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Surface modified nevirapine nanosuspensions for viral reservoir targeting: *In vitro* and *in vivo* evaluation

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ABSTRACT

Most of the time HIV virus escape immunological burden exerted by antiretroviral drugs and develops resistance against therapy. For complete eradication of virus from body one has to use long term chemotherapies, which results in drug toxicity, drug resistance and eventually poor patient compliance. Nevirapine (NNRTI, non nucleoside reverse transcriptase inhibitor) nanosuspensions were developed and surface modified with serum albumin, polysaccharide and polyethylene glycol to enhance its targeting potential. The biodistribution studies revealed improved antiretroviral drug accumulation in various organs of rat for nanosuspensions as compared to the plain drug solution when administered intravenously. Nanosuspension after surface modification showed further enhancement in accumulation. Higher MRT values of surface coated nanosuspension in brain, liver and spleen as compared to pure drug solution ensured enhanced bioavailability and prolonged residence of the drug at the target site.

1. Introduction

Nanocarriers, a delivery technology where the drug is encapsulated within a delivery system of <1000 nm in diameter, are being actively investigated for various disease conditions (Date et al., 2007; Kayser and Kiderlen, 2003). Nanoparticles are considered to have great potential for selective and controlled drug delivery of drugs to target cells and organs. The particle material, size and surface charge of tailor made nanocarriers can regulate biodistribution and target specific localization of nanosystems in the body (Blunk et al., 1993, 1996; Buckton, 1995; Davis et al., 1986). Furthermore, the speed of drug release from nanoparticles is also controlled by these factors. Specific engineering of nanosystems can promote transportation to across a variety of biological barriers including blood brain barrier (BBB) offering very interesting opportunities for delivery of antiretroviral drugs for HIV/AIDS to cellular reservoirs (Desormeaux and Bergeron, 2005; Govender et al., 2008; Ham et al., 2009; Mirchandani and Chien, 1993).

Strategies are currently being investigated to overcome limitations of presently available antiretroviral chemotherapy, which include the identification of new and chemical modification of existing chemical entities, the examination of various dosing regimens, as well as the design and development of novel drug delivery systems that can improve the efficacy of both existing and new antiretroviral drugs (ARVs). More specifically, in the past decade there has been an explosion of interest in the development of carrier systems for the incorporation of ARV drugs as a way of circumventing the problems described above and optimizing the treatment of HIV/AIDS patients (Desormeaux and Bergeron, 2005; Govender et al., 2008; Ham et al., 2009; Mirchandani and Chien, 1993; Schafer et al., 1992; Shah and Amiji, 2006; Vyas et al., 2006). As penetration of ARV drugs into the viral reservoir sites is restricted, a high dose has to be given with consequent intolerance (Amiji et al., 2006) and toxicity. In addition, many drugs cannot adequately reach or reside in these sites in sufficient concentrations and for the necessary duration to exert the therapeutic response (Vyas et al., 2006). Studies involving ARVs drug loaded nanoparticles for targeting to the macrophages have consequently emerged (Baert et al., 2009; van't Klooster et al., 2010).

This subsequent research paper focuses on formulation development of poorly soluble antiretroviral drug nevirapine rendering it as aqueous nanosuspensions (NVP-NS) by using high pressure homogenization technique. As a non nucleotide reverse transcriptase inhibitor, it is an ideal candidate for prophylactic use, as it interferes in viral replication cycle by inhibiting proviral DNA synthesis and ultimately disturbing an integration of viral genome in host's DNA. The surface of nanocrystals were modified by serum albumin, polyethylene glycol 1000 and polysaccharide. All nanosuspension formulations were evaluated *in vitro* and *in vivo*

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to study effect on surface modification. Uptake studies were carried out to evaluate their targeting potential. Toxicity studies were performed to evaluate their safety profile.

2. Materials and methods

2.1. Materials

Nevirapine (a non-nucleoside reverse transcriptase inhibitor, NNRTI was procured as a gift sample from Alkem Laboratories, Mumbai, India. Stabilizers like Poloxamer 188 (BASF GmbH, Ludwigshafen, Germany), Tween 80 (Uniquema, Everberg, Belgium), Plasdone (International Specialty Products, Mumbai, India) Volpo L4 (Croda GmbH, Nettetal, Germany) and polyvinyl pyrollidone (K-25, Signet Chemical Corporation, Mumbai, India) were received as gift samples. Dextan 60 was obtained as free sample from Pharmacosmos A/S, Holbaek, Denmark. Polyethylene glycol 1000, dimethyl sulfoxide (DMSO), yellow MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, a tetrazole), formalin and bovine serum albumin (Merck, Mumbai, India). ^{99m}Tc was obtained by separation from parent ^{99}Mo which was procured from Board of Radiation and Isotope Technology (B.R.I.T.), Mumbai, India. Hanks buffered salt solution (HBSS), fluid thioglycollate medium, Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute medium (RPMI), Fetal Calf Serum (FCS) were purchased from Himedia, Mumbai, India. Citrated human plasma (German Red Cross, Germany), Sepharose 2B and Immobiline DryStrips (Amersham Pharmacia Biotech, Uppsala, Sweden). Acrylamide (Serva, Heidelberg, Germany), N,N,N0,N0-tetramethylethylenediamine, ammonium persulfate and piperazine diacrylamide (BioRad, Munich, Germany). The BCA reagent-kit (Pierce, Rockford, USA).

