Characterization of anti HIV-1 activity of curcumin and its analogues and their delivery by apotransferrin nanoparticles

DOCTOR OF PHILOSOPHY

By

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Thesis submitted for the degree of

Doctor of Philosophy in Biotechnology

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DECLARATION

I hereby declare that the work presented in this thesis entitled ***Characterization of anti HIV-1 activity of curcumin and its analogues and their delivery by apotransferrin nanoparticles*** is entirely original work and was carried out by me in the Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, Hyderabad, under the supervision of Prof. Anand. K. Kondapi. I further declare that to the best of my knowledge this work has not formed the basis for the award of any degree or diploma of any university or institution.

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CERTIFICATE

This is to certify that this thesis entitled *"Characterization of anti HIV-1 activity of curcumin and its analogues and their delivery by apotransferrin nanoparticles"* is a record of bonafide work done by Mr. Upendhar Gandapu, a research scholar for Ph.D. programme in the Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad under my guidance and supervision.

The thesis has not been submitted previously in part or full to this or any other University or Institution for the award of any degree or diploma.

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List of Abbreviations

HIV	-	Human Immunodeficiency Virus
AIDS	-	Acquired Immune Deficiency Syndrome
WHO	-	World Health Organization
HAART	-	Highly active antiretroviral therapy
SIV	-	Simian immunodeficiency virus
FIV	-	Feline immunodeficiency virus
LTR	-	Long terminal repeats
PR	-	Protease
IN	-	Integrase
RT	-	Reverse transcriptase
NRTIs	-	Nucleoside Analogue Reverse Transcriptase Inhibitors
NNRTI	-	Non- Nucleoside Analogue Reverse Transcriptase Inhibitors
NtARTIs	-	Nucleotide analog reverse transcriptase inhibitors
AML	-	Acute Myelogenous Leukemia
FDA	-	Food and Drug Administration
Pls	-	Protease inhibitors
CCD	-	Catalytic Core Domain
EV	-	Elvitegravir
TP	-	Tipranavir
AV	-	Adefovir
GPCR	-	G-protein coupled receptors
MM	-	Molecular Mechanics
MDS	-	Molecular Dynamics Simulation
FEP	-	Free Energy Perturbation
RCM	-	Relaxed Complex Method
Mtb	-	Mycobacterium Tuberculosis
KEGG	-	Kyoto's Encyclopedia of Genes and Genomes
SWISSPROT	-	Swiss Protein Data Base
FASTA	-	Fast all sequences
BLAST-P	-	Basic Local Alignment Tool for Proteins
CPU	-	Central Processing Unit

3D	-	Three-Dimensional
DNA	-	Deoxyribonucleic acid
RNA	-	Ribonucleic acids
NMR	-	Nuclear Magnetic Resonance
PDB	-	Protein Data Bank
SCRs	-	Structurally Conserved Regions
VR	-	Variable Regions
CADD	-	Computer Aided Drug Designing
SA	-	Simulated Annealing
RMSD	-	Root Mean Square Deviation
RMSF	-	Root Mean Square Fluctuation
ADT	-	Auto Dock Tools
NCBI	-	National Center for Biotechnology Information
VMD	-	Visual Molecular Dynamics
PMDB	-	Protein Model Data Bank
SPDV	-	Swiss PDB Viewer
Pfam	-	Protein family
e-value	-	Expectation Value
Log P	-	Logarithm of Partition Coefficient
TPSA	-	Topological Surface Area
MW	-	Molecular weight
nON	-	Number of hydrogen donors
nOHNH	-	Number of hydrogen acceptors
nrotb	-	Number of rotatable bonds
µL		Microlitre
µg		Microgram
µM		Micromolar
µg		Microgram
mM		Milligrams
BSA		Bovine serum albumin
cDNA		Complementary DNA
CO2		Carbon dioxide
DMSO		Di methyl sulphoxide
DNA		Deoxy ribonucleic acid

dNTPs		Deoxynucleotidetriphosphates
dsDNA		Double-stranded DNA
HMM	-	Hidden Markov Model
TopoIIβ	-	Topoisomerase IIβ
TopoII α	-	Topoisomerase II α
TNF-α	-	Tumor necrosis factor alpha
IL-1β	-	interleukin-1 β
COX-2	-	Cyclooxygenase-2
FBS	-	Fetal Bovine Serum
mRNA	-	Messenger RNA
PBS	-	Phospahte buffer saline
PCR	-	Polymerase chain reaction
MTT	-	3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide

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CHAPTER I:

INTRODUCTION

1.1 HIV-1

Human immunodeficiency virus is major causative agent for development of acquired Immuno Deficiency Syndrome (AIDS), a condition in human's low immunity and opportunistic infections. The AIDS has evolved as a ruinous epidemic in recent times around the globe. United States Center for Disease Control (USCDC) in 1981 reported five unique pneumonia patients who had common symptoms of Pneumonia along with impaired T cell functions to antigenic stimulation (T cell dysfunctions) and immunodeficiency (MMWR, 1981). These distinctive symptoms were later identified as AIDS related symptoms. The major cause of T cell impairment was the virus infects CD4+ T cells in majority of infected patients. This virus was distinct from already known human T cell leukemia virus (HTLV). HTLV viruses often cause T-lymphocytes immortalization where as in AIDS infections; CD4+ T cells undergo cell death in the course of disease progression. Eventually, the causative agent of AIDS was recognized as a retrovirus by three individual groups of scientists at the same time (Barre-Sinoussi et al, 1983; Gallo et al, 1984; Levy et al, 1984) after that it was named as Human immunodeficiency virus type 1 (HIV-1) (Coffin et al, 1986). After recognizing HIV-1 another virus was identified based on the virus sequence variability and definite antigenicity to previously identified HIV-1 strains and it was named as Human immunodeficiency virus type 2 (HIV-2) (Clavel et al, 1986).

1.2 Epidemiology

According to the 2012, UNAIDS estimation, so far 27 million people were died (1.6 million in 2012) around the world since its identification in 1981 and 35.3 (32.2–38.8) million people were still living with HIV and there was 2.3 (1.9–2.7) million new HIV-1 infections have reported in 2012 around the globe. In India, about 2.5-3.5 million people are currently surviving with HIV-1 (NACO,2012). The epidemiologic studies have suggested that the major routes of transmission of AIDS are sexual contact, contaminated blood and mother to child transmission. AIDS was initially reported in homosexual and bisexual men and intravenous drug users (Gottlieb et al, 1981; Masur et al, 1981; Siegal et al, 1981).

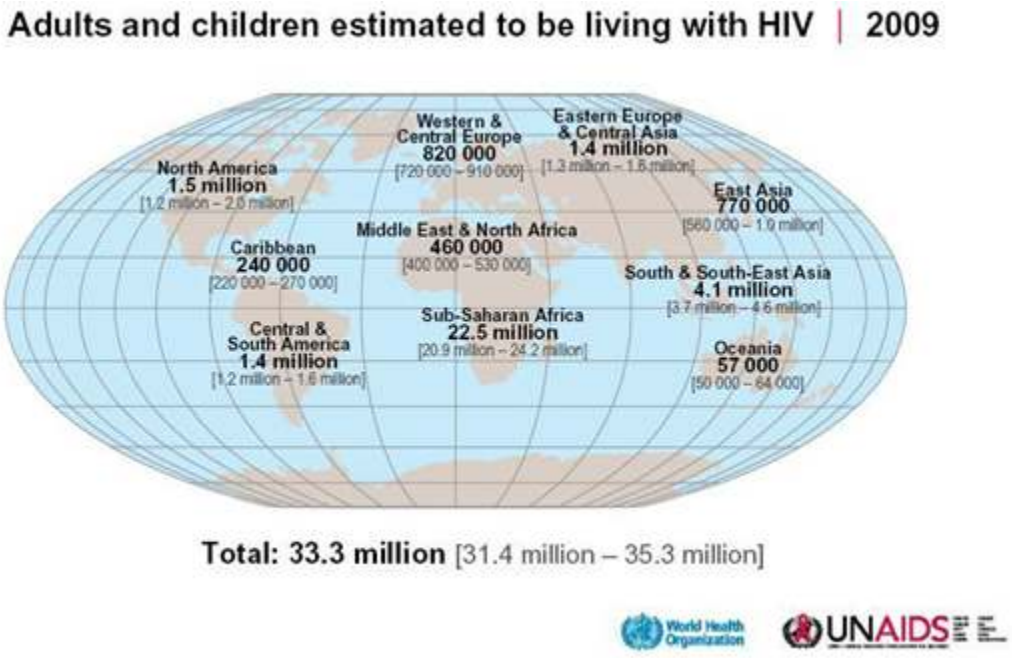


Fig 1.1: ***Estimated number of people living with HIV-1 infection in differentparts of the world in the year 2009 (Adapted from UNAIDS report, 2009).***

The infectious virions of HIV-1 are present in HIV-1patients body fluids like blood plasma, cerebrospinal fluids, semen, vaginal fluids, and breast milk, and the major roots of HIV-1 transmission is by sexual, blood to blood and mother to child transmissions. In acute infection of HIV-1the viral load in the blood plasma can be diagnosed within few weeks of HIV-1 infection after that this viral load then reduced due to the active antiviral immune response of person. The $CD4^+$ T Lymphocytes will rapidly deplete during the course of HIV-1 infection. In asymptomatic infected persons the $CD4^+$ cells count will be > 500 $CD4^+$ cells/µl and in symptomatic patients this can be< 200 $CD4^+$ cells/µl. The progress of HIV-1 infection to AIDS in virus infected patients depends on clinical state of person.

