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Nitrobenzocyclophosphamides as Potential Prodrugs for Bioreductive Activation: Synthesis, Stability, Enzymatic Reduction, and Antiproliferative Activity in Cell Culture

Zhuorong Li,[a,†] Jiye Han,[a] Yongying Jiang,[a] Patrick Browne,[b] Richard J. Knox[b] and Longqin Hu[a,*]

[a]*Department of Pharmaceutical Chemistry, Ernest Mario School of Pharmacy, Rutgers, the State University of New Jersey, 160 Frelinghuysen Road, Piscataway, NJ 08854, USA*

[b]*Enact Pharma PLC, Porton Down Science Park, Salisbury, Wiltshire SP4 0JQ, UK*

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Abstract—In efforts to obtain potential anticancer prodrugs for gene-directed enzyme prodrug therapy using *Eschericia coli* nitroreductase, a series of four benzocyclophosphamide analogues were designed and synthesized incorporating a strategically placed nitro group in a position *para* to the benzylic carbon for reductive activation. All four analogues were found to be stable in phosphate buffer at pH 7.4 and 37 °C and were good substrates of *E. coli* nitroreductase with half lives between 7 and 24 min at pH 7.0 and 37 °C. However, only two analogues **6a** and **6c**, both with a benzylic oxygen in the phosphorinane ring *para* to the nitro group, showed a modest 33–36-fold enhanced cytotoxicity in *E. coli* nitroreductase-expressing cells. These results suggest that good substrate activity and the *para* benzylic oxygen are required for activation by *E. coli* nitroreductase. Compounds **6a** and **6c** represent a new structure type for reductive activation and a lead for further modification in the development of better analogues with improved selective toxicity to be used in gene-directed enzyme prodrug therapy.
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Introduction

Cyclophosphamide (**1**) is an anticancer prodrug, which has to be activated by cytochrome P-450 enzyme in the liver.[1–3] As shown in Scheme 1, hepatic cytochrome P-450 oxidation converts cyclophosphamide to 4-hydroxycyclophosphamide (**2**). Ultimate conversion to the cytotoxic alkylating species, phosphoramide mustard (**5**), is initiated by ring opening of **2** to produce aldophosphamide (**3**). The formation of **5** from **3** proceeds by general base-catalyzed β-elimination. Enzymes are not required for conversions following the initial hydroxylation in the liver.[3] The aldehyde moiety in **3** can serve as a substrate for aldehyde dehydrogenase and the corresponding carboxylic acid product is less prone to β-elimination. Aldehyde dehydrogenase is widely distributed in normal human tissues and has been found in cyclophosphamide-resistant tumor cells. But, most malignant tumor cells seem to have very little of this enzyme. Therefore, it is believed that the detoxication by aldehyde dehydrogenase might be responsible for its tumor selectivity as well as drug-resistance in resistant tumor cells.[4] The α,β-unsaturated aldehyde acrolein (**4**) is a potent electrophile and the causative agent of the bladder toxicity associated with cyclophosphamide.[5]

In the last four decades, modifications of cyclophosphamide led to the design and synthesis of many cyclic and acyclic phosphoramidate alkylating agents.[1,6,7] However, these extensive structure–activity relationship studies failed to produce better drugs than cyclophosphamide.

Prodrug design is an important strategy that has been proven to work for many drugs in improving their undesirable physico-chemical and biological properties.[8–10] Recently, prodrug strategies have also been used in targeted drug delivery including antibody-directed enzyme prodrug therapy (ADEPT) and gene-directed enzyme prodrug therapy (GDEPT). In these approaches, an enzyme is delivered site-specifically by

*Corresponding author. Tel.: +1-732-445-5291; fax: +1-732-445-6312; e-mail: longhu@rci.rutgers.edu

†Current address: Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences, Tiantan, Beijing 100050, China.

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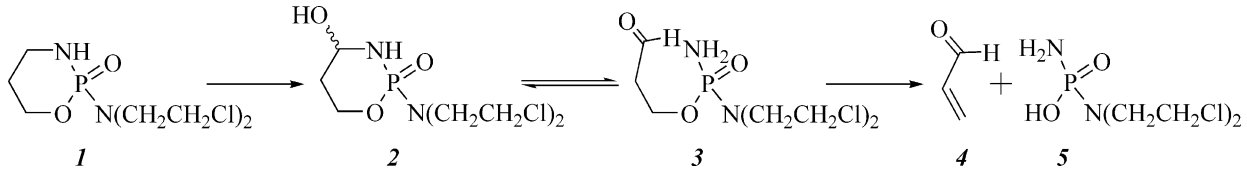
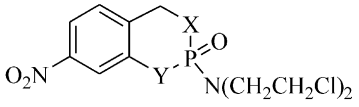


Scheme 1. Mechanism of activation of cyclophosphamide (**1**) in the liver.

chemical conjugation or genetic fusion to a tumor specific antibody or by an enzyme gene delivery system into tumor cells. This is then followed by the administration of a prodrug, which is selectively activated by the delivered enzyme at the tumor cells. A number of these systems are in development and have been reviewed.[11–15] Among the enzymes under evaluation is a bacterial nitroreductase from *Escherichia coli*. This FMN-containing flavoprotein is capable of reducing certain aromatic nitro groups to the corresponding amines or hydroxylamines in the presence of a cofactor NADH or NADPH.[16–18]

To increase tumor selectivity and overcome tumor-resistance, we hope to move the site of activation from the liver to tumor tissues through structural modification of cyclophosphamide and incorporation of a trigger-activation mechanism that could be activated by a reductive enzyme such as *E. coli* nitroreductase used in the enzyme prodrug therapies mentioned above. In this paper, we report the synthesis, stability, enzymatic reduction, and cellular antiproliferative activity of four nitrobenzocyclophosphamide analogues **6a–d** incorporating a strategically placed nitro group as the trigger (Fig. 1).



6a-d	X	Y
a	O	O
b	NH	O
c	O	NH
d	NH	NH

Fig. 1. Nitrobenzocyclophosphamide analogues designed.

