 mL/min, λ = 254 nm); t_R = 10.06 min) was prepared and used as the starting material for the preparation of phospha sugar derivatives.

2.2.2 Preparation of 4-bromo-3-methyl-1-phenyl-2-phospholene 1-oxide (Phospha sugar MBMPP):

To a chloroform (3 ml) solution of 3-methyl-1-phenyl-2-phospholene 1-oxide (192 mg, 1.00 mmol, 1.0 eq.) and N-bromosuccinimide (NBS, 213.6 mg, 1.20 mmol, 1.2 eq.) was added dropwise a chloroform (3 ml) solution of 2,2'-azobisisobutyronitrile (AIBN, 24.6 mg, 0.15 mmol, 0.15 eq.) at 60 °C and the reaction mixture was refluxed for 6 h under an Ar atmosphere. The reaction mixture was neutralized with saturated $NaHCO_3$ aqueous solution (10 ml), washed with water (10 ml) and saturated NaCl solution (10 ml), and dried over with anhydrous sodium sulfate. The solvent of the filtrate was evaporated under a reduced pressure to give an oily residual material. The residue was purified by column chromatography on silica gel by using chloroform and methanol (20 : 1) as the eluent to give 4-bromo-3-methyl-1-phenyl-2-phospholene 1-oxide (176 mg, 0.65 mmol) in 65% yield; *Rf* = 0.42 ($CHCl_3$:MeOH = 20 : 1); MS (*m/z*), 284.0(M - H^+); ^{1}H-NMR ($CDCl_3$, 300 MHz), δ (ppm)= 2.22 (s, 1H, C3-CH_3), 2.65-3.10 (m, 2H, C5), 4.96-5.17 (dd, 1H, C4), 5.91-6.38 (dd, 1H, C2), 7.49-7.87(m, 5H, Ph).

2.2.3 Preparation of 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (Phospha sugar DBMPP):

To a mixture of 3-methyl-1-phenyl-2-phospholene 1-oxide (0.265 g, 1.38 mmol, 1.0 eq.) and manganese dioxide (0.239 g, 2.75 mmol, 2.0 eq.) in dichloromethane (5 ml) was added dropwise dichloromethane (5 ml) solution of bromine (0.4 ml, 7.8 mmol, 5.7 eq.), and the reaction was stirred for 12 h under an Ar atmosphere. After reduction of excess bromine with saturated sodium sulfite solution, then the reaction mixture was extracted with chloroform (10 ml x 3). The organic layer was neutralized with saturated $NaHCO_3$ aqueous solution (10 ml), washed with saturated NaCl solution (10 ml), and dried over with anhydrous sodium sulfate. After removal of the solvent under a reduced pressure, the residue was purified by column chromatography on silica gel using chloroform and methanol (30 : 1) as the eluent to give DBMPP (0.376 g, 1.07 mmol) in 78% yield; R_f = 0.54 ($CHCl_3$: MeOH = 30 : 1); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ = 2.09 (s, 3H, C3-CH_3), 2.44-2.58 (m, 2H, C5), 2.60-2.85 (m, 2H, C4), 4.66-4.68 (dd, 1H, C2), 7.49-7.80 (m, 5H, Ph); MS (*m/z*): 351.5 (MH^+, 60), 353.5 (MH^+, 100), 355.5 (MH^+, 40).

2.2.4 Preparation of 2,3,4-tribromo-3-methyl-1-phenyl-2-phospholene 1-oxide (Phospha sugar TBMPP):

To 4-bromo-3-methyl-1-phenyl-2-phospholene 1-oxide (0.751 g, 2.64 mmol) being dissolved in carbon tetrachloride (10 mL) was heated to over 75 °C and bromine (1.03 mL 20.0 mmol) in CCl_4 was added. After stirring the reaction mixture at 80 °C for 8 h, 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 to give TBMPP (0.250 g, 0.562 mmol) in 21% yield; MS (*m/z*): 429.4 (MH^+, 30), 431.4 (MH^+, 66), 433.4 (MH^+, 100)

2.3 Biology (MTT *in vitro* evaluation)

Phospha sugar derivatives (MBMPP, DBMPP, TBMPP, etc.) in the compound library were prepared and tested by the MTT method for antitumor activity against the K562 (human chronic myelogenous leukemia), U937 (human acute myelogenous leukemia), etc. cell lines. Cells were seeded in 24-well flat-bottomed microplates at a density of 3×10^4 per well and incubated at various concentrations of phospha sugar derivatives 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 phospha sugar derivatives 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.

3. Results and Discussion

Sugar derivatives, whose oxygen atom in the hemiacetal ring of Haworth projection is replaced by a carbon, nitrogen, or sulfur atom are called pseudo sugars (Fig. 1). Some pseudo sugars, such as carba, aza, and thia sugars, respectively, are analogues of naturally occurring or synthesized products, and are well investigated, and many of them are known to be biologically active compounds.

