MACROPHAGE TARGETING USING NANOTECHNOLOGY FOR ANTI-HIV THERAPY

by

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ABSTRACT OF THE DISSERTATION

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Professor Patrick J. Sinko

Macrophages (MΦ) are involved in a number of pathological conditions, such as HIV infection/AIDS, tuberculosis, tumor development and atherosclerosis. The macrophage mannose receptor (MR) is expressed on the cell surface of tissue MΦ where it mediates the internalization of glycoproteins and glycoconjugates via both the endocytic and phagocytic pathways. The theme of the current study is to develop a nano-sized therapeutic delivery system that targets the MΦ MR. A series of mannosylated NCs were designed and synthesized to systematically evaluate the effects of mannose copy number, mannose ligand spacing and poly (ethylene glycol) (PEG) size on NC uptake via MR. Targeted nanocarriers (NCs) bearing mannose moieties were evaluated for targeting in MΦ cell lines. The first study demonstrated three important structural requirements for optimal mannose-targeted NC cellular uptake - two copies of the targeting ligand mannose were required, a 46.5 Å PEG spacer in between ligands was optimal and 12 kDa PEG size. Furthermore, the effect of MΦ polarization on NC uptake was also investigated. Rat peritoneal MΦs were polarized into classically activated (M1) and
alternatively activated (M2a) phenotypes. The Western blot results showed the highest expression of MR on the M2a phenotype. Using confocal microscopy and a fluorescence microscope, 12-fold higher uptake of NC (2 copy mannose) was observed in M2a MΦ as compared to M1 MΦ, suggesting great potential in selective MΦ cellular targeting. Drug-loaded NCs were prepared using a nucleoside reverse transcriptase inhibitor (AZT) or a protease inhibitor (RTV) conjugated to a NC with 2 copies of mannose linked by a PEG$_{12}$ (46.5 Å) spacer. Anti-HIV activities of the drug conjugated NCs were assessed using U937/HIV-2$_{MS}$ cells and HIV-1 infected human monocyte derived MΦs and were based on a reduction in viral matrix protein p24 production. The results showed significant p24 reduction with EC$_{50}$’s ranging from 5.7 uM to 25 uM indicating significant anti-HIV activity. Taken together, the studies presented in this dissertation identified the optimum configuration of drug loaded NCs for targeting the MΦ MR. Furthermore, the positive anti-HIV activities suggest that the NC approach holds great promise for receptor targeted drug delivery to HIV-infected MΦs.
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1 Chapter 1. Structural Optimization of Mannosylated Nanocarriers for Macrophage Targeting

1.1 Introduction

Human immunodeficiency virus (HIV) infection and acquired immune deficiency syndrome (AIDS) have caused the death of more than 30 million people worldwide becoming a major global health and social concern over the last thirty years. It was found that highly active antiretroviral therapy (HAART) reduced plasma viremia to clinically undetectable levels (less than 50 copies per milliliter plasma) within two months of the initiation of treatment [1, 2]. However, the eradication of HIV-1 infection is still not achievable because of the persistence of virions in reservoir sites even after HAART treatment occurs in extremely low frequency ($10^6$–$10^7$ latently infected cells per individual, i.e., 0.1–1 cell per million lymphocytes) [3]. Upon the discontinuation of treatment, there is a relapse of infection from the reservoir sites. The two major cellular reservoirs in the natural host are latently infected resting CD4$^+$ T cells and macrophages [4]. CD4, the primary receptor for HIV, is a glycoprotein expressed on the surface of T helper cells, regulatory T cells, monocytes, macrophages and dendritic cells. HIV infection is initiated by the interaction between gp120 of HIV and CD4 on the target cells or with the chemokine co-receptors CXCR4 and CCR5 [5]. HIV also can undergo carbohydrate-mediated binding to the endocytosis receptor or phagocytosis receptor on MΦs. Infected MΦs are found in all tissues including the brain, lungs, gut, liver, spleen, and lymphoid tissues.

Targeted drug delivery is an important focus in the treatment for AIDS since
specific cell populations are involved in HIV infection. Most anti-HIV agents have poor physical and chemical properties and bioavailability problems stemming from poor permeability and stability [6, 7]. Nanotechnology-based delivery systems have been developed to improve therapeutic efficacy in HIV/AIDS by delivering drugs to cellular and viral reservoirs. The systems have the ability to incorporate, protect and promote the absorption of anti-HIV drugs. The most common NCs include polymeric nanoparticles, solid lipid nanoparticles (SLNs), liposomes, nanoemulsions, dendrimers, and drug conjugates [8, 9]. It has been demonstrated that nanoparticle indinavir (IDV) packaged in bone marrow-derived MΦs (BMM) markedly improved the bioavailability and pharmacokinetics of IDV, suggesting the potential utility in the treatment of HIV-1 infection [10].

Receptor-mediated, targeted drug delivery is a promising approach for achieving efficient therapy with minimal systemic toxicity. MΦs express a range of receptors including scavenger receptors, formyl peptide receptors, integrins, mannose receptors, galactose receptors and Fc-receptors that could be utilized for receptor-mediated drug targeting and internalization. Targeting MΦs using peptides or nanoparticles (NP) with ligands such as mannose, galactose, tuftsin and fMLF has been reported in the literature [11-13]. Previously, our lab has successfully targeted MΦs via the formyl peptide receptor (FPR) using a novel PEG-based delivery system[14, 15]. By targeting FPR using multivalent N-formyl-Met-Leu-Phe (fMLF) NCs, significant cellular uptake and organ distribution such as accumulation in the liver, kidney, and spleen was achieved in mice [14, 15].
The mannose receptor (MR, MRC-1), a 175-kDa integral membrane protein, localizes on sinusoidal liver cells, peripheral and bone marrow MΦs, and dendritic cells. The MR, consisting of a cysteine rich domain, fibronectin type II domain, a multiple C-type lectin like domain (CTLDs) (also named carbohydrate recognition domains) and a transmembrane domain, is a type I transmembrane protein which has C-terminus on the cytoplasmic side of the membrane. MR is capable of recognizing and internalizing mannose, fucose and N-acetylglucosamine terminated molecules [16, 17]. Natural ligands include the carbohydrates on many bacteria and virus surfaces with terminal mannose units displayed in a repeated pattern. The MR can also bind and mediate the internalization of a wide variety of pathogenic microorganisms including HIV and the fungi Candida albicans, parasites such as Leishmania donovani and bacteria including Pneumocystis carinii.

The multiple CTLDs in a single polypeptide of the MR mediate interactions with its mannosylated ligands and also contribute to a clustering effect for ligand binding, which appears to be necessary for achieving high affinity binding [18]. CTLD 4-8 are involved in binding to multivalent ligands and must be present to achieve the optimum affinity ($K_d=40$–90 nM) [19-22]. The MR multivalency binding effect has been shown in several studies, suggesting that clustering of multiple CTLDs within the single polypeptide backbone of a drug delivery system may be important for increasing high affinity binding of oligosaccharide moieties [17].

Enhanced uptake and internalization of mannosylaed gelatin NPs was observed in the uptake into J774 cell and into MΦ tissues via MR than the uptake of unconjugated NPs [23, 24]. The mannan-coated NPs showed 50% higher uptake at 37° and over 2 fold
at 4° than those of the uncoated NPs [25]. Mannosylated liposomes have also been investigated as vectors for targeted delivery. Compared to the cell uptake of plain liposomes, enhanced cellular uptake into J774.A1 МΦ cells of mannosylated liposomes has been observed [26]. Furthermore, stavudine loaded liposomes conjugated with mannose or galactose showed increased cellular uptake compared to plain liposomes and free stavudine[12, 27, 28].

HIV binds to MR expressed on both dendritic cells and МΦs mainly via mannose oligosaccharides. Approximately 50% of the carbohydrates on gp120 of HIV are terminally mannosylated [29]; and a mannosylated terminal is a common pattern of many pathogenic organisms including bacteria, fungi and viruses [30]. It has been demonstrated that the gp120-binding protein is a membrane-associated mannose-binding lectin [31], and the uptake of HIV into МΦs occurs independently of the membrane-associated CD4 molecule [32]. HIV can bind to MR and enter МΦs but this pathway does not lead to HIV viral replication [33].

Once bound to MR, HIV is internalized and enters the cytoplasm by endocytosis or phagocytosis and is released from early endosomes or phagosomes and presented by MHC II to T cells [34]. MR is known to take up and present mannosylated antigens to T cells [35]. Furthermore, МΦ MRs are able to mediate transmission of bound HIV to co-cultured T cells [36].

PEGylation is the process of covalently attaching chains of poly (ethylene glycol) (PEG) to proteins or other drugs. PEG polymers are amphiphilic, nontoxic and are eliminated by a combination of renal and hepatic pathways [11]. Along with reduced immunogenicity and antigenicity, PEGylation is a well-known method for increasing the
solubility, blood circulation half-life and physical stability [37] [38]. Wattendorf et al postulated that PEG forms a protective shell hindering the reaction with immune cells such as dendritic cells and MΦs, both of which are the cells targeted for infection by HIV [39, 40]. The ideal MΦ targeted NCs should carry PEG polymers and would able to bind to receptors on MΦ. The specific receptor targeting to selected cells further decreases the undesired drug toxicity.

In this study, novel NCs consisting of a PEG-based polymer, imaging agent, and multivalent mannose targeting agents were designed and synthesized. To avoid the capping effect in ligand binding to the MR, the mannose copy numbers at NCs is carefully designed and studied. The uptake mechanism of NCs into MΦs was determined to be through MR-mediated endocytosis. The optimum NC structure for MΦ targeting required two copies of the targeting ligand, mannose with a 46.5 Å spacer between the two mannoses and a PEG\textsubscript{12KDa} scaffold.

1.2 Materials and Methods:

1.2.1 Materials

Amide Sieber resin was obtained from Anaspec (Fremont, CA), Fmoc-gamma-Abu-OH, Fmoc-Serine-OH, Fmoc-Cysteine-OH were purchased from Chem-impex (Wood Dale, IL), mPEG\textsubscript{x}-maleimide (x=5KDa, 12KDa, 20KDa, 30KDa, 40KDa) were obtained from NOF (Tokyo, Japan), α-D-Mannose pentaacetate, boron trifluoride diethyl etherate, N,N-Diisopropylethylamine (DIPEA) were obtained from Sigma Aldrich, Trifluoroacetic acid
(TFA) was obtained from Fisher Chemical. Amino-dPEG$_6$-acid, amino-dPEG$_{12}$-acid and amino-dPEG$_{20}$-acid were purchased from Quanta biodesign (Powell, Ohio).

### 1.2.2 Serine-Mannose pentaacetate (Ser(Man) in brief) synthesis

Fmoc-L-Ser-OH and $\alpha$-$\delta$-Mannose pentaacetate in molar excel were dissolved in anhydrous dichloromethane. Boron trifluoride etherate is added dropwise. The reaction mixture is stirred for 20 hours under argon, diluted with dichloromethane and washed with 1M HCl and water. The organic phase is dried over MgSO$_4$. The crude mixture was purified using flash chromatography on silica gel, resulting in the final protected mannosylation product (Ser(Man)) at a yield of 70%.

### 1.2.3 Synthesis of Nanocarriers

Multiple copy mannose NCs (Figure 1-1): FITC-Gaba-PEG$_{12}$-Ser(Man)-Gaba-Gaba-Cys-amide, FITC-Gaba-Ser(Man)-PEG$_{12}$-Ser(Man)-Gaba-Gaba-Cys-amide, FITC-GABA-Ser(Man)-PEG$_{12}$ -Ser(Man)-PEG$_{12}$-Ser(Man)- Gaba-Gaba-Cys-amide, and FITC-Gaba-Ser(Man)-PEG$_{12}$-Ser(Man)-PEG$_{12}$-Ser(Man)-PEG$_{12}$-Ser(Man)-Gaba-Gaba-Cys-amide were synthesized on solid phase using a Nautilus auto synthesizer (Figure 1-2, 1-4, 1-7, 1-10) NovaSynTG Sieber resin was used in the amino acid coupling with molar ratio 1:4:4:8 (resin: amino acid: coupling reagent (HOAT, HATU): basic reagent (DIPEA)) for four hours reaction time. Serine-mannosepentaacetate and PEG$_{12}$ amino acid were conjugated to the peptidic core as regular amino acid coupling with molar ratio 1:2:2:4 (resin: amino acid: coupling reagent (HOAT, HATU): basic reagent (DIPEA)) in Nautilus peptide auto synthesizer for 12 hours reaction time. Fmoc group was removed by using 20% piperidine in NMP. NMP was also used as the washing solvent. MALDI
TOF/MS and analytical reverse phase high-performance liquid chromatography (RP-HPLC) were performed in between amino acid couplings to confirm the molecular weight and purity. Five equivalents of fluorescein isothiocyanate (FITC) was dissolved in NMP and reacted to the N terminal of the peptide in solid phase for 24 hours. The acetyl group of the mannosepentaacetate at the serine moiety was removed by using sodium methoxide in methanol anhydride in solid phase for 1 hour. Peptides were cleaved from the resins by using the cleavage solution (85% DCM, 2% DODT, 1% TIS, 12% TFA) reacted for 1.5 hours. The cleaved peptides were then precipitated in 50% cold ether and 50% hexane in volume to remove the scavengers and impurities. Semi-preparative HPLC (RP-HPLC, C18 column) was performed to purify the synthesized NCs. Purified NCs were then lyophilized.

