

Nanoparticle-based drug delivery to improve the efficacy of antiretroviral therapy in the central nervous system

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Abstract: Antiretroviral drug therapy plays a cornerstone role in the treatment of human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome patients. Despite obvious advances over the past 3 decades, new approaches toward improved management of infected individuals are still required. Drug distribution to the central nervous system (CNS) is required in order to limit and control viral infection, but the presence of natural barrier structures, in particular the blood–brain barrier, strongly limits the perfusion of anti-HIV compounds into this anatomical site. Nanotechnology-based approaches may help providing solutions for antiretroviral drug delivery to the CNS by potentially prolonging systemic drug circulation, increasing the crossing and reducing the efflux of active compounds at the blood–brain barrier, and providing cell/tissue-targeting and intracellular drug delivery. After an initial overview on the basic features of HIV infection of the CNS and barriers to active compound delivery to this anatomical site, this review focuses on recent strategies based on antiretroviral drug-loaded solid nanoparticles and drug nanosuspensions for the potential management of HIV infection of the CNS.

Keywords: HIV/AIDS, blood–brain barrier, protease inhibitors, efflux transporters, drug targeting

Introduction

There are an estimated 35.3 million people infected with human immunodeficiency virus (HIV) worldwide, according to the latest numbers from the Joint United Nations Programme on HIV/AIDS (UNAIDS).¹ Although the number of new infections has shown a trend of decrease since 2001, the number of infected individuals keeps on growing, mainly because of the success of and increasing access to antiretroviral drugs. The introduction of combination antiretroviral therapy (cART) around 1996 had a huge impact on the management of acquired immunodeficiency syndrome (AIDS), and resulted in increased quality of life and lifespan of seropositive individuals.^{2,3} However, several limitations of currently available drugs, namely their toxicity and poor pharmacokinetics, the need to be used for prolonged periods of time (if not chronically), and the emergence of viral resistance, still jeopardize the optimal efficacy of cART.⁴ Of particular importance is the poor bioavailability of several anti-HIV drugs at viral reservoir sites, such as the central nervous system (CNS), in special brain macrophages and microglia cells, when using available dosage forms. Low cerebrospinal fluid: blood plasma (CSF:BP) concentration-ratio values have been reported for various drugs commonly used in the management of HIV/AIDS.⁵ For example, protease inhibitors (PIs) bind extensively to plasma proteins, and are substrates of permeability glycoprotein (P-gp) and other important efflux transporters present at the blood–brain barrier (BBB),

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which combined severely limit their uptake into the brain.⁶ Apart from impairing the elimination of the virus from the CNS, the low penetration ability of the BBB by antiretroviral drugs is associated with higher CSF viral loads, which can be critical in the development of neurological disorders.⁷

The use of drug nanocarriers has been advocated as potentially beneficial in delivering antiretroviral drugs across the BBB and into the CNS.^{8–13} Nanotechnology-based systems may additionally provide interesting features, such as enhanced intestinal absorption (following oral administration), improved toxicity profiles, increased drug stability, prolonged drug residence in the body (namely at the CNS), circumvention of efflux pumps (including those at the BBB), and selective drug delivery to specific cells (eg, HIV-target cells). Even if other types of nanocarriers have also been proposed for the management of HIV infection of the CNS, such as dendrimers,¹⁴ nanoemulsions,^{15,16} liposomes,¹⁷ micelles,¹⁸ and nanogels,¹⁹ this review will focus on solid nanoparticles (NPs) of polymeric, macromolecular, lipid, or metallic nature. Also, strategies based on antiretroviral drug nanosuspensions are overviewed.

HIV and the CNS: infection and barriers to viral eradication

Lentiviruses, such as HIV, are able to infect all structures in the nervous system. Soon after primary viral transmission, HIV is able to invade the CNS by means of peripherally infected leukocytes, mainly monocytes. These cells are able to cross the BBB, acting as “Trojan horses”. Other mechanisms, such as direct infection or transcytosis of endothelial cells, are also possible, but usually considered of minor importance.²⁰ Once at the CNS, the virus is able to replicate and further infect macrophages, astrocytes, CD4⁺ T cells, and microglia, thus triggering the release of a cascade of host-derived inflammatory molecules and HIV-encoded neurotoxic proteins (eg, gp-120, transactivator of transcription [Tat], and Vpr).²¹ In particular, macrophages and microglia seem to be the most important cell populations sustaining HIV infection of the CNS.²⁰ Inflammation and cytotoxicity often lead to neurocognitive and motor disorders, even in patients undergoing cART.²² Neurocognitive impairment is frequently symptomatic (mostly memory, learning, and executive function impairment) and associated with initial severe immunosuppression, but usually mild if treatment has been introduced early.²³ Severe HIV-associated dementia is rare, even though the life expectancy of AIDS patients is increasing, and linked to high virus levels in the CSF.²⁴ Viral encephalopathy typically encompasses immune activation

(encephalitis), with such histological features as gliosis, microglial nodules, perivascular macrophage accumulation, and the presence of multinucleated giant cells.²⁵ Also, inflammation results in increased leakiness of the BBB, which enhances the ability of further viral invasion of the CNS. Adding to the virus effects on the CNS, concomitant use of medication (including some antiretroviral drugs), the presence of other neurological (eg, cerebrovascular disease) and nonneurological (eg, hepatitis C infection, atherosclerosis) medical conditions, and aging-associated neurodegenerative disease may exacerbate neurological disorders.²⁵

The CNS is recognized as an anatomical viral reservoir, where HIV persists in long-lived cells, namely microglia, with more stable kinetics.²⁶ This results in increased difficulty of viral eradication by cART and the emergence of different and drug-resistant HIV strains. Added to this, the poor ability of several antiretroviral drugs to penetrate the CNS (Table 1) further limits eradication.⁵ Indeed, antiretroviral drugs may not reach the CNS in sufficient levels or may even be excluded (ie, by such efflux transporters as P-gp) from this site at the BBB level. At the same time, the inflammatory response triggered by HIV infection of the CNS induces changes to the BBB, resulting in tight-junction (TJ)

