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Novel nanoformulation to mitigate co-effects of drugs of abuse and HIV-1 infection: Towards the treatment of NeuroAIDS

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Abstract

Drug abuse (e.g. Methamphetamine-Meth or Cocaine-Coc) is one of the major risk factors for becoming infected with HIV-1 and studies show that in combination, drug abuse and HIV-1 lead to significantly greater damage to CNS. To overcome these issues, we have developed a novel nanoformulation (NF) for drug-abusing population infected with HIV-1. In this work, a novel approach was developed for the co-encapsulation of Nelfinavir (Nel) and Rimcazone (Rico) using layer-by-layer (LbL) assembled magnetic nanoformulation for the cure of neuroAIDS. Developed NF was evaluated for BBB transmigration, cell uptake, cytotoxicity and efficacy (p24 assay) in HIV-1 infected primary astrocyte (HA) in presence or absence of Coc and Meth. Developed magnetic nanoformulation (NF) fabricated using the LbL approach exhibited higher amounts of drug loading (Nel) and (Rico) with 100% release of both the therapeutic agents in a sustained manner for 8 days. NF efficacy studies indicated a dose-dependent decrease in p24 levels in HIV-1 infected HA (~55 %) compared to Coc+Meth treated (~50 %). The results showed that Rico significantly subdued the effect of drugs of abuse on HIV infectivity. NF successfully transmigrated (38.8±6.5 %) across *in-vitro* BBB model on the application of an external magnetic field and showed >90% of cell viability with efficient cell uptake. In conclusion, our proof of concept study revealed that sustained and concurrent release of sigma σ 1 antagonist and antiHIV drug from the developed novel sustained release NF can overcome the exacerbated effects of drugs of abuse in HIV infection and may solve the issue of medication adherence in the drug-abusing HIV-1 infected population.

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Keywords

NeuroAIDS; Drugs of abuse; Magnetic nanoparticle; Sustain release formulation; Blood-Brain-Barrier (BBB)

1. INTRODUCTION

HIV-1 can enter cells of the central nervous system (CNS) and cause progressive combined cognitive and motor impairment in infected individuals. Macrophage and microglial cells are the main sources of HIV-1 infection in the CNS though astrocytes are the most abundant kind of cells in the CNS and connect the cells of the brain to a compound intercellular complex. Since astrocytes are important cells for CNS function, their role in the context of HIV-1 neuropathogenesis should be studied (Díaz, et al., 2015). HIV infection and neuroinflammatory responses lead to the development of various neurological disorders collectively termed as HIV associated neurocognitive disorders (HAND) (Díaz, et al., 2015; Lindl, Marks, Kolson, & Jordan-Sciutto, 2010; Nair, Jayant, Kaushik, & Sagar, 2016). While the use of Highly Active Antiretroviral Therapy (HAART) has significantly reduced HIV-1 associated dementia (the most severe form of HAND), the milder forms of HAND continue to be a major health concern (Ances & Clifford, 2008; Joska, Gouse, Paul, Stein, & Flisher, 2010; Kaushik, Vabbina, et al., 2016). In addition, drugs of abuse promotes the virus replication promotion and have deleterious effects on CNS and its environment (Buch, et al., 2012; Lucas, Cheever, Chaisson, & Moore, 2001). In the USA, psychostimulants such as Cocaine (Coc), Methamphetamine (Meth), and other illicit drugs are highly abused among the HIV-infected population. Studies have demonstrated that Coc and/or Meth ingestion results in the acceleration of HIV infection and progression towards NeuroAIDS (Ciccarone, 2011; Ferris, Mactutus, & Booze, 2008; Gannon, Reichard, & Fantegrossi, 2014). CNS cells exposed to drug abuse, release abnormal levels of pro-inflammatory mediators e.g. cytokines and chemokines, resulting in neuroinflammation (Borgmann & Ghorpade, 2015; Ramesh, MacLean, & Philipp, 2013). It also affects permeability of the BBB that promotes enhanced transmigration of HIV-infected leukocytes/monocytes into the CNS (V. S. R. Atluri, et al., 2015; Ramirez, et al., 2009).

Despite the need, there is no FDA-approved pharmacotherapy against psychostimulant dependence in HIV patients that can be employed to target polydrug abuse (Stoops & Rush, 2013). Sigma-1 ($\sigma 1$) receptors have been well documented as a protein's target for Coc involved in the toxicity and stimulant actions of Coc (Narayanan, Mesangeau, H Poupaert, & R McCurdy, 2011). Already, it has been shown that sigma ($\sigma 1$) receptor antagonists can attenuate or block the behavioral sensitization caused by Meth in rat models (Maurice & Su, 2009). The increased activation of dopamine receptors is accountable for most of the neurotoxic effects of Meth, including psychomotor stimulation and addiction. Therefore, a single antagonist that can block or mitigate the effects of drug abuse (e.g. Meth and Coc) can act as a promising therapeutic agent for polydrug users. As per previous studies, Rimcazole dihydrochloride (Rico) acts as a potent antagonist of $\sigma 1$ receptors in a dose-dependent manner. It shows good affinity for the dopamine transporters and has capability to inhibit dopamine uptake, therefore it may also act as a Meth antagonist. Considering the dual

antagonistic property, we decided to test the efficacy of Rico and co-deliver it with antiretroviral drug (Nelfinavir, a protease inhibitor), across the BBB into the CNS to control HIV infection, mitigate the stimulatory and neurotoxic effects of drug abuse.