PEGylation was performed in liquid phase by reacting 5 equivalent mPEGx-maleimide (x= 5, 12, 20, 30, or 40 KDa) to the cysteine moiety at the NCs in 1 mL of phosphate-buffered saline (PBS, pH 7.4). The reaction was stirred overnight at room temperature. Sephadex G-50 or Sephadex G-15 column was used to purify the PEGylated NCs. Aliquots of the purified PEGylated NCs were collected and characterized using MALDI-TOF/MS (Figure 1-10-Figure-1-14). The collected PEGylated NCs were then lyophilized.

NCs with various PEG linker lengths ranging from 25.1 Å to 75.2 Å were synthesized to study the effect of length on cellular uptake. Fmoc-PEG6-OH (25.1 Å), Fmoc-PEG12 –OH (46.5 Å), or Fmoc-PEG20-OH (75.2 Å) were used as the linkers in between mannose moieties of NCs. The NCs were characterized by RP-HPLC and MALDI-TOF/MS.
1.2.4 Cellular Uptake and Analysis of NCs

Cell line

J774 cells, which were originally isolated from murine МΦ tumor cells, are heterogeneous in the expression of functional MRs. J774 clones (A1, B1, C1, A4, C2) were selected based on their distinct and stable ability to endocytose mannose BSA. The J774.E clone showed the highest MR activity and the highest total number of binding sites [41].

Cell Uptake Analysis

J774.E was a gift from Dr. Philip D. Stahl, Washington University (St. Louis, WA). The cells were cultured in medium RPMI 1640 containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin at 37°C in 5% CO₂-95% air atmosphere. Cells were removed from the flasks by treating them with 0.05% trypsin-EDTA at 37°C for 3 minutes. To induce a higher degree of cell differentiation, poly-D-Lysine was utilized to coat the 24 wells plate. J774.E cells were adjusted to 1 x 10⁵ cells/ml. Corresponding control products were incubated with cell medium and were allowed to adhere (2.5 x10⁴ cells/well) to 24 well plates. All incubations with test articles occurred over a 1 hour period. Aliquots of acid wash solution (0.5 M sodium chloride and 0.2 M acetic acid (pH 2.5)) at room temperature were added to each well for 3 minutes (1X). Phosphate buffered saline (PBS) was used to wash cells (3X). A fluorescence microscope was used to observe cellular uptake. Following microscopy, cells were lysed with 1 N NaOH overnight and neutralized with 1 N HCl the next day. The quantification of cellular uptake was determined by using fluorescence plate reader (excitation: 485 nm, emission:
517 nm). Each cell well was normalized against the cell protein amount as determined with each lysate using the Bradford protein assay reagent purchased from Pierce.

### 1.2.5 Uptake Inhibition by Mannan

The cellular uptake mechanism was initially investigated using an inhibition study. The studies were performed by incubating J774.E cells with NCs in the presence of a mannose polymer and known MR inhibitor, mannan. Mannan was pre-incubated with cells for 40 minutes and were then added to the incubation with cells at 37 °C, pH =7.0 for 1 h. Washing steps and cellular uptake quantification were performed as described previously.

### 1.2.6 Temperature-Dependent Study

To confirm the uptake of NCs into MΦs via MR receptor mediated endocytosis, J774. E cells were incubated with NCs for 1 hour at 37 °C (in a CO₂ free incubator) or 4 °C. At the end of the incubation period, cells were washed with PBS buffer (3X) and the acid-wash solution (1X) (0.5 M sodium chloride and 0.2 M acetic acid (pH 2.5)). The Bradford protein assay and a fluorescence reader were used to quantify cellular uptake.

### 1.2.7 Uptake Image by Fluorescence Microscopy

J774.E cells were incubated with the two- and four-copy mannose NC along with their control NCs (i.e., lacking mannose). The fluorescence microscope images of both controls showed no signal. A weak fluorescence signal, suggesting weak cell uptake, was observed for the four-copy mannose NC (Figure 1-19). A stronger fluorescence signal was observed for two-copy mannose NC uptake. The presence of punctate fluorescence suggests that the mannose bearing NCs were internalized via endocytosis (Figure 1-19).
1.2.8 Confocal Microscopy

J774.E cells were seeded in Laboratory-Tek II 4-chamber cover glasses slides. When the cells reached 80% confluence they were incubated with 80 nM NCs in 20 mM HEPES-buffered (pH 7.4) Hanks’ balanced saline solution (HBSS) for 1 hour at 37°C. The incubation media included a general fluid endocytosis marker rhodamine B-labeled dextran (10,000 MW) and the nuclear dye DAPI. Washing steps were performed as described previously. The confocal microscopy was performed on a Leica TCS SP2 spectral confocal microscope using the XYZ mode. A middle cell section is shown in each image (Figure 1-20).

1.3 Results:

The synthesis of NCs with 1-4 mannose copy number: FITC-Gaba-PEG$_{12}$-Ser(Man)-Gaba-Gaba-Cys-amide(Figure 1-2), FITC-Gaba-Ser(Man)-PEG$_{12}$-Ser(Man)-Gaba-Gaba-Cys-amide(Figure 1-4), FITC-GABA-Ser(Man)-PEG$_{12}$-Ser(Man)-PEG$_{12}$-Ser(Man)-Gaba-Gaba-Cys-amide (Figure 1-6), and FITC-Gaba-Ser(Man)-PEG$_{12}$-Ser(Man)-PEG$_{12}$-Ser(Man)-PEG$_{12}$-Ser(Man)-Gaba-Gaba-Cys-amide (Figure 1-8) were analyzed and purified by RP-HPLC and characterized by MALDI-TOF/MS (Figure 1-4, Figure 1-5, Figure 1-7, Figure 1-9). The PEGylated (PEG 5KDa, 12KDa, 20 KDa, 30 KDa or 40 KDa) NCs were purified by sephadex column and characterized by MALDI-TOF/MS (Figure 1-10, Figure 1-11, Figure 1-12, Figure 1-13, Figure 1-14).

1.3.1 Temperature Dependent Uptake of PEGylated NCs

In three separate experiments, NCs incubated at 37 °C showed a nearly two fold increase
in fluorescence compared to cells incubated at 4°C suggesting NC uptake into MΦs is energy dependent consistent with a receptor-mediated mechanism (Figure 1-15). A substantial portion of cell-associated NCs at 4°C were bound to the cell surface but were not internalized. Control NCs (without mannose) showed a similar pattern of low uptake at both temperatures.

1.3.2 Uptake Inhibition by Mannan

The specificity of uptake was investigated using the mannose polymer, mannan. J774.E cells were incubated with 80 nM two-copy mannose NC at 37 °C in the presence of 5 mg/ml mannan. Non-mannosylated NCs were used as a negative control in the presence or absence of mannan. Mannan led to a 65.1% decrease in NC uptake, while there was only a slightly difference for the control NCs in the presence or absence of mannan (Figure 1-16).

1.3.3 Effect of Mannose Copy Number at NC in Cellular Uptake via MR

The effect of mannose copy number on NC uptake was evaluated. Uptake of NCs with 1-4 copies of mannose was significantly higher than that of the corresponding control NCs without mannose indicating MR-dependent uptake. The two-copy mannose NC showed the highest uptake (taken as 100%), followed by the one-copy (50%), three-copy (40.6%) and four-copy (23.4%) NCs (Figure 1-17). One possible reason for the reduction in internalization of the multiple mannose copy NCs is a “capping effect” on the cell surface. To investigate this possibility, fluorescence and confocal microscopy were used with the four-copy mannose NC.

1.3.4 Effect of NC Size in Cellular Uptake via MR
To evaluate the effect of NC size on MΦ uptake, NCs were conjugated using 5 K, 12K, 20 K, 30K or 40 K mPEG-maleimide scaffolds. MALDI-TOF/MS was utilized to confirm the molecular weight of purified PEGylated NCs (Figure 1-10Figure 1-11Figure 1-12Figure 1-13Figure 1-14). The PEGylated NCs were incubated with J774.E cells for 1 h at 37 °C. Increasing PEG scaffold size from 5 kDa to 12 kDa enhanced (41.17%) the uptake of the two-copy NC (p < 0.05). However, when the PEG scaffold increased to 20 kDa, 30KDa and 40KDa, NC uptake decreased by 32.3 %, 35.29 % and 52.94% (p <0.05), respectively (Figure 1-18). Based on these results it appears that among the NC scaffolds studied, the 12 kDa was the optimal size as it relates to MR-mediated cell uptake.

1.3.5 Effect of PEG linker length in cellular uptake via MR

The effect of linker length was also studied using three different linkers (PEG₆, PEG₁₂ and PEG₂₀) holding the mannose copy number constant at two. The uptake of mannosylated NCs (80 nM) with the three linkers into J774.E cells was evaluated. The uptake conditions and washing steps were the same as mentioned previously. The optimal NC cell uptake occurred with the dPEG₁₂ (46.5Å) linker, followed by dPEG₂₀ (75.2 Å) linker and the dPEG₆ (25.1 Å) linker (Figure 1-21).

1.4 Discussion

Targeting the MR is particularly attractive because of its relatively narrow expression pattern primarily found on liver cells, peripheral and bone marrow MΦs, and dendritic cells. Of course, the latter two cell types are also important targets of HIV
infection. This is highly significant since these two cell types have also been implicated in the persistence of HIV infection and the difficult task of eradicating HIV-1 from these reservoir sites.

Several investigators have attempted to target the MR. The design and synthesis of multivalent mannose glycoconjugates that bind to MR has been clearly demonstrated suggesting that the targeting aspect of the approach is feasible [46, 47]. The MR C-type lectin like domains (CTLDs) can bind mannose, fucose and N-acetylglucosamine with the coordination of Ca\(^{2+}\) [44]. However, only CTLD 4 of MR is able to bind sugars although CTLD 5 might support ligand recognition by CTLD 4 [48]. Using proteolysis experiments, some CTLDs were found to be in close contact to each other in pairs (CTLD 1 and 2, CTLD 4 and 5, CTLD 7 and 8). The exposed linker regions were found to separate the domains of CTLD 3 and 6 from their neighboring domains [48]. The three dimensional arrangement of MR has been investigated using single particle electron microscopy and hydrodynamic measurements on analytical ultracentrifugation [49]. At least two alternative models, an extended model and a more compact conformation were proposed to regulate differences in ligand selectivity [50]. It was suggested that the cysteine-rich and FNII domains of MR fold back in order to bind to the CTLDs. Environmental pH and ligand binding regulate the conformational switch between the compact and extended structure of MR. The bent and globular configuration plays an important role in ligand recognition and ligand binding [50]. The long-term goal of this research is to exploit the structural uniqueness of the MR for the selective delivery of therapeutic agents.
In order to probe the unique properties of MR and the influence of NC configuration on CTLD binding, NCs containing one-, two-, three- or four-copies of mannose along with their control NCs (without mannose moieties) were successfully designed and synthesized. They were characterized using MALDI-TOF/MS (Figure 1-3, Figure 1-5, Figure 1-7, Figure 1-9) and HPLC. Results showed interesting patterns of NC total cell association and uptake. First, the mannose analog-inhibitable cell uptake of all four mannosylated NCs was significantly higher than that of the corresponding non-mannosylated control NCs. These studies and the temperature dependence and inhibition results collectively indicate that the initial goal of targeting the MR was achieved (Figure 1-17). Further, the co-localization of MR and the clathrin adaptor protein suggest a mechanistic role for clathrin dependent endocytosis of the MR [52]. The MR is present in both early and late endosomal compartment. While most of the intracellular MR was found in early endosomes little found in late endosomes or lysosomes [52]. In this study, the cellular uptake of NCs showed MR-mediated endocytic uptake (Figure 1-16, Figure 1-20). The MR bound ligand is found to be internalized into early endosomes with the MR. The colocalization of MR with the clathrin adaptor protein, alpha-adaptin, suggests that the MR might recycle from the cell membrane through clathrin-coated vesicles [52, 58]. The acidification environment and degrading enzymes in endosomes or lysosomes provides a great potential for controlled drug release. The MR-mediated binding and internalization could be further utilized in designing a novel drug delivery system.

