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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.

5. *In vitro* protein adsorption

The 2D-PAGE analysis has been performed in the first dimension using Multiphor II (GE Healthcare, Germany) on 18 cm IPG BlueStrips (Serva, Germany, pH gradient from 3 to 10) as discussed in our previous paper (Shegokar et al., 2011). In short, the second dimension, SDS–PAGE, was performed on the Protean II xi multi-cell (Bio-Rad laboratories GmbH, Germany). The crosslinked acrylamide gels were (linear gradient of 8–16%) used in this study. After the second dimension step, the gels were silver stained and scanned using a laser densitometer. Identification of the proteins was performed by comparing the spot location using reference available maps (ExPASy, Proteomics Server). This study helped us to predict *in vivo* fate of nanoparticle to some extent and select suitable formulation for animal study.

6. Cytotoxicity studies

Cytotoxicity check on nevirapine bare and surface modified nanosuspensions was carried out using MTT cytotoxicity assay in J774.A12 murine macrophage cell line (procured from National Centre for Cell Sciences, Pune, India). The J774 A-1 cell line derived murine macrophage cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 100 U of penicillin/ml and 100 μg of streptomycin/ml at 37 °C in humidified incubator with 5% CO_2 was used for determination of the cytotoxicity assay.

The drug concentrations selected for cytotoxicity studies was based on therapeutic plasma levels of nevirapine. The three concentrations were prepared for the study at half of therapeutic dose (1 μg/ml), therapeutic dose (2 μg/ml) and double the therapeutic dose (4 μg/ml). The J774.A12 macrophages (1×10^5) cultured in 96 flat well bottom culture plates containing (200 μl of DMEM) and incubated at 37 °C overnight. Next day, the cells washed and resuspended in RPMI (supplemented with 10% FCS, 100 U of penicillin/ml and 100 μg of streptomycin/ml) at 37 ± 2 °C in humidified incubator 5% CO_2 (Forma Scientific Inc., USA). After 12 h, the cells were washed with the DMEM thrice and a mixture of 80 μl of fresh DMEM and 20 μl of the respective test drug concentration was added. After 24 h treatment, 10 μl (2 mg/ml) MTT solution was added to culture plates and incubation was continued for next 6 h at 37 ± 2 °C. At end of 6 h, the MTT reaction was terminated by addition of acid propanol (1 M HCl:isopropanol in 1:24 ratio). At the end of incubation period, the medium was removed and the converted dye was solubilized in acidic propanol and measured spectrophotometrically at 570 nm. The percent cytotoxicity ($n = 3$) was calculated using following formula.

$$\%\,\text{Cytotoxicity} = \frac{100 - \text{PBS(Optical density)}}{\text{PBS(Optical density)} - \text{Test(Optical density)}} \times 100$$

7. Pharmacokinetic and biodistribution studies

7.1. Radiolabelling of nanosuspension

Bare and surface modified nevirapine nanosuspensions were labeled with ^{99m}Tc using stannous chloride ($SnCl_2$) as a reducing agent. $^{99m}TcO_4^-$ (~1.2 mCi) was mixed with 0.1 ml of stannous chloride solution in 0.1 N HCL and the pH of solution was adjusted to 6.9–7.1 by using Tris buffer. The nevirapine drug solution (NVP-DS) or nanosuspension (1 ml) was added to this mixture and incubated for 30 min at room temperature on shaker. Labeling efficiency was determined by thin layer chromatography (TLC) on silica gel coated fiber sheets (10 cm, Gelman Science Inc., USA) in acetone. As the free $^{99m}TcO_4^-$ moves with the solvent (Rf = 0.9) while the radiolabelled formulation remain at the point of application. Drop of the radiolabelled formulations was applied at one end of TLC strip and it was developed in acetone. The strip was removed from mobile phase when solvent front researched to 8 cm from the application point. The strip was then cut into two (1/3 (lower), 2/3 (upper)) halves and the radioactivity in each segment was determined in a well-type gamma-ray counter (gamma-ray scintillation counter, Type CRS 23C, Electronics Corporation of India Ltd., Mumbai, India). Labeling efficiency (%) was calculated using following equation:

$$\text{Radiolabelling efficiency}\,(\%) = \frac{\text{Count at lower end}}{\text{Count at upper end} + \text{lower end}} \times 100$$

The check on % radiolabelling of complex at different pH (4, 5, 6, 7, 8), time of incubation (5, 10, 20, 30 and 40 min) and at varied concentration of $SnCl_2$ (50, 250, 500, 1000 and 2500 μg/ml) was performed at room temperature. Also the transchelation stability, *in vitro* stability in PBS and plasma of radiolabelled nanosuspension was performed as discussed in our previously published results (Shegokar and Singh, 2011).

8. Instrument details

Millennium MPS Gamma camera (General Electrical, USA) system equipped with LEGP detector was used for the study. All the data was processed using Genieacq acquisition system.

8.1. Blood clearance

Healthy Wister rats (250–300 g) were selected as animal model and free access was given to drinking water and food. Freshly prepared radiolabelled bare and surface modified nanosuspensions and drug solution (1.2 mCi) was injected via the tail vein. After administration, blood was collected from retro-orbital puncture in test tube containing 0.3% sodium citrate solution at 5, 15, 30, 45 min, 1, 4, 8 and 24 h. Radioactivity was measured per gram of sample using well-type gamma scintillation counter. The pharmacokinetic parameters like C_{max}, T_{max}, area under zero-moment curve (AUC), area under first-moment curve (AUMC), mean residence time (MRT) were determined from the drug concentration-time profile.

8.2. Tissue distribution studies

Rats were anesthetized as discussed in our previous report (Shegokar and Singh, 2011). Radiolabelled drug solution, bare and surface modified nanosuspensions (1.2 mCi) were administered via the tail vein to Wister rats. After administration of dose, the animals were sacrificed using inhalation of high amount of anesthetic ether at 1, 4, 8 and 24 h. The rats were dissected and tissues like brain, lung, liver, kidney, spleen, heart and thymus were separated and washed thrice with Ringer's solution to remove any adherent debris and dried using tissue paper. The organs were weighed and triturated to form tissue homogenate. The radioactivity corresponding to per gram of organ was measured using well-type gamma scintillation counter. The results were expressed after decay correction; the organ activity of the nanoparticles was reported as percentage of the injected dose per gram of wet weight (% ID/g).

