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The preparation of 2,6-disubstituted pyridinyl phosphine oxides as novel anti-cancer agents

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ABSTRACT

A series of 2,6-dimethoxylpyridinyl phosphine oxides have been synthesized and examined for their antitumor activity. 2,6-Dimethoxy-3-phenyl-4-diphenylphosphinoylpyridine **2** has been employed as the lead compound for this study. We found out that the presence of phosphine oxide on the 2,6-dimethoxylpyridine ring is important for the antitumor activity; the presence of bromine on this core leads to a further enhancement of its antitumor activity. This is the first reported work on the antitumor activity of the 2,6-dimethoxy-3,5-dibromopyridinyl phosphine oxide **5b** towards MDAMB-231 breast cancer and SKHep-1 hepatoma cell lines.

Only a few previous reports have been published on the preparation of phosphine oxides and on the characterization of their antitumor activity. Examples of studies reporting the antitumor activities of phosphine oxides are those reported by Babbitt et al. and Spasokova et al. Babbitt and co-workers reported the preparation of tris(2,2-dimethyl-1-aziridinyl) phosphine oxide (TEPA-132) for treatment of leukemia cells in 1967.[1] The Spasovska's group reported the synthesis of platinum(II), platinum(IV), and palladium(II) complexes of amino substituted phosphine oxides (dimethyl aminomethylphosphine oxide and methyl bis(aminomethyl)phosphine oxide in 1990.[2] The pyridinyl compounds have been reported to show biological activity. On the other hand, Kleeman and his researchers reported that the 2,6-disubstituted pyridines can function as herbicides.[3] The reported studies aroused our interest to explore the antitumor activities using simple pyridinyl phosphine oxides. To begin with our assumption, compound **1** was produced and test as reference. In consideration of the biological activity exhibited by phosphine oxide and 2,6-disubstituted pyridines, we develop herein pyridinyl phosphine oxides based on the lead compound 2,6-dimethoxy-3-phenyl-4-diphenylphosphinoylpyridine **2** (Fig. 1), and report the antitumor activities of the synthesized compounds towards MDAMB-231 breast cancer and SKHep-1 hepatoma cell lines, whereas the biological activities of this type of phosphine oxides had not been reported previously. In order to further probe with our assumptions, we employed our previously reported synthetic scheme[4] for making compounds. As a strategic consideration, we firstly started the present study with the easily available 2,6-dimethoxypyridine (**3**), which followed our patented route of making pyridinyl phosphine ligands. The presence of substitution (i.e. methoxyl group) at positions 2 and 6 can avoid the interference of the pyridinyl nitrogen in the subsequent compounds synthesis in the reported scheme.

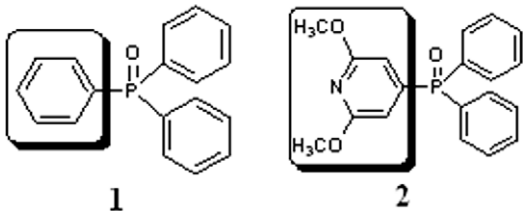

Figure 1. Chemical structures of triphenylphosphine oxides and 2,6-dimethoxy-3-phenyl-4-diphenylphosphinoylpyridine.

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Using the commercially available compound 2,6-dimethoxypyridine (**3**) as a starting material, 3-bromo-2,6-dimethoxypyridine (**4a**) were synthesized by adding bromine in carbon tetrachloride slowly to **3** at temperature between −30 and −40 °C to give the product with 76% yield, while 3,5-dibromo-2,6-dimethoxypyridine (**4b**) was the side product in this reaction. To a solution of **4a** or **4b** in THF, lithium diisopropylamide (LDA) in THF at −78 °C was added, followed by the addition of chlorodiphenylphosphine to produce 3-bromo-2,6-dimethoxy-4-diphenylphosphino-pyridine or 3,5-dibromo-2,6-dimethoxy-4-diphenylphosphino-pyridine of up to 95% yield. Dropwise addition of hydrogen peroxide to a acetone-dichloromethane (1:1) solution of the above compounds at 0 °C gave 3-bromo-2,6-dimethoxy-4-diphenylphosphinoylpyridine (**5a**) or 3,5-dibromo-2,6-dimethoxy-4-diphenylphosphinoylpyridine (5**b**) with up to 96% yield. 2,6-Dimethoxy-3-phenyl-4-diphenylphosphinoylpyridine (**6a**) was synthesized via a Suzuki coupling of **6a** with phenyl boronic acid with a 89% yield, while **6b** is the side product produced in this reaction. All products were characterized by ^{1}H, ^{31}P, ^{13}C NMR and high-resolution mass spectrometry. All of the synthetic intermediates are air-stable, and the overall synthetic steps are easy to handle. Scheme 1 shows the synthetic scheme for compounds **4**–**7**.[5] Details of the synthetic methods for the intermediates can be referenced to our previous work in 2001.[4]