After establishing that the mannosylated NCs target the MR, the objective was to begin to probe the relationship between NC scaffold size and uptake into target cells. The NC
scaffold plays a critical role in the potential efficacy that can be achieved as it will have to carry the drug payload. In fact, in order to keep the NC dose as low as possible, the NC will likely have to carry multiple drug molecules. The PEGylated NCs were incubated with J774.E cells for 1 h at 37 °C. The current NC cell uptake results showed that increasing PEG size from 5 kDa to 12 kDa enhanced uptake by 41.2 % (p < 0.05). However, when the PEG scaffold size increased to 20 kDa, 30kDa and 40kDa, NC uptake decreased by 32.3 %, 35.29 % and 52.94% (p < 0.05), respectively (Figure 1-18). This suggests that the 12 kDa was the optimal size among the five sizes that were investigated. It has been estimated that the molecular sizes of 5 kDa, 12kDa, 20 kDa, 30kDa and 40 kDa PEG molecules are about 2nm, 5nm, 7nm, 8nm and 10nm [11]. Through size-exclusion chromatography and gel electrophoresis, it has been shown that each ethylene glycol unit of PEG molecule interacts and binds to two to three water molecules, which make PEG appear to be 3 to 9 times larger than a soluble protein with a comparable molecular weight [53]. Using this estimate the optimal NC diameter is 5nm (corresponding to 12kDa). If hydration is accounted for the final size range is 15-45 nm. The current results are consistent with previous NC reports in the literature. For example, previous studies on the effect of size on NC cellular uptake have shown that nanoparticles that were <50 nm exhibited significantly better uptake than nanoparticles > 50 nm [54, 55]. Moreover, Aoyama et.al concluded that receptor-mediated endocytosis is size-dependent with an optimal size ~25 nm, which corresponds well to our current uptake results (Figure 1-18)[56, 57].
Given the potential complexity of NC binding to MR especially when consider the roles of the CTLDs, our next objective was to explore the influence of flexibility and ligand spacing on binding and uptake. The carbohydrate-binding sites at the distal end of the mannose binding protein (MBP) are separated from each other by 53 Å (rat) [42] and 45 Å (human) [43]. The most important carbohydrate-binding domain among the eight on the MR is CTLD-4. The carbohydrate recognition domain (CRD) of MBP-A showed very similar monosaccharide specificities for binding mannose, GlcNAc, fucose and galactose [44, 45]. To evaluate the effect of NC spacer length on NC cellular uptake via MR, three spacer lengths, dPEG₆ (25.1 Å), dPEG₁₂ (46.5 Å), dPEG₂₀ (75.2 Å) were evaluated. Simulation modeling (Insight II) was performed on the three spacers to further confirm linker size. The modeling results confirmed the length information provided by Quanta Biodesign (data not shown). The cellular uptake result showed that the NC with the dPEG₁₂ spacer had the optimum MR uptake among the three NCs that were studied. This is possibly significant since the 46Å length of the dPEG₁₂ spacer corresponds to the distance between MR CTLDs in human MBP, which has a very similar atomic structure to MΦ MR. The flexibility and length of the spacers also could influence the inter- and intra-MR binding properties of the NCs. Since a dramatic reduction in uptake was observed with 3- or 4-copy mannosylated NCs, the possible mechanisms for this phenomenon were explored. Fluorescence microscopy clearly demonstrated that a capping effect occurred at the cell surface when NCs were functionalized with four-copies of mannose (Figure 1-19). It is well known that multivalent binding and lattice formation on the cell surface are required for cap formation, an energy dependent process [51]. It is possible that a NC ligand could bind to more than two MR peptides on the cell
membrane, forming a lattice-like NC-MR on cell surface. The cross-linked NC-MR aggregate is then swept to one end of the cell to form a cap, which would interfere and inhibit receptor-ligand internalization [51]. This result suggests that the development of mannosylated NCs that target the MR will require careful optimization as a delicate balance between size, ligand spacing and flexibility, payload size, etc will ultimately effect the cellular disposition of the NC.

1.5 Conclusion

In the current studies, the optimum structure of mannosylated NCs for targeting MΦs via MR was assessed. A series of NCs with 1-4 copies of mannose were designed and synthesized. The results suggest that the two-copy mannose NCs with a PEG$_{12}$ spacer (46.5Å) and a scaffold of PEG$_{12kDa}$ result in optimal interactions with the MR on MΦs. We are now in the process of extending these studies by systematically conjugating therapeutic payloads that will selectively release their cargos intracellularly.
Figure 1-1 Structure of PEGylated Nanocarrier
Figure 1-2 Structure of Mannosylated NC (One Mannose)

Molecular Weight: 1613.7941
Figure 1-3 MALDI-TOF/MS Spectrum of Mannosylated NC (One Mannose)

1635.54 Daltons
Figure 1-4 Structure of Mannosylated NC (Two Mannose)

Molecular Weight: 1863.0120
Figure 1-5 MALDI-TOF/MS Spectrum of Mannosylated NC (Two Mannose)
Figure 1-6 Structure of Mannosylated NC (Three Mannose)
Figure 1-7 MALDI-TOF/MS Spectrum of Mannosylated NC (Three mannose)
Figure 1-8 Structure of Mannosylated NC (Four Mannose)

Molecular Weight: 3560.8651
Figure 1-9 MALDI-TOF/MS Spectrum of Mannosylated NC (Four Mannose)

3582.6 Daltons
Figure 1-10 MALDI-TOF Mass Spectrum of PEG<sub>5KDa</sub>-NC

6885.1
Figure 1-11 MALDI-TOF Mass Spectrum of PEG$_{12KDa}$-NC

13923.5 Daltons
Figure 1-12 MALDI-TOF Mass Spectrum of PEG20KDa-NC

23328 Daltons

Mass (m/z)

% Intensity

23328 Daltons
Figure 1-13 MALDI-TOF Mass Spectrum of PEG\textsubscript{30KDa-NC}

32872.1 Daltons Mass (m/z)
Figure 1-14 MALDI-TOF Mass Spectrum of PEG_{40KDa-NC}

46336 Daltons
PEGylated NC (2 mannose): PEG12K-Cysteine-Gaba-Gaba-Ser(Man)-PEG12-Ser(Man)-Gaba-FITC
PEGylated Control NC (no Mannose): PEG12K-Cysteine-Gaba-Gaba-Ser-PEG12-Ser-Gaba-FITC

Figure 1-15 Temperature-Dependent Cellular Uptake Study of PEGylated NCs

PEG12K-NCs were added in incubation medium HBSS and incubated at either 4°C (on ice) or 37 °C, pH =7.0 for 1 h. The means ± SD for three independent experiments are shown for each value. (*, statistically significant difference between 4 °C and 37 °C treatments, p < 0.05.)
Uptake of NCs in J774.E cells at 37 °C after 40 minutes of incubation in the absence and presence of 5 mg/ml mannan. The means ± SD for three independent experiments are shown for each value. (Statistically significant difference between the J774.E cells incubated with and without mannan * p < 0.05.)
Figure 1-17 Effect of Mannose Moiety Copy Number at NCs in Cellular Uptake via MR

1-4 copy mannose NCs and their corresponding control NCs (without mannose moieties) were incubated in the J774.E cells at 37 °C for 1 hr. The means ± SD for three independent experiments are shown for each value. (*, statistically significant difference between treatments, p < 0.05. Man: abbreviation of Mannose)
PEGx-Cystein-Gaba-Gaba-Ser(Man)-PEG12-Ser(Man)-Gaba-FITC (x=5, 12, 20, 30, 40 KDa)
Control: PEGx-Cystein-Gaba-Gaba-Ser-PEG12-Ser-Gaba-FITC (x=12, 20 KDa)

Figure 1-18 Effect of NC size in Cellular Uptake via MR

PEGx-NCs (x= 5, 12, 20, 30, 40KDa) were incubated in J774.E cells at 37 °C for 1 hr. The means ± SD for three independent experiments are shown for each value. (*, statistically significant difference between treatments, p < 0.05.)
Figure 1-19 Fluorescence Microscopy Images of Mannosylated NC uptake into J774.E cells

The cellular uptake of mannosylated NC (2 copy mannose or 4 copy mannose) uptake into J774.E cells at 80 nM was observed by fluorescence microscopy. The result images showed significant signal in the cells incubated with 2-copy mannose NC than the ones incubated with 4-copy mannose NC. Punctuate green color spots of the 2-copy mannose NC microscope image revealed the endocytosis uptake mechanism.
Figure 1-20 Confocal Microscopy of NC Uptake into J774.E Cells

Two-copy mannose NC (80 nM) was incubated with J774.E cells in HBSS at 37 °C for 1 hr, washed twice with PBS, once with acid solution. The live cells were scanned in XYZ mode at 0.5µm thickness of the Z-axis. Shown are the same mid section of each of the two treatments. The overlap image clearly showed the co-localization of FITC labeled NC (green color) and fluid endocytosis marker, rhodamine dextran (red color), further confirming that the NCs were internalized into cells via endocytosis.
The length effect of spacers: PEG$_6$ (25.1 Å), PEG$_{12}$ (46.5 Å), or PEG$_{20}$ (75.2 Å) between mannose moieties at NCs (Cys-Gaba-Gaba-Ser(Man)-PEG spacer-Ser(Man)-Gaba-FITC) was studied in cellular uptake into MΦs via MR. The means ± SD for three independent experiments are shown for each value. (*, statistically significant difference between treatments, p < 0.05.)
2 Targeting Polarized Rat Macrophages Using Mannosylated Nanocarriers

2.1 Introduction

Resting macrophages (MΦs) in tissues are activated in response to changes in the tissue environment [59], mostly through cytokines secreted by damaged tissue cells, bacterial products and recruited immune cells. Activation (i.e., polarization) in the tissues is affected by the type, timing and concentration of the stimuli. Two main activated (polarized) phenotypes are known, the classically activated (M1) phenotype is critical for host defense and the alternatively activated (M2) phenotype is critical for injury resolution and wound healing. M2 MΦs that can be furthered subdivided into M2a, M2b, and M2c cells and M2 are considered to be more heterogeneous than M1 cells [60-62]

Among the three subtypes, only M2a has been studied in detail and is considered the prevailing M2 phenotype. Polarized MΦs are characterized by the expression of surface proteins and production of cytokines [63]. Activated MΦs remain plastic and respond to a changing environment in order to modulate their activities [64]. Under pathological conditions excessive pro-inflammatory M1 response can result in collateral tissue damage and impaired tissue healing while an excessive M2 response can lead to tissue fibrosis. Abnormal MΦ activities have been implicated in a variety of pathological states. Specific reviews have been written on M1/M2 polarization in bacterial infection (Benoit M et al, 2008), in the pathogenesis of atherosclerosis [65], in obesity and metabolic syndrome [66], in tumor progression [67] and in HIV infection [60, 68].
M1 and M2 MΦs mediate different but effective immune responses against invading pathogens [61]. M1 MΦs support Th1 associated effector function, while M2 MΦs support Th2 associated effector function [69]. M1 MΦs are both inducer and effector cells in Th1 type inflammatory responses that produce pro-inflammatory cytokines and kill microorganisms and tumor cells [70]. M2 MΦs produce anti-inflammatory mediators. Several pathogens including HIV and M. tuberculosis use these activation pathways to facilitate dissemination and pathogenesis [71].

The enhanced microbicidal capacity and increased secretion of classical inflammatory cytokines, such as IL-1β, IL-6, and TNF, were observed in M1 polarized MΦs [72]. M1 MΦs often produce cytokines that drive viral replication and contribute to MΦ-mediated tissue injury [73-75]. IL-4 and IL-13 are two signature cytokines produced by Th2 cells, mast cells, and basophils, which are also the major inducers of the “alternative activation” of MΦs [69, 76]. M2 polarization participates in activities including suppression of inflammation, promoting angiogenesis, enhancement of phagocytosis, elimination of parasites, wound healing, tissue remodeling and tissue repair [67, 77-79]. In vitro, M1 phenotype is induced by IFN-γ, tumor necrosis factor-α (TNF-α) or LPS whereas M2a by IL-4/IL-13 [69, 70, 80].

To carry out their functions, monocytes and MΦs express a variety of pattern recognition receptors (PPRs) that recognize pathogen-associated molecular patterns (PAMPs) [81] and other receptors involved in normal physiological functions (Taylor PR et al, 2005). MΦs polarization affects the pattern of receptor expression. M2 MΦs express high levels of non-opsonic receptors and can be characterized by scavenger receptors, mannose receptors (MR), and galactose receptors. The expression of MR and
its endocytic function are upregulated by M2 type cytokines IL-4 [82] and IL-13 [83], but down-regulated by the M1 type cytokine IFN-γ [84]. The high level of MR expression in M2a cells serves as a marker for the M2a phenotype and provides a potential target for delivering therapeutic agents to M2a MΦs.

The concept of MΦ polarization, analogous and corresponding to the Th1/Th2 polarization of T helper cells, has changed our thinking about targeting MΦs. Examples of two MΦ-infected diseases, Mycobacterium tuberculosis (M.tb) and HIV infection, as well as fibrosis are briefly discussed below. In these diseases, M2 MΦs play a pivotal role. In M.tb disease, MΦs are often the only cell type infected. MΦs are polarized toward an M1 type during the early phase of M.tb infection as revealed by two transcriptomic studies [71, 85]. As the disease progresses, the susceptibility to M.tb increases with elevated levels of M2 type inducers such as IL-4 and IL13 [86-89]. At the intermediate stage of M.tb infection, M2a cells became the dominant phenotype, accompanied by high levels of M2 type inducers present in the lung tissues [85, 90, 91]. In a majority of tuberculosis patients infected MΦs are of the M2a type. Therefore, treating TB by targeting M2a may produce a more efficacious result.

