Development of a macrophage-based nanoparticle platform for anti-retroviral drug delivery

Running title: Macrophage-based anti-retroviral therapy

Huanyu Dou,1,2, Christopher J. Destache,1,2,6 Justin R. Morehead,1,2, R. Lee Mosley,1,2 Michael D. Boska,1,2,3 Jeffrey Kingsley,1,2,5 Santhi Gorantla,1,2 Larisa Poluektova,1,2 Jay A. Nelson,1,2,3 Mahesh Chaubal,7 Jane Werling,7 James Kipp,7 Barrett E. Rabinow,7 and Howard E. Gendelman1,2,4,*

1Center for Neurovirology and Neurodegenerative Disorders, 2Departments of Pharmacology and Experimental Neuroscience, 3Radiology, 4Internal Medicine, and 5Pediatrics, University of Nebraska Medical Center, Omaha, NE 68198-5880, 6Creighton University School of Pharmacy and Health Professions, Omaha, NE 68178, and 7Baxter Healthcare Corporation, Round Lake, IL 60073 USA

Key words: macrophages, nanoparticles, anti-retroviral drugs, human immunodeficiency virus, nonobese severe combined immunodeficient mice, immune reconstitution, indinavir

*Corresponding author

Howard E. Gendelman, MD

Department of Pharmacology and Experimental Neuroscience

Center for Neurovirology and Neurodegenerative Disorder

985880 Nebraska Medical Center

Copyright © 2006 American Society of Hematology
University of Nebraska Medical Center, Omaha, NE 68198-5880;
Phone 402-559-8920; FAX 402-559-3744; email: hegendel@unmc.edu

**Word Counts:** 159 words in Abstract, and 4870 words in Text.
Abstract

Complex dosing regimens, costs, side effects, biodistribution limitations, and variable drug pharmacokinetic patterns have affected the long-term efficacy of anti-retroviral medicines. To address these problems, a nanoparticle indinavir (NP-IDV) formulation packaged into carrier bone marrow derived macrophages (BMM) was developed. Drug distribution and disease outcomes were assessed in immune competent and HIV-1 infected humanized immune deficient mice, respectively. In the former, NP-IDV formulation contained within BMM was adoptively transferred. After a single administration, single photon emission computed tomography, histology and RP-HPLC demonstrated robust lung, liver, and spleen BMM and drug distribution. Tissue and sera IDV levels were greater than or equal to 50 μM for 2 weeks. NP-IDV BMM administered to HIV-1 challenged humanized mice revealed reduced numbers of virus-infected cells in plasma, lymph nodes, spleen, liver, and lung, as well as, CD4⁺ T cell protection. We conclude that a single dose of NP-IDV, using BMM as a carrier, is effective and warrants consideration for human testing.
Introduction

Despite the significant impact of anti-retroviral therapy (ART), the worldwide HIV-1 pandemic continues to grow\(^1\text{-}^3\). An estimated 40 million people globally are virus infected, with the majority from the developing world\(^4\text{-}^6\). Although ART has reduced disease morbidity and increased life expectancy, drug expenses, treatment failures, and dosing complexities limit global access\(^7\text{-}^9\). Multiple daily dosing regimens and untoward secondary side effects diminish achievement of significant long-term HIV-1 suppression in infected people\(^10\text{-}^12\). Additionally, continuous viral suppression requires maintenance of therapeutically effective drug concentrations\(^13\text{-}^15\). Most significantly, elimination of viral reservoirs in the infected human host has not yet been achieved\(^16\text{-}^17\).

To address these challenges to effective anti-retroviral delivery, we designed a novel BMM pharmacologic NP delivery system. This system could provide a strategy to achieve therapeutic efficacy, improve drug distribution to areas of active viral replication, and extend dosing intervals. Because of the small size of the NP and their highly stable nature, NP could be packaged within macrophages for subsequent systemic trafficking and sustained drug distribution. We reasoned that such a cell-based drug delivery system could reflect the patterns of viral replication and improve therapeutic outcomes.

To test this idea, we loaded IDV nanosuspension into BMM and administered i.v. into naive mice. Cell tissue distribution was tracked by single photon emission computed tomography (SPECT) and \(T_2^*\) weighted MRI of radio- and super paramagnetic iron oxide- (SPIO; Feridex) labeled BMM, and confirmed by histology. Reverse phase-high performance liquid
chromatography (RP-HPLC) IDV measurements showed sustained high drug levels in body tissues, sera, and urine. To assess anti-retroviral responses, NOD/SCID mice reconstitution with human peripheral blood lymphocytes (PBL) was used to generate humanized (hu)-PBL-NOD/SCID mice. Pharmacokinetic behavior and immune and anti-retroviral activities were monitored after HIV-1_{ADA} infection and a single dose of NP-IDV-BMM. Sustained anti-retroviral therapeutic responses with concomitant immune reconstitution were seen up to 14 days. These results provide proof of concept for the use of NP delivery in ART and open the possibility of developing such regimens for those with limited drug access and high disease prevalence.

