Letters

Nitroaryl Phosphoramides as Novel Prodrugs for *E. coli* Nitroreductase Activation in Enzyme Prodrug Therapy

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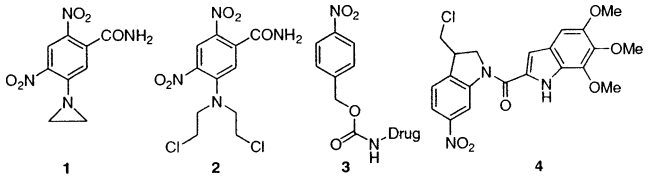
Abstract: Cyclic and acyclic nitroaryl phosphoramide mustard analogues were activated by *E. coli* nitroreductase, an enzyme explored in GDEPT. The more active acyclic 4-nitrobenzyl phosphoramide mustard (**7**) showed 167 500× selective cytotoxicity toward nitroreductase-expressing V79 cells with an IC_{50} as low as 0.4 nM. This is about 100× more active and 27× more selective than CB1954 (**1**). The superior activity was attributed to its better substrate activity (k_{cat}/K_m 19× better than **1**) and/or excellent cytotoxicity of phosphoramide mustard released.

Introduction. Prodrug design is an important strategy that has been proven to work for many drugs in improving their undesirable physicochemical and biological properties.[1−3] 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 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.[4] Among the enzymes under evaluation is the *nfsB* gene product of *Escherichia coli*, an oxygen-insensitive flavin mononucleotide-containing nitroreductase (NTR). This flavoprotein is capable of reducing certain aromatic nitro groups to the corresponding hydroxylamines in the presence of the cofactor NADH or NADPH.[5−7]

Reduction of aromatic nitro groups to hydroxylamines or amines represents a very large electronic change and can provide an efficient "switch" that can be exploited to generate potent cytotoxins.[8] This approach was used for the targeted delivery of toxic alkylating species to hypoxic tumor cells.[9,10] For activation by *E. coli* nitroreductase, four classes of prodrugs have been described: dinitroaziridinylbenzamides (e.g., **1**, CB1954), dinitrobenzamide mustards (e.g., **2**, SN 23862,), 4-nitrobenzylcarbamates (**3**), and nitroindolines (e.g., **4**).[8,11,12] Of the four classes, the first two are considered the most promising when used in conjunction with NTR.[13] Compound **1**, currently under Phase I clinical trial in conjunction with the virally delivered NTR enzyme,[14] has high selectivity (>1000-fold) in cell lines transfected with NTR and has potent and long-lasting inhibition of nitroreductase-transfected tumors in mice. The related mustard **2** has similar selectivity and good bystander effects in animal models. Here, we would like to report a class of nitroaromatics that are excellent substrates of NTR and that release a known highly cytotoxic phosphoramide mustard or like-reactive species upon NTR-reduction.



Chemistry. The design of our nitroaromatics is based on the activation mechanism of the clinically useful anticancer drug cyclophosphamide. Cyclophosphamide is an anticancer prodrug, which has to be activated by the cytochrome P-450 enzyme in the liver.[15−17] Hepatic cytochrome P-450 oxidation converts cyclophosphamide to 4-hydroxyphosphamide, which ultimately forms the cytotoxic alkylating species, phosphoramide mustard, following ring opening and β-elimination. Our efforts have been focused on designing phosphoramide analogues incorporating site-specific activation mechanisms such as nitro reduction in order to move the site of activation from liver into the tumor tissues.

Among the nitroaryl phosphoramides designed and synthesized were compounds **5**, **6**, and **7**, each with a strategically placed nitro group on the benzene ring in the para position to the benzylic carbon. Compound **5** is a cyclophosphamide analogue with the cyclophosphamide ring fused with a benzene ring and a nitro group placed in the para position to the benzylic carbon. Compound **6** is a 4-nitrophenyl-substituted cyclophosphamide analogue, and **7** is an acyclic nitrobenzyl phosphoramide mustard. The nitro group is a strong electron-withdrawing group (Hammett σ_p electronic parameter = 0.78) and is converted to an electron-donating hydroxylamino group ($\sigma_p = -0.34$) upon NTR-reduction. This large difference in electronic effect ($\Delta\sigma_p = 1.12$) is exploited to effect the formation of the highly cytotoxic phosphoramide mustard or like-reactive species as shown in Scheme 1. After reduction by NTR, the resulting hydroxylamines **8**, **10**, and **12** will relay their

