Amino Acid Ester Prodrugs of the Antiviral Agent 2-Bromo-5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole as Potential Substrates of hPEPT1 Transporter

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Amino acid ester prodrugs of 2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (BDCRB) were synthesized and evaluated for their affinity for hPEPT1, an intestinal oligopeptide transporter. Assays of competitive inhibition of [^{3}H]glycylsarcosine (Gly-Sar) uptake in HeLa/hPEPT1 cells by the amino acid ester prodrugs of BDCRB suggested their 2- to 4-fold higher affinity for hPEPT1 compared to BDCRB. Further, promoieties with hydrophobic side chains and L-configuration were preferred by the hPEPT1 transporter.

Introduction

Nucleoside analogues are one of the most important classes of antiviral drugs.[1,2] However, their clinical utility is often hampered by poor biopharmaceutical properties.[3] Several nucleoside drugs exhibit poor oral bioavailability and are highly toxic due to their inability to distinguish between normal cells and target cells.[1,2,4] Thus, strategies that can alleviate these effects will be of immense benefit. In this regard, prodrug approaches have been extensively adopted to improve pharmaceutical properties of drugs.[5,6] Prodrugs targeted to nutrient transporters have been developed in order to improve oral bioavailability, since nutrient transporters are expressed in the gastrointestinal (GI) tract and shown to be vital in the transport of nutrients and various therapeutically important drugs.[7−11] Of these, oligopeptide transporters such as hPEPT1 are promising targets since they are highly expressed in the GI tract and are known to transport a wide variety of di- and tripeptides as well as β-lactam antibiotics, ACE inhibitors, and other peptidomimetic agents.[12−16] It has been demonstrated a posteriori that the 3- to 5-fold higher oral bioavailability of valacyclovir and valganciclovir compared to acyclovir and ganciclovir, respectively, is attributable to their transport by oligopeptide transporters.[7−9] Recently, prodrug approaches termed "pronucleotides" have been designed for intracellular delivery of nucleoside monophosphates in order to obviate drug resistance arising from decreased nucleoside kinase activity.[17−19]

The antiviral agent 2-bromo-5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (BDCRB) is a member of a novel class of benzimidazole ribonucleosides that are potent inhibitors of human cytomegalovirus (HCMV) replication with low cellular toxicity at concentrations inhibiting viral growth.[20,21] The antiviral mechanism of action of BDCRB is unique and involves inhibition of viral DNA processing and virus assembly.[22] However, BDCRB like certain other nucleoside agents exhibits modest oral bioavailability mainly due to extensive first-pass effects.[23] The oral bioavailability of an L-riboside structural analogue, 2-isopropylamino-5,6-dichloro-1-(β-L-ribofuranosyl)benzimidazole (maribavir), was found to be high, indicating the inherent stabilization of the glycosidic bond in vivo.[24,25] Such stabilization of the glycosidic bond via structural alterations of the riboside moiety suggested that modulation of the free hydroxyl groups may result in glycosidic bond stabilization as well. Further, on the basis of recent reports on the potential of amino acid ester prodrugs of floxuridine as substrates of hPEPT1,[26,27] it was hypothesized that amino acid ester prodrugs of BDCRB could also be potential substrates of the hPEPT1 transporter. The syntheses of amino acid ester prodrugs of BDCRB were therefore undertaken in order to (a) investigate their affinity for and transport by the intestinal oligopeptide transporters, and (b) determine if the promoiety affords stabilization of the glycosidic bond of BDCRB.

In this report, we describe the synthesis of a variety of amino acid ester prodrugs of BDCRB and their affinities for the hPEPT1 transporter. The amino acids that were used as promoieties included the aliphatic amino acids L-valine, D-valine, and L-isoleucine; the aromatic amino acids L-phenylalanine, its analogues *p*-chloro-L-phenylalanine, *p*-methoxy-L-phenylalanine, and *p*-ethoxy-L-phenylalanine, as well as D-phenylalanine; the secondary amino acid L-proline; and the polar amino acids L-aspartic acid, D-aspartic acid, and L-lysine. The amino acid promoieties selected in this study represent a broad range of physicochemical and structural properties. BDCRB prodrugs were evaluated for their affinity for hPEPT1 by determination of their

