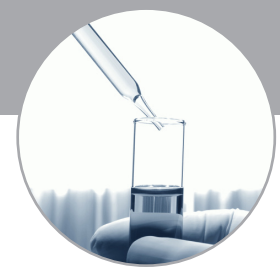

Tools for eradicating HIV in the brain: prodrug dimeric inhibitors of P-gp

Despite positive developments with the use of combination antiretroviral therapy, a major impediment to limiting the neurocognitive effects of HIV and eradicating HIV brain reservoirs is the penetration of these therapies across the blood–brain barrier (BBB). The focus of our work, therefore, has been to develop tools to significantly improve the penetration of antiretroviral agents to sites of HIV reservoirs, with an emphasis on the CNS. To this end, we have developed an innovative chemical approach – dimeric prodrugs of the antiretroviral agents themselves with a traceless tether. These dimeric prodrugs were designed to serve two purposes: inhibition of P-gp, the major drug efflux protein at the BBB, by occupying two substrate binding sites in the transporter; and prodrug dimers that gain entry into the endothelial cells at the BBB would revert to their monomeric forms in the reducing environment of the cytosol due to breakdown of the traceless tether, thus delivering the therapy. We have demonstrated the feasibility of this design by dimerizing the P-gp substrate and antiviral agent abacavir with a traceless tether. Abacavir dimers displayed potent inhibition of P-gp in two different cellular settings and reverted to active abacavir in the reducing environment of HIV-infected T cells, also leading to antiviral activity. Overall, these experiments point to the excellent promise for future use of dimeric prodrug inhibitors of P-gp for brain penetration of a wide range of CNS-active agents that are substrates of P-gp.

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HIV is believed to enter the CNS as either cell-free particles or via infected monocytes. The consequences of this penetration on individuals infected with HIV are profound and far reaching, spanning neurocognitive impairment to the generation of persistent reservoirs of HIV in the brain. Combination antiretroviral therapy (ART) has dramatically reduced mortality among HIV-infected individuals; however, HIV-associated neurocognitive disorders still remain a serious problem.

Although viral plasma loads have been reduced to undetectable levels in HIV patients treated with combination ART, a tremendous obstacle to the total eradication of HIV is the occurrence and persistence of reservoirs of the virus [1]. These reservoirs contain latent and/or replicating forms of the virus, and are found in a number of cell types and anatomical sites, including resting memory $CD4^+$ T cells, macrophages and the CNS [2]. These sanctuaries of HIV occur in sites where antiretroviral access is limited [3], pointing to the need for effective entry of ART into these sites. Whereas ART can significantly lower plasma viral loads, recent studies have demonstrated that only ART with effective CNS penetration has the ability to lower cerebrospinal fluid viral loads [4]. Furthermore, recent studies have shown that ART with increased CNS penetration also resulted in increased cognition in HIV-infected subjects [5]. These data underscore the importance of optimal ART penetration for viral eradication and limiting HIV-induced neurocognitive impairment.

Although latent reservoirs of HIV may not have active replication, they retain a replicative capacity. Recent efforts aimed at eradicating HIV, therefore, have focused on purging the last sanctuaries of HIV infection by combining ART with agents that force viral expression from latent reservoirs [6,7]. In this way, HIV induction would expose latently infected cells to virus- and immune-mediated death, whereas ART would block any further viral spread. Such strategies, however, would again strongly depend on ART to reach and completely inhibit all new infection events in all HIV sanctuaries. Therefore, there is a great need to develop tools to significantly improve the penetration of antiretroviral agents to sites of HIV reservoirs with an emphasis on the CNS.

The ABC transporter, P-gp, limits the penetration of antiretrovirals at the blood–brain barrier

The inability of combination ART to fully protect against HIV-induced nervous system disease and block the accumulation of reservoirs of the virus, such as those in the brain, is due to the limited ability of many ART agents to cross the blood–brain barrier (BBB) – a system of capillary endothelial cells that protects the brain from damaging substances found in the bloodstream [8,9]. This lack of penetration is due to a number of physiochemical properties of drugs, such as

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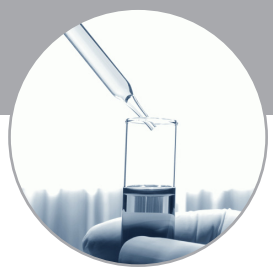


protein binding, size, lipophilicity and ionization. Additionally, features of cerebral capillaries themselves contribute strongly to the lack of ART penetration, including tight junctions between the endothelial cells and the presence of drug transporters at the BBB. Two classes of transporters have currently been identified that limit the accumulation of antiretrovirals used for HIV treatment: multidrug resistance proteins of the ABC family and organic ion transporters [10]. ABC transporters, including **P-gp**, ABCG2 and MRP1–3, are localized to the apical membrane of the capillary endothelial cells. The ABC transporters limit the accumulation of lipophilic agents in the brain by effluxing the compounds back into the bloodstream [11].