Human breast cancer MDAMB-231 and hepatoma SKHep1 cell lines and consented non-malignant hematological disorder bone marrow cells during presentation (female, 45 years old and male, 56 years old) were used for preliminary anti-cancer screening and toxicology analysis purpose. A concentration of 1×10^5/mL of human cells seeded in the 96 wells microtitre plates for 24 h were used. The tested compounds were prepared as a stock concentration of 100 mM in DMSO. All the tested compounds were added at a starting concentration of 100 μM, followed by a serial of twofold dilutions and incubated for a further period of 48 h. Afterwards, the evaluation of possible antiproliferative and/or cytotoxicity of our novel synthesized compounds was assayed by the one step ATP lite assay purchased from PerkinElmer (Netherlands) according to the technical manual provided (Table 1). Morphological changes of cancer cells include cell rounding, bubbling of membrane, lost of attachment property and shrinkage with the apoptotic features were recorded under inverted microscopy study (Fig. 2).[6–8]

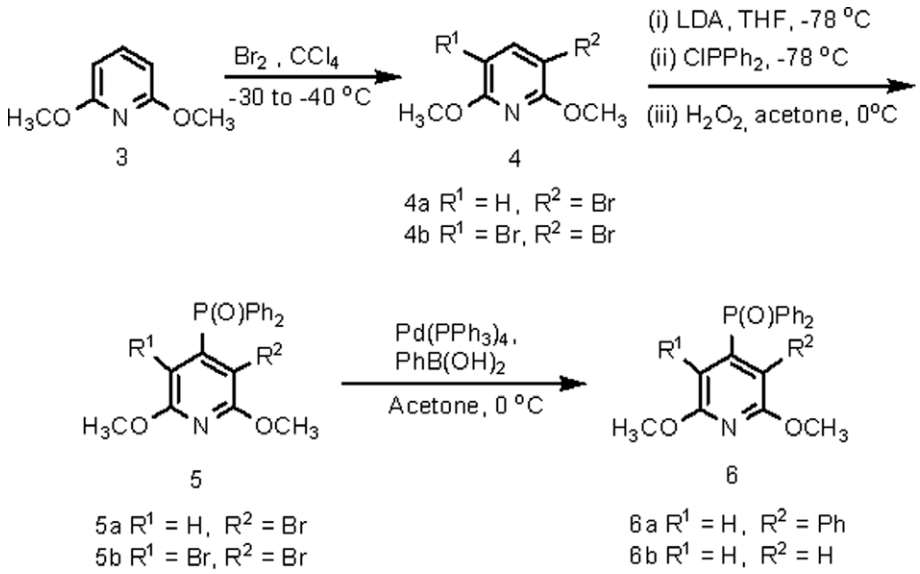
Table 1
Growth inhibitory concentration (IC_{50}) of compound **5b** on the MDAMB-231 and SKHep-1 human cancer cells as well as two non-malignant human hematological disorder bone marrow cells [BM(1) and BM(2)]

	MDAMB-231	SKHep-1	BM(1)	BM(2)
*Compound **5b***				
IC_{50}: (μM)	47.50 ± 2.11	38.53 ± 3.13	54.17 ± 5.51	58.13 ± 4.85
Cisplatinum				
IC_{50}: (μM)	115.53 ± 9.30	87.33 ± 5.01	144.53 ± 8.96	132.30 ± 5.24
Relative activities: **5b** > **5a** > **6a** > **6b** (or **2**)				

Cisplatinum was used as a positive reference antitumor agent. Results are based on the one step ATP lite assay which documented the intracellular adenosine triphosphate level. Reported results represent the mean ± standard deviation from three mean values of triplicate experiments in which each experiment had triplicate tests and a mean was calculated. The three independent experiments gave similar results. Please refer to the text for details.

