CHEMMEDCHEM

DOI: 10.1002/cmdc.200700328

Development of Pyrrolo[2,1-*c*][1,4]benzodiazepine β-Galactoside Prodrugs for Selective Therapy of Cancer by ADEPT and PMT

Ahmed Kamal,*[a] Venkatesh Tekumalla,[a] Anita Krishnan,[a] Manika Pal-Bhadra,[a] and Utpal Bhadra*[b]

*The pyrrolo[2,1-c][1,4]benzodiazepines (PBDs) are a class of well-studied DNA-interactive agents with a potential for use in the treatment of cancer. The clinical utility of these molecules is limited because of the lack of selectivity for tumor tissues, high reactivity of the pharmacophoric imine functionality, low water solubility, and stability. To address the shortcomings, especially the lack of selectivity, associated with the molecules, two new β-galactoside prodrugs of PBDs have been synthesized and evaluated for their potential use in selective therapy of solid tumors by ADEPT and PMT protocols. The preliminary studies reveal the prodrugs to be much less toxic compared to the parent moieties. These prodrugs are activated by E. coli β-galactosidase (EC 3.2.1.23) to form the active cytotoxic moiety signifying their utility in ADEPT of cancer. One of the significant outcomes of the present study is the toxification of the prodrug **1a** by the endogenous β-galactosidase of human liver cancer cells (Hep G2) to form the cytotoxic moiety, enabling selective therapy of hepatocellular carcinoma. Another important property of these molecules is their enhanced water solubility and stability, which are essential for a molecule to be an effective drug.*

Introduction

Most anticancer agents used in the chemotherapy of cancer lack selectivity towards tumor cells, leading to severe side effects and dose limitation. The selectivity of traditional cytotoxic agents is mainly based on the difference in proliferation rate between malignant and normal cells. Therefore, the development of anticancer agents with improved selectivity towards cancerous tissues is one of the most important challenges of modern drug design. Chemists have long been seeking a chemotherapeutic agent for the treatment of cancer that would selectively kill cancerous cells without causing any side effects. A practical approach would be to selectively deliver the available cytotoxic agents in and around the tumor cells, maximizing its concentration around the tumors while at the same time minimizing its concentration in healthy tissues.

In recent years tumor targeted prodrug therapy has been investigated extensively to enhance the selectivity of the cytotoxic agents. In such a strategy, the cytotoxic agent is administered in the form of its prodrug that is considered to be relatively less toxic to normal cells, but is selectively activated by a biochemical process resulting in specific cytotoxicity in tumor tissues. Several strategies have been explored in this direction, including the activation by enzymes, which are tissue specific or could be delivered to the tumor cells through antibody and gene delivery approaches. Antibody directed enzyme prodrug therapy (ADEPT)[1–12] is an approach where a monoclonal antibody is employed to selectively deliver an enzyme, to the tumor cells, which subsequently activates a less toxic prodrug to drug. Whereas, prodrug monotherapy (PMT)[13–16] is another strategy where the selectivity is achieved by the confined presence of an enzyme in and around the tumor cells which activates the prodrug. The human cytosolic β-glycosidase[17,18] is reported to possess both, β-D-galactosidase and β-D-glucosidase activity. The expression of β-galactosidase in normal liver tissues is much lower compared to the hepatocellular carcinoma cell line (Hep G2).[19,20] The X-Gal assay[21] to assess the quantity of intracellular β-galactosidase in Hep G2 cells confirmed the presence of this enzyme in optimum quantities (Figure 1). Therefore Hep G2 cells were studied, as potential targets for selective therapy by PMT strategy. These findings prompted us to explore the possibility of exploiting the endogenous β-galactosidase of human liver cancer cells for the targeted delivery of the β-galactoside prodrugs to human hepatocellular carcinoma, Hep G2 cells, using the PMT strategy.

There has been considerable interest in DNA binding molecules because of their involvement in carcinogenesis and their potential use as antitumor agents as well as probes of DNA structure.[22,23] The pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs)[24] such as tomamycin, anthramycin, and DC-81 are a class of naturally occurring tricyclic antitumor antibiotics that bind to

[a] *Dr. A. Kamal, V. Tekumalla, A. Krishnan, Dr. M. Pal-Bhadra*
Department of Chemical Biology
Indian Institute of Chemical Technology
Hyderabad 500 007 (India)
Fax: (+91) 40-2719-3189
E-mail: ahmedkamal@iict.res.in

[b] *Dr. U. Bhadra*
Functional Genomics and Gene Silencing Group
Centre for Cellular and Molecular Biology
Uppal Road, Hyderabad-500007 (India)
Fax: (91) 40-27160311
E-mail: utpal@ccmb.res.in

Supporting information for this article is available on the WWW under http://www.chemmedchem.org or from the author.

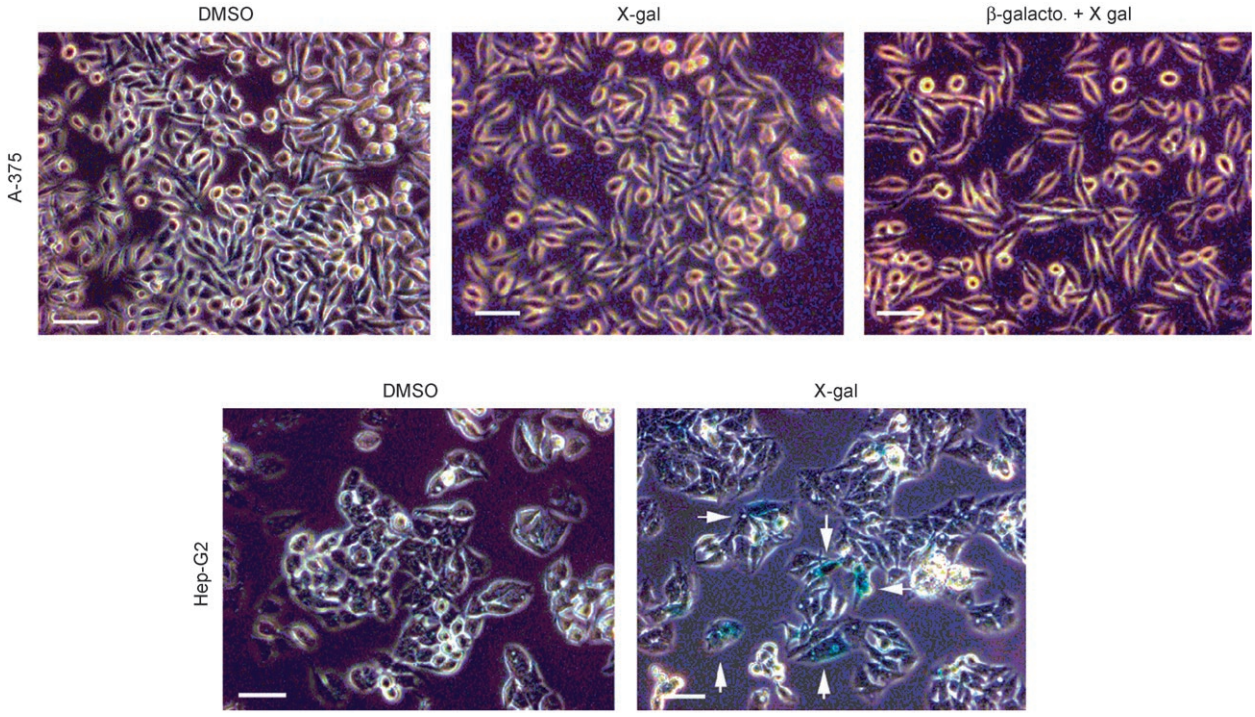
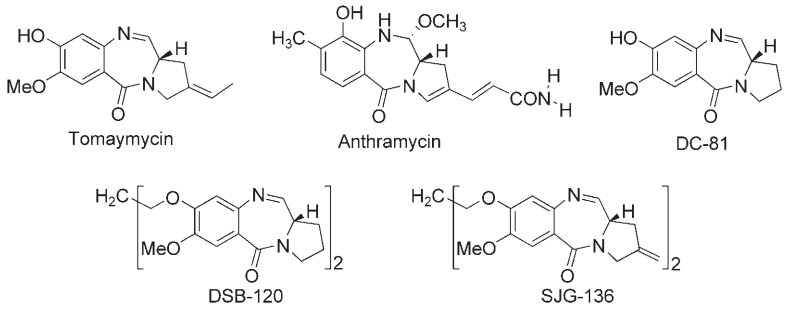
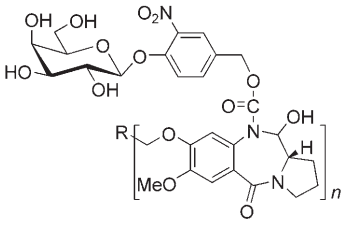