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Table 1. Inhibition of [^{3}H]Gly-Sar Uptake in HeLa/hPEPT1 Cells and Calculated LogP of BDCRB and Various Amino Acid Ester Prodrugs of BDCRB

prodrugs	IC_{50} (μM), average ± SEM[a]	IC_{50} ratio[b]	CLogP[c]
BDCRB, **1**	447 ± 35.4	na[d]	2.04
L-Val-BDCRB, **4a**	166 ± 8.8	2.7	2.59
D-Val-BDCRB, **4b**	342 ± 18.0	1.3	2.59
L-Ile-BDCRB, **4c**	121 ± 2.4	3.7	3.12
L-Phe-BDCRB, **4d**	218 ± 22.4	2.1	3.08
D-Phe-BDCRB, **4e**	282 ± 10.2	1.6	3.08
p-chloro-L-Phe-BDCRB, **4f**	142 ± 16.8	3.1	3.79
p-methoxy-L-Phe-BDCRB, **4g**	300 ± 13.2	1.5	3.01
p-ethoxy-L-Phe-BDCRB, **4h**	96 ± 0.7	4.7	3.53
L-Asp-BDCRB, **4i**	260 ± 24.0	1.7	−1.35
D-Asp-BDCRB, **4j**	421 ± 38.4	1.1	−1.35
L-Pro-BDCRB, **4k**	537 ± 25.2	0.8	2.37
L-Lys-BDCRB, **4l**	477 ± 35.5	0.9	1.36
Valacyclovir	431 ± 12.7	na	−1.21

[a] Mean and standard error of the mean derived from three separate experiments. [b] Parent/prodrug. [c] Calculated using ChemDraw Ultra 7.0. [d] na: not applicable.

ability to inhibit [^{3}H]Gly-Sar uptake in HeLa cells overexpressing hPEPT1.

Results and Discussion

Uptake Inhibition Assays. IC_{50} values calculated from the inhibition data using nonlinear regression analysis for BDCRB and its amino acid ester prodrugs are listed in Table 1. In our assay system, valacyclovir, a known substrate of hPEPT1[9] that was used as a positive control, exhibited high affinity ($IC_{50} = 431$ μM) for hPEPT1 transporter (Table 1). All amino acid ester prodrugs of BDCRB tested also exhibited high affinity (96−476 μM) for hPEPT1 transporter. Except for D-Val, L-Pro, L-Lys, and D-Asp prodrugs, all amino acid ester prodrugs of BDCRB exhibited significantly higher affinity for hPEPT1 than BDCRB ($p < 0.01$). Interestingly, BDCRB, which has no structural similarities to known hPEPT1 substrates, also exhibited affinity that was similar to valacyclovir ($IC_{50} = 447$ μM). None of the other nucleoside agents tested to date (acyclovir, ganciclovir, AZT, and floxuridine) exhibited any affinity for hPEPT1 transporter.[9,10,26,27] The high affinity of BDCRB compared to the other nucleoside agents may in part be due to its highly hydrophobic heterocycle.

The structure of the promoiety influenced the affinity of BDCRB prodrugs for hPEPT1. Generally, amino acid prodrugs of BDCRB with aliphatic (**4a, 4c**), aromatic (**4d, 4f**−**h**), or acidic (**4i**) side chains showed higher affinity than those with basic (L-Lys, **4l**) or constrained (L-Pro, **4k**) side chains. A modest inverse linear correlation of IC_{50} values and CLogP for BDCRB and its prodrugs was evident ($r^2 = 0.65$, Figure 1; Asp prodrugs which were distinct outliers were excluded), suggesting that hydrophobicity of the promoiety plays a significant role in the affinity of the prodrug for hPEPT1 transporter. For BDCRB and its Phe-analogue prodrugs only, this inverse linear correlation was substantially higher; $r^2 = 0.89$. These results are consistent with earlier literature studies with hPEPT1.[26,28] These findings corroborate our hypothesis that amino acid ester prodrugs in general would exhibit enhanced affinity for hPEPT1.[9,10,26,27]

The stereochemistry of the amino acid promoiety also influenced the affinity of the prodrugs for hPEPT1.

