FLUORESCENT MAGNETIC NANOPARTICLE AS ANTI-HIV DRUG CARRIER FOR RODENT BRAIN TARGETING

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ABSTRACT

BACKGROUND: HIV-1 still remains one of the leading life-threatening diseases in the world despite the introduction of Highly Active Antiretroviral Therapy (HAART). Since most of the drugs have less penetrance into viral reservoir sites such as brain, development of an active targeted drug delivery system is essential to increase the efficacy of drug targeting to the brain. In this regard, we developed a multifunctional iron oxide magnetic nanoparticle (MNP) bound to anti-HIV drug azidothymidine 5'-triphosphate (AZTTP) (MNP-AZTTP) tagged with fluorescent probe for noninvasive imaging in our lab for targeting to brain. Our initial observation on *in vitro* study indicated that MNP-AZTTP have significantly improved cellular uptake and drug delivery across the BBB model.

METHODOLOGY: The synthesis of MNP was prepared by reverse-phase evaporation method. MNP-AZTTP was encapsulated into liposomes with fluorescent probe incorporation. This MNP-AZTTP was further characterized with respect to particle size, polydispersity index (PDI) and surface charge. The drug dissolution study was done through dialysis at different time intervals (30min to 2 days) measured by HPLC. The initial *in vivo* characterization was carried out in mice by injecting MNPs through i.v. injection and retained within the 0.2 Tesla magnetic fields for 30min, 1hr, and 2hrs with anesthesia assistance. At the end of the treatment, mice were sacrificed and brain tissues were harvested for co-localization studies of drug and MNP by fluorescent detection on frozen section slides of brain tissue.

RESULTS: Preliminary data showed that MNP-AZTTP are of 118±15.2 nm size with 0.15 PDI and zeta potential of the particles was 10.3 ± 2.2 mV. Further, drug dissolution study displayed significantly sustained release property over free drugs. *In vivo* analysis indicated that within 2hr of injection the fluorescent MNP-AZTTP reached the brain while the signal from control group was negligible. Dose dependent study has revealed that 5mg/kg of MNPs are ideal for pharmacokinetics study in mice.

CONCLUSIONS: This novel formulation will be able to deliver multiple anti-HIV drugs more efficiently to rodent brain and also help us to detect the site of drug target in a noninvasive manner through imaging process.

INTRODUCTION

Despite significant advances in highly active antiretroviral therapy (HAART), the prevalence of neuroAIDS remains high. This is mainly attributed to inability of antiretroviral therapy (ART) to cross the blood–brain barrier (BBB), thus resulting in insufficient drug concentration within the brain[1]. The selective permeability of the BBB is due to the distinct morphology and enzymatic properties of endothelial cells that enable them to form complex tight junctions with minimal endocytic activity [2]. Previous studies report that delivery of ART to the brain is limited especially due to the physical structure of the BBB, presence of efflux pumps and higher expression of metabolizing enzymes, which makes BBB an effective barrier against many antiretroviral drugs[3]. In recent years, advent of nanotechnology has stimulated the development of innovative systems for the delivery of drugs and diagnostic agents. It is now possible to synthesize, characterize and specifically tailor the functional properties of nanoparticles for various clinical as well as diagnostic applications. Additionally, nanoformulations of small drugs delivered systemically are more efficacious and less toxic than the same drug delivered in free form. This effectiveness of nanoparticle based drug delivery systems is attributed to their small size, controlled time release of the drug and modification of drug pharmacokinetics and biodistribution profile[4]. In this regard, one type of nanoparticle that has gained increasing interest is magnetic nanoparticles, which mainly consist of nano-sized iron oxide particles (magnetite; Fe_3O_4 or maghemite; γ-Fe_2O_3). The use of magnetic nanoparticles contributes to a precise delivery of drugs to the exact site (eg, inflammation, cancer) by application of an external magnetic field[5]. We report herein development of magnetic azidothymidine 5'-triphosphate (AZTTP) liposomal nanoformulation and its ability to transmigrate across an *in vitro* BBB model by application of an external magnetic field. Our *in vitro* study indicated successful transmigration of AZTTP in trans-well culture model of BBB and also showed therapeutic efficacy against virus[6]. In the present *in vivo* study, we hypothesize that this magnetically guided nanoformulation can transverse the BBB by direct transport or via monocyte-mediated transport. Magnetic AZTTP liposomes were prepared using a mixture of phosphatidyl choline and cholesterol. The average size of prepared liposomes was about 150 nm with maximum drug and magnetite loading efficiency of 54.5% and 45.3%, respectively. Further, magnetic AZTTP liposomes were checked for transmigration across an mouse BBB model using direct or monocyte-mediated transport by application of an external magnetic field. The results show that apparent permeability of magnetic AZTTP liposomes was 3-fold higher than free AZTTP. Thus, we anticipate that the developed magnetic nanoformulation can be used for targeting active nucleotide analog reverse transcriptase inhibitors to the brain by application of an external magnetic force and thereby eliminate the brain HIV reservoir and help to treat neuroAIDS.

