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Short communication

AZT 5′-triphosphate nanoformulation suppresses human immunodeficiency virus type 1 replication in peripheral blood mononuclear cells

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Inefficient cellular phosphorylation of nucleoside and nucleotide analog reverse transcriptase inhibitors (NRTIs) to their active nucleoside 5′-triphosphate (NTPs) form is one of the limitations for human immunodeficiency virus (HIV) therapy. We report herein direct binding of 3′-azido-3′-deoxythymidine-5′-triphosphate (AZTTP) onto magnetic nanoparticles (Fe_3O_4; magnetite) due to ionic interaction. This magnetic nanoparticle bound AZTTP (MP-AZTTP) completely retained its biological activity as assessed by suppression of HIV-1 replication in peripheral blood mononuclear cells. The developed MP-AZTTP nanoformulation can be used for targeting active NRTIs to the brain by application of an external magnetic force and thereby eliminate the brain HIV reservoir and help to treat NeuroAIDS. *Journal of NeuroVirology* (2009) **15,** 343–347.

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Nucleoside and nucleotide analog reverse transcriptase inhibitors (NRTIs) remain as an important component of the combination antiretroviral therapy (ART). However, the low conversion of NRTIs into their active nucleoside 5′-triphosphate (NTP) form results in the accumulation of NRTIs, which leads to the development of drug resistance, toxicity, and ultimately compromising the effectiveness of this therapy (Antonelli *et al,* 1996; Kohli *et al,* 2007). In order to solve the problem of insufficient drug activation, previous attempts have been made to directly deliver nucleoside 5′-monophosphates or their protected prodrug derivatives (Wagner *et al,* 2000). Administration of active phosphorylated form of NRTI offers an advantage by bypassing the first step of intracellular phosphorylation; however, it poses a challenge in terms of protecting the NTPs from cellular phosphatases and neutralizing the electronegative charge of NTP, thus this approach offered very limited success (Vinogradov *et al,* 2005). More recently, nanomaterial based drug carriers are being explored for delivery of NRTIs in their active phosphorylated forms (Vinogradov, 2007). Previous few reports have shown increased drug activity and reduced cytotoxicity of 5′-triphosphates of NRTI encapsulated within the nanogel carriers or liposomes or erythrocytes (Vinogradov *et al,* 2005; Szebeni *et al,* 1990; Magnani *et al,* 1994). However, target specific delivery of NRTI through an effective carrier would provide significant therapeutic benefit and also reduce clinical toxicities associated with high doses of NRTIs.

In recent years, nano-sized magnetic particles (Fe_3O_4, magnetite) have been increasingly used as carrier for binding proteins, enzymes, or drugs (Saiyed *et al,* 2003). Drug-loaded magnetic nanocarrier offers advantage in terms of site-specific targeting (by application of an external magnetic field), tissue retention, and sustained release of drugs. Magnetically guided drug delivery systems have been successfully used to increase the efficacy and reduce the toxic side effects associated with chemotherapy (Ito *et al,* 2005). We and others have also previously reported successful immobilization of several clinically and biotechnologically important proteins and enzymes onto magnetic nanoparticles

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(Saiyed *et al,* 2007; Koneracká *et al,* 2006). However, site-specific targeting of active NRTIs bound to magnetic nanocarriers has not been reported. Therefore, we hypothesize that active NRTIs can directly bind to magnetic nanoparticles by ionic interaction and provide an effective magnetically guided nanoformulation that can inhibit HIV-1 replication. In the current study, we report for the first time direct binding of 3′-azido-3′-deoxythymidine-5′-triphosphate onto magnetic nanoparticles (MP) and this bound AZTTP (MP-AZTTP) completely retained its biological activity as assessed by inhibition of human immunodeficiency virus (HIV)-1 replication in peripheral blood mononuclear cells (PBMCs).