Figure 1. Endogenous β-galactosidase activity in human hepatocarcinoma Hep G2 and melanoma A375. The enzyme activity and pattern of expression were determined by X-gal staining. A375 and Hep G2 cells were cultured with DMSO and stained and incubated with X-gal for 48 h. The majority of Hep G2 cells turned blue after staining because of the presence of endogenous β-galactosidase whereas A375 cells stained negative with X-gal thus indicating no enzyme activity. Further A375, cultured with exogenous β-galactosidase and stained with X-gal, also did not exhibit a positive staining.



minor groove DNA sites spanning three base pairs, through a covalent bond to the exocyclic C2-N2 of the central guanine.[25] A synthetic dimer[26,27] of DC-81, DSB-120 exhibited a better in vitro cytotoxicity, enhanced DNA binding affinity, and sequence specificity compared to the natural product DC-81. Further studies have led to the design and synthesis of another PBD dimer,[28] SJG-136, containing C2/C2′ exomethylene functionalities. This molecule shows significant in vivo potency and has been selected for clinical studies.[29,30] However in vivo studies with DSB-120 in the murine ADJ/PC6 plasmacytoma model were not encouraging and the low therapeutic index observed was considered to be partly due to the reaction of the molecule with cellular thiol containing molecules[31] prior to reaching the tumor site. The other reason could be low water solubility leading to poor bioavailability. Apart from the development of new pyrrolobenzodiazepine molecules[32–36] with enhanced anticancer activity, we have been interested in the overall development of this class of molecules leading to their improved efficacy as anticancer agents. Thurston and coworkers[37,38] have recently reported pyrrolobenzodiazepine prodrugs for the selective therapy of cancer by ADEPT and gene-directed enzyme prodrug therapy (GDEPT) strategies, by employing carboxypeptidase G2 (CPG2) and nitroreductase enzymes. The PBDs have been described to be better suited molecules for ADEPT strategy compared to mustards, the prodrugs which were studied extensively.[39]

To address the limitations associated with this class of compounds along with improvement of selectivity towards cancer tissues, the development of their glycoside prodrugs has been initiated in this laboratory. This work is also influenced by the recent results achieved by gylcosidic prodrugs[40] of anticancer agents for use in ADEPT. The present study is primarily intended to improve the selectivity of PBDs towards cancer tissues, through β-galactosidase[21,41–50] based ADEPT and PMT strategies. In this regard we have designed, synthesized, and evaluated two new β-galactoside prodrugs **1a** and **1b**, based on the pyrrolobenzodiazepine ring system for selective therapy of solid tumors and to improve the efficacy in a wide-ranging



1a R = Ph, *n* = 1
1b R = CH_2, *n* = 2

PBD galactoside prodrugs

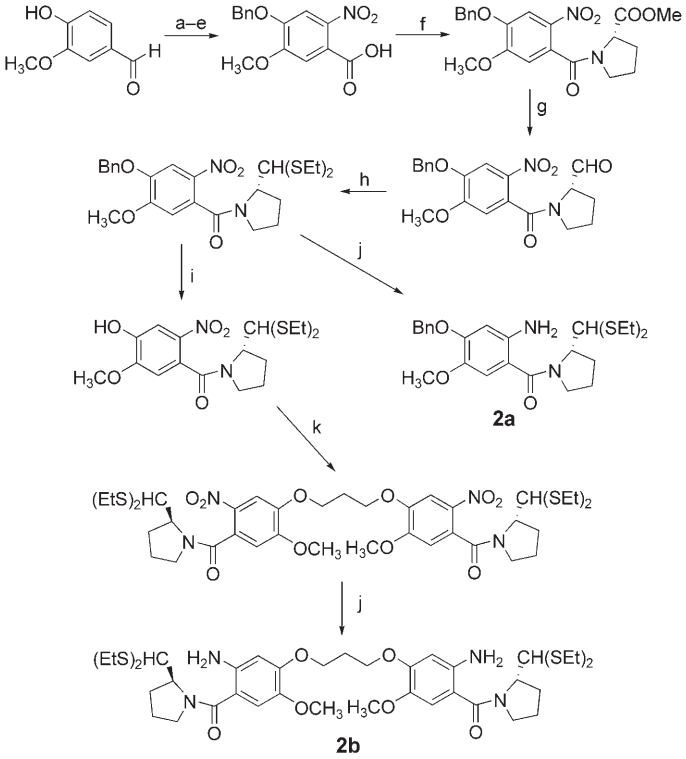
manner, such as improving water solubility and stability. As predicted, these new prodrugs fulfill the criteria of selective activation with the selectivity or activation factor (QIC_{50}) ranging from 100–400-fold, and the activity approaching that of the parent drug, when exposed to the enzyme β-galactosidase. Significantly enhanced stability and water solubility in comparison to the parent moiety are the other important features of these prodrugs.

Results and Discussion

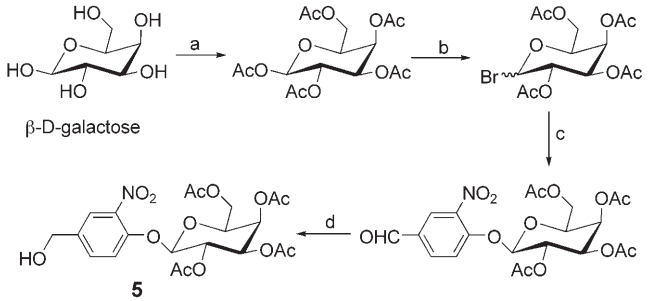
Chemistry

The synthesis of PBD precursors[51–53] [(2-amino-4-benzyloxy-5-methoxy-1,4-phenylene)carbonyl](2*S*)-pyrrolidine-2-carboxaldehyde diethylthioacetal (**2a**) and 1,1′-[[(propane-1,3-diyl)dioxy]-bis[(2-amino-5-methoxy-1,4-phenylene) carbonyl]]-bis[(2*S*)-pyrrolidine-2-carboxaldehyde diethylthioacetal (**2b**) was carried out employing vanillin as the starting material. Vanillin on oxidation followed by esterification, benzylation, and nitration was hydrolyzed to give nitro acid which was coupled with proline ester and reduced to obtain the aldehyde which was protected as carboxaldehyde diethylthioacetal, followed by reduction of the nitro group to provide the amine precursor **2a**. The nitro thioacetal intermediate was debenzylated and dimerized using 1,3-dibromopropane in acetonitrile to give the nitro thioacetal dimer that was reduced to yield **2b** (Scheme 1). The synthesis of β-galactoside promoiety[54] (**5**) was carried out starting from D-galactose which was peracetylated followed by anomeric bromination and coupling with 4-hydroxy-3-nitrobenzaldehyde. The coupled moiety was reduced to afford the required β-galactoside self immolative moiety (Scheme 2).