Due to the selective semi-permeability of the BBB, desired therapeutic concentrations of the antiretroviral (ARV) drugs and potent drug abuse antagonist have negligible ability to reach the CNS, leading to efficacy issues, high dosing frequency, and regimen adherence leading to neurocognitive issues (Ingersoll & Cohen, 2008). Our group has already demonstrated that magnetic nanocarriers loaded with anti-HIV drugs can be facilitated across the BBB upon applying non-invasive external static magnetic field, without hampering the integrity of the BBB (V. S. R. Atluri, et al., 2016; Ding, et al., 2014; R. Jayant & Nair, 2016; R. D. Jayant, et al., 2015; Kaushik, Jayant, & Nair, 2016; Kaushik, Jayant, Nikkhah-Moshaie, et al., 2016; Kaushik, Jayant, Sagar, & Nair, 2014; Nair, et al., 2013; Saiyed, Gandhi, & Nair, 2009; Tomitaka, et al., 2017). Even though the MNPs have large surface areas, loading higher amounts of different classes of drugs onto a single nanoparticle is still a daunting task. To overcome the challenge of multi-drug loading on a single nanoparticle's surface, we have explored layer-by-layer (LbL) self-assembly technique (Decher, 1997; Hammond, 2012). Based on our previous success in LbL-based NF development as demonstrated in our previous work (R. Jayant, McShane, & Srivastava, 2009; R. Jayant & Srivastava, 2007; R. D. Jayant & Madhavan, 2016; R. D. Jayant, McShane, & Srivastava, 2011), we have developed a novel nanoengineered sustained release magnetic NF to deliver Nel and Rico across the BBB under the influence of an externally applied non-invasive static magnetic field. The rationale of developing this multi-therapeutic nanoformulation is to increase the adherence of medication to HIV-1 infected drug abusing population, which is a major concern for the current HIV-1 therapy. Therefore, we expect that by formulating such sustained release nanoformulation can serve as platform technology for more effective HIV therapy and may be useful for the treatment of neuroAIDS in drug-abusing HIV patients.

2. MATERIALS AND METHODS

2.1 Cell culture and reagents

Primary human astrocytes (HAs) were procured from ScienCell Research laboratories (Carlsbad, CA; Cat. # 1800-5) and grown in astrocyte medium (ScienCell laboratories, Cat. #1801) containing 2% fetal bovine serum (ScienCell Cat. #0010), 1% astrocyte growth supplement (ScienCell Cat. #1852), and penicillin/streptomycin (ScienCell Cat. #0503). HIV-1_{Ba-L} (clade B) (NIH AIDS Reagent Program Cat. # 510) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

2.2 HIV-1 infection of HA

A standard lab protocol was used for infecting the HA cells with HIV with minor modifications (V. S. Atluri, Kanthikeel, Reddy, Yndart, & Nair, 2013). Briefly, on 4th passage HAs (1×10^5 cells) cells were grown overnight in 6-well plates in Minimum Essential Media (MEM); Polybrene (10 µg/ml) treatment for 6 hrs before the infection was done to activate the cells. Optimized dose (20 ng) of HIV-1 clade B virus was used to infect the cells overnight and then the cells were washed with phosphate-buffered saline (PBS) to

remove the unbound virus, fresh media was added and cells were further incubated for total of 5 days.

2.3 Coc and Meth dose optimization

To study the effects of drugs of abuse (Meth and Coc) on HIV infectivity and the antagonist activity of Rico, dose optimization studies were performed with HAs cells. HIV-infected (5 days post infected, DPI, control) HA cells were treated with different concentrations of Meth (10, 25, 50, 100 and 250 μ M) and Coc (0.1, 1, 2.5, 5 and 10 μ M). On the 6th day, the HIV infected cells were treated with drugs (Coc, Meth or Rico alone or in combination) and incubated for 48 hrs. The culture supernatant was collected after 48 hrs and the p24 antigen was estimated using an ELISA kit (ZeptoMetrix Corp. Cat # 0801200). Non-HIV infected cells were used as control samples for all experiments. MTT assay (details are given in section 2.12 below) was performed to check the viability of the cells after drug treatment, which helped in determining the optimal dose with higher infectivity and less toxicity.

