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Synthesis and structure–activity relationships of nitrobenzyl phosphoramide mustards as nitroreductase-activated prodrugs

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ABSTRACT

A series of nitrobenzyl phosphoramide mustards and their analogs was designed and synthesized to explore their structure–activity relationships as substrates of nitroreductases from *Escherichia coli* and trypanosomes and as potential antiproliferative and antiparasitic agents. The position of the nitro group on the phenyl ring was important with the 4-nitrobenzyl phosphoramide mustard (**1**) offering the best combination of enzyme activity and antiproliferative effect against both mammalian and trypanosomatid cells. A preference was observed for halogen substitutions *ortho* to benzyl phosphoramide mustard but distinct differences were found in their SAR of substituted 4-nitrobenzyl phosphoramide mustards in *E. coli* nitroreductase-expressing cells and in trypanosomatids expressing endogenous nitroreductases.

Targeted prodrug therapy has been extensively investigated to improve the selectivity of cytotoxic agents toward tumor cells.[1–4] By design, the prodrug remains inactive until activated by biochemical mechanisms unique to cancerous cells, resulting in site-specific release of cytotoxic moieties. Among the mechanisms explored, enzyme-mediated activation of prodrugs is of great promise.[1,5–9] Enzymes used in the targeted activation can be of endogenous origin, such as DT-diaphorase, β-glucuronidase, prostate-specific antigen (PSA) and cytochrome P450 enzymes, or derived from an exogenous source, such as the bacterial carboxypeptidase and nitroreductase (NTR). Delivery of prokaryotic enzymes into cancerous cells can be carried out by antibody-directed enzyme prodrug therapy (ADEPT), where tumor-specific antibodies are coupled to the 'exogenous' protein by either direct chemical conjugation or by expression as a recombinant fusion protein.[1,10,11] Alternatively, gene-directed enzyme prodrug therapy (GDEPT) can be used, where the gene encoding the activator is targeted to and then expressed in the cancerous cell.[12–15] In both systems, delivery of the exogenous enzyme/gene is followed by the administration of a prodrug which then undergoes activation in the targeted cells to produce the toxin. Both ADEPT and GDEPT can utilize NTRs, enzymes that catalyze reduction of an aromatic nitro group to the corresponding hydroxylamine. The large electronic change resulting from conversion of the electron-withdrawing nitro to the electron-donating hydroxylamino group provides an effective 'switch' mechanism for the activation of prodrugs and the subsequent release of cytotoxic agents.[16,17]

The NTR enzymes used in cancer prodrug design belong to the oxygen-insensitive Type I class. These are associated with prokaryotes and a subset of protozoan parasites, but are absent from most higher eukaryotes.[18] This difference in distribution is the basis of drug selectivity for many nitroaromatic antimicrobial drugs[19] and is currently being exploited to develop improved anti-trypanosomatid treatments and some of our nitrobenzyl phosphoramide mustard prodrugs have been shown to be activated by nitroreductases from *Trypanosoma brucei*.[20] Here, we report the synthesis and structure–activity relationships of a range of nitrobenzyl phosphoramide mustards as prodrugs activated by NTRs from *Escherichia coli*, *T. brucei*, *Trypanosoma cruzi*, and *Leishmania major*. This work reveals that there are subtle differences between the bacterial and parasite NTRs and identifies compounds with potential for the treatment of several parasitic diseases and cancer.

Our efforts have been focused on the design of phosphoramidates to deliver the cytotoxic phosphoramide mustard alkylating agent to targeted cells. Our strategy was to introduce an electron-withdrawing nitro group onto the benzyl phosphoramide