**Materials and methods**

**Preparation and characterization of nanoparticles**

IDV suspensions were prepared by high-pressure homogenization. The surfactant coating of the IDV crystals were comprised of Lipoid E80, an egg phosphatide mixture of phosphatidylcholine, phosphatidylethanolamine, and the hydrolyzed lyso (single aliphatic chain). Lipoid E80, 1.2% w/v coated the particles. The nanosuspension was formulated at an alkaline pH at 8.5. 1.2 g IDV free-base was added to the phospholipid dispersion and a pre-suspension prepared using an Ultraturrax rotor-stator mixer for 4 minutes to reduce particle size. An isotonic buffer solution was made by dissolving 1.8 g sodium chloride and 0.28 g sodium phosphate dibasic in 200 mL of water. The pre-suspension was homogenized at 15,000 psi for 40 passes. The final mean NP size of the suspension was 1.6 μm, with 99% of the particles (by volume) less than 8.4 μm. The process was optimized for temperature, pressure and homogenization cycles. Particle size was optimized to minimize dissolution before and during macrophage uptake and measured using...
light scattering and suspension stability assays and assessed by stress and short-term stability
tests. The NP suspension was made in a concentration at $10^{-2}$ M. Lissamine™ rhodamine B 1,2-
dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (rDHPE, Invitrogen,
Carlsbad, CA) was used to label NP-IDV, which appeared as red fluorescence.

**BMM isolation and culture**

Mice femur bone marrow was dissociated into single cell suspensions and cultured for 10 days
supplemented with 2 mg/ml macrophage colony stimulating factor (MCSF, a generous gift from
Wyeth, Inc., Cambridge, MA). Cells were analyzed using a FACSCalibur (BD Biosciences, San
Jose, CA), and 98% of cells were found to be CD11b⁺.

**NP-IDV uptake and release**

BMM were cultured at $5 \times 10^5$/ml with NP-IDV ($5 \times 10^{-4}$ M). NP-IDV uptake was assessed
without medium change for 72 hr and BMM samples collected at 10, 30, 45, 60, 90, and 120
min, and every 2 h thereafter. NP-IDV/IVD release from BMM, with or without media changes,
was evaluated as a function of time. Time course release was performed with a single wash-out
after 12 h of NP-IDV exposure to BMM. Additionally, BMM were cultured with NP-IDV for 12
h with daily fresh medium changes. BMM and medium samples were then collected each day
between medium changes.

**NP-IDV-BMM preparation and delivery**

BALB/c mice (Charles River Laboratory, Inc., Wilmington, MA), male at 4-5 weeks of age were
used as BMM donors. BMM were incubated with $5 \times 10^{-4}$ M of either rDHPE-NP-IDV or NP-
IDV for 12 h. Following NP-IDV loading, a single i.v. dose of NP-IDV-BMM (20×10^6) were transferred to naive recipients for pharmacokinetic and anti-retroviral efficacy studies.

**Single photon emission computed tomography (SPECT) analysis of BMM migration**

To assess cell migration by SPECT, BALB/c 5-8 weeks, male mice (Charles River Laboratory, Inc., Wilmington, MA) were used in experiments (n=4). BMM were labeled with 111indium oxyquinoline (indium oxine, Amersham Healthcare, Arlington Heights, IL) at a dose of 600 μCi per 30×10^6 cells in 1 ml of RPMI-1640/10mM HEPES for 45 min at 37°C. Cells were extensively washed and resuspended in HBSS. Labeling efficiency as determined by γ-scintillation spectrometry (Packard Instrument Company, Meriden, CT) was routinely 70% - 80% of total input isotope. Each recipient received 5-7 x 10^6 111In labeled BMM. Mice were anesthetized with 0.5% - 1% isoflurane delivered in a 2:1 mixture of nitrous oxide and oxygen. Image acquisition was accomplished with a γ-scintillation camera detector fitted with a 1-mm pinhole collimator and interfaced with image acquisition software (A-SPECT, GammaMedica, Northridge, CA). Briefly for each animal, 64-one minute, equiangular exposures over a 360° axis of rotation were acquired at each time point. Acquired exposures were reconstructed into a single 3-dimensional tomogram. Each region of interest (ROI) within the processed tomograms was circumscribed by electronic bit maps to contain lung, liver, or spleen. Relative activities for each were determined. After acquisition of SPECT images for final time point, animals were sacrificed, tissues excised, weighed, and submitted for γ-scintillation spectrometry to determine the intensity of 111In signal in each tissue.
MRI tracking of Feridex labeled BMM

