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ProTides of BVdU as potential anticancer agents upon efficient intracellular delivery of their activated metabolites

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ABSTRACT

Nucleosides represent a major chemotherapeutic class for treating cancer, however their limitations in terms of cellular uptake, nucleoside kinase-mediated activation and catabolism are well-documented. The monophosphate pro-nucleotides known as ProTides represents a powerful strategy for bypassing the dependence on active transport and nucleoside kinase-mediated activation. Herein, we report the structural tuning of BVdU ProTides. Forty six phosphoramidates were prepared and biologically evaluated against three different cancer cell lines; murine leukemia (L1210), human CD8 T-lymphocyte (CEM) and human cervical carcinoma (HeLa). Twenty-fold potency enhancement compared to BVdU was achieved against L1210 cells. Interestingly, a number of ProTides showed low micromolar activity against CEM and HeLa cells compared to the inactive parent BVdU. The ProTides showed poor, if any measurable toxicity to non-tumourigenic human lung fibroblast cell cultures. Separation of four pairs of the diastereomeric mixtures and comparison of their spectral properties, biological activities and enzymatic activation rate is reported.

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the amino acid ester 5, Scheme 2. The formation of the key phosphorochloridate was monitored by $^{31}$P NMR. Next, the ary laminoacetyl phosphorochloridate 6 was reacted with either BVdU or its carboxy methyl ester precursor 2 in the presence of 1-methylimidazole (NMI), Scheme 2. Overnight reactions at ambient temperature generated crude materials that were purified by column chromatography to provide low to moderate yields typical of previous phosphoramidate ProTide syntheses (3.4–28%) [15]. Each of the phosphoramidate compounds was generated as a pair of diastereoisomers at the phosphate center, in roughly 1:1 ratio, as revealed by the two closely spaced peaks in the $^{31}$P NMR spectrum.

It has been reported before that the biological activity of phosphoramidates can be dependent on the configuration of the phosphorus centre [16,17]. Single phosphorus centre diastereoisomers were separated from their corresponding racemic mixtures of compounds (35, 36, 47 and 49) using a combination of gradient column chromatography and preparative thin liquid chromatography. Obtaining four pairs of fast eluting (f) and slow eluting (s) diastereoisomers; (35f/35s, 36f/36s, 47f/47s and 49f/49s, respectively), offered the opportunity to compare their spectroscopic, anti-proliferative and enzymatic activation rate profiles. Fig. 2A, shows the $^{31}$P NMR of the diastereomeric mixture of 36 and that of its single diastereoisomer components separated (36f and 36s), Fig. 2B and C, respectively. It is noteworthy that in the case of proline-based ProTides (39 and 51), we obtained single P diastereoisomers, however the reasons behind this observed stereoselectivity still need to be investigated.

A comparison between the $^1$H NMR spectra of the diastereomeric pair (49f and 49s) revealed a characteristic pattern difference of the benzylic methylene protons. Fig. 3. For the 49f diastereoisomer, the two protons display a singlet signal, Fig. 3A, while the 49s diastereoisomer shows a double doublet signal, Fig. 3B. Similar findings were reported before for a partially separated racemic mixture of another BVdU ProTide analogue using preparative HPLC and were previously explained through conformational studies [10]. The three aromatic rings (nucleoside base, benzyl ester and naphthyl) are stacked in $\pi-\pi$ interactions in the case of the Sp diastereoisomer. This imparts relative rigidity of this conformation and justifies the observed non-equivalent doublet of doublet splitting NMR pattern of the benzylic methylene hydrogens. On the other hand, the Rp counterpart does not show such interaction among the aromatic rings, resulting in the greater flexibility of the benzylic methylene group reducing the magnetic differences between the two protons and hence, they appear as a singlet signal. Therefore, by combining the NMR and the conformational data, we can propose the Rp configuration to the fast-eluting diastereoisomer 49f and, consequently, the Sp absolute configuration to the slow-eluting diastereoisomer 49s.