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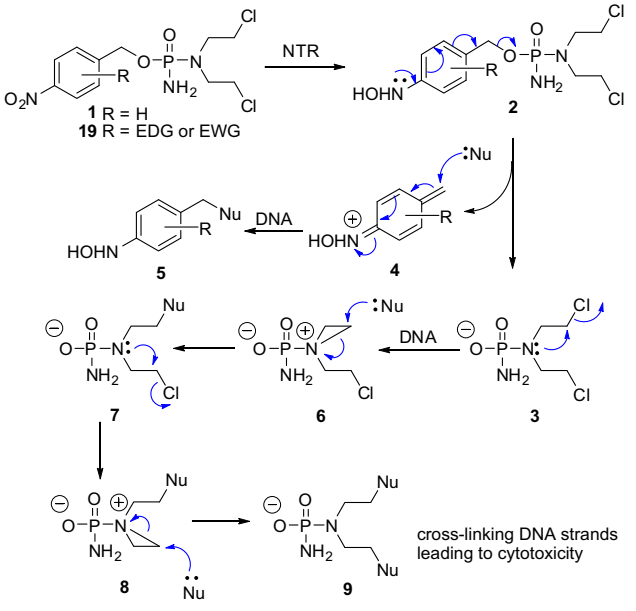
mustard that could then undergo bioreducutive activation by Type I NTRs (Scheme 1). Upon reduction, the nitro group in compound **1** is converted to the corresponding electron-donating hydroxylamino group as shown in compounds **2** resulting in redistribution of electrons within the structure. This triggers the cleavage of the benzylic C–O bond linking the phosphoramide functionality and thus promotes the release of the cytotoxic phosphoramide mustard (**3**), the active metabolite of cyclophosphamide.

We previously reported that 4-nitrobenzyl phosphoramide mustard (**1**) was an excellent substrate for nfsB, an *E. coli* Type I NTR. This compound exhibited excellent antiproliferative activity against V79 cells expressing nfsB with a subnanomolar IC_{50} and a selectivity ratio of more than 160,000.[17,21] Previous SAR studies using derivatives of **1** demonstrated that the benzylic C–O bond is crucial for prodrug activation.[17,21] Here, we focused on modifications to the mustard, the linker, and the aromatic ring to gain a better understanding of the electronic and steric effects involved in enzyme substrate activity and subsequent drug release.

A series of phosphoramide mustard-based analogs of compound **1** was first designed and synthesized. These included 2- and 3-nitrobenzyl (**12**, **13**), α-methylated 4-nitrobenzyl (**14A**, **14B**), and 5-nitropyrid-2-yl methyl (**15**) phosphoramide mustards, 4-nitrobenzyl carbamate (**16**) and sulfamidite (**17**) mustards plus the 4-nitrobenzyl phosphoramide bromomustards (**18**). The arylmethyl phosphoramide mustards **12**–**15** and **18** were synthesized starting from the corresponding arylmethyl alcohol in a 3-step one-pot procedure as published previously.[17] The carbamate and sulfamidite analogs **16** and **17** were synthesized by reacting 4-nitrobenzyl alcohol with phosgene or thionyl chloride followed by quenching with nitrogen mustard.

A second series of substituted 4-nitrobenzyl phosphoramide mustards **19a**–**19l** was designed to introduce various functional groups like electron-donating or electron-withdrawing groups to the aromatic benzene ring in order to modulate the electron density of the aromatic ring system to affect the release of cytotoxic phosphoramide mustard **3** via 1,6-elimination and to allow the 4-hydroxylamino arylmethyl phosphoramide mustard intermediate **2** to permeate across cell membranes and release the phosphoramide mustard **3** inside neighboring cells, thus, increasing bystander effect. The substituted 4-nitrobenzyl phosphoramide mustards **19a**–**19l** were synthesized starting from the corresponding



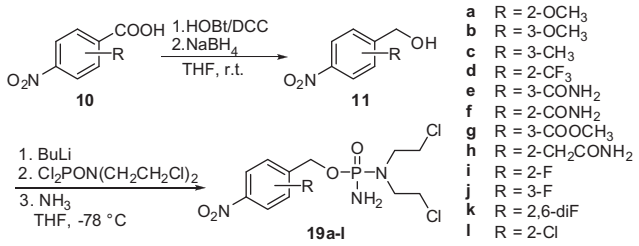
Scheme 1. Proposed mechanism of activation of 4-nitrobenzyl phosphoramide mustards.