BMM were labeled with super paramagnetic iron oxide (SPIO) nanoparticles (Feridex, Berlex Laboratory, Wayne, NJ) by culturing at 2 mg of Feridex/10⁷ cells/ml of complete media for 1 hr at 37°C. Cells were washed twice with DMEM and each recipient mouse received 1 x 10⁷ Feridex-labeled BMM in 200 μl i.v. Four mice were used for each group (5-8 weeks old of male BALB/c mice form Charles River Laboratory, Inc. Wilmington, MA). Greater than 95% cells were labeled with SPIO particles as evidenced by the presence of Prussian blue stain. The presence of Feridex-labeled BMM in tissues was evaluated by MRI whereby SPIO particles in tissue induce increased magnetic relaxivity of tissue water which is strongly field dependent, directly related to cell density, and produces a corresponding loss of image signal. The measurements were performed with a 7Tesla system (Bruker 21 cm Biospec operating Paravision 3.0.2). High resolution 3D gradient recall echo MRI scans of mouse body were acquired using a 25 mm birdcage volume coil covering a region from the neck to the hips with acquisition parameters of TE=3 ms, TR = 50 ms, 30% echo, flip angle = 45 degrees, NA = 2, field of view = 35 x 25 x 50 mm with a resolution of 256 x 128 x 128 (voxel size = 137 x 195 x 390 um) reconstructed to 256 x 256 x 128, total acquisition time = 30 min. Signal intensity was normalized to an external standard to account for signal drift over time. After injection of Feridex labeled cells, 3D gradient recall echo images were acquired every 30 min for 6.5 h, at 24 h and on days 3, 5, and 7 thereafter. Signal intensity, normalized to an external standard, was measured within anatomical ROI to determine the rate of labeled cell influx or efflux. R₂* (1/T₂*) decreased in proportion to the density of labeled cells in tissue. We used this to calculate the average cell density from normalized signal loss using the equation: Cell Density = α ln(M_unlabeled) - ln(M_SPIO).
M_{unlabeled} is the normalized signal intensity before injection of Feridex labeled cells, and M_{SPIO} is the normalized signal intensity at each time point after injection.

**IDV measurements**

BALB/c mice (n=4), male and 5-8 weeks old, were used for all experiments (Charles River Laboratory, Inc., Wilmington, MA). A single dose of IDV, NP-IDV or NP-IDV-BMM was administered intravenously and tissues (spleen, liver, lung, kidney), sera, and urine were harvested. Briefly, tissues were homogenized in 60% methanol (ml/g), maintained at 4°C overnight and clarified by centrifugation at 20,000xg for 15 min at 4°C. Supernatants were collected and added to glass tubes containing 1 ml diethyl ether. Tubes were mixed for 30 seconds and maintained on ice for 30 min. The ether layer was evaporated to dryness under a nitrogen stream at room temperature. The residue was reconstituted in 100µL of mobile phase [10 mM ammonium dihydrogen phosphate with 1 mM 1-heptanesulfonic acid at pH 4.8 mixed with acetonitrile 65:35 (v/v)]. Rehydrated samples were clarified by centrifugation at 20,000 x g for 5 min. Triplicate 35 µl aliquots of each sample were injected for RP-HPLC analysis. A C4 reversed-phase column with 5µm particle size packing (Phenomenex, Torrance, CA) was used, and analytes were measured at 210 nm. Data was analyzed using chromatographic software (EZStart, Shimadzu), and peak area was integrated, concentrations of IDV were determined compared to a standard concentration curve of IDV in mobile phase. Processing and analyses were validated using known concentrations of IDV and spiking drug into homogenized tissue samples from naive animals.
hu-PBL NOD/SCID mice

Four-week-old male NOD-C.B-17 SCID mice (n=4/group) were purchased from the Jackson Laboratory (Bar Harbor, Maine). Animals were injected once i.p. with rat anti-CD122 Ab (0.2 mg/mouse) and twice with rabbit asialo-GM1 Ab (0.2 mg/mouse) (Wako, Richmond, VA) 2 days before and 3 days after reconstitution with 20-50 x 10^6 human PBL.

HIV-1 infection and NP-IDV-BMM treatments

Hu-PBL NOD/SCID mice (n=4) were treated with single dose of NP-IDV-BMM (1-2 x 10^7) on day 5 after PBL reconstitution. Mice were injected i.p. with HIV-1ADA at 10^2 TCID_{50}/mouse immediately after NP-IDV-BMM administration. Mice were sacrificed 7 and 14 days after viral challenge.

Flow cytometric immunophenotypic analyses

Cells were isolated from hu-PBL NOD/SCID mouse spleens (n=4-6/group). Splenocytes were analyzed with antibodies for mouse and human leukocyte common antigen (LCA; FITC anti-CD45), human CD4 (allophycocyanin anti-CD4), and human CD8 (phycoerythrin anti-CD8) (Boehringer Mannheim, Germany).

ELISA tests

HIV-1p24 concentrations were determined by enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Advanced BioScience Laboratories, Kensington, MD) and HIV-1p24 standard curves. Values were considered positive when the readings of the absorbance in the tested samples were 2-fold higher than those in control samples.
Histopathology and image analyses

Immunohistochemical analysis used antibodies to Vim, HIV-1p24, or CD3. Feridex labeled cells were stained with Prussian blue. Antibody to CD11b (BD Pharmingen, San Diego, CA) was used to visualize macrophages by immunofluorescence. Images were obtained by Optronics digital camera (Buffalo Grove, IL) with MagnaFire 2.0 software (Goleta, CA) and processed by Adobe® Photoshop 7.0 software. Immune-positive cells were enumerated as absolute numbers/field/section in seven sections per mouse.