2.4 Antagonist (Rico) to drug of abuse (Meth and Coc) ratio optimization

Sigma receptors are known to be involved in the toxic effects induced by drugs of abuse such as Coc and Meth (with moderate affinity). Different concentrations of Rico were added to HIV-infected HAs 1hr prior to Coc and Meth treatment to determine the effective concentration of Rico to block/attenuate the activity of Coc and/or Meth by binding to the sigma-1 receptor. We have used various concentration ratios of Rico and Coc and/or Meth (1:1, 1:2, 1:3 and 1:4) for the purpose. To see the activity of Rico against Coc- and/or Meth treated HIV infected cells, p24 antigen production was measured in these cells using the p24 antigen ELISA kit (ZeptoMetrix Corp. Cat # 0801200).

2.5 Flow cytometry for the quantification of sigma-1 receptor expression

To see the effects of Coc and Meth exposure on the expression profile of sigma-1 receptors on CNS cells, HA cells were treated with optimal doses of Coc and Meth for 48 hrs and then cells were washed with PBS and harvested. Cells were counted and aliquoted (1×10^6 cells) in polystyrene falcon conical tubes (12 $\times$ 75 mm; BD Biosciences, catalog # 352058). Cells were blocked with heat-inactivated human serum and incubated on ice for 10 minutes. Subsequently, the cells were spun down (2000 rpm for 5 mins) and the supernatant was discarded. Cells were incubated in the dark with anti-sigma-1 antibody (1:500 dilution, Cat # PAS-30372, Thermo Fisher, USA) on ice for 20 mins, followed by washing with wash buffer twice and fixed with 4% paraformaldehyde. Cells that were positive for the sigma-1 receptor were analyzed using BD Accuri flow cytometer (Ann Arbor, MI). A total of 10,000 events were collected for each treatment sample and the results were analyzed using the FlowJo software (Tree Star, Inc.; Ashland, OR).

2.6 Synthesis of MNPs, characterization and drug binding studies

The ultra-small MNPs (Fe_3O_4) were synthesized using the co-precipitation method in our previously published protocol (R. D. Jayant, et al., 2015). Nanoparticles size was determined using Transmission Electron Microscopy (TEM, Phillips CM-200 200 kV at 80 kV). Briefly, a small drop of highly diluted MNPs solution was spread on a carbon support film (400

mesh Cu grids Type B, Ted Pella, Inc., USA) and used for analysis. Dynamic light scattering (DLS) (Zetasizer NanoZS, Malvern) was carried out at 25°C to measure the size distribution, hydrodynamic radius, and surface charge measurements of the MNPs. For the drug binding studies, 1 mg of MNPs were added to 1mg/mL solution of Nel [protease inhibitors (PIs)] in PBS buffer (pH 7.4) and amount or % binding of drugs to the MNPs was determined by calculating the concentration of Nel in the unbound fractions (supernatant) of the mixture by UV spectrophotometry at 260 nm at different time intervals at RT. A similar binding protocol was performed to calculate Rico % binding at UV absorbance of 240 nm.

2.7 Layer-by-layer (LbL) assembly on MNPs

Loading of both the therapeutic agents (Nel and Rico) on to the magnetic nanoparticle were completed using previously established LbL protocol (R. D. Jayant, et al., 2015). The polyelectrolyte used for the experiment was Dextran Sulphate sodium (DS, Polycation: 2mg/ml in 0.15M NaCl, Mol wt-500,000, Sigma Aldrich), Nel (Polyanion: 1mg/ml in PBS, Mol. Wt. 287.213g/mol) and Rico (in PBS, Mol. Wt. 394.38). Briefly, a DS solution (2 mg/ml) was dissolved in 0.15 M NaCl at pH-7.4. For 1st layer coating, 100µl (1mg/ml) of DS solution was added to 1mg of the Nel-loaded MNPs and were kept on a vertical rotator under constant rotation (200 rpm) for 20 minutes. To separate out the unbound DS, nanoparticles solution was washed 3 times with Milli-Q water using centrifugation at 2500 rpm for 5 mins. Successively, 100µl (1mg/ml) of negatively charged drug (i.e. Nel or Rico, polyanions solution) was added to form a second layer of drug on to the magnetic nanocarriers and again kept on vertical rotator under constant rotation (200 rpm) for 20 minutes. The steps mentioned above were repeated twice to have two bilayer coatings of drugs on MNPs surface. The surface charge of the nanoformulation was measured using the zeta potential analyzer after rinsing and prior to depositing the second DS layer. The final assembled magnetic nanoformulation (NF) was washed *via* distilled water by centrifugation and used for *in vitro* release and other efficacy related studies.

2.8 In-vitro drug release study

In vitro drug release studies of finally assembled NF were performed using a micro Float-A-Lyzer dialysis device (MWCO-100 KD) containing 50 mL of 0.01M PBS buffer (pH 7.4) at 37°C. At predetermined time intervals, 500 µL samples of released drug were collected and substituted with a fresh PBS solution every time to maintain the sink condition. The % cumulative release of each drug was determined *via* UV spectrophotometry at λ_{max} of 260 nm and 240 nm for Nel and Rico, respectively.