8.3. Toxicity studies

Acute and repeated toxicity test were carried for NVP-DS, NS and BNS. All animals were deprived of food for 8 h prior to treatment and 2 h after the treatment. Free access to water was given throughout the study period. The test formulations (72 mg/kg – two times higher than calculated therapeutic dose) were administered intravenously in divided doses over a period of 24 h for acute toxicity studies. For repeated dose toxicity study, formulations were administered daily over a period of 14 days to deliver dose of 36 mg/kg/day of nevirapine intravenously. One group received

appropriate dilutions (in divided doses) of the plain drug while one group was maintained as untreated/control for each test. Effect of the treatment on important physiological functions of animals such as locomotion, behavior, respiration and pharmacologic signs like convulsions and vomiting were noted for all the experimental animals over period of 28 days. All the animals in each of the experimental groups were humanly sacrificed using anesthetic ether at the end of 28 days. The rats were dissected and major tissues like brain, lungs, liver, kidneys, heart, spleen and thymus were removed. The organs were preserved in 10% neutral buffered formalin solution till the histology was performed. Schedule 'Y', Drug and Cosmetic Act 1945 (Amendments) was adopted for determination of repeated dose toxicity of developed nanosuspensions. Toxicity study protocols were approved by institutional ethical committee for animal use and care. All animal experimentations were performed in compliance with the guidelines for the care and use of laboratory animals in the animal house of C.U. Shah College of Pharmacy, Mumbai, India.

9. Results and discussion

9.1. Preparation and characterization of bare and surface modified nanosuspensions

In our previously reported work, we developed nevirapine nanosuspensions (uncoated) with a mean particle size of 457.6 ± 10.2 nm with narrow PI of about 0.578 ± 0.176 and size distribution below 2000 nm. A decrease in crystallinity of nevirapine was observed after nanonization. The cellular uptake studies and tissue distribution studies performed on nanocrystals confirmed targeting potential of developed nanosuspensions (Shegokar and Singh, 2011).

In this work, an attempt was made to surface modify nevirapine nanocrystals to make them more selective in targeting to specific site in body. It is well documented that plasma protein adsorbs on nanoparticles affect the *in vivo* biodistribution of nanoparticles (Blunk, 1994). Literature reports use of various surface modifiers like polymers (Santander-Ortega et al., 2006), polysaccharide (Kang et al., 1994), folates (Anderson et al., 1999), and antibodies (Deng and Balthasar, 2007; Klibanov et al., 1991), etc. An effort was made to modify the surface by physical adsorption of dextran 60, PEG 1000 and serum albumin at various concentrations starting from 0.1 to 2% (w/w).

Dextran 60, a polysaccharide showed concentration dependant increase in particle size of nanosuspension. At higher concentration it was able to increase the particle size to 520.3 nm i.e. coating thickness of 40–50 nm around particle. Dextran was tried at 0.1, 1 and 2% (w/w), the increase in the particle size for dextran 60 surface modified nanosuspensions was observed from 468.9 to 520.3 nm at 0.1, 1 and 2% concentration. Dextran 60 gave highest surface adsorption at 1% (w/w) concentration while at lower concentration of 0.1% (w/w) did not show any distinct increase in particle size. Adsorption of dextran on particle surface increased nanocrystal size to 520.3 nm at 1% concentration. The particle size increase with dextran at concentration of 1% and 2% (w/w) was almost comparable with no increase in polydispersity index (=0.538). Surface modification with lower molecular weight (dextran 40) and higher molecular weight dextran (dextran 70) at above concentration showed increase in particle size but stability was very poor with significant degree of aggregation.

Surface modification with serum albumin was tried at three different concentrations of 0.25, 0.5 and 1% (w/w). After 24 h incubation at temp $37 \pm 2\,^{\circ}C$, serum albumin gave increase in mean particle size of about 50–60 nm (PCS diameter = 515.8 nm) at 1% concentration. Whereas at lower concentration of 0.25 and 0.5% (w/w) the mean particle size increased from 480.9 to 495.2 nm, respectively. Increase in concentration of BSA more than 1% caused aggregation of particles.

PEGs are well known for their stealth effect (Immordino et al., 2006; Wattendorf et al., 2008). PEG 300, 400 and 600 were tried at 2, 5 and 10% w/w concentration. All the low molecular weight PEGs were not able to show any significant change in particle size after incubation while higher molecular weight PEG 1000 at 0.25, 0.5 and 1% showed concentration dependant increase in particle size. At 0.25% w/w concentration the particle size of nanoparticles increased by 15–20 nm. PEG1000 at 1% w/w concentration, gave highest physical adsorption leading to increase in particle size to 520 nm after incubation at $37 \pm 2\,^{\circ}C$.

Further increase in incubation time beyond 24 h did not affect particle size. So throughout all experimentation the time 24 h was kept constant for incubation. Similarly lowering the temperature had no much impact on particle size and higher temperature was not found suitable for stabilizing nanosuspensions. It was assumed that the entire surface modifier at its suitable concentration (1%, w/w) was adsorbed completely on the surface of nanosuspension.

The polydispersity index for surface modified nanosuspensions DNS, BNS, PNS at 90° and 20 °C temperature was also noted to be 0.528, 0.418 and 0.493, respectively. The PI indices showed that the dispersion is mix of polydispersed phase. The bare and surface modified nanosuspensions were odorless white dispersions, pH ranging between 6.85 ± 0.17. Drug content of all bare and surface modified nanosuspension was found to be in range of 96–98%.

9.2. Zeta potential

The zeta potential tested in Milli-Q water was observed to be around −33 mV for surface modified (−32.21 mV for BNS, −31.34 mV for PNS, −32.56 mV for DNS) and −35.21 mV for bare nanosuspensions, respectively. Surface modification did not affect the zeta potential (−32 mV) confirming that the surface was modified without changing the stability of system.

9.3. Atomic force microscopy

AFM images of the bare and surface modified nanosuspension were obtained in non contact mode. After deposition on the mica surface and glass surface all the samples showed a spherical form. Fig. 1 shows the AFM image of coated and uncoated nanosuspensions. Slight aggregation could be due to drying steps. Surface modified nanosuspension with serum albumin, PEG 1000 and dextran showed formation of layer (Fig. 1: fluorescent lining around particle) on the surface of nanocrystal, which was completely absent in AFM figure of bare nanosuspension. Particle size data showed comparable results to that of PCS measurements.

9.4. Cellular uptake studies

Pure drug solution, bare and surface modified nanosuspensions were added to cell culture to observe the interaction between nanocarriers and macrophage cells. Fig. 2(left) shows the cellular uptake of bare and surface modified nanosuspension in macrophages. The NS were easily taken up by primary macrophages, and showed time dependant uptake kinetics. Nanonized drug in form of nanosuspension showed higher uptake only within 5 min as compared to NVP-DS. This could be due to higher permeability of nevirapine to cells (Yazdanian and Glynn, 1998).