ProTides 8–53 described above were evaluated for their cytostatic activity against a panel of three established tumour cell lines in vitro: L1210 (murine leukemia), CEM (human CD4 T-lymphocyte), and Hela (human cervix). In each case a thymidine kinase-deficient (TK−) mutant of the parent cell line is included to probe the effect of TK deficiency on the cytostatic activity of the test compounds.

BVDU and NB1011 were prepared from 5-iodo-2-deoxyuridine 1, which was used to prepare the carboxymethyl ester derivative, 2 via Heck reaction with methyl acrylate in the presence of palladium acetate. Hydrolysis of 2 was carried out using NaOH followed by acidification to get the carboxylic acid derivative 3, which was treated with N-bromosuccinimide (NBS) to give BVDU [11], Scheme 1.

The target BVDU ProTides were all prepared using the extensively described phosphorochloridate chemistry [12,13]. The phenyl phosphorochloridate 4a was used to introduce the phenolic aromatic masking unit of the ProTide. For the naphthyl analogues, 1-naphthol was reacted with phosphoryl chloride to give the required dichloridate 4b, Scheme 1. The second component of the ProTide motif is an amino acid ester (general formula, 5), if not commercially available, was prepared by esterification of the appropriate amino acids using standard methods [14]. The ary laminoacetyl phosphorochloridate (general formula, 6), were prepared by reacting the aryl phosphorochloridate 4a or 4b with the amino acid ester 5, Scheme 2. The formation of the key phosphorochloridate was monitored by $^{31}$P NMR. Next, the ary laminoacetyl phosphorochloridate 6 was reacted with either BVdU or its carboxy methyl ester precursor 2 in the presence of 1-methylimidazole (NMI), Scheme 2. Overnight reactions at ambient temperature generated crude materials that were purified by column chromatography to provide low to moderate yields typical of previous phosphoramidate ProTide syntheses (3.4–28%) [15]. Each of the phosphoramidate compounds was generated as a pair of diastereoisomers at the phosphate center, in roughly 1:1 ratio, as revealed by the two closely spaced peaks in the $^{31}$P NMR spectrum.

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pounds and the degree to which the ProTides could bypass this dependence. The murine L1210 cell line was included because these tumour cells can be used in a mouse tumour model. Also, a normal human non-tumourigenic primary monolayer cell line (lung fibroblast HEL cells) is included for comparative purposes, and BVdU as a positive parental drug control, Table 1. For the murine leukemia cell line (L1210), alanine-based ProTides were amongst the most active compounds with compound 23 (L-alaninyl benzyl ester naphthoxy phosphoramidate, IC$_{50}$ = 1.8 µM) showing 20-fold increase in potency compared to BVdU (IC$_{50}$ = 3.8 µM). Interestingly, while BVdU itself does not show any noteworthy anti-proliferative activity against the human T-lymphocyte cell line (CEM) (IC$_{50}$ > 100 µM), most of its phosphoramidate derivatives showed a greatly enhanced activity with compound 37 (L-phenylalaninyl ethyl ester naphthoxy phosphoramidate, IC$_{50}$ = 4.8 µM) being the most active. While BVdU is poorly cytostatic against the human cervical carcinoma (HeLa) (IC$_{50}$ = 160 µM), compounds 33 (L-valinyl cyclohexyl ester naphthoxy phosphoramidate, IC$_{50}$ = 7.2 µM) and 36 (L-tryptophanyl ethyl ester naphthoxy phosphoramidate, IC$_{50}$ = 7.8 µM) were found to boost the antiproliferative activity significantly. Interestingly, BVdU and its phosphoramidate ProTide derivatives generally showed poor, if any cytotoxicity against the human lung fibroblast cell cultures displaying minimal cytotoxic concentration (MCC) values of $\gg$ 100 µM in the vast majority of the test compounds, Table 1. These findings point to a considerable extent of selectivity of the synthesised ProTides, which is potentially beneficial from a drug development viewpoint.

It has been noticed before that BVdU, in contrast to most other thymine-based nucleoside analogues, often shows an increased

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**Scheme 2.** Synthesis of BVdU phosphoramidate analogues; Reagents and conditions: a) Et$_3$N, anhydrous DCM, –78 °C, 2–5 h, b) NMI, anhydrous THF, –78 °C to r.t., 16–18 h.

