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Original article

Novel phosphoramidates with porphine and nitrogenous drug: One-pot synthesis and orientation to cancer cells

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ABSTRACT

One-pot synthesis of novel phosphoramidates with porphine and nitrogenous drug was accomplished. In the absence of light, MTT test showed that they killed the BEL-7402 liver cancer cells effectively in vitro. The cell viability studied on normal liver and cancer cells showed that porphine phosphoramidates selectively kill the cancer cells, which was in sharp contrast with the non-porphine containing compound 4-formylphenyl *N*,*N*-bis(2-chloroethyl)-phosphoramidate. These results, coupled with the cell uptake test showing that they could differentiate the tumor cells from the normal cells by their selective accumulation in cancer cells, gave strong support to the notion that the introduction of porphine moiety in these molecules was responsible for the effectiveness and cell differentiability of these porphine phosphoramidates.

1. Introduction

Phosphorates are widely present in life forms as important physiological function molecules [1–3]. Researchers have found that phosphoramidates could also be used as drugs because of their good bioactivities [4–6]. For example, cyclophosphamide and ifosfamide have been used as anticancer drugs in the treatment of breast cancer, lung cancer, ovarian cancer, etc. [7,8]. These drugs could kill tumor cells effectively. However, one major problem associated with them is that they can not distinguish tumor cells from normal cells. Thus, improving the recognition ability of these phosphoramidate drugs to tumor cells has become one of the major challenges for medicinal chemists [9–11].

Porphines could selectively accumulate in tumor tissues than in normal tissues. Based on this fact, scientists have used porphines in photodynamic therapy (PDT) as sensitizers [12–18]. Recognizing this unique property of porphines, scientists have introduced porphines moiety into the drugs to improve the selectivity of the drugs for the tumor cells. As a result the side effects of the drug could potentially be alleviated when the drug is administrated at the same dosage. Though there have been many synthetic methods available for the synthesis of substituted porphines [19], the typical low yields, the poor solubility of porphines in both water and organic solvents make the synthesis of drugs bearing porphines moiety quite challenging. In addition the sensitive nature of some drugs toward acids and/or bases can make their synthesis even more complicated. In recent years, the synthesis of anticancer drugs bearing porphines has attracted a lot of interest from chemists and medicinal scientists alike because of their high affinity to tumor cell and also for their synthetic challenges [20–24]. For example, Napoli synthesized meso-tetrakis(4-phosphonatophenyl)porphine, a type of porphine containing phosphates, by the reaction of diethyl(4-formylphenyl)phosphorate and pyrrole in refluxing propanoic acid [25]. Ganesan prepared 5,10,15,20-tetrakis (4-phosphonoxy phenyl)porphine by the oxidation of 5,10,15,20-tetrakis(4-phosphitephenyl)porphine, which was synthesized by the reaction of 5,10,15,20-tetrakis(4-hydroxyphenyl)porphine and di-*tert*-butyl-*N*,*N*-diethylphosphoramidite [26]. However, as far as we know, there is still nothing reported on the preparation of porphine containing phosphoramidates.

In this paper, we synthesized five phosphoramidates with *meso*-substituted AB_3 porphine and ifosfamide mustards (**1**–**5**) shown in Scheme 1 by a simple one-pot phosphonation of hydroxyl substituted porphine with $POCl_3$, and subsequent amidation with 2-chloroethylamine. The biological activities of porphine phosphoramidates against normal liver cells (L-02) and cancer cells (BEL-7402) were tested by the MTT method, which were compared with those of the corresponding phosphoramidates without

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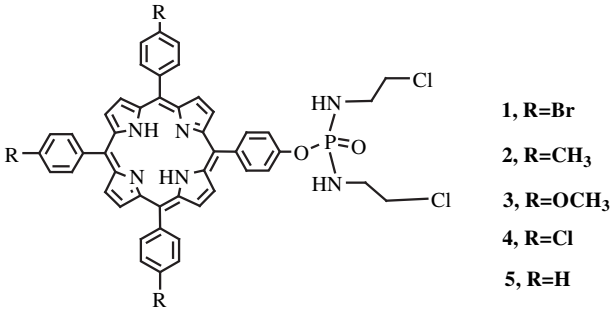


Scheme 1. The structures of porphine phosphoramidates (**1**–**5**).

porphine. These results, coupled with cell viability test and the cell uptake test show that phosphoramidates bearing porphine not only had considerable cytotoxicity against liver cancer cell but they can also differentiate the tumor cells from the normal cells by their selective accumulation in cancer cells.