As shown in Scheme 3, the PBD-β-galactoside prodrugs were synthesized by coupling PBD intermediates **2a**–**b** with the β-galactoside promoiety (**5**). The coupling was carried out via isocyanates of PBD-amine intermediates, using triphosgene, triethylamine, and a catalytic amount of dibutyltin dilaurate to obtain the carbamates **3a**–**b**. Deprotection of the diethylthioacetal group using mercuric chloride and calcium carbonate provides the carbinolamine carbamates **4a**–**b** upon cyclization. Finally the deacetylation of the acetates using NaOMe at 0-5 °C affords the desired target compounds **1a**–**b**.



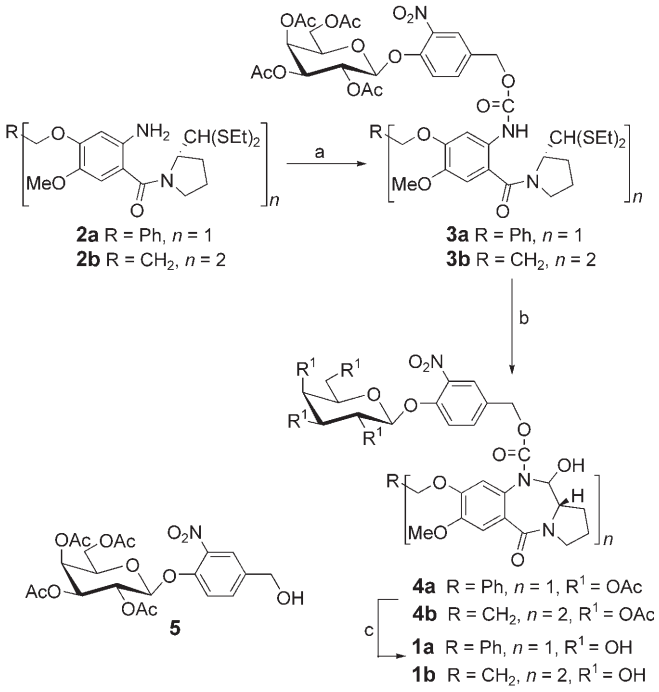
Scheme 1. Preparation of PBD precursors. Reagents and conditions: a) NH_2SO_3H, $NaClO_2$, H_2O, 1 h; b) H_2SO_4, MeOH, 15 h; c) BnBr, K_2CO_3, acetone, reflux, 12 h; d) HNO_3-H_2SO_4, $SnCl_4$, CH_2Cl_2, −25 °C, 5 min; e) 2 N LiOH, THF, MeOH, H_2O (3:1:1), RT, 12 h; f) C_6H_6, $SOCl_2$, L-prolinemethyl ester hydrochloride, THF/H_2O, Et_3N, 0 °C; g) DIBAL-H, CH_2Cl_2, −78 °C, 45 min; h) EtSH-TMSCl, $CHCl_3$, RT, 16–18 h; i) BF_3.OEt-EtSH, CH_2Cl_2, 12 h; j) $SnCl_2$·2H_2O, MeOH, reflux, 4 h ; k) 1, 3-dibromopropane, K_2CO_3, acetonitrile, reflux, 12 h.



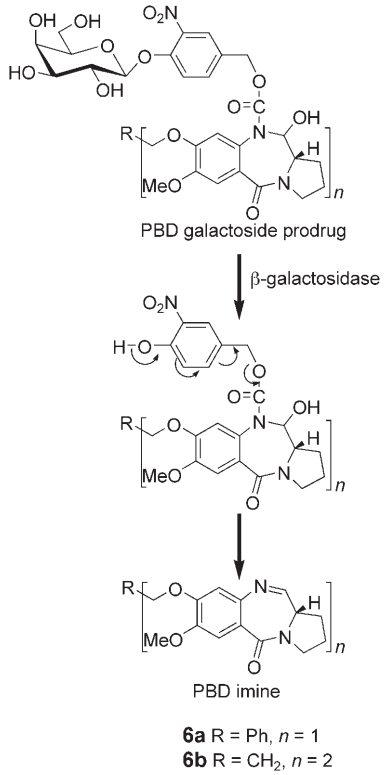
Scheme 2. Preparation of β-galactoside promoiety. Reagents and conditions: a) Ac_2O, CH_3COONa, reflux, 2 h; b) CH_2Cl_2, HBr-CH_3COOH, 3 h; c) CH_3CN, Ag_2O, 4-hydroxy-3-nitro benzaldehyde, 3 h; d) $CHCl_3$, $(CH_3)_2CHOH$, $NaBH_4$, 3 h.

Stability and Activation

The galactoside prodrugs **1a** and **1b** were found to be stable when incubated at a pH of 7.4 and 37 °C, and tested by subjecting aliquots of the mixture to HPLC analysis. The prodrugs were stable for longer than the time needed for activation by the enzyme β-galactosidase. The prodrugs were found to be good substrates for *E. coli* β-galactosidase enzyme and were self immolative after the cleavage of the β-galactoside bond



Scheme 3. Synthesis of PBD β-galactoside prodrugs. Reagents and conditions: a) Et_3N, $CO(COCl_3)_2$, 25 min, RT, b) 5, dibutyltin dilaurate, RT, 6–8 h, yields **3a**: 78%, **3b**: 67%; b) $HgCl_2$, $CaCO_3$, CH_3CN/H_2O, 4:1, 12 h, RT, yields **4a**: 75%, **4b**: 76%; c) MeOH, NaOMe, 30 min, 0–5 °C, yields **1a**: 85%, **1b**: 50%.



Scheme 4. Pyrrolobenzodiazepine β-galactoside prodrug activation. Representation of enzymatic toxification of the PBD-glycoside prodrugs: a) β-galactosidase catalyzed hydrolysis of the glycoside moiety; b) 1, 6-elimination of the self-immolative spacer to form the cytotoxic drug.

leading to the complete conversion to their parent moieties (Scheme 4), in 75 and 90 min respectively (Figures 2 and 3).

Biology: Selective Anticancer Activity

The two prodrugs **1a** and **1b** were evaluated for their antiproliferative activity by using 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) assay.[55] Two different cell lines, A375 (Table 1) and Hep G2 (Table 2) were selected to probe the activation ability of the prodrugs by ADEPT and PMT strategies respectively. The two prodrugs **1a** and **1b** exhibited a highly reduced cytotoxicity, compared to the parent imines **6a** and **6b** respectively, against the A375 tumor cell line. In the experiments directed to investigate the antiproliferative nature of the prodrugs in the presence of the enzyme β-galactosidase against A375 cell lines, the prodrug **1a** was found to be non-toxic (IC_{50} = 422 μmol) (Figure 4a), whereas in the presence of *E. coli* β-galactosidase enzyme (ADEPT) the prodrug inhibited cell growth efficiently and the IC_{50} value was found to be 1.2 μmol, with an activation factor (QIC_{50}) of 350 (Figure 4b). Likewise the prodrug **1b** was also found to be relatively less toxic (IC_{50} = 9.74 μmol) (Figure 4c). In the presence of the enzyme β-galactosidase **1b** showed an IC_{50} value of 0.09 μmol, with the activity approaching that of the active parent moiety (0.06 μmol) and an activation factor (QIC_{50}) of more than 100 (Figure 4d).