substituted 4-nitrobenzoic acids **10** as shown in Scheme 2. Our earlier efforts demonstrated that reduction of methyl 4-nitrobenzoate with DIBAL at −78 °C in THF afforded less than 40% of the desired alcohol product with about 50% of the starting material recovered. By using a modified procedure of sodium borohydride reduction of activated esters,[22] we were able to obtain the desired alcohols **11** in moderate yields. Typically, the *N*-hydroxybenzotriazoyl (HOBt) activated esters of **10** were formed in situ followed by reduction with sodium borohydride in THF. To avoid the problem of benzyl benzoate ester formation, we added the solutions of OBt activated esters dropwise to a suspension of sodium borohydride in THF and we were able to achieve high yield of product formation. The substituted 4-nitrobenzyl phosphoramide mustards **19a**–**19l** were synthesized via the 3-step one-pot procedure in which the alcohols were first deprotonated with *n*-BuLi followed by phosphorylation with bis(2-chloroethyl)phosphoramidic dichloride and then aminolysis.[17,23]

The compounds synthesized were evaluated in a series of growth inhibition experiments against mammalian cells and trypanosomatid parasites. In experiments involving mammalian cells, inhibition data obtained with control lines (V79 and SKOV3) were compared with values generated using variants expressing *E. coli* nfsB ($V79^{NTR+}$ and $SKOV3^{NTR+}$). The parasites used in screening were the extracellular, bloodstream trypomastigote of *T. brucei*, the intracellular, amastigote of *T. cruzi* (the two medically relevant parasite forms that replicate in the human host) and the *L. major* promastigote, the form present in the sandfly gut.

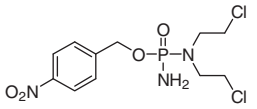
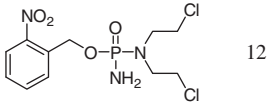
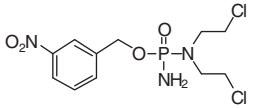
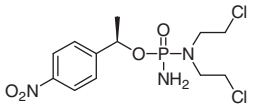
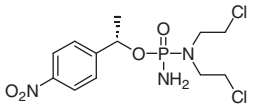
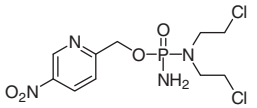
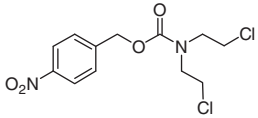
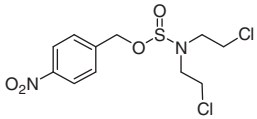
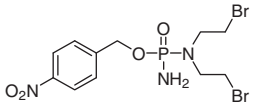
Table 1 illustrates the structure and activities of the first series of nitroarylmethyl phosphoramidates and analogs. When the positional effect of the nitro group on the benzene ring was investigated, bacterial enzyme and mammalian antiproliferative activities were highest when using **1**, where the nitro substituent is in the *para*-position. Lower activity levels were observed when the nitro group was moved to the *ortho*- (**12**) or *meta*-positions (**13**): the 2-nitro analog (**12**) was the worst substrate, presumably due to the steric effect of the *ortho* substitution. When enzyme activities were compared between the positional isomers, only a 2 to 5-fold difference was detected whereas a 225- to 325-fold difference was observed in their antiproliferative properties against mammalian cells expressing *E. coli* nfsB. This suggests that intracellular phosphoramide mustard release occurs more readily from **1** than from the other analogues, thus enhancing its antiproliferative effect. Analysis of effects against trypanosomatid pathogens revealed that both **1** and **13** were reduced by the *T. brucei* NTR (TbNTR), but only **1** displayed any antiparasitic activity in the concentration range tested and this was restricted to *T. brucei* bloodstream forms.

