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Design, synthesis, and biological evaluation of pyrrolobenzodiazepine-containing hypoxia-activated prodrugs

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ABSTRACT

The ability of various pyrrolobenzodiazepine(PBD)-containing cytotoxic compounds to function as hypoxia-activated prodrugs was assessed. These molecules incorporated a 1-methyl-2-nitro-1*H*-imidazole hypoxia-activated trigger (present in the clinically evaluated compound TH-302) in a manner that masked a reactive imine moiety required for cytotoxic activity. Incubation of the prodrugs with cytochrome P450-reductase under normoxic and hypoxic conditions revealed that some, but not all, were efficient substrates for the enzyme. In these experiments, prodrugs derived from PBD-monomers underwent rapid conversion to the parent cytotoxic compounds under low-oxygen conditions while related PBD-dimers did not. The ability of a given prodrug to function as an efficient cytochrome P450-reductase substrate correlated with the ratio of cytotoxic potencies measured for the compound against NCI460 cells under normoxic and hypoxic conditions.

Tumor-activated prodrugs of various cytotoxic molecules have been explored for many years as a means to mitigate the (often severe) dose-limiting side effects associated with the parent compounds.[1] One such tumor-specific targeting strategy employs prodrugs that activate only in the presence of low oxygen levels as a means to subsequently liberate the potent, biologically active parent molecules.[2] These entities seek to exploit the hypoxic environment common to most solid tumors and are not expected to form toxic moieties in well-oxygenated normal tissues. Several hypoxia-activated prodrugs have progressed to clinical testing including tirapazamine, AQ4N,[3] PR104,[4] and evofosfamide (TH-302).[5] The latter compound contains a 1-methyl-2-nitro-1*H*-imidazole group that undergoes one-electron bioreduction to produce a radical anion intermediate (Scheme 1).[2,5] In the presence of low oxygen levels (i.e., hypoxia), this intermediate subsequently fragments to generate a cytotoxic bromo-isophosphoramide mustard (Br-IPM) capable of effecting potent cell killing via DNA alkylation/cross-linking.[6] However, under normoxic conditions typically found in non-cancerous tissues, the radical anion is quenched without release of the cytotoxic moiety (Scheme 1).[2,5] As part of our efforts to maximize the therapeutic index associated with antibody-drug conjugates (ADCs), we explored whether the 1-methyl-2-nitro-1*H*-imidazole hypoxia-activated trigger present in TH-302 could be successfully employed with cytotoxic molecules commonly utilized as ADC payloads. In this report, we describe our initial studies of molecules comprising such combinations.

The pyrrolobenzodiazepine (PBD)-containing compounds **1**–**3** are well-known cytotoxic agents that exert their cell-killing effects via DNA alkylation and, in the case of compound **3**, DNA cross-linking (Fig. 1).[7,8] The most potent members of this compound class (e.g., compound **3**) have been widely utilized as ADC payloads, and several conjugates employing such cytotoxic entities are currently undergoing clinical assessment.[9] As shown in Scheme 2, we envisioned incorporating the 1-methyl-2-nitro-1*H*-imidazole hypoxia-activated trigger into the PBD chemical structure via an N-10 linked carbamate moiety (structure **4**). This attachment configuration requires cleavage of the carbamate group to liberate the reactive PBD imine moiety known to form covalent adducts with guanine residues in the DNA minor groove (i.e., conversion of structure **4** to structure **5**).[8] In the absence of such cleavage, the intact PBD-containing prodrugs were expected to exhibit significantly attenuated cytotoxicity properties owing to their inability to effectively bind to and/or covalently modify DNA.[10,11]

One-electron reduction of the 1-methyl-2-nitro-1*H*-imidazole hypoxia-activated trigger is believed to be mediated by the cytochrome P450-reductase enzyme (EC 1.6.2.4).[2] Accordingly, we initiated our studies by examining whether various PBD-containing

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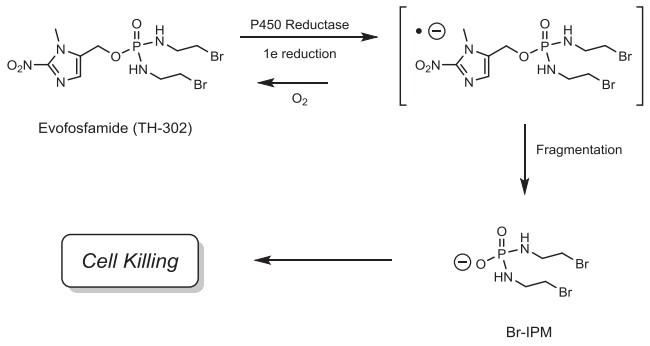


Scheme 1. TH-302 mechanism of action.