1.3 HIV-1 virion, genomic structure and viral gene expression

HIV-1 belongs to genus lentivirus and retroviridae family. It is an enveloped virus and spherical in shape with around 100 nm in size. The envelope covers the cone shaped core composed of viral capsid (CA) protein. Inside CA protein and two identical positive RNA strands are present in closely bonded with reverse transcriptase and nucleocapsid protein (NC). The inner portion of the viral membrane contains a lipid bilayer of myristoylated matrix protein (MA). This matrix protein is important for the integrity of the virion. The viral core contains viral proteins like Vif, Vpr and Nef. the virion also associate with some of the host cell proteins such as MHC molecules, actin, coflinmoesin, ezrin,and actin binding cellular proteins (Ott et al, 1996; Ott et al, 2000). The envelope HIV-1 is made up of

72 glycoprotein spikes of envelope glycoproteins gp120 and gp41 on the surface of the virion. (Figure-1.3.1).

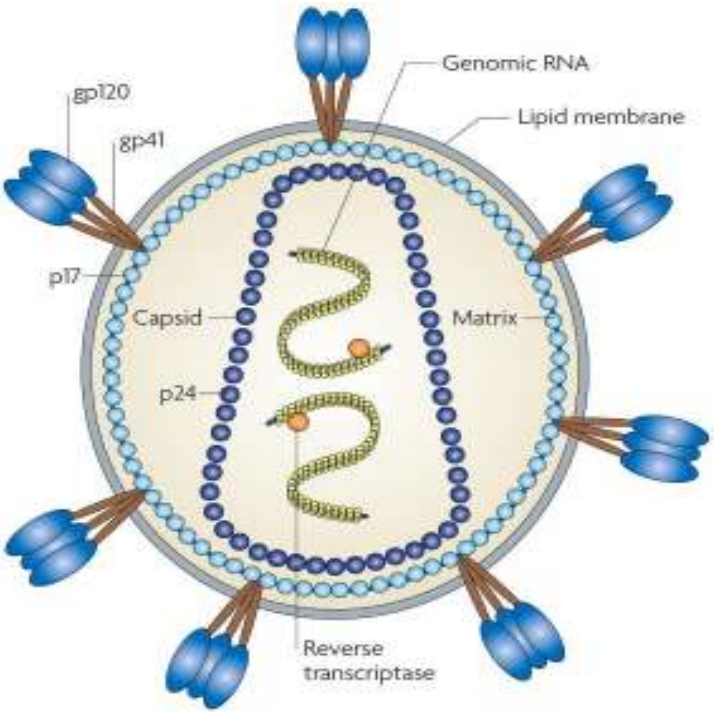


Fig 1.3.1: ***Schematic representation of HIV-1 virion structure (***Adapted from (Hedestam et al., 2008)***).***

The genomic size of the HIV is about 9.8kb with open reading frame coding 2 structural genes (Gag, ENV) and three enzymes(RT,IN,PR) and six accessory genes that help to regulate viral replication. The genome contains Long Terminal Repeat (LTR) sequences at both ends (5′ and 3′) of the viral genome (Figure-1.). The 5' LTR acts as a viral promoter and is essential for viral gene expression. The LTR of HIV-1 is divided in to three regions: U3 (nucleotides - 454 to – 1 relative to the transcription start site), R (nucleotides + 1 to + 60) and U5 (nucleotides + 60 to +181). The U3 and R regions have four functional regions which participate in regulation of LTR mediated transcription. One is trans-activation response element

(TAR) (nucleotides + 1 to + 60) found within R region, second is core promoter region (nucleotides - 78 to - 1), the third region core enhancer region (nucleotides - 104 to – 79) and fourth one is modulatory region (nucleotides - 454 to – 105). From nucleotides – 340 to – 184 a negative regulatory element (NRE) is present in the modulatory region, deletions in NRE increases LTR-directed transcription (Rosen et al, 1985; Siekevitz et al, 1987). LTR containstwo to three NFκB binding sites in core enhancer region and three to four Sp1 binding sites in core promoter region depending upon the genetic subtype (Pereira et al, 2000). The TATA box (nt - 22 to – 27), initiator (nt - 7 to + 30), and Sp1 binding sequences (nt - 46 to – 78) are participated in positioning the RNA polymerase at the right site for initiating transcription. Several cellular factors such as nuclear factor of activated T cells (NFAT), upstream stimulating factor (USF), NFκB, cellular proto-oncogene c-Myb, and chicken ovalbumin upstream promoter transcription factor (COUP) have binding sites in the modulatory region and regulate LTR promoter activity (Pereira et al, 2000). A high level of virus replication is observed in the presence of these transcription factors in activated T cells and differentiated macrophages. The viral genome encodes for group specific antigen (gag), polymerase (pol) and envelope (env) poly-proteins, the genome contains open reading frames that encodes 15 proteins. Along with these proteins, HIV-1 encodes another two spacer peptides p1 and p2, which are not completely understood. All unspliced genomic RNA and singly spliced transcripts like gag-pol, Viral protein r (Vpr),Virion infectivity factor (Vif), Viral protein u (Vpu) and Envelope protein (env) require Regulator of virion gene expression (Rev) protein for their export from the nucleus to cytoplasm

(Frankel et al, 1998). The gag protein is a 55 kDa precursor protein and is cleaved by viral protease into several smaller functional peptides, during maturation process. The pol mRNA is expressed as a160 kDa gag-pol fusion protein. During protein synthesis both gag-pol polyproteins are encoded by the same mRNA but they translated into different proteins by the usage of alternative reading frames.

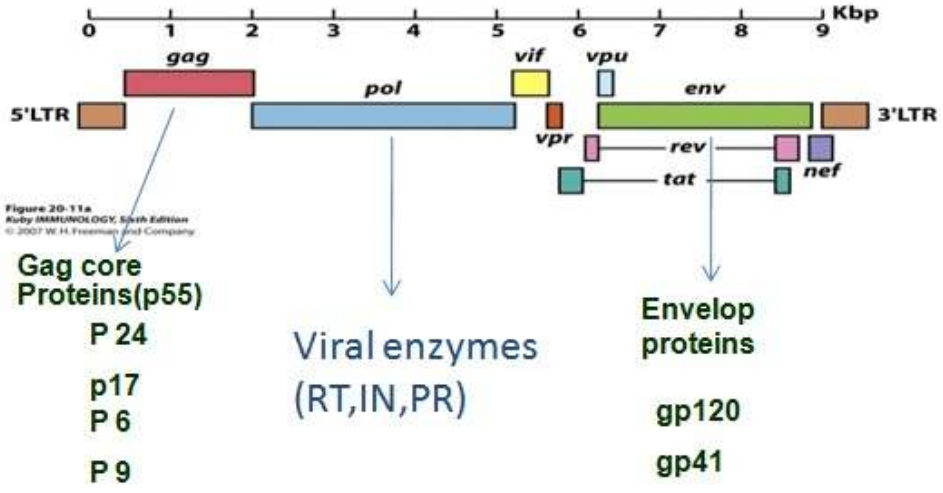


Fig 1.3.2: ***The HIV-1 proviral genome transcripts and proteins. Here, Black lines denote unspliced and spliced transcripts, above which coding sequences are given, with start codons indicated (Adapted from Peterlin et al, 2003).***

The envencodes the envelope glycoproteins gp120 (SU) and gp41 (TM). The mRNA of env is a singly spliced RNA, initially it is translated into an 88kDa precursor protein, further it is glycosylated to become 160 kDa protein (gp160). The cellular endo-proteases such as human protein convertase 6 (hPC6) cleaves the gp160to become a mature envelope glycoproteins gp120 and gp41 (Miranda et al, 1996).The Nef, Tat, and Rev viral mRNAs are fully spliced and are expressed during viral

replication. The structural, enzymatic proteins and factors of HIV-1 that regulate virion infectivity are singly spliced or unspliced. The transport of un-spliced and singly spliced viral mRNAs from the nucleus to cytoplasm are regulates by Rev Protein.

