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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Toward Eradicating HIV Reservoirs in the Brain: Inhibiting P-Glycoprotein at the Blood−Brain Barrier with Prodrug Abacavir Dimers

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Supporting Information

ABSTRACT: Eradication of HIV reservoirs in the brain necessitates penetration of antiviral agents across the blood−brain barrier (BBB), a process limited by drug efflux proteins such as P-glycoprotein (P-gp) at the membrane of brain capillary endothelial cells. We present an innovative chemical strategy toward the goal of therapeutic brain penetration of the P-gp substrate and antiviral agent abacavir, in conjunction with a traceless tether. Dimeric prodrugs of abacavir were designed to have two functions: inhibit P-gp efflux at the BBB and revert to monomeric therapeutic within cellular reducing environments. The prodrug dimers are potent P-gp inhibitors in cell culture and in a brain capillary model of the BBB. Significantly, these agents demonstrate anti-HIV activity in two T-cell-based HIV assays, a result that is linked to cellular reversion of the prodrug to abacavir. This strategy represents a platform technology that may be applied to other therapies with limited brain penetration due to P-glycoprotein.

INTRODUCTION

Although viral loads have been dramatically reduced in HIV patients using highly active antiretroviral therapy (HAART), a significant hurdle to the total eradication of HIV is the presence of reservoirs of the virus.[1,2] These HIV reservoirs are found in locations such as the central nervous system, macrophages and lymphocytes and occur, in large part, due to the limited ability of antiretroviral therapies to enter these sites.[3,4] In the brain, for instance, the failure of many HAART agents to block the accumulation of reservoirs of the virus is largely a result of limited penetration across the blood−brain barrier (BBB).[5] This lack of penetration is due to a number of physiochemical properties of the drugs and also to the presence of drug transporters at the BBB. These transporters include multidrug resistance proteins (MDRs) of the ATP-binding cassette (ABC) family that are localized to the apical membrane of brain capillary endothelial cells.[5−7] Of these ABC transporters, P-glycoprotein (P-gp) has been our focus as it is expressed at particularly high levels in brain capillaries and is currently implicated in the transport of more HAART compounds than other MDRs.[5] P-gp is also expressed at other reservoirs of HIV besides the BBB, including macrophages and lymphocytes.[8,9]

P-gp is an ATP-dependent, integral membrane protein that transports a large variety of hydrophobic agents out of the plasma membrane into the extracellular milieu.[10,11] One proposed functional model is that P-gp may reduce intracellular drug concentrations by acting as a "hydrophobic vacuum cleaner", effectively increasing drug efflux by recognition and removal of compounds from the membrane before they reach the cytosol to elicit their effects.[12,13] *In vitro* studies have demonstrated that the HIV protease inhibitors (PIs) saquinavir, amprenavir, nelfinavir, ritonavir, and indinavir, and the HIV reverse transcriptase inhibitor (RTI) abacavir are substrates of P-gp.[5,14−20] The results of these *in vitro* studies have been confirmed *in vivo*. For example, significantly increased levels of abacavir and the PIs listed above (20-fold, and 7.4- to 36.3-fold, respectively) have been found in the brains of dosed P-gp-null mice versus dosed wild-type mice.[16,18,21] Thus, P-gp actively limits the brain penetration of antiretroviral drugs used to treat HIV-infected patients. Therefore, therapies that include inhibition of P-gp represent promising treatment strategies for HIV patients and their use may ultimately lead to eradication of the cellular and anatomical reservoirs of HIV.

Numerous studies have pointed to the existence of at least two spatially distinct substrate binding sites within the transmembrane domain of P-gp that function in transport or regulation of transport.[22−26] Furthermore, in the recently solved crystal structure of mouse P-gp, the drug binding site appears to be formed by the contacts between transmembrane helices leading to a large and fluid internal cavity that is able to accommodate the binding of multiple molecules.[27] To exploit this multiplicity of binding sites within the transporter region of P-gp, we have developed an approach to convert P-gp substrates into potent,