2.9 Intracellular NF uptake studies

Primary human astrocytes (HAs) (1×10^6 cells) were seeded on cover slips in six-well plates for 24 hrs. After incubation, equal amount of NF cells added to cells and treated for 3 hrs in the presence or absence of external magnetic force. After rinsing the cells with PBS (pH 7.4), they were fixed in 4% paraformaldehyde for 30 min at RT. Coverslips were mounted on the slides with Prolong®Gold Antifade Reagent (Invitrogen, USA) and fluorescent intensity was analyzed with Confocal Laser Scanning Microscope (Leica Microsystems, Germany) at excitation 488–543 nm and emission 560 nm using 20× objective as previously described by us (V. S. Atluri, et al., 2013).

2.10 *In-vitro* blood-brain barrier (BBB) preparation and NF transmigration studies

Primary human brain microvascular endothelial cells (HBMECs, ScienCell, Cat # 1000) and primary human HA (ScienCell, Cat # 1800) cells were cultured as per the provider's protocol. The *in-vitro* BBB model was established as described earlier by Persidsky et al (Persidsky, et al., 1997) and modified by us. In brief, the *in-vitro* BBB model was established using bi-compartmental transwell culture plate (Cat# 3415, Corning Life Sciences, USA). In a sterile 24 well cell culture plate and transwell were coated with PLL (1µg/mL), with a pore density of 2×10^6 pores/cm² and a cell growth area of 0.33 cm², 2×10^5 HBMECs and HAs were grown to confluence on the upper chamber and underside of the lower chamber, respectively. After the transmigration assay was completed, the paracellular transport of FITC-dextran (average MW. 70,000, Cat#46945, Sigma Aldrich, USA) was measured in order to assess the effects of NF on the integrity of the *in vitro* BBB model as previously described by us (V. S. Atluri, et al., 2013). Integrity of the BBB membrane was calculated *via* measuring TEER (transendothelial electrical resistance) using Millicell ERS microelectrodes system (Millipore). Transmigration studies with drug-loaded MNPs and control MNPs were conducted on the 5–7th day of the BBB culture, depending upon the achievement of ideal BBB integrity as established by TEER measurement. Samples (Plain MNPs or NF) were added to the apical chamber of transwell in presence or absence of magnet field (0.8 T) in incubator at 37°C. Magnet was placed below the trans-well and % transmigration of MNPs/NF was analyzed using an ammonium thiocyanate-based photometric assay.

2.11 *In vitro* efficacy evaluation of NF in the presence and absence of drugs of abuse

To confirm the inhibition effect of Rico on drugs of abuse (Coc and Meth) and to check the antiviral efficacy of NF against HIV infectivity, we conducted the p24 antigen assay using a standard protocol. HAs (10×10^6 cells) were infected with HIV-1Ba-L (NIH AIDS Research and Reference Reagent Program, # 510) overnight as described earlier (V. S. Atluri, et al., 2013), washed with PBS (pH 7.4), and further incubated with and without Coc+Meth treatment, plus NF treatment at a concentration of 100 µg/mL. The culture supernatants from days 1st, 2nd, 3rd, 5th, and 8th day after 5 DPI (total 14 of HIV infection) were quantified for p24 antigen (pg/ml) using p24 ELISA kit (ZeptoMetrix, NY).

2.12 *In vitro* cytotoxicity assay

NF cytotoxicity was evaluated using cell proliferation MTT assay kit (Catalog # G 3580, Promega, USA). Briefly, HAs 5×10^3 cells per well were seeded in 96-well plates and incubated for 24 hrs at 37°C. After incubation, old culture medium was replaced with 100 µl fresh medium and different concentrations of NF (50–250 µg/mL) were added and treatment was performed for 24 hrs and 48 hrs. After treatment, 20 µL of MTT solution (5mg/ml in PBS) was added into each treated well and incubated for 2 hrs at 37°C. Lastly, 100 µL of stop solution (20% SDS in 50% Dimethyl Formamide) was added into each treated well and absorbance was measured at 550 nm using microplate reader (Synergy HT, Multi-mode microplate reader, BioTek Instrument, Inc., Winooski, Vermont, USA)

2.13 Statistical analysis

Experiments were performed in three independent experiments with triplicates and the values obtained were averaged. All the results were expressed as mean $\pm$ standard error of the mean (SEM). Statistical analysis of two groups was performed by Student's t-test, while statistical analysis of more than two groups was analyzed using one-way ANOVA followed by Bonferroni's multiple comparison test. Differences were considered significant at $p < 0.05$. Data analysis was performed with GraphPad Prism software (La Jolla, CA).