1.4 Proteins coded by HIV-1

HIV-1 genome encodes for 15 different proteins. In maturation stage of viral life cycle, viral protease cleaves poly-proteins to produce mature form of viral proteins. HIV-1 proteins can be categorized into four classes based on their biological functions such as structure, catalytic activities and regulation of viral gene expression.

1.4.1 Structural proteins

In the first three structural genes*gag*, *pol*, and *env*, was synthesized as poly-protein precursors, later they were cleaved into mature proteins by viral or cellular protease. The 55-kD gag precursor ($Pr55^{Gag}$) is self-assembled on the cell membrane to form an immature viral particle and cleaved into the structural proteins by protease:(MA), capsid (CA), nucleocapsid (NC), and p6 during maturation (Gheysen et 1989; Wiegers et al., 1998). *pol* has 241 nucleosides overlapping with *gag*. The 160-kd Gag-pol polyprotein ($Pr160^{Gag\text{-}pol}$) is processed into protease, transcriptase, and integrase by autocatalysis (Jacks et al., 1988b). The glycosylated 160-kD Env precursor is proteolytically digested by cellular enzymes into surface (SU) glycoprotein the gp120 and transmembrane (TM) glycoprotein the gp41 (Chan & Kim, 1998a; Earl, Moss, &Doms, 1991).

1.5.2 Enzymatic proteins

The gag-pol (p160) is the precursor of polymerase (pol) polyprotein that undergoes proteolytic cleavage to produce viral enzymes reverse transcriptase (RT), integrase (IN) and protease (PR).

1.5.2.1Protease (PR, p10)

HIV-1 protease is an aspartyl protease and it cleaves the gag-pol, gag, pol and Nef precursor proteins to produce mature viral proteins. It is a homodimer protein and like other aspartyl proteases its active site contains Asp-Thr-Gly(catalytic triad) conserved residues at positions 25 to 27 (Pargellis et al, 1994). The enzyme has a flexible flap region and it closes down on the active site after binding of the substrate. The hydrophobic substrate cleft identifies different peptide sequences of viral poly-proteins and cleaves to produce completely processed, mature proteins (Shao et al, 1997).

1.5.2.2 Reverse transcriptase (RT, p51/p66)

HIV-1 reverse transcriptase(RT) is a heterodimeric protein contains 51 kDa and 66 kDa subunits. The p51 subunit has both RNA dependent DNA polymerase activity and DNA dependent DNA polymerase activity and the p66 subunit contains another small 15 kDa (p15) subunit which is covalently bound to the p55 subunit. The p15 has Rnase H activity and it is useful for the removal of RNA template after synthesis of DNA strand (RNA/DNA hybrid) and this allows the synthesis of full-length viral cDNA. The p51 and p15 subunits are produced by incomplete proteolytic cleavage of pol polyprotein (Lowe et al, 1988). RT produces a double stranded DNA copy (viral cDNA) of viral genomic RNA. The RNA dependent DNA polymerase activity,

DNA dependent DNA polymerase activity and Rnase H activity of the active reverse transcriptase enzyme are useful for the full-length viral cDNA synthesis. The RT lacks 3' → 5' exonuclease activity and this frequently leads to the error prone cDNA synthesis (Roberts et al, 1988; Preston et al, 1988) which leads to drug resistant viruses.

1.5.2.3 Integrase (IN, p32)

The 3' end of the pol polyprotein encodes integrase enzyme. It consist three structural domains: the amino terminal domain (NTD), catalytic core domain (CCD) and carboxy terminal domain (CTD). These domains are important for enzyme catalysis. Integrase catalyzes the integration of viral cDNA(proviral DNA) into the host's chromosomes. Some reports suggest that CCD of integrase binds to the reverse transcriptase enzyme and may help in reverse transcription (Zhu et al, 2004). Cellular proteins such as Integrase interactor 1 (INI1), Lens Epithelium Derived Growth Factor (LEDGF, p75), Embryonic ectoderm Development protein (EED) and Heat Shock Protein 60 (HSP 60) bind to the integrase and stimulate enzymatic activities of integrase (Kalpana et al, 1994; Parissi et al, 2001; Cherepanov et al, 2003 and Violot et al, 2003).

1.5.3 Regulatory proteins

HIV-1 encodes six regulatory proteins encoded by *tat*, *vif*, *vpr*, *vpu*, *nef*, and *rev*, which are translated by spliced mRNA.

Tat (Trans-activator of transcription) is responsible for the activation of HIV gene expression and up-regulation of the level of transcription of HIV dsRNA, in conjunction with RNA polymerase II (Frankel &Pabo, 1988; Herrmann & Rice, 1995; Jones &Peterlin, 1994).

Vif (Viral infectivity factor) inhibits the antiviral activity of human cellular enzyme APOBEC family by directly binding and triggering ubiquitination and degradation (H. M. Liu et al., 1995; J. H. Miller, Presnyak, & Smith, 2007).

Nef means "Negative regulatory factor", it helps for increasing the viral infection and replication by regulating host cellular machinery and it can promote the survival of infected cells by down-regulating the surface molecules expressed in host immune cells such as MHC molecules presented on APCs and T Lymphocyte cells (Goldsmith, Warmerdam, Atchison, Miller, & Greene, 1995; Leonard, Filzen, Carter, Schaefer, & Collins, 2011; Salghetti, Mariani, &Skowronski, 1995).

Rev indicates "Regulator of virion" and it promotes the exportation and expression of the precursor proteins by overcoming the host RNA splicing mechanisms (GreweUberla, 2010; Levin et al., 2010).

Vpr represents "Viral protein R", and it is required for efficient viral replication and is important for virus replication in non-dividing cells such as macrophages (Vodicka, Koepp, Silver, &Emerman, 1998). Vpr leads to immune dysfunction and immune suppression in proliferating cells by inducing G2 cell cycle arrest and

apoptosis.(Bukrinsky&Adzhubei, 1999; Muthumani, 2006).

Vpu known as "Viral protein U" and it is participates in virus release (Klimkait, Strebel, Hoggan, Martin, & Orenstein, 1990; Neil, Eastman, Jouvenet, &Bieniasz, 2006; Terwilliger, Cohen, Lu, Sodroski, &Haseltine, 1989).

Some proteins produced from gag and envelope (env) polyproteins are involved in the virion structure construction. The gag (p55) polyprotein gives rise to p17 (MA), p24 (CA), p2, p7 (NC), p1 and p6. The cellular protease processes the envpolyprotein to a non-covalent complex of gp120external glycoprotein (SU) and agp41 transmembrane glycoprotein (TM). Membrane associated gag binds to two copies of viral genomic RNA along with cellular and viral proteins that trigger budding of virion from infected cell surface (Bryant et al, 1990

1.6 HIV-1 life cycle and possible drug targets

Viral life cycle comprises of several steps, which can be targeted by anti-HIV-1 drugs. These steps include entry, reverse transcription, integration, transcription, assembly and budding (figure-1.5)

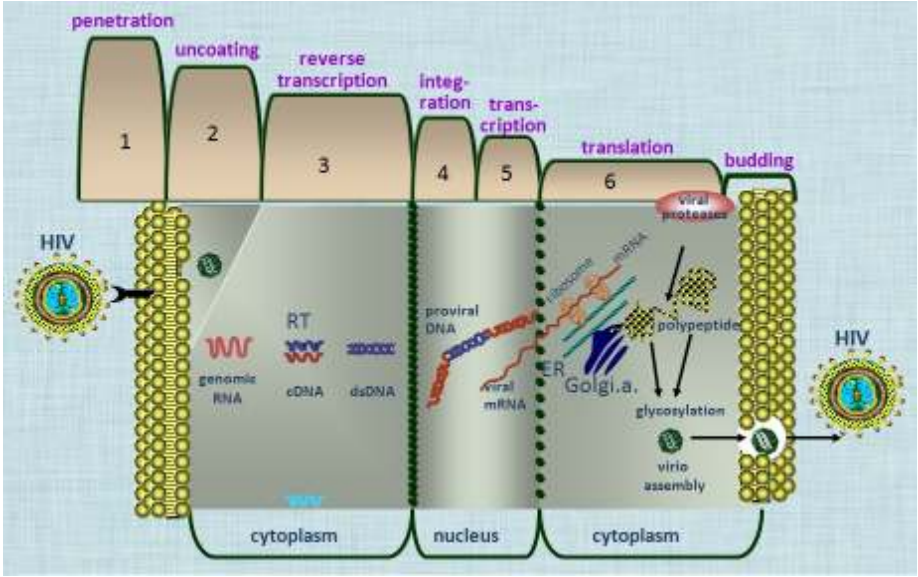


Fig 1.5: ***A schematic representation of HIV-1 life cycle (Adapted from Han etal, 2007)***

1.6.1 Entry

The entry of HIV-1 into the target cell is a multi-step process. In the first the viral surface envelope protein gp120 interacts with CD4 of host cell membrane and induces conformational changes that promote the binding of gp120 to selective chemokine receptors. The gp120 and CD4 interaction induces a conformational change in gp41transmembrane protein and it is a coiled coil protein, projects its three fusion peptide domains into the plasma membrane of the target cell. With this process, a hairpin is formed this leads to fusion of virion and target cell membranes and subsequent release of viral core into the cell interior (Figure-1.8) (Chan et al, 1998).