2. Results and discussion

2.1. Chemistry

Since Adler–Longo method and Lindsey method are the reactions of choice for the synthesis of AB_3 porphines [27,28], initially, we planned to synthesize the desired porphine phosphoramidates through the direct condensation of pyrrole with two different types of substituted benzaldehyde, one bearing the desired phosphoramidates, under acid conditions. (Scheme 2).

As such, 4-formylphenyl *N*,*N*-bis (2-chloroethyl)phosphoramidate (**6**) has to be prepared first (Scheme 3). Compound **6** was readily synthesized by treating *p*-hydroxybenzaldehyde with $POCl_3$ and followed by amidation with chloroethylamine according to the literature procedures [29–31].

Unfortunately, the synthesis of the porphine phosphoramidate (**1**) by the reaction of compound **6**, 4-bromobenzaldehyde and pyrrole according to the Adler–Longo procedure in refluxing propanoic acid failed. The failure might be due to the fact that the P–N bonds of **6** were not stable under the acid conditions at high temperature [32].

Next, compound **1** was attempted by using Lindsey procedure at room temperature. We were delighted to discover that compound **6** was stable under standard Lindsey conditions at room temperature. However, the reaction of compound **6**, 4-bromobenzaldehyde and pyrrole under the Lindsey conditions, gave 5, 10, 15, 20-tetra(4-bromophenyl)porphine as the main product.

Finally, we decided to synthesize compound **1** according to a different route by utilizing the monohydroxyl substituted tetraphenylporphine **7** as shown in Scheme 4, since monohydroxyl substituted tetraphenylporphines are well known and can be easily synthesized. Phosphonation of the hydroxyl groups on the monohydroxyl substituted tetraphenylporphines with $POCl_3$ and amidation with 2-chloroethylamine would produce the desired porphine containing ifosfamide mustards. By simply changing the substituents on the monohydroxyl tetraphenylporphine from Br to Me, MeO, Cl, and H, we can synthesize all five different porphine phosphoramidates via a single strategy.

The reaction of 5, 10, 15-tri(4-bromophenyl)-20-(4-hydroxyphenyl)porphine (**7**) with excess amount of $POCl_3$ should give the tetraphenylporphine phosphorodichloridate (**8**). It was reported that 20 equiv of $POCl_3$ was needed and the excess $POCl_3$ had to be removed by vacuum distillation for such transformation [29–31]. Indeed, when the hydroxyl porphine **7** was reacted with 20 equiv $POCl_3$, the starting material **7** disappeared completely after 2 h, and porphine phosphorodichloridate **8** was formed. However, the attempt to remove the excess of $POCl_3$ from the reaction mixture by vacuum distillation failed because the amines within the porphine structure reacted with the $POCl_3$ at the reflux temperature [33]. This result was confirmed with LC-MS analysis of the reaction mixture. The molecular ion peak of (5,10,15,20-tetraphenylporphinato) dichlorophosphorous chloride at *m*/*z* 713 showed that only the hydroxyl group of 5,10,15,20-tetraphenylporphine could react with $POCl_3$ at room temperature, and more complicated products were observed at 50 °C. Above results suggested that it was difficult to remove the excess of $POCl_3$ from the mixtures of porphine phosphorodichloridate **8** and $POCl_3$ by vacuum distillation. Attempts to purify the reaction mixture via column chromatography failed too because of the instability of porphine phosphorodichloridate **8** on column.

Fortunately, much to our delight, the phosphonation of the hydroxyl porphine **7** with 1.2 equiv $POCl_3$ and 1.8 equiv triethylamine gave only one product, i.e., porphine phosphorodichloridate **8** after 4 h at −20 °C. It was also found that the compound **8** could be used without further purification for the amidation step. Addition of 3 equiv of 2-chloroethylamine hydrochloride and 6 equiv of triethylamine to the in situ generated porphine phosphorodichloridate **8** gave porphine phosphoramidate **1** in 5% yield after 4 h at −20 °C. The yield of porphine phosphoramidate **1** was increased to 10% when the temperature was raised from −20 °C to 20 °C. subsequent investigation revealed that the yield of porphine phosphoramidate **1** was profoundly influenced by the amount of 2-chloroethylamine hydrochloride and triethylamine and the results are summarized in Table 1.