Received: July 22, 2011
Published: August 25, 2011

dx.doi.org/10.1021/ja206867t | J. Am. Chem. Soc. 2012, 134, 2976−2980

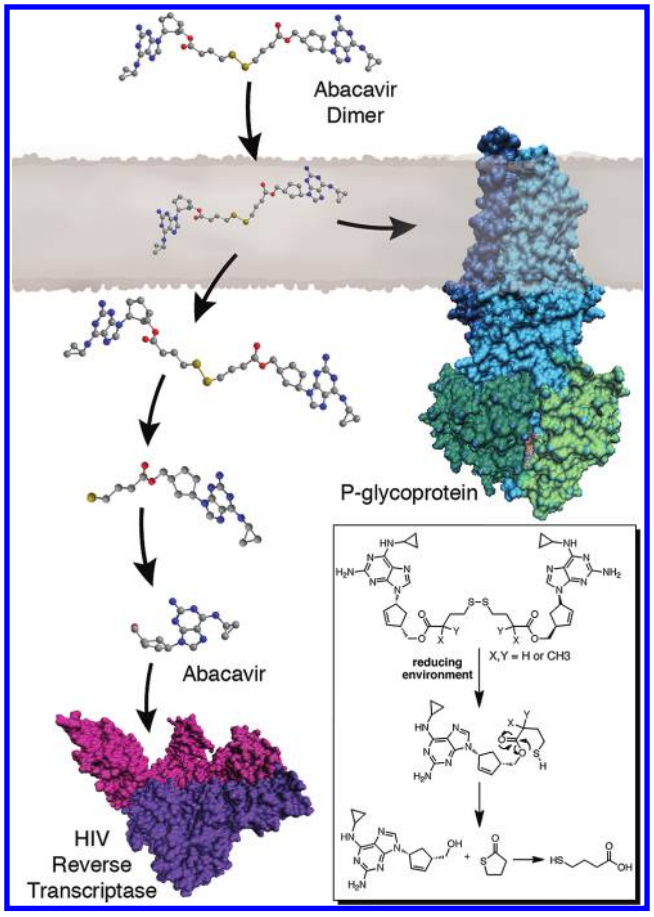

Figure 1. Design of P-gp inhibitors: dimeric prodrugs containing a traceless linker. These novel dimeric prodrug P-gp inhibitors also revert to the monomeric drugs within the reducing environment of the cell (inset) using a traceless linker strategy.

dimeric P-gp inhibitors.[28,29] Dimerization of P-gp substrates should increase the affinity for drug binding sites, thus, lowering the off rate, resulting in inhibition rather than efflux. In this study, we describe a novel design, using an HIV therapeutic, in which a dimeric P-gp inhibitor also functions as a prodrug with the potential to revert back to the corresponding monomeric drug in the reducing environment of the cell (Figure 1 and inset). This reversion is an important feature of our design in that the P-gp inhibitor is also a prodrug of the therapeutic agent itself. Herein, we disclose a set of dimeric prodrug agents based on the HIV RTI abacavir, that were designed to function in two ways: (1) as inhibitors of P-gp, the major drug efflux protein at the BBB, by occupying multiple binding sites within the transporter and (2) as prodrug dimers that upon entry into cells would revert to their monomeric forms in the reducing environment of the cytosol, thus, delivering the therapy (Figure 1).

RESULTS

Our dimeric prodrug strategy requires a tethering group that would be removed completely from the monomers once inside the cell, hence, a "traceless" tether. The addition of a disulfide within the tether is a key component to the designed breakdown of the dimeric agents. Under reducing conditions, such as those that exist in the cytosol, the disulfide linkage within the tether will reduce and the remaining tether would rearrange to regenerate

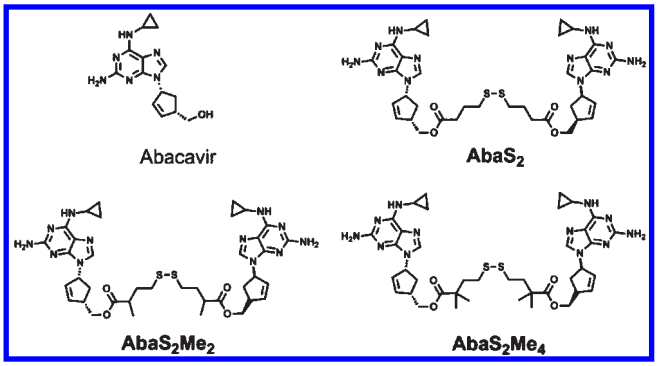

Figure 2. Structures of abacavir and abacavir prodrug homodimers: **AbaS$_2$**, **AbaS$_2$Me$_2$**, and **AbaS$_2$Me$_4$**.