3. RESULTS

3.1 HIV infectivity in HAs treated with Coc and/or Meth in combination with Rico

We studied the effects of exposure of Coc and Meth on HIV infectivity, HA cells were infected with HIV and treated with different concentrations of Coc and Meth. In the quantitative estimation of p24 antigen production, we observed a significant increase in HIV level in Coc (at 1 μ M concentration) and Meth (at 25 μ M) in HA cells. We determined that these concentrations were optimal concentrations and used them for further experiments (as shown in Fig. 1a & c). Additionally, we studied the cytotoxicity of various concentrations of Coc (0.1–10 μ M) and Meth (10–250 μ M). Results showed that Coc (up to 5 μ M) and Meth (up to 50 μ M) treatment (as shown in Fig. 1b & d, cell viability $\geq 80\%$) did not show any significant cytotoxicity. HAs cells were treated with the optimal concentrations of Coc (1 μ M) and Meth (25 μ M), and then Rico (the antagonist) was added in different ratios (1:1, 1:2, 1:3 and 1:4 ratios) to study its effect in reducing the exaggerating effect of drug of abuse on HIV infection. We observed significant reduction in HIV infectivity ($\sim 1.5 \pm 0.25$ fold) in Coc and Rico (up to 2 μ M) and Meth and Rico (up to 50 μ M) treated samples. We concluded that the optimal concentrations of Rico to inhibit the activity of Coc and Meth were 2 μ M and 50 μ M, respectively. We used these Rico concentrations to attenuate HIV infection levels alone and on co-exposure with Coc and Meth together. The 50 μ M Rico concentrations showed significant attenuation in p24 levels (Fig. 2-i). We did not find any significant cytotoxicity with the optimal concentrations of Coc (1 μ M) + Meth (25 μ M) co-exposed to HIV-infected HAs.

3.2 Expression of sigma-1 receptor expression in HA treated with Coc and/or Meth

Fluorescence-activated cell sorting (FACS) analysis was performed to assess σ -1 receptor expression upon treatment with the drugs of abuse. Coc and Meth treatment for 48 hrs showed 4.09% and 5.49% expression respectively, whereas the co-treatment of Coc and Meth resulted in a synergistic increase in the receptor expression (15%) as shown in Fig 2 ii.

3.3 Nanoformulation preparation, characterization, and drug release studies

LbL-assembled NF loaded with Nel and Rico was developed as illustrated in Fig. 3a. The MNPs were synthesized, characterized (size, shape, charge) and analyzed for drug binding efficacy. TEM morphological characterization showed a round structure with an average size of 10 ± 3 nm (Fig. 3b). Dynamic light scattering (DLS) analysis also confirmed that MNPs have a hydrodynamic size of 12 ± 5 nm, with excellent dispersion properties (polydispersity index > 0.18 in aqueous medium). The effects of drug loading and LbL deposition on

nanoformulation hydrodynamic diameter size was also evaluated using DLS. The loading efficiency of Nel ($31 \pm 2\%$) and Rico ($35 \pm 4\%$) were obtained in non-LbL MNPs formulation. Results showed that (Fig. 3c), in the case of Nel, application of two layers (2BL) of Dextran sulfate sodium leads to ~ 1.9 times ($30 \mu\text{g}$ to $58.5 \mu\text{g}/\text{mg}$ MNPs) higher drug loading compared to 1BL formulations. Similar results were obtained with Rico (2BL), as drug loading increased ~ 1.4 times ($32 \mu\text{g}$ to $47 \mu\text{g}$). LbL-deposition was established using zeta (ζ) potential analysis; results showed that the ζ -potential value for drug MNPs loaded with Nel was 16.5 ± 1.5 mV, charge reversed upon adsorption of drug DS coating to -22.3 ± 1.5 mV. Continuous charge reversal occurred upon consequent layer deposition (Fig. 3d), thus confirming that layer deposition occurred as anticipated. *In-vitro* drug release studies show that uncoated MNPs released 100% Rico and Nel in 5 hrs and 6 hrs respectively (as shown in Fig. 3e). Results proved that application of LbL-assembly (1BL & 2BL) on MNPs increased the drug release duration from 120 hrs to 192 hrs for Nelfinavir and from 108 hrs to 180 hrs for Rico (Fig. 3f).

3.4 *In vitro* BBB development and NF transmigration studies

Transmigration studies of samples (Plain MNPs and NF) were performed using developed *in-vitro* BBB model. The BBB formation and its integrity were confirmed using TEER values calculation. After achieving desired (300 ± 10 ohms/cm²) TEER values, plain MNPs and NF were added to different BBB transwell under ± 0.8 T magnetic field as shown in Table.1.