Although other ABC transporters, such as ABCG2 and MRP1–3, have been implicated in the active transport of a subset of HIV reverse-transcriptase inhibitors (RTIs) and protease inhibitors (PIs) [10], this spotlight is primarily focused on P-gp, as it is highly expressed in brain capillaries and currently implicated in the transport of many ART compounds. P-gp is an integral membrane protein that is a member of the large ABC superfamily of membrane transporters (**Figure 1**) [12]. P-gp transports a large variety of hydrophobic agents out of the plasma membrane or the cytoplasm into the extracellular milieu. One proposed model is that P-gp acts as a 'hydrophobic vacuum cleaner' and reduces drug accumulation within cells through drug efflux from the membrane [13]. Importantly, P-gp contains at least two drug-binding sites that are localized to the transmembrane regions of the transporter [14].

Key Terms

P-gp: Multidrug resistance transporter of the ATP-binding cassette family.

Prodrug: Therapeutic agent that is in an inactive form but becomes metabolized to its active form after administration.

Traceless tether: Compound that is used to link two agents together in a reversible fashion.

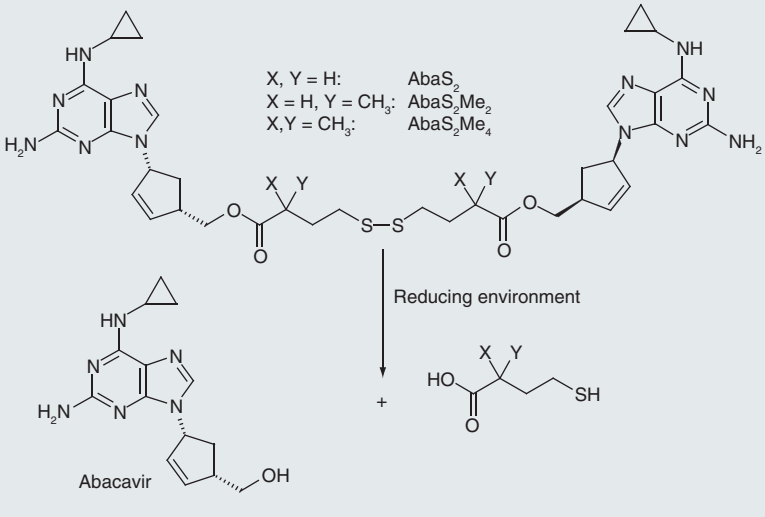


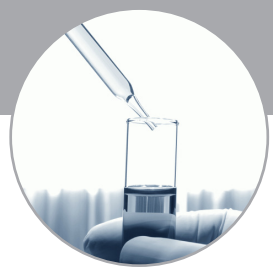
Figure 1. Reversal of the prodrug dimeric inhibitors of P-gp to therapeutic abacavir.

A number of HIV PI drugs currently used, such as saquinavir, amprenavir, nelfinavir, ritonavir and indinavir, and the RT **prodrug** inhibitor, abacavir, have been shown to be P-gp substrates through *in vitro* experiments [10]. In a number of cases, these results have also been confirmed in *in vivo* experiments. Pharmacological inhibition of P-gp has also provided promising results in animals. Cotreatment *in vivo* with PIs and known P-gp inhibitors (nelfinavir with LY335979, and saquinavir with ritonavir) was also found to result in increased brain penetration of the PIs [9,15]. However, ritonavir – a mixed P-gp/cytochrome P450 CYP3A inhibitor – has a number of off-target interactions, including cytochrome P450 CYP2D6 and extensive undesirable drug–drug interactions [16], thereby opening the door for other innovations in the area of P-gp inhibition with ART. These lines of evidence strongly support the hypothesis that P-gp is involved in limiting the brain penetration of antiretroviral drugs used to treat HIV-infected patients.

Development of dimeric prodrug inhibitors of P-gp

Together, the data presented above suggest that therapies involving inhibition of P-gp constitute a promising addition to treatment strategies for HIV patients. With this in mind, a new therapeutic focus has been to develop inhibitors of P-gp based on P-gp substrates themselves. One strategy that has demonstrated promise for P-gp inhibition is the crosslinking of two monomeric substrates of P-gp. For instance, the dimerization of P-gp substrates, such as stipiamide [17], emetine [18] and quinine [19], produced low micromolar inhibitors of P-gp efflux. Mechanistic studies with some of these dimeric agents provide strong evidence for binding within the transporter region of P-gp. Inhibition may occur, therefore, by the dimeric agents occupying the multiple binding sites within the transporter region of P-gp, thereby leading to prolonged occupation of these sites. Significantly, reversion of the taxol-resistant phenotype in MCF-7/DX1 cells that overexpress P-gp has been reported with one of the dimers of quinine [19].

More recently Namanja *et al.* have extended this dimerization strategy to the anti-HIV therapy, abacavir, a known P-gp substrate [20]. In this case, a dimerization scheme was devised to include the use of a **traceless tether** (**Figure 1**). The tether was designed to break down in the reducing environment of the cell, due to the



presence of a central disulfide moiety adjacent to the two ester linkages to abacavir. In this way, the abacavir dimers were designed to function as both P-gp inhibitors and as prodrugs of abacavir.

