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Synthesis and biological evaluation of novel pyrrolo[2,1-*c*][1,4]benzodiazepine prodrugs for use in antibody-directed enzyme prodrug therapy

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Abstract—The design, synthesis and evaluation of four novel pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) prodrugs (**1a**,**b** and **2a**,**b**; Fig. 1) for potential use in carboxypeptidase G2 (CPG2)-based antibody-directed enzyme prodrug therapy (ADEPT) is reported. Although all four prodrugs were shown to be less cytotoxic than the released parent PBDs **3** and **4**, the urea prodrugs **1b** and **2b** were found to be too unstable for use in ADEPT, whereas carbamates **1a** and **2a** are both stable in an aqueous environment and are good substrates for CPG2.

A major limitation of the use of cancer chemotherapy results from the lack of tumour specificity shown by most anticancer drugs. Many clinically used agents act predominantly through an antiproliferative mechanism which leads to damage of normally multiplying cells such as those of the bone marrow and gut. Therefore, chemotherapy is often linked to severe side effects due to the destruction of healthy tissue.[1] One strategy to overcome this problem involves the use of non-biologically active prodrug derivatives of cytotoxic agents that can be selectively activated at the tumour site.[2] In the antibody-directed prodrug therapy (ADEPT) approach,[3] an antibody–enzyme conjugate is used to localize an enzyme at the tumour site. A prodrug form of a cytotoxic agent, that can be converted to the active agent by the antibody-linked enzyme, is then administered systemically leading to selective release of the cytotoxic agent at the tumour site. An important feature of this system is that the releasing enzyme is of non-human origin (e.g., bacterial), thus avoiding release of the cytotoxic agent at other sites in the body. It has also been suggested that a bystander effect may enhance the efficacy of treatment, with the cytotoxic agent produced within the tumour diffusing out to neighbouring cells.

One such system is presently in clinical trials and involves a fusion protein conjugate of the A5B7 F(ab')(2) antibody and the enzyme carboxypeptidase G2 (CPG2) targeted against colorectal carcinoma expressing carcinoembryonic antigen (CEA).[4] This is being used in conjunction with a nitrogen mustard prodrug ZD2767P (**5**, Fig. 2). However, with this combination of agents, therapeutic efficacy may be limited due to rapid repair of the DNA adducts formed after release of the cytotoxic agent, a commonly occurring phenomenon with mustard-based drugs.[4] To address this issue, we have designed and synthesized two novel families of pyrrolobenzodiazepine (PBD)-based prodrugs (**1a**,**b** and **2a**,**b**; Fig. 1) that are not only more potent (i.e., picomolar) compared to mustard-based prodrugs but their adducts may be more resistant to repair,[5,6] thus reducing the probability of clinical resistance developing.

The pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs) are a family of antitumour antibiotics that includes the natural products anthramycin and DC-81.[7] They exert their cytotoxicity by covalently bonding to the exocyclic