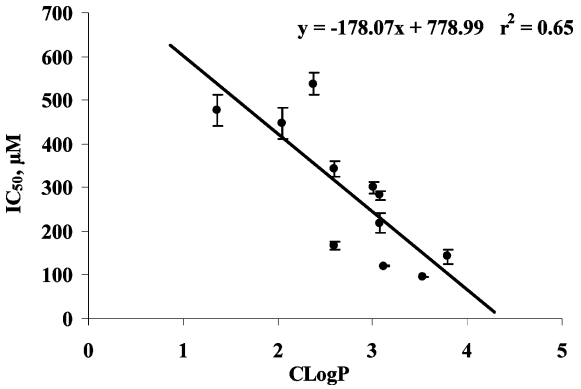


Figure 1. Correlation between IC_{50} and CLogP for BDCRB and its amino acid ester prodrugs. IC_{50} expressed as mean ± SEM ($n \geq 3$). CLogP estimated using ChemDraw Ultra 7.0 and CLogP program (version 3.0) from BioByte Corp. (Claremont, CA).

Thus, L-amino acids exhibited 1.3- to 2-fold higher affinity for hPEPT1 than their D-counterparts. These results are similar to those reported for L- and D-amino acid ester prodrugs of acyclovir, AZT, and floxuridine prodrugs[9,26] and are also consistent with the stereochemical preferences observed with di- and tripeptides.[29,30]

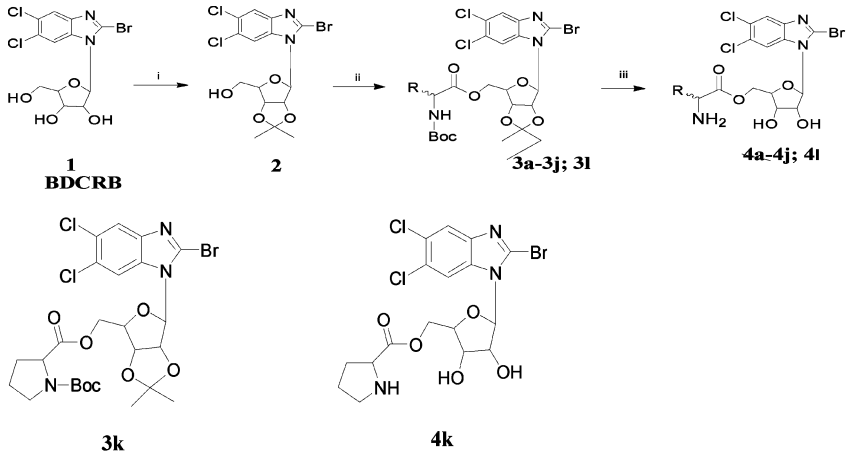
In conclusion, the novel amino acid ester prodrugs of BDCRB synthesized exhibited increased affinity for hPEPT1 transporter. The metabolism of BDCRB and its prodrugs in various biological systems are currently being investigated in order to determine direct hPEPT1-mediated transport of the prodrugs and the extent of glycosidic bond stabilization afforded by the amino acid promoiety.

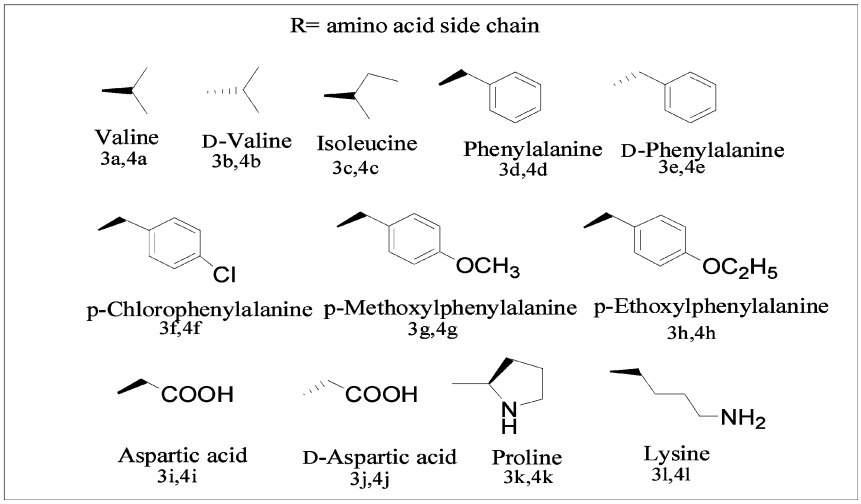
Experimental Section

Synthesis of 2′,3′-*O*-Isopropylidene-BDCRB, 2. To a stirred solution of BDCRB (100 mg, 0.25 mmol) in dry acetone (15 mL) was added *p*-toluenesulfonic acid monohydrate (477 mg, 2.5 mmol). The mixture was stirred for 1 h at room temperature and poured into ice-cold 10% Na_2CO_3 solution (20 mL). The mixture was evaporated to a small volume under reduced pressure. The residue was extracted with ethyl acetate, washed with water and brine, and dried over $MgSO_4$. 2′,3′-*O*-Isopropylidene-BDCRB was purified by column chromatography.