Results and Discussion

Design and proposed mechanism of activation

Compounds **6a–d** are cyclophosphamide analogues with the cyclophosphamide ring fused with a benzene ring, where a nitro group is placed in the position *para* to the benzylic carbon. The nitro group here serves as an electronic trigger. It is a strong electron-withdrawing group and is converted to an electron-donating amino or hydroxyamino group upon reduction. Scheme 2 illustrates the potential mechanism of activation for nitrobenzocyclophosphamide **6a–d** designed incorporating this strategically placed nitro group as the trigger. After reduction by an enzyme such as the bacterial nitroreductase, the resulting hydroxyamines or amines **7a–d** will relay their electrons to the *para*-position and facilitate the cleavage of the benzylic C-O/NH bond, producing the cytotoxic intermediates (**8a–d**). These intermediates **8a–d** resemble the phosphoramide mustard (**5**) produced in the activation process of cyclophosphamide **1** and could be the ultimate cytotoxic alkylating agent. In addition, **8a–d** also possess additional electrophilic centers that potentially could form cross-links with functionally important macromolecules, providing additional mechanism for cytotoxicity.

Synthesis

The dioxaphosphorinane analogue **6a** was synthesized as shown in Scheme 3 starting from 2-methyl-5-nitrophenol (**10**). Acetylation with acetic anhydride followed by bromination with *N*-bromosuccinimide afforded 2-acetoxy-4-nitrobenzyl bromide (**11**) in 76% yield for the two steps. Complete hydrolysis of both the ester and the bromide in **11** using $CaCO_3$ in H_2O–dioxane (1:1) gave 2-hydroxy-4-nitrobenzyl alcohol (**12**) in 82% yield. Subsequent triethylamine-mediated cyclization with bis(2-chloroethyl)phosphoramidic dichloride gave the desired 7-nitro-2-[bis(2-chloroethyl)amino]-1,3,2-benzodioxaphosphorinane-2-oxide (**6a**) in 55% yield. The overall yield for the synthesis of **6a** before optimization is 34%.

The benzo[e]cyclophosphamide analogue **6b** was synthesized using the Gabriel synthesis of primary amines by converting the bromide **11** via intermediate **13** to 2-hydroxy-4-nitrobenzylamine (**14**) in 32% yield as shown in Scheme 4. Subsequent triethylamine-mediated cyclization with bis(2-chloroethyl)phosphoramidic dichloride gave the desired 7-nitro-2-[bis(2-chloroethyl)amino]-1,3,2-benzoxazaphosphorinane-2-oxide **6b** in 62% yield. The overall yield before optimization for the synthesis of **6b** is 15%.

The benzo[e]cyclophosphamide analogue, 7-nitro-2-[bis(2-chloroethyl)amino]-3,1,2-benzoxazaphosphorinane-2-oxide (**6c**), and the diaza analogue, 7-nitro-2-[bis(2-chloroethyl)amino]-1,3,2-benzodiazaphosphorinane-2-oxide (**6d**), were synthesized starting from 2-methyl-5-nitroaniline (**15**) using the similar series of reactions discussed above for the synthesis of **6a** and **6b** and are shown in Schemes 5 and 6. The overall yields for the synthesis of **6c** and **6d** before optimization were 4.5 and 6.8%, respectively. The overall yields of these syntheses are limited by formation of the phosphorinane ring system. The yields reported in literature for the cyclization and formation of similar systems varies from 15% to

around 50%.[19–23] Structures of the synthetic intermediates and products were confirmed by IR, NMR, and mass spectrometry.

Stability

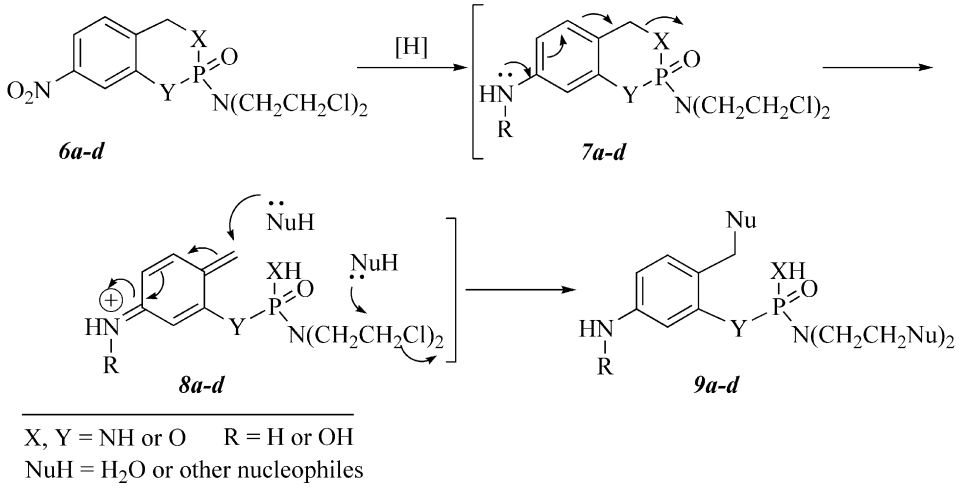
All four nitrobenzocyclophosphamide analogues were incubated in pH 7.4 phosphate buffer at 37 °C. HPLC analysis of the incubation mixtures showed no significant changes of the analogues over a period of 4 days (<10%, data not shown), suggesting that the compounds are very stable under these conditions.