Tuberculosis is also associated with HIV-infection, another disease in which MΦs are infected by a microbial organism. Among the 1.4 million deaths in 2010, 0.35 million deaths were from HIV-associated TB (WHO Global Tuberculosis Control Report 2011). Next to CD4+ T cells, MΦs are the second most important cell type infected by HIV. Infected MΦs support much less viral replication than infected T cells. For this and other reasons, unlike virus-replicating T cells, virus-replicating MΦs are not killed by HIV-1.
The lifespan of tissue MΦs range from weeks to months or longer and continuously replenished by precursor monocytes. As a result, infected MΦs not only constantly supply new virus but also constitute an important viral reservoir. MΦs also play important role in viral transmission to other cell types and to another host. The effect of MΦ polarization on HIV-1 activity is more complicated, which we are just beginning to understand. HIV-1 infection and replication are two related but different outcomes of virus-host interaction, both affected by MΦ polarization [61] [62]. Frequently, the timing of infection relative to the time of activation is critical. Compared to monocytes, resting MΦs show increased susceptibility to HV-1 infection. However, upon polarization to all phenotypes, susceptibility decreases, as does viral replication capacity. There is evidence suggesting that HIV-1 infection subtly affects MΦ activity and M2a marker appear to be associated with the dominant viral replicating MΦs [92, 93]. HIV Nef accessory protein preferentially targets and activate M2, but not M1, MΦs [94]. In activated M2 MΦs, Nef also shifts the phenotype toward the inflammatory M1 type. M1 MΦs are more potent in CD4+ T cell activator than M2 cells. The importance of CD4+ T cell activation in HIV disease is suggested by studies from SIV infection in non-human primates. It was found that what distinguishes no disease in SIV infected natural hosts (African green monkeys & the sooty mangabey) and fetal disease in non-natural hosts (Asian rhesus macaques) is the CD4+ T cell activation level (Lackner et al, 2009). Although our knowledge about MΦ polarization in HIV-1 infection is limited at this point, available evidence suggests that a cell surface marker that is shared by resting and M2a MΦs is preferable. Indeed general targeting of all phenotypes of MΦ through targeting monocytes works well in animal HIV infection models [95-97]. In contrast to the two MΦ-infected diseases, there
is clear understanding that M2 phenotype is critical for fibrosis diseases and targeting to this particular population of MΦs may reverse the pathological condition [98, 99]

To target resting or M2 MΦs, the MR is a good choice. MR is expressed on resting MΦs and expression is elevated on M2a cells, the prevailing M2 MΦ phenotype. Mannose receptor (MR, MRC-1), a 175-kDa integral membrane protein, is expressed on sinusoidal liver cells, peripheral and bone marrow MΦs, and dendritic cells. MR recognizes and internalizing mannose, fucose and N-acetylglucosamine on the termini of polycarbohydrates [16, 17, 100]. The receptor does so using its eight C-type lectin like domains (CTLDs) two of which have appreciable binding affinity for mannose. Since the main natural ligands are repeated polycarbohydrates on many bacteria and virus surface with terminal mannose units, MR-microbial mannose interaction is multivalent, resulting in an enhanced total binding affinity (the avidity effect). MR mediates phagocytosis of a wide variety of pathogenic microorganisms including HIV and the fungi Candida albicans, parasites such as Leishmania donovani and bacteria such as Pneumocystis carinii.

Previously, we constructed and optimized a novel MR-targeting nanocarrier (NC). In this study, the feasibility of using the NC to specifically target M2a MΦs is assessed. Significantly, close to 20-fold higher mannosylated NC uptake into M2a cells as compared to M1 cells was observed, demonstrating that targeting a specific MΦ cell population with M2a phenotype can be achieved using our mannosylated NC approach.
2.2 Materials and Methods:

2.2.1 Materials

Amide Sieber resin was purchased from Anaspec (Fremont, CA), Fmoc-gamma-Abu-OH, Fmoc-Serine-OH, Fmoc-Cysteine-OH from Chem-impex (Wood Dale, IL), α-D-Mannose pentaacetate, boron trifluoride diethyl etherate, 4',6-diamidino-2-phenylindole (DAPI), N,N-Diisopropylethylamine (DIPEA) and sodium methoxide in methanol from Sigma Aldrich (Cat. # 403067), trifluoroacetic acid from Fisher Chemical and amino-dPEG$_{12}$-acid from Quanta Biodesign (Powell, Ohio).

Recombinant rat interferon gamma was purchased from Millipore (Cat. # IF006) and recombinant rat IL-4, IL-10 and IL-13 from R&D System (Minneapolis, MN). Antibodies used were iNOS (BD Bioscience, Cat. # 610432), Cox-2 (Abcam, Cat. # 15191), Arg-1(BD bioscience, Cat. # 610708), MR (Abcam, Cat. # 64963), Gal-3(R&D Systems, AF1245) and actin (SC.1615) and HRP-conjugated anti-goat IgG (SC-2020) from Santa Cruz Biotechnology Inc.; HRP-conjugated anti-rabbit IgG (NA934V) and anti-mouse-IgG (NA931V) from GE Healthcare; and HRP-conjugated anti-chicken IgY (SA1-300) from Affinity BioReagents. Mac-3 antibody (ab22506) was from Abcam.

2.2.2 Quantitative Real-Time PCR

Total RNA was extracted from MΦ cells using an RNeasy Mini kit (Qiagen, Valencia, CA) and RNA reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturers' protocols. Standard curves were generated using serial dilutions from pooled randomly selected cDNA samples. Samples from each treatment were analyzed and results presented
relative to glyceraldehyde-3-phosphate dehydrogenase (GADPH) mRNA expression. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a 7900HT Thermocycler using 96-well optical reaction plates according to manufacturer's protocol. All PCR primer sequences were generated using Primer Express 3.0 (Applied Biosystems) and primers were synthesized by Integrated DNA Technologies (Coralville, IA). A minimum of three samples was analyzed for each experimental group, and all samples were run in duplicate. Primer sequences were: inducible nitric oxide synthase (iNOS): (F) TGGTGAAAGCGGTGT TCTTTG, (R) ACGCGGGAAGCCATGA; Arginase-1: (F) CCAAGCCAAAGCCCA TAGAG (R) TCCTCGAGGGCTTCCCTTAG and GAPDH: (F) CCTGGAGAAACC TGCGAAGTAT, (R) CTCGGCCCGCTTGCTT.

2.2.3 Western blotting

MΦ cells were extracted and lysed using lysis buffer consisting of 20 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl$_2$, 1 mM diethylene triamine pentacetic acid, 1 mM phenylmethylsulfonylenediamine, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 2 mM sodium orthovanadate and protease inhibitor cocktail (Sigma, St. Louis, MO). Protein concentrations were assayed using a BCA Protein Kit (Pierce, Rockford, IL) with bovine serum albumin as the standard. Proteins were separated on 4~12% polyacrylamide gels (Invitrogen) and then transferred onto nitrocellulose membranes. Nonspecific binding was blocked by incubation of the blots at room temperature with blocking buffer (5% nonfat dry milk, 10 mM Tris-base, 200 nM
sodium chloride, and 0.1% Tween 20) for 60 min. Membranes were then incubated overnight at 4°C with monoclonal mouse anti-mouse iNOS antibody (1:500, BD), polyclonal rabbit anti-mannose receptor antibody (1:1000, Abcam), polyclonal goat anti-Gal3 antibody, or monoclonal mouse anti-Arginase 1 antibody (1:1000, BD). This was followed by incubation with goat anti-mouse horseradish peroxidase (HRP) (Cell Signaling, Cat. #7076), donkey anti-goat IgG-HRP (Santa Cruz, Cat. #2020), or anti-rabbit IgG HRP (Cell Signaling, Cat. #7074) for 1 h at room temperature (1:10,000). Binding was detected using ECL Plus (GE Healthcare, Piscataway, NJ).

### 2.2.4 Preparation of Nanocarriers

Fmoc-Serine-OH was conjugated to mannose-pentaacetate in an organic reaction condition. α-D-Mannose pentaacetate (1.06 g, 2.72 mmol) and Fmoc-Ser-OH (1.08 g, 3.28 mmol) are dissolved in 50 ml dichloromethane and 3.1 ml (24.6 mmol) boron trifluoride etherate is added. The reaction mixture is stirred for 20 hours in dry atmosphere, diluted with 160 ml dichloromethane and washed with 20 ml of 1M HCl and 20 ml water. The organic phase is dried over anhydrous MgSO₄. After evaporation of the solvent on a rotary evaporator and purification of the crude mixture (2.03 g) by flash chromatography on silica gel (eluent: methanol: dichloromethane, 5:95, v/v), 1.25 g (70%) of product, Serine-Mannosepentaacetate, was obtained.

Mannosylated nanocarriers, FITC-Gaba-Ser(Man)-PEG₁₂-Ser(Man)-Gaba-Gaba-Cys-amide was synthesized in solid phase using Nautilus auto synthesizer (Figure 2-4). NovaSynTG Sieber resin was used in the amino acid coupling with molar ratio 1:4:4:8 (resin: amino acid: coupling reagent (HOAT, HATU): basic reagent (DIPEA)) for four
hours reaction time. Serine-mannosepentaacetate and PEG_{12} amino acid were conjugated to the peptidic core as regular amino acid coupling with molar ratio 1:2:2:4 (resin: amino acid: coupling reagent (HOAT, HATU): basic reagent (DIPEA)) in Nautilus peptide auto synthesizer for 12 hours reaction time. Fmoc group was removed using 20% piperidine in NMP. NMP was also used as the washing solvent. MALDI TOF/MS and analytical reverse phase high-performance liquid chromatography (RP-HPLC) were performed in between amino acid couplings to confirm the correct molecular weight and purity of the intermediates. Five equivalent of fluorescein isothiocyanate (FITC) was dissolved in NMP and reacted to the N terminal of the peptide in solid phase for 24 hours. Acetyl group of the mannosepentaacetate at the serine moiety was removed using sodium methoxide in methanol anhydride in solid phase for 1 hr. Peptides were cleaved from the resins in the cleavage solution (85% DCM, 2% DODT, 1% TIS, 12% TFA) for 1.5 hrs. The cleaved peptides were then precipitated in 50% cold ether and 50% hexane in volume to remove the scavengers and impurities. Semi-preparative HPLC (RP-HPLC, C18 column) was performed to purify the synthesized NCs. Purified NCs were then lyophilized.

2.2.5 Rat peritoneal macrophage isolation and polarization

Male Sprague–Dawley rats (jugular vein cannulated, weighing 200–225 g and 8-9 weeks of age) were purchased from Harlan. The rats were allowed to acclimatize to the animal facility for a minimum of 5 days prior to use. The animals were euthanized using CO\textsubscript{2} asphyxiation. 20 ml ice-cold PM1 perfusion medium (0.5 mM EGTA, pH 7.2, Ca\textsuperscript{++}-Mg\textsuperscript{++} free) was injected into peritoneal cavity for three times and collected. The peritoneum of
the rat was massaged for 30 seconds and the solution inside the abdominal cavity containing peritoneal MΦs was withdrawn. The collected MΦs were washed three times with PM1 using centrifugation at 300 g/4° C for 8 minutes. The settled MΦs were suspended and seeded in 24-well plates (Corning Glass) or covered glass chambers (LabTek; Nunc) at 1 x 10^6 cells/well in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS. After 4 hrs of culture, MΦs were washed three times with DMEM containing 1% FBS and the cells were cultured in the same medium for 48 hrs. The cells were then treated with IFN-gamma (20 ng/ml), IL-4 (10 ng/ml) +IL13 (10 ng/ml), or IL-10 (10 ng/ml) for 48 hours for M1, M2a and M2c polarization, respectively.

2.2.6 Cellular Uptake Study

Cellular uptake was studied using confocal microscopy. Polarized rat peritoneal MΦs were incubated for 1 hour with phenol red-free DMEM containing 1% FBS, 120 nM of FITC-labeled mannosylated NC or control non-mannosylated NC, the general fluid endocytosis marker Rhodamine-dextran and the nuclear dye DAPI. In some uptake experiments, the polarized cells were preincubated for 40 minutes with medium contained additionally 3 mg/ml of mannan (MR ligand serving as a competitive inhibitor) and the cells were further incubated for 1 hr after the addition of NCs. After incubation with NCs, the attached cells were washed twice with cold phosphate buffered saline (PBS), twice with acid wash solution (0.5 M sodium chloride and 0.2 M acetic acid, pH 2.5) and twice with cold PBS. The cells were immediately used for confocal microscopy. Quantitative uptake was determined using software described below.
2.2.7 Confocal Microscopy

Rat polarized peritoneal MΦs seeded in chambers of Laboratory-Tek II 4-chamber cover glass slides were incubated with medium containing NC for 1 hr at 37°C and washed as described above. Confocal microscopy was done on a Leica TCS SP2 spectral confocal microscope using the XYZ mode. Stacks of images 0.41 µm in thickness were collected along the Z-axis. Image of a middle section of a Z-stack was used to represent a treatment in Fig.8. Leica LAS AF Lite software was used to analyze the images in order to quantify uptake of FITC-labeled NCs, in which fluorescence intensity in unit of pixels per µm² of a number of randomly chosen cells in five fields of each phenotype was summarized and expressed as fold of that cells without incubation with FITC-labeled NCs.