Due to the significant antitumor activity of aziridinyl phosphine oxides and transitional metal complexes of the amino phosphine oxides reported, we have tried to employ the simple and easily available triphenylphosphine oxides (**1**) as the leads. However, this compound was found to exhibit low activity. Surprisingly, the replacement of one phenyl group of triphenylphosphine oxide with 2,6-dimethoxypyridine (**2**) was demonstrated to significant improve the antitumor activity towards MDAMB-231 breast cancer and SKHep-1 hepatoma cell lines. It is interesting to recognize that both phosphine oxide and 2,6-dimethoxypyridine moieties are important to account for the activity with reference to compounds **1** and **3**. The addition of a bromo group to the core structure **2** can improve the antitumor activity (compound **5a**: mean growth inhibitory percentage ~30% on both MDAMB-231 and SKHep-1 at 100 μM when compared with control). Compound **5b**, which bears two bromo groups, exhibits a significant enhanced apoptotic activity than that of **5a** (bears 1 bromo group only) (Fig. 2). In contrast to compound **5**, pyridinyl phosphine compound does not exhibit any antiproliferative activity on MDAMB-231 and SKHep-1 human cancer cells.

To understand the possible underlying mechanism of how compound **5b** could induce cancer cell death, both MDAMB-231 and SKHep-1 human cancer cells were pre-incubated with 10 μM of z-VAD-fmk, a pan-capsase inhibitor, before the addition of 70 μM of compound **5b**. After 48 h of incubation, cell viability was deter-



Scheme 1. Synthetic pathway for pyridinyl phosphine oxide.

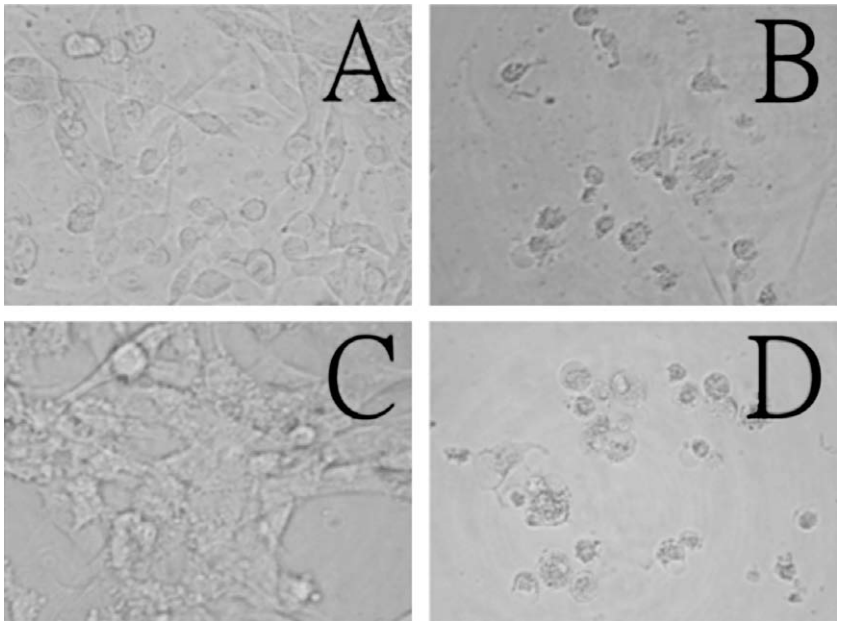


Figure 2. Morphological changes of (B) MDAMB-231 and (D) SKHep-1 human cancer cells after a 48 h incubation with compound **5b** (60 μM) when compared with the untreated (A) MDAMB-231 and (C) SKHep-1 human carcinoma cells.