As shown in Table 1, the yield of porphine phosphoramidate mustard **1** was increased from 10% to 50% when the amounts of 2-chloroethylamine and triethylamine increased from 3 equiv to 6 equiv–5 equiv and 10 equiv, respectively (Run 3). Prolonging the reaction time from 8 h to 24 h had little effect on the yield (Run 4). Further increasing the amounts of 2-chloroethylamine hydrochloride and triethylamine did not raise the yield of porphine phosphoramidate **1** either (Run 5).

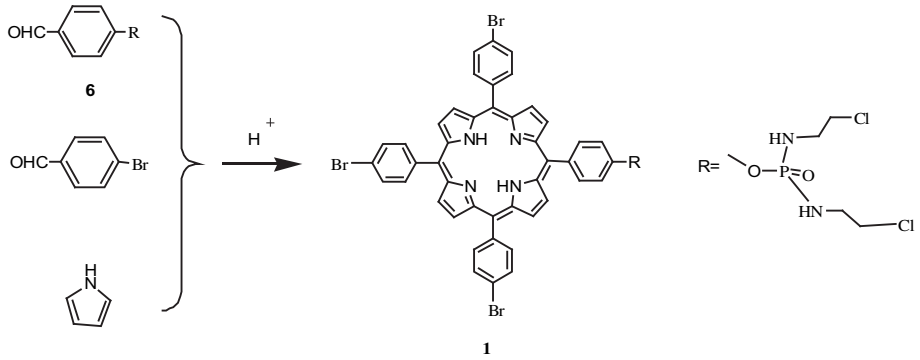
With a satisfactory protocol for the synthesis of the desired porphine containing ifosfamide mustard in hand, compounds **1**–**5** were synthesized accordingly from their corresponding monohydroxyl substituted tetraarylporphines in yields of 42–50%.

2.2. Cytotoxicity assays

In the absence of light, the porphine phosphoramidates **1**–**5**, 4-formylphenyl *N*,*N*-bis(2-chloroethyl)phosphoramidate **6**, and haematoporphine dihydrochloride (HPD) were tested in vitro against liver cancer cell BEL-7402 by the MTT method [23,24] and the IC_{50} values of the liver cancer cell BEL-7402 were listed in Table 2.

Compound **6** and HPD were used as the references to truly gauge the effectiveness of the five porphine phosphoramidates since compound **6** had the same phosphoramide structure as **1**–**5**, and HPD was a known drug used in the treatment of cancer. Test results showed that porphine phosphoramidates **1**–**5** had much lower IC_{50} values than 4-formylphenyl *N*,*N*-bis (2-chloroethyl)phosphoramidate **6**, and were nearly as effective as HPD while the Br-substituted compound was found to be the most effective one, affording a IC_{50} value at 8.41 μM. Other four compounds gave values ranging from 11.42 to 20.32. The low IC_{50} values observed for these five compounds suggested they all can be potential anticancer drugs.

To compare the toxicity of the porphine phosphoramidates to the tumor and normal cells, porphine phosphoramidates **1**–**5** and compound **6** were tested via MTT test in liver cancer cells BEL-7402



Scheme 2. Synthetic route of porphine phosphoramidate via Adler or Lindsey procedures.

and normal liver cells L-02 (in the absent of light), respectively, and the cell viability results were shown in Scheme 5.

In the case of compound **6**, we noted that the cell viability of the normal liver cells L-02 was lower than the liver cancer cells BEL-7402, which was expected because tumor cells tends to be more difficult to kill than normal cells. However, with compounds **1**–**5**, cell viabilities of normal liver cells L-02 were higher than those of the liver cancer cells BEL-7402 when compounds **1**–**5** were given at the same dosage as in the case of **6**. This reversal of the trend showed that porphine phosphoramidates could selectively kill the cancer cells. Clearly, the inclusion of a porphine structure motif is responsible for this.

2.3. Cancer cell selectivity

In order to further understand the cancer cell-oriented accumulation of the synthetic porphine phosphoramidates **1**–**5**, their accumulations in the BEL-7402 tumor and the normal cells L-02 were investigated by the cell uptake tests [34]. The amounts of the intracellular porphine phosphoramidates **1**–**5** absorbed by the liver cancer cells BEL-7402 and the normal cells L-02 were shown in Scheme 6.