Introduction of a methyl group at the benzylic carbon of **1** resulted in two diastereomers (**14A**, **14B**) that were separable on silica gel. This modification did not significantly affect the compound's biological properties in the mammalian system: the two diastereomers had similar enzymatic activities against the bacterial enzyme as compared to **1** and gave IC_{50} values approximately 2- to 3-fold higher when screened against $V79^{NTR+}$ and $SKOV^{NTR+}$



Scheme 2. Synthesis of substituted 4-nitrobenzyl phosphoramide mustards.

Table 1
Nitroreductase activation of nitroarylmethyl phosphoramidates and analogs in V79 cells and trypanosomal parasites

Compd		nfsB Activity[a](nmol NADH oxidized/min/mg)	V79 cells, 72 h exposure			SKOV3 cells, 18 h exposure			TbNTR activity[a] (nmol NADH oxidized/min/mg)	Trypanocidal activity (IC_{50}, μM)		
			IC_{50}[b] (μM), NTR^-	IC_{50}[b] (μM) NTR^+	Ratio[c] (NTR^-/NTR^+	IC_{50}[b] (μM), NTR^-	IC_{50}[b] (μM) NTR^+	Ratio[c] (NTR^-/NTR^+)		*T. cruzi*	*T. brucei*	*L. major*
	1	1283	59	0.0008	73,750	943	0.099	9525	246	3.4	>10	>30
	12	256	>100	0.18	>556	>1000	5.23	>190	19	>10	>10	>30
	13	683	>100	0.26	>385	947	55	17	369	>10	>10	>30
	14A	1405	57	0.002	28,500	>1000	0.28	>3500	34	>10	>10	>30
	14B	916	65	0.002	32,500	>1000	0.49	>2000	52	>10	>10	>30
	15	ND	75	0.009	8,333	ND	ND	ND	ND	1.6	ND	8.3
	16	ND[d]	45	1.6	28	>300	16	>18	1122	>10	>10	16.23
	17	ND	62	2.9	21	>300	>300	ND	ND	ND	ND	ND
	18	ND	6	0.0001	60,000	1102	0.09	12244	ND	ND	ND	ND

[a] Nitroreductase substrate activity expressed as the initial velocity (nmol of NADH oxidized/min/mg) in the presence of either *E. coli* nfsB or *T. brucei* nitroreductase (TbNTR) by following the change in absorption at 340 nm.
[b] V79 cells were transfected with vector only while a bicistronic construct coding for the *E. coli* nfsB and a puromycin selective marker was introduced to form the $V79^{NTR+}$ line. IC_{50} values are the concentration required to reduce the cell number to 50% of control after the cells were exposed to the drug. The standard errors of all assays were within 10% of the mean between replicates at a given concentration and 10–20% for the fitted IC50 values.
[c] Ratio of IC_{50} values (NTR^-/NTR^+) as an indication of activation by *E. coli* nitroreductase.
[d] Was not determined.

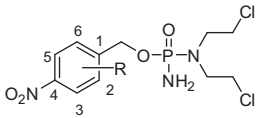
cells. In contrast, the two diastereomers were not efficiently reduced by the parasite enzyme and displayed no antiparasitic activity at concentrations tested. Replacing the phenyl ring in **1** with a pyridinyl ring (**15**) generated a compound with significant antiproliferative activity against the $V79^{NTR+}$ cells, although the IC_{50} value is 10-fold higher than that for **1**. This compound also had growth inhibitory activity against bloodstream form *T. brucei* and *L. major* promastigotes, in these cases better than parental structure: the pyridinylmethyl-containing compound **15** gave an IC_{50} of 1.6 μM against *T. brucei* and 8.3 μM against *L. major* while the benzyl-containing compound **1** yielded values of 3.4 μM against *T. brucei* and no activity (>30 μM) toward *L. major*. Replacement of the phosphoramidate with carbamate (**16**) or sulfamidate (**17**) was not expected to affect enzyme substrate activity, but was found to have reduced antiproliferative activity against mammalian NTR expressing cells by 2 to 3 orders of magnitude, indicating the phosphoramidate functionality was critical to prodrug activation and the resulting cytotoxicity. Similarly, the 4-nitrobenzyl carbamate analogue **16** was efficiently reduced by the parasite Type I NTR but this did not translate to a significant anti-trypanosomatid activity: **16** did not affect trypanosomal growth at concentrations up to 10 μM, while a low leishmanicidal activity was observed (16.2 μM). As **16** was metabolized by the TbNTR in vitro, inefficient uptake by the parasite and transport into the mitochondrion, the organelle where this enzyme is located, may account for the poor anti-trypanosomatid activity. Alternatively, cleavage of the benzylic C–O bond following nitro reduction and subsequent elimination of the nitrogen mustard may be inefficient for this compound. Replacement of chlorine with bromine in the mustard portion (**18**) resulted in an improvement in cytotoxicity but not selectivity against $V79^{NTR+}$ cells. When screened against SKOV3 cells, compound **18** were shown to be similar in cytotoxicity and selectivity against $SKOV3^{NTR+}$ cells.