Chemical reduction of nitrobenzocyclophosphamides

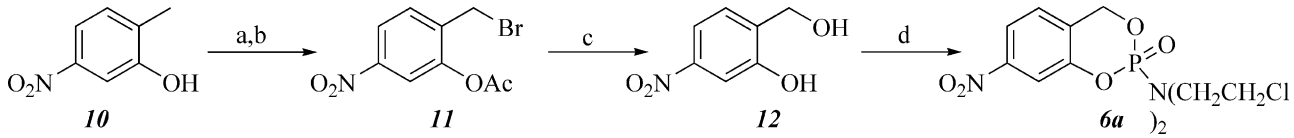
We used catalytic hydrogenation or $NaBH_4$ in the presence 10% Pd/C in methanol to selectively reduce the nitro group and then characterized the reduced product with NMR and high resolution MS.[24] In the case of compounds **6a** and **6c**, where the benzylic carbon is attached to an ester oxygen, the reduction gave a complex product mixture, suggesting that the corresponding reduced products were not stable and may undergo the cleavage reactions proposed in Scheme 2. However, when the benzylic carbon is attached to a phosphoramide nitrogen in the case of **6b** and **6d**, we were able to isolate the corresponding reduced aminobenzocyclophosphamides **20b** and **20d** in 97% and 52% yield, respectively (Scheme 7). In addition, both **20b** and **20d** were found to be similarly stable as compared to their precursors under the same stability testing conditions used above.

Substrate activity for *E. coli* nitroreductase

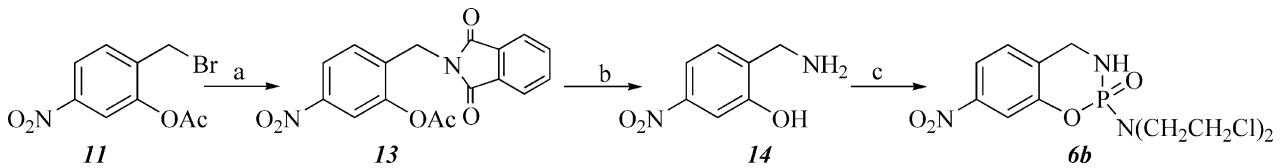
The four nitrobenzocyclophosphamide analogues were evaluated as substrates of *E. coli* nitroreductase by incubation of each compound (0.2 mM) in 10 mM phosphate buffer, pH 7.0 at 37 °C in the presence of 1 mM NADH as the cofactor. The reaction was initiated by the addition 1.8 μg *E. coli* nitroreductase. Aliquots were withdrawn at various time intervals, quenched with acetonitrile and stored frozen prior to HPLC analysis. The half lives calculated based on the disappearance of the substrate are tabulated in Table 1. All four compounds were found to be substrates of *E. coli* nitroreductase with half lives between 7 and 24 min, though not as good a substrate as CB1954, which has a half life of 5 min under the same assay conditions.



Scheme 2. Proposed mechanism of activation of nitrobenzocyclophosphamides **6a–d** by bioreduction.



Scheme 3. Synthesis of the dioxaphosphorinane analogue **6a**. Reagents and conditions: (a) Ac_2O (10 equiv), pyridine (1.2 equiv), 0 °C to rt, 6 h, 91%; (b) NBS/CCl_4 (1.0 equiv), hν, rt, 14 h, 83%; (c) $CaCO_3$ (5.2 equiv), dioxane–H_2O (1:1), reflux, 3 h, 82%; (d) bis(2-chloroethyl)phosphoramidic dichloride (1.0 equiv), Et_3N (2 equiv), EtOAc, rt, 18 h, 55%.



Scheme 4. Synthesis of the benzo[e]cyclophosphamide analogue **6b**. Reagents and conditions: (a) potassium phthalimide (1.2 equiv), 18-crown-6 (0.1 equiv), rt, 20 h, 52%; (b) NH_2NH_2 (2.4 equiv), CH_2Cl_2–CH_3OH (1:1), rt, 14 h, 61%; (c) bis(2-chloroethyl)phosphoramidic dichloride (1.0 equiv), Et_3N (2 equiv), EtOAc, rt, 14 h, 62%.

CB1954, an excellent substrate of *E. coli* nitroreductase, is currently in clinical trials and was used as a control in our experiments.[18,25] It should be noted that compound **6d** only reached an end point of 58% while all other compounds reached end points of less than 10% (Fig. 2). This behavior of compound **6d** in reduction by nitroreductase is not understood. Since each of our four compounds contains a chiral phosphorus center, and because we used racemic mixtures in our assays, one possibility is that one enantiomer might not be as good of a substrate of *E. coli* nitroreductase as the other. It is also possible that the nitroreductase enzyme was being inhibited by the product formed upon reduction by the enzyme. No loss of substrate was observed when the *E. coli* nitroreductase was replaced with 50 μg/mL of the human enzyme NQO1 and it was concluded that these compounds are not substrates for this enzyme.

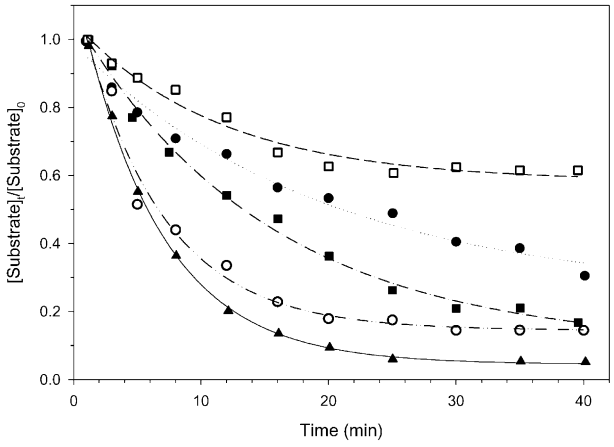
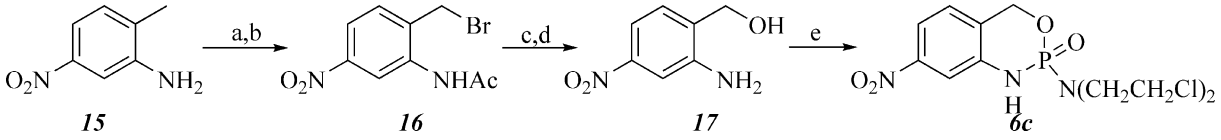