As shown in Scheme 6, significant concentration differences of the porphine phosphoramidates between the liver cancer cells BEL-7402 and normal cells L-02 were observed. The fact that the uptakes of liver cancer cells BEL-7402 were higher than liver normal cells L-02 gave proof to that the selective accumulation of porphine phosphoramidates in liver cancer cells BEL-7402 did occur. This could be the reason for the selective killing of the tumor cells.

3. Conclusion

In conclusion, we have developed a practical and efficient one-pot approach to synthesize porphine containing ifosfamide mustard and 5 different porphine phosphoramidates are synthesized. In the absence of light, the bioactivity tests show that the porphine phosphoramidates are potent anticancer reagents, with one being as effective as the commercial anticancer drug HPD. They not only can selective accumulate in cancer cells but also can selective kill the tumor cells. Investigations are currently under way to elucidate the molecular mechanism for the intracellular accumulation of these porphine phosphoramidates in liver cancer cells BEL-7402.

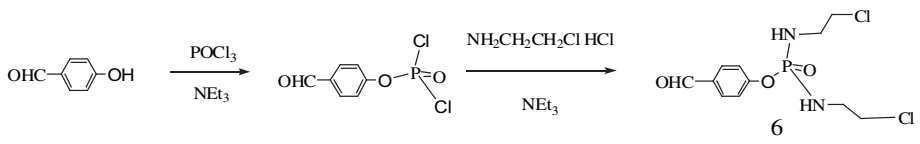
4. Experimental section

4.1. Chemistry

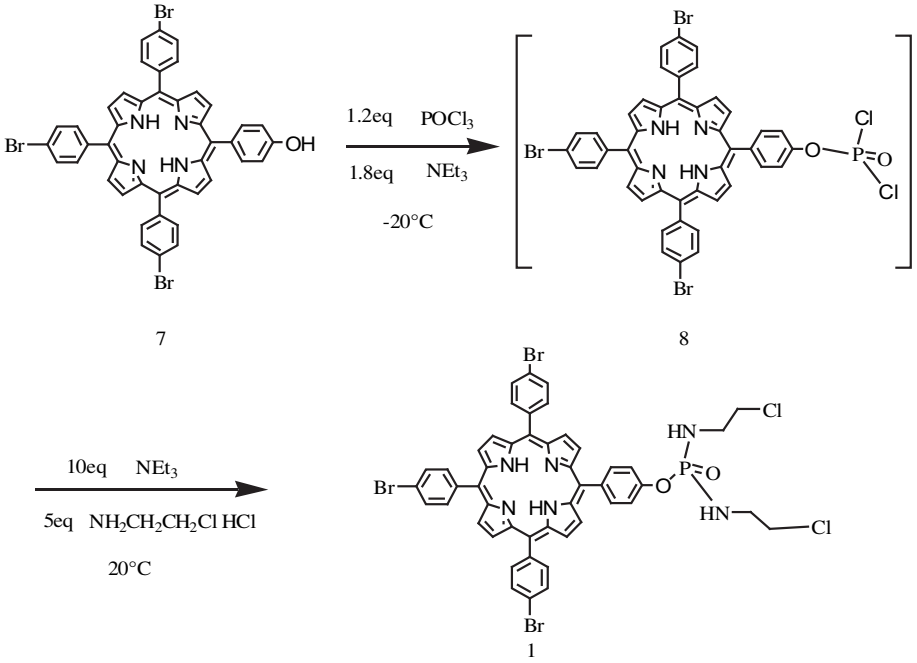
^{1}H NMR and ^{31}P NMR spectra were recorded on a Varian NOVA-400 spectrometer (400 MHz 1H), and the chemical shifts were reported with tetramethylsilane (TMS) as the internal standard in $CDCl_3$ solvent. Mass spectra were obtained on a Shimadzu QP-5000 mass spectrometer and Agilent 1100 LC-MS. Elemental analyses were obtained on a Vario El III elementary analyzer. Fluorescence emission was recorded on a Hitachi F-4500 Fluorescence Spectrophotometer. Cell numbers were measured on a CASY Cell Counter and Analyzer. CH_2Cl_2, pyrrole and $POCl_3$ were distilled before use. 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Sigma Chemical Co. (USA). The cell lines BEL-7402 and L-02 were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Science. Other chemicals were used as received.