Table 1 Selected drugs currently used in combination antiretroviral therapy and their ability to reach the central nervous system, as reflected by the cerebrospinal fluid: blood plasma (CSF:BP) concentration ratio in humans (expressed as mean values or mean range values from cited references)

Drug classes	Drugs	CSF:BP	References
Nucleoside reverse-transcriptase inhibitors	Zidovudine	0.5	27
	Didanosine	0.21	28
	Stavudine	0.16–0.40	29,30
	Lamivudine	0.06–0.23	30,31
	Abacavir	0.18–0.36	32
	Emtricitabine	0.26	33
Nucleotide reverse-transcriptase inhibitors	Tenofovir	0.05	33,34
Nonnucleoside reverse-transcriptase inhibitors	Nevirapine	0.63	30
	Efavirenz	0.003–0.01	35
	Etravirine	0.01	36
Protease inhibitors	Saquinavir	≤0.002	37,38
	Indinavir	0.11	30
	Ritonavir	0.001–0.005	38
	Nelfinavir	Undetectable in CSF	39
	Atazanavir	0.002–0.014	40
	Fosamprenavir	0.012	41
Entry inhibitors	Darunavir	0.01	42
	Enfuvirtide	Undetectable in CSF	43
Integrase inhibitors	Maraviroc	0.028	44
	Raltegravir	0.01–0.61	45
	Dolutegravir	0.5	46

modulation and increased leakiness to different molecules and even circulating cells (eg, HIV-infected monocytes).⁴⁷ Alongside the BBB, other anatomical barriers also pose significant hurdles that limit drug penetration of the CNS, namely the blood–CSF barrier at the choroid plexuses, and to a minor extent the CSF–brain barrier. Blood plasma protein binding has also been held responsible for poor bioavailability of several antiretroviral drugs, such as efavirenz (99.5% binding) and various PIs.⁴⁸ Different distribution patterns of HIV were observed at different areas of the CNS of patients undergoing cART, with higher levels of viral ribonucleic acid (RNA) being observed in the caudate nucleus, while the lowest concentrations were observed in the frontal cortex and CSF.⁴⁹ This fact may reflect, at least partially, the heterogeneous distribution of antiretroviral drugs in the CNS. Therefore, strategies enhancing antiretroviral drug levels and distribution in the CNS are required.

Drug delivery to the CNS: focus on nanotechnology

The CNS is a unique and complex environment with restricted anatomical access, mainly due to such brain barriers as the BBB.⁵⁰ The BBB is an active, dynamic, and complex interface between the blood and the CNS that has neuroprotective functions, regulates the in-and-out brain transport of different molecules and cells (eg, leukocytes), and maintains the homeostasis of the brain microenvironment.^{51,52} This strict access is mainly due to the presence of TJs between endothelial cells of blood capillaries interfacing with the CNS (Figure 1).⁵³ The role of the BBB is crucial for neuronal activity and proper functioning of the CNS. Thus, when drug delivery to this anatomical site is required, overcoming the BBB blocking action is one of the main hurdles in order to improve and develop effective treatments without the need to administer directly into the CNS or use high drug doses with increased risks of adverse side effects.⁵⁴

There are different options for molecules to cross the BBB, which could be strategically used for drug delivery purposes. In the case of the transport of low-molecular-weight molecules, there are two possibilities: 1) diffusion, either passive or facilitated across aqueous channels, and 2) active transport, which is mediated by such carriers as proteins (carrier-mediated transport). As for macromolecules, their transport includes receptor-mediated transcytosis (RMT), nonspecific adsorptive-mediated transcytosis (AMT), and cell-mediated transcytosis. RMT involves the endocytosis of macromolecules specifically bound to a receptor on the endothelial surface of the BBB, their diffusion across the

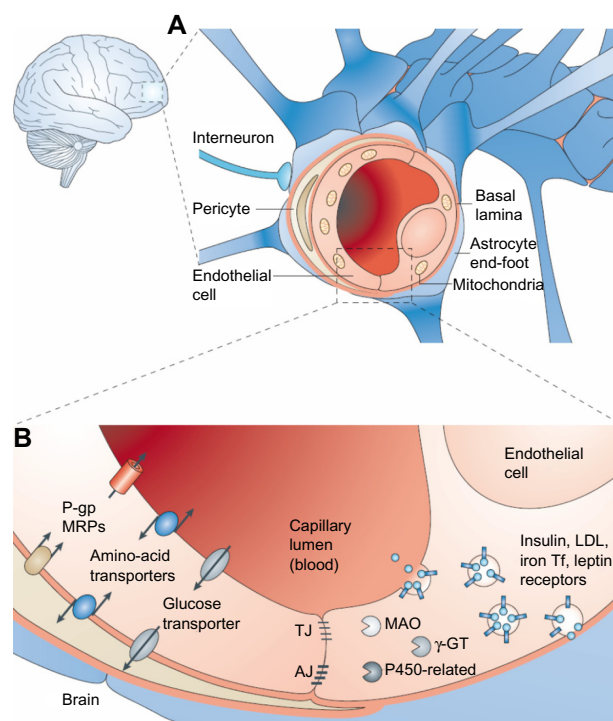


Figure 1 The blood–brain barrier (BBB).