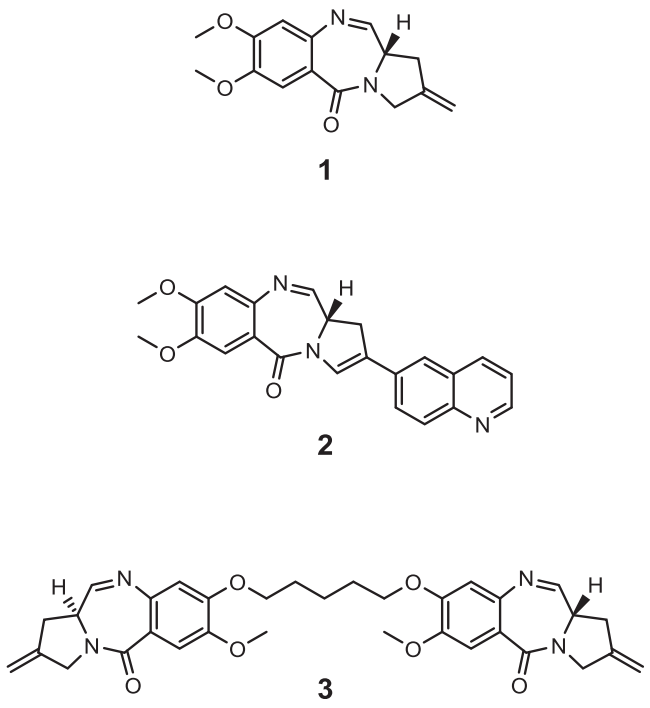
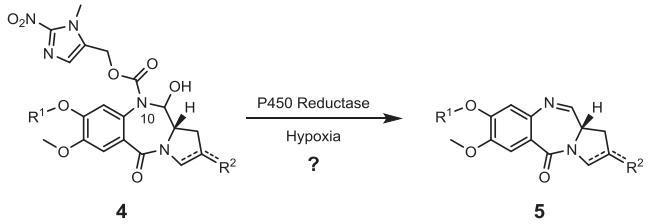


Fig. 1. Structures of parent compounds **1**–**3**.



Scheme 2. General design and MOA of PBD-containing hypoxia-activated prodrugs.

cytotoxic molecules bearing N-10 carbamate prodrugs derived from the 1-methyl-2-nitro-1*H*-imidazole moiety could function as efficient substrates for this enzyme. As shown in Table 1, incubation of the relatively simple compound **6** (Fig. 2) with the P450-reductase enzyme under normoxic conditions for 2 h did not result in appreciable transformation of the molecule. This result demonstrated good stability of the 1-methyl-2-nitro-1*H*-imidazole-containing N-10 carbamate moiety to aqueous conditions at neutral pH.[12] In contrast, similar incubation of compound **6** with P450-reductase under low oxygen conditions resulted in complete disappearance of the prodrug and the corresponding generation of the parent PBD-monomer **1**. These observations were consistent with the P450-reductase-mediated cleavage of the N-10 carbamate present in **6** via 1-methyl-2-nitro-1*H*-imidazole reduction and subsequent fragmentation/immolation (c.f., Schemes 1 and 2). As expected based on this mechanism, a closely-related compound that lacked the imidazole nitro group (and was thus a poorer electron acceptor as compared with **6**) did not generate parent compound **1** in the presence of the P450-reductase enzyme under either normoxic or hypoxic conditions (compound **7**, Table 1). Notably, the extent of prodrug cleavage observed for compound **6** was similar to that observed for TH-302 when the latter molecule was incubated with P450-reductase in a low oxygen environment (Table 1).

Encouraged by the preceding results, we examined whether prodrugs derived from larger and more potent PBD-containing compounds could also function as effective P450-reductase substrates. As shown in Table 1, the quinoline-containing prodrug **8** also underwent efficient transformation to the corresponding parent compound **2** when incubated with P450-reductase under hypoxic conditions. However, the larger PBD-dimer-containing prodrug **9** did not undergo a similar transformation and instead exhibited an inert reactivity profile that appeared indistinguishable from that of the corresponding des-nitro analog (Table 1, compare compounds **9** and **10**). Consistent with these observations, computational docking studies conducted with the previously disclosed P450-reductase crystal structure[13] confirmed that prodrugs **6** and **8** could be accommodated in the enzyme's active site while the larger prodrug **9** could not (Fig. 3A and B, see Supporting Information for additional details). Taken together, these observations indicate that some, but not all, PBD moieties containing the 1-methyl-2-nitro-1*H*-imidazole prodrugs can function as substrates for the P450-reductase enzyme.

We next explored the impact that the 1-methyl-2-nitro-1*H*-imidazole prodrugs had on the cytotoxic properties of the PBD-containing compounds into which they were incorporated. As shown in Table 2, all of the parent PBD compounds exhibited measurable cell-killing activity when tested against the NCI460 cell line under normoxic conditions (compounds **1**–**3**). As expected based on their mechanisms of action, the cell-culture potencies of these PBD-containing molecules loosely correlated with their ability to interact with DNA as determined by a semi-quantitative in vitro alkylation assessment (Table 2, more alkylation = improved cytotoxic potency).[14] In contrast, the N-10 prodrug-containing compounds exhibited attenuated DNA binding activities relative to the corresponding parent molecules (compare **6** with **1**, **8** with **2**, and **9** with **3**, Table 2). In the case of prodrugs **6** and **8**, the attenuation was virtually complete with no appreciable DNA alkylation observed during the course of the in vitro experiment. These outcomes were consistent with the inability of the two prodrugs (which lack the reactive imine moieties present in the parent PBD structures) to form guanine-mediated covalent adducts with DNA.[8] As shown in Table 2, the lack of DNA alkylation displayed by **6** and **8** translated into weaker cytotoxic activity for the two compounds against the NCI460 line with each exhibiting a similar magnitude of potency loss. The precise mechanisms by which compounds **6** and **8** exert their cytotoxic effects are not known with certainty at this time but may involve their binding to DNA in a non-alkylative manner.

In contrast, the PBD-dimer-derived prodrug **9** retained measurable DNA alkylation activity in the in vitro assessment. This result was not entirely surprising since **9** contains a PBD-monomer bearing a reactive imine moiety which can covalently modify DNA if the remainder of the molecule does not interfere with binding in the DNA minor groove.[8] Importantly however, the extent of in vitro DNA alkylation measured for prodrug **9** was less than that observed for parent compound **3**, and the former molecule