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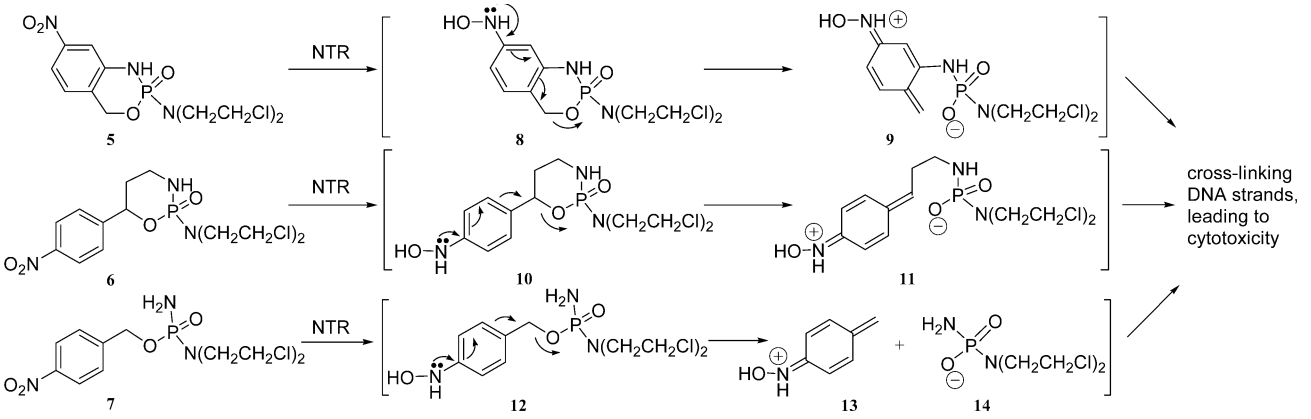
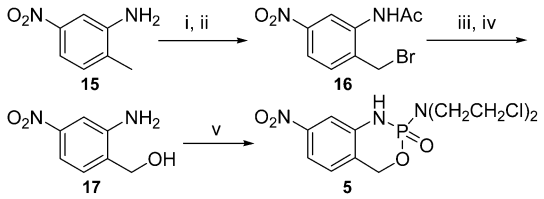
Scheme 1. Proposed Mechanism of Activation of Nitroaryl Phosphoramides by Bioreduction

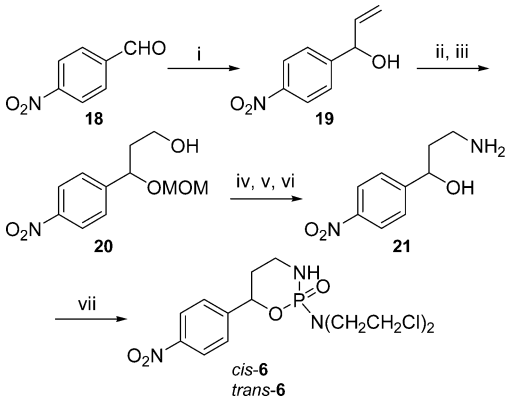


Scheme 2. Synthesis of 7-Nitrobenzocyclophosphamide **5**[a]



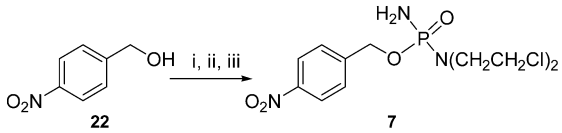
[a] Reagents and conditions: (i) Ac_2O, pyridine, 6 h, 84%; (ii) NBS/CCl_4, *hv*, 20 h, 56%; (iii) $CaCO_3$, dioxane−H_2O, Δ, 3 h, 42%; (iv) 6 N HCl, rt, 16 h, 100%; (v) bis(2-chloroethyl)phosphoramidic dichloride, Et_3N, EtOAc, 48 h, 23%.