Magnetic nanoparticles were prepared by coprecipitating divalent and trivalent iron ions by alkaline solution and treating under hydrothermal condition as described earlier by us (Saiyed *et al,* 2007). The resultant black magnetite particles were washed repeatedly with deionized water and dispersed in Tris-EDTA buffer (pH 7.5). Transmission electron microscopy (TEM) examination revealed the mean particle size to be 40 nm (Figure 1). For the binding experiment, different ratios of magnetic nanoparticles and AZTTP (eENZYME, Maryland, USA) were mixed in TE buffer pH 7.5, followed by incubating the mixture on a shaker (50 to 100 rpm) for 2 h at room temperature. After incubation, the magnetic particles bound with AZTTP were attracted by application of an external magnetic field. The supernatant containing the unbound AZTTP was collected and the pellet was resuspended in appropriate volume of TE buffer pH 7.5 and stored at 2°C to 8°C until further use. The binding efficiency (μg AZTTP/mg of magnetic nanoparticles) was determined spectrophotometrically at 267 nm ($\varepsilon = 11{,}650\ M^{-1}cm^{-1}(1/M\ 1/cm)$) by measuring the amount of AZTTP in the unbound fraction. The difference between the total AZTTP added and unbound AZTTP was used to calculate the amount of AZTTP bound to the magnetic nanoparticles.

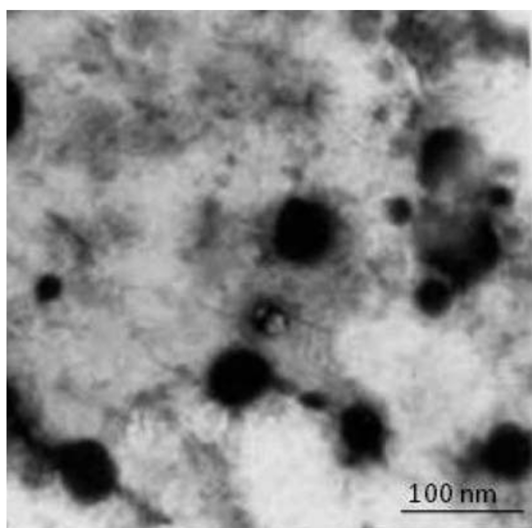

Figure 1 TEM micrograph of magnetite particles. The average particle size is ~40 nm.

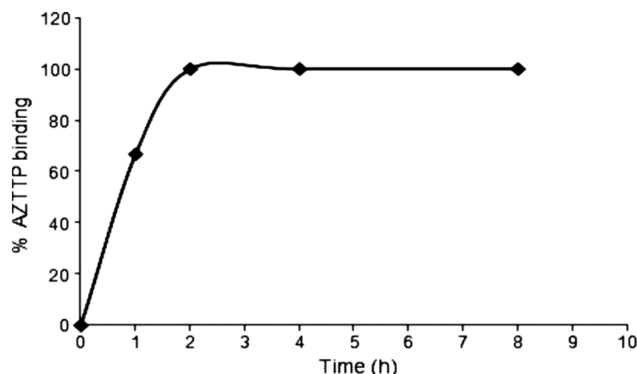

Figure 2 Time kinetics of AZTTP binding onto magnetic nanoparticles. Binding ratio used for the experiment was 3 mg magnetic nanoparticles to 0.1 mg AZTTP.

Figure 2 shows the time kinetics and percent direct binding of AZTTP to magnetic nanoparticles. The binding was standardized by a series of experiments and the data presented in Figure 2 show that ratio of 3 mg magnetic nanoparticles to 0.1 mg of AZTTP produced 100% binding in 2 h. After the reaction time was optimized, the AZTTP binding efficiency was estimated by using different ratios, 1:0.02, 1:0.04, 1:0.06, 1:0.1, 1:0.2, and 1:0.3 of magnetic nanoparticles and AZTTP, respectively. Data presented in Figure 3 show the adsorption isotherm of AZTTP binding on magnetic nanoparticles. The result obtained indicates the binding efficiency in the range of 30 to 35 μg AZTTP per milligram of magnetic nanoparticles. Thus, our data shows that AZTTP binds efficiently to magnetite nanoparticles.