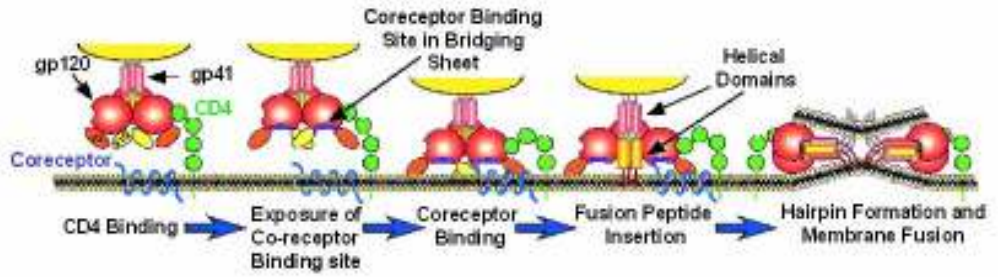


Fig 1.6.1.: ***A model for HIV-1 entry (Adapted from Doms et al, 2000)***

1.6.2 Reverse transcription

After the release of viral core in to the target cell, the virion gets uncoated to liberate reverse transcription complex from the virion core into the cytoplasm. The reverse transcriptase complex consists of two viral genomic RNAs, $tRNA^{Lys}$ primer, viral proteins RT, IN, MA, NC, Vpr and several host proteins. In this step phosphorylation of matrix (MA) protein take place by a MAP kinase. Cyclophilin A, Viral proteins Nef and Vif are essential for efficient reverse transcription process (Greene et al, 2002). The $tRNA^{Lys}$ hybridizes to the HIV-1 RNA by binding to the primer-binding site (PBS) and RT synthesizes a short fragment of DNA through U5 and R regions and stops at the end of U3 region of 5' LTR.

The Rnase H activity of RT digest the RNA present in the form of RNA/DNA hybrid at 5' LTR region, this facilitates the release and further binding of synthesized small fragment of 5' LTR DNA (U3 to PBS) to the R region of 3' LTR. Then, the RT extends R region and first negative DNA strand (complementary DNA) is synthesized (Figure-1.9). The genomic RNA strand is digested till polypurine tract of negative strand DNA by Rnase H activity of RT. The undigested RNA acts as a

primer for synthesis of complementary positive strand DNA. At last, synthesis of positive DNA strand and 5' LTR from PBS to double stranded DNA with 5' and 3' LTRs (viral cDNA) this completes the reverse transcription process (Panganiban et al, 1988; Kim et al, 1989). Any molecules that bind to the catalytic site of RT inhibits its catalytic functions and blocks HIV-1 replication. Termination of cDNA synthesis by nucleoside and nucleotide analogues is also an excellent strategy for inhibition of viral replication.

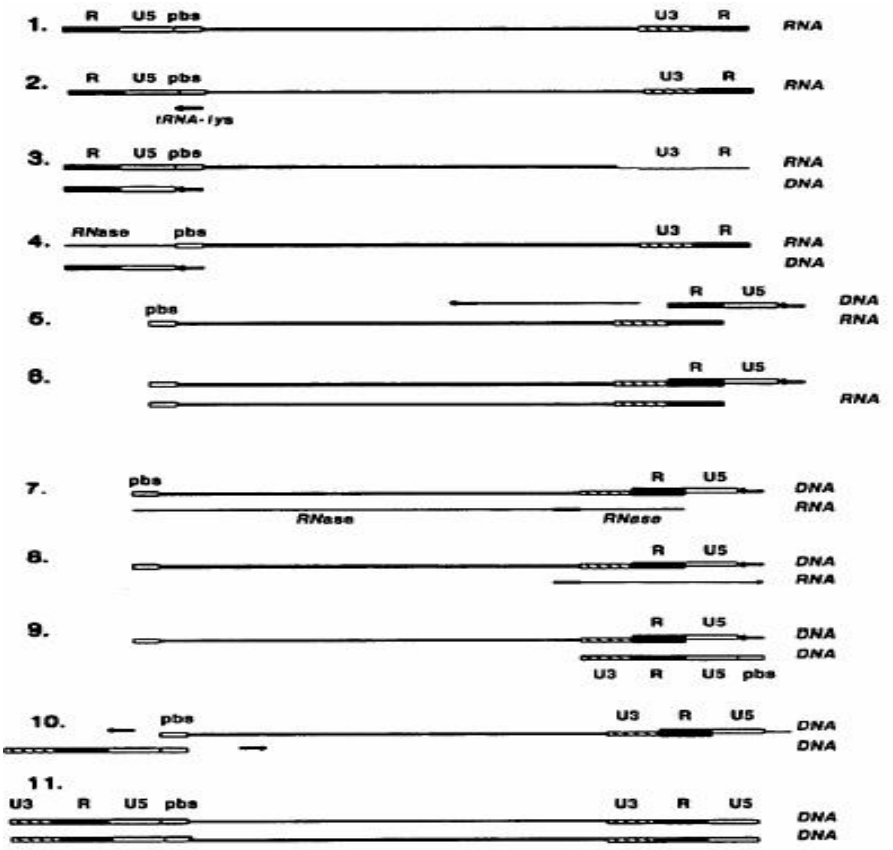


Fig 1.6.2:*Synthesis of HIV-1 cDNA from its genomic RNA in reverse transcription (Wang et al, 2000)*

1.6.3 Integration

After reverse transcription, RT bound viral double stranded cDNA, integrase, MA, Vpr along with several cellular factors form the pre-integration complex (PIC). Viral cDNA is primed for integration in cytoplasm by integrase-mediated removal of two nucleotides at the 3'- ends of viral DNA (3' processing). PICs are transported into the nucleus due to the karyophilic properties of viral proteins IN, MA, Vpr and a DNA flap (a triple helical DNA doman) generated due to the discontinuous synthesis of viral positive DNA strand (Bukrinsky et al, 1993; Heinzinger et al, 1994; Gallay et al, 1997; Zennou et al, 2000). After nuclear import of PICs, integrase catalyses the integration of double stranded viral DNA into the host chromosome. In this process, viral 3'- OH DNA ends (generated by3'- processing) attack phosphodiester bonds on each strand of host DNA with a five base pair stagger. Strand transfer leaves a five-base single stranded gap at each junction between integrated viral DNA and host DNA and a two-base flap at 5'- ends of viral DNA (Figure-1.10). Unpaired 5'- ends of viral DNA are released and single-stranded gap is filled with cellular DNA repair enzymes (Yoder et al, 2000; Daniel et al, 2004). Inside the nucleus, linear cDNA integrates into the chromatin and form provirus. Some linear cDNAs are converted into one-LTR and two-LTR circles by homologous recombination (HR) and non-homologous end joining (NHEJ) . All circular viral DNA forms cannot produce infectious virus though they may have little transcriptional activities (Pommier et al, 2005). Viral cDNAs with free 5' and 3' LTR ends are integrated into different chromosomal locations, which form pro-viruses (Wu et al, 2001).