4.1.1. General procedure for the synthesis of porphine phosphoramidate (**1**–**5**, Scheme 4)

A solution of hydroxyl porphine (0.1 mmol, 1.0 equiv) and $POCl_3$ (0.12 mmol, 1.2 equiv) in CH_2Cl_2 under nitrogen was cooled to −20 °C. NEt_3 (0.18 mmol, 1.8 equiv) in CH_2Cl_2 was added dropwise while maintaining the temperature at −20 °C. The reaction mixture was stirred for 4 h at −20 °C before dry 2-chloroethylamine hydrochloride (0.5 mmol, 5 equiv) was added. The final mixture was stirred at −20 °C for 15 min and then warmed slowly to room temperature for another 8 h. The solution was washed once with aqueous saturated $NaHCO_3$, once with water and dried over anhydrous Na_2SO_4. After removing the solvent under vacuum, the crude product was purified by column chromatography (CH_2Cl_2:ethyl acetate, 5:1).



Scheme 3. Synthetic routes of 4-formylphenyl *N*,*N*-bis(2-chloroethyl)phosphoramidate.



Scheme 4. Synthetic routes of porphine phosphoramidates.

*4.1.1.1. 5,10,15-tri(4-Bromophenyl)-20-(4-phenyl)porphine N,N-bis(2-chloroethyl) phosphoramidate (**1**).* The compound was prepared according to the general procedure mentioned above, purified and isolated as a purple solid; yield 50%. MS: *m/z* 1070. ^{1}H NMR ($CDCl_3$): *δ*, ppm, 8.84 (s, 8H), 8.17 (d, 2H, *J* = 8 Hz), 8.06 (d, 6H, *J* = 8 Hz), 7.90 (d, 6H, *J* = 8 Hz), 7.63 (d, 2H, *J* = 8 Hz), 3.77 (t, 4H, *J* = 5.2 Hz), 3.52–3.59 (m, 4H), 3.43–3.48 (m, 2H), −2.87 (s, 2H). ^{31}P NMR ($CDCl_3$): *δ*, 11.77. Anal.Calcd. for $C_{48}H_{36}Br_3Cl_2N_6O_2P$, %: C, 53.86; H, 3.39; N, 7.85; Found: C, 54.03; H, 3.42; N, 7.64.

*4.1.1.2. 5,10,15-tri(4-Methylphenyl)-20-(4-phenyl)porphine N,N-bis(2-chloroethyl) phosphoramidate (**2**).* The compound was prepared according to the general procedure mentioned above, purified and isolated as a purple solid; yield 45%. MS: *m/z* 876. ^{1}H NMR ($CDCl_3$): *δ*, ppm, 8.86 (s, 6H), 8.83 (s, 2H), 8.18 (d, 2H, *J* = 8 Hz), 8.09 (d, 6H, *J* = 8 Hz), 7.61 (d, 2H, *J* = 8 Hz), 7.56 (d, 6H, *J* = 8 Hz), 3.75 (t, 4H, *J* = 5.2 Hz), 3.52–3.61 (m, 4H), 3.43–3.48 (m, 2H), −2.81 (s, 2H). ^{31}P NMR ($CDCl_3$): *δ*, 10.80. Anal.Calcd. for $C_{51}H_{45}Cl_2N_6O_2P$, %: C, 69.94; H, 5.18; N, 9.60; Found: C, 70.02; H, 5.09; N, 9.75.

*4.1.1.3. 5,10,15-tri(4-Methoxyphenyl)-20-(4-phenyl)porphine N,N-bis(2-chloroethyl) phosphoramidate (**3**).* The compound was prepared according to the general procedure mentioned above, purified and isolated as a purple solid; yield 42%. MS: *m/z* 924. ^{1}H NMR ($CDCl_3$): *δ*, ppm, 8.88 (s, 6H), 8.83 (s, 2H), 8.19 (d, 2H, *J* = 8 Hz), 8.12 (d, 6H, *J* = 8 Hz), 7.62 (d, 2H, *J* = 8 Hz), 7.30 (d, 6H, *J* = 8 Hz), 3.77 (t, 4H, *J* = 5.2 Hz), 3.53–3.59 (m, 4H), 3.43–3.46 (m, 2H), −2.80 (s, 2H). ^{31}P NMR ($CDCl_3$): *δ*, 10.80. Anal.Calcd. for $C_{51}H_{45}Cl_2N_6O_5P$, %: C, 66.31; H, 4.91; .N, 9.10. Found: C, 66.41; H, 4.75; N, 9.24.

Table 1
Reaction condition screening.