Notes: (A) The BBB is formed by endothelial cells at the level of the cerebral capillaries. These endothelial cells interact with perivascular elements, such as the basal lamina and closely associated astrocytic end-feet processes, perivascular neurons (represented by an interneuron here), and pericytes to form a functional BBB. (B) Cerebral endothelial cells are unique in that they form complex tight junctions (TJs) produced by the interaction of several transmembrane proteins that effectively seal the paracellular pathway. These complex molecular junctions make the brain practically inaccessible for polar molecules, unless they are transferred by transport pathways of the BBB that regulate the microenvironment of the brain. There are also adherens junctions (AJs), which stabilize cell–cell interactions in the junctional zone. In addition, the presence of intracellular and extracellular enzymes, such as monoamine oxidase (MAO), γ -glutamyl transpeptidase (γ -GT), alkaline phosphatase, peptidases, nucleotidases, and several cytochrome P450 enzymes, endow this dynamic interface with metabolic activity. Large molecules, such as antibodies, lipoproteins, proteins, and peptides can also be transferred to the central compartment by receptor-mediated transcytosis or nonspecific adsorptive-mediated transcytosis. The receptors for insulin, low-density lipoprotein (LDL), iron transferrin (Tf) and leptin are all involved in transcytosis. Reprinted by permission from Macmillan Publishers Ltd: *Nat Rev Drug Discov*. Cecchelli R, Berezowski V, Lundquist S, et al. Modelling of the blood–brain barrier in drug discovery and development. © 2007.⁵³

Abbreviations: P-gp, P-glycoprotein; MRP, multidrug resistance-associated protein family.

endothelium, and exocytosis on the opposite site.⁵⁰ AMT, also known as pinocytosis, is mediated by electrostatic interactions between positively charged ligands and the negatively charged membranes of the BBB.⁵⁵ Cell-mediated transcytosis refers only to immune cell-mediated transport.⁵⁶ Finally, active efflux transport is another important type of molecule transfer across the BBB that limits brain penetration. This last mechanism involves extrusion of drugs from the CNS back to the blood, thus representing a major impediment to drug therapy.^{52,57} Until now, the most extensively characterized efflux transporters at the BBB have been multidrug resistance-associated proteins (MRPs), of which P-gp is the main representative.^{58–60} P-gp has a high affinity for a wide

range of cationic and lipophilic compounds, and thus limits the transport of many drugs, including cytotoxic anticancer drugs, antibiotics, hormones, and antiretroviral PIs.^{61,62}

Among others, nanotechnology-based strategies have been useful in overcoming the BBB, as they can generally improve the permeability characteristics of different pharmacologically active agents, making them able to achieve target sites.⁶³ NPs are great examples of drug carriers, due to their versatile and tunable properties, such as large surface:volume ratio, surface charge, and small and controllable size,⁶⁴ which promote their permeation through the BBB and facilitate drug delivery to the CNS.⁶⁵ Moreover, NPs can be made nontoxic, biodegradable, and biocompatible, depending on proper material selection and manufacturing.⁵² NPs have also a customizable surface that can be modified in order to target different cells or tissues, according to their final intent. These characteristics can contribute to increased drug bioavailability and decreased peripheral toxicity and side effects.^{56,57,64,66} Charge and hydrophobicity of the NP surface influence the adsorption of plasmatic proteins, and as a consequence their uptake and/or rate of transcytosis.⁶⁷ In the particular case of CNS delivery, NP coating with particular surface stabilizers may be of interest in achieving increased brain drug levels. The best-characterized one is polysorbate 80, a nonionic surfactant that has been shown to be effective as a brain-delivery enhancer in different types of NPs, such as polymeric NPs^{68,69} and solid-lipid NPs (SLNs).⁷⁰ Once in the blood circulation, polysorbate 80-coated NPs adsorb different apolipoproteins, thus mimicking lipoproteins in their RMT pathway into the CNS.⁷¹

Engineered NPs targeting the CNS can be obtained by surface coupling of specific molecules that provide them the ability to overcome the BBB and eventually also reach HIV-reservoir sites, depending on the type of functionalization and incorporated ligands. Targeting mechanisms could either be passive or active. In this last case, transporter-mediated delivery is a strategy commonly used to allow NPs to cross the BBB based on the principle that peptides or other molecules may use specific transporters expressed on endothelial cells at the BBB. These molecules usually recognize and bind to target receptors or antigens, which may be overexpressed or selectively expressed by particular cells or tissue components.⁶³ In the case of the AMT mechanism, the most prominent candidates used as NP surface-targeting moieties are cell-penetrating peptides. These ligands facilitate enhanced intracellular drug delivery through endocytosis or by the formation of a transient structure with the cell membrane.⁷² The HIV-1 Tat peptide is one of the most widely

tested cell-penetrating peptides.¹⁰ This peptide possesses certain regions, known as protein-transduction domains, that can promote migration through biological membranes. The BBB-permeation mechanism is independent of transporters and receptor-mediated endocytosis, but depends on the fusion of β -galactosidase to the Tat peptide.^{73,74} Another peptide that is also commonly used for achieving brain targeting is glutathione. This endogenous tripeptide possesses antioxidant properties and plays a central role in the detoxification of intracellular metabolites. Glutathione has been shown to possess the ability to enhance drug delivery to the brain as mediated by liposomes.⁷⁵

One of the most widely studied proteins for targeted drug delivery is transferrin (Tf). The transport of this endogenous protein is mediated by the Tf receptor – known to be expressed in the luminal membrane of the capillary endothelium of the BBB, among other body regions – which can also be targeted by specifically designed antibodies.^{76–78} Although the mechanism of this approach is not yet fully understood, results from multiple studies of drug targeting and delivery to the brain with these types of moieties confirm its feasibility (reviewed by De Boer and Gaillard).⁶¹ As described earlier for the mechanism of polysorbate 80-coated NP-mediated CNS drug delivery, apolipoproteins, namely apolipoprotein E (ApoE), are obvious candidates for surface functionalization of NPs. ApoE is involved in the transport of lipids into the brain via low-density lipoprotein receptors, which are essential for maintaining cholesterol homeostasis in the brain.⁷⁹ ApoE not only binds to various receptors on the BBB (eg, the low-density lipoprotein receptor-related protein [LRP]-1) but also to receptors in other regions of the CNS, which may be an additional advantage for subcompartmental HIV therapy. Both LRP-1 and LRP-2 have been explored in order to target drugs to the brain.⁸⁰