2.3 Results:

2.3.1 Synthesis and Characterization of Mannosylated Nanocarriers

In our previous study, the MΦ MR-targeting mannosylated nanocarrier (NC) was optimized. In this study, FITC-labeled NC and rat polarized peritoneal MΦs were used to assess the feasibility to target MΦs with a particular phenotype. The NC synthesis was done mostly in solid phase. Fmoc-serine-mannosepentacetate was first synthesized in liquid phase from Fmoc-serine-OH and the hydroxyl groups protected mannose, mannosepentaacetate (Figure 2-1). ESI/MS showed correct molecular weight of Fmoc-serine-mannose pentaacetate, and HPLC result demonstrated high yielding (78%) and purity of the conjugate (Figure 2-2Figure 2-3). Subsequent NC synthesis from Fmoc-serine-mannose pentaacetate was performed as described in Chapter 1 and a companion manuscript. MALDI-TOF/MS data and semi-preparative HPLC result in Figure
2.3.2 Characterization of Polarized Rat Peritoneal Macrophages

Isolated rat primary peritoneal MΦs (PMs) were polarized into M1 and M2a cells by IFN-γ and IL-4/IL-13, respectively. The rare M2b and M2c phenotype induced by immunocomplex and IL10, respectively, was not studied. Expression of iNOs and Arg-1 proteins, the markers for M1 and M2a phenotypes, respectively, was quantified at the mRNA level using real time qRT-PCR and at protein level using Western Blotting. Figure 2-7 shows the mRNA expression level of the M1 marker iNOs after the treatment with each of the two types of inducers for 6, 24, 48 and 72 hrs. Significant and dramatically increased iNOs mRNA expression at all time points was only seen for IFN-γ-treated PMs with the highest expression occurred at 24 hr. Figure 2-8 shows the mRNA expression level of the M2a marker Arg-1 after the treatment with each of the two types of inducers for 6, 24, 48 and 72 hrs. Significant and dramatically increased Arg-1 mRNA expression at three of the four time points was only seen for IL4/IL13-treated PMs with the highest expression occurred at 48 hr. Figure 2-9 shows the protein expression levels of the M1 marker iNOs and M2a markers Arg-1 and MR and the housekeeping protein actin after the treatment with each of the two types of inducers for 24, 48 and 72 hrs. In agreement with the qRT-PCR results, the M1 marker iNOs protein was expressed at highest level at 48 hr only in PMs treated with the M1 inducer IFN-γ. The M2a marker Arg-1 and MR proteins were expressed at highest levels at 48 only in PMs treated with
the M2a inducer IL4/IL13. The characterization of marker expression at both the mRNA and the protein levels thus verified the polarized M1 and M2a phenotypes of treated PMs.

2.3.3 MR-mediated NC Uptake in Rat Polarized Macrophages

Cellular uptake of the FITC-labeled mannosylated NC into rat polarized PMs was studied using confocal microscopy. In agreement with the order of the expression levels of M2a marker proteins (Figure 2-9), the highest intracellular green fluorescence in images presented in Figure 2-10 is seen in M2a cells, followed by that in resting and the lowest in M1 MΦs. The intracellular green fluorescence assumed a punctate appearance in both resting and M2a MΦs, which in Figure 2-11 co-localizes with the red general endocytosis marker of Rhodamine-Dextrane but not all red punctate fluorescence is co-localized with the green punctate fluorescence. This suggests that uptake occurred by endocytosis and follows a particular receptor-mediated endocytosis pathway. Quantification of intracellular green fluorescence (Figure 2-12) shows that, compared to resting cells not treated with the NCs, treated resting cells had ~19 fold-, treated M1 cells about 3 fold- and treated M2a cells about 46-fold higher fluorescence. Since in Western Blotting M1 cells had no detectable MR, resting cells expressed a low level and M2a cells the highest expression level of MR protein, the correlation between MR expression level with uptake strongly suggests that NC uptake was mediated by the MR. To confirm this, a competitive inhibition uptake was carried, in which the uptake was conducted in the absence and presence of non-toxic concentration of mannan, a mannose polymer known to specifically bind to MR. The data in Figure 2-13 shows that mannan completely
abolished NC uptake into M2a cells, suggesting that MR exclusively mediates NC uptake.

2.4 Discussion

MΦ polarization is intimately related to immune response orchestrated by T helper cells. Resting MΦs undergo M1 and M2 polarization to acquire inflammatory and anti-inflammatory activities, respectively, corresponding to Th1 and Th2 activation of helper T cells [101-103]. M2a is the prevailing M2 subtype, balancing the M1 inflammatory reaction [104]. Chronic excess of either M1 or M2 activity leads to pathological states and diseases, so do the infections by a number of microorganisms [61, 65-68, 105]. As illustrated earlier in the three examples of diseases, targeting MΦ with M2 phenotype is justifiable to delivery drugs to treat infections or to modulate MΦ activities. It may be possible to curb both excessive M1 and M2 activity through up-regulating and down-regulating M2 activity, respectively.

In this study, it was found that three M2a markers, MR, Arg1 and Gal-3, were expressed at elevated levels in M2a in Western Blotting (Fig. 2-5). Among them, Gal-3 was least specific for M2a since it was expressed at low levels in resting and M1 cells as well, which is in good agreement with others’ [80, 104, 106, 107]. Arg1 was the most specific for M2a with no detectable expression in resting and M1 cells, which is explainable by the protein’s function. In M2a MΦs, Arg-1 antagonizes M1a phenotype. It shifts arginine metabolism to the production of ornithine and polyamines and inhibits NO production by competing with iNOS for arginine [77]. Therefore, expression of the M1 marker iNOS and M2a marker Arg1 is oppositely regulated. That is, iNOS and Arg1 is a
pair of good M1 and M2a markers, which was confirmed in their mRNA expression levels shown Fig. 2-3 and Fig. 2-4, respectively. In the two figures, an all-or-none expression pattern of the two is observed. Taken together, therefore, our data confirmed that the rat peritoneal MΦs were well polarized into M1 and M2a phenotypes.

To target M2 MΦs, MR is a good choice. As shown by others [108] and confirmed in this study (Fig. 2-3, Western Blotting), no MR was detected in M1-polarized rat peritoneal MΦs (PMs) while moderate and high levels were found in resting and M2a cells, respectively. The absence of detectable amount of MR in M1 cells is highly desirable for M2 MΦ targeting. M1 cells are inflammatory and unintended engagement of an M2-targeting drug delivery nanocarrier (NC) to M1 cells in the body might jack up local inflammation to result in tissue damage. MR expression is also relatively specific for MΦs [46, 109]. In contrast, monocytes do not express significant level of MR. There are other mannose-recognizing receptors including Endo180, DC-SIGN, L-SIGN, and SIGNR [46], whose cell type expression patterns are similar to that of MR. Therefore, using mannose in a NC as the MR-targeting moiety may bind to these other receptors but will not grossly change MΦ targeting specificity.

MR has been used to target MΦs and ligand used for targeting is mannose. Salman et al. (2008) reviewed mannosylated liposomes for treatment of tuberculosis and leishmaniasis, vaccination and non-specific immunostimulation. Mannosylated emulsions have been used to deliver hydrophobic drugs to liver MR-expressing non-parenchymal cells [110]. Mannosylated albumin displayed on tetanus toxoid-loaded liposome was used as an adjuvant [111]. Efficacy for HIV-1 infection has been demonstrated with drug-loaded mannosylated gelatine nanoparticles [112] and mannosylated dendrimers [113].
Mannosylation enhanced uptake is generally much higher in DCs than in MΦs. DC-targeted, mannosylated conjugates have been tested in several small-scale clinical trials to boost cellular immunoresponse to cancer with promising results [46]. In addition to drug cargo effect on HIV-1 infection, multivalent mannose itself on a NC can also have anti-HIV activity. Pollicita M et al [114] found that carbohydrate-binding agents that bind to MR can compete with HIV-1 gp120 binding to MR, inhibiting MΦ infection by HIV-1 and viral transmission to CD4+ T cells. However, use of multivalent mannosylated NCs may have unfavorable consequences since Langerin protein specifically expressed on Langerhans cells (LCs) is another mannose receptor. de Witte L et al [115] found that langerin protects LCs from HIV-1 infection and prevents LC transmission of HIV-1 to CD4+ T cells by capturing/internalizing/degrading HIV-1, which was negated by multivalent mannan.

To our knowledge, this study represents the second attempt to target a specific MΦ population with a particular phenotype (M2a) after a recent publication [116]. Complementary to their finding is our extensive characterization of the resting, M1 and M2a phenotypes, which may explain the higher M2a to M1 specificity in cellular uptake of this study (~12-fold, Figure 2-12) than the ~6-fold recently reported. We were also able to observe that the uptake was completely abolished by a MR competitive inhibitor (mannan, Figure 2-13). In addition, we uniquely show that the MR-mediated uptake is via a particular endocytosis pathway (Figure 2-10, Figure 2-11), which is in agreement with known observation that many natural ligands for MR are internalized through endocytosis. The intracellular fate of mannosylated NC needs to be delineated as the fate determines the topological destination of intracellular delivery. It is known that, after
endocytosis of natural ligands, MR is then recycled back to cell surface while the content of the endosome is delivered to the lysosomes [46]. It is possible that a non-natural mannosylated NC is trapped in the endosomes, unable to reach the cytosol/nucleus compartment. If this is the case and the destiny is the cytosol/nuclear compartment, either an endosomal escape or a lysosomal escape strategy needs to be incorporated into the NC’s design. MR also mediates phagocytosis [109, 117]. How MR mediates both endocytosis and phagocytosis and what ligand property dictates this dichotomy is known. Since phagocytosis can accommodate much bigger particles and hence a much higher cargo capacity, this is another area that should be studied for targeting MR.

In summary, our previously optimized and unique mannosylated NC is shown in this study to be well suited for targeting MΦs with M2a phenotype involved in a variety of diseases. Further studies are needed to test therapeutic cargo delivery in several disease models.
Results:

Figure 2-1 Scheme of Fmoc-Serine (Mannose pentaacetate) reaction

Chemical Formula: C_{32}H_{35}NO_{14}
Molecular Weight: 657.6186
MALDI/MS spectrometry was performed to characterize the conjugation of Fmoc-Serine-Mannosepentaacetate (N-[(9H-Fluoren-9-ylmethoxy)carbonyl]-O-(2,3,4,6-tera-O-acetyl-a-D-mannopyranosyl)-L-serine. The 680.15 daltons represent the [657.6+Na]^+ molecular weight.
Reverse phase HPLC (C18 column) was used to evaluate the retention time of Fmoc-Serine (Mannose pentaacetate). With 1mL/min flow rate, the mobile phase gradient was from 5% to 100% in 30 minutes. UV detector was used in the HPLC system at wavelength 254 nm. (Stationary phase: 0.05 v/v% TFA in H₂O, Mobile phase: 0.05 v/v% TFA in acetonitrile)
Figure 2-4 Structure of the FITC labeled Mannosylated NC

Molecular Weight: 1863.0120
MALDI-TOF/MS confirmed the correct molecular weight at 1862.47 Dalton of mannosylated NC (2 copy mannose).
A. HPLC characterization UV spectrum at 254 nm

B. HPLC characterization Fluorescence spectrum (ex. 485 nm, em. 517 nm)

Figure 2-6 HPLC Profile of Mannosylated NC

Reverse phase HPLC (C18 column) was used to evaluate the retention time and purity of the mannosylated NC. With 1mL/min flow rate, the mobile phase gradient was from 5% to 100% in 6 minutes. (A) HPLC UV spectrum of mannosylated NC was performed at 254 nm UV wavelength using UV detector. (B) HPLC Fluorescence spectrum of mannosylated NC was performed at the ex. 485 nm, em. 517 nm using Fluorescence detector of HPLC system. (Stationary phase: 0.05 v/v% TFA in H₂O, Mobile phase: 0.05 v/v% TFA in acetonitrile)
iNOS mRNA expression in IFN-γ or IL4/IL13 treated rat peritoneal Mφs for 6-72 hours was characterized using qRT-PCR (n=5). 65 fold, 28 fold, 13 fold and 19 fold difference in iNOS expression between IFN-γ and IL4/IL13 treated Mφs was observed in 6 hours, 24 hours, 48 hours and 72 hours (*, t test statistically significant difference between treatment, p < 0.05).
Arg-1 mRNA expression in IFN-γ or IL4/IL13 treated rat peritoneal MΦs for 6-72 hours was characterized using qRT-PCR (n=5). 32 fold, 135 fold and 265 fold difference in Arg-1 mRNA expression were observed in between IFN-γ and IL4/IL13 treated MΦs at 24 hours, 48 hours and 72 hours, respectively. (*, t-test statistically significant difference between treatment, p < 0.05 )
Western blot was performed to analyze iNOS, MR, Arg-1 and Gal-3 expression in M\(\Phi\)s with different phenotypes. The M\(\Phi\)s treated with IFN-\(\gamma\) has relatively high iNOS expression at 48 hour and 72 hour, suggesting M1 M\(\Phi\)s. The M\(\Phi\)s treated with IL4/IL13 have relatively high MR and Arg-1 expression at 48 hours and 72 hours, suggesting M2a M\(\Phi\)s. t Results shown are representative of two independent experiments.
Resting МΦ, М1 МΦ and M2a МΦ were used in cellular uptake study. Mannosylated NC (120 nM), DAPI dye, and Rhodamine-dextran were incubated with three different phenotypes of МΦs for one hour. PBS buffer and acid solution were used to wash away unspecific binding. The uptake results were observed using confocal microscopy. The detail of uptake procedure is described in the methods.
Rat peritoneal M2a MΦ was incubated using 120 nM mannosylated NC, DAPI nuclei dye and the general fluid endocytosis marker, rhodamine-dextran for one hour. PBS buffer and acid solution were used to wash away unspecific binding. The overlapped image showed the endocytosis pathway of NC cellular uptake.