In the Hep G2 cell lines the activation factor (QIC_{50}) of the prodrug **1a** was found to be very low (2.5), contrary to that observed in A375 cell lines (350). The prodrug **1a** was found

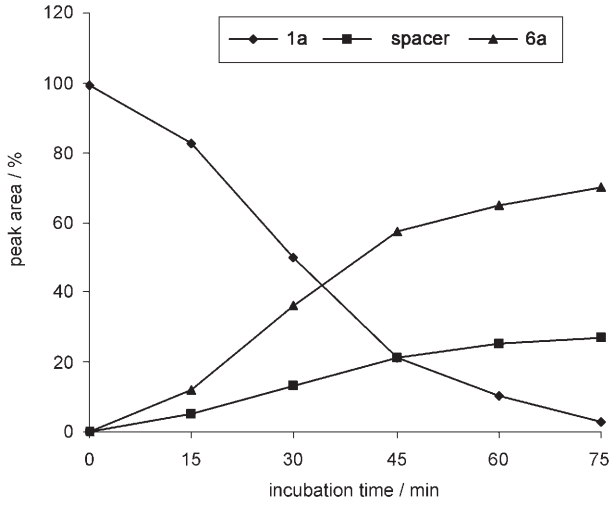


Figure 2. A graph showing a decrease in the percentage peak area of the prodrug **1a**, with an increase in the area of parent moiety **6a** and self-immolative spacer with respect to time in the presence of *E. coli* β-galactosidase enzyme (2 U μmol^{-1}).

to show an IC_{50} value of 14.5 μmol, close to the IC_{50} value of the prodrug when activated with β-galactosidase (5.52 μmol), whereas the parent imine **6a**, was found to possess an IC_{50} value of 8.56 μmol (Figure 4e). A drastic decrease in the activation factor (QIC_{50}) of the prodrug from the A375 to the Hep G2 cell line indicates the activation of the prodrug by the endoge-

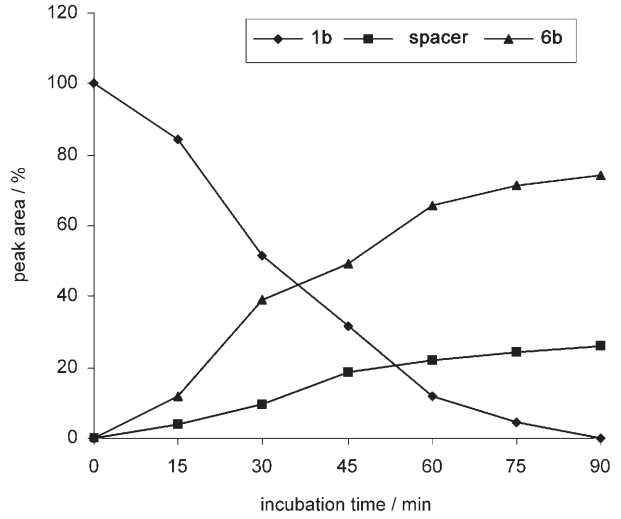


Figure 3. A graph showing a decrease in the percentage peak area of the prodrug **1 b**, and an increase in the area of parent moiety **6 b** and self-immolative spacer with respect to time in the presence of *E. coli* β-galactosidase enzyme (4 U μmol^{-1}).

Table 1. Cytotoxicity of the prodrugs in A375 cell line.

Prodrug	IC_{50}[a]	IC_{50}[a] in the presence of β-galactosidase	Activation factor QIC_{50}	IC_{50}[a] of the parent PBD
1 a	422.9	1.2	352.4	0.37
1 b	9.74	0.09	108.2	0.06

[a] IC_{50} values given as [μmol]

Table 2. Cytotoxicity of the prodrugs in Hep G2 cell line.

Prodrug	IC_{50}[a]	IC_{50}[a] in the presence of β-galactosidase	Activation factor QIC_{50}	IC_{50}[a] of the parent PBD
1 a	14.11	5.52	2.5	8.56
1 b	> 200	0.87	> 100	0.94

[a] IC_{50} values given as [μmol]

nous β-galactosidase (PMT). In Hep G2 cells, contrary to **1 a** the prodrug **1 b** was observed to be less toxic even at a concentration beyond 200 μmol, whereas in the presence of β-galactosidase its IC_{50} value was 0.87 μmol, equivalent to that of the parent imine (0.94 μmol) (Figure 4 f and g). The high-hydrophilic/low-lipophilic nature of the molecule arising from two sugar moieties is anticipated to be the reason; preventing the transport of the molecule across the lipoprotein cell membrane, and thereby preventing the interaction of the prodrug with the endogenous enzyme.

The pharmacophoric N10-C11 imine functionality of PBD molecules is essential for its reaction with DNA to form covalent adduct with N2 of guanine, leading to cytotoxicity. The imine functionality was found to be reactive towards thioalcohol containing molecules present in the cells, forming adducts before it interacts with DNA, making them less effective. In the prodrug molecules the imine functionality is protected as carbinolamine carbamate averting its reaction with DNA to form a covalent bond. The prodrugs get converted into active cytotoxic imine or carbinolamine moiety by the enzyme β-galactosidase, enabling them to interact with DNA to form the covalent bond. The delivery of PBD molecules as prodrugs not only serves the purpose of selective delivery but also as an approach to prevent their interaction with nontarget molecules to a great extent. The experimental results clearly indicate that both the N10 carbamate galactoside prodrugs of PBDs, **1 a** and **1 b**, as latent forms of imine functionality that have also resulted in molecules with a potential for use in selective therapy of solid tumors by ADEPT and the prodrug **1 a** was found to be suitable for PMT of hepatocellular carcinoma. The molecules were also found to possess enhanced aqueous solubility and stability.

Conclusions

In summary, the two new PBD galactoside prodrugs **1a** and **1b**, that were synthesized by masking the pharmacophoric N10-C11 imine functionality were found to be good substrates for β-galactosidase and significantly less cytotoxic than the parent compounds. The prodrugs demonstrated a very good aptitude for their use in targeted therapy of solid tumors, by ADEPT protocol, with the best activation factor (QIC_{50}) reported so far by PBD prodrugs. In addition, **1a** was also found to be suitable for use in PMT of hepatocellular carcinoma, demonstrating that PBD monomers and conjugates are good candidates for targeted delivery by PMT. Furthermore, these molecules were found to possess enhanced water solubility and stability compared to the parent moieties improving their prospects enormously, as new drugs for cancer treatment. Based on the interesting results that have been achieved in this investigation, it is considered that compound **1b** shall be taken up for detailed investigations, including the in vivo studies.

Experimental Section

General: Chemicals were procured from Alfa Aesar (Lancaster Chemicals Ltd) and Sigma Aldrich Chemical Co. Enzyme *E. coli* β-galactosidase was obtained from Sigma Aldrich Chemical Co. Reaction progress was monitored by thin layer chromatography (TLC) using GF254 silica gel with fluorescent indicator on glass plates. Visualization was achieved with UV light and iodine vapor unless otherwise stated. Chromatography was performed using Acme silica gel (100–200 mesh). ^{1}H NMR spectra were recorded on an Inova 500 MHz, Bruker FT 300 MHz, and Unity 400 MHz spectrometer. Spin multiplicities are described as s (singlet), br s (broad singlet), d (doublet), and m (multiplet). Coupling constants are reported in Hertz (Hz). ESI spectra were recorded on Agilent 1100 series mass spectrometer in ESI mode positive ion trap detector. High-resolution mass spectra (HRMS) were recorded on high resolution QSTAR XL Hybrid MS/MS mass spectrometer. IR spectra were recorded on Thermo Nicolet Nexus 670 spectrometer. Specific rotation was recorded on Horiba Sepa-300 polarimeter. The green fluo-