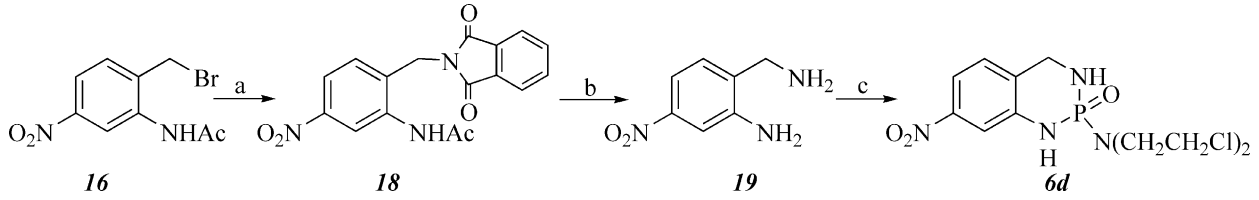
Fig. 2. The disappearance of **6a** (●), **6b** (○), **6c** (■), **6d** (□), and CB1154 (▲) during reduction by *E. coli* nitroreductase as monitored by HPLC. Substrate (0.2 mM) was incubated with 1.8 μg of *E. coli* nitrorectase in 10 mM phosphate buffer, pH 7.0 in the presence of 1 mM of NADH at 37 °C in a total volume of 250 μL.

Antiproliferative activity in cell culture

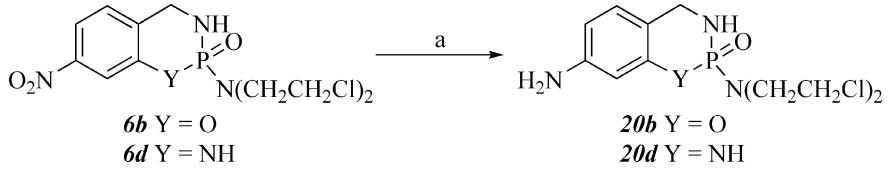
Compounds **6a–d** were assayed for their cytotoxicity against cells expressing either *E. coli* nitroreductase (T116) or the human quinone oxidoreductase enzyme NQO1 (hDT7). Cells were Chinese hamster V79 cells that had been transfected with a bicistronic vector encoding for the *E. coli* nitroreductase or the human quinone oxidoreductase protein and puromycin resistance protein as the selective marker. F179 cells were transfected with vector only and were used as the controls. The cells were exposed for 72 h to each test compound and the maximum concentration used was 100 μM. With the exception of compound **6b**, which has an IC_{50} of 61 μM in the control cells, most compounds were not cytotoxic at 100 μM in the control cells. The IC_{50} and the ratios of IC_{50} (F179/T116) of our test compounds are tabulated in Table 1. In calculating the ratio of IC_{50}, the value of 100 μM was used for those compounds with an undetermined $IC_{50} > 100$ μM so the ratio is an underestimate. As shown in Table 1, compounds **6a**, **6c**, and **6d** are not very cytotoxic in their own right and were not activated by endogenous mammalian enzymes (at least not those found in V79 cells). The results with the *E. coli* nitroreductase-expressing cells is much more interesting. Generally, the T116 cells are more cytotoxically effected by our test compounds than the control cells. All compounds except **6d** tested show ratios >1 indicating activation by *E. coli* nitroreductase. Compounds **6b** and **6d** were found to have similar IC_{50} values in cells expressing or not expressing



Scheme 5. Synthesis of the benzo[e]cyclophosphamide analogue **6c**. Reagents and conditions: (a) Ac_2O (10 equiv), pyridine (1.1 equiv), rt, 6 h, 84%; (b) NBS/CCl_4 (1.2 equiv), hv, rt, 20 h, 56% based on recovered starting material; (c) $CaCO_3$ (6 equiv), dioxane–H_2O (1:1), reflux, 3 h, 42%; (d) 6 N HCl, rt, 16 h, 100%; (e) bis(2-chloroethyl)phosphoramidic dichloride (1.0 equiv), Et_3N (2 equiv), EtOAc, rt, 48 h, 23%.



Scheme 6. Synthesis of the diazaphosphorinane analogue **6d**. Reagents and conditions: (a) potassium phthalimide (1.5 equiv), 18-crown-6 (0.1 equiv), rt, 24 h, 65%; (b) 6 N HCl, 50 °C, 5 h, 64%; (c) bis(2-chloroethyl)phosphoramidic dichloride (1.0 equiv), Et_3N (2 equiv), EtOAc, rt, 3 h, 35%.



Scheme 7. Hydrogenation of analogues **6b** and **6d**. Reagents and conditions: (a) H_2, 10% Pd/C, 97% for **20b**, 52% for **20d**.

E. coli nitroreductase even though both were reduced by *E. coli* nitroreductase as shown in our enzyme assays. Both of these compounds contain a benzylic nitrogen, instead of a benzylic oxygen, *para* to the nitro group. Chemical reduction of **6b** and **6d** produced stable amine products that are not expected to be alkylating agents. On the other hand, **6a** and **6c** with benzylic oxygen at the *para* position to nitro group gave no clearly identifiable products upon chemical reduction. **6a** and **6c** were found to be over 30-fold more cytotoxic in *E. coli* nitroreductase-expressing cells with IC_{50} values of around 3 μM. These results indicate that *E. coli* nitroreductase-reduction was an important first step but not sufficient for enhanced cytotoxicty in *E. coli* nitroreductase-expressing cells. Although we have not determined experimentally the reactive species responsible for the enhanced cytotoxicity in *E. coli* nitroreductase-expressing cells, we believe that nitroreductase converts **6a** and **6c** to their corresponding amino or hydroxylamine analogue **7a** and **7c**, which would then follow the electron 'push and pull' mechanism outlined in Scheme 2 to produce the observed cytotoxicity. It should be pointed out that the 33–36-fold activation shown by **6a** and **6c** in *E. coli* nitroreductase-expressing cells is about 100 fold less than that shown by CB1954. However, our benzocyclophosphamides represent a new chemo type for reductive activation by nitroreductases and further structure modification of **6a** and **6c** might lead to the development of a new generation of cyclophosphamide analogues that are activated by *E. coli* nitroreductase and shown to have enhanced cytotoxicity in *E. coli* nitroreductase-expressing cells. These new analogues might then be used in conjunction with nitroreductase in enzyme prodrug therapy in the treatment of cancer.