Statistical analysis

Animal studies were performed with 4-8 animals per group. Significance amongst groups was determined by two-tailed unpaired nonparametric Mann-Whitney test. $P$ values $< 0.05$ were deemed significant. Analysis was done using GraphPad Prism version 4.0a for Macintosh (GraphPad Software).

Results

Characteristics, uptake and cell-based release of NP-IDV

The NP are drug crystals, prepared from the free base, that are coated with phospholipids, which stabilize the nanocrystals and prevent aggregation. The volume-weighted mean size of NP-IDV was approximately 1.6 μm and ellipsoid in morphology. Scanning electron microscopy (SEM) performed on NP-IDV suspensions revealed distinct, smooth-surfaced particles, without gross defects, which maintained structural integrity after loading into cells (Figure 1a). Our previous study of Confocal Z-scan (H. Dou et al., submitted) and transmission electron microscopy
(TEM) images demonstrated the localization of NP-IDV in the BMM cytoplasm (Figure 1b, black arrow head). Entry occurred < 1 h, thereby supporting endocytosis as the likely mechanism for cell entry (Figure 1c). Lissamine™ rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (rDHPE) labeling was accomplished by adding the fluorescent phospholipid described in the article. We did not study the effects of including the labeled phospholipid on surface properties. However, based on the amount of added tracer, the thickness of the phospholipid coating derived from our experience with many other drugs, the number of labeled phospholipid molecules represents a very small fraction of the total coating material. The rDHPE labeled NP-IDV (rDHPE-NP-IDV) produced red-fluorescence. More than 95% of BMM had taken up rDHPE-NP-IDV after 12 h of incubation (Figure 1d). A linear correlation of NP-IDV uptake from $1 \times 10^{-6}$ M to $5 \times 10^{-4}$ M established an upper threshold concentration of $5 \times 10^{-4}$ M, which afforded significant in vitro attenuation of HIV-1 replication without cytotoxicity (data not shown). Light and fluorescence microscopy showed identical intracellular patterns of rDHPE-NP-IDV and NP-IDV (Figure 1c and 1d). The majority of BMM showed high levels of NP uptake after 6 h of culture in the presence of $5 \times 10^{-4}$ M NP-IDV. RP-HPLC assays used to measure intracellular drug concentrations confirmed that intracellular levels of NP-IDV increased during 12 h of incubation (Figure 1e).

Alkaline pH limited the solubility of NP-IDV and improved its physical stability. After NP-IDV was ingested by the BMM, reductions in phagosomal pH enhanced drug release (data not shown). Two very distinct physicochemical steps are involved in the release of intracellular NP-IDV including the initial dissolution of the NP in the cell and subsequent diffusion of the released IDV into the extracellular media. From the release profile of NP-IDV during the initial 12 h after loaded into BMM, an initial burst-release of extracellular IDV at levels approximating
30% of total intracellular concentration was observed within 4 h after fresh media (Figure 1f). In the absence of media changes, a slow diffusion of IDV occurred over 3 days establishing an environment of IDV equilibrium suggesting that diffusion or possibly recycling rather than active transport was responsible for extracellular drug transport. With sequential media changes, intracellular and extracellular levels of IDV progressively diminished and reached a nadir by day 6 (Figure 1g). At this time IDV levels fell below the limit of detection, likely through achieving molecular equilibration.

**BMM tissue migration: A “Trojan horse” for drug delivery**

Parallel BMM migration and viral tissue tropism provide a first step toward proof of concept that the drug, when carried inside the cell, can be transported to tissues of interest where ongoing viral replication occurs. If NP-IDV-BMM could act as “Trojan horses” for transport of drug into tissues known to be targets for HIV, it would direct ART into diseased body regions. To address this question, we measured \(^{111}\)indium-labeled BMM migration by SPECT following adoptive transfer of cells to naive animals. For each recipient, cell migration was analyzed by SPECT at 6 and 24 h and every other day thereafter up to 7 days. Relative counts per cm\(^3\) for each ROI were determined and corrected for radioactive decay. Within 6 h of injection, the largest cell concentrations of BMM were detected in lungs based on radioactive intensity (Figure 2a). Quantitative analysis of BMM density from tomographic images (Figure 2b) showed significant (\(P<0.05\)) accumulation of radiolabeled BMM in lung at 6 h after adoptive transfer compared to other tissues (spleen, liver, and kidney). By day 1, radiolabeled BMM were significantly diminished (\(P<0.001\)) from lung with concomitant increases in liver and spleen (Supplemental 1). BMM levels in liver and spleen remained relatively constant, but not significantly different
from days 1 through 7 (Figure 2b). Histological examination of tissues after adoptive-transfer of Feridex-labeled BMM is shown in Figure 2c.