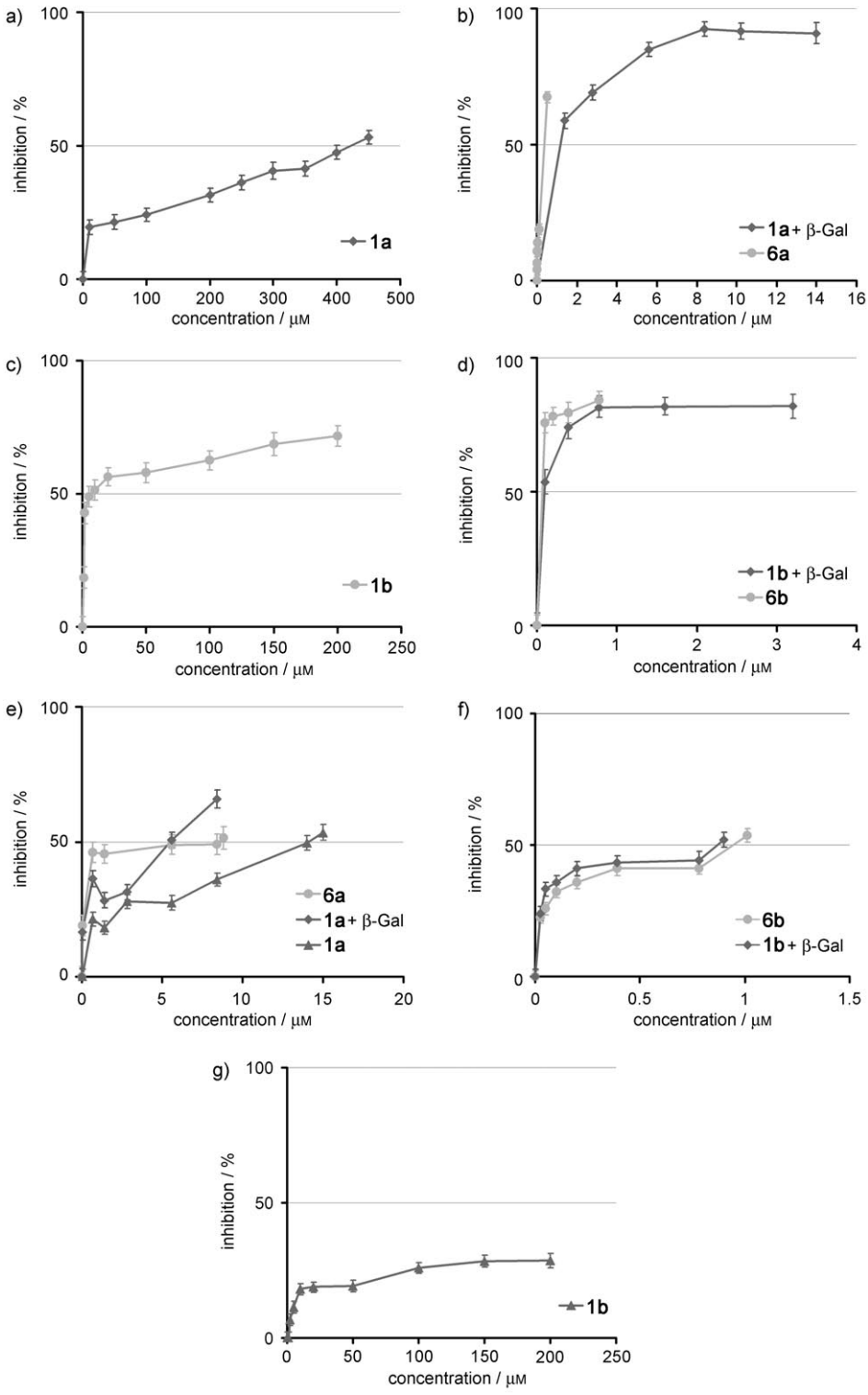


Figure 4. Antiproliferative activity of **1a**, **1a** + β-gal, **6a**, and **1b**, **1b** + β-gal, **6b** were studied in A375 cells with an intracellular label CFSE, which upon cell division, is divided equally between daughter cells, with sequential halving of fluorescence intensity. Flow cytometry was used to assess the MFI of the drug treated and vehicle treated control cells. The maximal and minimal fluorescence were scaled to a percentage level and 50% inhibition was calculated by plotting a graph of the serial dilutions of the drug concentrations. **1a** (422 μM) and **1b** (9.74 μM) showed minimal inhibition in A375 (graphs a and c respectively) proving to be nontoxic, whereas in the presence of enzyme exhibited IC_{50} values of 1.2 μM and 0.09 μM respectively (graphs b and d). In Hep G2 cells, **1a** in the presence of endogenous enzyme (although the activity range is much below the pH of culture medium 7.2) showed an effective inhibitory concentration of 14.5 μM. In the presence of the exogenous enzyme the IC 50 value was 5.52 μM (graph e). The IC_{50} value of **1b** in the presence of enzyme was 0.87 μM, (graph f) although in its absence **1b** was not able to inhibit much (graph g). Each data point represents the mean of triplicate cultures from two representative experiments.

rescence of CFSE was excited at 488 nm, FACS Calibur, Becton Dickinson, Heidelberg, Germany.

{[2-Amino-*N*-(4-β-D-2,3,4,6-tetra-*O*-acetylgalactopyranosyloxy-3-nitrophenyl) methoxycarbonyl-4-benzyloxy-5-methoxy-1,4-phenylene]carbonyl}(2*S*)-pyrrolidine-2-carboxaldehyde diethylthioacetal (3a)

Triethylamine (0.6 mL, 4.29 mmol) and triphosgene (0.19 g, 0.64 mmol) were added to compound **2a** (0.9 g, 1.95 mmol) taken in dry CH_2Cl_2 (25 mL), and stirred. After 25 min CH_2Cl_2 was evaporated under reduced pressure and the reaction mixture was dissolved in tetrahydrofuran (30 mL), and filtered through a sintered funnel leaving behind a white crystalline solid. The tetrahydrofuran from the filtrate was evaporated and the residue was redissolved in CH_2Cl_2 (25 mL). To this solution, **5** (0.97 g 1.95 mmol) and a catalytic amount of dibutyltin dilaurate were added and stirred for 6–8 h. The reaction mixture was washed with brine, dried over anhydrous Na_2SO_4, and purified by column chromatography to get **3a** (yield 1.49 g, 1.51 mmol, 78%). mp 85–86 °C; $[\alpha]_D^{25} = +25.0$ ($c = 0.55$, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta =$ 1.36–1.77 (m, 6H), 1.86–2.33 (m, 16H), 2.59–2.80 (m, 4H), 3.54–3.61 (m, 2H), 3.83 (s, 3H), 4.04–4.28 (m, 3H), 4.62–4.75 (m, 2H), 5.04–5.21 (m, 6H), 5.45–5.59 (m, 2H), 6.92 (s, 1H), 7.29–7.60 (m, 7H), 7.84 (d, $J = 2.26$ Hz, 1H), 7.93–7.98 (m, 1H), 9.15 ppm (br s, 1H); ESI-MS: *m/z* 1008 $[M+Na]^+$; HRMS (ESI): $[M+Na]^+$ calcd for $C_{46}H_{55}N_3O_{17}NaS_2$ *m/z* 1008.2865, found *m/z* 1008.2906; IR: $\tilde{\nu} =$ 3296, 2925, 1751,1622, 1596, 1533, 1452, 1369, 1226, 1168, 1065 cm^{-1}. Anal. Calcd for C_{46} H_{55} N_3 O_{17} S_2: C, 56.03; H, 5.62; N, 4.26%. Found: C, 56.38; H, 5.33; N, 4.39%.

1,1′-{[(Propane-1,3-diyl)dioxy]-bis[(2-amino-*N*-(4-β-D-2,3,4,6-tetra-*O*-acetylgalacto pyranosyl-oxy-3-nitrophenyl)methoxycarbonyl-5-methoxy-1,4-phenylene)carbonyl]}-bis[(2*S*)-pyrrolidine-2-carboxaldehyde diethylthioacetal] (3b)

Compound **3b** was synthesized following the protocol for **3a**

using **2b** (1.3 g, 1.66 mmol), triethylamine (1.02 mL, 7.30 mmol), triphosgene (0.32 g, 1.09 mmol), **5** (1.65 g, 3.32 mmol), and a catalytic amount of dibutyltin dilaurate, to get **3b** (yield 2.36 g, 1.11 mmol, 67%). mp 80–81 °C; $[\alpha]_D^{25} = +2.7$ ($c = 0.55$, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 1.20$–1.35 (m, 12H), 1.55–2.44 (m, 34H), 2.59–2.79 (m, 8H), 3.53–3.60 (m, 4H), 3.81 (s, 6H), 4.04–4.35 (m, 10H), 4.63–4.73 (m, 4H), 5.04–5.18 (m, 8H), 5.45–5.58 (m, 4H), 6.90 (s, 2H), 7.34–7.39 (m, 2H), 7.54–7.59 (m, 2H), 7.82–7.90 (m, 4H), 9.18 ppm (br.s, 2H, NH); ESI-MS: *m/z* 1853 $[M+Na]^+$; HRMS (ESI): $[M+Na]^+$ calcd for $C_{81}H_{102}N_6O_{34}NaS_4$ *m/z* 1853.5217, found *m/z* 1853.5270; IR: $\tilde{\nu} = 3337$, 2925, 2855, 1753, 1621, 1530, 1372, 1225, 1075 cm^{-1}. Anal. Calcd for C_{81} H_{102} N_6 O_{34} S_4: C, 53.11; H, 5.61; N, 4.59%. Found: C, 52.85; H, 5.42; N, 4.74%.