Nanosuspension was taken up fast till 1 h after that the uptake process reached plateau. At end of 2 h, a 2.76 fold increase in drug concentration was observed for bare nanosuspension to that of NVP-DS suggesting that nanonized drug could cross cellular barrier

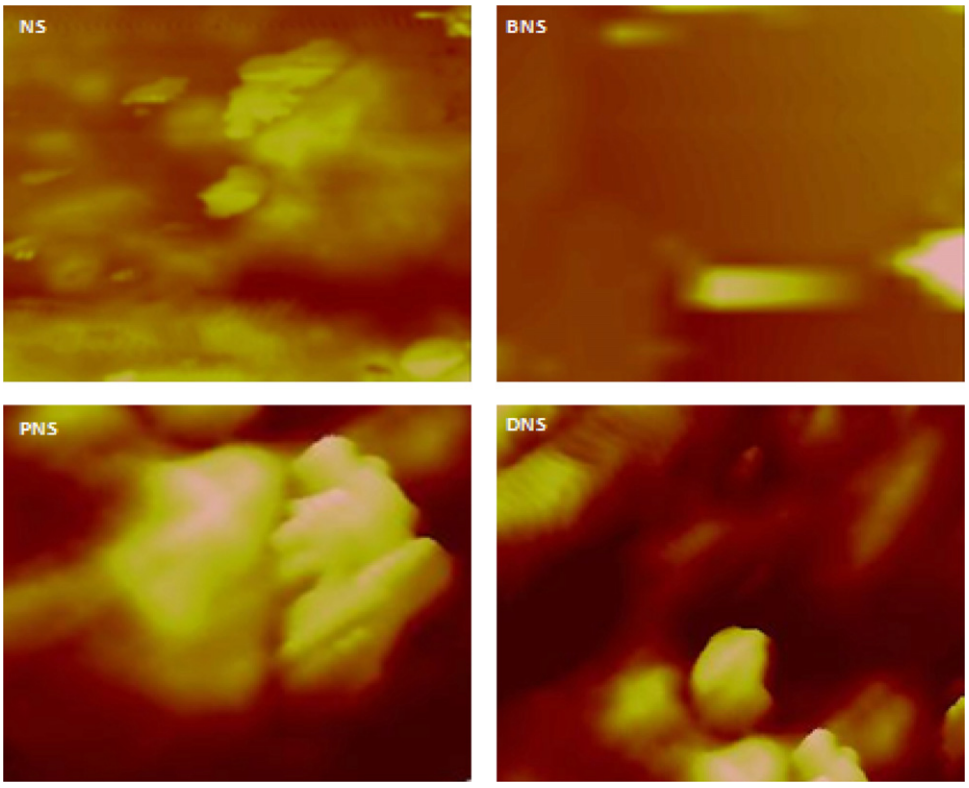

Fig. 1. Atomic force microscopy images of nevirapine bare (NS) and surface modified nanosuspensions using serum albumin (BNS), polyethylene glycol 1000 (PNS) and dextran (DNS).

and enter very fast in to the cell. This could be due to the particle size of nanosuspensions (457–550 nm) which is ideal for faster macrophage uptake.

PEGylated nanosuspension (PNS), showed decreased cellular uptake of 2.38 μg/million cells at end of 2 h. PEGylation is well reported for avoiding opsonization and rendering stealth property to nanoparticles (Owens and Peppas, 2006). PEG on the surface of nanosuspension might have provided hydrophilic protective layer which could have repelled the absorption of opsonin proteins via steric repulsion forces, thereby blocking and delaying the first step in the opsonization process. The reduced uptake of stealth nanosuspension could be due slight low value of zeta potential which was decreased after adsorption of PEG on surface forming a hydrophilic cloud. This could prevent further interactions with biological components. The nanosuspensions modified with PEG could reduce the uptake by primary macrophages which were in accordance with the literature reports (Yoncheva et al., 2005). Surface modification of nanosuspensions using dextran (DNS) showed increased cellular drug concentration levels after macrophage uptake. The formulation DNS showed 3.36 times higher cellular uptake than of free drug and 1.22 times higher for that of NS ($p < 0.001$) at 2 h respectively. Surface modified DNS formulation achieved the highest drug concentration in macrophages within 60 min which bare nanosuspension was not able to attain. This could be probably due to carbohydrates coating present on the surface of nanosuspension allowing macrophage-selective uptake. Dextran, being a polysaccharide might have facilitated cellular uptake. Surface modification of nanocrystals using serum albumin (BNS formulation) showed 3.84 fold increase in cellular uptake as compared to pure drug and 1.39 fold higher than that of nanosuspensions ($p < 0.001$). This could be probably due to efficient recognition by the receptors on macrophages.

However, there was apparent difference in cellular drug concentration of NS and surface modified nanosuspensions indicating

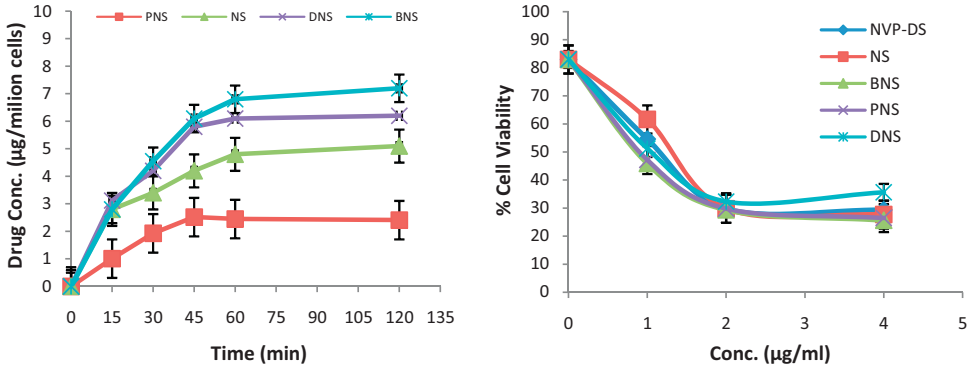

Fig. 2. Left: effect of incubation time on the % phagocytosis of bare (NS) and surface coated nevirapine nanosuspensions (BNS, DNS and PNS) incubated with primary macrophages. Right: cytotoxicity profiles of nevirapine bare and surface modified nanocarriers when incubated with J744.A12 murine macrophages as determined by MTT assay.

Table 1
Amounts of identified proteins on nevirapine bare nanosuspension (NS), polyethylene glycol modified nevirapine nanosuspension (PNS), serum albumin modified nanosuspension (BSA) and dextran modified nanosuspension (DNS).

Proteins identified	Nanosuspensions			
	NS	PNS	BNS	DNS
Apo A1	+++	+++	+++	+++
Apo A2	+	+	+	+
Apo A4	+++	+++	+++	+++
Apo C2	++	++	++	++
Apo C3	+	+	+	+
Apo E	+	+	+	++
Apo J	++	++	+++	+++
Albumin	+++	+++	+++	++
Immunoglobulin light chain	++	+++	++	++
Immunoglobulin heavy chain alpha	++	+++	+++	+++
Immunoglobulin heavy chain gamma	+++	+++	+++	++
Fibrinogen alpha chain	+	++	+	+
Fibrinogen beta chain	+	++	+	+
Fibrinogen gamma chain	+	+	++	++
Alpha-1-antichymotrypsin	+	+	++	+
Alpha-1-antitrypsin	++	++	++	++

−, no protein; +, small amount of protein; ++, medium amount of protein; +++, high amount of protein.

that the surface modification does affect the intracellular distribution of formulation. A higher intracellular content of a drug via endocytosis of a carrier may remarkably increase the therapeutic effect against the target cells (Na et al., 2003). The excessive uptake of batch BNS and DNS by macrophages as compared with the bare NS holds a promise for the specific receptor-mediated cellular endocytosis. It was found that surface modification using albumin significantly ($p < 0.001$) increased cellular uptake of nevirapine than polysaccharide and PEG coated nanosuspension. Nevirapine bare and surface modified nanosuspension facilitated the cellular uptake (NS < PNS < DNS < BNS) thereby effectively increasing the drug concentration inside cellular compartments enabling to understand their *in vivo* behavior. It is possible to localize the drug to specific intracellular targets like lysosomes, cytoplasm, mitochondria, etc.