Reaction run	2-chloroethylamine/equiv	Triethylamine/equiv	Time/h	Yield %
1	3	6	8	10
2	3	6	24	13
3	5	10	8	50
4	5	10	24	50
5	10	20	8	50

*4.1.1.4. 5,10,15-tri(4-Chlorophenyl)-20-(4-phenyl)porphine N,N-bis(2-chloroethyl) phosphoramidate (**4**).* The compound was prepared according to the general procedure mentioned above, purified and isolated as a purple solid; yield 49%. MS: *m/z* 937. ^{1}H NMR ($CDCl_3$): *δ*, ppm, 8.84–8.87 (m, 8H), 8.18 (d, 2H, *J* = 8 Hz), 8.13 (d, 6H, *J* = 8 Hz), 7.75 (d, 6H, *J* = 8 Hz), 7.64 (d, 2H, *J* = 8 Hz), 3.77 (t, 4H, *J* = 5.2 Hz), 3.53–3.60 (m, 4H), 3.44–3.49 (m, 2H), −2.86 (s, 2H). ^{31}P NMR ($CDCl_3$): *δ*, 11.32. Anal.Calcd. for $C_{48}H_{36}Cl_5N_6O_2P$ %: C, 61.52; H, 3.87; N, 8.97. Found: C, 61.38; H, 3.79; N, 9.08.

*4.1.1.5. 5,10,15-Triphenyl-20-(4-phenyl)porphine N,N-bis(2-chloroethyl)phosphoramidate (**5**).* The compound was prepared according to the general procedure mentioned above, purified and isolated as a purple solid; yield 46%. MS: *m/z* 834. ^{1}H NMR ($CDCl_3$): *δ*, ppm, 8.83–8.86 (m, 8H), 8.21 (dd, 6H, *J* = 1.2, 8 Hz), 8.18 (d, 2H, *J* = 8 Hz), 7.72–7.78 (m, 9H), 7.60 (d, 2H, *J* = 8 Hz), 3.73 (t, 4H, *J* = 5.2 Hz), 3.51–3.60 (m, 4H), 3.42–3.46 (m, 2H), −2.80 (s, 2H). ^{31}P NMR ($CDCl_3$): *δ*, 11.01. Anal.Calcd. for $C_{48}H_{39}Cl_2N_6O_2P$, %: C, 69.15; H, 4.71; N, 10.08. Found: C, 69.44; H, 4.91; N, 9.89.

*14.1.2. Synthesis of 4-formylphenyl N,N-bis (2-chloroethyl)]phosphoramidate (**6**, Scheme 3)*

A solution of NEt_3 (3 mmol, 1.0 equiv) was slowly added to a mixture of 4-hydroxybenzaldehyde (3 mmol, 1.0 equiv) and $POCl_3$ (60 mmol, 20 equiv) in CH_2Cl_2. The mixture was stirred for 1 h at 20 °C. Subsequently 10 mL of ether was added. The solids were removed by filtration. The solvents were removed, and 10 mL of ether was added again. The remaining solids were removed, and the solvent was evaporated. The excess of $POCl_3$ was removed in vacuum. A greenish oil (4-formylphenyl phosphorodichloridate) was obtained in the yield of 51%.

Subsequently NEt_3 (1 mmol, 10 equiv) and 2-chloroethylamine hydrochloride (0.5 mmol, 5 equiv) were added at 20 °C. 4 h later the volatiles were removed, and ether was added again. The remaining solids were removed, and the solvent was evaporated. The crude product was purified by column chromatography and isolated as a yellow liquid. Yield 52%. MS: *m/z* 325, ^{1}H NMR ($CDCl_3$): *δ*, ppm,9.95 (s, 1H), 7.87 (d, 2H, *J* = 8.4 Hz), 7.40 (d, 2H, *J* = 8.4 Hz), 3.59–3.62 (m, 6H), 3.35–3.40 (m, 4H). ^{31}P NMR ($CDCl_3$): *δ*, 11.65.

Table 2
IC_{50} values of five porphine phosphoramidates.[a]

IC_{50}	1	2	3	4	5	6	HPD
μM	8.41(7.48 – 9.35)	11.42(9.13 – 13.70)	17.33(16.25 – 19.50)	20.32(18.18 – 22.46)	14.41(10.80 – 15.61)	>150	8.93(7.44 – 10.42)

[a] IC_{50} values represent the mean from at least three independent experiments.