Due to their role in HIV infection of the CNS, monocytes/macrophages can be interesting targets for therapeutic NPs. Monocytes/macrophages have various surface receptors, namely to mannose residues, which can help them in the process of recognition and endocytosis of NPs. Hence, NPs functionalized with mannose/mannan are better phagocytosed by these cells.⁸¹ Also, bradykinin type II (B_2) receptors are primarily expressed in neuronal and vascular tissue, which makes these interesting targets for drug delivery into the CNS. B_2 -receptor agonists may be effective in targeting the BBB by mediating the opening of TJ (through a calcium-mediated mechanism) at the brain–microvascular endothelial cell (MEC) interface. In addition, RMP-7, which is a synthetic linear pseudopeptide that functions as a B_2 -receptor

agonist, could be used as a targeting BBB molecule.⁸² Furthermore, exposure to an electromagnetic field (EMF) can also augment the permeability of drugs across the BBB, since it could cause significant and temporary alterations in the structure of this natural barrier. Generally, higher EMF power yields increased permeability, but the long-term effects of these changes to the BBB may be hazardous.⁸³

As efflux transporters may greatly limit the residence of antiretroviral drugs in the CNS, another possible strategy to enhance inward flux across the BBB is the inhibition of these efflux-transport systems.⁶¹ As mentioned before, P-gp is the most widely studied representative of all the BBB efflux-system proteins,⁸⁴ and prevents the accumulation of a remarkably wide range of substrates in the brain endothelial cells through potent adenosine triphosphate-driven pumping.⁸⁵ Therefore, improved drug uptake into the CNS could be achieved by altering the MRP1 function at the BBB, as shown in a pioneer *in vivo* study by using MRP1-knockout mice, where brain-to-plasma ratios of a considerable number of drugs increased five- to 50-fold.⁸⁶ Consequently, many MRP1 inhibitors have been developed since,⁸⁷ but their toxicity has been an important limitation.⁸⁸ Therefore, other options need to be considered, and small interfering RNA (siRNA) directed against MRP1 has been shown to be promising as a new reversal agent for avoiding the undesired effects of MRP1 inhibitors.^{89,90} In order to improve physical stability, resistance to nuclease degradation, intracellular penetration, and efficacy and toxicity profiles, NPs can be used to deliver siRNA. This is an alternative way to temporarily disable the efflux mechanisms of the BBB and thus reach the CNS. After the administration and silencing effect of siRNA, the required drugs (such as antiretroviral drugs) can be administered and reach the CNS, thus circumventing MRP1 efflux.

Antiretroviral drug-loaded nanoparticles for CNS delivery

Various nanocarrier-mediated solutions have been proposed in order to increase antiretroviral drug delivery to the CNS. For example, the ability of nanostructured lipid carriers (NLCs) to potentially mediate the brain delivery of zidovudine, a frequently used nucleoside reverse-transcriptase inhibitor that has related toxic effects and a very short plasma half-life (around 1 hour), was studied *in vitro* by Joshy and Sharma.⁹¹ Spherical NLCs comprising a mixture of Compritol® 888 ATO (Gattefossé, Saint-Priest, France), stearic acid, and oleic acid and prepared using a solvent-diffusion method, with a final size range of 170–500 nm, were developed and shown to be taken up by the C6 brain cell

line after 2 hours of incubation, as assessed by fluorescent microscopy. Rhodamine-loaded NLCs associated with the cells and deposited either on the outer side of the plasmatic membrane or in the cytosol. Although preliminary, the ability of these NLCs to promote intracellular trafficking at a representative cell line of the BBB suggests that these nanocarriers may be a promising system to enhance zidovudine brain uptake. In another study, atazanavir was loaded into stearic acid-based SLNs for enhanced brain delivery by Chattopadhyay et al.⁹² SLNs produced by a thin-film hydration technique based on a microemulsion possessed negative charges (zeta potential of approximately -18 mV) and a mean particle-size range between 150 and 250 nm. Besides confirming the low toxicity potential of SLNs in hCMEC/D₃ cell line, a human brain microvessel endothelial cell line (HBMEC), up to a concentration corresponding to 200 nM of atazanavir, this group also demonstrated significantly higher drug-permeation ratios through endothelial cell monolayers compared to atazanavir in aqueous solution after 2 hours of incubation. The hCMEC/D₃ cell line, which is an extensively characterized human brain endothelial cell line, mimics most of the key features of the BBB, and thus represents a useful model for screening drug candidates and carrier systems for CNS drug delivery.^{92,93} Despite the interesting *in vitro* results obtained by this group, future *in vivo* work is required in order to demonstrate the potential efficacy of proposed SLNs to deliver atazanavir to the brain.

In a study by Kuo and Su,⁹⁴ both polymeric (polybutylcyanoacrylate [PBCA] and methyl methacrylate–sulfopropyl methacrylate [MMA-SPM]) NPs and SLNs (comprising mixtures of tripalmitin and cocoa butter) were developed as carriers for stavudine, delavirdine (a nonnucleoside reverse-transcriptase inhibitor), and saquinavir, and characterized for their ability to modulate drug permeability across an *in vitro* BBB model. Nanocarriers were produced by emulsion polymerization (PBCA NPs), free radical polymerization (MMA-SPM NPs) or microemulsion method (SLNs). The BBB model comprised a dual-chamber setup separated by a confluent HBMEC monolayer on a microporous polycarbonate membrane, and cultured on human fibronectin-coated dishes with endothelial cell medium. These studies showed that even though increases in particle size from 90 to 185 nm (PBCA NPs), 5 to 70 nm (MMA-SPM NPs), and 140 to 320 nm (SLNs) yielded a decrease in the permeability coefficient, all three NP types were efficacious carriers of all tested drugs and improved the BBB permeability by three- to 16-fold compared to the compounds in solution. Later, Kuo and Lee⁸² also studied the possibility of using MMA-SPM