Confirmation of the SPECT data set was provided by T_{2}\(^*\) weighted MRI and histology using Feridex labeled BMM. MRI tests demonstrated increased hyperdensities of BMM in spleen and liver compared to kidney from 0, 3, 6 and 24 h (Figure 3a, b, c and d). Histological quantification of Prussian blue stained cells (Feridex-labeled BMM) showed the highest numbers of cells in spleen at days 1 and 7 (721±98 and 625±126, respectively) and was distributed primarily within the resident macrophage areas surrounding germinal centers. By day 7, a five-fold increase of BMM was found in spleen compared to liver (625±126 vs. 159±37). Feridex-labeled cells were not detected in lymph nodes collected at day 1 after adoptive transfer of labeled BMM via i.v. infusion. However, by day 7, BMM were detected in both cervical and mesenteric lymph nodes (data not shown).

**BMM carriage of NP-IDV: Parallel of tissue distributions for BMM and IDV**

It was reasoned that BMM loaded with NP-IDV would show parallel distributions of BMM labeled with \(^{111}\)In and Feridex (Figures 2 and 3). Therefore, we engaged the optical properties of red fluorescent rDHPE-NP-IDV to investigate in vivo drug delivery. In this manner, BMM were easily identified under fluorescent microscopy (Figure 4a). The images reflected robust labeling of BMM by rDHPE-NP-IDV. This enabled drug visualization in tissue sections.

Double fluorescent immunohistochemistry performed in tissue sections demonstrated that all red rDHPE-NP-IDV-BMM were identified by CD11b immunopositive macrophages (Figure 4a, yellow). CD11b cells were both rDHPE-NP-IDV positive and negative. The rDHPE-NP-IDV-labeled BMM were widely distributed in the lymph nodes (data not shown), as well as in
the liver and lung. Both rDHPE-NP-IDV and CD11b-positive yellow cells were most numerous in spleen. All together, these findings provide clear evidence that the NP-IDV loaded BMM traffic to areas of active HIV-1 replication.

**Tissue IDV levels after single dose NP-IDV-BMM administration**

RP-HPLC assays were employed to measure IDV levels in tissue, sera, and urine. In these experiments a solution of IDV sulfate (Figure 4b), free NP-IDV (Figure 4c), or nanoparticle indinavir packaged into BMM (NP-IDV-BMM) (Figure 4d and e) were administered into animals as a single dose. IDV and NP-IDV showed equivalent drug levels, time distribution and tissue patterns with peak concentrations at 1 h and rapid elimination by 6 h (Figure 4b and c). In all animals that received IDV solution or free NP-IDV, no significant difference in serum drug levels was measured (data not shown). In contrast, administration of NP-IDV-BMM at drug concentrations identical to that of IDV, elicited sustained IDV in tissue and sera for up to 10 days. More importantly, NP-IDV-BMM delivery showed drug levels in sera >4- and 50-fold of clinical effective plasma concentrations (5-10 μM)\textsuperscript{19-22} at 24 h and 10 days (Figure 4e). Urine levels demonstrated drug excretion over time. NP-IDV-BMM showed no demonstrable toxicity to the animals when measures of weight, behavior, or tissue pathology were used to compare untreated controls and animals that received IDV or NP-IDV (data not shown). To demonstrate release of drug from cells, replicate experiments were performed where IDV was extracted from isolated spleen cells and drug concentrations compared to tissue levels. These assays demonstrated that greater than 30% and 47% of IDV were released from cells at days 1 and 5, respectively. The relative bioavailability of IDV measured in tissues from mice receiving NP-IDV-BMM showed peak levels by day 1 in kidney and lung, and day 3 in liver (Figure 4d).
Rapid progressive elimination of IDV was observed in lung with 600 μM at 6 h and 106 μM (Figure 4d) on day 1 following cell injection. Spleen IDV levels were sustained over 14 days and peaked at 3 days after administration. Drug levels were greater than 50 μM in lymph nodes during the two-week analysis. IDV plasma concentrations reached a peak at day 10 (Figure 4e). Interestingly, NP-IDV-BMM was not detected in the kidney (by rDHPE-NP-IDV staining, data not shown) and IDV levels in kidney were relatively low at each time point suggesting that the drug was likely secreted in urine. These results confirmed that IDV was secreted from cellular stores, as it was present at significant levels in sera and urine and shown to be released from spleen cells, thus providing initial proof of concept for therapeutic drug testing in animal models of human disease (see below).

**Anti-retroviral responses of NP-IDV-BMM in HIV-1 hu-PBL-NOD/SCID mice**

To determine the effects of NP-IDV-BMM administration on long-term antiviral responses, HIV-1p24 antigens were measured in serum by ELISA analysis and in tissue by immunohistochemical tests in HIV-1 challenged hu-PLB-NOD/SCID mice. HIV-1p24 antigen levels were reduced by 23% and 51% between treated and untreated mice at days 7 and 14, respectively (Figure 5a). Numbers of virus-infected cells were next measured in microscopic sections of spleen tissue. CD3+ T cells and HIV-1p24 antigens were readily identified in the empty white pulp of the spleen 14 days after viral challenge in untreated mice (Figure 5b). There was a reduction of HIV-1p24+ cells in the treated mice (Figure 5c) as compared to the untreated animals.