In summary, a series of four benzocyclophosphamide analogues were designed and synthesized incorporating a strategically placed nitro group in a position *para* to the benzylic carbon for reductive activation. All four analogues were found to be stable in phosphate buffer at pH 7.4 and 37 °C. They are all good substrates of *E. coli* nitroreductase with half lives between 7 and 24 min at pH 7.0 and 37 °C. However, only two compounds **6a** and **6c**, both with a benzylic oxygen in the phosphorinane ring *para* to the nitro group, showed a modest 33–36-fold enhanced cytotoxicity in *E. coli* nitroreductase-expressing cells. The other two analogues **6b** and **6d**, each with a benzylic nitrogen in the same position, showed no selective toxicity in cells expressing the *E. coli* nitroreductase enzyme. Chemical reduction of **6b** and **6d** resulted in the isolation of stable amine products. These results suggest that good substrate activity is not sufficient and a benzylic oxygen in the phosphorinane ring *para* to the nitro group is required for reductive activation of nitrobenzocyclophosphamide analogues by *E. coli* nitroreductase. Compounds **6a** and **6c** represent a new structure type for reductive activation and a new lead for further modification in the development of better analogues with much improved selective toxicity to be used in gene-directed enzyme prodrug therapy.

Table 1. *E. coli* nitroreductase (NR)-activation of nitrobenzocyclophosphamides **6a–d**

Compd	NR assay $t_{1/2}$ (min)[a]	IC_{50} (μM)[b]			Ratio[c] (F179/T116)
		F179	hDT7	T116	
6a	24	>100	>100	2.7	>36
6b	11	61	48	48	1.3
6c	13	>100	>100	3.0	>33
6d	7.8[d]	>100	>100	>100	~1
CB1954	5.0	>100	1.7	0.036	>2777

[a]Half lives of reduction by *E. coli* nitroreductase were determined using 0.2 mM of substrate in 10 mM phosphate buffer (pH 7.0) in the presence of 1 mM of NADH at 37 °C in a total volume of 250 μL. The reaction was initiated by the addition of 1.8 μg of *E. coli* nitroreductase. Aliquots were withdrawn and analyzed by HPLC.
[b]IC_{50} values are the concentration required to reduce cell number to 50% of control. Cytotoxicity was assayed against cells expressing either *E. coli* nitroreductase (T116) or human quinone oxidoreductase NQO1 (hDT7). Cells were Chinese hamster V79 cells that had been transfected with a bicistronic vector encoding for the *E. coli* nitroreductase or the human quinone oxidoreductase protein and puromycin resistance protein as the selective marker. F179 cells were transfected with vector only and were used as the controls.
[c]Ratio of IC_{50} values (F179/T116) as an indication of activation by *E. coli* nitroreductase.
[d]The catalysis seemed to reach an end point of 58%.

Experimental

General methods

Solvents were either ACS reagent grade or HPLC grade. Unless otherwise stated, all reactions were magnetically stirred and monitored by thin-layer chromatography (TLC) using 0.25 mm Whatman precoated silica gel plates. TLC plates were visualized using either 7% (w/w) ethanolic phosphomolybdic acid or 1% (w/w) aqueous potassium permagnate containing 1% (w/w) $NaHCO_3$. Flash column chromatography was performed using silica gel (Merck 230–400 mesh). Yields refer to chromatographically and spectroscopically (^1H NMR) homogeneous materials, unless otherwise noted. All reagents were purchased at the highest commercial quality and used without further purification.

Infrared spectra were recorded with a Perkin–Elmer model 1600 series FTIR spectrometer using polystyrene as an external standard. Infrared absorbance is reported in reciprocal centimeters (cm^{-1}). All ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer at ambient temperature and calibrated using residual undeuterated solvents as the internal reference. Chemical shifts (300 MHz for ^1H and 75 MHz for ^{13}C) are reported in parts per million (δ) relative to CD_3OD (δ 3.3 for ^1H and 49.0 for ^{13}C). Coupling constants (*J* values) are given in hertz (Hz). The following abbreviations were used to explain the multiplicities: s = singlet; d = doublet; t = triplet; q = quartet; p = quintet; m = multiplet; br = broad. Mass spectral data were obtained from the University of Kansas Mass Spectrometry Laboratory (Lawrence, KS).

Acetic acid, 2-bromomethyl-5-nitrophenyl ester (11). 2-Methyl-5-nitrophenol **10** (2.5 g, 13 mmol) was dissolved in 50 mL of acetic anhydride (10 eq) and immersed in an

ice water bath. After the addition of pyridine (2 mL, 1.2 equiv), the reaction mixture was stirred at room temperature for 6 h. Excess acetic anhydride was removed under reduced pressure and the residue was dissolved in 100 mL of CH_2Cl_2, washed with satd $NaHCO_3$, water, dried over Na_2SO_4. 2-Methyl-5-nitrophenyl acetate was obtained as a white solid (2.9 g, 91%). Mp 68–72 °C, ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.04 (d, 1H, J = 8.4 Hz), 7.93 (s, 1H), 7.40 (d, 1H, J = 8.4 Hz), 2.37 (s, 3H), 2.29 (s, 3H); MS (FAB$^+$) m/z (relative intensity) 196 (MH$^+$, 12.9), 195 (50.8), 152 (54.1), 135 (70.5), 119 (100).

2-Methyl-5-nitrophenyl acetate (2.9 g, 14.9 mmol) and *N*-bromosuccinimide (2.65 g, 14.9 mmol) were suspended in 50 mL of carbon tetrachloride, and photolyzed with a 300 watt lamp under N_2 for 14 h. The reaction mixture was then diluted with 50 mL of methylene chloride, washed with water and brine, dried over anhydrous Na_2SO_4. The residue after removal of solvents was purified through flash column chromatography to afford the desired product **11** (3.27 g, 83%). Mp 76.5–78 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.09–8.04 (m, 2H), 7.60 (d, 1H, J = 8.4 Hz), 4.44(s, 2H), 2.43 (s, 3H). MS (FAB$^+$) m/z (relative intensity) 196 (MH$^+$-Br, 7.9), 195 (82.3).