The ability of the abacavir dimers to function as prodrugs was analyzed by monitoring the release of a monomer from a series of three abacavir dimers in human plasma and with reducing agent **(Table 1)**. While $AbaS_2$ and $AbaS_2Me_2$ reverted to monomers in the presence of plasma, interestingly, $AbaS_2Me_4$ demonstrated only low levels of ester hydrolysis (<10%) after 100 h [20]. Although ester hydrolysis was inhibited by tetramethylation, the reductive pathway to monomer production was found to decrease only approximately 1.5-fold for $AbaS_2Me_4$ as compared with $AbaS_2Me_2$. These data are significant as they demonstrate that that it is possible to halt the breakdown of dimeric agents in human plasma, while still allowing for release of monomer through the reductive pathway within the cytosol.

The potency of the above abacavir dimers has been evaluated in a P-gp-overexpressing human T-cell line (12D7-MDR) **(Table 1)**. Dimers of abacavir demonstrated potent inhibition of P-gp-mediated efflux of the fluorescent substrate, NBD-abacavir, with submicromolar inhibition obtained with $AbaS_2Me_4$ [20]. Similarly, the abacavir dimers inhibited P-gp efflux in a rat brain capillary model of the BBB. Overall, these data strongly confirm that reversibly dimerizing the known P-gp substrate, abacavir, leads to potent inhibitors of P-gp.

Since abacavir in its dimeric form ($AbaS_2Me_4$) displayed no activity in cell-free RTI assays, this compound should only demonstrate antiviral acitivity in cells if it had first reverted to therapeutic abacavir within the cellular reducing environment. To test this hypothesis, two HIV-infected T-cell lines, 12D7 and MT-2 cells, were treated with $AbaS_2Me_4$ and HIV titer and HIV-induced toxicity were monitored. In each of these *in cyto* experiments, $AbaS_2Me_4$ displayed potent antiviral activity, which was between 2.5- and four-fold the activity observed with abacavir itself [20]. These results provide a strong indication that this abacavir dimer reverted to the monomeric therapy within the cells.

Conclusion

Our initial work with dimers of P-gp substrates laid the foundation for a new strategy to produce P-gp inhibitors. More recently, we added an enhanced feature to the dimeric structures in the form of a traceless tether. By linking two therapies that are P-gp substrates with a reversible tether, we developed a novel class of compounds: prodrug dimeric inhibitors of P-gp. Our first demonstration of the feasibility and therapeutic potential of this overall strategy was with a dimeric abacavir agent, such as $AbaS_2Me_4$. $AbaS_2Me_4$ displayed submicromolar inhibition of P-gp efflux in a T-cell line, inhibited P-gp in a brain capillary model of the BBB, and was found to revert to therapeutic abacavir *in vitro* and *in cyto*; in the latter case, potent anti-HIV activity was obtained. These experiments are an excellent demonstration of the power of including a traceless linker within dimeric inhibitors of P-gp.

Future perspective

Eradication of HIV reservoirs will necessitate a varied arsenal of strategies. Dimeric prodrug inhibitors of drug efflux transporters, such as P-gp, are one such tool to significantly improve the brain penetration of antiviral agents. The dimeric nature of the agents allows for two therapies to be crosslinked, for instance a PI and an RTI, thereby producing a combination antiviral treatment in one dimeric molecule. It is important to note that limited brain penetration of therapeutic agents, due to P-gp efflux, is by no means limited to antiviral therapies. The dimeric prodrug approach described in this spotlight represents a platform technology that may be applied to a range of therapies, including anticancer and antipsychotic agents.

Financial & competing interests disclosure

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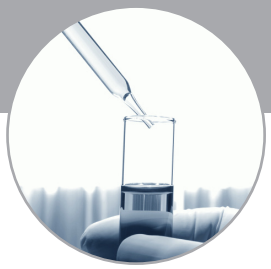
Table 1. Stability of abacavir dimers and inhibitory activity against P-gp.

Compound	$t_{1/2}$ plasma (h)	$t_{1/2}$ DTT (h)	IC_{50} 12D7-MDR T cells (μM)
Abacavir			>200
$AbaS_2$	1.6 ± 0.1	8.8 ± 0.4	4.9 ± 0.1
$AbaS_2Me_2$	7.1 ± 1.6	11.7 ± 0.3	2.1 ± 0.2
$AbaS_2Me_4$	>100	17.2 ± 0.1	0.7 ± 0.1

DTT: Dithiothreitol; MDR: Multidrug resistant.
Data taken from [20].



Executive summary

- The persistence of HIV reservoirs, such as those in the CNS, presents a major obstacle to the eradication of HIV.
- The inability of many of the current antiviral agents to effectively penetrate the CNS contributes to the establishment of viral reservoirs and limits the tools available to eradicate them.
- The presence of efflux transporters, such as P-gp, at the blood–brain barrier plays a significant role in limiting antiviral penetration of the CNS through active efflux.
- A successful strategy is presented to block P-gp and promote cellular delivery of antiviral agents based on dimeric prodrug inhibitors of P-gp.
- The dimeric prodrug approach, based on the therapies themselves, represents a platform technology that may be applied to other therapies with limited brain penetration due to efflux transporters.

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