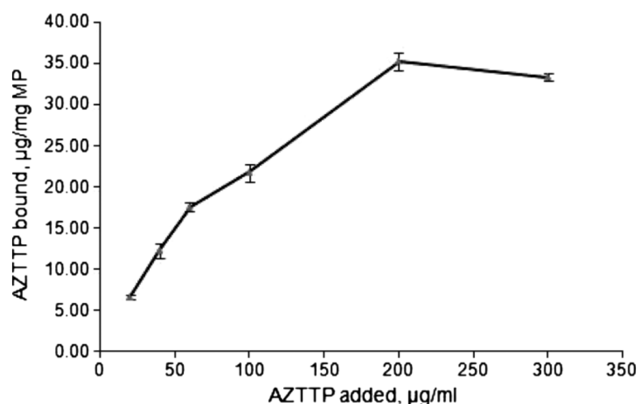

Figure 3 Binding isotherm for AZTTP on magnetic nanoparticles. Binding ratios used were 1:0.02, 1:0.04, 1:0.06, 1:0.1, 1:0.2, and 1:0.3 of magnetic nanoparticles and AZTTP, respectively. The incubation time used for binding reaction was 2 h. Binding efficiency (μg AZTTP/mg magnetic nanoparticles) was determined using spectrophotometric method by measuring the amount of AZTTP in the unbound fraction.

Table 1 List of primers used for real-time PCR

Primer	Sequence
β-Actin	5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′
	5′-AGTCATAGTCCGCCTAGAAGCATTTGCGGT-3′
HIV-1 LTR	5′-GGCTAACTAGGGAACCCACTG-3′
	5′-CTGCTAGAGATTTTCCACACTGAC-3′

In order to examine whether MP-AZTTP was equally efficacious as unbound or free AZTTP, the anti-HIV effect of MP-AZTTP was evaluated in an *in vitro* model system using HIV-infected PBMCs. Briefly, PBMCs (1×10^6 cells/ml) were infected with HIV-1 IIIB [X4] (NIH AIDS Research and Reference Reagent Program catalog no. 398) at a concentration of $10^{3.0}$ $TCID_{50}$/ml cells for 3 h, as described by the HIV-1 IIIB supplier. Cells were washed and cultured in the presence of free AZTTP and MP-AZTTP (10 to 100 nM) for 7 days, the culture supernatants were quantitated for p24 antigen by enzyme-linked immunosorbent assay (ELISA) kit (Zeptometrix, Buffalo, NY). Additionally, after a postinfection period of 24 h, cells were analyzed for gene expression of LTR-R/U5 by quantitative real-time polymerase chain reaction (PCR) using specific primers for HIV-1 LTR (long terminal repeat) gene. β-Actin served as an internal control (Table 1). Relative mRNA species expression was quantitated and represented as transcript accumulation index ($TAI = 2^{-\Delta\Delta CT}$) as described earlier by us (Nair *et al,* 2009).

Data presented in Figure 4a show the HIV-1 p24 antigen levels in the culture supernatants on day 7 post infection. A dose-dependent decrease in p24 antigen production was evident at 10 nM (183 ± 8.8 ng/ml, 18% decrease; $P < .05$), 50 nM (107 ± 7.04 ng/ml, 52% decrease; $P < .004$), and 100 nM (15 ± 0.9 ng/ml, 93% decrease; $P < .0006$) MP-AZTTP treatment by HIV-1–infected PBMCs compared to the untreated control cultures (221.4 ± 4.5) (Figure 4a). The anti-HIV activity of free AZTTP (positive control) and MP-AZTTP was found to be similar under identical culture conditions at various doses. In addition to p24 antigen decrease by MP-AZTTP treatment, we also examined the suppression of LTR-R/U5 gene expression, which represents early stages of reverse transcription of HIV-1. Our results (Figure 4b) demonstrate that MP-AZTTP at 10 nM ($TAI = 0.82$; $P < .01$), 50 nM ($TAI = 0.23$; $P < .001$), and 100 nM ($TAI = 0.01$; $P < .0005$) concentrations significantly down-regulated viral replication as determined by LTR suppression in HIV-1–infected PBMCs compared to control cultures treated with HIV-1 alone. The suppression of LTR-R/U5 gene expression with MP-AZTTP treatment was comparable to that of free AZTTP. These results suggest that AZTTP bound to magnetic nanoparticles (MP-AZTTP) is able to exert significant anti-HIV effects comparable to free AZTTP.