(11*S*)-10-(4-β-D-2,3,4,6-tetra-*O*-acetylgalactopyranosyloxy-3-nitrophenyl methoxycarbonyl-11-hydroxy-7-methoxy-1,2,3,10,11,11a-hexahydro-5*H*-pyrrolo[2,1-*c*][1,4]-benzodiazepin-5-one (4a)

Compound **3a** (1.4 g, 1.42 mmol) was taken in CH_3CN-H_2O as a 3:1 mixture (15 mL) to which $CaCO_3$ (0.35 g, 3.55 mmol) and $HgCl_2$ (0.84 g, 3.12 mmol) were added and stirred for 12 h. After the completion of the reaction, the mixture was filtered through a celite bed. Acetonitrile was evaporated from the filtrate and extracted with ethyl acetate and water. The ethyl acetate extract was dried with anhydrous Na_2SO_4 and the solvent was evaporated. The compound was purified by column chromatography to get **4a** (yield 0.94 g, 1.07 mmol, 75%). mp 110–111 °C; $[\alpha]_D^{25} = +96.9$ ($c = 0.65$, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta = 1.55$–2.20 (m, 16H), 3.43–3.75 (m, 3H), 3.93 (s, 3H), 4.10–4.23 (m, 3H), 4.90–5.16 (m, 6H), 5.41–5.56 (m, 2H), 5.59 (d, $J = 9.82$ Hz, 1H), 6.63 (s, 1H), 7.23–7.42 (m, 8H), 7.57 ppm (s, 1H); ESI-MS: *m/z* 902 $[M+Na]^+$; HRMS (ESI): $[M+Na]^+$ calcd for $C_{42}H_{45}N_3O_{18}Na$ *m/z* 902.2590, found *m/z* 902.2604; IR: $\tilde{\nu} = 3441$, 2922, 2852, 1752, 1623, 1534, 1461, 1374, 1227, 1050 cm^{-1}. Anal. Calcd for C_{42} H_{45} N_3 O_{18}: C, 57.34; H, 5.16; N, 4.78%. Found: C, 56.98; H, 5.31; N, 5.08%.

1,1′-{[(Propane-1,3-diyl)dioxy]-bis(11*S*,11a*S*)-10-(4-β-D-2,3,4,6-tetra-*O*-acetylgalactopyranosyloxy-3-nitrophenyl)methoxycarbonyl-11-hydroxy-7-methoxy-1,2,3,10,11,11a-hexahydro-5*H*-pyrrolo[2,1-*c*][1,4]-benzodiazepin-5-one} (4b)

Compound **4b** was synthesized following the protocol for **4a** using **3b** (2 g, 1.09 mmol), $CaCO_3$ (0.54 g, 5.45 mmol), and $HgCl_2$ (1.33 g, 4.90 mmol) to get **4b** (yield 1.33 g, 0.822 mmol, 76%). mp 132–133 °C; $[\alpha]_D^{25} = +123.75$ ($c = 0.8$, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 1.95$–3.35 (m, 34H), 3.37–4.33 (m, 22H), 4.84 (d, $J = 12.84$ Hz, 2H), 4.97–5.15 (m, 4H), 5.30 (d, $J = 13.56$, 2H), 5.46 (d, $J = 3.02$ Hz, 2H), 5.52–5.66 (m, 2H), 5.72 (d, $J = 9.82$ Hz, 2H), 6.86 (s, 2H), 7.12–7.44 (m, 6H), 7.52 ppm (s, 2H); ESI-MS: *m/z* 1641 $[M+Na]^+$; HRMS (ESI): $[M+Na]^+$ calcd for $C_{73}H_{82}N_6O_{36}Na$ *m/z* 1641.4667, found *m/z* 1641.4672; IR: $\tilde{\nu} = 3448$, 2929, 1755, 1626, 1538, 1435, 1373, 1233, 1068 cm^{-1}. Anal. Calcd for C_{73} H_{82} N_6 O_{36}: C, 54.14; H, 5.10; N, 5.19%. Found: C, 54.45; H, 5.32; N, 5.42%.

General procedure for the preparation of 1a and 1b

Carbinolamine carbamates (0.9 g, 1.02 mmol **4a** or 1 g, 0.61 mmol **4b**) were dissolved in methanol (15 mL) and catalytic amount of NaOMe was added at 0 °C and stirred for 30 min to obtain the final compounds. Compound **1a** was purified by column chromatography using methanol/chloroform (1:9) as eluent, followed by reverse phase preparative HPLC purification using a (4:6) methanol-water system. Compound **1b** was purified by reverse phase preparative HPLC, using C-18 reverse phase column and, methanol/water (1:1) as mobile phase.

(11*S*)-10-(4-β-D-galactopyranosyloxy-3-nitrophenyl)methoxycarbonyl-11-hydroxy-7-methoxy-1,2,3,10,11,11a-hexahydro-5*H*-pyrrolo[2,1-*c*][1,4]-benzodiazepin-5-one (1a)

Yield 0.61 g; (85%); mp 137–139 °C; $[\alpha]_D^{25} = +36.6$ ($c = 0.6$, CH_3OH); ^{1}H NMR (CD_3OD, 500 MHz): $\delta = 1.97$–2.17 (m, 4H), 3.39–3.84 (m, 9H), 3.89 (s, 3H), 4.91–5.17 (m, 5H), 5.66 (d, $J = 9.73$ Hz, 1H), 6.87 (s, 1H), 7.24–7.47 (m, 8H), 7.57 ppm (m, 1H); ESI-MS: *m/z* 734 [M + Na] $^+$; HRMS (ESI): $[M+Na]^+$ calcd for $C_{34}H_{37}N_3O_{14}Na$ *m/z* 734.2173, found *m/z* 734.2158; IR: $\tilde{\nu} = 3369$, 2926, 1712, 1623, 1532, 1460, 1382, 1277, 1069 cm^{-1}. Anal. Calcd for C_{34} H_{37} N_3 O_{14}: C, 57.38; H, 5.24; N, 5.90%. Found: C, 57.02; H, 5.50; N, 6.24%.

1,1′-{[(Propane-1,3-diyl)dioxy]-bis(11*S*,11a*S*)-10-(4-β-D-galactopyranosyloxy-3-nitrophenyl)methoxycarbonyl-11-hydroxy-7-methoxy-1,2,3,10,11,11a-hexahydro-5*H*-pyrrolo[2,1-*c*]-[1,4]benzodiazepin-5-one} (1b)

Yield 0.39 g; (50%); mp 102–103 °C; $[\alpha]_D^{25} = +65.0$ ($c = 0.3$, CH_3OH); ^{1}H NMR (CD_3OD, 500 MHz): $\delta = 1.99$–2.20 (m, 8H), 2.23–2.30 (m, 2H), 3.42–3.54 (m, 4H), 3.58–3.66 (m, 4H), 3.71–3.78 (s, 6H), 3.82–3.90 (m, 8H), 3.92–3.96 (m, 2H), 4.03–4.29 (m, 4H), 4.91–5.07 (m, 4H), 5.22 (d, $J = 11.73$ Hz, 2H), 5.70 (d, $J = 8.97$ Hz, 2H), 6.96 (s, 2H), 7.21 (s, 2H), 7.32–7.72 ppm (m, 6H); ESI-MS: *m/z* 1305 $[M+Na]^+$; HRMS (ESI): $[M+Na]^+$ calcd for $C_{57}H_{66}N_6O_{28}Na$ *m/z* 1305.3822, found *m/z* 1305.3802; IR: $\tilde{\nu} = 3413$, 2954, 1622, 1534, 1464, 1435, 1318, 1273, 1072 cm^{-1}. Anal. Calcd for C_{57} H_{66} N_6 O_{28}: C, 53.35; H, 5.18; N, 6.55%. Found: C, 53.02; H, 5.42; N, 6.83%.