Efficiency of engraftment of human PBL in hu-PBL-NOD/SCID mice was evaluated by vimentin (Vim) immunohistochemistry to assess numbers of human cells. Vim positive cells
were unchanged in NP-IDV-BMM animals and untreated control (Figure 5d). HIV-1p24 immunopositive cells were readily visualized in HIV-1\textsubscript{ADA} infected hu-PBL-NOD/SCID mice and co-localized with Vim in lymph nodes, spleen, liver, and lung. Although engraftment efficiencies for human lymphocytes varied amongst the hu-PBL-NOD/SCID mice, NP-IDV-BMM administration did not affect human lymphoid cell reconstitution (Figure 6a). The largest numbers of human PBL (Vim\textsuperscript{+} cells/field/section) were observed in lymph nodes and spleen and were significantly higher than that found in liver and lung, respectively (Figure 6a, $P<0.01$). To determine whether NP-IDV-BMM treatment affected the numbers of productively infected cells, HIV-1p24 antigen positive cells were assessed as a percent of total human lymphocytes (Vim) (Figure 6b). Decreased numbers of HIV-1 infected cells were observed following NP-IDV-BMM treatment. This was significantly apparent in cervical lymph nodes ($P<0.01$), spleen ($P<0.01$), liver ($P<0.05$), and lung ($P<0.01$) (Figure 6b) on day 14, reflecting a long-term robust anti-retroviral response elicited by NP-IDV-BMM administration. Although mesenteric lymph nodes (M-LN) of NP-IDV-BMM treated mice showed remarkable reduction in HIV-1 infected cells, no statistical differences were observed by days 7 and 14 after viral infection.

**Immune restoration after NP-IDV-BMM therapy**

To assess whether cell delivery of anti-retroviral drugs could initiate immune restoration, we evaluated human CD4 and CD8 T cell populations after HIV-1 infection and single dose administration of NP-IDV-BMM in humanized NOD/SCID mice. Despite reduced numbers of human lymphoid cells in the model, the relative proportions of CD4\textsuperscript{+} and/or CD8\textsuperscript{+} populations paralleled those observed in HIV-1 infected humans\textsuperscript{33}. Engraftment of human PBL was readily demonstrated in spleen, lymph nodes, liver, and
lung. Spleen cells were isolated from HIV-1 challenged hu-PBL-NOD/SCID mice at day 7 and 14. The human CD4\(^+\) and CD8\(^+\) T cells were analyzed against total numbers of (mouse and human) CD45\(^+\) cells (Figure 7a). Numbers of PBL reconstitution were increased from day 7 to 14. Intravenous administration of 10\(^7\) NP-IDV-BMM showed no differences in the ability to reconstitute PBL between untreated (control) and NP-IDV-BMM-treated groups (Figure 7b).

The CD4\(^+\)/CD8\(^+\) human T-cell ratios were reduced after HIV-1 challenge. This progressive loss of human CD4\(^+\) T lymphocytes observed in the spleens of hu-PBL-NOD/SCID HIVE mice was previously observed by our group\(^9,22\). By day 7 after infection, CD4\(^+\) T-cells accounted for 42.2\% and 45.8\% of human T cells in controls (untreated animals) and NP-IDV-BMM treated mice, respectively (Figure 7c). However despite the progressive loss of CD4\(^+\) T cells in both groups by day 14, a significant increase of CD4\(^+\) T cells (24.4\%) was obtained in NP-IDV-BMM treated mice compared to the untreated group (7.6\%) (Figure 7c, \(P<0.05\)), suggesting that NP-IDV-BMM enhanced human CD4\(^+\) T cell survival. At days 7 to 14, the percentage of CD8\(^+\) T cells to human T cells were 57.8\% and 92.4\% in untreated mice and 54.2\% and 75.6\% in NP-IDV-BMM treated animals, respectively (Figure 7d). Thus, no differences were detected in CD8\(^+\) T cells in either NP-IDV-BMM treated and control mice at day 7. However, by day 14, the numbers of CD8\(^+\) T cells in untreated compared to treated HIV-1-infected hu-PBL-NOD/SCID animals were increased (\(P<0.05\)). NP-IDV-BMM treatment provided a significant increase of CD4\(^+\)/CD8\(^+\) cell ratios at day 14 as compared to controls at day 14 (Figure 7e, \(P<0.01\)).