Results showed that there was no significant difference in TEER values, with respect to drug loaded MNPs NF when compared to plain (uncoated and unloaded) MNPs (Table-1). Effect of external magnetic field on NF BBB transmigration, was verified by measuring the amount of MNP transmigrate across the BBB membrane in basolateral chamber. Fig 4a showed that $40 \pm 4.5\%$ of plain MNPs and $38.8 \pm 6.5\%$ of NF crosses the BBB in the presence of magnetic fields after 2 hrs. In absence of external magnetic force, only $3.0 \pm 1.5\%$ plain MNPs or NF were able to cross the BBB after 2 hrs of treatment. Thus showing that the application of an external magnetic field can help deliver the NF across the BBB. To further evaluate the integrity of the BBB after NF transmigration under magnetic field, paracellular transport of FITC-dextran experiment was performed. Results showed that $8.5 \pm 3.5\%$ FITC molecules transmigrated (apical to basolateral chamber) in NF treated well compared to $\sim 100\%$ negative control (without any BBB cells) as shown in Fig. 4b. Thus, proves that transmigration of NF under an external magnetic field does not affect BBB integrity. Additionally, the effects of longer treatments of magnetic field for higher NF transport across the BBB was also studied and results showed that longer magnetic treatment period (up to 6 hrs, using 0.8 T) does not lead to higher transmigration (Fig. 4c).

3.5 *In vitro* antiviral efficacy of NF in the presence of drugs of abuse, cell uptake and cytotoxicity studies

To overcome the exacerbated effects of drugs of abuse in HIV infection, NF efficacy was determined *via* quantification of p24 antigen level in infected HA cells. HAs were infected with HIV for 5 days, further same cells were treated everyday with optimized doses of Coc (1uM) and Meth (25 uM) from 6th day to 13th day (Total NF treatment time was 8 days). NF

was added (100 µg/mL) on 5 day after infection (DAI) to HIV infected cells in presence or absence of Meth or Coc or Meth+Coc and the supernatant was collected on 6th, 7th, 8th, 10th and 13th days of treatment (8 days of NF treatment) to check the antiviral inhibitory efficacy of the NF. ELISA p24 assay results (Fig. 5-A) showed a significant increase in the p24 viral protein production when compared with HIV-infected control cells over the entire treatment period of 8 days. Results showed that Rico release, along with Nel from the NF, attenuates the additive viral activity of HIV in the presence of drugs of abuse (Coc+Meth). The sustained-release ability of the NF was evaluated over a treatment period of 8 day. Result showed ~ 50 % (550–650 pg/mL to 210–250 pg/mL) reduction in viral load over a period of 8 days after NF treatment. Thus, confirms NF capability to attenuate the drug abuse effect and suppress the viral replication in HIV infection.

Further, to study the qualitative NF cell uptake, different concentrations (50–200 µg/ml) of NF were tested in HAs cell over a period of 24 hrs. Results (Fig. 5-B) showed representative confocal image of NF (100µg/ mL), additionally flow cytometry studies were performed for quantitative analysis, result showed that maximum cell uptake took place within 6 hrs and longer incubation time (24 hrs) did not results in higher cell uptake (percentage of cells positive for FITC-tagged NF, data not showed). Consequently, we also evaluated the cytotoxicity of the developed NF using the MTT cytotoxicity assay on HAs cells using different concentrations for 24 and 48 hrs treatment period. Results (Fig. 5-C) showed that none of the tested NF (10, 20, 50 and 100 µg/ml) showed any significant cytotoxicity at cellular and morphological levels and the percentage of viable cells were comparable to the control group (untreated cells), thus, suggesting that developed NF is nontoxic in nature and is safe to be used for *in-vivo* applications.

4. DISCUSSION

Abusing drugs is very common phenomenon amongst HIV-positive individuals, as nearly as 50% of PLWH/A are reported to have histories of drug and/or alcohol disorders (Bing, et al., 2001; Rabkin, McElhiney, & Ferrando, 2004). Substance abuse is allied with marked deterioration in key health behaviors and consequences include non-adherence, immunosuppression, augmented sexual behaviors, and augmented burdens on health care (Conigliaro, et al., 2004; Kelley & Petry, 2000; Masson, Sorensen, Phibbs, & Okin, 2004). It's known that HIV seropositive drug users have greater age-matched morbidity and mortality when equated to non-drug using HIV positive patients (Durvasula & Miller, 2014). Substance abuse among PLWH/A has been associated with nonadherence to HAART. Alcohol and drug abuse disorders are consistently linked with poor HAART adherence and poor immunologic and virologic consequences (Binford, Kahana, & Altice, 2012).

HAART has considerably enhanced the prognosis of HIV-1 patients and reduced the prevalence of HAD and its associated asymptomatic neurocognitive impairment. In addition to HAART, the pursuit for effective neuroprotective agents or anti-drugs of abuse agents is critical to combat with the additive effects on HIV progression. Sigma-1 receptors are extensively dispersed in memory-associated cortical and motor-related brainstem areas (hypothalamic nuclei, dentate gyrus of the hippocampus, and facial nucleus), which are the same sites for HAND or HIV/gp120-induced neuronal damage (Samikkannu, et al., 2014;

Zhang, et al., 2012). Developing approaches that will eliminate or reduce the effects of individual adherence could upsurge the effectiveness of treatment strategies against HIV transmission. Considering these possibilities, our work involves testing the hypothesis of concurrently delivering of sigma receptor antagonist (Rico) and an antiretroviral drug (Nel) into CNS, to simultaneously provide neuroprotection in neuropathy and neurotoxicity induced by HIV viral proteins (gp120 or Tat) in presence of drugs of abuse (Meth and Coc), with the ultimate purpose of decreasing HIV viral load from the brain.