Scheme 3. Synthesis of 6-(4-Nitrophenyl)cyclophosphamide **6** Isomers[a]



[a] Reagents and conditions: (i) CH_2=$CHMgBr$, THF, −78 °C to −60 °C, 4 h, 95%; (ii) MOMCl/DIEA/CH_2Cl_2, 0 °C to rt, 24 h, 95%; (iii) B_2H_6/THF, 0 °C, 5 h, then 3 N NaOH, 30% H_2O_2, 30 min, 78%; (iv) MsCl/ Et_3N, 0 °C, 15 min then NaN_3, 15-crown-5 (cat.), DMF, 91%; (v) PPh_3, THF−H_2O, 72%; (vi) CBB followed by HOAc, CH_2Cl_2, −20 °C, 76%; (vii) bis(2-chloroethyl)phosphoramidic dichloride, Et_3N, EtOAc, 48 h, *cis*-**6**, 34%, *trans*-**6**, 33%.

Scheme 4. Synthesis of 4-Nitrobenzyl Phosphoramide Mustard **7**[a]



[a] Reagents and conditions: (i) BuLi,,-78 °C; (ii) bis(2-chloroethyl)phosphoramidic dichloride, THF, −78 °C, 2 h; (iii) NH_3, −78 °C, 30 min, 53% for the three steps.

electrons to the para-position and facilitate the cleavage of the benzylic C−O bond, producing the anionic cytotoxic species phosphoramide mustard (**14**) or like-reactive species (**9** and **11**). Structurally, the phosphoramide portion in **9** and **11** closely resembles phosphoramide mustard (**14**), the reactive alkylating agent produced following the metabolic activation of cyclophosphamide in the liver, and could also be the ultimate cytotoxic alkylating agent. In addition, compounds **9**, **11**, and **13** possess additional electrophilic centers that might form adducts with functionally important macromolecules, providing additional mechanisms for cytotoxicity. The mechanism of activation proposed here is similar to that proposed for the hypoxic reductive activation of 4-nitrobenzyl *N*,*N*,*N*′,*N*′-tetrakis(2-chloroethyl)phosporodiamidate[9] and the reductive activation of 4-nitrobenzyl carbamates.[12,18]

Compounds **5**, **6**, and **7** were synthesized by the reaction sequences shown in Schemes 2−4. Bromination of acetylated 2-methyl-5-nitroaniline (**15**) followed by hydrolysis and triethylamine-mediated cyclization with bis(2-chloroethyl)phosphoramidic dichloride gave compound **5** (Scheme 2).[19,20] For the synthesis of compound **6**, a Grignard reaction with vinylmagnesium bromide followed by protection and hydroboration−oxidation was used to convert 4-nitrobenzaldehyde (**18**) to monoprotected 1,3-diol **20**. Subsequent activation, S_N2 displacement, and reduction afforded 1,3-amino alcohol **21**. Cyclization of **21** with bis(2-chloroethyl)phosphoramidic dichloride gave our desired product **6** as a pair of cis and trans diastereomers, which were separated on flash silica gel column chromatography (Scheme 3). Here, *cis* and *trans* refer to the relative orientation of the 6-(4-nitrophenyl) and the 2-oxo substituents on the 1,3,2-oxazaphosphorinane ring. Compound **7** was synthesized according to a modified literature procedure (Scheme 4).[10] All new compounds were fully characterized by IR, ^1H, ^{13}C, and ^{31}P NMR and high-resolution mass spectrometry.

Results and Discussion. Two types of cells were used to evaluate the antiproliferative activities of our compounds in combination with *E. coli* nitroreductase: the V79 Chinese hamster fibroblast cells and the SKOV3 human ovarian carcinoma cells. The Chinese hamster V79 cells were transfected with a bicistronic vector encoding for the NTR protein and puromycin resistance protein as the selective marker. The corresponding V79 cells, transfected with vector only, were used as controls. The SKOV3 human ovarian carcinoma cells were infected with a replication-defective adenovi-

Table 1. >IC_{50} Values (μM) and IC_{50} Ratio (NTR−/NTR+) for Compounds **5**, **6**, and **7** in Comparison to Compound **1**[a]