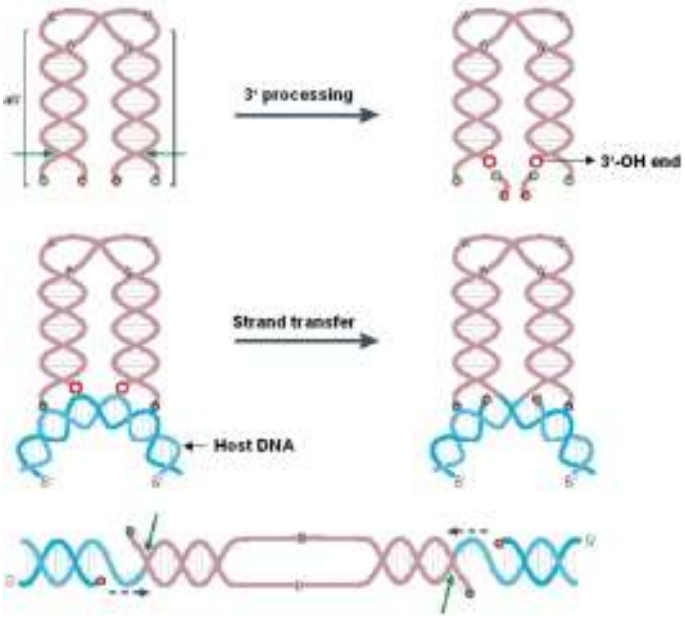


Fig 1.6.3: ***HIV-1 integrase catalytic reactions (Adapted from Pommier et al,2005).***

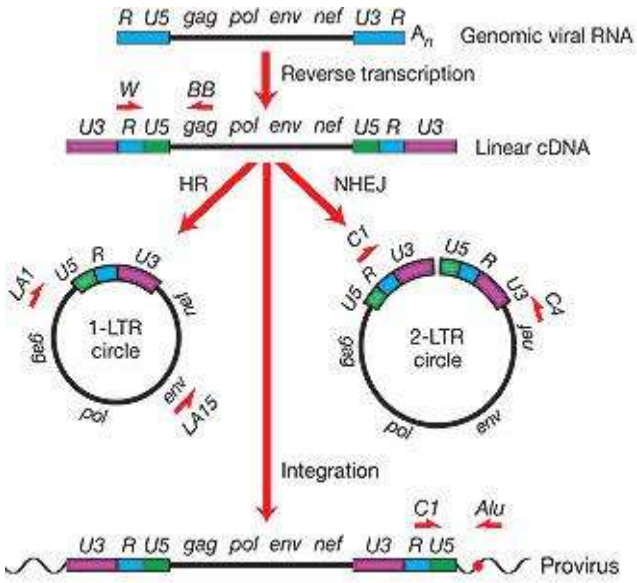


Fig 1.6.4: ***Linear and circular forms of viral cDNAs formed during HIV-1integration (Adapted from Jacque et al, 2006)***

Several cellular factors such as barrier to auto integration factor (BAF) and HMG1 (Y) are essential for efficient integration (Chen et al, 1998). The inner-nuclear-envelope protein emerin, a lamin associated protein interacts with viral DNA and is required for proper localization of viral DNA before its integration into the chromatin (Jacque et al, 2006). LEDGF interacts with integrase and target viral DNA to active genes and A/T rich sequences during integration (Cherepanov et al, 2003; Ciuffi et al, 2005). Molecules that inhibit the enzymatic activity of integrase directly inhibit HIV-1 replication. The diketoacids that inhibit HIV-1 integrase activity are under clinical development (Billich et al, 2003).

1.6.4 Viral transcription

The integrated proviruses, present in transcriptionally active sites produce viral RNA transcripts due to the LTR-mediated transcription using viral and cellular factors. Proviruses, which are integrated into the heterochromatic regions lack transcriptional activators that bind to the HIV-1 LTR and thus frequently lead to latency. Even though several hypotheses have been previously described, exact mechanism of viral latency is not yet understood. In the integrated provirus, RNA polymerase II (RNAPII) binds to 5' LTR promoter and assembles initiation complexes to begin transcription. After initiation of transcription, cyclin T1associated Tat binds to TAR region of LTR promoter and recruits cyclin dependent kinase 9 (Cdk9). This interaction leads to hyper phosphorylation of C-terminal domain of RNAP II by Cdk9 and increases the processivity of the enzyme and promotes transcription elongation (Kao et al, 1987). Cdk9 is auto-phosphorylated at serine and threonine

residues present in C-terminus and allow high affinity interaction of Tat, P-TEFb and TAR (Garber et al, 2000). Unspliced genomic RNA and singly spliced transcripts are exported from nucleus to cytoplasm in Rev dependent manner. Cellular proteins CRM1/exportin-1, which interact with NES of Rev and RanGTP, a nucleus-cytoplasm shuttling protein are involved in this viral transcript export (Cullen et al, 1998). Molecules that interfere with Tat transactivation function have potent therapeutic applications. In this step, Tat is an attractive target for the development of anti-HIV drugs because inhibition of Tat would decrease the viral replication in early stages of viral life cycle.

1.6.5 Maturation and budding

Maturation and budding is one of the least studied areas of HIV-1 life cycle. Viral assembly begins with the production of structural proteins, viral core and envelope. Viral protease cleaves polyproteins gag (p55) and gag-pol (p160) to completely processed proteins and produce mature viral proteins to form the mature virions. This process occurs in the late phase of virus budding. A nucleoprotein complex is formed by the interaction of p7 with packaging sequence (ψ) of genomic RNA, located between 5' splicing donor site and initiation codon of gag. Splicing removes packaging sequences from all RNAs except genomic RNA because of which only genomic RNA is incorporated into the virion. Nucleoprotein complexes come in close proximity with plasma membrane and are associated with envelope proteins. The myristoylated MA is essential for interaction of nucleoprotein complexes with the plasma membrane. Progeny virions are produced by infected cell by budding

from the plasma membrane. Viral protease is a good candidate to develop novel anti-HIV drugs and several protease inhibitors are currently in use and are also in clinical development.

1.7 Antiretroviral therapy

After successful clinical trials, in 1987 the AZT was approved by FDA to treat AIDS patients, after that several nucleoside inhibitors were also approved by FDA and made available to HIV-1 infected patients. Initially single drug therapy (monotherapy) was used to treat HIV infected patients and found it was not always effective in reducing viral RNA levels and this lead to disease progression and emergence of drug resistant variants. Hence, in the late 1990s the scientists found a combination of effective drug therapy in decreasing AIDS progression. this therapy includes combination of three or more antiviral drugs and they were used to treat HIV-1 infected patients and it was named as Highly Active Anti-Retroviral Therapy (HAART).The nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitor (NNRTI), protease inhibitor (PI) and fusion inhibitor were employed in this HAART therapy. Using this HAART therapy effectively altered the viral prognosis in HIV-1 infected patients from high morbidity and mortality to chronic and manageable but still intricate disease. Because of the availability of several antiretroviral drugs the life expectancy for AIDS patients is improvised and HAART regimens have become the standard of care for AIDS patients in developed countries (Palella et al, 1998; Matthews et al, 2004).Even though HAART therapy is effective in controlling the HIV-1 infection and

progression of AIDS, in several cases this it has failed to improve the life of HIV-1 infected patients due to several limitations.

Table 1.1: FDA approved AIDS drugs (Adapted from *www.aidsmeds.com*).

Yr. Of approval	Brand name	Generic name
Nucleoside reverse transcriptase inhibitors (NRTIs)		
1987	Retrovir	Zidovudine (AZT)
1991	Videx	Didanosine (ddI)
1992	Hivid	Zalcitabine (ddC)
1994	Zerit	Stavudine (d4T)
1995	Epivir	Lamivudine (3TC)
1997	Combivir	Lamivudine+ Zidovudine
1998	Ziagen	Abacavir
2000	Trizivir	Abacavir + lamivudine + zidovudine
2000	Videx EC	Didanosine (ddI)
2001	Viread	Tenofovir disoproxil
2003	Emtriva	Emtricitabine (FTC)
2004	Epzicom	Abacavir+ Lamivudine
2004	Truvada	Emtricitabine+ Tenofovir
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)		
1996	Viramune	Nevirapine
1997	Rescriptor	Delavirdine (DLV)
1998	Sustiva	Efavirenz
Protease inhibitors (Pis)		
1995	Invirase	Saquinavir
1996	Norvir	Ritonavir
1996	Crixivan	Indinavir (IDV)
1997	Viracept	Nelfinavir
1997	Fortovase	Saquinavir Mesylate
1999	Agenerase	Amprenavir
2000	Kaletra	Lopinavir+ Ritonavir
2003	Reyataz	Atazanavir
2003	Lexiva	Fosamprenavir
Fusion inhibitors		
2003	Fuzeon	Enfuvirtide (T20)

1.7.1 Limitations of HAART therapy

HAART has efficiently decreased rapid progression towards AIDS and morbidity, but the treated patients face several problems during the combination therapy. Long-term treatment with anti-HIV drugs leads to problems such as toxic effects in patients, emergence of drug resistant variants and weak immune responses towards viral antigens due to the suppression of plasma viremia. Existence of transcriptionally silent latent viruses is a major difficulty in eradication of virus from infected patients (Finzi et al, 1999).