NPs for the codelivery of all three antiretroviral drugs. They developed polymeric NPs that were also grafted with RMP-7 (surface functionalization performed by carbodiimide chemistry with poly[ethylene glycol] [PEG]-COOH to allow cross-linking with RMP-7) in order to promote BBB crossing by modulating endocytosis and TJ opening. This strategy was shown to be mildly effective in improving the permeability of considered drugs across another *in vitro* BBB model (monolayer of HBMECs regulated by human astrocytes in a dual-chamber setup). For example, drug-permeability coefficients were increased approximately 1.4-, 2.1- and 1.4-fold for stavudine, delavirdine, and saquinavir, respectively, when compared to values obtained for similar NPs not bearing RMP-7 at their surface. Finally, the impact of EMF exposure on the permeability of saquinavir across the BBB model, as mediated by drug incorporation in the same type of polymeric NPs and SLNs, was studied by Kuo and Kuo.⁸³ Higher EMF frequency yielded larger permeability coefficients when both drug-loaded nanocarriers and free saquinavir were used, while square waves produced greater permeability than sine and triangle waves. Moreover, and also important to note, higher EMF power caused apoptosis of HBMECs, a fact that can impair the usefulness of this additional strategy due to toxicity issues.

Albumin is a matrix-forming macromolecule commonly used in the production of drug nanocarriers, mainly due to its advantageous biodegradability and low toxicity properties. Albumin NPs encapsulating zidovudine have been proposed for brain delivery of this polar nucleoside;⁷⁷ alongside, surface modification of NPs with PEG was performed in order to reduce their rapid removal from blood circulation. The NP surface was further modified by anchoring Tf ligand with the purpose of enhancing CNS uptake. The biodistribution of zidovudine was studied in Wistar rats after intravenous administration of unmodified and Tf-modified PEG-albumin NPs with an equivalent dose of 54.4 mg drug/kg of body weight. Experimental results showed significant enhancement of brain drug localization for Tf-PEG-albumin NPs compared to PEG-albumin NPs (21.1% \pm 1.8% versus 9.3% \pm 0.9%, respectively, of the total drug recovered at 4 hours postadministration). This study confirmed the potential use of Tf modification of nanocarriers in order to enhance brain drug targeting for HIV therapy.

In order to target macrophages, either at the CNS or the mononuclear phagocyte system, Kaur et al⁸¹ developed didanosine-loaded gelatin NPs with mannan coating. These macromolecular (gelatin) NPs were produced by double desolvation, and in order to coat them, they were incubated

with a mannan solution. Besides providing sustained drug release, this nanosystem improved *in vitro* drug uptake by macrophages, with a fivefold increase in cell-associated levels after 2 hours' incubation compared with didanosine in solution. Fluorescence microscopy also confirmed NP cell internalization. Further, didanosine levels in the brain were increased by 12.4-fold upon subcutaneous administration of mannan-coated NPs compared to didanosine in solution (Figure 2).⁸¹ Also, brain accumulation of didanosine was higher than when plain NPs (ie, without mannan surface modification) were used. These results seem to support the positive influence of mannose residues in the ability of NPs to reach the CNS, probably by a monocyte/macrophage-mediated mechanism.

Poly(L-lactic acid) NPs conjugated with Tat peptide were also studied in order to increase the transport of ritonavir across the BBB and into the CNS.^{73,74} This strategy was envisioned in order to bypass the known efflux action of P-gp on this particular PI. NPs were produced by an emulsion-solvent evaporation technique and Tat functionalization achieved by using an epoxy conjugation method, where the NP surface is activated by epoxy compound followed by Tat conjugation. NPs were shown to be effective *in vitro* in inhibiting HIV-1 infection of monocyte-derived macrophages through the reduction of cytopathic effects, HIV-1 p24 protein secretion, and production of progeny virions.⁷⁴ Also, P-gp intact (wild-type) mice were injected via the tail vein with either ritonavir-loaded NPs or the drug in solution (45 mg/kg ritonavir in both cases).⁷³ Obtained data showed an increase in the ritonavir brain parenchyma:capillary ratio over time (Figure 3), without disruption of BBB integrity, in animals that received drug-loaded Tat-conjugated NPs; after 2 weeks, brain drug level with Tat-conjugated NPs was 800-fold higher than that with the drug in solution, and about sevenfold higher than unconjugated NPs. Rao et al proposed that Tat-conjugated NPs were transported to the parenchyma without influencing the integrity of the BBB, which suggests that this transport could occur due to transcytosis across the endothelium of the brain vasculature. Moreover, these NPs maintained potentially therapeutic drug levels in the brain for a sustained period (14 days) with a single-dose intravenous administration.⁷³ Therefore, Tat-NPs may constitute an effective way of delivering anti-HIV-1 drugs to the CNS.

In another approach, Mahajan et al⁹⁵ proposed the use of Tf-conjugated quantum rods (QRs; average length and diameter of approximately 25 nm and 5 nm, respectively, as assessed by transmission electron microscopy) in order to enhance the brain delivery of saquinavir. QRs were composed