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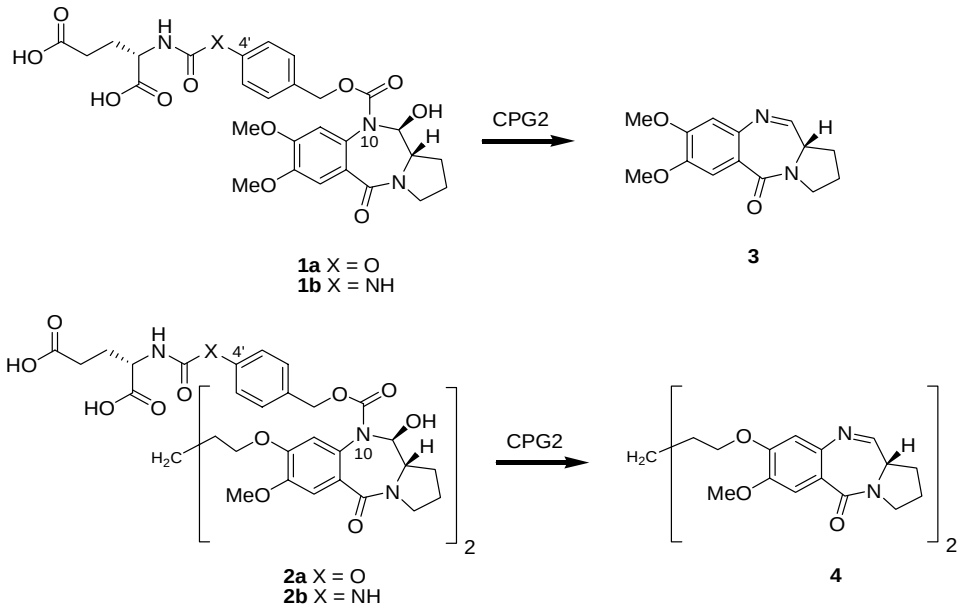
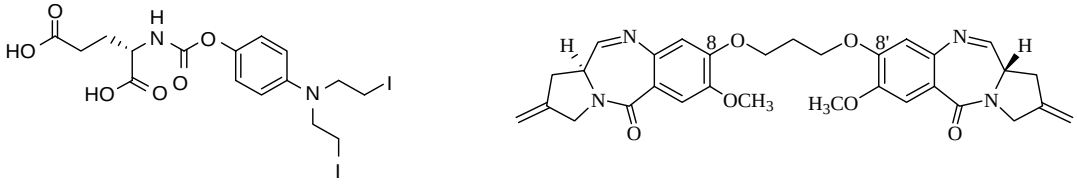


Figure 1. Carbamate (**1a** and **2a**) and urea (**1b** and **2b**) PBD ADEPT prodrugs and their conversion to parent monomer (**3**) and dimer (**4**) PBDs.

C2–NH_2 group of guanine residues in the minor groove of DNA through their N10–C11 imine functionality.[7] This leads to a number of biological effects including the inhibition of transcription[8,9] and of enzymes binding to cognate sites.[9,10] The PBD monomers have significant in vitro cytotoxicity,[11] and it has been demonstrated that joining two PBD moieties through a linker (via their C8-positions) leads to PBD dimers capable of interstrand DNA cross-linking.[12–14] One example of a PBD dimer, SJG-136[15] (**6**, Fig. 2), is now being evaluated in Phase 1 clinical trials.[5,16] One interesting property of PBD dimers is that the interstrand cross-linked adducts they form in the minor groove of DNA appear to be highly resistant to repair,[5,6] and it is this feature that may lead to ADEPT prodrugs with distinct advantages over ZD2767P (**5**, Fig. 2). To explore the potential of using these extremely cytotoxic molecules in prodrug systems, we initially demonstrated the possibility of converting PBD monomers into nitroreductase-sensitive prodrugs.[17] We now report the design, synthesis and evaluation of four model self-immolative CPG2 PBD prodrugs (**1a**,**b** and **2a**,**b**, Fig. 1) formed from the PBD monomer **3** and dimer **4** (Pentamethylene linker between PBD units), respectively, potentially suitable for use in CPG2-based ADEPT therapy. Prodrugs of this type should have an advantage over existing mustard-based prodrugs both in terms of potency and/or resistance to DNA repair.

The new agents (**1a**,**b** and **2a**,**b**) are prodrugs of the known PBD monomer (**3**)[18] and dimer (**4**),[12] respectively, where the DNA-interactive N10–C11 functionality necessary for biological activity is masked with an L-glutamic acid CPG2 substrate attached through either a carbamate or ureidic linkage (i.e., X = O or NH, respectively) to the 4′-position of an N10-benzyloxycarbonyl PBD. In the case of **1a** and **1b**, cleavage of the substrate by CPG2 releases either a 4′-hydroxy- or 4′-aminobenzyloxycarbonyl intermediate **7** (X = O or NH, respectively) which then undergoes 1,6-elimination to release the cytotoxic PBD **3** (Fig. 3). The PBD dimer prodrugs **2a** and **2b** release PBD dimer **4** by an identical mechanism, except that two N10-progroups are released rather than one.