	V79 cells, 72 h drug exposure			V79 cells, 1 h drug exposure			SKOV3 cells, 18 h drug exposure		
compound	IC_{50},[b] NTR−	IC_{50},[b] NTR+	ratio[c]	IC_{50},[b]NTR−	IC_{50},[b] NTR+	ratio[c]	IC_{50},[b]NTR−	IC_{50},[b]NTR+	ratio[c]
5	>100	3.0	>33	—[d]	—[d]	—[d]	550	55	10
cis-**6**	832	0.029	28 690	>100	0.34	>294	680	4.7	145
trans-**6**	608	0.027	22 519	>100	0.17	>588	>1000	5	>200
7	67	0.0004	167 500	>100	0.01	>10 000	>1000	1.2	>833
							>1000	6.2[e]	>161[e]
1	254	0.036	7056	>100	0.31	>323	>1000	4.7	>213
							625	52[e]	12[e]

[a] Cells were exposed to each test compound for a given amount of time, and a standard cell viability assay was performed 72 h after the addition of test compound. Initially, the maximum concentration used was 100 μM in the case of V79 cells and 1000 μM in the case of SKOV3 cells. When necessary, assays were repeated at higher drug concentrations to obtain accurate IC_{50} values. [b] IC_{50} values are the concentration required to reduce cell number to 50% of control after the cells were exposed to the drug for the indicated time. [c] Ratio of IC_{50} values (NTR−/NTR+) as an indication of activation by *E. coli* nitroreductase. [d] Not determined. [e] SKOV3 cells for these experiments were infected at a lower infection ratio of 10 pfu/cell while the others in the same column were infected at a higher infection ratio of 100 pfu/cell. Cell lines: V79 (Chinese hamster fibroblast) and SKOV3 (human ovarian carcinoma).

rus vector vPS1233 expressing NTR,[21] and the uninfected SKOV3 cells were used as controls.

The IC_{50} values and their ratios (NTR−/NTR+) of compounds **5**, **6**, and **7** are provided in Table 1 in comparison with that of compound **1**. In calculating the ratio of IC_{50}, the value of 100 or 1000 μM was used for those compounds with an undetermined IC_{50} >100 or >1000 μM so the ratio was an underestimate. None of our test compounds were very cytotoxic in the absence of NTR, and they were not activated by endogenous mammalian enzymes, at least not those found in V79 and SKOV3 cells. In all cases, the NTR+ cells were more cytotoxically affected by the test compounds than the control cells and all gave ratios >1, indicating activation by NTR. For the 72 h drug exposure, the benzene ring-fused cyclophosphamide **5** was >33× more cytotoxic in the nitroreductase-expressing Chinese hamster V79 cells than in the control V79 cells that were transfected with a vector only, while compound **1** showed 7000× selectivity toward nitroreductase-expressing V79 cells under the same conditions. The IC_{50} values of the 6-(4-nitrophenyl)-substituted cyclophosphamide isomers (*cis*-**6** and *trans*-**6**) were about the same as that of compound **1** in nitroreductase-expressing V79 cells while both were 3−4× less toxic to V79 cells not expressing NTR. Thus, the advantage of *cis*-**6** and *trans*-**6** over compound **1** is their 22 000−28 000× selective cytotoxicity in targeting nitroreductase-expressing V79 cells. This selectivity is 3−4× better than compound **1**. The acyclic 4-nitrobenzyl phosphoramide mustard **7** turned out to be the most active compound with IC_{50} as low as 0.4 nM and a selectivity ratio of 167 500 against nitroreductase-expressing V79 cells upon 72 h exposure. This shows that **7** is 100× more active and 27× more selective than compound **1** in targeting nitroreductase-expressing V79 cells.

More importantly, compound **7** was quickly activated and had long lasting cytotoxic effects in *E. coli* nitroreductase-expressing Chinese hamster V79 cells. When the nitroreductase-expressing V79 cells were briefly exposed to each test compound for 1 h, **7** showed the best activity with an IC_{50} as low as 10 nM, while *cis*-**6**, *trans*-**6**, and compound **1** had IC_{50}'s around 300 nM. The fast activation kinetics and the lasting cytotoxic effects upon activation could potentially overcome an important hurdle known in the development of the enzyme−prodrug therapy using *E. coli* nitroreductase.[4]

In SKOV3 human ovarian cancer cells infected with 100 pfu/cell of adenovirus expressing NTR, compound **7** showed an IC_{50} that is about 4× lower than that of compound **1** and compound **6** isomers. When the level of NTR expression was made more limiting by infection with just 10 pfu/cell of the adenovirus, although both IC_{50}'s were higher, the differential increased such that the IC_{50} for compound **7** was about 9× lower than that for compound **1**. The higher IC_{50}'s of all prodrugs in the SKOV3 cells could be due to the possibly lower levels of NTR expressed in the virally transfected SKOV3 as compared to the stably transfected V79 cells. Another contributory factor could be a greater DNA repair capability, or resistance to DNA damage-induced apoptosis, in the human ovarian tumor cells.