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**Fig. 2.** A) $^{31}$P NMR of the racemic mixture 36, B) slow eluting diastereoisomer, 36s, C) fast eluting diastereoisomer 36f.

**Fig. 3.** $^1$H NMR spectra of the benzylic methylene protons (CH$_2$) of the isolated diastereoisomers; (a) 49f, (b) 49s.
cystostatic activity against thymidine kinase-deficient tumour cell lines. The molecular/biochemical basis for this phenomenon is still unclear [18]. Here, a pronounced increase of cystostatic activity was also observed for several BVdU ProTides (i.e. 8, 16, 21 for L1210 and the majority of compounds for CEM and HeLa). These observations are of particular interest since certain forms of drug resistance of cancer cells have been reported to be caused by thymidine kinase deficiency (i.e. drug resistance against 5-FdUrd- and 5-trifluoromethyl-dUrd-treated cancers) [19,20]. It would therefore be reasonable to suggest the use of such BVdU ProTides to treat tumours that became refractory to FdUrd/CfFdUrd treatment.

All compounds 8–53, were evaluated as mixtures of two phosphate diastereoisomers (Rp and Sp in 1:1 ratio). Interestingly, we were able to separate four pairs of diastereoisomers, 35f/35s, 36f/36s, 47f/47s and 49f/49s, which offered the opportunity to compare their relative biological activity. Table 1. It could be concluded that in general, the fast eluting diastereoisomer 35f showed a relatively better cystostatic activity across the three cell lines than its slow eluting diastereoisomer 35s. Apart from 35f/35s, the other three pairs of diastereoisomers, 36f/36s, 47f/47s and 49f/49s, displayed very similar activity profiles across the three cell lines. In two cases, 36f and 49f, the activity of the parent racemic mixture (1:1 ratio) and that of its single diastereomeric components

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* MCC or minimal cytotoxic concentration (µM) required to achieve a microscopically visible cell morphology.
(36f/36s and 49f/49s, respectively) were evaluated. In both cases the anti-proliferative activity were very similar, Table 1.

The side products from the catabolic conversion of the BVdU ProTides to BVdU-MP are amino acids, and phenol or naphthol. Whereas the release of naturally occurring amino acids should not be a matter of concern in terms of potential side-effects, the release of naphthol or phenol might be. However, it should be noted there are currently two ProTide derivatives approved for clinical use (i.e. TAF, or tenofovir alafenamide [21,22] and Sofosbuvir [2]) that release phenol upon conversion to the parent compound without measurable toxic side-effects. Also, given the increased activity of some of the BVdU prodrugs against TK-deficient tumour cell lines, the use of BVdU ProTides may perhaps be more efficient in suppression of TK-based resistance development. Generally, the activity profiles suggested that our library of phosphoramidates were able to release the nucleoside monophosphate within intact cells and afford pronounced cytostatic activity in both the wild-type and TK- cancer cell lines, as shown in Table 1.

Nucleoside analogues are challenged by numerous inherent and acquired cancer resistance mechanisms that can significantly limit

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**Scheme 3.** Proposed activation pathway of phosphoramidate 35; i) esterase or carboxypeptidase-type enzyme, ii) and iii) spontaneous, iv) phosphoramidase-type enzyme.

**Fig. 4.** $^{31}P$ NMR spectra of ProTide 35f (A) and ProTide 35s (B) over time (every 7 min) after treatment with carboxypeptidase Y (5 mg in acetone-D/Trizma) showing the signals of different metabolites.
their effectiveness. ProTides are specifically designed to overcome some key cancer resistance pathways and thereby achieve a superior antineoplastic effect. To exert their anticancer activity, the ProTides are metabolised to release the free nucleoside monophosphate form, which will then generate the corresponding active forms, di- and/or triphosphates. The proposed intracellular activation route of the ProTides has been described for other ProTide families [23] and is exemplified by compound 35 in Scheme 3. The proposed mechanism of activation of the ProTides involves a first enzymatic activation step (i) mediated by a carboxypeptidase-type enzyme that hydrolyses the ester of the aminoacyl moiety to produce the intermediate A. Next, a spontaneous cyclization (ii) displacing the aryl moiety via an internal nucleophilic attack of the carboxylate residue on the phosphorus center to yield B. In a third step (iii), the unstable ring is hydrolysed to release the intermediate C. The last step (iv) involves a phosphoramidase-type enzyme, which cleaves-off the amino acid to generate the corresponding nucleoside monophosphate D, Scheme 3. This assay was designed to verify whether an enzymatic cleavage of the ester motif would be sufficient to trigger the first steps of the activation route and generate the intermediate C [24].