1.7.1.1 Drug toxicity

More patients are experiencing drug toxicities, which make their treatment intolerable. Side effects like skin rashes, diarrhoea, nausea, vomiting and lipodystrophy are common problems faced by HAART treated patients (Weller et al, 2001). Nucleoside reverse transcriptase inhibitor triphosphates inhibit polymerase function of DNA polymerase - γ (DNA pol- γ), which is important for mitochondrial DNA replication. This causes mitochondrial dysfunction and there by leads to mitochondrial toxicity (Lewis et al, 1995; Lim et al, 2001). Long term use of nucleoside reverse transcriptase inhibitors lead to the toxic effects such as cardiac dysfunction, skeletal myopathy, lipodystropy, lactic acidosis and mitochondrial toxicity (de la Asuncion et al, 1998; Brinkman et al, 1999; Lewis et al, 2000; Brinkman et al, 2000; Johnson et al, 2001; Lewis et al, 2003).

1.7.1.2 Latent viral reservoirs

HAART also improves total number of $CD4^+$ T cell count in infected patients. Initially, it improved the hope of eradication of HIV-1 within two to three years of therapy from infected individuals (Perelson et al, 1997; Ho et al, 1995a). However, later it was shown that even patients taking potential antiretroviral therapy for as long as 30 months, and had undetectable viral load harbored a latent reservoir of resting $CD4^+$ T cells carrying replication competent virus (Finzi et al, 1999; Wong et al, 1997). Since the latent reservoirs are established very early during the primary infection, potent antiretroviral therapy fails to clear infection (Chun et al, 1998). The latently infected cells have half-life of 6 to 44months and small bursts of viremia due to the activation of latently infected cells can replenish the viral reservoirs (Zhang et al, 1999; Finzi et al, 1999; Ramratnam et al, 2000). After the knowledge about viral reservoirs in infected patients, scientists are unable to predict the time of eradication of HIV-1.

1.7.1.3 Emergence of drug resistant variants

Emergence of drug resistant HIV-1 variants during the drug therapy is another major difficulty in eradication of virus from the patients (Martinez-picado et al, 2000). Some of the mutated HIV-1 variants contribute to resistance and are selected during the therapy to replicate in presence of drug (Condra et al, 1995). Lengthy exposures to antiretroviral agents also lead to the emergence of multi drug resistant variants (MDRs), which are resistant to more than one anti-retroviral drugs. A recent report showed that many patients are infected with virus that is already resistant to several antiretroviral drugs at the time of diagnosis (Little et al, 2002). The main reason for

emergence of drug resistant variants is low fidelity of viral reverse transcriptase enzyme, which frequently lead to high mutation rate in progeny viruses released from the infected cells.

1.7.1.4 Weak immune responses during HAART

During antiretroviral therapy, HIV-1 specific CD8 and memory T cell number is decreased. Suppression of plasma viremia during antiretroviral therapy, leads to low humoral and cell-mediated immune responses due to low levels or absence of antigenic stimulation (Ogg et al, 1998; Pitcher et al, 1999; Weekes et al, 2006).

1.8 Strategies to improve the efficacy of antiretroviral therapy

Because of the difficulties in regular HAART treatment, patients need to be treated with different drug combinations of HAART regimens. Current therapy provides clinical improvement in majority of the patients but fails to eradicate the virus from treated patients (Saag et al, 1999). Therefore additional therapeutic approaches are needed to effectively treat infected patients.

1.9 Natural molecules against human diseases.

Nature has always provided a source of large number of novel molecules with unique structures for various ailments. Many plants, terrestrial, marine and microorganisms are very important source of clinically active natural products against several human diseases. Several of potent drug molecules that have been discovered during 1981 to 2002 consist around 28% of natural products origin and 24% were synthetic and

natural mimic molecules (semi synthetic) (Newman et al, 2004). This indicates that natural products are very important source of new drug candidates and for developing new potent drugs with chemical modifications. The secondary metabolites of natural organisms are important drug candidates for drug discovery. Several natural products were approved to treat human diseases like blood coagulation, metabolic disorders, neurological syndromes, cancers, immune dysregulation, bacterial, fungal, parasitic and viral infections etc. Plenty of the natural compounds with unique chemical structures are isolated from plant species, Compounds such as noscapine (narcotine), morphine, and papaverine, codeine are clinically used for the treatment of neuronal disorders like parkinson's disease (Chin et al, 2006). Quinine a natural product is isolated from the bark of *Cinchonaofficianalis*and its analogues are used to treat malaria (Tagboto et al,2001). Huperzine isolated from a club moss *Huperziaserrata* is useful for treating cholinergic neurodegenerative diseases including Alzhiemer's disease (Akhondzadeh et al, 2006). Hemisuccinatecarbenoxolone sodium is a semi-synthetic derivative of glycyrrhetic acid isolated from licorice (*Glycyrrhizaglabra*) is used to treat gastric and duodenal ulcers (Dewick et al, 2002). In recent years, many such drug molecules from natural products were approved to treat several human diseases and there are so many drugs are currently in clinical trials.

1.10 Natural molecules against HIV-1

Various studies showed that many natural bioactive compounds isolated from various plant species, flora and fauna and their derivatives have the potential for

development of effective anti HIV 1drugs with less cytotoxicity. The number of compounds exhibiting anti HIV-1 activity and isolated from natural sources is increasing steadily. Calanolide A, a comarin isolated form Callophyllymlanigerum, DSB and 3-hydroxymethyl-4-methyl DCK are phase II clinical candidates with potential to come up as drugs for treatment of HIV infection. A variety of alkaloids (Michellamine,atropisomericanpthlisoquinoline,MichellamineB,tetrahydroxylindolizidinealkaloid,castanospermine),Flavonoids(8hydroxyguanine,6,8diprenylaromadendrin,6,8diprenylkaempferol,robustaflavone,hinokiflavone),phenolics(punicalin,punicalagin cornusin-A, corilangin have been found to posess HIV-inhibitory activity (inder pal singh et.al) .One of such potent natural molecule is curcumin it contain many pharmalogical activities including anti HIV-1 activity (Zhihua et.al 1993)

1.11 Curcumin

Curcumin is a yellow polyphenolic natural molecule present in the Indian spice turmeric (curcuma longa), it constitutes around 3–4% of the total turmeric.Since ancient time in the south and southeast tropical Asian countries the turmeric has been used as a spice to give the specific flavor and yellow color to curries (Eigner and Scholz, 1999). Curcumin become popular when it was found that it act as a therapeutic agent for various illnesses. A large number of *in vitro* and *in vivo* studies in both animals and man have indicated that Curcumin has strong antioxidant, anti-inflammatory, anti-carcinogenic, anti-microbial, anti-parasitic and other activities including anti HIV-1 activity (Zhihua.et.al 1993).

1.11.1 Curcumin against HIV-1

It was found that curcumin effectively inhibits type I Human Immunodeficiency Virus (HIV) replication by inhibiting long terminal repeat (LTR) directed gene expression (Jiang et al., 1996a; Li et al.,1993) and could able to inhibit p24 antigen production in cells infected with HIV-1(Li et al., 1993).Some studies demonstrated that the anti hiv-1 activity of curcumin against HIV-1 integrase mutant and wild-type integrase, and some Insilco studies showed that the curcumin is active against HIV-1 integrase and protease.

1.11.2 Limitations to curcumin:

However, the curcumin possesses diverse pharmacologic effects but its clinical usefulness of curcumin is limited because of poor solubility in water, highly unstable, undergoes rapid hydrolytic degradation at alkaline conditions and its rapid systemic elimination

1.12 Nanotechnology –Targeted Drug delivery through Apotransferrin loaded Nanoparticles:

In order to improving the bioavailability of curcumin and should bring this natural product to the forefront of promising therapeutic agents, numerous approaches were tried earlier. These include usage of adjuvants and encapsulation in liposomes or Nanoparticles of various compositions, though these delivery systems are

biocompatible, they mostly lack target specificity. To enhance specificity and targeted drug delivery, many drug-loaded materials were conjugated with Apotransferrin / transferrin proteins, which were abundantly expressed in actively proliferating cells. In order to enhance specificity and targeted drug delivery, doxorubicin loaded Apotransferrin nanoparticles were developed. **(**Krishna et.al, 2009). Showed encapsulation of doxorubicin with Apotransferrin enables preferential localization into targeted cells through receptor-mediated endocytosis (Qian et al., 2002). Encapsulation of drugs with these proteins enables preferential localization into targeted cells through receptor-mediated endocytosis.