9.5. In vitro protein adsorption

It has been seen from phagocytic uptake studies, that bare and surface modified nanosuspensions can accumulate in several times higher concentration in primary macrophages. Before performing *in vivo* studies, plasma protein adsorption patterns of the four formulations viz. NS, PNS, BNS and DNS were studied qualitatively and quantitatively (Shegokar et al., 2011). The protein adsorption data showed very high amounts of adsorbed apolipoproteins for all nanosuspensions (NS, BNS, DNS) including PEGylated nanosuspensions (PNS). In case adsorbed concentration of PEG is not optimum, no stealth effect will be observed. Also presence of Apo J, Alpha-1-antitypsin, immunoglobulin heavy chain gamma, immunoglobulin light chain and albumin were found in very high concentration. From the Apolipoprotein family ApoA2, ApoC2, ApoC3 and ApoE could also be detected. The 2-D PAGE analysis served as a useful tool to predict roughly *in vivo* organ distribution pattern. Nanosuspensions (both bare and surface modified) would facilitate lymphatic transport and efficient internalization of drug particles in cells. An overview about the adsorbed proteins is given in Table 1.

9.6. Cytotoxicity studies

Fig. 2(right) shows the percent viability of murine macrophages (J744.A12) after incubation with bare and surface modified nanosuspension in comparison with NVP-DS. Cell exposure to higher concentration of nevirapine showed faster cell death, but high count of viable cells were observed up to 2 μg/ml. Similar results were obtained for three surface modified nanosuspensions. As surface modifiers used in this work are pharmaceutically safe materials and showed no added cytotoxicity when tested in murine macrophages against NVP-DS and NS. Even several time higher cellular uptake of bare and surface modified nanosuspension (data from uptake studies) did not cause any additional cytotoxicity. It can be seen that both nevirapine solution and nevirapine nanosuspension could target at cellular level, can be administered at higher dose without increasing risk of cytoxicity.

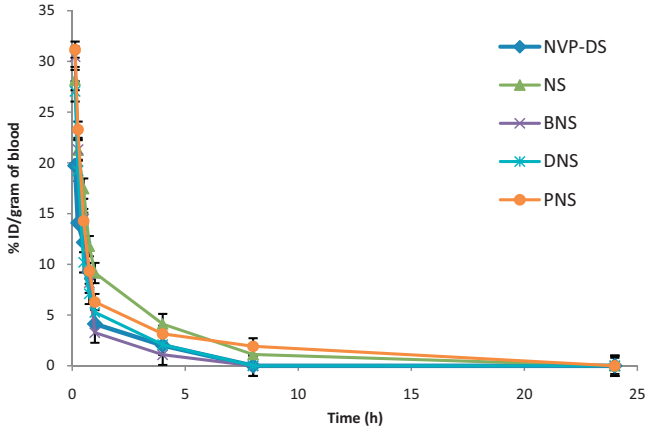

Fig. 3. Blood clearance profiles of radiolabelled nevirapine bare (NS) and surface modified nanosuspensions (BNS, DNS and PNS) compared to pure drug solution (NVP-DS) at different time intervals in rat.

9.7. Pharmacokinetics and tissue distribution studies

Nevirapine bare and surface modified nanosuspensions were labeled using ^{99m}Tc using stannous chloride reducing method (Arulsudar et al., 2003) at pH (6–7). Parameters like lebelling temperature and time was optimized and it was observed that increase in said variables had no marked effect on radiolabelling. The stannous chloride at concentration of 1000 μg gave highest radiolabelling at pH 6.5–7 after 30 min incubation. Radiolabelled nanosuspensions were evaluated for *in vitro* (in PBS and plasma) and *in vivo* stability as discussed in our previous paper (Shegokar and Singh, 2011). Nevirapine nanosuspension both bare and surface modified showed excellent stability *in vitro* and *in vivo*.

9.8. Blood pharmacokinetics

The radioactivity was high enough within few minutes of administration and sharply decreased after 4 h and reached below detectable limit at 24 h suggesting extended circulation in vascular network. The pharmacokinetic curves for ^{99m}Tc-labeled nevirapine bare and surface modified nanosuspensions were as shown in Fig. 3. Highest gamma count was observed to be 25.17 ± 3.54, 31.17 ± 2.15, 27.05 ± 3.05, 30.47 ± 2.47 and for NS, PNS, DNS and BNS, respectively and 20.08 ± 2.04 for NVP-DS. Superior gamma count was observed for surface modified formulations as compared to NS. The AUC_0^{24} of 20.867, 23.394, 30.89, 35.893 counts h/g was observed for BNS, DNS, PNS and NS was observed, respectively, comparatively high as compared to NVP-DS (20.483). Table 2 summarizes the pharmacokinetic parameters for nevirapine nanosuspensions.

Table 2
Pharmacokinetic parameters and AUCs of intravenously injected radiolabelled nevirapine bare (NS) and surface modified nanosuspension (BNS, DNS and PNS).

Tissue	NVP-DS		
	AUC (0–t)	AUMC (0–∞)	MRT (h)
Blood	20.483	28.275	1.306
Brain	–	–	–
Lungs	40.288	179.29	4.45
Liver	217.485	3711.252	5.747
Spleen	120.253	548.663	4.563
Kidneys	121.71	1223.88	9.096
Heart	66.175	818.164	10.67
Thymus	2.743	4.258	1.544

Tissue/formulation	NS			BNS		
	AUC (0–t)	AUMC (0–∞)	MRT (h)	AUC (0–t)	AUMC (0–∞)	MRT (h)
Blood	35.893	83.608	1.959	20.867	19.052	0.889
Brain	–	–	–	194.735	1425.13	7.043
Lungs	25.068	108.24	4.317	191.42	2023.7	9.516
Liver	385.0	2862.65	7.153	565.28	9173.49	13.378
Spleen	307.305	5088.16	13.468	450.245	9609.129	16.1015
Kidneys	90.921	425.39	4.678	69.513	366.26	4.406
Heart	76.703	366.735	4.781	39.418	151.882	3.853
Thymus	11.573	26.669	2.304	17.033	43.457	2.551

Tissue/formulation	PNS			DNS		
	AUC (0–t)	AUMC (0–∞)	MRT (h)	AUC (0–t)	AUMC (0–∞)	MRT (h)
Blood	38.09	129.94	3.057	23.394	39.745	1.519
Brain	–	–	–	–	–	–
Lungs	88.07	847.386	8.901	91.75	1210.46	11.367
Liver	575.63	10642.7	14.66	545.89	7967.32	12.283
Spleen	339.5	5186.67	12.665	336.715	2729.38	13.455
Kidneys	103.24	1203.114	10.248	66.073	283.503	4.291
Heart	90.165	700.443	7.313	57.168	176.01	3.078
Thymus	9.488	23.849	2.513	11.965	31.604	2.638