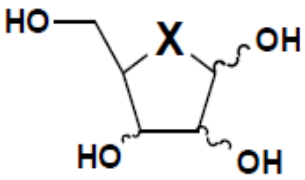


Fig. 1. Pseudo sugars : X=CH_2, NH, S, P(O)R, etc.

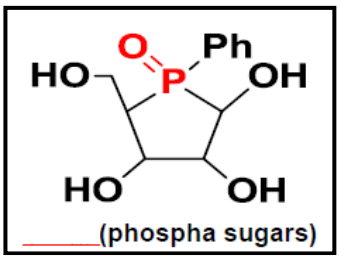


Fig. 2. An example of phospha sugar (pentofuranose).

Phospha sugars are also one category of the pseudo sugar analogues (Fig. 2), which have a phosphorus residue instead of the oxygen atom in the hemiacetal ring. However, phospha sugars are not yet found in nature, and the synthesis and the characterization of them are not yet so well studied in spite of their potentially important biological activities [1]. We have successfully synthesized various kinds of phospha sugar derivatives from heterocycles (using new synthetic methods) and/or carbohydrates (using more traditional synthetic methods for preparation of pseudo sugars) to construct a compound library [2], and then evaluated their biological activities [3].

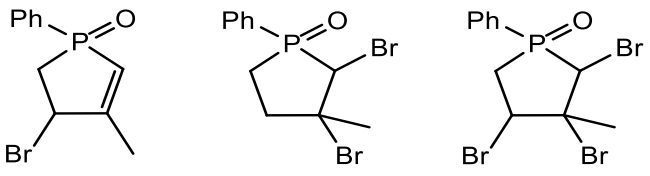
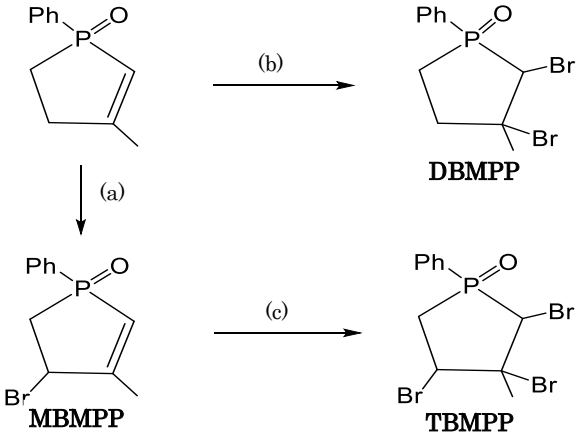


Fig. 3. Mono- (left: MBMPP), di- (middle: DBMPP), and tri-bromo- (right: TBMPP) deoxyphospha sugars.

We have found that deoxybromophospha sugar derivatives (Fig. 3 and Scheme), as well as some substituted phospha sugar analogues such as anhydrophospha sugar derivatives, exerted strong, selective, and tumor cell specific antitumor activities by MTT *in vitro* evaluation against various kinds of leukemia cells via apoptosis induction, with little or no damage to normal cells [4]. The phospha sugars DBMPP and TBMPP have excellent antitumor activities against various kinds of leukemia cells such as the K562 (Fig. 4) and U937 cell lines, as well as solid cancer cells such as stomach cancer, lung cancer, etc.



Scheme. Preparation of 4-bromo-3-methyl-1-phenyl-2-phospholene 1-oxide (mono-bromo-methyl-phenyl unsaturated phospha sugar (MBMPP)), 2,3-dibromo-3-methyl-1-phenylphospholane 1-oxide (dibromo-methyl-phenyl phospha sugar (DBMPP)), and 2,3,4-tribromo-3-methyl-1-phenylphospholane 1-oxide (tribromo-methyl-phenyl phospha sugar (TBMPP)) from 3-methyl-1-phenyl-2-phospholene 1-oxide. Reagents and reaction conditions: (a) NBS, AIBN, $CHCl_3$, reflux, 6h; (b) NBS, MnO_2, AIBN, $CHCl_3$, reflux, 6h; (c) Br_2, CCl_4, reflux, 6h.

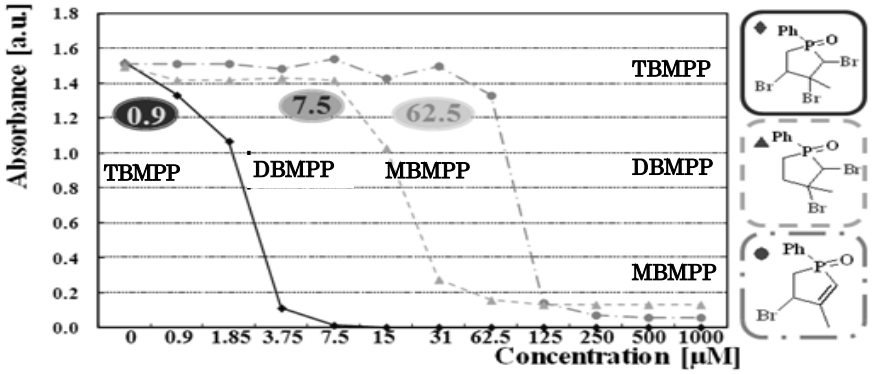


Fig. 4. MTT *in vitro* evaluation against K562 cells.