two molecules of the monomeric therapeutic agent (Figure 1, inset). Although a similar strategy has been previously used to link a single therapeutic agent to cell delivery agents such as folate and cell penetrating peptides,[30−32] we now describe a dual therapy strategy targeting P-gp. Specifically for abacavir, the primary hydroxyl groups of two monomers are linked via ester linkages containing a central disulfide unit (**AbaS$_2$**, Figure 2) and evaluated in terms of P-gp inhibition, rate of reductive monomer release, and stability of the ester linkages in human plasma. The role of additional methyl units adjacent to the ester carbonyl in the tethers was also evaluated for stability (**AbaS$_2$Me$_2$** and **AbaS$_2$Me$_4$**, Figure 2).

Briefly, the dimers **AbaS$_2$**, **AbaS$_2$Me$_2$**, and **AbaS$_2$Me$_4$** were synthesized by treating PyBOP activated bis-carboxylic acids with abacavir in the presence of DIEA and DMAP. The abacavir dimers were purified to homogeneity by reverse phase HPLC and structures were confirmed by ^{1}H NMR, ^{13}C NMR, and MALDI-TOF mass spectrometry (see Supporting Information); **AbaS$_2$Me$_2$** was obtained as a mixture of stereoisomers that was used without further separation.

We evaluated the potency of the abacavir dimers in a P-gp overexpressing T lymphoblastoid cell line (12D7-MDR).[33] Inhibition of P-gp-mediated transport of the fluorescent substrates calcein-AM and NBD-abacavir (NBD-Aba)[34] was measured as the increase in cellular fluorescence by flow cytometery. All dimers demonstrated potent inhibition of P-gp mediated efflux of fluorescent substrates (Table 1), whereas monomeric abacavir was only minimally effective at a concentration of 500 μM. Likewise, the tether alone or the reduced product of the tether showed no inhibition up to a concentration of 200 μM. Methylation improved the activity of the **AbaS$_2$Me$_2$** and **AbaS$_2$Me$_4$** as compared to **AbaS$_2$**, with **AbaS$_2$Me$_4$** being more than 700-fold more potent than the starting monomeric abacavir at inhibiting P-gp-mediated efflux from cells.

To elucidate the mechanism of efflux inhibition by abacavir dimers, we probed their ability to compete for transporter binding sites with a known photoactive substrate of P-gp, [^{125}I]-iodoarylazidoprazosin (^{125}I-IAAP). Crude Sf9 membranes expressing P-gp were incubated with ^{125}I-IAAP and the abacavir dimers followed by photo-cross-linking.[35] Covalent modification of P-gp by ^{125}I-IAAP was monitored by autoradiography of the resulting SDS−PAGE gels as a function of increasing concentrations of **AbaS$_2$**, **AbaS$_2$Me$_2$**, and **AbaS$_2$Me$_4$**. All dimers competed for ^{125}I-IAAP binding sites on P-gp in a concentration-dependent manner with IC_{50} values in the nanomolar range (Table 1), with **AbaS$_2$Me$_4$** being 1500-fold more potent than monomeric

dx.doi.org/10.1021/ja206867t | J. Am. Chem. Soc. 2012, 134, 2976–2980

Table 1. Inhibition of P-gp-Mediated Efflux in 12D7 T-cells and P-gp Substrate Binding Competition *in Vitro*

compound	IC_{50} (μM) 12D7-MDR (Calcein-AM)	IC_{50} (μM) 12D7-MDR (NBD-Aba)	IC_{50} [^{125}I]IAAP competition
abacavir	>500	>500	>100 μM
$AbaS_2$	4.8 ± 0.1	4.9 ± 0.4	170 ± 42 nM
$AbaS_2Me_2$	2.4 ± 0.1	2.1 ± 0.2	62 ± 24 nM
$AbaS_2Me_4$	0.6 ± 0.1	0.7 ± 0.1	65 ± 28 nM