Determination of purity of the prodrugs by HPLC: Purity of the prodrugs **1a** and **1b** was determined by applying (25 μL) the samples to a prepacked reverse phase column (Luna 5 μm, 250× 4.60 mm, C18 (**2**), Phenomenex), using Shimadzu LC-10AT vp HPLC system equipped with a SPD-10 A vp UV visible detector, at 254 nm absorbance, with a flow rate of 1 mL min^{-1} at room temperature. A mixture of acetonitrile/water (3:7) for **1a** and (4:6) for **1b**, was used as mobile phase. The samples were prepared by dissolving (1 μmol) the prodrugs in (3 mL) mobile phase.

Stability of prodrugs in the absence and presence of *E. coli* β-galactosidase: The enzyme was purchased from Sigma Chemical Co., and used as received. The prodrugs **1a** and **1b** (1 μmol) were incubated in (3 mL) 0.05 M phosphate buffer of pH 7.4 at 37 °C. Samples of the incubated compounds (25 μL) were analyzed, and were found to be stable. The prodrugs **1a** and **1b** under similar conditions were treated with *E. coli* β-galactosidase enzyme (2 units for **1a** and 4 units for **1b**) and incubated at 37 °C. Aliquots (25 μL) of the reaction mixture were analyzed, immediately after the addition of the enzyme and at time intervals of every 15 min by HPLC. In addition to the prodrug compounds, progressively increasing peaks of the reaction products were also present in all the chromatograms. The peak corresponding to prodrug **1a** with a retention time of 5.30 min decreased, with the progressive increase of peaks at retention times of 2.40 and 7.86 min, corresponding to the spacer and the parent PBD moiety **6a**, respectively. Similarly, the peak corresponding to prodrug **1b** with a retention time of 4.15 min was observed to decrease, with a progressive increase of the peak at a retention time of 2.60 min, corresponding to the spacer, and a peak with a retention time of 3.39 min that corresponds to the parent PBD moiety **6b**. Prodrug **1a** is activated within a period of 75 min whereas **1b** takes 90 min to activate completely. Reverse phase HPLC with a flow rate of 1 mL min^{-1}, a prepacked reverse phase column (Luna 5 μm, 250×4.60 mm, C18 (2), Phenomenex), and Shimadzu LC-10AT vp HPLC system equipped with a SPD-10 A vp UV visible detector were used, at 254 nm absorbance.

X-Gal assay: Cells were grown in 30 mm petriplates till they reach appropriate confluency. The cells were then fixed with 0.05% glutaraldehyde for 5 min at room temperature. Cells were then washed with 1X PBS twice and incubated in PBS for 10 min to remove all traces of fixative. To the fixed cells X-Gal solution (1 mg mL^{-1} X-Gal in 5 mmol $K_4Fe(CN)_6 \cdot 3H_2O$, 5 mmol $K_3Fe(CN)_6$, 1 M

$MgCl_2$, 1 XPBS) was added and incubated at 37 °C for 24 h at slightly acidic pH. The color change in substrate in Hep G2 cells was observed with an Axiovert Live Cell Microscope and captured in RGB mode to record the change in color. The A375 cells, which showed no blue color were also captured as negative controls.

Anticancer activity of the compounds (CFSE proliferation assay): Stocks of 5 mM CFSE (Sigma Chemicals) were prepared with DMSO. The cells were resuspended in prewarmed PBS/0.1% BSA at a final concentration of 1×10^6 cells/mL and 2 μL of 5 mM stock CFSE solution was added per milliliter of cells for a final working concentration of 10 μM. The cells were incubated with the dye at 37 °C for 10 min. The staining was quenched by the addition of five volumes of ice-cold culture media to the cells. The cell suspension was incubated for 5 min on ice. The cells were pelleted by centrifugation and washed by resuspending in the fresh media for a total of three washes. In vitro cell cultures were set up with fresh medium. After the cells attach and spread well, they are subjected to treatment of increasing concentrations of the drugs and enzymes. Compounds **1a**, **6a** were administered in 0.035–14 μM concentration range, whereas **1b**, **6b** were administered in 0.025–3.2 μM concentrations. Enzyme β-galactosidase, 2 U μM^{-1} for **1a** and 4 U μM^{-1} for **1b**, was added for activation. After 36 h of incubation, allowing sufficient time for at least one round of cell division, cells were harvested and analyzed with a flow cytometer to estimate mean fluorescence intensity. Proliferation analysis had to be performed within a certain maximum permissible time as death was initiated in cells incubated with drug for a longer time period. Moreover the initiation of death lowers the pH of the media (below 6.8) that quenches the fluorescence of CFSE. The green fluorescence of CFSE was excited at 488 nm (FACS Calibur, Becton Dickinson, Heidelberg, Germany). At least ten thousand cells were analyzed per sample and each staining experiment was repeated four times. Data analysis was performed on fluorescence intensities that excluded cell autofluorescence and cell debris. CELLQuest analysis software was used for fluorescence determination and was used to generate the histogram overlays.

Acknowledgements

The authors V. Tekumalla and A. Krishnan are grateful to CSIR, New Delhi, for the award of research fellowship.

Keywords: antitumor agents · glycosides · prodrugs · pyrrolobenzodiazepines · selective therapy