**Discussion**

We used macrophages as a carrier of NP for anti-retroviral drug delivery. The advantages of this system include its stability, controlled release, and enhanced drug bioavailability. This owes to
the fact that particles of such small size are efficient in crossing permeable barriers when packaged into cells. The significance of this work is reflected by its interdisciplinary approaches to assess anti-retroviral responses of drug nanoformulations. The work progressed from analyses of cell biology, to imaging, to biochemical, and pharmacologic approaches in investigations of disease outcomes. Humanized mice permitted analysis of human T cell subsets and viral replication ensuring translation of works from cell culture to “proof of concept” in therapeutic responses. Although IDV therapy can elicit the emergence of clinically significant viral isolates\textsuperscript{24-28}, the NP-IDV BMM delivery system employed under the conditions developed in this report induced significant viral inhibition. Moreover, the animal models employed herein have already demonstrated bench-to-bedside translational potential in ART and adjunctive therapies for HIV-1 infection and virus-associated dementia\textsuperscript{9,29}.

Nanotechnology has revolutionized modern-day pharmacology\textsuperscript{30-34}. The abilities of carriers to alter their size, shape, and composition allows incorporation of drugs with variable biochemical properties\textsuperscript{35}. One example is liposomes, hydrophobic drug-encapsulating lipid containers of submicron size that are commonly used in clinical practice. More recently, drug delivery systems including polymeric micelles\textsuperscript{36,37} and block copolymers have emerged and appear more promising for use in cancer chemotherapy, infectious diseases, photodynamic and gene therapies\textsuperscript{38-40}. Nanoformulations have a number of advantages over more conventional oral administrations of free drugs as they increase systemic bioavailability, attenuation of early drug degradation and solubility. There is precedent for drug nanoformulations in improving delivery and untoward side effects of heparin\textsuperscript{41}, enalaprilat\textsuperscript{42}, tobramycin\textsuperscript{43}, and several of the antituberculosis medicines\textsuperscript{44}. To this end, polylactide-co-glycolide and alginate have already been approved for human use as carriers for drug encapsulation\textsuperscript{45-48}. 
Our novel NP system utilizes a cellular vehicle for NP-drug delivery and shows promise for clinical use. *First*, the cell delivery system relies on the natural phagocyte system for IDV-NP drug delivery. Indeed, macrophages target disease sites where active HIV-1 replication occurs and enhance the amount of drug present at sites of active disease. *Second*, single dosing positively affects therapeutic outcomes. NP-IDV-BMM formulations promote sustained “local” drug release in HIV-1 target tissues for periods equal to or greater than two weeks. *Third*, toxicities were not evident during the administration period in animal behavior, histopathology, weight, or immune function. *Fourth*, NP-IDV-BMM increases the therapeutic index of established drugs by affecting bioavailability and tissue distribution. In all assays performed, drug levels exceeded the therapeutic dose in tissue and sera and were cleared through secretion in the urine. In comparison with classical multiple daily therapies, NP-IDV-BMM showed significant migration into HIV-1 infected areas and delivered ART at levels far exceeding the therapeutic dose. NP-IDV was recently shown to increase brain tissue levels of IDV up to 20X of solution drug in murine HIV-1 encephalitis$^{49,50}$. A ready explanation for the steep drops in IDV serum levels when compared to what is seen in tissue is not yet available. These changes are now being investigated by extending the period of observation beyond one month. At the present time the observed data may reflect a second wave of drug secretion from tissue to circulation beyond what was seen at day 14.

Treatment of HIV-1 associated disease requires life long therapy. In contrast to multiple daily dose regimens, NP-IDV-BMM has improved efficacy. If similar approaches can be employed in combination therapy, a likely possibility, such NP systems could affect drug usage in the developing world. We are currently planning a nanoformulation trial for HIV-1 infected patients who have failed conventional therapy or in whom oral administration cannot be
administered or absorbed adequately. For this work a “modified” leukophoresis system for monocyte-enriched PBMC fractionation would be performed at the time of drug administration during the cell separation procedure. This would serve to minimize cell manipulations. The numbers of autologous monocytes required for treatment in humans still awaits cell-dose response measures and pre-clinical drug biodistributions. All together, the stated improvements in anti-retroviral dosing, economy, sustained release, and drug bioavailability could make ART nanoformulations attractive for human use.

Acknowledgements

We thank Robin Taylor for excellent administrative support. This work was supported by Baxter Pharmaceuticals and from National Institute of Health Grants 2R37 NS36126, PO1 NS31492, 2R01 NS034239, P20RR15635, PO1 NS43985 and P01MH64570.