In addition, we also examined the nonspecific cytotoxicity of MP-AZTTP to PBMCs and our results showed that MP-AZTTP even at the highest concentration (100 nM) was not cytotoxic to PBMCs as evaluated by XTT cell viability assay (Table 2).

Previous studies have reported direct coupling of proteins, enzymes, or drugs to magnetic nanoparticles in the presence of carbodiimide. This mechanism of protein coupling to magnetic particles has been attributed to the presence of amphoteric hydroxyl (-OH) group on the surface of the magnetite particles (Bacri *et al,* 1990). However, results reported herein demonstrate that AZTTP can directly bind on magnetite nanoparticles without the

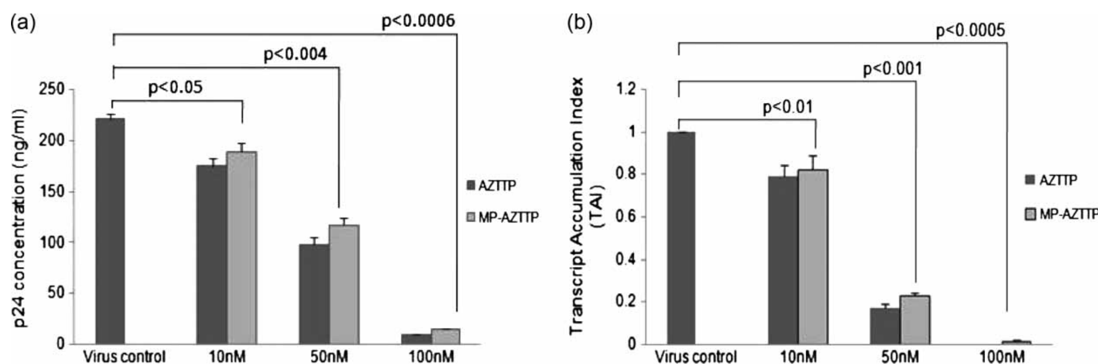

Figure 4 (**a**) MP-AZTTP inhibits p24 production. PBMCs (1×10^6 cells/ml) obtained from normal subjects were infected with native HIV-1 IIIB (NIH AIDS Research and Reference Reagent Program catalog no. 398) at a concentration of $10^{3.0}$ $TCID_{50}$/ml cells for 3 h and washed 3 times with Hanks' balanced salt solution (GIBCO-BRL, Grand Island, NY) before being returned to culture with and without AZTTP or MP-AZTTP (10 to 100 nM) for 7 days. The culture supernatants were quantitated for p24 antigen using a p24 ELISA kit (ZeptoMetrix, Buffalo, NY). The data represent the mean $\pm$ SE of three independent experiments and is expressed as ng/ml. Statistical analysis was done using Student's *t* test. (**b**) MP-AZTTP inhibits HIV-1 replication. PBMCs (1×10^6 cells/ml) were infected with native HIV-1 IIIB ($\times 4$) (NIH AIDS Research and Reference Reagent Program catalog no. 398) at a concentration of $10^{3.0}$ $TCID_{50}$/ml cells for 3 h and washed 3 times with Hanks' balanced salt solution (GIBCO-BRL, Grand Island, NY) before being returned to culture with and without AZTTP or MP-AZTTP (10 to 100 nM) for 24 h. The RNA was extracted, reverse transcribed, and followed by quantitative real-time PCR against the LTR-RU5 and the housekeeping gene, β-actin, as the internal controls. The data represent mean $\pm$ SE of three independent experiments. Statistical analysis was done using Student's *t* test.