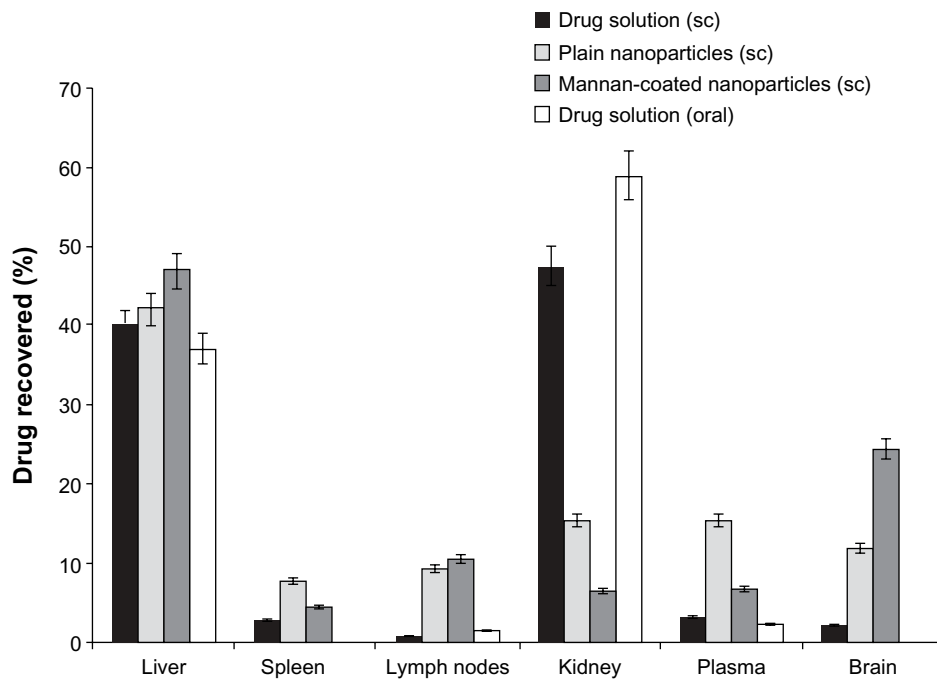


Figure 2 Tissue distribution of didanosine (after 12 hours of administration) delivered as mannan-coated nanoparticles, plain nanoparticles, and in solution (mean ± standard deviation, n=3).

Notes: Reproduced from *Acta Pharmaceutica*, 2008, vol 58, No 1, page 71. Kaur A, Jain S, Tiwary AK. Mannan-coated gelatin nanoparticles for sustained and targeted delivery of didanosine: in vitro and in vivo evaluation.⁸¹

Abbreviation: SC, subcutaneous.

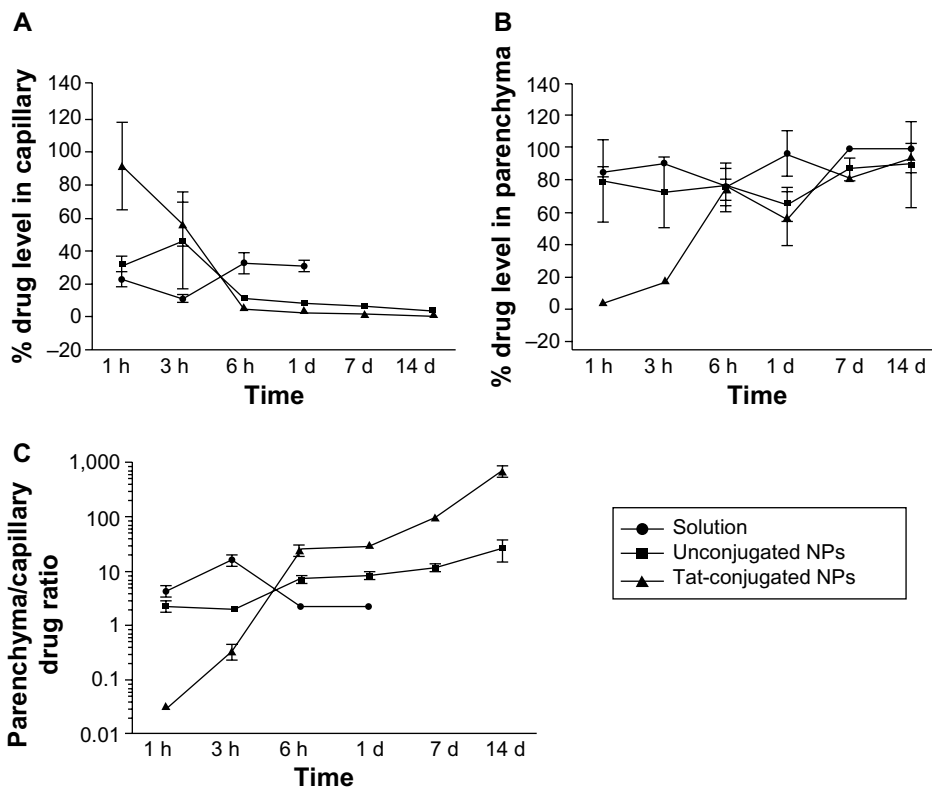


Figure 3 Changes in the fraction of ritonavir distribution between brain capillaries and parenchyma over time following intravenous drug administration.

Notes: The fractions of the total brain drug present in (A) brain capillaries and (B) brain parenchyma, and (C) the ratio of drug distribution between the brain parenchyma/capillaries in different groups is presented. The capillary drug levels in the solution group were undetectable beyond 1 day after administration. Data are represented as means ± standard error of the mean (n=4). *P<0.05 (Student's t-test) between Tat-conjugated and unconjugated nanoparticles (NPs). Reprinted from *Biomaterials*, 2008;29: Rao KS, Reddy MK, Horning JL, Labhasetwar V TAT-conjugated nanoparticles for the CNS delivery of anti-HIV drugs. 4429–4438. © (2008) with permission Elsevier.⁷³

Abbreviations: h, hour(s); d, day(s); Tat, transactivator of transcription.

of a thin zinc sulfide layer over a cadmium/selenium core nanocrystal and coated with mercaptosuccinic acid; saquinavir and Tf were further conjugated to surface carboxyl groups by carbodiimide chemistry and stabilized by mixing with poloxamer 407 (Figure 4). The nanocarrier was tested in an *in vitro* BBB model comprising primary cultures of HBMECs and normal human astrocytes in a double-chamber setup. An HBMEC monolayer was grown in the apical side of a porous polyethylene terephthalate membrane, while the basolateral side was covered with an astrocyte monolayer. Additionally, HIV-1-infected peripheral blood mononuclear cells (PBMCs) were placed in the receptor chamber in order to evaluate the antiretroviral efficacy of different treatments. Results showed that QRs did not affect the integrity of the BBB, and presented reduced cytotoxicity of up to 40 nM concentration in saquinavir; also, functionalization with Tf led to enhanced uptake of QRs by HBMECs and PBMCs compared to nonfunctionalized QRs. Moreover, when the above BBB model was used, a significant decrease of HIV-1 replication in PBMCs was observed for Tf-saquinavir QRs compared to both the free drug and non-Tf-functionalized saquinavir QRs. These observations seem to support the view that the proposed QRs may provide an efficient way to promote the transport of saquinavir across this barrier and reduce the viral load at the CNS. Nevertheless, the mechanism of release of conjugated drugs from the carrier is not clear, even though the authors claim that degradation of the nanosystem as a whole is involved.⁹⁶ Comparable results were also reported for Tf-conjugated QRs carrying amprenavir.⁹⁶ In addition, these QRs present the ability to be used in real-time tracking of particle crossing through the *in vitro* BBB model, as well as a potential use in diagnostic imaging, due to their intrinsic optical properties.⁹⁷ Of further note, nano-