Figure 2-11 Fluorescence Microscopy Images of NC Uptake-Colocalization
Mannosylated NC (120 nM) was incubated with rat peritoneal MΦs for 1 hour at 37°C. PBS and acid solution were used to wash away unspecific binding. Quantification of the uptake is described in the methods. (2.39 fold difference between resting MΦ and M2a type, 11.84 fold difference between M1 and M2a type MΦ, * t-test statistically significant difference between treatments, p < 0.05)
3mg/ml of mannan was incubated with M2a MΦ for 40 minutes, followed by adding 120 nM mannosylated NC into incubation for 1 hour at 37 °C. PBS and acid buffer wash was used to wash way unbound and non-specific bound NCs. Quantification of the uptake is described in the methods. (98.5 fold difference, * statistically significant difference between treatments, p < 0.01)
3 Chapter Three – Delivery of anti-HIV drug AZT to infected macrophages with mannosylated nanocarriers (NC)

3.1 Introduction

HIV, mostly HIV-1, is the most studied virus in the history. Every conceivable aspect of the infection, including drug and gene delivery, has been intensely explored. The combinatorial chemotherapy regimen, highly active anti-retroviral therapy (HAART), introduced in 1995 has changed HIV-1 infection from a fatal disease to a chronic condition [118]. Nevertheless, viral persistence due to the existence of viral reservoirs and sanctuary sites render the disease incurable. The therapy also takes a toll on patients. Stringent adherence to therapy with high pill burden, reduction in therapy efficacy over time stemmed from viral mutation and severe side effects plaque patients, resulting in high incompliance rate. In addition, current HAART has only a small margin of safety in viral suppression [119]. Consequently, new drugs and therapies are urgently and constantly sought. Many current and new developing drugs suffer from poor oral absorption, low solubility and instability, variability in bioavailability, insufficient cellular uptake and rapid drug elimination. For all these reasons, drug and gene delivery has become one prominent strategy to improve HIV therapy with broad participation [120, 121]. Among various drug delivery systems, nanocarriers (NCs) possess many advantageous properties [122]. At least 24 NC-based therapeutic products have been approved for clinical use, liposomal drugs and polymer–drug conjugates being the two major classes [123]. NCs being developed for HIV intervention include polymeric
nanoparticles, solid lipid nanoparticles, nanoemulsions, liposomes, dendrimers and drug conjugates [8, 9].

CD4⁺ T cells and MΦs are the two main cell types infected by HIV. HIV replicates only in activated cells with different consequences. Infected and activated CD4⁺ T cells die soon from direct viral and cytotoxic T cell killing [124]. Apoptosis of bystanders of uninfected CD4⁺ T cells has also been reported [125]. In contrast, infected and activated MΦs do not die; they continuously sustain low level of viral replication. Since CD4⁺ T cells are the master immune cells affecting all immune functions and they lack unique non-immune cell surface markers, NC drug delivery for HIV intervention has been attempted mostly for MΦs. The objective of HIV chemotherapy and drug delivery is to maintain drug concentrations at sufficient levels in all CD4⁺ T cells and MΦs so productive replication in infected cells can be aborted and new infection of uninfected cells can be prevented.

HIV and host biology also dictates drug delivery to certain parts of the body. HIV-1 accumulates and replicates mainly in the lymphoid tissues. Under normal conditions, only 2% of T cells are in the blood and the rest 98% resides in the non-blood compartment, notably the lymphoid tissues [119]. Anatomically, the gut mucosa and peripheral lymph nodes (LNs) are the main lymphoid tissues. The gut mucosa (i.e., gut epithelium plus underneath lamina propria) harbors the largest collection of lymphoid tissues in the body named gut-associated lymphoid tissues (GALT), accounting for ~40% of all lymphocytes [126]. Naïve T cells constantly circulate between blood, tissue interstices, and secondary lymphoid organs that include GALT, peripheral and mesenteric
LNs, and the spleen. LNs are particularly important in HIV infection where immune cells are densely packed. It is in the secondary lymphoid organs (the induction sites) where naive T have the highest chance to encounter antigens such as HIV, to be activated, &/or to undergo clonal expansion and to differentiate into CD4⁺ and CD8⁺ effector memory cells. Like gut mucosa, LNs are a main site where HIV-1 replicates and forms a major reservoir [127]. Although the frequency of HIV-1-infected MΦs in gut mucosa is low (0.06% of lamina propria mononuclear cells), the extraordinary size of the gut mucosa makes intestinal MΦs a prominent viral reservoir [128]. Most activated T cells home to extralymphoid tissues (effector sites) in the presence or absence of inflammation [129] and the gut mucosa receives a large share of CD4⁺ effector memory cells. The gut is also the main site of CD4⁺ T cell depletion due to high expression of CCR5 and relatively highly activated state in gut mucosa. Gut CD4⁺ effector memory T cells undergo profound depletion during the acute phase and subsequent stages of the disease [126, 130-132], even in patients under long term effective HAART [133]. One consequence of the depletion is the disruption of gut mucosal barrier, leading to translocation of microbes and their products that promotes systemic activation and systemic destruction of CD4⁺ T cell [131]. The importance of CD4⁺ T cell activation in HIV disease is learned from studies of SIV infection in non-human primates. What distinguishes the non-progressive nature in the natural hosts (African green monkeys & the sooty mangabey) and disease progression in the non-natural hosts (Asian rhesus macaques) is the activation level; baseline CD4⁺ T cells activation was observed in the former vs. persistent activation in the later [126]. The pivotal role of LNs in HIV disease is demonstrated by excellent therapeutic result of delivery of HIV drugs to only this site via MΦ-targeting [10, 95, 96,
At present, limited effort has been made to deliver drugs to the other site, the gut mucosa, although the benefit of delivery to this site has been predicted [126, 136].

Relatively MΦ-specific receptors have been used to target MΦs. Among them, mannose receptor (MR) is the choice by many groups [112-114, 137, 138]. The ligand used is often multi-valent mannose. Chapter 1 and 2 described the construction, characterization and MΦ targeting of our novel mannosylated peptidyl nanocarriers (NCs) aimed at MR. This Chapter describes the construction, characterization and test of two new drug-loaded conjugates, the AZT- and RTV-conjugated NCs (AZT-NC and RTV-NC). They were designed as prodrugs. An ester bond was used as a releasable linker between a drug and the NC. AZT (zidovudine) is a HIV nucleoside reverse transcriptase inhibitor while RTV (ritonavir) is a HIV protease inhibitor, both approved for clinical use [139-141] [142]. They were selected as model therapeutic compounds because of their very different physicochemical properties.

A great deal of effort has been made in developing two anti-HIV activity assays suitable for MΦs. With the availability of two assays, we were able to test the prodrugs’ anti-HIV activity. The preliminary data shows that the two conjugates indeed act as prodrugs with moderate MR-targeting specificity.
3.2 Methods and material:

3.2.1 Materials

Amide Sieber resin was purchased from Anaspec (Fremount, CA), Fmoc-gamma-Abu-OH, Fmoc-Serine-OH and Fmoc-Cysteine-OH from Chem-impex (Wood Dale, IL), α-D-Mannose pentaacetate, boron trifluoride diethyl etherate, Succinic acid and N,N-Diisopropyylethylamine from Sigma Aldrich and Trifluoroacetic acid from Fisher Chemical. Amino-dPEG$_{12}$-acid was purchased from Quanta biodesign (Powell, Ohio). AZT, RTV, MT-2 cells and U937/HIV-2MS cells were obtained from NIH AIDS Research and Reference Reagent Program. The cell line was originally contributed by Dr. Phyllis Kanki [143]. Human monocytes and serum were purchased from Sanguine Biosciences (Santa Monica, CA) and p24 ELISA kit from ABL Inc (Rockville, MD) and cell culture reagents from Invitrogen (Grand Island, NY).

Nanocarrier (NC) synthesis

3.2.2 Synthesis of Serine-Mannose pentaacetate (Ser(Man))

Fmoc-L-Ser-OH (1.08 g, 3.28 mmol) and α-D-Mannose pentaacetate in molar excel were dissolved in 50 ml anhydrous dichloromethane. 3.1 ml (24.6 mmol) boron trifluoride etherate is added drop wise. The reaction mixture is stirred for 20 hours under argon, diluted with 160 ml dichloromethane and washed with 20 ml of 1M HCl and 20 ml water. The organic phase is dried over MgSO$_4$. The crude mixture (2.03 g) was
purified using flash chromatography on silica gel, resulting in the final protected mannosylation product (Ser(Man)) at a yield of 70%.

### 3.2.3 Synthesis of Peptidyl Core

Stepwise solid-phase synthesis of peptidyl core Fmoc-Gaba-Ser(Man)-PEG$_{12}$-Ser(Man)-Gaba-Gaba-Cys-amide (Figure 3-1) and its non-mannosylated control (Fmoc-Gaba-Ser-PEG$_{12}$-Ser-Gaba-Gaba-Cys-amide)(Figure 3-2) was carried out on a Nautilus Auto Synthesizer using NovaSynTG Sieber resin. Reagent molar ratio of Gaba coupling (4 hr reaction) was 1:4:4:8 (resin: Gaba: coupling reagent (1-Hydroxy-7-azabenzotriazole (HOAT))/2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU)): basic reagent N,N-Diisopropylethylamine (DIPEA)) and the ratio for the coupling of both Ser(Man) and PEG$_{12}$-amino acid (12 hr reaction) was 1:2:2:4 (resin: Ser(Man)/PEG$_{12}$-amino acid: coupling reagent (HOAT/HATU): DIPEA).

Fmoc protection group was removed and the resin washed with 20% piperidine in N-Methyl-2-pyrrolidone (NMP). MALDI-TOF/MS (Figure 3-3) and analytical reverse phase high-performance liquid chromatography (RP-HPLC)(Figure 3-4) were performed after each coupling step to confirm the correct molecular weight and to determine purity.

After synthesis of peptidyl cores, acetyl group of the Ser(Man) in the cores was de-protected (removed) in solid phase for 1 hr with 10 mg sodium methoxide dissolved in 4 ml methanol anhydride.

### 3.2.4 Drug-Alanine Conjugation

Drug (AZT or RTV) (1mmole) was conjugated to Boc-Alanine-OH (1mmole) in 1ml NMP containing 1.25 mmole of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide
hydrochloride (EDCI) and 10 mg of N,N-Dimethylpyridin-4-amine (DMAP). The reaction mixture is stirred for 24 hours in dry atmosphere. The Drug-Alanine-BOC products were purified by flash chromatography and their structures (Figure 3-5 Figure 3-6) were confirmed by ESI/MS (Figure 3-7), MALDI-TOF/MS (Figure 3-9) and HPLC (Figure 3-8). Deprotection of Drug-Alanine-BOC was carried out for 30 minutes in 5ml TFA/Dichloromethane (DCM) (1:1 volume ratio) with constant stirring. Fresh chromatography was used again to purify the resultant Drug-Alanine-OH. The structures of Drug-Alanine-OH were confirmed by MALDI-TOF/MS and HPLC.

### 3.2.5 Conjugation of Drug-Alanine-OH with Peptidyl Core to form final NC

The N terminus of the synthesized Peptidyl Core on resin (0.04 mmole) was reacted with succinic anhydride (0.2 mmole) in 3ml DCM anhydride containing DIPEA (0.4 mmole) for 1.5 hour, followed by three washes with NMP. The reaction was monitored using MALDI-TOF/MS (Figure 3-3) and HPLC (Figure 3-4). The resultant succinimidyl Peptidyl Core on resin (0.04 mmole) was then reacted with Drug-Alanine-OH (0.08 mmole) for 24 hours in 4 ml NMP containing 0.12 mmole of 6-Chloro-Benzotriazole-1-yl-oxy-tris-Pyrrolidino-phosphonium hexafluorophosphate (Pyclog) and 0.24 mmole of DIPEA. The structures of products, the final NCs, were confirmed by MALDI-TOF/MS (Figure 3-12 Figure 3-13 Figure 3-14 Figure 3-15) and HPLC (Figure 3-16.). The NCs were cleaved from the resin for 1.5 hrs using the cleavage solution (85% DCM, 2% DODT, 1% TIS, 12% TFA). The cleaved NCs were then precipitated in 50% cold ether/50 % hexane in large volume to remove the scavengers and impurities used in the cleavage reaction. Semi-preparative RP-HPLC (C18 column) was performed to purify the
synthesized NCs, followed by lyophilization.