2-Hydroxy-4-Nitrobenzyl alcohol (12). Compound **11** (200 mg, 0.7 mmol) dissolved in 2 mL of dioxane, was mixed with 5.2 equiv of $CaCO_3$ in 2 mL of H_2O and the reaction mixture was heated to reflux for 3 h. After the disappearance of starting material as shown by TLC, dioxane was removed by evaporation and the residue was treated with 5 mL of 2 N HCl and extracted with 30 mL of EtOAc. The combined extract was washed with brine (3×30 mL) and dried over anhydrous Na_2SO_4. Final separation through flash column chromatography afforded the desired product **12** (101 mg, 81.9%). Mp 145–149 °C. ^{1}H NMR (300 MHz, $CDCl_3$), δ 7.70 (s, 1H), 7.69 (d, 1H, J = 9.0 Hz), 7.34 (d, 1H, J = 9.0 Hz), 4.81 (s, 2H), 4.53 (s, 1H), 2.20 (s, 1H). MS (EI) m/z (relative intensity) 169 (41.6, M$^+$), 151 (100), 105 (54.4), 77 (78.4).

7-Nitro-2-[bis(2-chloroethyl)amino]-1,3,2-benzodioxaphosphorinane-2-oxide (6a). Compound **12** (100 mg, 0.59 mmol) was dissolved in 1 mL of EtOAc and mixed with 2.0 equiv of Et_3N and a solution of bis(2-chloroethyl)phosphamidic dichloride (153 mg, 1.0 equiv) in 1 mL of EtOAc. The mixture was stirred at room temperature for 18 h. After removal of the precipitate through filtration, the filtrate was purified by flash column chromatography to give 1,3-dioxa analogue **6a** as an yellow oil (114.7 mg, 54.6%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.02 (d, 1H, J = 8.4 Hz), 7.92 (s, 1H). 7.32 (d, 1H, J = 8.4 Hz), 5.71–5.24 (m, 2H), 3.67 (t, 4H, J = 6.6 Hz), 3.55–3.46 (m, 4H); IR (neat) 2960–2820, 1520, 1420, 1340, 1260, 970, 840 and 726 cm^{-1}; MS (FAB$^+$) m/z (relative intensity) 355(MH$^+$, 12.6), 307(16.2), 289(8.9), 154(100); HRMS (FAB$^+$) m/z calcd for $C_{11}H_{14}Cl_2N_2O_5P$: 355.0017, found: 354.9992.

2-Acetoxy-4-nitro-α-phthalimido toluene (13). Compound **11** (3.9 g, 14.2 mmol) was dissolved in 50 mL of toluene and mixed with potassium phthalimide (2.63 g, 1.2 equiv) and 18-crown-6 (375 mg, 0.1 equiv). The suspension was stirred at room temperature for 20 h. The reaction mixture was then diluted with 50 mL of water and extracted with methylene dichloride. The CH_2Cl_2 extract was washed with 5% citric acid, saturated $NaHCO_3$, and H_2O. After drying over anhydrous Na_2SO_4 and removal of solvent, the residue was purified through flash column chromatography to give the desired product **13** (2.5 g. 52%). Mp 175–178 °C, ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.17–7.73 (m, 7H,), 4.89 (s, 2H), 2.47 (s, 3H). MS (FAB$^+$) m/z (relative intensive) 341 (MH$^+$, 5), 299 (7), 195 (33), 152 (39), 135 (100). HRMS (FAB$^+$) m/z calcd for $C_{17}H_{13}N_2O_6$: 341.0773, found: 341.0773.

2-Hydroxy-4-nitrobenzylamine (14). To a solution of compound **13** (2.5 g, 7.35 mmol) in 50 mL of 1:1 mixture of CH_2Cl_2 and CH_3OH was added 2.4 equiv of hydrazine. The reaction mixture was stirred at room temperature for 14 h. After removal of solvent under reduced pressure, the residue was treated with 6 N HCl (50 mL) and stirred at room temperature for 1 h. The filtrate was neutralized to pH = 7 with aqueous NaOH solution and extracted with EtOAc. The combined EtOAc extract was dried over anhydrous Na_2SO_4 and concentrated to dryness to afford the desired product **14** (0.752 g, 60.6%). Mp 210–215 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 7.45 (s, 1H), 7.42 (d, 1H, J = 8.1 Hz), 7.26 (d, 1H, J = 8.1 Hz), 4.00 (s, 2H). ^{1}H NMR (300 MHz, DMSO) δ 7.55 (d, 1H, J = 8.4 Hz), 7.43 (s, 1H), 7.36 (d, 1H, J = 8.4 Hz), 3.91 (s, 2H). MS (FAB$^+$) m/z (relative intensity) 169 (MH$^+$, 7), 154 (100), 136 (69). HRMS (FAB$^+$) m/z calcd for $C_7H_9N_2O_3$: 169.0613, found: 169.0613.

7-Nitro-2-[bis(2-chloroethyl)amino]-1,3,2-benzoxazaphosphorinane-2-oxide (6b). To a solution of compound **14** (752 mg, 4.47 mmol) and 2.0 equiv of Et_3N in 20 mL of EtOAc was added dropwise with stirring a solution of 1.0 equiv of bis(2-chloroethyl)phosphoramidic dichloride (1.16 g, 4.47 mmol) in 20 mL of EtOAc. After strirring was continued for 14 h, the precipitate was removed by suction filtration and the filtrate was concentrated under reduced pressure. The residue was purified by flash column chromatography to afford the desired product **6b** (974 mg, 61.9%). Mp 123–126 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.97 (d, 1H, J = 8.1 Hz), 7.90 (s, 1H), 7.29 (d, 1H, J = 8.1 Hz), 4.61–4.31 (m, 2H), 3.80 (s, 1H), 3.72–3.59 (m, 4H), 3.57–3.47 (m, 4H). IR (neat) 3100, 1480, 1304, 1175, 1045, 925 and 804 cm^{-1}; MS (FAB$^+$) m/z (relative intensity) 354 (MH$^+$, 3.3), 309 (6.5), 195 (28), 152 (68), 135 (90), 119 (100). HRMS (FAB$^+$) m/z calcd for $C_{11}H_{15}N_3O_4Cl_2P$: 354.0177, found: 354.0181.