In this study, an effective dose of Meth and Coc was optimized to study their effects on HIV infectivity and viral replication. The ratio of Rico (the antagonist) to attenuate the effects of Meth and Coc inhibition in the presence of HIV was optimized. Receptor expression results showed that co-treatment with Coc and Meth lead to higher expressions of sigma receptor. Ultra-small MNPs were synthesized by the co-precipitation method and NF was evaluated for % drug-loading, *in-vitro* release study, cytotoxicity, and *in-vitro* antiviral efficacy in the presence or absence of drugs of abuse in HA cells. Surface charge distribution of synthesized MNPs and developed NF was determined by measuring the ζ (zeta) potential. Results showed that the ζ values reversed on coating on addition of alternately charged moieties (Nel/DS coating + Rico/DS coating) on each layer deposition, thus demonstrating that multilayer build-up is achieved and drugs are loaded in the desired manner. Electrostatic interactions between magnetic nanoparticles and polyelectrolytes are key factor for the multilayer deposition. Due to high drug loading and LbL coating barrier, we expected that the drugs would release in a slow and sustained manner over a period of 7–10 days. Our results showed that 2BL NF releases both of the drugs over a period of 8 days compared, to 5–6 hrs on non-LbL NF. Thus, proves that LbL technique helps in achieving fine tuning of desired release rate of drugs and also helpful in reducing the initial burst release of drugs without affecting the release kinetics.

Further, cytotoxicity and cell uptake studies were done to assess the *in-vitro* biocompatibility of NF in HA cells. Different cytotoxicity parameters (e.g. cell viability, adhesion, proliferation, morphology and cellular uptake) were analyzed. The results showed good adhesion, growth, and morphology of treated cell with > 90 % cell viability over a period of 48 hrs. Moreover, the effects of the magnetic force on BBB transmigration and its integrity was observed, and we observe no BBB damage and > 40% of the NF migrated across the BBB on magnetic treatment for 2 hrs. Results confirm that our developed NF are proficiently taken up by HA cells, hence providing a platform to effectively deliver any drug cargo of interest. Finally, we have shown that NF release can reduce HIV replication (p24 antigen level) in the presence and absence of drugs of abuse (Coc and Meth) in HIV-infected HA cells. The NF were able to control the HIV replication for 8 days in a continuous manner without showing any cytotoxic effects to the treated HA cells, and the results correlated well with the *in vitro* drug release period.

5. Conclusion

Our work demonstrates that concurrent delivery of Rico and Nel can be achieved through LbL assembly technique. We also showed the magnetically guided delivery of developed NF across the BBB with sustained release up to 8 days with synergistic effects of both drugs

(Rico and Nel) in mitigating the effects of drug abuse in HIV infection. We believe that combination therapy of ARV drugs (i.e., [NNRTI+NRTI+PI] or [NRTI+PI +Integrase inhibitor]) with latency reactivating agents (e.g. Bryostatin, Disulfiram, PMA, etc.) will certainly enhance the treatment efficacy and help in the eradication of neuroAIDS in drug-abusing patients. To bring this platform nanotechnology technique to clinical level, we still need to check the efficacy of the developed nanoformulation *in-vivo* studies. Developing latent humanized HIV-1 animal model still poses a big challenge, our lab is working towards this problem and has generated few models based on HIV-SCID mouse model. For our future work, we will test the magnetic based transport of NF across BBB, bio-distribution, PK-PD studies and therapeutic efficiency of the developed NF.

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Figure. 1.

Dose optimization studies in HA: (A & B) Different concentrations of Meth (10–250 μM) were tested to study the synergistic effect on HIV infection and its concentration dependent cytotoxicity profile; (C & D). Different concentrations of Coc (0.1–10 μM) were tested to study the synergistic effect on HIV infection and its concentration dependent cytotoxicity profile; (E & F) Rimcazole (Rico) dose optimization in presence of Coc and Meth for attenuating the synergistic effect on HIV infection. (G) Rimcazole concentration dependent cytotoxicity profile. HA (1×10^5 cells) were grown in 6 well culture plates and were infected with 20ng of HIV-1 clade B. After overnight infection, unbound virus was washed out with PBS and cells were infected for total of 5 days. After 5 DPI (day of post infection) Meth (10, 25, 250, 500, 1000 μM) and Coc (0.1, 1, 2.5, 5, 10 μM) were added and supernatant from each treated well was collected after 48 hour (Total 7 days of HIV infection). HIV infection levels were measured by p24 ELISA. (*, $p < 0.05$; **, $p < 0.01$; NS-Not Significant).