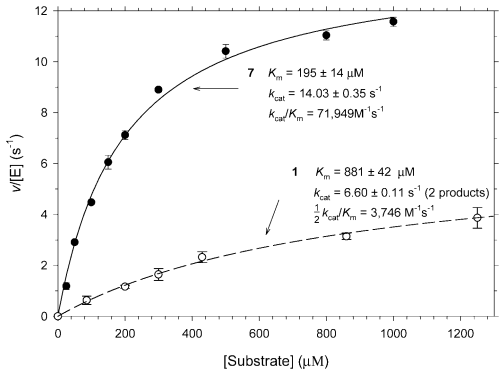


Figure 1. Enzyme kinetics: **7** (●) as a substrate of *E. coli* nitroreductase in comparison with compound **1** (○).

To demonstrate the positional requirement of nitro group para to the benzylic oxygen, analogues of **5** and **6** with ring O and NH transposed and an analogue of **7** with the nitro group moved to the meta position were also synthesized and evaluated. Even though those analogues of **5**, **6**, and **7** were NTR substrates, they were shown to have no significant selective cytotoxicity against nitroreductase-expressing V79 and SKOV3 cells (data not shown). These results indicate that nitroreductase reduction is an important first step but not sufficient for enhanced cytotoxicity in nitroreductase-expressing cells. This is consistent with the mechanism proposed in Scheme 1.

Furthermore, compound **7** was confirmed to be a much better substrate for NTR than compound **1** in enzyme assays. As shown in Figure 1, compound **7** has a K_m of 195 ± 14 μM, and a k_{cat} of 14.03 ± 0.35 s^{-1} while compound **1** has a K_m of 881 ± 42 μM and k_{cat} of 6.60 ± 0.11 s^{-1}, which were similar to the published values.[7]

Since compound **1** is reduced to a 1:1 mixture of 2-hydroxylamino and 4-hydroxylamino products by NTR and the 4-hydroxylamino is known to be the major cytotoxic product,[13,22] the productive $^1/_2k_{cat}$ of 3.30 s^{-1} is used for comparison purposes. This gave a specificity constant of 71 949 M^{-1} s^{-1} (k_{cat}/K_m) for compound **7** and 3746 M^{-1} s^{-1} ($^1/_2k_{cat}/K_m$) for compound **1**. Thus, compound **7** is 19 times better as a substrate of NTR than compound **1**. This at least partially contributed to the better activity and selectivity in cell culture assays of compound **7** in comparison with compound **1**.

It should also be noted that compound **7** upon reductive activation releases phosphoramide mustard, which is the active metabolite of the clinical drug cylcophosphamide.[15−17] The excellent activity of compound **7** in nitroreductase-expressing cells was unexpected considering the fact that only a 2-fold increase in cytotoxicity was observed for 4-nitrobenzyl *N*,*N*,*N*′,*N*′-tetrakis(2-chloroethyl)phosporodiamidate toward cancer cells under hypoxic conditions.[9] This suggests that either this class of compounds were poor substrates of the human reductase(s) present or the expression of these reductase(s) was limited under the hypoxic assay conditions used.

Conclusions. In summary, we have developed a superior class of nitroaryl phosphoramides as potential prodrugs for nitroreductase-mediated enzyme-prodrug therapy. These nitroaryl phosphoramides have low cytotoxicity before reduction and are converted to phosphoramide mustard or like-reactive species upon bioreduction. The excellent biological activity of these compounds correlates well with their substrate activity for *E. coli* nitroreductase and is consistent with the expected high cytotoxicity of the reactive species released upon reduction. Work is in progress in our laboratories to further evaluate the biological activity of these analogues as potential prodrugs for bioreductive activation.

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Supporting Information Available: Experimental section including spectroscopic data, and enzyme and cell culture assay conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

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