9.9. Gamma imaging

Gamma imaging showed clear distribution of pure drug, bare and surface modified nanosuspensions (Fig. 4). NVP-DS was mainly accumulated in kidney and bladder and was cleared from body in <24 h. Bare nanosuspensions mainly concentrated in RES organs and showed significant increase in radioactivity within 30 min. Surface modification of nanosuspensions further increased accumulation in RES organs which was more prominent at 1 and 8 h. The excretion via kidney and urinary bladder remained invisible for surface modified nanocarriers as compared to bare nanosuspensions for which excretion was observed at end of 8 h. Therefore, all the bare and surface modified nanosuspensions showed comparable accumulation in RES organs. At 24 h, the pure drug was completely excreted from body while bare nanosuspensions were able to retain in smaller amounts in RES organs and surface modified nanosuspension maintained detectable the levels. Surface modification of nanosuspensions significantly enhanced their targeting potential specifically to liver and spleen as compared to free drug and bare. This could be due to larger particle size (450–500 nm, LD99% 2200 nm) of nanosuspension which can be easily phagocytosed by macrophages. The nanosuspension surface modified with albumin showed higher radioactivity levels in brain, while other formulations did not. The radioactivity levels in individual organ were further confirmed by performing tissue distribution studies.

9.10. Tissue distribution

Comparative biodistribution profiles after *i.v.* administration of radiolabelled NVP bare and surface modified nanosuspensions are depicted in Fig. 5.

HIV virus mainly resides in various organs like spleen, lung, liver, brain and thymus (Chun and Fauci, 1999). The main objective of this work was to target active reservoirs in body using organ targeting approach. HIV virus enters central nervous system (CNS) and progressively replicate there leading to AIDS dementia complex (Lipton, 1998), the transport of anti-HIV agents into the CNS has been a research focus in drug discovery and delivery (Rao et al., 2009). Two physiological barriers viz. blood circulation system barrier (BCB) and the CNS (blood brain barrier, BBB) serve important biological barriers during absorption of nanoparticles, both barriers are formed of endothelial cells and allow transport via passive diffusion and carrier-mediated transport. BBB serves not only as a permeability barrier and but also a metabolic barrier for the transport of anti-HIV agents into the CNS (Li and Chan, 1999). The nevirapine nanosuspensions were able to cross BBB within less than 30 min and maintaining the levels up to 24 h after modifying the surface of nanocrystal with serum albumin. The capability of nevirapine to penetrate the BBB has been well documented previously (Gimenez et al., 2004; Streck et al., 2008), conversion into nanocrystals further facilitated transport of nevirapine in presence of surfactant. The AUC_{brain}/AUC_{blood} ratio of BNS was found to be 9.33. The formulation BNS showed 20.79 folds enhancement in gamma count at end of 1 h (with $AUC_0{}^{24}$ of 194.74 counts h/g) with prolonged MRT of 7.043 h (Fig. 6, top). The clearance was observed to be very slow for nanosuspension coated with serum albumin. None of the other nanosuspension formulation showed levels in brain.

The spleen and lymph nodes serves as important sites of local HIV replication during the latent period of AIDS (Embretson et al., 1993; Pantaleo et al., 1993). In spleen, bare nanosuspension itself showed higher $AUC_0{}^{24}$ of 307.305 counts h/g as compared to NVP-DS (120.253 counts h/g) as discussed in our previous paper, nanonization significantly increased cellular uptake of nanocrystals. Surface modification of nanocrystals further enhanced levels of gamma count. The $AUC_0{}^{24}$ for PNS and DNS formulation was

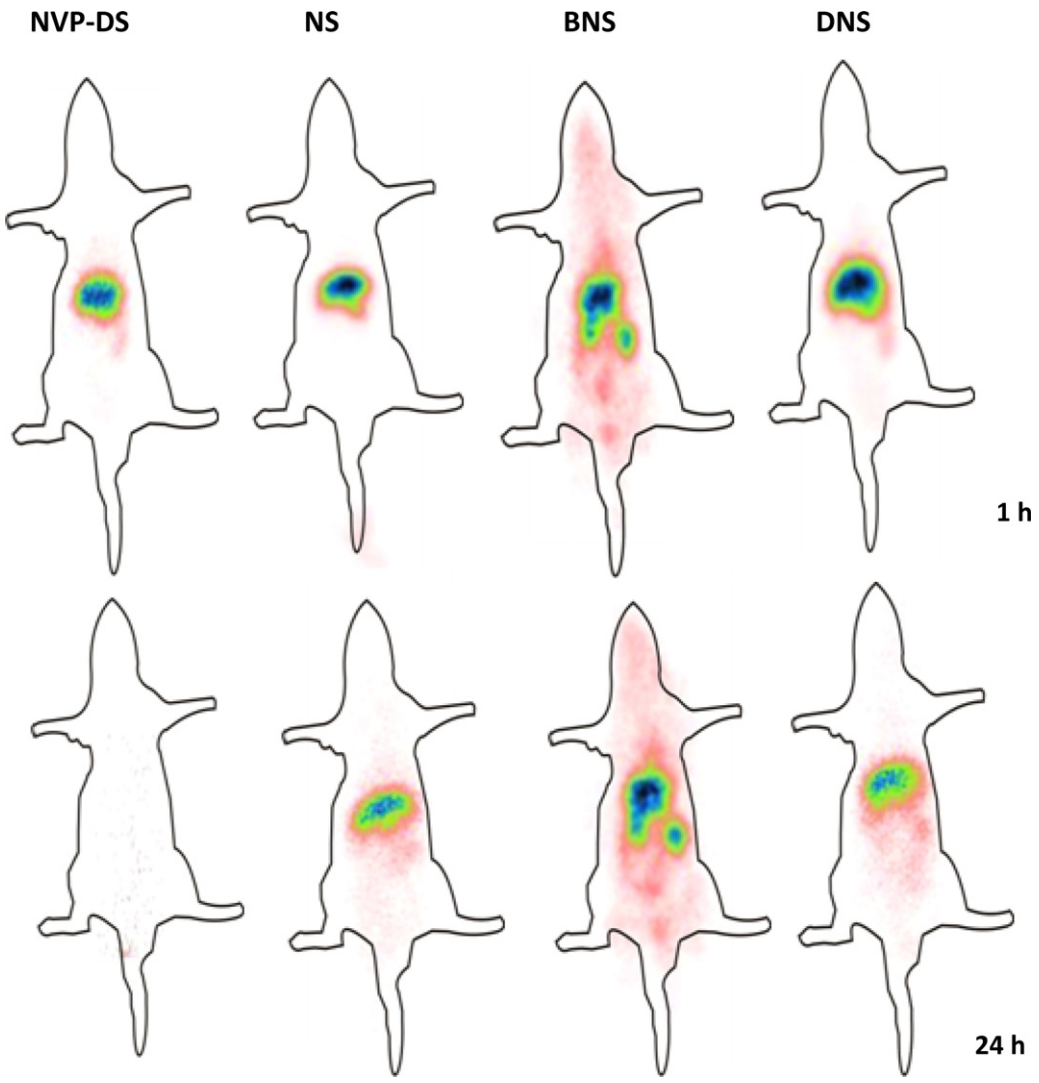

Fig. 4. Gammascintigrams depicting biodistribution of nevirapine bare (NS) and surface modified (BNS and DNS) radiolabelled nanosuspensions at 1 h (top) and 24 h (down) time intervals in rat compared to pure drug solution (NVP-DS).