Table 2 Cytotoxicity of AZTTP and MP-AZTTP

Samples	% Cell viability
Untreated control PBMCs	100
AZTTP treated PBMCs	96 ± 2.3
MP-AZTTP treated PBMCs	94.5 ± 4.9

Note. Cytotoxicity studies were performed using XTT cytotoxicity assay (Sigma Aldrich, St. Louis, MO, USA) using uninfected PBMCs. 100 nM AZTTP was used for this experiment.

requirement of coupling agents. This is possible because phosphate groups are known to have a strong interaction with iron oxide and oxyhydroxide in the pH range of 4 to 8 presumably via a Fe-O-P bond (Kreller *et al,* 2003). In our study, the mechanism of direct adsorption of AZTTP is due to the strong interaction of triphosphate groups of AZTTP with the Fe_3O_4 nanoparticles. This ionic interaction may also help to neutralize the electronegative charge of AZTTP and make their mass transport easier across the cellular barriers.

It has been reported that under normal circumstances AZTTP (the active form of AZT) is unable to cross the blood-brain barrier (BBB) (Vinogradov, 2007). On the contrary, AZT itself (nonphosphorylated form) is able to cross the BBB; however, its antiviral efficacy is limited due to poor intracellular phosphorylation to active form AZTTP, which acts as a chain terminator during reverse transcription of the viral RNA genome. AZT is converted to AZTTP by the action of three enzymes; thymidine kinase (TK), thymidylate kinase (TMPK), and nucleoside diphosphate kinase (Wohrl *et al,* 2005; Furman *et al,* 1986). However, the rate-limiting step is the phosphorylation of AZT monophosphate (AZTMP) to AZT diphosphate (AZTDP), catalyzed by the enzyme thymidylate kinase (TMPK), which is inefficient in human cells (Lavie *et al,* 1997).

Although highly active antiretroviral therapy (HAART) inhibits HIV-1 in the periphery to an undetectable level, virus persists in the brain reservoirs because of the inability of HAART to cross the BBB (Potula *et al,* 2008). Therefore, it is critical to efficiently deliver the antiviral drugs across BBB to eliminate the viral reservoirs in the brain. There are several approaches that are being pursued to deliver drugs across BBB, which includes use of lipid carrier, tagging drugs to ligands that cross the BBB through a carrier-mediated transport (CMT), or receptor-mediated transport (e.g., insulin, transferrin, etc.) (Pardridge, 2007). However, the transport of drugs across BBB still remains a challenge. Previous reports have shown successful delivery of anticancer drugs bound to magnetic nanoparticles to treat brain carcinoma (Chertok *et al,* 2008; Ito *et al,* 2005). Further, magnetic nanoparticles have been used as an imaging agent in the brain for diagnostic purposes (Riviere *et al,* 2007). In our studies, as a first step towards specific drug targeting to brain to eliminate the remaining HIV-1 reservoirs, we have developed for the first time a nanoparticle-based drug delivery system, which shows that AZTTP (an active form of NRTI) can be tagged to magnetic nanoparticles. In addition, our studies indicate that MP-AZTTP effectively inhibits HIV-1 in *in vitro* infection model. Further studies to examine whether MP-AZTTP can cross BBB under the influence of external magnetic force are being currently pursued in our laboratory. Therefore, the delivery of AZTTP using magnetic nanoparticles is expected to be more therapeutic and may reduce the risk of developing drug resistant viral strains and further reduces the clinical toxicities associated with the use of high doses of NRTIs. In summary, we have successfully developed an AZTTP nanoformulation bound to magnetic nanoparticles and this MP-AZTTP is able to exert significant anti-HIV effects similar to free AZTTP.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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