METHODS

Preparation of nanoformulation

Magnetic nanoparticle synthesis and AZTTP binding to nanoparticles were performed according to the procedure we previously described[6].In our previous study we determined the most optimal reaction conditions for production of MP-AZTTP. A reaction time of 2 hour with a ratio of 0.2 mg AZTTP to 3 mg magnetic nanoparticles (Fe_3O_4, magnetite) yields magnetic nanoparticles with 35 µg of AZTTP per mg of magnetic nanoparticles. Magnetic liposomes were prepared by reverse-phase evaporation method according to the procedure described earlier. L-α-Soya phosphatidyl choline (PC) and cholesterol (CHOL) were obtained from Sigma (St. Louis, MO). In a typical optimized procedure, PC and CHOL (1.2:1 mole ratio) were dissolved in chloroform, followed by evaporation of a solvent in a rotary evaporator resulting in formation of dry lipid film. After completely removing all solvent traces, the lipid film was rehydrated under constant shaking with 1 mL of 0.9% saline containing 3 mg of MP-AZTTP nanoparticles. This resulted in encapsulation of MP-AZTTP into the liposomes. The un-encapsulated magnetic nanoparticles were removed by magnetic decantation using a weak magnet. Fluorescent rhodamine labeled magnetic liposomes (Rho magnetic liposomes) were prepared by incorporation of 0.8 % of 1,2-distearoyl-sn-glycero-3-phosphoethnolamine-Ncarboxylfluorescein (CFPE, Cat#: 810332), Ex: 485/20nm, Em: 528/20nm during the formation of the lipid film.

Drug dissolution study of MP-AZTTP compare to free drug

Polymeric formulations can release the antiretroviral drugs in biological environment (*in vitro*). The drug release from the formulation depends on the structure of the drug and block copolymer as well as their ratio (drug loading). Therefore, by adjusting the copolymer structure and drug loading, we would be able to optimize the release profiles. The drug released from the nanoparticle was determined in PBS under sink condition using equilibrium dialysis. Dispersion of the nanoparticle in PBS (pH 7.4) was placed in the dialysis bag (Pur-A-Lyzer Maxi Dialysis Kit, SIGMA) and dialyzed against the respective buffer solution at 37˚ C. The released drug outside of dialysis bag were sampled at different time intervals (from 5min up to 14 days) and measured by high performance liquid chromatography (HPLC). Results were expressed as % of drug released from the pluronics relatively to the initial drug loading. Compared to free drug, the polymeric nanodrug showed significant sustained drug release character up to 14 days indicating better pharmacokinetics property *in vitro*. (in progress)

Time dependent MP-AZTTP drug delivery in mice

The fluorescent MP-AZTTP were injected (5 mg/kg) in normal BLAB/cJ mice through I.V injection. Mice were anesthetized after injection and put in the 0.2 Tesla (T) magnetic force for 30min -2hrs (Fig-2). At the end of incubation, moue is sacrificed and the brain tissue were used for immunohistochemistry and fluorescent detection of the particle.

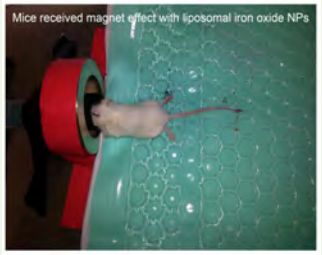


Fig.-1 Mice administered fluorescent MP-AZTTP with magnetic force of 0.2T.

Toxicological analysis of MP-AZTTP

Pathological analysis was done on tissue obtained from mice (Brian, liver and spleen) . Three groups of mice (control, MP-AZTTP treated, MP-AZTTP treated with magnetic force) were i.v injected with same amount of nanoformulation and PBS for control. Tissues were harvested after two weeks of injection, fixed in 4% formalin , embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E stain). Representative H&E staining images obtained form Leica Imager, M2 were presented in Fig.- 4.

Statistical analysis

Binding efficacy of MP-AZTTP was performed in replicate and repeated at least six times. Drug loading capacity was measured and expressed in µg drug/µmol polymer. Drug release study was performed separately for each batch of the formulation. The statistical analysis was done by two-tailed paired t-test. P values of ≤ 0.05 was considered as significant. The statistical analysis was done with GraphPad Prism software (GraphPad Prism software Inc. San Diego,CA).