This apotransferrin nanoparticle-drug delivery system also provides all the general advantages offered by nanoformulations such as appropriate size for cellular uptake, excellent water dispensability and improved intracellular localization. HIV-1 infected cells are known to express transferrin receptors, which bind transferrin or apotransferrin and transport into the cell. These receptors could be targeted for ligand-mediated transport of curcumin into the infected cells. Thus, exploiting apotransferrin as delivery system would pay a way for targeted delivery of curcumin. Since it is nanoparticle form, it would enhance bioavailability of curcumin and its activities

1.13 Rationale of study

The development of new drugs targeting HIV-1 infection is of utmost importance for the clinical use for HIV-1 patients. Curcumin which is a natural product can effectively suppress replication of HIV-1, but its clinical usefulness was limited

because of low bioavailability, rapid systemic elimination, rapid degradation etc. Therefore we aim to improve the bioavailabity of curcumin through transferrin receptor mediated endocytosis through formation of curcumin loaded apotransferrin nanoparticles drug delivery system. Since several curcumin analogues were also found to be affective against several human diseases, we further synthesized several curcumin analogues by taking the curcumin as starting material with a hope to produce a new potent molecules that has improved antiviral activity against HIV-1.After screening the anti viral activity of curcumin analogues we found four molecules that exhibit potent antiviral activity against HIV-1 wild-type virus, further apotransferrin nanoparticles were prepared by encapsulating the active curcumin analogues to study their enhanced antiviral activity. After that several comprehensive studies were performed to understand the molecular effect of active curcumin analogues on HIV-1 viral proteins

Based on the above rational the specific aims and objectives of my study are entitled as

1. **Development of curcumin loaded Apotransferrin nanoparticles as a potent anti HIV-1 formulation.**
2. **Evaluation of anti-HIV activity of soluble and nano formulation of curcumin analogues.**
3. **Characterization of inhibitory activity of anti-HIV active curcumin analogues against reverse transcriptase, protease and integrase.**

CHAPTER II:

Materials and Methods

Materials:

Biochemical Kits:

HIV-1 p24 ANTIGEN CAPTURE ASSAY kit (ABL, USA), SuperScript™ III First-Strand Synthesis System for RT-PCR (invitrogen,USA), HIV-1 RT assay(Roche), Hiv-1 integrase assay kit (Express biotech international, USA), HIV-1 Protease assay(AnaSpec, Inc)

Enzymes:

T4 DNA ligase ,Taq DNA Polymerase was purchased from Invitrogen;,

HIV-1 protease was purchased from AnaSpec,Inc.

Antibodies:

Anti-Human CD8 71 was purchased from Calbiochem India.

Chemicals:

DMSO, Diethyl ether, Apotransferrin, Curcumin, AZT was purchase from Sigma India

Tissue culture reagents:

Keratinocyte serum-free medium (Life Technologies, Gibco Ind), Dulbecco's modified Eagle medium (DMEM), RPMI 1640, fetal bovine serum (FBS), penicillin-streptomycin(P/S), L-glutamine, G418, puromycin, hygromycin B, and trypsin-ethylenediaminetetra-acetic acid (EDTA) were purchased from Invitrogen; phyto-hemagglutinin (PHA) was purchased from Sigma India; recombinant human interleukin-2 (IL-2) was purchased from Roche; Ficoll-Hypaque Plus was purchased from GE healthcare Bio-science AB (Life Technologies, Gibco,Ind).

2.2 Sources of HIV-1 viruses

Live viruses

All stock laboratory-adapted viruses were used provided by NIH AIDS Research and Reference Reagent Program. These viruses include

Table: 2.1

Virus	Co receptor	Group	Subtype	Origin	NIH-Cot log No
HIV-193RW024	R5X4	M	A	Ruwanda	2200
HIV-1 94UG103	R5X4	M	A	Uganda	2304
HIV-1 KNH1135	R5	M	A	Uganda	11245
HIV-1 92US657	R5	M	B	USA	2053
HIV-I IIIB	R5X4	M	B	USA	398
HIV-1 IN98026	R5	M	C	India	4168
HIV-1 IN98-022	R5	M	C	India	4167
HIV-1 IN93905	R5	M	C	India	3040
HIV-1 93IN101	R5	M	C	India	2900
pINDIE-C1(clone)	R5	M	C	India	

Infectious clones:

pINDIE-C1 plasmid was kindly provided by Dr.Mithra laboratory(NCCS puna)

Methodology:

2.3: Preparation and characterization of curcumin loaded Apotransferrin nanoparticles

2.3.1: Nanoparticles preparation:

Nanoparticles were prepared using a variation of the procedure described in Sai Krishna et al. (2009). 10mg of apotransferrin (Sigma-Aldrich, St. Louis, MO, USA) in 100 µl of phosphate-buffered saline was slowly mixed with 3.6 mg of curcumin (Sigma-Aldrich) in 100 µl DMSO and the mixture was incubated on ice for 5 min. The mixture of apotransferrin and curcumin was slowly added to 15 ml of olive oil at 4°C with continuous dispersion by gentle manual vortexing. The sample was sonicated 15 times at 4°C using a narrow stepped titanium probe of ultrasonic homogenizer (300V/T, Biologics Inc., Manassas, Virginia, USA). The sonication amplitude was 5 µm and the pulses were 30 sec long with an interval of 1 min between successive pulses. The resulting mixture was immediately frozen in liquid nitrogen for 10 min and was then transferred to ice and incubated for 4 h. The particles formed were pelleted by centrifugation at 6000 rpm for 10 minutes and the pellet was extensively washed with diethyl ether and dispersed in PBS. The particles' protein content was estimated by the Biurett method and the protein content was used to determine the amount of nanoparticles used for each experiment.

2.3.2: Nanoparticles characterization:

Structure and morphology of the nanoparticles were investigated using a scanning electron microscope (SEM, Philips FEI-XL 30 ESEM; FEI, Hillsboro, OR, USA) operated at 20 KV, atomic force microscopy (AFM; SPM400) and transmission

electron microscope (TEM, Techni) operated at 80 KV. For SEM the particles were gold coated, AFM sample was spin coated on glass cover slip, TEM sample was prepared by fixing the sample on 200 mesh type-B carbon coated copper grid (ICON) using 2% osmium tetroxide in 50 mM phosphate buffer followed by staining using phopshotugestic acid. Manufacturer's instructions were followed for data collection, and analysis of particles. This method was used to confirm the structure and morphology of the preparation used in each experiment.

2.4: Cell culture assays:

2.4.1. Propagation of cell lines:

SupT1 cells, U-937 cells were obtained from NIH AIDS Research and Reference Reagent Program VK2-E6/E7 cells were obtained from American Type Culture Collection (ATCC, USA). SupT1 cells and U-937 cells were cultured in RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, 100U/ml penicillin, and 100μg/ml streptomycin. VK2-E6/E7 cells were cultured in Keratinocyte Serum Free medium (GIBCOBRL17005042)with 0.1 ng/ml human recombinant EGF, 0.05mg/ml bovine pituitary extract, and additional calcium chloride 44.1 mg/L (final concentration 0.4 mM), 100U/ml penicillin, 100μg/ml streptomycin, Fresh peripheral blood mononuclear cells (PBMCs) were isolated from buffy-coats of healthy volunteer blood donors using a Ficoll-Hypaque Plus gradient centrifugation method and cultured in RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, 10U/ml IL-2, 100U/ml penicillin, and 100μg/ml streptomycin.

All cells were cultured in a humidified incubator with 5% CO_2 atmosphere at 37°C.

2.4.2. Nanoparticle localization assay

SUP-T1 T cells/ U937 macrophage cells/ VKT-E6/E7 vaginal epithelial cells obtained from the NIH-AIDS Reference and Reagents Program were used. The cells were incubated in the presence of apotransferrin-drug nanoparticles containing curcumin, or an equivalent amount of dissolved curcumin (sol-curcumin), for a period of 60 min. The amount of intracellular curcumin was assessed through laser confocal microscopy (Leica and ZEISS) employing the intrinsic fluorescence of curcumin.

2.4.3: Competition of transferrin receptor antibodies with the apotransferrin-drug nanoparticles

SupT1cells ($1x10^6$) were incubated in serum-free medium for 60 min in a 12-well plate. Nano-curc or sol-curc (equivalent to 5 μM curcumin) was added to the cells in either the presence or absence of monoclonal anti-human transferrin receptor antibodies (400ng/ml) and incubated for 2 h. After incubation, cells were washed three times with PBS and lysed in 1 ml of lysis buffer. The lysate was cleared by centrifugation at 12,000 rpm for 20 min at 4°C. Curcumin was quantified through its intrinsic fluorescent emission (λ Ex 458 nm and Em 530 nm) as measured by a fluorescence spectrometer (Shimadzu FL 2000; Shimadzu, Kyoto, Japan). In parallel, the cells were observed under a laser Confocal microscope.