2-Acetamido-4-nitrobenzyl bromide (16). To a solution of 2-methyl-5-nitroaniline **15** (3.04 g, 2 mmol) in 50 mL of $CHCl_3$ were added Ac_2O (10 equiv) and pyridine (1.78 mL, 1.1 equiv). The reaction mixture was stirred at room temperature overnight. After concentration under reduced pressure, the residue was dissolved in 100 mL of CH_2Cl_2, washed with water, satd $NaHCO_3$ and water,

and dried over anhydrous Na_2SO_4. After removal of solvent, the residue was triturated with CCl_4 to give the desired product 2-acetamido-4-nitrotoluene as a solid (3.36 g, 84%). Mp 154–155 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.76 (s, 1H), 7.94 (d, 1H, J = 8.1 Hz), 7.34 (d, 1H, J = 8.1 Hz), 7.09 (br, 1H), 2.37 (s, 3H), 2.26 (s, 3H).

2-Acetamido-4-nitrotoluene (1.0 g, 3.66 mmol) and *N*-bromosuccinimide (0.78 g, 1.2 equiv) were suspended in 100 mL of CCl_4 and photolized with a 300 watt lamp under N_2 for 20 h. After removal of solvent under reduced pressure, the residue was subjected to flash column chromatography to afford the desired product **16** (0.46 g, 55.6% after recovery of 0.2 g of starting material). Mp 187.5–189 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.84 (s, 1H), 8.00 (d, 1H, J = 8.4 Hz), 7.54 (br, 1H), 7.50 (d, 1H, J = 8.4 Hz), 4.52 (s, 2H), 2.32 (s, 3H); MS (FAB^+) m/z (relative intensity) 273 (MH^+, 5.6), 195 (25.7), 153 (33.1), 135 (100).

2-Amino-4-nitrobenzyl alcohol (17). Compound **16** (163 mg, 0.6 mmol) dissolved in 2 mL dioxane was mixed with a suspension of $CaCO_3$ (358.5 mg, 3.6 mmol) in 2 mL of water. The mixture was then heated up to reflux for 3 h until all starting material disappeared as monitored by TLC. After removal of solvent under reduced pressure, the residue was treated with 2 mL of 2 N HCl and extracted with CH_2Cl_2. The organic extract was dried over Na_2SO_4 and subjected to flash column chromatography to give 2-acetamido-4-nitrobenzyl alcohol (53.2 mg, 42.2%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.01 (d, 1H, J = 2.1 Hz), 8.87 (br, 1H), 7.91 (dd, 1H, J_1 = 2.1 Hz, J_2 = 8.1 Hz), 7.32 (d, 1H, J = 8.1 Hz), 4.82 (d, 2H, J = 5.7 Hz), 2.53 (t, 1H, J = 5.7 Hz), 2.24 (s, 3H). MS (FAB^+) m/z (relative intensity) 211 (MH^+, 7.5), 195 (34.0), 152 (42.0), 135 (100).

2-Acetamido-4-nitrobenzyl alcohol (53.2 mg, 0.316 mmol) was treated with 1 mL of 6 N HCl and the reaction mixture was stirred at room temperature overnight. After neutralization with 6 N aqueous NaOH solution to pH 10, the reaction mixture was extracted with EtOAc, dried over Na_2SO_4, purified through flash column chromatography to give desired product **17** (46 mg, 100%). Mp 178–180 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.56–7.51 (m, 2H, aromatic), 7.20 (d, 1H, J = 8.1 Hz, aromatic), 4.74 (d, 2H, J = 4.5 Hz), 4.52 (br s, 2H), 1.72 (t, 1H, J = 4.5 Hz). MS (EI) m/z (relative intensity) 168(M^+, 100), 150(60.8).

7-Nitro-2-[bis(2-chloroethyl)amino]-3,1,2-benzoxazaphosphorinane-2-oxide (6c). To a solution of **17** (46 mg, 0.27 mmol) in 0.5 mL of EtOAc were added with stirring Et_3N (54.6 mg, 0.54 mmol) and bis(2-chloroethyl)phosphoramidic dichloride (70.8 mg, 0.27 mmol) in 0.5 mL EtOAc. After 48 h, the precipitate was removed by suction filtration and the filtrate was concentrated under reduced pressure. The residue was purified through flash column chromatography to give the desired product **6c** as a yellow solid (21.6 mg, 22.5%). Mp 138–142 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.78 (dd, 1H, J_1 = 2.4 Hz, J_2 = 8.1 Hz), 7.69 (d, 1H, J = 2.4 Hz), 7.22 (d, 1H, J = 8.1 Hz), 6.57 (d, 1H), 5.56–5.07 (m, 2H), 3.69–3.62 (m, 4H), 3.48–3.39 (m, 4H). IR (neat) 3600–3000 (broad), 2930, 2860, 1600, 1520, 1450, 1340, 1220, 970, 880, 820, and 735 cm^{-1}. MS (FAB^+) m/z (relative intensity) 354 (MH^+, 4.9), 307 (20.0), 289 (12.6), 154 (100), 136 (98.8). HRMS (FAB^+) m/z calcd for $C_{11}H_{15}Cl_2N_3O_4P$: 354.0177, found: 354.0162.