The final drug-NCs are synthesized as prodrugs since the -OH of both AZT and RTV forms a critical part of the pharmacophores of both drugs and the pharmacophores were destroyed when the -OH groups were used to conjugate with the peptidyl core to form a releasable ester bond. Therefore, only released free drugs are expected to impart anti-HIV activity to the drug-NCs.

3.2.6 Cytotoxicity and Anti-HIV Activity of AZT-NC and RTV-NC

Unlike infected CD4\(^+\) T cells, tested HIV-infected MΦ and MΦ -like cell lines supports up-to 1000-fold less viral production as measured by a p24 production in our hands. Therefore, extremely high sensitivity is required for a MΦ anti-HIV activity assay. The only assay available, the RT activity assay, uses radioisotope. We developed a non-radioactive anti-HIV activity assay based on measuring viral p24 level in cell culture of the U937/HIV-2\(_{MS}\) cell line with a sensitivity of 3 pg/ml. The only drawback of this system is that it excludes testing RTV-NC since released free RTV boosts p24 production in this system, interfering with anti-HIV activity of the same compound. Therefore, most anti-HIV activity was performed using this U937/HIV-2\(_{MS}\) system for AZT-NC and its non-mannosylated NC and free AZT controls. An additional system based on using human monocyte-derived-MΦ (MDM) and the same p24 assay was also developed, which was used to test AZT-NC and RTV-NC, as well as their controls. In some experiments, MT2 CD4\(^+\) T cell line was used in place of MDM.
Cytotoxicity of AZT-NC, RTV-NC and their controls, free AZT, RTV and non-mannosylated NCs, was first assessed using the MTT cell viability assay [144] to determine the safe concentrations that can be used with the U937/HIV-2MS system.

Anti-HIV activity using the U937/HIV-2MS cell line/p24 system was done as follows. U937/HIV-2MS cells were cultured for five days in 96-well plates at 5 x 10⁴ cells/ml (200 µl/well) in the absence or presence of different concentrations of AZT-NC and its controls. At the end of the incubation, 100 µl of 600 fold-diluted conditioned medium of each well was used to determine the p24 level according the protocol of the p24 ELISA kit made by ABL Biosciences. The remaining wells of cells were used for MTT assay to determine the cytotoxicity of tested AZT-NC and its controls. Anti-HIV activity using the MDM/p24 system was done as follows. Human monocytes were plated into wells of 96-well plates at 8.33 x 10⁴ cell/well. The cells were cultured in RPMI medium supplemented with 10% of fetal bovine serum, 5% of human serum and 100-fold diluted (from 100x solution) of penicillin/streptomycin for seven days until the monocytes have differentiated into monocyte-derived MΦs (MDM). The MDMs were then used to test NCs and their controls at different concentrations for 5 days in the presence of MΦ-trophic HIV-1 strain Ba-L at a multiplicity of infection [133] of 0.08. At the end of the 5-day incubation period, 10 µl/well of the culture medium of the plates was taken as samples for p24 assay and the remaining wells with cells were used for the MTT cell viability assay.

3.2.7 AZT Release from AZT-NC in Cell Culture medium
To be consistent with the МΦ anti-HIV activity assays, the release of AZT of AZT-NC prodrug and its non-mannosylated control-NC occurred in cell culture for 5 days. At the end of the 5-day period the conditioned media were used to determine free AZT amount. To avoid contamination of HPLC instrument with HIV-2 released by U937/HIV-2<sub>MS</sub> cells, the U937/HIV-2<sub>MS</sub> cells were not used. In their place, MT2 CD4<sup>+</sup> T cells were used.

5 x 10<sup>4</sup>/ml of MT-2 cells were cultured for five days in RPMI medium containing 12.5 µM of free AZT, AZT-NC, or control AZT-NC. After five days, 300µl of each conditioned medium was collected, 90 µl of which was sampled for HPLC analysis. The sample was mixed with 100 µl methanol, centrifuged at 10000 rpm/4 °C for 30 minutes, evaporated to reduce the volume and analyzed for free AZT by RP-HPLC monitored at 266 nm. The amounts of released AZT (pmole) were calculated from area under the curve (AUC) of the RP-HPLC using a standard AZT curve generated with the AUCs of different amounts of free AZT injected into the RP-HPLC column.
3.3 Results:

3.3.1 Drug-NC synthesis

The NCs were made of a peptidyl core and drug-alanine-OH. The peptidyl core was constructed using solid phase synthesis while the drug-alanine-OH was constructed using solution phase synthesis. The peptidyl core has one PEG\textsubscript{12} spacer, two serine-mannose units and three γ-Aminobutyric acid (gaba) units and one cysteine. As determined by the studies described in Chapter One, the highly soluble and flexible PEG\textsubscript{12} spacer provides the optimal spacer length of 49.5 Å for the two serine-mannose units, which is optimal for MR-mediated peptidyl core uptake. The Fmoc group on the immediate precursors, Fmoc-gaba-Ser(Man)-PEG\textsubscript{12}-Ser(Man)-gaba-gaba-Cys and the control non-mannosylated Fmoc-gaba-Ser-PEG\textsubscript{12}-Ser-gaba-gaba-Cys, was removed and reacted with succinic anhydride to resulted in an active hydroxyl group at the N terminus (Figure 3-1Figure 3-2). The correct molecular weights of the peptidyl cores were confirmed by MALDI-TOF (Figure 3-3) and their high purity (82%) by HPLC (Figure 3-4).

The releasable ester bond between a drug (AZT or RTV) and a peptidyl core were synthesized, in which a drug was pre-conjugated to Boc-alanine-OH (Figure 3-5, Figure 3-6). ESI/MS confirmed the correct molecular weight of 438.44 daltons for, as an example, pre-conjugated AZT (Figure 3-7). After purification, Boc de-protection was performed. MALDI-TOF/MS confirmed the correct molecular weight of 792 daltons for pre-conjugated RTV (Figure 3-9). A significant peak shift in HPLC profiles showing Boc removal from pre-conjugated AZT was observed (Figure 3-8).
The resultant NH₂-alanine-AZT or NH₂-alanine-RTV was reacted to the succinate peptidyl cores to form a drug-bearing NC. Figure 3-10 and Figure 3-11 shows the synthesis scheme, structures and molecular weight of the drug conjugated NCs. The molecular weight of AZT-NC (1892.8 daltons) was confirmed by MALDI-TOF/MS (Figure 3-12), and its control NC (1568 daltons) was also confirmed (Figure 3-14). The RTV-NC and its control NC were also confirmed by MALDI-TOF/MS, showing the expected molecular weight at 2346 daltons (Figure 3-14) and 2021 daltons (Figure 3-15), respectively. A clear and single RP-HPLC peak at 239 nm for AZT-NC (Figure 3-16) and similar peak at 266 nm for RTV-NC (Figure 3-16) were observed, respectively.

3.3.2 Drug release from Drug-NCs

Since the most reproducible anti-HIV activity results were obtained using U937-HIV-2MS cells as compared to using human monocyte-derived MΦs (MDMs) and MT2 CD4⁺ T cells and since production of HIV-2 p24 by U937-HIV-2MS cells is boosted by RTV, the U937-HIV-2MS/p24 anti-HIV assay system that we developed could only be used for AZT-NC and its control-NC. Therefore, drug release from prodrug NCs was studied at the non-cytotoxic concentration of 12.5 µM for AZT-NC and its control-NC, not for RTV-NC and its control-NC. To avoid contamination of the instrument by HIV-2 released from U937-HIV-2MS cells, AZT release in cell culture was studied in MT2 CD4⁺ T cell culture under the same conditions as that of the anti-HIV assay using the U937-HIV-2MS/p24 assay system. The released (free) AZT was separated from intact AZT-NC and control-NC and quantified using HPLC against a standard curve generated from the AUC values of a series known AZT amounts injected into the same HPLC column. AZT release rates were 20.6% and 25.1%, in five days for control-NC and AZT-NC,
respectively. In human plasma, RTV-NC (39 µM) at 37°C released 20% free RTV in 25 hrs, which is roughly in agreement with the amount release in tissue culture since human plasma contains 100% serum while tissue culture medium contains only 10% bovine serum. In different pH buffers, RTV-NC released free RTV at a comparable rate to that in human plasma with a slight increase at acidic pH, indicating that human serum does not contain appreciable carboxylesterase activity and the mechanism of release was mainly chemical in nature. As discussed later, this is consistent with the lack such activity in human plasma.

3.3.3 Anti-HIV activity

The data presented in this section are not final as more repeats and new studies are in progress or planned. All three types of cells, the U937-HIV-2_MS MΦ-like cell line, the MT2 CD4+ T cell line and human monocyte-derived MΦs (MDMs), have been used. HIV P24 matrix protein was used as the indicator of viral production. P24 is made late in viral cycle so it can be used to test all known HIV drugs working at different stages of viral cycle. So far the best result was obtained with the U937-HIV-2_MS/p24 system. Data obtained with the other two types of cells are more erratic.

Figure 3-17, Figure 3-18, and Figure 3-19 represent typical cytotoxicity and anti-HIV activity obtained with the U937-HIV-2_MS/p24 system for free AZT (Figure 3-17), AZT-NC (AZT-P-M in Figure 3-18) and non-mannosylated control-NC (AZT-P in Figure 3-19). Cells were treated with no or different concentrations of each tested compounds. As the tested concentrations increased, MTT cell viability and p24 production responded differently. MTT activity kept steady then fell abruptly after a certain high concentration
while p24 production fell gradually following the early phase of a sigmoid dose-response curve as indicated by the high $R^2$ values of curve-fitting. The reduction in p24 value in the concentration range where no MTT activity reduction, therefore, is contributed solely by the anti-HIV activity of tested compounds, not by cytotoxicity. Note, the highest concentration in Figure 3-17 is 32 µM while those in Figure 3-18 and Figure 3-19 are 50 µM. Note also that, to make the curves less compact, the X-axis has been made linear but still represents log values.

Comparing the upper fitted curves (MTT activity on the right Y-axis) of Figure 3-17 to that of Figure 3-18 and Figure 3-19, it is seen that AZT is slightly more toxic than AZT-NC and control-NC, which suggests that intact prodrugs in the tested concentration range were not cytotoxic. The non-toxicity at 12.5 µM was seen with MT2 cells as well and was thus used in the above release study. Examination of the lower curves (p24 production on the left Y-axis) in Figure 3-17, Figure 3-18, and Figure 3-19, it is seen that as the concentration increased, p24 production in U937/HIV-2MS cells decreased indicating that all three have anti-HIV-2 activity. Curve fitting of the three lower curves using dose-response equation of the Prism graphing software showed $R^2$ values of 0.9639, 0.9866 and 0.9843 for AZT, AZT-NC and the control-NC, respectively. The EC$_{50}$ value derived from the curves are 6.7, 8.4 and 53.8 µM for AZT, AZT-NC and control-NC, respectively. Since the only difference between AZT-NC and its control-NC is the presence and absence of two copies of mannose, respectively, used to target MR (Chapter One), the result suggests that the interaction between AZT-NC and MR on U937/HIV-2MS cells leads to better viral replication suppression than without MR targeting. Alternatively, the difference may be explained by slightly higher release rate of
AZT-NC than that of Control-NC (see below).

The anti-HIV activity (p24 production reduction) can also be used to derive AZT release in the U937/HIV-2MS/p24 system. Applying the curve fitting (dose-response) equation to Fig. 3-9 b and c, the p24 values at the non-toxic 12.5 μM concentration for AZT-NC is 20.71 pg/ml and that for its control-NC is 22.77 pg/ml, representing a 34% and 28% reduction in p24 production, respectively. This is consistent with conclusion obtained from the EC50 values described above that AZT-NC has a moderately better anti-HIV activity than its non-mannosylated control-NC. Assuming the anti-HIV-2 activity of the control-NC is due exclusively to the released free AZT and that the intact prodrug has zero activity and applying the 28% reduction in p24 production value of control-NC to Figure 3-17, the changing (ever increasing) concentration of AZT released by 12.5 μM control-NC from the start of the five day incubation period (0 μM) to the end of the incubation period has produced anti-HIV-2 activity (the 28% reduction in p24 production) that is equivalent to the activity of a constant AZT concentration of 1.48 μM (Figure 3-17). Assuming further that AZT release from control-NC and the reduction in p24 production over time is linear in the early phase of the sigmoid curve (i.e., assuming the lower curve in Figure 3-19 is linear), the deduced free AZT concentration at the end of the incubation period is 2.96 μM for control-NC, or a releasing rate of 24% in 5 days, which is close to the 20.6% value obtained in MT2 culture. The estimate of the amount release for AZT-NC was not made since, as mentioned previously, mannose ligand-MR interaction may partially contribute to the 34% reduction in p24 production. In other words, while it may be valid for the control-NC, application of the p24 reduction value to Figure 3-17 may not be valid for AZT-NC.
Preliminary result using the MDM/p24 system also shows similar pattern of anti-HIV activities for AZT-NC, AZT control-NC, RTV-NC and RTV control-NC (Figure 3-20). Since MDM/p24 system gave high well-to-well variability, the p24 concentration in a well was normalized by the well’s MTT activity (Y-axis of the figure). It can be seen that statistically significant reduction of p24 production was only observed for mannosylated AZT-NC and RTV-NC. With more repeats, it is expected that the two non-mannosylated control-NCs will show statistically significant but less p24 production.