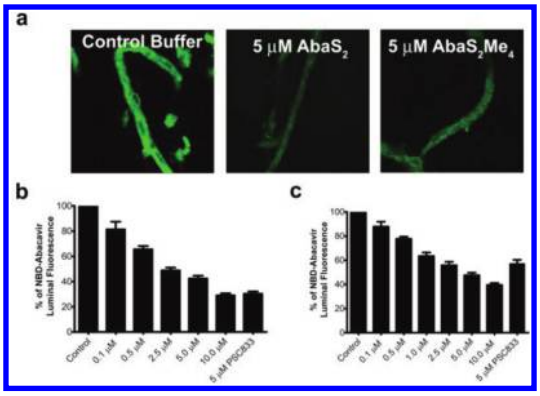

Figure 3. P-gp efflux is inhibited by prodrug abacavir homodimers in rat brain capillaries. (a) Rat brain capillaries were incubated in the presence or absence of 5 μM **$AbaS_2$** or **$AbaS_2Me_4$** in PBS (pH 7.4), followed by the addition of 2 μM NBD-abacavir in PBS (pH 7.4). Analysis was made by confocal scanning microscopy at 40× magnification. (b and c) Concentration dependent inhibition of P-gp transport of NBD-abacavir in rat brain capillaries by (b) **$AbaS_2$** and (c) **$AbaS_2Me_4$** using 5 μM PSC833 as the positive control.

abacavir. These data further support abacavir dimers as inhibitors of P-gp mediated efflux through interaction with the substrate binding domains.

Abacavir and other HIV therapeutics are actively effluxed by P-gp at the BBB. Given that our ultimate goal is to increase brain penetration of therapeutics by inhibiting P-gp at the BBB, we employed isolated rat brain capillaries that express endogenous levels of P-gp.[36] These experiments were designed to test the ability of abacavir dimers to interact with P-gp present at the BBB. When exposed to a fluorescent P-gp substrate, these capillaries concentrate fluorescence within the lumen by a process that is blocked by P-gp inhibitors.[36,37] This robust assay has been used previously to demonstrate transport and inhibition of P-gp with established P-gp inhibitors as well as transporter modulation.[7] For the assay, we incubated freshly isolated capillaries with a fluorescent substrate, NBD-abacavir, in the absence (control) and presence of **$AbaS_2$** or **$AbaS_2Me_4$**; the potent P-gp inhibitor, PSC833, served as a positive control. Accumulation of NBD-abacavir within the capillaries was visualized by confocal microscopy (Figure 3). Control capillaries showed intense NBD-abacavir fluorescence within the lumens, whereas exposure to 5 μM **$AbaS_2$** or **$AbaS_2Me_4$** substantially reduced luminal fluorescence (Figure 3a). Quantitation of luminal fluorescence showed concentration-dependent reductions with increasing concentrations of **$AbaS_2$** or **$AbaS_2Me_4$** (Figure 3b,c). Thus, the abacavir dimers are capable of inhibiting P-gp transport in a BBB model with endogenous levels P-gp expression.

Table 2. Reversion of Abacavir Prodrug Dimers to Monomeric Abacavir *in Vitro*

compound	$t_{1/2}$ (h) human plasma[a]	$t_{1/2}$ (h) DTT[b]
abacavir	NA	NA
$AbaS_2$	1.6 ± 0.1	8.8 ± 0.4
$AbaS_2Me_2$	7.1 ± 1.6	11.7 ± 0.3
$AbaS_2Me_4$	>100	17.2 ± 0.1

[a] Concentration of compounds was 60 μM in assay. [b] Concentration of compounds was 70 μM in assay.