Figure 2.

(A) *In vitro* Rico efficacy studies for attenuating Coc and Meth induced HIV infection:

HA (1×10^5 cells) were grown in 6 well culture plates and were infected with 20ng of HIV-1B for overnight. Unbound virus was washed with PBS and cells were infected for 5 days. After 5 day of infection, HIV infected astrocytes were pretreated (1hour) with Rimcazone (2 and 50 μ M) and then treated with optimized dose of Meth (25 μ M) and Coc (1 μ M). To study the Rico attenuation capacity, supernatant from each treated well was collected after 24 hour of treatment and HIV infection levels were measured by p24 ELISA. (*, $p < 0.05$; **, $p < 0.01$; NS-Not Significant); **(B) Flow-cytometry analyses to assess**

expression of sigma (σ 1) receptors: Primary astrocytes were treated with either cocaine and/or meth for 48 hour. Following treatment, cells were collected and stained with FITC labeled sigma receptor. Accuri (BD Biosciences) was used for fluorescence acquisition, and data were analyzed with FlowJo software (BD Biosciences). Red line indicates the untreated control, and blue line indicates the treated cells (test). Untreated control was gated based on the isotype control, and the treated cells (test) were gated based on the untreated cells.

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Figure 4.

(A) NF transmigration analysis using *in-vitro* BBB model: NF was added in the upper chamber of the BBB model, after 3 hour of treatment migrated plain MNP and NF in presence or absence of static magnetic (0.8 T) were calculated for iron content in the lower chamber. Results are expressed as mean $\pm$ SE of three independent experiments. Statistical significance was determined using unpaired Student's t-test. **(B) BBB integrity using FITC-dextran transport model:** FITC-dextran transport was measured in BBB model after 3 hour of magnetic treatment, FITC-dextran was added on the upper chamber of the insert. After 3 hour of incubation, relative fluorescence units (RFUs) from the basal chambers of the inserts were measured. Results are expressed as % FITC-dextran transport with respect to the untreated control cultures (–ve control= no cell and +ve control= with BBB cells) and represented as mean $\pm$ SE of independent experiments. **(C) Effect of time with respect to Nanoformulation BBB transmigration:** NF was added in the upper chamber of the BBB model, after every 1hour till 6 hour of treatment migrated NF in presence of static magnetic (0.8 T) were calculated for iron content in the lower chamber. Results are expressed as mean $\pm$ SE of three independent experiments. Statistical significance was determined using unpaired Student's t-test.

Figure. 5.

(A) NF efficacy in HIV-1 infected HA in presence of drug of abuse (Meth and Coc): HA (1×10^5 cells) were grown in 6 well culture plates and cells were infected with 20 ng of HIV-1 clade B for overnight. Unbound virus was washed with PBS and cell were infected for 5 days. On 6th day of infection, optimized Coc (1 µM) and Meth (25 µM) were added to cells and treated every day for next 8 days (total 14 days of HIV infection). Drug loaded NF (100 µg/ml) was added only once (on 6th day) to the respective wells and reduction in HIV infection levels were measured by using the p24 ELISA. Results were analyzed with respect to HIV-1 infection v/s HIV+(Meth+Coc) and HIV+(Meth+Coc) v/s NF treatment (*, p 0.05; **, p 0.01; ***, p 0.001; ****, p 0.0001; NS-Not Significant); **(B) Qualitative analysis of cellular uptake of FITC-tagged LbL-NF in HA by fluorescence microscopy:** FITC-tagged LbL-NF (concentration- 100 µg/mL) in HA after 6 hour of treatment. (A) Control cell bright field image; (B) Cell nuclei stained with DAPI (blue); (C) FITC tagged

drug loaded NF (green); (D) Composite image show green and blue fluorescence inside the cells, confirms the cellular uptake of the NF. Images taken at 10× magnification using Fluorescence microscopy (Zeiss, Wetzlar, Germany); (C) ***In-vitro* cytotoxicity of NF:** Results show the percentage of cells viability after treatment with different NF concentrations (10, 20, 50 and 100 µg/mL). HA (1×10^5 cells) were grown in 6 well culture plates and cells were infected with 20 ng of HIV-1 for overnight. Uninfected virus was washed and cells were infected for 7 days. On 5th day of infection, NF was added at different concentrations to the respective wells and incubated for 24 and 48h respectively. After total 6th and 7th day of post infection (DPI), cell viability was measured by using the MTT assay (*, p < 0.05; NS-Not Significant);

Table 1

Transendothelial electrical resistance (TEER) values of the *in vitro* BBB model before and after treatment with NF in the presence and absence of external magnetic force (0.8 tesla)

TEER values (ohms/cm ²)	Untreated Well (Control)	Plain MNPs	Nanoformulation (NF)
Before treatment	308.2±9.7	308.4±7.1	304.3±8.6
After Magnetic treatment	302.2±8.5	301.2±10.5	306.2±12.5