3.4 Discussion

The long-term objective of this chapter is to synthesize prodrugs of small chemical drugs to target MΦs in tissues that are critical for a particular disease. The immediate goal of this Chapter is, as an example, to synthesize and test in cell culture MR-targeting AZT and RTV prodrug nanocarriers and their control nanocarriers. As discussed below, in view of the low or non-existent carboxylesterase activity in the plasma and high lysosomal esterase activities, our hypothesis is that the low molecular weight (~ 2 KDa) prodrug-NCs will not be cleaved in circulation and but will easily cross the endothelium barrier to reach tissue interstices to release active drug (AZT or RTV) once internalized via MR. This strategy combines two well validated concepts, the prodrug [145, 146] and the targeting [147, 148]. The strategy is not new. Many have pursued the “targeted prodrug” approach [149, 150]. The main rationale of making prodrugs is to improve the parent drug’s pharmacokinetics while that of targeting is to improve a parent drug’s therapeutic index and efficacy. Prodrugs are a large category of US FDA approved drugs
[151] and an AZT prodrug has been marketed [152]. What is unique in this study is the use of MR as the target and our novel peptidyl core with the same aim of slow release and increased cellular uptake as the “targeted prodrug” approach. The peptidyl core affords flexible but precise control of targeting moiety copy number and space between multiple copies of the targeting moiety as well as increased drug loads with multiple drug molecules per nanocarrier.

MΦs by definition are tissue interstitial-resided and dispersed. It is extremely difficult to study them in vivo and difficult to study in vitro. They are usually studied in tissue culture using MΦ-like cell lines or human monocyte-derived MΦs (MDMs) [153]. MΦ-like cell lines have the drawback of not faithfully representing tissue MΦs while MDMs are highly variable from person to person and from time to time within a person. We noticed that, even with commercially supplied human monocytes that are antibody affinity-purified from extremely large volume of pooled blood using blood bank supply, there is significant batch-to-batch variation in the ability to support HIV-1 replication when these cells were induced to become MDMs. For HIV infection studies, MΦ present an additional challenge. Unlike CD4+ T cells, MΦs support extremely low level of HIV replication. In our hands, p24 production in MΦ-like cell lines is at about 1000-fold less level as that produced in MT2 CD4+ T cell line. Consequently, it is so difficult to study drug anti-HIV activity in MΦs that some published papers used CD4+ T cell lines, such as MT2, not MDMs or MΦ-like cells. There is only one assay, the RT assay using radioisotopes, has sufficient sensitivity. For this reason, we decided to develop our own MΦ anti-HIV activity assay. It took a great deal of effort to develop the non-radioactive
U937/HIV-2<sub>MS</sub>/p24 test system, after exploring several MΦ cell lines and MDMs. The MΦ cell lines that were tested and found unsuitable for p24-based non-radioactive anti-HIV activity assay include U937, U1 [154], BF24 [155], Mono Mac [156]. P24 made by HIV-2<sub>MS</sub> is adequately detected by the p24 ELISA kit and AZT is equally effective against HIV-1 and HIV-2 [157]. Therefore, this assay system is suitable to test the current hypothesis. The only drawback is that RTV boosts, instead of suppresses, p24 production in U937/HIV-2<sub>MS</sub> cells, making the assay unable to test RTV-NC. RTV-NC and its Control-NC were tested using the less satisfactory MDM/p24 system.

By design, the current prodrugs rely on carboxylesterase to cleave the carboxylate ester bond between the drug and the peptidyl core. Esterases belong to a very large collection of difficultly classified hydrolases [158-160], many of them having overlapping substrate specificity. For example, carboxylesterases cleave both aliphatic ester bond and amide bond. Pharmacologically, the most important group of esterases is the carboxylesterases, consisting no more than seven members in human [160, 161]. In addition to aliphatic ester and amide bonds, they cleave thioesters, phosphoric acid esters, and acid anhydrides. These enzymes are highly expressed in the liver, the small intestine, the kidneys and the lungs, but are also widely present at lower levels in various other tissues. In the plasma, however, the previously characterized carboxylesterase activity belongs to other types of esterases [160]. A more precise biochemical study, backed by proteomic evidence, ruled out the presence of carboxylesterate in human plasma [153]. At the cellular level, most carboxylesterases are localized in the endoplasmic reticulaulm (ER) and lysosomes [160]. Cytosolic localization has also been suggested but not certain [153, 162]. Within a cell, esterases are transported from ER via the Golgi apparatus to
late endosomes (pH = ~6) that derive from early endosomes or phagosomes and mature into lysosomes (pH = ~5) (Alberts, B et al, 2004). Therefore, MR-mediated endocytosis of AZT-NC and RTV-NC is likely to be eventually delivered to lysosomes where acidic carboxylesterases release AZT and RTV. The released drugs would have higher concentrations than the concentrations in the extracellular space. Since free drugs can diffuse into cells, the higher concentrations of released free drugs within the lumen of lysosomes can diffuse out to reach cytosol to exert their pharmacological effect. This may explain why AZT-NC is more active than its control-NC in the U937/HIV-2MS/p24 assay system and both AZT-NC and RTV-NC are more active than their respective control-NCs in the human MDM/p24 assay system. The alternative explanation of higher releasing rate for AZT-NC and RTV-NC than their respective control-NCs cannot be ruled out.

Directly measuring released free AZT from control-NC using HPLC in the MT2 cell culture as well as the deduction based on anti-HIV-2 activity under the U937/HIV-2MS/p24 assay conditions indicated that AZT release in five days is ~ 21-25% for both control-NC and AZT-NC at 12.5 μM which is roughly in agreement with the result for RTV-NC in human plasma. The release in cell culture and in human plasma is comparable to that in buffers, indicating the chemical nature of the slow release in plasma. Combined, the release data shows a slow release rate consistent with the expectation of low or non-existing carboxylesterase activity in human serum.

In summary, the data in this Chapter represent a work-in-progress support but our “prodrug targeting strategy” that the AZT-NC and RTV-NC prodrugs would not be significantly cleaved in circulation before renal clearance. During the circulation, the
small size of the prodrugs would allow for sufficient tissue penetration to reach MR on MΦs. This would be followed by internalization by endocytosis and lysosomal release of cleaved active AZT and RTV. To fulfill the long-term objective of this strategy, much more work, especially those using an animal disease model, lies ahead, in which the size of prodrugs, the releasable bond and the drug load can be tweaked to improve pharmacokinetics and pharmacodynamics.
Results

Figure 3-1 Structure of Succinimidyl Mannosylated Nanocarrier

Figure 3-2 Structure of Succinimidyl Nanocarrier (control)
Succinimidyl mannosylated NC was characterized using MALDI-TOF/MS. A \( [1556.75+K]^+ \) was obtained in the MALDI-TOF spectrum, confirming the molecular weight of succinimidyl mannosylated NC.
Reverse phase HPLC (C18 column) was used to determine the succinimidyl mannosylated NC. With 1mL/min flow rate, the mobile phase gradient was from 5% to 100% in 6 minutes. HPLC UV spectrum of mannosylated NC was obtained at 254 nm UV wavelength using UV detector. (Stationary phase: 0.05 v/v% TFA in H₂O, Mobile phase: 0.05 v/v% TFA in acetonitrile)
Figure 3-5 Scheme of AZT-Alanine-NH₂ Conjugation Reaction

Figure 3-6 Structure of RTV-Alanine-NH₂ Conjugation
Figure 3-7  Electrospray Spectrometry Ionization-Mass Spectrometry Characterization of AZT-Alanine-Boc

ESI/MS was performed to confirm the AZT-alanine-Boc prodrug unit. Peak 437.2 [M+ Cl^-] shown in the profile present the correct M.W. 438.
A. HPLC (C-18) profile of AZT-alanine-BOC (retention time: 4.2 minute)

B. HPLC (C-18) profile of AZT-alanine-NH₂ (retention time: 2.35 minute)

Figure 3-8 HPLC profile of (A) AZT-Alanine-Boc and (B) AZT-Alanine-NH₂

Analyze retention time and purity of AZT-alanine-BOC and AZT-alanine-NH₂ using reverse phase HPLC (C-18 column). A significant peak shift from 4.2 minute (Fig 3-5-a) to 2.35 minute (Fig 3-5-b) was observed from HPLC profiles with the removal of Boc protection group. With 1mL/min flow rate, the mobile phase gradient was from 5% to 100% in 6 minutes. HPLC UV spectrum of mannosylated NC was obtained at 239 nm wavelength using UV detector. (Stationary phase: 0.05 v/v% TFA in H₂O, Mobile phase: 0.05 v/v% TFA in acetonitrile)
The RTV-alanine-BOC was de-protected by removing BOC protection group. MALDI-TOF/MS spectrum confirmed the RTV-alanine-OH.
A. AZT conjugated mannosylated (two mannose) nanocarrier (AZT-NC)

B. AZT conjugated nanocarrier (Control NC)

Molecular weight 1894.0080

Figure 3-10 SchemeStructure of (A) AZT-NC (2 mannose) (B) AZT Control NC (no mannose)
C. RTV conjugated mannosylated (two mannose) nanocarrier

Molecular Weight: 2347.7109

D. RTV conjugated nanocarrier (Control)

Exact Mass: 2021.9651
Molecular Weight: 2023.4297

Figure 3-11 Scheme Structure of (A) RTV-NC (two mannose) (B) RTV Control-NC (no mannose)
Figure 3-12 MALDI-TOF/MS Spectrom of AZT-NC (AZT-mannosylated peptide)

1892 daltons of molecular weight was obtained from MALDI-TOF/MS, confirming the success of AZT-NC synthesis.
1568 daltons of molecular weight was obtained from MALDI-TOF/MS, confirming the success synthesis of Control-NC of AZT.
2369 daltons [2347+Na]$^+$ of molecular weight was obtained from MALDI-TOF/MS, confirming the success of RTV-NC synthesis.
2045 daltons $[2022+\text{Na}]^+$ of molecular weight was obtained from MALDI-TOF/MS, confirming the success of RTV-NC synthesis.
A. HPLC profile of AZT-NC-(mannose)$_2$ using C18 column

B. HPLC profile of RTV--NC-(mannose)$_2$ using C18 column

Figure 3-16 HPLC Profile of (A) AZT-NC and (B) RTV-NC using RP-HPLC (C18 column)

AZT-alanine-NH$_2$ and RTV-alanine-NH$_2$ have been characterized using reverse phase HPLC (C-18 column). A significant and lone peak at 2.8 minute (A) showed the product of AZT-NC-(mannose)$_2$. The clear peak at 3.9 minute (B) showed the product of RTV--NC-(mannose)$_2$. With 1mL/min flow rate, the mobile phase gradient was from 5% to 100% in 6 minutes. HPLC UV spectrum of AZT-NC-(mannose)$_2$ was obtaind at 239 nm wavelength using UV detector. HPLC UV spectrum of RTV-NC-(mannose)$_2$ was obtaind at 266 nm wavelength using UV detector. (Stationary phase: 0.05 v/v% TFA in H$_2$O, Mobile phase: 0.05 v/v% TFA in acetonitrile)
Figure 3-17 Anti-HIV Activity and Cytotoxicity of Free AZT

Macrophage-like U937/HIV-2\textsubscript{MS} cells were treated with different concentrations of free AZT for five days and HIV-2 viral matrix p24 protein in culture medium was determined (left Y-axis) as the indicator of viral replication level. The cells in wells were then assessed for their viability using the MTT assay (right Y-axis).
Figure 3-18 Anti-HIV Activity and Cytotoxicity of AZT-NC

Macrophage-like U937/HIV-2MS cells were treated with different concentrations of AZT-NC conjugate for five days and HIV-2 viral matrix p24 protein in culture medium was determined (left Y-axis) as the indicator of viral replication level. The cells in wells were then assessed for their viability using the MTT assay (right Y-axis).
Macrophage-like U937/HIV-2<sub>MS</sub> cells were treated with different concentrations of free Control NC for five days and HIV-2 viral matrix p24 protein in culture medium was determined (left Y-axis) as the indicator of viral replication level. The cells in wells were then assessed for their viability using the MTT assay (right Y-axis).
Figure 3-20 Anti-HIV Activity of Drug Conjugated NCs on HIV-1 Infected MDM Cells

Anti-HIV activity of drug conjugated NCs were evaluated in human monocyte derived MΦs (MDM) using P24 assay. Results shown are representative of three independent experiments. (*, statistically significant difference between treatments, p < 0.05.)
4 REFERENCE


5 Curriculum Vitae

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