*4.1.3. General procedure for the synthesis of monohydroxyl substituted porphines (**7**,**9**–**12**)*

The reactions was performed in a 500 mL three neck round bottom flask, which was charged with propionic acid (300 mL), benzaldehyde (26 mmol), substituted benzaldehyde (60 mmol), and pyrrole (80 mmol). The resulting solution was magnetically stirred in refluxing propionic acid for 35 min. The crude product was recrystallized from cold propionic acid and further purified by column chromatography (CH_2Cl_2).

*4.1.3.1. 5,10,15-tri(4-Bromophenyl)-20-(4-hydroxyphenyl)porphine (**7**).* The compound was prepared according to the general procedure mentioned above, purified and isolated as a purple solid; yield 7.1%. MS: *m*/*z* 867. ^{1}H NMR ($CDCl_3$): *δ*, ppm, 8.89 (d, 2H, *J* = 4.8 Hz), 8.81–8.83 (m, 6H), 8.05–8.07 (m, 8H), 7.88 (dd, 6H, *J* = 2, 8 Hz), 7.16 (d, 2H, *J* = 8 Hz), −2.86 (s, 2H). Anal.Calcd. for $C_{44}H_{27}Br_3N_4O$ %: C, 60.92; H, 3.14; N, 6.46; Found: C, 61.11; H, 3.40; N, 6.64.

*4.1.3.2. 5,10,15-tri(4-Methylphenyl)-20-(4-hydroxyphenyl)porphine (**9**).* The compound was prepared according to the general procedure mentioned above, purified and isolated as a purple solid; yield 6.7%. MS: *m*/*z* 673. ^{1}H NMR ($CDCl_3$): *δ*, ppm, 8.86 (s, 8H), 8.09 (d, 6H, *J* = 8 Hz), 8.03 (d, 2H, *J* = 8 Hz), 7.54 (d, 6H, *J* = 8 Hz), 7.10 (d, 2H, *J* = 8 Hz), 2.70 (s, 9H), −2.19 (s, 2H). Anal.Calcd. for $C_{47}H_{36}N_4O$,%: C, 83.90; H, 5.39; N, 8.33. Found: C, 83.62; H, 5.59; N, 8.30.

*4.1.3.3. 5,10,15-tri(4-Methoxyphenyl)-20-(4-hydroxyphenyl)porphine (**10**).* The compound was prepared according to the general procedure mentioned above, purified and isolated as a purple solid; yield 7.4%. MS: *m*/*z* 721. ^{1}H NMR ($CDCl_3$): *δ*, ppm, 8.87 (s, 6H), 8.52 (d, 2H, *J* = 8 Hz), 8.47 (s, 2H), 8.13 (d, 6H, *J* = 8 Hz), 7.53 (d, 2H, *J* = 8 Hz), 7.29 (d, 6H, *J* = 8 Hz), 4.16 (s, 3H), 4.11 (s, 6H), −2.76 (s, 2H). Anal.Calcd. for $C_{47}H_{36}N_4O_4$, %: C, 78.31; H, 5.03; N, 7.77; Found: C, 78.20; H, 4.87; N, 8.01.

*4.1.3.4. 5,10,15-tri(4-Chlorophenyl)-20-(4-hydroxyphenyl)porphine (**11**).* The compound was prepared according to the general procedure mentioned above, purified and isolated as a purple solid; yield 7.0%. MS: *m*/*z* 734. ^{1}H NMR ($CDCl_3$): *δ*, ppm, 8.90 (d, 2H, *J* = 4.4 Hz), 8.83 (s, 6H), 8.14 (d, 6H, *J* = 8 Hz), 8.06 (d, 2H, *J* = 8 Hz), 7.75 (d, 6H, *J* = 8 Hz), 7.21 (d, 2H, *J* = 8 Hz), −2.82 (s, 2H). Anal.Calcd. for $C_{44}H_{27}Cl_3N_4O$ %: C, 71.99; H, 3.71; N, 7.63. Found: 72.12; H, 3.76; N, 7.43.

*4.1.3.5. 5,10,15-Triphenyl-20-(4-hydroxyphenyl)porphine (**12**).* The compound was prepared according to the general procedure mentioned above, purified and isolated as a purple solid; yield 4.9%. MS: *m*/*z* 631. ^{1}H NMR ($CDCl_3$): *δ*, ppm, 8.84–8.89 (m, 8H), 8.22 (dd, 6H, *J* = 1.2, 8 Hz), 8.08 (d, 2H, *J* = 8 Hz), 7.73–7.78 (m, 9H), 7.22 (d, 2H, *J* = 8 Hz), −2.78 (s, 2H). Anal.Calcd. for $C_{44}H_{30}N_4O$,%: C, 83.79; H, 4.79; N, 8.88. Found: C, 83.94; H, 4.62; N, 9.03.