Mechanistic studies of the phospha sugar DBMPP against leukemia cells U937 by Western blotting showed that the phospha sugar suppresses the expression of "Accelerator Factors of Tumor Cells" such as FoxM1, KIS, Skp2, Aurora-B, etc. and accelerates the expression of "Suppressor Factors of Tumor Cells" such as $p27^{Kip1}$ and $p21^{Cip1}$. Together these effects, control tumor cell viability (Fig. 5).

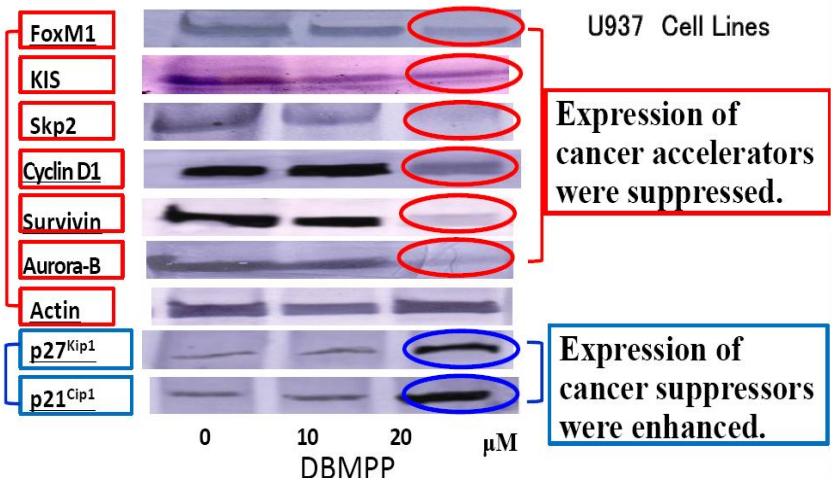


Fig. 5. Phospha sugar (DBMPP) suppresses the expression of "Accelerator Factors of Tumor Cells" and accelerates the expression of "Suppressor Factors of Tumor Cells", and thus controls tumor cell viability.

Further mechanistic studies of the phospha sugar TBMPP against leukemia cells by Western blotting showed that the phospha sugar enhanced the expression of IER5, and then suppressed the expression of Cdc25B. Cdc25B is an essential and common factor for the mitosis of tumor cells. Thus the present observations of mechanism imply that phospha sugar induces apoptosis at G2/M stage and inhibits the proliferation of various kinds of cancer cells (Fig. 6) [4, 5-7].

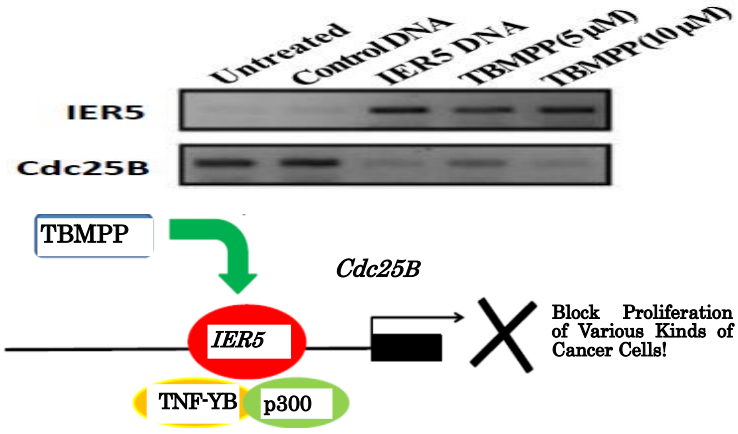


Fig. 6. Western blot analysis: Enhancement of IER5 expression and suppression of Cdc25B expression with phospha sugar TBMPP were observed.

The observed results regarding enhanced expression of IER5 and suppressed expression of Cdc25B by TBMPP as shown by Fig. 6 can explain why this phospha sugar exerts wide spectrum and selective as well as tumor cell specific antitumor activities against various kinds of tumor cells yet does not damage normal cells.

The *in vivo* evaluation with phospha sugar TBMPP was also performed successfully using a nude mouse transplanted K562 cells on the skin (Fig. 7).

4. Conclusion

Low-molecular-weight phospha sugar derivatives, e.g., DBMPP, TBMPP, etc., should be developed to be clinically useful. They provide novel and innovative, wide spectrum antitumor agents that target IER5/Cdc25B. With these innovative novel antitumor agents, as well as improved sugar dendritic Gd-DTPA complex MRI contrast agents [8], medical treatments for cancer patients will be improved. They may also be used to treat various kinds of tumors successfully with a single antitumor agent.

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