In the second series, various substitutions were introduced on the phenyl ring to optimize their NTR substrate activity and their antiproliferative and antiparasitic activity. As shown in Table 2, the introduction of an electron donating $–OCH_3$ *ortho* to the phosphoramide mustard substituent on the phenyl ring (**19a**) generated a substrate that showed preferential reduction by *E. coli* nfsB (NTR activity against **19a** was 5-fold higher than against **1**), and exhibited similar subnanomolar growth inhibitory effect towards $V79^{NTR+}$ cells. Additionally, both compounds displayed comparable toxicity to controls resulting in similar selectivity ratios. In contrast, the *T. brucei* NTR enzyme reduced both compounds at equivalent rates with **19a** showing a slight improvement in antiparasitic activity against *T. brucei* and *L. major*. When $–OCH_3$ (**19b**) or $–CH_3$ (**19c**) substituents were introduced meta to phosphoramide mustard substituent, a slight reduction (3.8-fold) in antiproliferative activity against $V79^{NTR+}$ cells was observed while sensitivity of the control cells remained unchanged. Neither of these compounds displayed any antiparasitic activity and both were considered very poor substrates for TbNTR. Introduction of electron-withdrawing $–CONH_2$ (**19e**) and $–COOCH_3$ (**19g**) at the *meta*-position resulted in a 6- to 8-fold reduction in *E. coli* nfsB enzymatic activity, translating to a lower antiproliferative activity towards $V79^{NTR+}$ and $SKOV3^{NTR+}$ cells. A similar effect was seen when $–CONH_2$ (**19f**) and $–CH_2CONH_2$ (**19h**) substituents were added to the *ortho*-position. Introduction of an electron withdrawing CF_3 at the *ortho*-position (**19d**) caused a 5-fold decrease in antiproliferative activity towards $V79^{NTR+}$ cells while having no appreciable difference in its effect on control cells. When screened against SKOV3 cells, a reduction in sensitivity was observed in both *E. coli* nfsB expressing and non-expressing lines, resulting in **19d** displaying lower selectivity than **1**. Interestingly, all trypanosomatid lines were more sensitive to **19d** than to **1** and this correlated with an increased rate of reduction by the parasite NTR.