almost similar (~335 counts h/g), however, surface modification with serum albumin resulted in highest AUC_0^{24} value for BNS, i.e. 450.245 counts h/g with prolonged MRT of 16.10 h in spleen (for NVP-DS the MRT was 4.56 h) (Fig. 6, middle). Bare nevirapine nanosuspension showed almost four times higher AUC_0^{24} than that of plain drug solution. In liver, nevirapine bare nanosuspensions showed AUC_0^{24} of 385 counts h/g which was much higher than that of NVP-DS (217.49 counts h/g) maintaining higher MRT 7.153 h as compared to NVP-DS (5.747 h). Surface modification of nanosuspension with polysaccharide, polyethylene glycol and serum albumin resulted in higher accumulation and remained comparable to each other (~540–570 counts h/g) (Fig. 6, bottom). Higher levels of accumulation after surface modification in spleen and liver could be due to increased particle size (~500 nm) which is ideal for cellular uptake by MPS cells. Radioactivity distribution profiles obtained in spleen are in accordance with cellular uptake studies and as predicted in *in vivo* tissue distribution results concluded from 2-D PAGE study. Surface modified formulations with serum albumin and polysaccharide showed several times enhanced cellular and organ uptake as compared to NVP-DS and NS.

Radioactivity in heart for bare and surface modified nanosuspension remained high for bare NS and PNS while was slightly lower for BNS and DNS. In lungs, radioactivity was increased for surface modified nanosuspensions and the highest accumulation was observed for BNS (AUC_0^{24} 191.42 counts h/g, MRT = 9.52 h) followed by DNS and PNS. Thus, surface modified nanosuspensions showed marked effect as compared to bare NS on kinetics in lung. In kidney, highest AUC_0^{24} was observed for NVP-DS (AUC_0^{24} 121.71 counts h/g) followed by PNS (AUC_0^{24} 103.24 counts h/g) and NS (AUC_0^{24} 90.92 counts h/g). While highest MRT of 10.25 h was noted for PNS as compared to other surface modified nanosuspension formulations (MRT = 4–5 h). Thymus plays crucial role in immunity related diseases via T cell maturation (Ferri et al., 2006; Ho Tsong Fang et al., 2008). Nevirapine bare nanosuspension showed accumulation in thymus but with lower gamma count, accumulation was further enhanced by surface modification of nanosuspension with serum albumin and dextran. Thymus showed lower levels of gamma counts with MRT of 2.638, 2.551, 2.513 and 2.304 h for DNS, BNS, PNS and NS, respectively. BNS showed highest AUC_0^{24} of 17.033 counts h/g with $AUMC_0^{\infty}$ 43.458 counts h/g. The bare as well as surface modified nanosuspensions showed access to thymus. Substantial levels of gamma count were obtained for DNS followed by BNS and the levels could be sustained up to 4 h. Surface modification had marked effect on AUC_0^{24} and MRT in thymus.

Significant differences in biodistribution profiles of NVP-DS, NS and surface modified nanosuspension (BNS, DNS and PNS) were

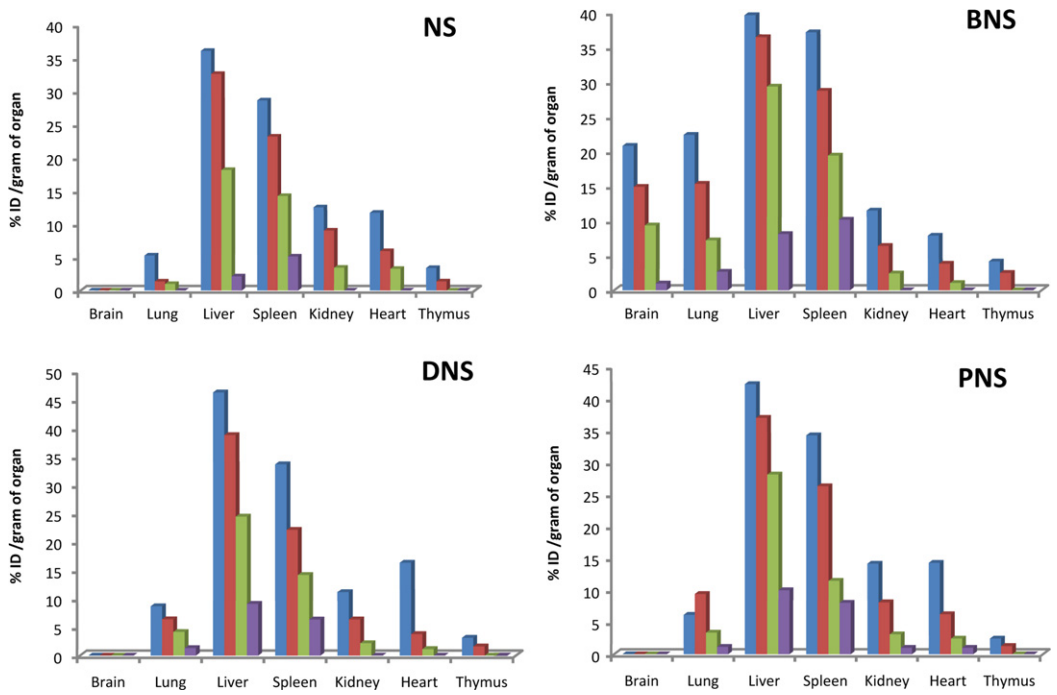

Fig. 5. Radioactivity in different organs after intravenous administration of bare and surface modified nevirapine nanosuspensions using serum albumin (BNS), PEG 1000 (PNS) and dextran (DNS) at 1 h ( ), 4 h ( ), 8 h ( ) and 24 h ( ).