[1] K. D. Bagshawe, *Br. J. Cancer* **1987**, *56*, 531–532.
[2] K. D. Bagshawe, *Drug Dev. Res.* **1995**, *34*, 220–230.
[3] W. A. Denny, *Cancer Invest.* **2004**, *22*, 604–619.
[4] L. F. Tietze, T. Feuerstein, *Curr. Pharm. Des.* **2003**, *9*, 2155–2175.
[5] L. F. Tietze, T. Feuerstein, *Aust. J. Chem.* **2003**, *56*, 841–854.
[6] W. A. Denny, *Eur. J. Med. Chem.* **2001**, *36*, 577–595.
[7] M. Jung, *Mini-Rev. Med. Chem.* **2001**, *1*, 399–407.
[8] G. Xu, H. L. McLeod, *Clin. Cancer Res.* **2001**, *7*, 3314–3324.
[9] K. N. Syrigos, A. A. Epenetos, *Anticancer Res.* **1999**, *19*, 605–614.
[10] G. M. Dubowchik, M. A. Walker, *Pharmacol. Ther.* **1999**, *83*, 67–123.
[11] I. Niculescu-Duvaz, C. J. Springer, *Adv. Drug Delivery Rev.* **1997**, *26*, 151–172.
[12] L. N. Jungheim, T. A. Shepherd, *Chem. Rev.* **1994**, *94*, 1553–1566.
[13] K. Bosslet, J. Czech, D. Hoffman, *Tumor Target.* **1995**, *1*, 45–50.
[14] A. K. Sinhababu, D. R. Thakker, *Adv. Drug Delivery Rev.* **1996**, *19*, 241–273.
[15] K. Bosslet, R. Straub, M. Blumrich, J. Czech, M. Gerken, B. Sperker, H. K. Kroemer, J-P. Gesson, M. C. Koch, *Cancer Res.* **1998**, *58*, 1195–1201.
[16] F. M. H. deGroot, E. W. P. Damen, H. W. Scheeren, *Curr. Med. Chem.* **2001**, *8*, 1093–1122.
[17] M. de Graaf, I. C. Van Veen, I. H. Van Der Meulen-Muileman, W. R. Gerritsen, H. M. Pinedo, H. J. Haisma, *Biochem. J.* **2001**, *356*, 907–910.
[18] M. de Graaf, H. M. Pinedo, R. Quadir, H. J. Haisma, E. Boven, *Biochem. Pharmacol.* **2003**, *65*, 1875–1881.
[19] V. Paradis, N. Youssef, D. Dargère, N. Bâ, F. Bonvoust, J. Deschatrette, P. Bedossa, *Hum. Pathol.* **2001**, *32*, 327–332.
[20] B. Y. Lee, E. C. Goodwin, W. J. Kleijer, D. Dimaio, E. S. Hwang, *Aging Cell* **2006**, *5*, 187–195.
[21] A. Inada, C. Nienaber, S. Bonner-Weir, *Diabetologia* **2006**, *49*, 1120–1122.
[22] D. E. Thurston, A. S. Thomson, *Chem. Br.* **1990**, *26*, 767–772.
[23] D. E. Thurston, M. S. Puvvada, S. Neidle, *Eur. J. Cancer Part A* **1994**, *30*, 567–568.
[24] A. Kamal, M. V. Rao, N. Laxman, G. Ramesh, G. S. K. Reddy, *Curr. Med. Chem.: Anti-Cancer Agents* **2002**, *2*, 215–254.
[25] D. E. Thurston in *Molecular Aspects of Anticancer Drug-DNA Interactions, Vol. 1* (Eds.: S. Neidle, M. J. Waring), MacMillan, Basingstoke, Hants, UK, **1993**, pp. 54–87.
[26] D. S. Bose, A. S. Thompson, J. A. Ching, J. A. Hartley, M. D. Berardini, T. C. Jenkins, S. Neidle, L. H. Hurley, D. E. Thurston, *J. Am. Chem. Soc.* **1992**, *114*, 4939–4941.
[27] A. Kamal, N. V. Rao, *Tetrahedron Lett.* **1995**, *36*, 4299–4302.
[28] S. J. Gregson, P. W. Howard, J. A. Hartley, N. A. Brooks, L. J. Adams, T. C. Jenkins, L. R. Kelland, D. E. Thurston, *J. Med. Chem.* **2001**, *44*, 737–748.
[29] J. A. Hartley, V. J. Spanswick, N. Brooks, P. H. Clingen, P. J. McHugh, D. Hochhauser, R. B. Pedley, L. R. Kelland, M. C. Alley, R. Schultz, M. G. Hollingshead, K. M. Schweikart, J. E. Tomaszewski, E. A. Sausville, S. J. Gregson, P. W. Howard, D. E. Thurston, *Cancer Res.* **2004**, *64*, 6693–6699.
[30] P. H. Clingen, I. U. De Silva, P. J. McHugh, F. J. Ghadessy, M. J. Tilby, D. E. Thurston, J. A. Hartley, *Nucleic Acids Res.* **2005**, *33*, 3283–3291.
[31] M. I. Walton, P. Goddard, L. R. Kelland, D. E. Thurston, K. R. Harrap, *Cancer Chemother. Pharmacol.* **1996**, *38*, 431–438.
[32] A. Kamal, G. Ramesh, N. Laxman, P. Ramulu, O. Srinivas, K. Neelima, A. K. Kondapi, V. B. Sreenu, H. A. Nagarajaram, *J. Med. Chem.* **2002**, *45*, 4679–4688.
[33] A. Kamal, P. S. M. M. Reddy, D. R. Reddy, E. Laxman, *Bioorg. Med. Chem.* **2006**, *14*, 385–394.
[34] A. Kamal, R. Ramu, V. Tekumalla, G. B. R. Khanna, M. S. Barkume, A. S. Juvekar, S. M. Zingde, *Bioorg. Med. Chem.* **2007**, *15*, 6868–6875.
[35] A. Kamal, P. Ramulu, O. Srinivas, G. Ramesh, P. P. Kumar, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4791–4794.
[36] A. Kamal, B. S. N. Reddy, G. S. K. Reddy, G. Ramesh, *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1933–1935.
[37] L. A. Masterson, V. J. Spanswick, J. A. Hartley, R. H. Begent, P. W. Howard, D. E. Thurston, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 252–256.
[38] M. J. Sagnou, P. W. Howard, S. J. Gregson, E. Eno-Amooquaye, P. J. Burkeb, D. E. Thurston, *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2083–2086.
[39] K. D. Bagshawe, *Expert Rev. Anticancer Ther.* **2006**, *6*, 1421–1431.
[40] L. F. Tietze, F. Major, I. Schuberth, *Angew. Chem.* **2006**, *118*, 6724–6727; *Angew. Chem. Int. Ed.* **2006**, *45*, 6574–6577.
[41] G. M. Dubowchik, M. A. Walker, *Pharmacol. Ther.* **1999**, *83*, 67–123.
[42] R. Abraham, N. Aman, R. von Borstel, M. Darsley, B. Kamireddy, J. Kenten, G. Morris, R. Titmas, *Cell Biophys.* **1994**, *24/25*, 127–133.
[43] J. P. Gesson, J. C. Jacquesy, M. Mondon, P. Petit, B. Renoux, S. Andrianomenjanahary, H. D. T. Van, M. Koch, S. Michel, F. Tillequin, J. C. Florent, C. Monneret, K. Bosslet, J. Czech, D. Hoffmann, *Anti-Cancer Drug Des.* **1994**, *9*, 409–423.
[44] L. F. Tietze, R. Hanneman, W. Buhr, M. Logers, P. Menningen, M. Lieb, D. Starck, T. Grote, A. Doring, I. Schuberth, *Angew. Chem.* **1996**, *108*, 2840–2842; *Angew. Chem. Int. Ed. Engl.* **1996**, *35*, 2674–2677.
[45] M. J. Ferguson, F. Y. Ahmed, J. Cassidy, *Drug Resist. Updates* **2001**, *4*, 225–232.
[46] M. Y. Torgov, S. C. Alley, C. G. Cerveny, D. Farquhar, P. D. Senter, *Bioconjugate Chem.* **2005**, *16*, 717–721.
[47] L. F. Tietze, M. Lieb, T. Herzig, F. Haunert, I. Schuberth, *Bioorg. Med. Chem.* **2001**, *9*, 1929–1939.
[48] S. Andrianomenjanahary, X. Dong, J. C. Florent, G. Gaudel, J. P. Gesson, J. C. Jacquesy, M. Koch, S. Michel, M. Mondon, C. Monneret, P. Petit, B. Renoux, F. J. Tillequin, *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1093–1096.
[49] A. K. Ghosh, S. R. Khan, D. Farquhar, *Chem. Commun.* **1999**, 2527–2529.

[50] E. Bakina, D. Farquhar, *Anti-Cancer Drug Des.* **1999**, *14*, 507–515.
[51] D. R. Langley, D. E. Thurston, *J. Org. Chem.* **1987**, *52*, 91–97.
[52] S. M. Courtney, D. E. Thurston, *Tetrahedron Lett.* **1993**, *34*, 5327–5328.
[53] D. Subhas Bose, G. B. Jones, D. E. Thurston, *Tetrahedron* **1992**, *48*, 751–758.
[54] A. K. Ghosh, S. Khan, F. Marini, J. A. Nelson, D. Farquhar, *Tetrahedron Lett.* **2000**, *41*, 4871–4874.
[55] A. B. Lyons, C. R. Parish, *J. Immunol. Methods* **1994**, *171*, 131–137.

Received: November 14, 2007
Revised: December 20, 2007
Published online on February 5, 2008