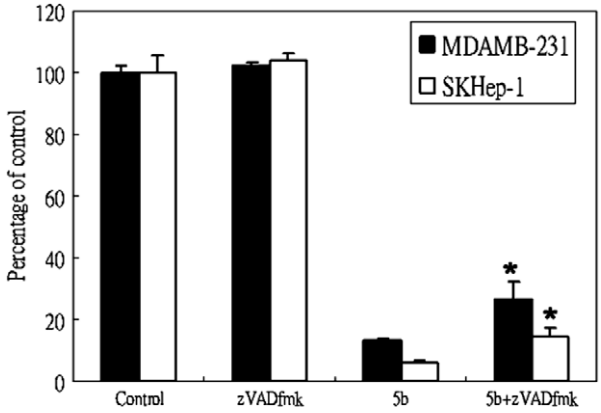


Figure 3. Analysis of the possible reversal effects of a pan caspase inhibitor zVADfmk (10 μM) on the cytotoxicity of compound **5b** at a concentration of 70 μM on MDAMB-231 and SKHep1 human carcinoma cells after 48 h of incubation. Reported results represent the mean ± standard deviation from triplicate tests. The Figure shows a representative experiment taken from three independent experiments giving similar results. Asterisk indicates that there is a statistical significant (p value <0.05, student's t-test) between the compound **5b** loaded cells and the compound **5b** plus zVADfmk loaded cells.

mined again by the one step ATP lite assay system. As shown in Figure 3, even preincubation of the pan caspase inhibitor could significantly reverse the growth inhibitory effect of compound **5b** on both cancer cell lines and the mean percentage of survival cells was found to increase near a double for MDAMB-231 while there was near a triple for that of SKHep-1 human carcinoma cells, the treatment itself could not recover the cell viability to the control level. As caspase pathway is only one of the possible ways to mediate the signal that is initiated by phosphine oxide treatment, we believe that there would be any other possibilities being included as well.

Collectively, our experimental results show that caspase dependent apoptotic pathway is recruited in our compound **5b** induced cancer cell death on both MDAMB-231 breast carcinoma cells and SKHep-1 hepatoma cells in vitro. Interestingly, a differential selective effects between the human cancer cell lines and primary cultured non-malignant hematological disorder bone marrow cells was observed, suggesting the benefits for the development of this class of compounds as novel anti-tumor agents.

In conclusion, we have discovered the new usage (i.e. antitumor studies in this work) of our reported pyridinyl type phosphorine oxide moieties. Further work using this type of compounds and their derivatives are in-progress.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.02.091.

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5. *NMR spectral data. 3-Bromo-2,6-dimethoxypyridine (**4a**)*: ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.90 (s, 3H, OCH_3), 3.99 (s, 3H, OCH_3), 6.23 (d, J = 8.5 Hz, PyH), 7.64 (d, J = 8.5 Hz, PyH); ^{13}C NMR (125 MHz, $CDCl_3$): δ 53.9, 54.4, 95.5, 102.9, 143.8, 158.6, 162.3; mass spectrum (ESI): 218.09 $[M(Br^{79})+H]^+$ and 220.09 $[M(Br^{81})+H]^+$. *3,5-Dibromo-2,6-dimethoxypyridine (**4b**)*: ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.99 (s, 6H, OCH_3), 7.86 (s, PyH); ^{13}C NMR (125 MHz, $CDCl_3$): δ 55.3, 96.2, 146.2, 158.3; mass spectrum (ESI): 295.89 $[M(Br^{79})(Br^{79})+H]^+$, 297.89