Direct halogen substitution on the phenyl ring exhibited interesting SAR in terms of antiproliferative and antiparasitic activity. Fluorine substitution at the *ortho*- (**19i**) or *meta*- (**19j**) positions on the phenyl ring had different effects on the activation and release of cytotoxic phosphoramide mustard. When screened against TbNTR, both compounds generated a significantly higher enzymatic activity than **1**. However, in the case of **19j**, this increased turnover when compared to **1**, was not reflected in an increased trypanocidal capacity, although an improvement in leishmanicidal activity was detected. In contrast, the increased enzymatic activity observed with **19i** did correlate with increased parasite toxicity in all the pathogens tested. Similarly, $V79^{NTR+}$ and $SKOV3^{NTR+}$ cells were more susceptible to **19i**, generating IC_{50} values slightly lower than both **1** and **19j**. The fact that **19j** was a better substrate of NTR but had lower antiparasitic effect than **19i** suggests that the difference in antiproliferative and trypanocidal activity is the result of the structural effects of the fluorine substitutions on the second step of activation and eventual release of cytotoxic phosphoramide mustard rather than the first step of nitro reduction. This preference of 2-F substitution could be due to the balanced electronic inductive and resonance effect of fluorine: although electron-withdrawing fluorine decreased the electron density of hydroxylamino intermediate **2**, and thus might impact the 1,6-elimination process, fluorine also facilitated the cleavage of benzylic C–O bond through its electron-donating resonance effect. Introduction of two fluorines, one each side of the benzylic carbon (**19k**) maintained the subnanomolar antiproliferative activity towards $V79^{NTR+}$ cells. However, **19k** was also 4-fold more cytotoxic towards $V79^{NTR-}$ and $SKOV3^{NTR-}$ cells. Substitution of a chlorine at the *ortho*-position (**19l**) produced a reduction in antiproliferative activity against $V79^{NTR+}$ and $SKOV3^{NTR+}$. This combined with increased toxicity toward the appropriate control lines resulted in lower selectivity for **19l**. However, this compound was reduced at a similar rate as **19i** and was a highly effective antiparasitic agent. Against bloodstream form *T. brucei*, **19l** was 425-fold more effective at inhibiting pathogen growth than **1** with an IC_{50} value of 8 nM and gave IC_{50} values between 3 and 10 μM for *T. cruzi* and *L. major*. The antiparasitic activity was increased further by incorporating two fluorine substituents, one on each side of the benzylic carbon (**19k**). Out of all the mustards analyzed, **19k** was the most effective substrate for TbNTR and displayed the highest potency against all parasite forms: **19k** was 485-fold more effective at inhibiting pathogen growth than **1**, with an IC_{50} value of 7 nM against bloodstream form *T. brucei* and about 1 μM against *T. cruzi* and *L. major*.

For gene-directed prodrug therapy (GDEPT) of cancer, it is unlikely that all tumor cells will be transfected by the vector and thus not all tumor cells will express the prodrug-converting enzyme. Consequently, some tumor cells would not be directly exposed to the active agent released from its corresponding prodrug form upon enzymatic activation. Therefore, the ideal active agent is able to diffuse into the intercellular fluid and kill neighboring tumor cells through the bystander effect, which is crucial to the success of GDEPT as the effect of the drug is amplified. The bystander effect (BE_{50}) of compounds (**1**, **19d**, **19i**, **19j**, **19k** and **19l**) were measured in SKOV3 cell lines by quantitating the percentage of NTR^+ cells in a mixed population of NTR^+ and NTR^- cells to produce an IC_{50} midway on a log scale (geometric mean) between those in either NTR^+ or NTR^- cell type alone; BE_{50} is a parameter used to compare the bystander effect of different compounds similar to the TE_{50} reported previously.[15,21] As shown in Table 2, the results showed that the introduction of one fluorine at either *ortho*- or *meta*-positions and a CF_3 or chlorine at the *ortho*-position improved the bystander effect by ~2-fold while the introduction of two fluorines at the ortho positions decreased the bystander effect as compared to the unsubstituted compound **1**. Therefore, out of all the derivatives synthesized, the 2-fluoro-4-nitrobenzyl phosphoramide mustard

Table 2
Nitroreductase activation of substituted 4-nitrobenzylmethyl phosphoramide mustards in V79 cells, SKOV3 cells, and trypanosomal parasites