2-Acetamido-4-nitro-α-phthalimido toluene (18). A solution of compound 16 (45.9 mg, 0.168 mmol) in 2 mL of THF was mixed with 1.5 equiv of potassium phthalimide (146.6 mg) and a catalytic amount of 18-Crown-6 (4.4 mg, 0.1 equiv). The reaction mixture was stirred at room temperature for 24 h. After removal of solvent, the residue was taken up in 20 mL of CH_2Cl_2, washed with 5% citric acid, satd $NaHCO_3$, and water, and dried over Na_2SO_4. Purification through flash column chromatography afforded the desired product **18** (37.2 mg, 73.3% after recovery of 5 mg of starting material). Mp 221.3–224 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.97 (s, 1H), 7.96–7.76 (m, 6H), 4.88 (s, 2H), 2.39 (s, 3H). MS (FAB^+) m/z (relative intensity) 340 (MH^+, 6.2), 307 (16.9), 289 (9.9), 273 (4.0), 154 (100), 136 (67.2).

2-Amino-4-nitrobenzylamine (19). Compound **18** (50 mg, 0.15 mmol) was suspended in 2 mL of 6 N HCl and stirred at 50 °C for 5 h. After filtration to remove the solid, the filtrate was neutralized to pH 10 and extracted with EtOAc. The EtOAc extract was dried over anhydrous Na_2SO_4. Removal of EtOAc afforded the desired compound **19** (15.7 mg, 63.8%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.51 (dd, 1H, J_1 = 2.4 Hz, J_2 = 8.1 Hz), 7.49 (d, 1H, J = 2.4 Hz), 7.15 (d, 1H, J = 8.1 Hz), 3.97 (s, 2H).

7-Nitro-2-[bis(2-chloroethyl)amino]-1,3,2-benzodiazaphosphorinane-2-oxide (6d). To a solution of **19** (358 mg, 2.14 mmol) in 8 mL of EtOAc were added with stirring Et_3N (433 mg, 4.28 mmol) and bis(2-chloroethyl)-phosphoramidic dichloride (554 mg, 2.14 mmol) in 2 mL of EtOAc. After the reaction mixture was stirred for an additional 3 h, the precipitate was removed by suction filtration and the filtrate was concentrated under reduced pressure. The residue was purified through flash column chromatography to give the desired product **6d** as a yellow solid (263 mg, 34.6%). Mp 168–169.5 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.74 (dd, 1H, J_1 = 2.4 Hz, J_2 = 8.4 Hz), 7.65 (d, 1H, J = 2.4 Hz), 7.16 (d, 1H, J = 8.4 Hz), 6.23 (br s, 1H), 4.46–4.12 (m, 2H), 3.66 (t, 4H, J = 5.7 Hz), 3.48–3.37 (m, 4H), 3.24 (br s, 1H). MS (FAB^+) m/z (relative intensity) 324 (MH^+, 4.2), 307 (17.9), 289 (10.4), 273 (4.6), 154 (100), 147(58.2), 136 (68.7). HRMS(FAB^+) m/z calcd for $C_{11}H_{17}Cl_2N_3O_2P$: 324.0435, found: 324.0435.

Stability test of benzocyclophosphamides (6a, 6b, 6c, 6d) in aqueous buffer. A 2 mg sample of a benzocyclophosphamide (**6a**, **6b**, **6c** or **6d**) was dissolved in 2 mL of 50 mM sodium phosphate buffer (pH = 7.40) containing 10% DMSO and incubated at 37 °C. At different time intervals, aliquots were withdrawn and subjected to reversed phase HPLC analysis (C_{18} analytical column, gradient elution from 5–80% acetonitrile containing 0.1% TFA at a flow rate of 1 mL/min).

Enzyme assays

Substrate (0.2 mM) was incubated with 1 mM of NADH at 37 °C in 10 mM phosphate buffer (pH 7.0) in a total volume of 250 μL. The reaction was initiated by the addition of 1.8 μg of *E. coli* nitroreductase. Aliquots were withdrawn and analyzed by HPLC. The half life of reduction by *E. coli* nitroreductase was calculated based on the disappearance of the substrate.

Plasmid vector construction

Bicistronic eukaryotic expression vectors containing the coding regions for either human NQO1 or *E. coli* nitroreductase together with puromycin acetyl transferase (conferring puromycin resistance) driven from a single CMV promoter was constructed by cloning into the XhoI site of the vector pIRES-P (EMBL:Z75185) using conventional techniques. Insert orientation and identity were confirmed by diagnostic restriction digests and dideoxy sequencing using a Sequenase II kit (Amersham Pharmacia Biotech, St Albans, Herts, UK).

Cell culture and transfection

V79 Chinese hamster lung fibroblasts were grown in monolayer culture in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum and 4 mM glutamine (all from GibcoBRL, Life Technologies Ltd, Paisley, Scotland, UK). Cells were maintained in a humidified atmosphere at 37 °C with 5% CO_2 and subcultured twice weekly by trypsinization. All cells were determined to be free of mycoplasma. Plasmid vectors were transfected into cells by calcium phosphate coprecipitation (Profection, Promega, Southampton, Hampshire, UK) and positive clones were selected in growth medium containing 10μg/mL puromycin and maintained under selective pressure. Individual clones were screened for either NQO1 or NR activity and a suitable nitroreductase (designated T116) or NQO1 (designated hDT7) expressing clone selected for further use. Transfection of the empty vector supplied a suitable puromycin-resistant control cell line (designated F179).

Growth inhibition was measured by the sulforhodamine B method.[26] Cells in exponential phase of growth were trypsinized, seeded in 96-well plates at a density of 500 cells per well (100 μL) and permitted to recover for 24 h. For testing, the compounds were dissolved in dimethyl sulfoxide to give 100 mM stock solutions. These were diluted into medium to 300 μM, that were then serially diluted in situ (8- of 3-fold) giving final concentrations of 100–0.046 μM. Cells were then incubated with the drug for 3 days at 37 °C. The plates were fixed and stained with sulforhodamine-B (SRB), before reading optical absorption at 570 nm; results were expressed as percentage of control growth. The cytotoxicity of each compound was expressed as that concentration producing 50% inhibition of cell growth (IC_{50}) compared with cells incubated with medium only and evaluated by interpolation from the dose response curve.

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