The abacavir prodrug dimers were designed first to inhibit P-gp at the cell membrane and then release therapeutic abacavir within the intracellular reducing environment. The ester linkages of the dimers, therefore, must have sufficient stability to withstand esterases within blood, but still be sufficiently reactive through the reduced tether mechanism in the cytosol (Figure 1, inset). **$AbaS_2$**, **$AbaS_2Me_2$**, and **$AbaS_2Me_4$** were evaluated for their stability to plasma esterases and reducing conditions using human plasma and dithiothreitol (DTT), respectively. Each dimer was incubated with either human plasma (55%) or DTT (10 mM) at 37 °C, and the loss of dimer and appearance of monomer were monitored by reverse phase HPLC. In plasma, the unhindered abacavir dimer **$AbaS_2$** displayed a relatively rapid reversion to monomer with a half-life ($t_{1/2}$) of 1.6 h (Table 2). The addition of methyl groups to the abacavir dimer tethers affected susceptibility to plasma esterases; two methyl groups (**$AbaS_2Me_2$**) increased stability 4-fold, whereas the addition of four methyl groups (**$AbaS_2Me_4$**) strikingly resulted in less than 10% ester hydrolysis after 100 h. Under reducing conditions, **$AbaS_2$** reverted to monomer with a $t_{1/2}$ of 8.8 h (Table 2). Although ester hydrolysis was reduced by methylation, the reductive pathway to monomer production was much less affected. **$AbaS_2Me_2$** displayed a similar rate of breakdown to monomer as **$AbaS_2$**, whereas only a 1.5-fold decrease in monomer release was observed for **$AbaS_2Me_4$** as compared to **$AbaS_2Me_2$**. These data are significant as they demonstrate that it is possible to slow or halt the breakdown of dimeric agents in human plasma, while still allowing release of monomer through the reductive pathway within cells.

We hypothesize that antiviral activity will only be afforded by the breakdown of **$AbaS_2Me_4$** to the active RTI abacavir, as **$AbaS_2Me_4$** did not demonstrate intrinsic RTI activity (see Supporting Information). To ascertain that an abacavir prodrug dimer would have anti-HIV activity due to reversion to monomeric therapeutic within the reducing environment of cells, we employed two cell-based assays of anti-HIV activity: HIV titer in 12D7 cells and HIV-induced toxicity in MT-2 cells (Figure 4).[38] The 12D7 and MT-2 cells were infected with HIV-1_{LAI} using a previously described protocol. Antiretroviral activity was assayed by monitoring the production of HIV p24 by ELISA in the cell-free culture medium of infected cells exposed to varying concentrations of **$AbaS_2Me_4$** and monomeric abacavir after 12−14 days.[39] In addition, an MTT assay was employed to determine the ability of **$AbaS_2Me_4$** to protect MT-2 cells from the cytopathic effect of HIV-1_{LAI} after 7 days. We confirmed that **$AbaS_2Me_4$** was stable in the culture medium used for the above antiviral cell-based experiments. In both assays, **$AbaS_2Me_4$** demonstrated a dose-responsive increase in antiviral activity within 2.5- to 4-fold of the abacavir monomer activity (Figure 4). Together, these data indicate that the observed cellular antiviral activity is due to reversion of the