RESULTS

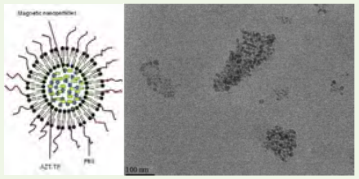


Fig.-2. Schematic representation and TEM image of nanodrug MP-AZTTP

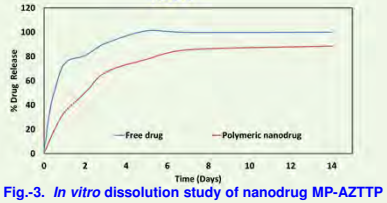


Fig.-3. *In vitro* dissolution study of nanodrug MP-AZTTP

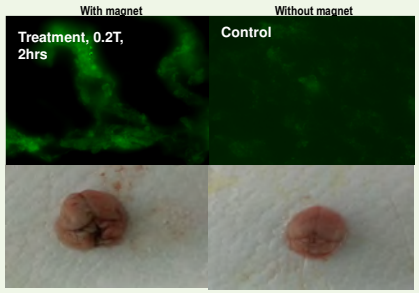


Fig.- 4. Immunohistochemistry of the brain after administration of MP-AZTTP. (Fluorescence detection)

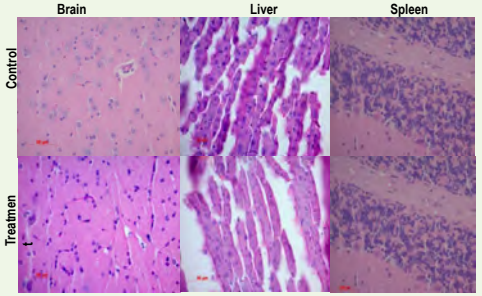


Fig.-5. Immunohistochemical analysis of the brain, liver and spleen in control and treated mice.

SUMMARY AND CONCLUSIONS

➢Preliminary characterization of the particle indicated that MP-AZTTP are of 118±15.2 nm size with 0.15 PDI and zeta potential of the particles was 10.3 ± 2.2 mV (Fig-2).

➢The new MP-AZTTP formulation was further characterized *in vitro* in order to obtain the integrity of the formulation. A drug dissolution study was performed with MP-AZTTP compare to free drug (AZT). The study indicated that there was sustained release of drug from MP-AZTTP formulation from 30min up to 2days compare to free drug (Fig-3) .

➢The representative images showed the significant intensity of fluorescence from i.v. injection compare to control (mice received fluorescent particle but no magnetic force). As the nanodrug were tagged with fluorescent, detection of fluorescence in the brain tissue section indicated targeted drug delivery of the tested formulation in the brain reservoir (Fig.-4) .

➢The data also indicated the gradual optimization and standardization of time of targeted delivery and strength of magnet required for this MP-AZTTP to obtained the drug delivery at the brain. Thus, this is the first time we are reporting of nanodrug delivery within 2hrs of injection with 0.2T magnet.

➢A dose dependent study was also performed with different concentration (0.5 through 8mg/kg) of MP-AZTTP in mice with 2hrs incubation in 0.2T magnet (Fig.-1). It was observed that 5mg/kg was optimum concentration for mice for potential drug delivery in the brain (data not shown) .

➢There was no apparent pathological change were observed in brain, liver and spleen tissues obtained form mice receiving MP-AZTTP and control. The immunohistochemical analysis of the tissues did not show any significant inflammation and cytotoxicity related pathology in those tissues (Fig.-5).

DISCUSSION

Formulation development and testing is an iterative process involving repetitive attempts to improve different parameters to obtain an adequate nanodrug. *In vitro* testing of the formulation majorly depends on the uptake, release, biological stability, transmigration and non-toxicity 2hrs. More over, the nanodrug has not shown any cytotoxicity in our histochemical analysis of the mouse tissues treated with drug. The serum cytotoxicity analysis is in progress. This study will definitely help us to close the gap between limitation and perfection of this nanodrug. Based on the result, similar therapeutic approaches can be investigated towards other HIV reservoirs. to cells. Based on these results, successive reformulations with different HAART drugs combination were made in response to the results of the biological assays. The selection of the formulation were depend on its biological efficacy against HIV and cytotoxicity. In the past few years our laboratory has employed range of methods to fabricate the nanoformulations that are more target oriented. In the present study, we have found that MNP based nanodrug is very stable and good for long term study. The drug dissolution study up to two days also showed a sustained release of drug from nanoformulation compare to free drug. This nandrug also has ability to target delivery to the brain.

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