[M(Br^{79})(Br^{81})+H]$^+$ and 299.89 [M(Br^{81})(Br^{81})+H]$^+$. *3-Bromo-2,6-dimethoxy-4-diphenylphosphinoylpyridine (**5a**)*: ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.85 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 6.34 (d, J = 15 Hz, 1H, PyH), 7.35–7.32 (m, 4H, PhH), 7.45–7.42 (m, 2H, PhH), 7.56–7.52 (m, 4H, PhH); ^{13}C NMR (125 MHz, $CDCl_3$): δ 54.3, 55.1, 98.9, 108.4, 128.9, 130.4, 131.3, 132.1, 132.2, 146.0, 146.8, 160.0, 161.9; ^{31}P NMR (125 MHz, $CDCl_3$): δ 31.6; mass spectrum (ESI): 441.8 [M+Na]$^+$. *3,5-Dibromo-2,6-dimethoxy-4-diphenylphosphinoylpyridine (**5b**)*: ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.99 (s, 6H, OCH_3), 7.44–7.47 (m, 4H, PhH), 7.54–7.56 (m, 2H, PhH), 7.70–7.74 (m, 4H, PhH); ^{13}C NMR (125 MHz, $CDCl_3$): δ 55.1, 101.2, 128.5, 128.9, 132.1, 132.3, 132.5, 132.6, 133.3, 144.1, 144.8, 158.9, 159.0; ^{31}P NMR (125 MHz, $CDCl_3$): δ 32.9; mass spectrum (ESI): 495.93 [M(Br^{79})(Br^{79})+H]$^+$, 497.93 [M(Br^{79})(Br^{81})+H]$^+$ and 499.93 [M(Br^{81})(Br^{81})+H]$^+$. *2,6-Dimethoxy-3-phenyl-4-diphenylphosphinoylpyridine (**6a**)*: ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.85 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 6.34 (d, J = 15 Hz, 1H, PyH), 7.08–6.95 (m, 6H, PhH) 7.35–7.32 (m, 4H, PhH), 7.45–7.42 (m, 2H, PhH), 7.56–7.52 (m, 4H, PhH); ^{13}C NMR (125 MHz, $CDCl_3$): δ 54.0, 54.3, 105.5, 105.6, 105.7, 119.6, 119.7, 127.3, 128.4, 128.5, 128.6, 131.7, 131.8, 132.5, 133.8, 133.9, 145.7, 146.4, 161.4, 161.5, 161.8, 161.9; ^{31}P NMR (125 MHz, $CDCl_3$): δ 27.6; mass spectrum (ESI): 416.17 [M+H]$^+$; 438.15 [M+Na]$^+$. *2,6-Dimethoxy-4-diphenylphosphinoylpyridine (**6b**)*: ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.88 (s, 6H, OCH_3), 6.52 (d, J = 12 Hz, 2H, PyH), 7.51–7.53 (m, 4H, PhH) 7.61–7.63 (m, 2H, PhH), 7.64–7.66 (m, 4H, PhH); ^{13}C NMR (125 MHz, $CDCl_3$): δ 54.4, 104.4, 129.2, 132.5, 132.6, 132.9, 133.0, 163.9, 164.0; ^{31}P NMR (125 MHz, $CDCl_3$): δ 28.9; mass spectrum (ESI): 340.11 [M+H]$^+$.

6. (a) Cell lines and cell culture. Human breast cancer MDAMB-231 and hepatoma SKHep-1 cell lines were used for preliminary anti-cancer screening of these compounds. Both cancer cell lines were routinely maintained in RPMI cell culture medium supplemented with 5% heat inactivated fetal bovine serum and penicillin/streptomycin antibiotics (complete cell culture medium). The cells were incubated in a humidified cell culture incubator at 37 °C with 5% CO_2.; (b) Antiproliferation of phosphosine oxides. Cancer cells were removed from 75 cm^3 sterile cell culture flasks with onefold trypsin and neutralized with fetal bovine serum. After washing with phosphate buffered saline and centrifugation, cancer cells were re-suspended in complete cell culture medium at a concentration of approximately 1 × 105/mL and counted manually using a haematocytometer under an inverted microscope. Cancer cells seeded in the 96 wells microtitre plates for 24 h were prepared for the phosphine oxides screening. The compounds were dissolved in molecular biology grade dimethylsulfoxide (DMSO). Cisplatinum was used as the positive reference and added at a starting concentration of 167 μM. Compounds were added at a starting concentration of 100 μM and followed by a serial of twofold dilutions and incubated for a further 48 h. The effective concentration of DMSO in each case was approximately 0.1% by volume. Untreated control received either total complete cell culture medium or 0.1% DMSO. Afterwards, the evaluation of possible anti-proliferative potential of our synthesized compounds was evaluated by the one step ATP lite assay cell proliferation kit purchased from PerkinElmer (Netherlands). The one step ATP lite assay is a homogeneous experiment of determining the number of viable cells in culture based on quantitation of the ATP present, which is an indicator of metabolically active cells. The homogeneous assay procedure involves adding the single reagent directly to cells cultured in serum-supplemented medium. This results in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. The amount of ATP is directly proportional to the number of cells present in culture.; (c) Morphological investigation of cancer cells. MDAMB-231 and SKHep-1 human carcinoma cells were seeded at a concentration of 1 × 105/mL. After 24 h, phosphine oxides were added (compound **5b** at 60 μM) and incubated for 48 h. Any morphological changes were compared with the vehicle control under the inverted microscope.
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