The known carbamate and urea progroups **9a** (X = O) and **9b** (X = NH) were prepared by modification of literature procedures,[19] allowing the preparation of gram quantities of each (Scheme 1). These were attached to the monomer and dimer PBD precursors **8** and **11**, prepared using standard methodology developed in our laboratory (Schemes 1 and 2). This was accomplished by first converting the PBD precursors to isocyanates in situ which were then reacted with the progroups to give the carbamates (**10a**,**12a**) and ureas (**10b**,**12b**), respectively. De-protection of the silyl ethers, then cyclisation using either pyridinium dichromate or



ADEPT Mustard Prodrug ZD2767P (**5**) PBD Dimer SJG-136 (**6**)

Figure 2. Structures of the mustard ADEPT prodrug ZD27767P (**5**) and PBD dimer SJG-136 (**6**).

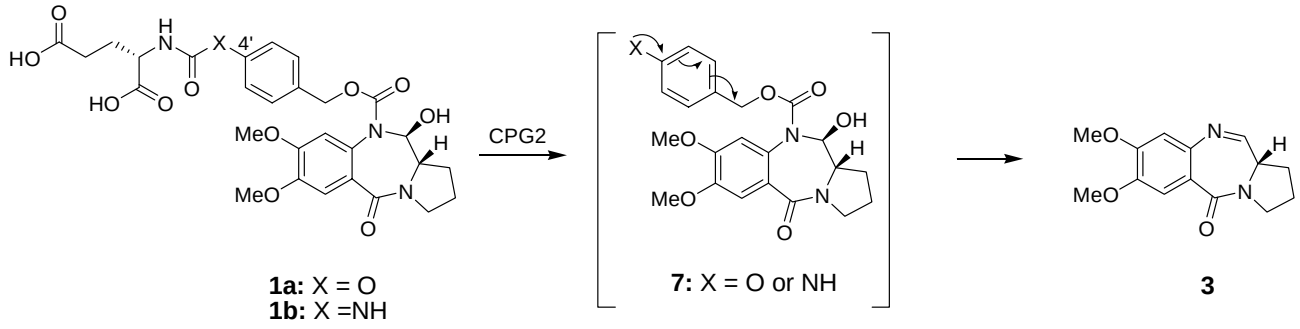
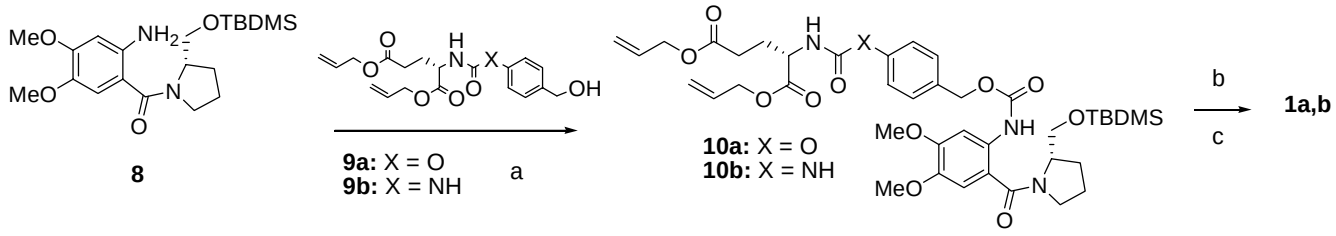
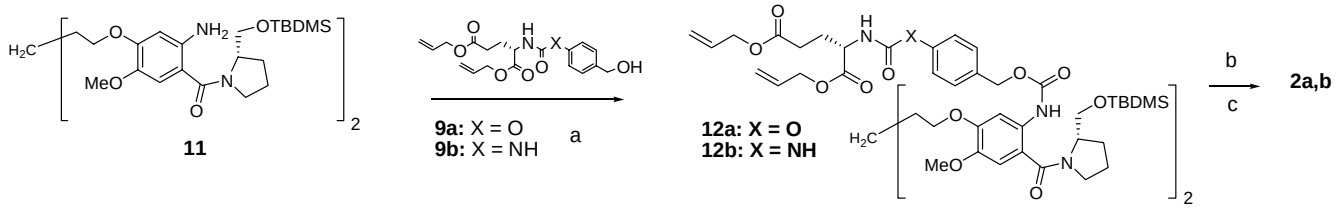


Figure 3. Schematic representation of release of the cytotoxic PBD monomer **3** from prodrugs **1a** and **1b** through CPG2-mediated 1,6-elimination.