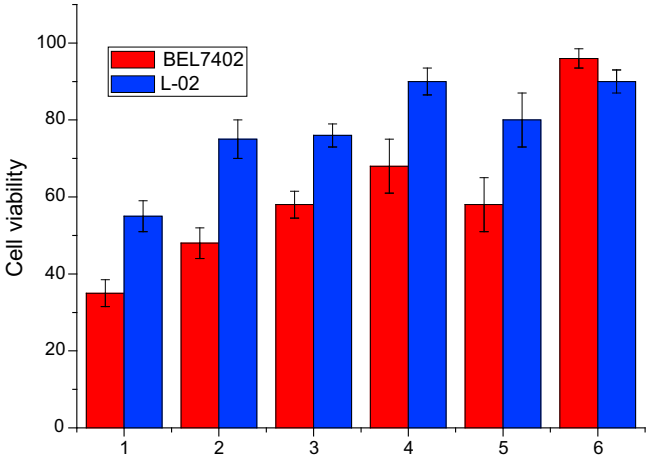
4.2. Bioactivity

4.2.1. Cytotoxicity assays

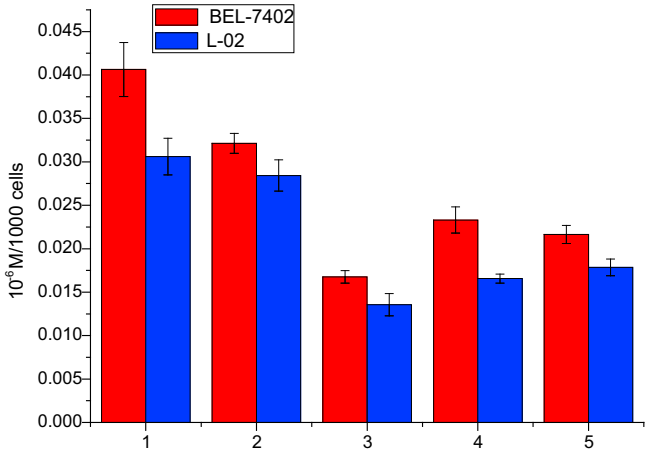
The cytotoxicity effects of the compounds on BEL-7402 cell were determined by using the MTT assay. BEL-7402 cells were plated at a concentration of 5000 cells per well on a Costar 96-well plate and allowed to attach overnight. Conjugate stock solutions were prepared in DMSO at a concentration of 50 mM. These stocks were diluted to give a final concentration range from 2.5 μM to 100 μM. The cells were then incubated 48 h in the dark. 20 μL MTT (5 mg/mL in PBS) was added to each well followed by 3 h incubation. The formazan crystals were dissolved in DMSO. The absorbance was measured in an enzyme linked immunoabsorbent assay plate reader (Bio-Tek) at a wavelength of 570 nm. Concentration 50% cell death (IC_{50}) was determined for the various compounds tested. L-02 cells were tested as above.

4.2.2. Cell uptake assays

The uptake test for liver cancer cells BEL-7402 and normal liver cells L-02 was carried out according to the following processes. Liver cancer cells BEL-7402 (or normal liver cells L-02) were seeded on a Costar 24-well plate and allowed to attach overnight, and then



Scheme 5. Cell viability of BEL-7402 and normal L-02 cells in the presence of 10 μM porphine phosphoramidates **1**–**5** and 4-formylphenyl *N*,*N*-bis(2-chloroethyl)phosphoramidate **6**.



Scheme 6. Uptake of 5 porphine phosphoramidates at 5 μM by BEL-7402 cells and L-02 cells for 24 h.

exposed to 5 μM porphine in medium for 24 h. At the end of the loading period, the medium was removed, and the cells were washed three times with ice-cold PBS (phosphate buffered saline) and dissociated from the plate using enzyme free cell dissociation solution and measured in 1.5 mL DMSO solution. Standard concentration–absorbency curve are established on a Hitachi F-4500 Fluorescence Spectrophotometer using excitation/emission wavelengths of 420 and 650 nm, respectively. So the intracellular accumulation of conjugate was determined by measuring the fluorescence emission of porphines and the data were the average of at least three independent experiments. Cell numbers were measured using the CASY Cell Counter and Analyzer.

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