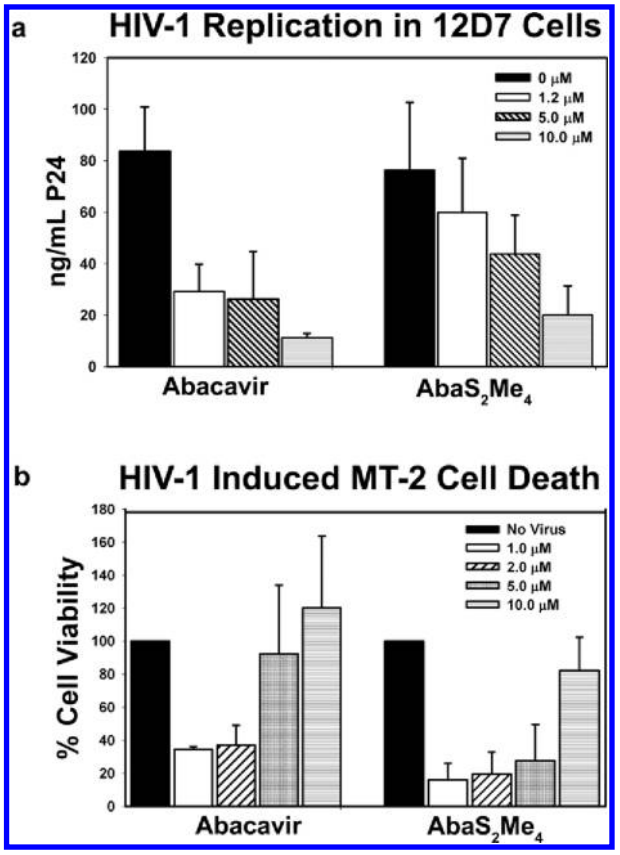

Figure 4. (a) Inhibition of HIV-1 replication in the presence or absence of increasing concentrations of abacavir or **AbaS$_2$Me$_4$**. IC_{50} values of approximately 0.6 and 2.5 μM were obtained for abacavir and **AbaS$_2$Me$_4$**, respectively. (b) Inhibition of HIV-induced cell death with increasing concentrations of abacavir or **AbaS$_2$Me$_4$**. IC_{50} values of approximately 2.5 and 6.5 μM were obtained for abacavir and **AbaS$_2$Me$_4$**, respectively.

dimer into therapeutic abacavir within the reducing environment of the cell.

DISCUSSION

Eradication of HIV reservoirs in the brain will necessitate the penetration of antiviral agents across the BBB, through processes that either evade P-gp,[40−43] or block its activity directly. Herein, we have focused on this latter strategy with dimerized P-gp substrates that were designed to have two functions: to inhibit P-gp efflux at the BBB, and to act as prodrugs and revert to the functional, monomeric therapeutic within the reducing environment of the cytosol. The HIV RTI abacavir was chosen for these experiments as *in vivo* experiments have clearly demonstrated the role of P-gp in limiting the entry of abacavir into the brain.[18] Prodrug abacavir dimers were synthesized, therefore, using a "traceless tether" that was designed to respond to a reducing environment and regenerate abacavir through molecular rearrangement. Additional methyl groups were added to the tether in an effort to tune the rate of breakdown of the prodrugs under a variety of conditions.

We have demonstrated that the prodrug abacavir dimers are potent P-gp inhibitors both in cell culture and, notably, in a brain capillary model of the BBB. The significantly reduced susceptibility of the prodrugs to plasma esterases with increased methylation state of the dimeric tethers, combined with only small changes in reductive monomer release, clearly demonstrate that we can engineer the rate of release of monomer from the prodrugs. Importantly, the abacavir dimer **AbaS$_2$Me$_4$** demonstrated anti-HIV activity in two separate T-cell-based HIV assays, while this agent was itself inactive in an *in vitro* reverse transcriptase (RT) assay. These data strongly suggest that the observed cellular antiviral activity of **AbaS$_2$Me$_4$** is linked to the reversion of this prodrug dimer to the RT-active monomeric abacavir.

Dimeric prodrugs of antiretroviral agents, such as those described herein, have interesting potential for use in conjunction with HAART, as a number of these therapies are substrates of P-gp. Co-administration of the dimeric prodrugs with monomeric drugs would allow for accumulation of the therapeutic agent within the brain via two pathways: enhanced entry of monomeric drug through inhibition of P-gp at the BBB, and breakdown of the dimeric P-gp inhibitors within endothelial cells at the BBB to provide additional monomeric therapeutic. This approach precludes the need for additional P-gp inhibitors with HAART and, as such, lowers the risk of possible drug toxicities.[44] Since combination therapy is the hallmark of HAART treatment due to drug resistance found with single therapy regimes, this dimeric prodrug design provides the opportunity to cross-link different antiviral agents that are P-gp substrates, such as abacavir and saquinavir, into heterodimeric prodrug inhibitors. This overall strategy represents a platform technology that may be readily applied to other therapies with limited brain penetration due to P-gp efflux activity, from the anticancer agents topotecan and taxol to the antischizophrenia drugs quetiapine and paliperidone.

ASSOCIATED CONTENT

Supporting Information. Experimental procedures, synthetic schemes, characterization and reverse transcriptase data. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

We acknowledge the National Institutes of Health and the Purdue Research Foundation for support of this work. We dedicate this work to Professor Ronald Breslow on the occasion of his 80th birthday.

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