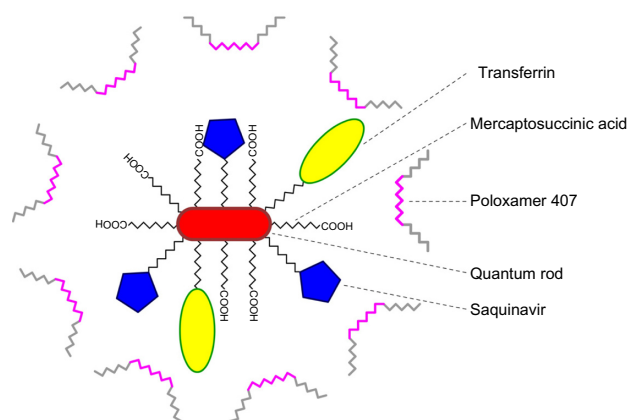


Figure 4 Schematic representation of the quantum rod-based nanosystem for the delivery of saquinavir.

Note: Data from Mahajan et al.⁹⁵

plexes have also been obtained by the same group by using quantum dots (QDs) produced with the same materials and presenting a similar structure as QRs (in this case, Tf was not conjugated and poly[diallyldimethylammonium chloride] was used to provide a positive surface charge to QDs) and siRNA targeting the expression of matrix-degrading metalloproteinase type-9.⁹⁸ This protein plays a significant role in disrupting the BBB, and its expression is triggered by HIV-1 infection of the CNS. Results using the aforementioned *in vitro* BBB model showed that proposed siRNA-QD nanoparticles were able to silence gene expression in HBMECs and thus maintain the integrity of the membrane. Although not directly targeting the virus, this delivery system may provide an interesting auxiliary approach in preventing continuous viral invasion of the CNS.

Antiretroviral drug nanosuspensions for CNS delivery

Added to the previous examples of different nanocarriers, size reduction to the low-micrometer/nanometer scale of antiretroviral drugs, and their eventual surface modification, may also be an interesting strategy of enhancing drug delivery to the CNS. For example, Shegokar and Singh⁹⁹ prepared nanosuspensions of nevirapine by high-pressure homogenization and proceeded with surface modification with serum albumin, PEG 1000, or dextran 60 by physical adsorption in solution (final diameters around 500 nm). When tested *in vivo* in a rat model, particles modified with albumin presented the ability to accumulate in the brain compared to nevirapine in solution or other drug nanosuspensions (in the previous cases, no drug was detected at this anatomical site). The area under the curve in the brain ($AUC_{\text{brain}}/AUC_{\text{blood}}$) ratio of albumin-modified nanosuspensions was 9.33. Although no specific mechanistic explanation for the observed enhancement of brain drug levels was provided by the authors, increased macrophage uptake of albumin-modified nevirapine particles over other formulations may be at least partially associated with differential patterns of adsorbed proteins at the particle surface.^{99,100} A recent study by Dash et al¹⁰¹ further demonstrated the potential of using nanosuspensions of atazanavir and ritonavir obtained by high-pressure homogenization and using poloxamer 188 as a stabilizer for neuroprotection in a humanized HIV-infected animal model (nonobese diabetic/severe combined immunodeficiency- γ_c^{null} mice). Weekly intravenous administration of nanosuspensions allowed the eliciting of neuroprotective responses (as assessed by reduced neuronal, synaptic, and astrocyte damage), alongside the reduction of viral loads and maintenance

of CD4⁺ cells in peripheral blood. A further study by the same group using the same *in vivo* model confirmed these observations and demonstrated its relation with tissue and blood serum levels of atazanavir, ritonavir, and efavirenz when administered as nanosuspensions.¹⁰² Again, the role of monocytes/macrophages on the enhanced CNS delivery of the antiretroviral drugs was emphasized.

Indeed, monocytes/macrophages may be an ideal physiological “shuttle” for brain delivery of antiretroviral drugs, due to their phagocytic nature, which allows extensive uptake (depending on coating, size, shape, and charge) and sustained release (for days to weeks) of different antiretroviral drug particles.^{103–105} Additionally, these immune cells possess the ability to readily migrate across the BBB or at least transfer their drug content to endothelial cells at the BBB, as recently shown *in vitro* (Figure 5).¹⁰⁶ The previous possibilities have been systematically explored by investigators at the Nebraska Medical Center, Omaha, NE, USA.^{103–109} Drug incorporation into cells was performed by simply incubating cells with particles in culture media for 8–12 hours, followed by cell washing and removal of free drug. Researchers observed that nanoformulated indinavir could be delivered into the brain of an HIV-1 encephalitis rodent model up to around 20-fold higher levels when incorporated into bone marrow-derived macrophages after a single dose administered intravenously.¹⁰⁹ Moreover, treatment with this cell-based nanoformulation was able to release indinavir in a continuous fashion over 2 weeks and reduce HIV replication in brain regions presenting signs of encephalitis. This may well be associated with the role played by used drug-carrier cells in the inflammatory response at HIV-replication sites, thus providing a natural targeting mechanism for antiretroviral drugs. One important aspect of using monocytes/macrophages for the delivery of nanosized antiretroviral drugs to the CNS is related to the potential of drug-induced toxicity to cell carriers. Even if toxicity to human monocytes/macrophages and HBMECs has been shown to be generally low *in vitro* when cells were incubated with different ritonavir, indinavir, and efavirenz particles up to concentrations of 100 μM , a considerable decrease in cell viability was observed for levels of 500 μM .¹¹⁰ Moreover, toxicity (viability, release of inflammatory mediators, and reduction of the transendothelial electrical resistance of primary HBMEC monolayers) was dependent on the combination of used antiretroviral drugs and nanosuspension parameters, namely surface charge, surfactants (used as stabilizers), and particle shape and size. Although these concentrations may not be observed *in vivo*,^{104,106,109} special