Endnotes

References


**Figure 1. NP-IDV synthesis, cell uptake and release.** (a) SEM (magnification ×12,000) analysis showed smooth surfaces of NP with sizes of approximately 1.6 um. (b) TEM (original magnification ×20,000) demonstrated uptake of NP-IDV into BMM (arrowhead). (c) BMM cytoplasm appeared dark by light microscopic examination due to uptake and concentration of NP after culture in the presence of NP-IDV for 12 h. (d) Fluorescence microscopy of BMM co-cultured with rDHPE-NP-IDV (red) confirmed intracellular localization of NP-IDV. Ingested NP appeared as red fluorescent dots and showed intensity located within cytoplasm. (e) Levels of IDV were assayed by HPLC from lysates of cultured BMM sampled at specified times. (f) After single washout, extracellular (media) and intracellular (BMM) IDV levels were determined. (g) With subsequent media changes, intracellular and extracellular levels of IDV progressively diminished until reaching a nadir at day 6, when IDV levels fell below the limit of detection.
Figure 2. BMM tissue distribution assessed by SPECT and histological tests. (a) BMM migration and tissue distribution of $^{111}$In-labeled BMM are illustrated by SPECT analysis. Planar presentations of the tomographic images from a representative mouse show that radiolabeled BMM initially accumulate in the lung (Lng), liver (Liv) and spleen (Spl). (b) To quantify BMM migration, ROI were circumscribed and radioactive counts determined as a function of time (days) after adoptive transfer. (c) Histological analysis of Feridex-labeled BMM, as determined by Prussian blue stained (blue cells), were consistent with SPECT data. BMM in liver and lung was less than in observed in spleen after day 7. Magnification is 100x and 400x.
Figure 3. BMM tissue distribution assessed by MRI tests. MRI tests were used to track BMM migration. Feridex was administered to BMM in vitro. After Feridex labeling the BMM were
administered i.v. to immune competent mice. Signal loss is shown in the spleen (arrow) over time from 0, 3, 6 and 24 h (a, b, c and d) after adoptive transfer demonstrating cell migration.
Figure 4. NP-IDV tissue distribution and pharmacokinetics. (a) Sections of spleen, liver and lung from mice after day 5 post transfer of rDHPE-NP-IDV labeled BMM were stained for
CD11b and examined by fluorescence microscopy. Higher magnification inserts demonstrate the presence of rDHPE-NP-IDV (red) co-localized in the cell cytoplasm of CD11b⁺ cells (green). BMM (yellow), were abundantly present in spleen, but were less in liver and lung. (b-e) IDV distribution in targeted tissues and body fluids were assessed in mice treated with a single i.v dose of (b) IDV sulfate solution, (c) cell-free NP-IDV, or (d and e) NP-IDV-BMM. In contrast to IDV concentration nadirs within 6 h post treatment in mice treated with IDV sulfate solution or NP-IDV, levels were prolonged in tissues and plasma over 14 days in mice treated with NP-IDV-BMM. Magnification is 100x and 400x.
Figure 5. Anti-retroviral activities of NP-IDV-BMM in HIV-1 infected hu-PBL NOD/SCID mice. (a) Serum HIV-1p24 levels (Mean ± SEM) from HIV-1 challenged hu-PBL-NOD/SCID mice untreated (control) or treated with NP-IDV-BMM after days 7 and 14. Spleen sections from (b) untreated or (c) NP-IDV-BMM treated mice were immunostained for CD3 (pink) and HIV-1p24 (brown). CD3⁺ T cells were observed at comparable frequencies, whereas HIV-1p24 positive cells were vastly diminished in NP-IDV-BMM treated mice. *P<0.05 compared to untreated controls. Magnification is 200x and insets 400x. (d) Distribution of human PBL (Vim⁺) and HIV-1 infected cells (HIV-1p24⁺) were evaluated in lymph nodes, spleen, liver, and lung from HIV-1 infected mice that were untreated or treated with NP-IDV-BMM. Magnification is 200x.
Figure 6. Quantitation of HIV-1p24 expressing cells in NP-IDV-BMM-treated HIV-1 infected hu-PBL NOD/SCID mice. (a) Numbers of human PBL in cervical lymph nodes (C-LN), M-LN, spleen, liver, and lung were determined from Vim⁺ immunostained sections. (b) Numbers of HIV-1p24⁺ cells were normalized to total Vim⁺ cells and expressed as the mean percent (± SEM). *P<0.05 and **P<0.01 compared to untreated controls.
Figure 7. T cell subset analyses in hu-PBL NOD/SCID mice after NP-IDV-BMM-treatment. (a) Flow cytometric histograms of splenocytes from mice 14 days following NP-IDV-BMM treatments were used to determine the numbers of mouse CD45 cells and human reconstituted CD4$^+$ and CD8$^+$ T cells. (b) The level of human lymphocyte reconstitution in mouse spleens was determined as compared to mouse CD45$^+$ cells. (c and d) Frequencies of (c)
CD4⁺ and (d) CD8⁺ T cells were assessed as percentages of total human T cells. (e) Ratios of CD4⁺/CD8⁺ T cells increased significantly in NP-IDV-BMM treated group compared to control animals. *P<0.05 and ** P<0.01 compared to untreated controls.
Development of a macrophage-based nanoparticle system for anti-retroviral drug delivery

Huanyu Dou, Christopher J Destache, Justin R Morehead, R L Mosley, Michael D Boska, Jeffrey Kingsley, Santhi Gorantla, Larisa Poluektova, Jay A Nelson, Mahesh Chaubal, Jane Werling, James Kipp, Barrett Rabinow and Howard E Gendelman