Scheme 1. Reagents and conditions: (a) i—triphosgene, TEA, toluene, N_2, rt, 2 h; ii—**9a** or **9b**, TEA, DCM, N_2, rt, 18 h, 56%, 45%; (b) AcOH/THF/H_2O, 3/1/1, rt, 3 h, X = O 91%, X = NH 99%; (c) i—pyridinium dichromate, 4 Å sieves, DCM, rt, 2 h, X = O, 62%; X = NH, 57%; ii—$Pd(PPh_3)_4$, pyrrolidine, DCM, rt, X = O 90%, X = NH 87%.



Scheme 2. Reagents and conditions: (a) i—triphosgene, TEA, N_2, toluene, rt, 2 h; ii—**9a** or **9b**, TEA, DCM, N_2, 48 h, X = O, 60%; X = NH, 44%; (b) AcOH/THF/H_2O, 3/1/1, rt, 3 h, X = O, 72%, X = NH 75%; (c) i—BAIB, TEMPO, DCM, rt, X = O, 73%, X = NH 64%; ii—$Pd(PPh_3)_4$, morpholine, DCM, 18 h, X = O, 78%; X = NH, 77%.

diacetoxyiodobenzene/TEMPO followed by final deprotection of the diallyl esters using $Pd(PPh_3)_4$ gave the target compounds **1a**,**b** and **2a**,**b** in good yields.

Stability studies were performed using high-performance liquid chromatography. Both prodrug/parent pairs (**1a**,**b** and **3**; **2a**,**b** and **4**) could be readily separated and detected using a Phenomenex™ column (C18 5 μM, 25 cm × 0.46 cm) with a mobile phase of H_2O (with 0.1% trifluoroacetic acid)/acetonitrile 70:30 (1 ml/min) and detection at 254 nM. Stability was assessed over a 24 h period in distilled water at both room temperature and 37 °C. Differences in the rate of conversion of prodrugs to parent PBDs were observed between the carbamate and urea series. The urea prodrugs **1b** and **2b** were unstable at both room temperature and 37 °C, and were almost completely converted into the parent PBDs within 24 h. Conversely, the carbamate prodrugs **1a** and **2a** were relatively stable at room temperature and underwent only 7% and 3% conversion, respectively, after 24 h at 37 °C.

The in vitro cytotoxicity of the prodrugs was assessed in the LS174T human colon cell line (Table 1). The results were found to reflect the stability of the prodrugs, with the unstable urea compounds (**1b** and **2b**) giving relatively poor cytotoxicity differentials between prodrugs and parents (i.e., approximately 2:1 for both after 1 h exposure). However, the carbamate prodrugs (**1a** and **2a**)

Table 1. In vitro cytotoxicity data (IC_{50}[a]) for prodrugs (**1a**,**b** and **2a**,**b**) and parent (**3** and **4**) PBDs in LS174T cells after 1 h and continuous exposure

Compound	1 h exposure (μM)	Continuous exposure (μM)
1a	>100	20 ± 1.2
1b	28 ± 5.9	1.6 ± 0.2
3	14 ± 1.5	1.4 ± 0.1
Compound	1 h exposure (nM)	Continuous exposure (nM)
2a	200 ± 15	21 ± 2.3
2b	6 ± 1	0.8 ± 0.2
4	13 ± 2.4	0.47 ± 0.09

[a] Results are expressed as mean IC_{50} (μM) values ± standard error for PBD monomers (**1a**,**b** and **3**) and as mean IC_{50} (nM) values ± standard error for PBD dimers (**2a**,**b** and **4**). All values are the mean of three separate experiments.