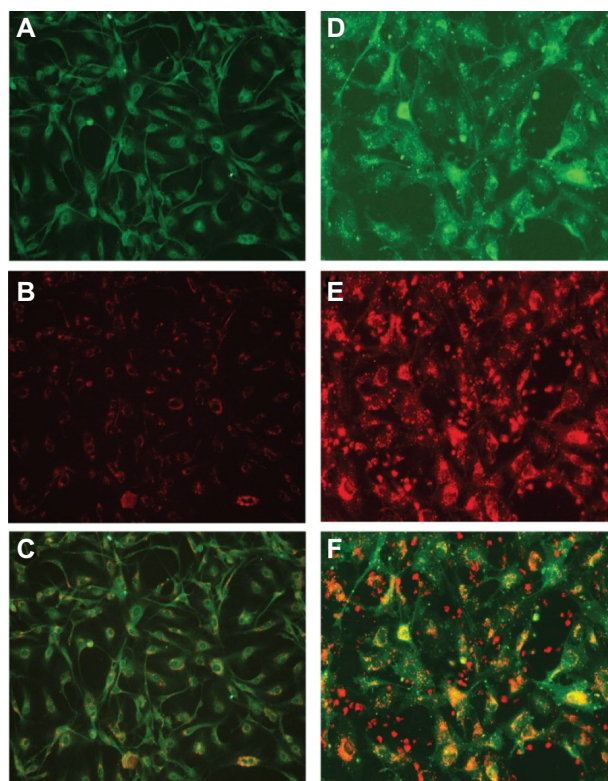


Figure 5 Uptake of antiretroviral drugs formulated as nanosuspensions by human brain microvascular endothelial cells (HBMECs) following endothelial mononuclear phagocyte cocultivation.

Notes: (A–C) Primary HBMECs were exposed for 2 hours to conditioned media from monocyte-derived macrophages loaded with rhodamine-labeled efavirenz nanosuspension (300 nm and +7.4 mV; obtained by sonication and using poly[lactide-co-glycolide], poly[vinyl alcohol] and cetrimide as stabilizers); HBMECs were then washed thrice with phosphate-buffered saline to remove macrophage-conditioned media, and analyzed by fluorescence microscopy. (B and C) Endothelial cell uptake of nanosuspensions (orange-yellow) released by monocyte-derived macrophages. Staining with the endothelium-specific marker von Willebrand factor (green, A) confirmed the endothelial nature of primary HBMECs. (D–F) Primary HBMECs labeled with Vybrant 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbonyl perchlorate (green, D) were cocultured for 2 hours with monocytes loaded with rhodamine-labeled ritonavir (613 nm and –26.7 mV); obtained by high-pressure homogenization and using poloxamer 188 and N-(carbonyl-methoxy-polyethylene glycol 2000)-1,2-sn-glycero-3-phosphoethanolamine as stabilizers, washed to remove monocytes, and analyzed by fluorescence microscopy. (E and F) Endothelial cell uptake of nanosuspensions (orange-yellow) during endothelial monocyte cocultivation. Copyright ©2012. Dove Medical Press. Reproduced from Kanmogre GD, Singh S, Roy U, et al. Mononuclear phagocyte intercellular crosstalk facilitates transmission of cell-targeted nanoformulated antiretroviral drugs to human brain endothelial cells. *Int J Nanomedicine*. 2012;7:2373–2388.¹⁰⁶

attention should be paid to these issues in order to reduce the potential onset of safety issues.

Conclusion and remaining challenges

Tremendous progress has been achieved in the field of HIV/AIDS management over the last few decades. The development of different antiretroviral drugs alongside the introduction of cART allowed a shift of the infection from rapidly progressive to a chronic disease. Even so, a cure is

still elusive, and refinement of current therapy is required. Restriction of CNS infection by HIV and the management of its consequences are limited by poor antiretroviral pharmacokinetics, which are often associated with poor compound permeation of the BBB. Nanotechnology-based solutions have shown the potential to provide efficient and safe tools to circumvent these problems, and in particular solid drug nanocarriers and drug nanosuspensions may be preferential, due to their ability to prolong systemic circulation, provide anatomical and/or cell targeting, increase crossing and reduce drug efflux at the BBB, and enhance intracellular drug levels at HIV-target cells. Moreover, the inherent toxicity of many drugs currently being used in cART may be reduced.

Despite the work developed so far, important questions remain. One limitation of current strategies is related to the need for systemic delivery of NPs in order to allow them to reach the BBB and deliver drugs into the CNS. Interestingly, we are not aware of any study considering the delivery of nanosystems by alternative routes with the purpose of antiretroviral therapy. The intranasal delivery of nanomedicines may be an interesting option due to the direct nose-to-brain transport, which bypasses the BBB.¹¹¹ Another issue is related to the accumulation of antiretroviral drugs and even nanocarriers at the CNS and their potential neurotoxicity, particularly when long-term regimens are needed. The biodistribution patterns of NPs once in the CNS, alongside their long-term fate, also require additional understanding.¹¹² Indeed, the field of nanotoxicology is growing exponentially, and the potentially deleterious effects of nanomedicines crossing the BBB require further clarification.¹¹³ Finally, the true impact and the possibility of viral eradication with the use of nanotechnology-based cART needs to be assessed in vivo, while optimization of dosages and schedules is needed. Answering these and other questions adequately is mandatory in order to open the way to the translation of such therapeutics to exploratory human clinical trials. The different investigations so far conducted and reviewed in this manuscript, alongside the prolific work being developed in the field, assure that these issues will be addressed in coming years.

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Disclosure

The authors report no conflicts of interest in this work.

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