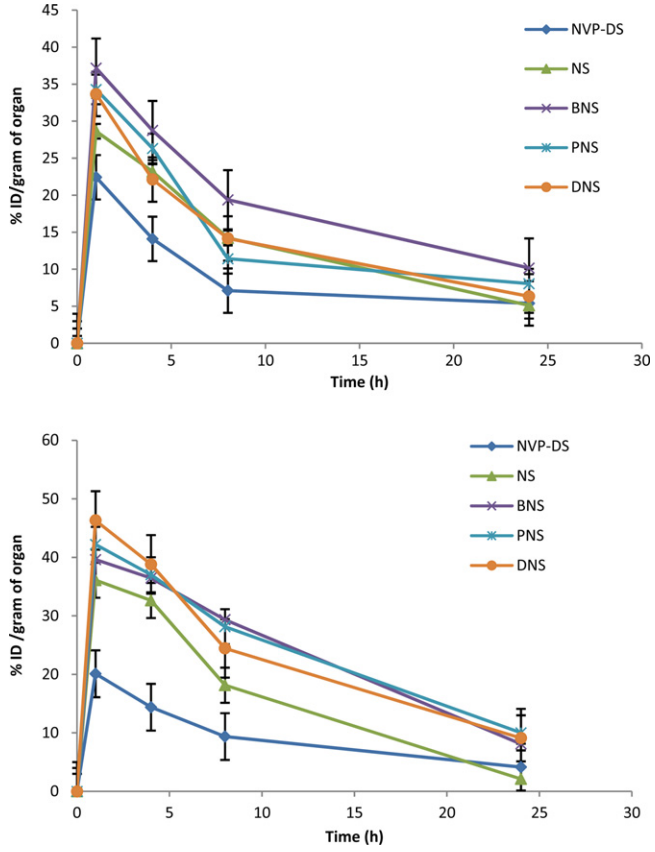

Fig. 6. Comparative radioactivity profiles of drug solution (NVP-DS), bare (NS) and surface modified nanosuspensions (BNS, PNS and DNS) of nevirapine in brain (top), spleen (middle) and liver (down) at different time intervals in rat.

observed. Surface modification of nanosuspensions significantly enhanced targeting specifically to brain, liver and spleen as compared to free drug. Highest radioactivity was observed in all organs at 1 h and declined after that for respective formulation. Organs biodistribution results indicated that nanosuspension clearance from blood is mainly due to liver. This could be explained by two classically described uptake mechanisms by Kupffer cells and other macrophages (Barratt, 2000). The developed uncoated nanosuspension was able to successfully target spleen, where as surface modification of these systems further enhanced the levels in spleen even after 24 h.

ANOVA showed significant differences among AUCs of the formulations studied. Nevirapine nanosuspension experimental group has higher value of F than the tabulated value (>2.85 at 95% confidence limit, $p = 0.05$), the differences of mean gamma count between various tissues were significantly different. However, the F ratios of BNS were much higher than those of NS and NVP-DS indicating the overall differences of mean were more significant. The developed nanosuspension of nevirapine showed potential for targeting organs which serve as active and latent cellular reservoirs during HIV infection without any imposing any toxic effects to other organs. Surface modification can further help in directing nanoparticles to desired target site.

9.11. Toxicity studies

Animals from acute toxicity experimental groups showed no signs of abnormal behavioral or physiological reactions after dose administration over the period of 14 days (acute toxicity studies). No mortality was observed for any group. The food consumption, water intake and weight gain was observed to be within normal ranges even after administration of double of the therapeutic dose of bare and serum albumin coated nanosuspensions. Histological studies further confirmed the absence of any pathological changes in vital organs of animals as compared to the untreated control group. Figs. 7–9 represent histological photomicrographs of spleen, liver and brain, from different experimental groups. Thus, bare and

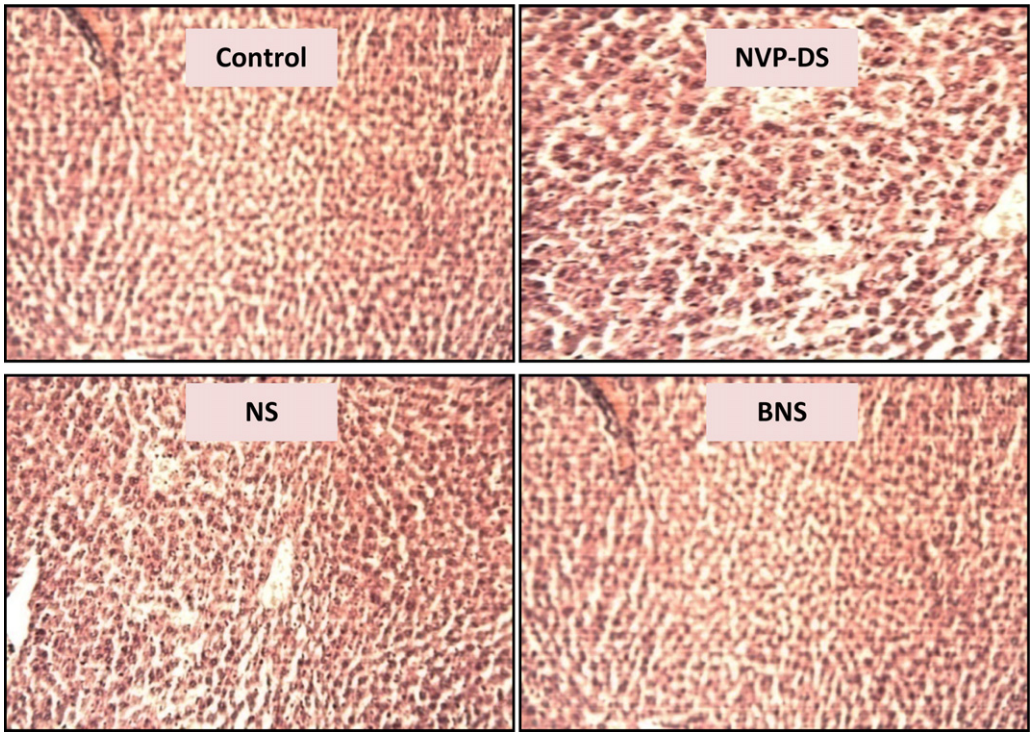

Fig. 7. Photomicrograph of spleen of rat from different experimental groups of repeated dose toxicity study for nevirapine drug solution (NVP-DS) and nanosuspension (NS and BNS).

surface modified nanosuspensions as well as the excipients used in formulations were absolutely safe even at higher dose than that of therapeutic dose level in the experimental animals.

None of the animals from repeated dose toxicity experimental groups showed abnormal behavioral or physiological changes on the day of dose administration and thereafter over the period of 28 days (repeated toxicity, dose 36 mg/kg/day). No changes in food consumption, water intake and weight gain was observed and no mortality was recorded. Histological examination of various tissues indicated no sign of any pathological changes in the treatment groups as compared with the untreated control group. It was observed that some mild degenerative changes took place