2.2. Methods

2.2.1. Preparation of bare nanosuspensions

Aqueous nanosuspensions of nevirapine (NS) were produced using cold high pressure homogenization technique (Avestin C50, Canada) in continuous mode. In short, coarse nevirapine powder 2.0% (w/w) was dispersed in 2.8% (w/w) of surfactant solution Tween 80 (1%), VolpoL4 (0.9%), Plasdone (0.1%), Poloxamer 188 (0.5%), PVP (0.3%) and pre-mixing was performed using high shear mixing (Remi over head stirrer, 1200 rpm) followed by pre-mixing (Ultra Turrax T25, Janke and Kunkel GmbH, Germany) for 1 min at 9500 rpm. Coarse suspension was subjected to homogenization using Avestin C50 at high pressure of 25,000 psi for 30 min.

2.2.2. Surface modification of nanosuspensions

Prepared nanosuspensions were surface modified by simple physical adsorption method (Santander-Ortega et al., 2006). The surface modifiers like serum albumin, PEG 1000 and dextran 60 at 1% (w/w) concentration were allowed to adsorb on the surface of nanosuspensions at 37 ± 2 °C for 12 h to produce BNS, PNS and DNS, respectively. The prepared bare and surface modified nanosuspensions were evaluated for particle size and subjected to *in vivo* testing.

3. Characterization of bare and surface modified nanosuspensions

3.1. Photon correlation spectroscopy (PCS)

PCS was performed using the Beckman particle size analyser N5 (Beckman Inc., USA). The analysis yields the mean size of the sample, which is intensity weighted mean diameter of the bulk population and the polydispersity index (PI), which is a measure for the width of the size distribution. The nanosuspension samples were diluted in doubled distilled water and three measurements were performed at 20 °C under a fixed angle of 90° angle. Mean value of three measurements is reported.

3.2. Atomic force microscopy measurements (AFM)

AFM measurements were performed on freshly prepared nevirapine bare and surface modified nanosuspensions. Silanized mica was prepared by dropping 0.1% of 3-aminopropyltriethoxysilane (APTES) solution onto a mica surface. The experiments were conducted at room temperature using triangular silicon nitride tip (spring constant 30–80 N/m; resonance freq. ~340 kHz). The NS was diluted in water and droplets of 40 μl were deposited onto a small mica disk. The AFM images were captured using Veeco Digital Instruments Multimode Nanoscope IV in tapping mode.

3.3. Zeta potential measurements

The surface charge of the particles was assessed by zeta potential measurements using a Brookhaven Zeta PALS, BI-ZETAMAN (Ver.1). NVP-NS was diluted in Milli-Q water, and total ten different measurements were performed at room temperature and mean value is reported.

4. Cellular uptake studies

Phygocytic uptake studies were performed for bare and surface modified nanosuspensions at PERD Research Centre, Ahmedabad, India. The primary peritoneal macrophages elicited by injecting thioglycolate medium (intra-peritoneally, 10 ml) to Wistar rat (male, 10–15 week old). Three days later, 10 ml freshly prepared HBSS was injected to peritoneal cavity of rat and massaged for 3 min under anesthesia. HBSS was recovered from abdomen and subjected to centrifugation at 13,000 rpm at 4 °C to obtain cell concentrate. The confluent layer of primary macrophages (1×10^6 cells/ml) was allowed to form layer at 37 ± 2 °C and 5% CO_2 in sterilized culture plates containing glass cover slips in presence of RPMI 1640 medium supplemented with fetal bovine serum (10%). Formation of monolayer was monitored by examining under microscope. Formed confluent layer was washed twice using HBSS to remove nonadherent cells. After second wash, transport medium was exchanged with test nanosuspensions (250 μl). Phosphate buffer saline (pH 7.4) served as positive control. The culture plates were incubated in a controlled environment at a temperature of 37 ± 1 °C, phagocytosis was terminated by immersing the plate in an ice bath after 15, 30, 45, 60 and 120 min. At specified intervals, the cell monolayer was washed thrice using ice-cold phosphate-buffered saline [pH 7.4] to remove non-adherent particles and macrophages were separated from the medium by centrifuging at 2000 rpm for 15 min. The cells were lysed using hypotonic solution centrifuged again at 10,000 rpm for 15 min. The absorbance of collected supernatant and cell lysate was determined using UV Visible Double Beam spectrophotometer 2201 (Systronics, India) at 313 nm. The percent cellular uptake was calculated using following formula:

$$\text{Uptake efficiency}\,(\%) = \frac{W_{\text{sample}}}{W_{\text{total}}} \times 100$$

where W_{sample} is the amount of drug phagocytosed and W_{total} is the total amount of drug in the added to culture.