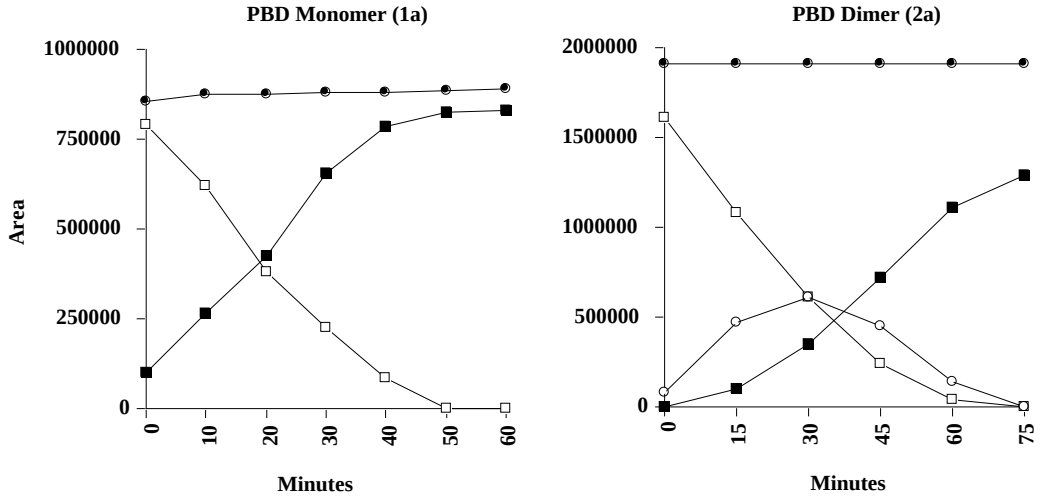


Figure 4. Conversion of the carbamate PBD monomer (**1a**) and PBD dimer (**2a**) prodrugs into parent PBDs (**3** and **4**, respectively) by CPG2 at 37 °C. Left-hand panel: □ = **1a**, ■ = **3**, ● = control; Right-hand panel: □ = **2a**, ■ = **4**, ● = control; ○ = mono-protected PBD dimer intermediate. Note: control = incubation of prodrugs in the absence of CPG2; *Y* axis = relative areas under HPLC peaks.

gave much better cytotoxicity differentials between the parent PBDs and prodrugs, with differentials of >7.1 and >15.4, respectively, for 1 h exposure rising to >14.3 and >44.7 for continuous exposure. All of the compounds examined were between 5- and 27-times more cytotoxic on continuous exposure compared to 1 h incubation, presumably due to both increased exposure time and hydrolysis of the prodrugs.

Finally, the carbamate monomer and dimer prodrugs **1a** and **2a** were incubated with 1 U of CPG2 and both compounds were shown to be good substrates (Fig. 4). The monomer prodrug **1a** was completely converted into the cytotoxic parent PBD **3** within 50 min with no apparent chemical degradation observed in the assay buffer (100 mM Tris–HCl/260 μM ZnCl, pH 7.3) in the absence of CPG2 over the same time period. Similarly, the dimer prodrug **2a** was completely converted into the cytotoxic PBD dimer **4** in 75 min with no chemical degradation in the absence of CPG2. Interestingly, in the latter case, it was possible to observe the formation of an intermediate (○, Right-hand panel, Fig. 4) thought to be the mono-protected PBD dimer with only one glutamic acid residue cleaved. As anticipated, this intermediate converted into the fully de-protected PBD dimer **4** during the course of the experiment. To confirm the potential value of these prodrugs in ADEPT therapy, the monomer (**1a**) and dimer (**2a**) prodrugs were incubated with CPG2 in the presence of LS174T cells for 1 h. This reduced the IC_{50} values for **1a** and **2a** by 7.6- and 9.0-fold, respectively, thus confirming their transformation into cytotoxic species.

In conclusion, these results demonstrate that it is possible to synthesize N10-protected PBD prodrugs suitable for CPG2-based ADEPT therapy. The prodrugs are, in the case of the more stable carbamate derivatives **1a** and **2a**, significantly less cytotoxic than the parent compounds, and are good substrates for CPG2, being rapidly converted into the cytotoxic monomer and dimer parent PBDs upon exposure to enzyme. As the released PBD dimer (**4**) and related analogues (e.g., SJG-136, **6**) are known to produce DNA interstrand cross-links that are difficult for cancer cells to repair,[5,6] the potential exists to develop second-generation ADEPT prodrugs that are less prone to the development of resistance compared to mustard-based agents. Further work is underway to study the behaviour of the prodrugs in human tumour xenografts and to improve the stability of the prodrugs and the cytotoxicity of the released PBDs.

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