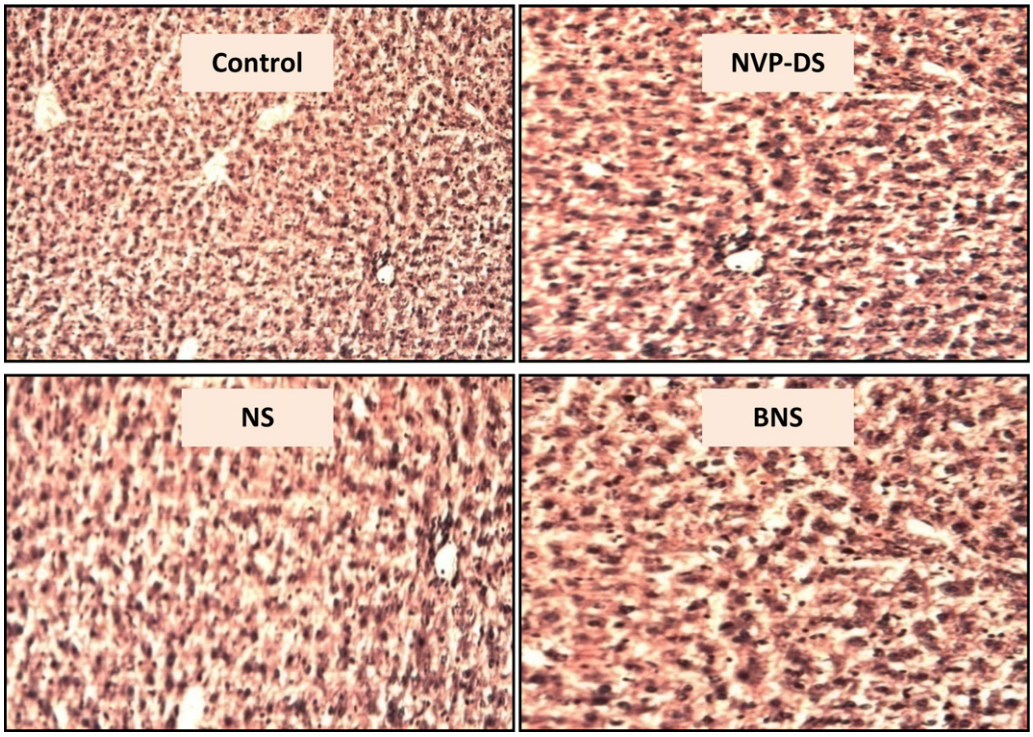

Fig. 8. Photomicrograph of liver of rat from different experimental groups of repeated dose toxicity study for nevirapine drug solution (NVP-DS) and nanosuspension (NS and BNS).

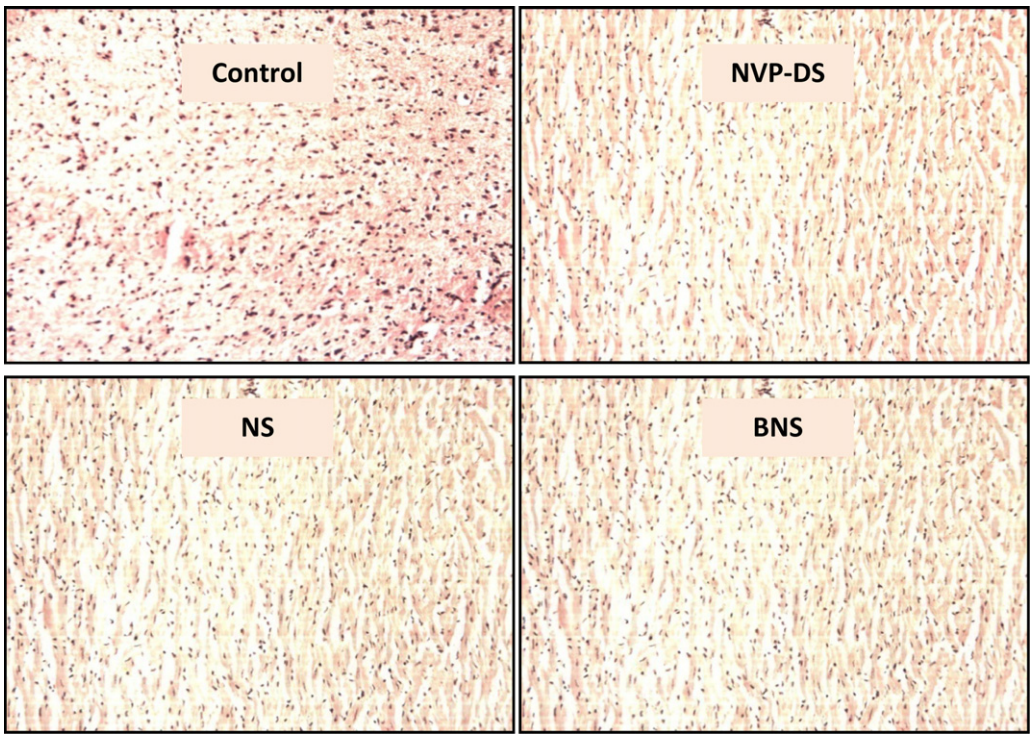

Fig. 9. Photomicrograph of brain of rat from different experimental groups of repeated dose toxicity study for nevirapine drug solution (NVP-DS) and nanosuspensions (NS and BNS).

Table 3
Histopathological observations for repeated dose toxicity in different organs of rat after intravenous administration of bare and surface modified nevirapine nanosuspensions.

Tissue/s	Observations			
	Control	NVP-DS	NS	BNS
Brain	NAD	NAD	NAD	NAD
Lungs	NAD	NAD	NAD	NAD
Liver	NAD	NAD	NAD	NAD
Kidneys	NAD	Mild swelling of tubules with early degeneration		
Spleen	NAD	Mild congestion	Mild congestion	Mild congestion
Heart	NAD	NAD	NAD	NAD
Thymus	NAD	NAD	NAD	NAD

NAD, no abnormalities detected.

in liver after repeated administration of the NS, BNS and NVP-DS for 28 days. Liver showed very mild degenerative changes and swelling for NVP-DS and nanosuspension experimentation group. As all antiretrovirals are well known for their severe toxicity profile, these mild changes included degeneration and proliferation of Kupffer cells and cytoplasmic degenerative changes. However it was assumed that before 28th day, if any degenerative changes which might have occurred prior to 28 days they had disappeared over the time slowly indicating restoration of normal function of the tissues. Thus, the histological changes which took place were self limiting and were recovered on discontinuation of the treatment (Table 3). Thus, it can be concluded that the developed bare and coated nanosuspensions were safe to be administered over the required therapeutic schedule with some mild self limiting pathological changes.

10. Conclusion

Nevirapine nanosuspensions (<500 nm) were successfully prepared by using cold high pressure homogenization technique. Nanosuspensions were surface modified to achieve organ targeting. Enhanced cellular uptake of bare and surface modified nanosuspensions ensured their targeting potential at cellular level. Improved therapeutic efficacy may be attributed to higher drug levels and increased bioavailability of drug in spleen, liver and thymus without added cytotoxicity. Serum albumin surface modified nevirapine nanosuspensions could cross blood brain barrier and accumulate in brain for more than 24 h, which opens new perspective for treatment of AIDS dementia. Macrophage uptake study revealed the potential of prepared nanosuspension for targeting intracellular compartments. This type of delivery system is expected to provide better therapeutic effects by preferential concentration at target site maintaining the local drug concentration for longer periods of time due to surface modification to achieve reduced viral load. Nanonization of nevirapine significantly improved its *in vivo* behavior and targeting potential to cellular HIV reservoirs. An optimized nanosuspension formulation of nevirapine is expected to have fast onset and long duration of drug accumulation in viral reservoirs with reduced side effects.

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