1, 19a-l

Compd	R	nfsB Activity[a] (nmol NADH oxidized/min/mg)	V79 cells, 72 h exposure			SKOV3 cells, 18 h exposure					TbNTR Activity[a] (nmol NADH oxidized/min/mg)	Trypanocidal activity (IC_{50}, μM)[b]		
			IC_{50}[b] (μM) NTR^-	IC_{50}[b] (μM) NTR^+	Ratio[c]	IC_{50}[b] (μM) NTR^-	IC_{50}[b] (μM) NTR^+	Ratio[c]	IC_{50}[b] (μM) 25% NTR^+ 75% NTR^-	BE^d_{50}		*T. brucei*	*T. cruzi*	*L. major*
1	H	1283	59	0.0008	73,750	943	0.099	9,520	0.98	5.6%	246	3.4	>10	>30
19a	2-OCH_3	6978	41	0.0005	82,000	847	0.023	3680	ND	ND	239	1.2	>10	15.6
19b	3-OCH_3	2444	48	0.003	16,000	641	0.44	1460	ND	ND	0	>10	>10	>30
19c	3-CH_3	894	48	0.003	16,000	477	0.45	1060	ND	ND	158	>10	>10	>30
19d	2-CF_3	ND[e]	50	0.004	12,500	172	0.31	570	1.01	2.8%	654	0.15	7.97	5.88
19e	3-$CONH_2$	211	>100	0.03	>3,330	>1000	6.75	>150	ND	ND	ND	ND	ND	ND
19f	2-$CONH_2$	ND	>100	0.04	>2,500	>100	0.8	>125	ND	ND	ND	ND	ND	ND
19g	3-$COOCH_3$	167	69	0.1	690	435	0.43	1000	ND	ND	ND	ND	ND	ND
19h	2-CH_2CONH_2	ND	>100	0.05	>2,000	>100	1.1	>90	ND	ND	ND	ND	ND	ND
19i	2-F	ND	49	0.0004	122,750	266	0.08	3,290	0.37	3.9%	832	0.27	9.54	4.72
19j	3-F	ND	48	0.003	16,000	246	0.21	1,200	0.48	4.2%	1116	3.2	>10	9.05
19k	2,6-diF	ND	17	0.0008	20,880	257	0.17	1520	1.37	9.2%	1454	0.007	0.99	1.29
19l	2-Cl	ND	26	0.002	13,100	140	0.12	1,120	0.54	3.2%	706	0.008	9.14	3.10

[a] Nitroreductase substrate activity expressed as the initial velocity (nmoles of NADH oxidized/min/mg) in the presence of either *E. coli* nfsB or *T. brucei* nitroreductase (TbNTR) by following the change in absorption at 340 nm.
[b] V79 cells were transfected with vector only while a bicistronic construct coding for the *E. coli* nfsB and a puromycin selective marker was introduced to form the $V79^{NTR+}$ line. For SKOV3, NTR-expressing cells ($SKOV3^{NTR+}$) were compared with untransfected parental line. IC_{50} values are the concentration required to reduce the cell number to 50% of control after the cells were exposed to the drug. The standard errors of all assays were within 10% of the mean between replicates at a given concentration and 10–20% for the fitted IC50 values.
[c] Ratio of IC_{50} values (NTR^-/NTR^+) as an indication of activation by *E. coli* nitroreductase.
[d] BE_{50} values refer to percentage of NTR^+ cells in a mixed population of NTR^+ and NTR^- cells to produce an IC_{50} midway on a log scale (geometric mean) between those in either NTR^+ or NTR^- cell type alone.
[e] Was not determined.

19i showed the best profile in GDEPT of cancer with excellent activity, selectivity and improved bystander effect although the selectivity of **19i** was slightly lower than that of **1** in SKOV3 cells.

In summary, 4-nitrobenzyl phosphoramide mustards were identified as excellent substrates of nitroreductases from *E. coli* and *T. brucei*. In vitro cell culture assays showed that they were highly cytotoxic against NTR^+ mammalian cell lines and trypanosomes with some having relatively low cytotoxicity against non-type I NTR expressing cells. The excellent bystander effect and selectivity suggest that our nitroarylmethyl phosphoramide mustards are good candidates for use in combination with nitroreductase in ADEPT or GDEPT of cancer and as potential chemotherapeutic agents for the treatment of trypanosomal infections.

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