To probe the difference between the P diastereoisomer activation rate, Scheme 3, an enzymatic study using carboxypeptidase Y while monitoring the conversion by 31P NMR was performed. Each diastereoisomer of compound 35, 35f and 35s was dissolved in aceton-d6, in the presence of Trizma buffer (pH 7.6) and treated with carboxypeptidase Y to monitor the metabolic conversion of the ProTide using 31P NMR spectroscopy over time, Fig. 4. In the case of compound 35f, the experiment showed the hydrolysis of the starting material 35f (βP = 4.19) to the intermediate A (βP = 5.08), which is then processed to a compound of type C (βP = 7.09) through the putative intermediate B, Fig. 4A. Interestingly, in the case of compound 35s, the enzymatic study showed a relatively faster hydrolysis rate of 35s (βP = 4.13), which is converted directly to compound of type C (βP = 7.08), Fig. 4B. These findings demonstrate that phosphoramidate diastereoisomers are processed at different rates by carboxypeptidase-type enzymes.

In an attempt to better understand the difference in the enzymatic activation rate of both diastereoisomers of compound 35 in the enzymatic study, a molecular modelling simulation using the crystal structure of the carboxypeptidase Y (PDB: 1YSC) [25,26] was performed, Fig. S4 (supporting information, computational study section).

In summary, a series of forty six BVdU ProTides has been synthesised and have been biologically evaluated together with the parent BVdU nucleoside for their cytostatic activity in vitro against three different cancer cell lines; murine leukaemia (L1210), human T-lymphocyte (CEM) and human cervix carcinoma (HeLa) and for toxicity against human non-tumourigenic lung fibroblast (HEL) cell cultures. ProTide 23 showed twenty-fold better cytostatic activity than the parent BVdU against the L1210 cell line. Low micromolar activity against the CEM and HeLa tumour cell lines compared to inactive parent BVdU nucleoside is observed with a number of our ProTides. The anti-proliferative activity was retained or even enhanced against thymidine kinase-deficient cancer cell lines. Poor, if any cytotoxic activity was observed in HEL cell cultures pointing to a considerable degree of selectivity. Separation of four pairs of P diastereoisomers and the comparison of some spectral properties, anticancer cell activity and enzymatic activation features is described. Molecular modeling studies were conducted in an attempt to explain some of our findings.

Finally, we have demonstrated that the application of the phosphoramidate approach to brivudine (BVdU) results in an extensive source of potential anticancer agents.

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A. Supplementary data

Supplementary data (experimental procedures and spectroscopic characterisation data of the compounds 8–53 as well as the computational study) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.10.077. These data include MOL files and InChIKeys of the most important compounds described in this article.

References and notes

[13] General method for the preparation of BVdU ProTides: Phosphoramidates (8–53) were prepared from a stirrering solution of (E)-5-(2-bromovinyl)-2-deoxyuridine (BVdU) or (E)-5-(2-carboxethoxyvinyl)-2-deoxyuridine (2) (3.0 mol equiv) and the appropriate phosphochloridate (3.0 mol equiv) in anhydrous THF at –78 °C. NMI (5.0 mol equiv) was added dropwise over 1 min. After 30 min the reaction was left to rise to room temperature and stirred for 12 h. The solvent was removed under reduced pressure and the yellow oil obtained was dissolved in DCM, washed with 0.5 M HCl, and water. The organic layer was dried over MgSO4, filtered, reduced to dryness and purified by flash chromatography (dichloromethane/methanol 95/5).