Synthesis of Amino Acid Ester Prodrugs of BDCRB, 4a−**l.** Amino acid ester prodrugs of BDCRB were synthesized as described in Scheme 1. Boc-protected amino acids (Boc-L-Asp, Boc-D-Asp, Boc-L-Ile, Boc-L-Lys, Boc-L-Phe, Boc-D-Phe, Boc-*p*-chloro-L-Phe, Boc-*p*-methoxy-L-Phe, Boc-*p*-ethoxy-L-Phe, Boc-L-Pro, Boc-L-Val, and Boc-D-Val; 5 mmol), DCC (5 mmol), and DMAP (0.5 mmol) were allowed to react with 2′,3′-*O*-isopropylidene-BDCRB (**2**) (1 mmol) in 10 mL of dry *N*,*N*-dimethylformamide (DMF). The reaction mixture was stirred at room temperature for 24 h. The progress of the reaction was monitored by TLC. After 24 h, the reaction mixture was filtered and DMF was removed in vacuo. The residue was dissolved in ethyl acetate (30 mL) and washed with water (2 × 20 mL), saturated $NaHCO_3$ (2 × 20 mL), and brine (1 × 20 mL). The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The intermediates (**3a**−**l**) were purified using column chromatography. Pure intermediates were then treated with 4 mL of TFA:DCM (1:1). After 4 h, the solvents were removed in vacuo and the residues reconstituted with water and lyophilized. The TFA salts of amino acid prodrugs of BDCRB were obtained as white fluffy solids. All BDCRB prodrugs were obtained in reasonable yields (~40−50% overall) and purity (more than 95% pure). The observed molecular

Scheme 1. Synthetic Scheme and Structures of Amino Acid Ester Prodrugs of BDCRB[a]





[a] Reagents: (i) TsOH, acetone; (ii) N-*t*Boc-protected amino acids, DCC, DMAP; (iii) TFA, CH_2Cl_2.

weights (ESI-MS) of the prodrugs and proton nuclear magnetic resonance (^{1}H NMR) spectra were identical to that required by their structures. (See Supporting Information for details).

Cell Culture. HeLa cells (passage 100−120) from American Type Culture Collection (Rockville, MD) were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1 mM sodium pyruvate, and 1% l-glutamine. Cells were grown in an atmosphere of 5% CO_2 and 90% relative humidity at 37 °C.

Uptake Inhibition of [^{3}H]Gly-Sar in HeLa/hPEPT1 Cells. The affinity of BDCRB and its amino acid ester prodrugs for hPEPT1 transporter was evaluated by measuring their ability to inhibit the uptake of [^{3}H]Gly-Sar, a standard hPEPT1 substrate, in HeLa cells overexpressing hPEPT1 as described previously.[31,32] Briefly, HeLa cells were infected with adenovirus containing hPEPT1 to obtain HeLa cells overexpressing hPEPT1, and at 2 days postinfection, cells were washed twice with uptake buffer (pH 6.0, 145 mM NaCl, 3 mM KCl, 1 mM NaH_2PO_4, 1 mM $CaC1_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, and 5 mM MES) and incubated with 10 μmol/L Gly-Sar (9.94 μmol/L Gly-Sar and 0.06 μmol/L [^{3}H]Gly-Sar) and various concentrations (0.1−2 mM) of BDCRB or its amino acid ester prodrugs in 0.3 mL uptake buffer for 30 min at room temperature. After 30 min, the drug solutions were aspirated and the cells were washed three times with ice-cold uptake buffer and solubilized with 0.1% Triton X-100/0.1 N NaOH. Aliquots of the suspensions were then used for scintillation counting (Beckman LS-9000, Beckman Instruments, Fullerton, CA) and for protein assays.

Data Analysis. The IC_{50} values were calculated from the inhibition data using nonlinear regression analysis with Prism software. Statistical significance was evaluated using GraphPad Prism V.3.0 by performing one-way analysis of variance with posthoc Tukey's test to compare means.

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Supporting Information Available: Materials and methods; percent yield, HPLC purity, ^{1}H NMR, and ESI-MS data for intermediate **2** and compounds **4a**−**l**. This material is available free of charge via the Internet at http://pubs.acs.org.

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