2.4.4: Cytotoxicity assay

SUP-T1 cells ($0.2x10^6$/well) were seeded in a 96-well plate and incubated at 37°C for 4 h in a 5% CO_2 incubator (Forma Scientific, Marietta, OH, USA). These cells were treated with increasing concentrations of curcumin, either soluble or

incorporated into nanoparticles (nanoparticle curcumin was assessed by the amount of associated protein), and incubated for 16 hours. The cells were pelleted at 1200 rpm for 10 min and re-suspended in new medium. To this, 20 µl of 5 mg/ml MTT (Sigma-Aldrich) was added and incubated for 4 h. The cells were then pelleted at 1200 rpm for 20 minutes, the medium was removed, and the precipitate was dissolved in DMSO and read in an ELISA reader at 595 nm.

2.5: In vitro antiviral activity assay:

2.5.1 Isolation of PBMCs and activation

Fresh PBMCs were isolated from buffy-coats of healthy volunteer blood donors or HIV-1 patients using a Ficoll-Hypaque Plus gradient centrifugation method.

Briefly, whole blood was obtained from patients in anti-coagulant EDTA, then buffy-coats were separated through centrifugation at 2,000rpm at room temperature for 10min. The plasma in the upper layer were divided into aliquots in 1.5-ml tubes and stored at -80°C. The blood cells were diluted with same volume of PBS in 50ml tubes. The diluted blood were gently loaded on top of Ficoll-Hypaque Plus solution with the volume ratio at approximately 4:3 and centrifuged at 400×g without brakes at room temperature for 30min. PBMCs on the top of Ficoll layer were collected with a Pasteur pipette and transferred into a new 50ml tube. These PBMCs were washed with 50ml PBS for 3 times by centrifuging at 200×g for 10min at room temperature and discarding the supernatant. Finally, PBMC cell pellets were re-suspended with 1ml RPMI 1640 and cell number was counted. PBMCs were cryopreserved with 90% FBS+10% DMSO at a cell density of 1×10^7 per ml at -180°C for until use.

2.5.2 Propagation of HIV-$1_{(93IN101)}$ virus on SupT1 cells and on PBMCs

2.5.2.1 p24 quantification

After 96 hr of HIV-1 infection the viral core p24 antigen was quantified by HIV-1 p24 Antigen ELISA Kit (Advanced Bioscience Laboratories,Kensington, MD, USA) according to manufacturer's instructions. Supernatants of specimen were 10-fold diluted (1:10-1:1000). Optical density of each well was measured at 450nm by ELISA reader. The p24 concentration was calculated according to the standard curve obtained from the same experiment.

2.5.3: HIV-1 neutralization assay

SUP-T1 cells ($0.4x10^6$/ml) with 100% viability were seeded in RPMI 1640, 0.1% FBS on four 12-well plates. Increasing concentration of nano-curcumin, sol-curcumin, or nano-curcumin in the presence of anti-human transferrin receptor antibody (as indicated) were added to the cells which were then infected with HIV-$1_{93IN101}$ / HIV-1_{pINDIE} a final virus concentration equivalent to 1 ng of p24 per ml. The infected cells were incubated for 2 h at 37°C in a 5% CO_2 incubator. The cells were then pelleted at 350 x *g* for 10 min, the supernatant was discarded, and cells were washed with RPMI 1640 containing 10% FBS. The cells were resuspended in fresh complete medium and were incubated for a further 96 h. The supernatants were then collected and analyzed using a p24 antigen capture assay kit (Advanced Bioscience Laboratories, Kensington, MD, USA). The extent of infection in the absence of test compound was considered to be equivalent to 0% inhibition. Azidothymidine (AZT) was employed as a positive control.

2.6 Wide range of HIV-1 neutralizing activity

2.6.1 Propagation of HIV-1 isolates:

All HIV-1 subtype A, subtype B', and subtype C viruses were used provided by NIH AIDS Research and Reference Reagent Program and all viruses were revised in PHA-stimulated PBMCs and propagated in SupT1 cells.

2.6.2 Broad spectrum antiviral activity assay

The broad spectrum of antiviral activity of nano-curcumin and curcumin analogues was conducted using several HIV-1 isolates in Sup-T1 cells as mentioned above with HIV-1 $_{(93IN101)}$ strain (section 2.5.3).

2.7: qPCR and RT-PCR analysis of HIV-1 gag gene and host toisomerase II α toisomerase II β, COX-2, IL-1b and TNF-a expression

The effect of nano-curcumin on the expression of HIV-1 gag gene and host toisomerase II α expression and toisomerase II β, COX-2, IL-1b and TNF-a expression was analyzed by (i) semi-quantitative and (ii) quantitative-PCR.

2.7.1 HIV-1 infection to SupT1 cells in presence of curcumin and nano curcumin

SUP-T1 cells ($1x10^6$/ml) with 100% viability were seeded in RPMI 1640, 0.1% FBS on 6-well plates. 5μM concentration of nano-curcumin, sol-curcumin was added to the cells which were then infected with HIV-$1_{93IN101}$the final virus concentration equivalent to 1 ng of p24 / ml. The infected cells were incubated for 5 h at 37°C in a 5% CO_2 incubator. The cells were then pelleted at 350 x *g* for 10

min, the supernatant was discarded, and cells were washed with RPMI 1640 containing 10% FBS. The cell pellet was collected were used for mRNA Isolation and subsequent gene analysis.

2.7.2 HIV-1 genomic DNA and RNA Isolation

Genomic DNA (to monitor gag expression) and total RNA were isolated from treated samples using suppliers' (Qiagen, GmbH,Germany & Sigma-Aldrich respectively) protocol. cDNAs were synthesized using SuperscriptTM III first strand synthesis system(Invitrogen, Carlsbad, CA, USA)

2.7.3 cDNA synthesis

Viral cDNA was synthesized using SuperScript^{TM} III First-Strand Synthesis System for RT-PCR. Briefly, 13µl reaction volume with 5µg viral RNA, 1µl 10mM dNTP mix, 50ng random hexamers, and DEPC-treated water was firstly incubated at 65°C for 5min followed by chilling on ice for at least 1min. Reverse transcription was then performed with the addition of 4µl 5×First-strand buffer, 1µl 0.1M DTT, 1µl RNase OUT recombinant RNase inhibitor, and 200U SuperScriptIII RT. Reaction mixture was incubated at 25°C for 5min, followed by 50°C for 1h and heat-inactivation at 70°C for 15min. Finally, 2U E.coli RNase H was added into the mixture and incubated at 37°C for 20min to remove any RNA templates.

2.7.4 Primers:

Primers were designed based on the gene sequences available in PUBMED nucleotide database. Multiple alignment (wherever required) was performed using the ClustalW program. For each gene sequences of the forward and reverse primers

Table 2.3 Primers for RT and qPCR analysis of HIV-1 gag gene and host genes

name of primers	primer sequence 5'-3'
gag F	GCAGGGCCTATTGCACCAGGC
gag R	GGCCAGGTCCTCCCACTCCC
Topo II α F	GGGTTCTTGAGCCCCTTCACGA
Topo II α R	GTAGGTGTCTGGGCGGAGCAA
18S	GCTACCACATCCAAGGAAGGCAGC
18S R	CGGCTGCTGGCACCAGACTTG
Topo II β F F	GCCCAGTTGGCTGGCTCTGT
Topo II β F R	GCATGGGATGAGGATCCAGGCC
COX-2 R	AACAGGAGCATCCTGAATGG
COX-2 F	GGTCAATGGAAGCCTGTGATG
IL-1 β R	AGCTGATGGCCCTAAACAGA
IL-1 β R	TCTTTCAACACGCAGGACAG
TNF α R	AGCCCATGTTGTAGCAAACC
TNF α R	CCAAAGTAGACCTGCCCAGA

2.7.5 Analysis of HIV-1 gag gene and host toisomerase II α toisomerase II β, COX-2, IL-1b and TNF-a expression

The expression of topoisomerase IIα was determined by (i) semi-quantitative and (ii) quantitative-PCR. B) Quantity of viral cDNA synthesized was shown by both (i)semi-quantitative and (ii) real-time PCR using gag-specific primers. Template from normal SUP-T1 cells was used as negative control. Azidothymidine(AZT) was employed as a positive control and 18S was used as an internal control in both

experiments. Real-time PCR was performed on an ABI PrismH 7500 fast thermal cycler (Applied Biosystems, Foster, CA, USA). Each sample was run in triplicate in a final volume of 25 ml containing 1 ml of template (1:10 dilution), 10 pmol of each primer and 12 ml of Power SYBRH Green PCRmaster mix (Applied Biosystems). The real-time PCR results were presented as change in expression relative to control using target gene CT values normalized to that of 18S gene CT values based on the comparative CT method [41].IL-1 b, PGE2 and TNF a assays Cells (16105) were treated with either HIV, nano-curcumin, AZT, soluble-curcumin or apotransferrin for 4 h. Levels of IL-1b, PGE2 and TNF-a in the culture media were quantified using ELISA kits (R&D Systems, Minneapolis, MN, USA). The protocol was followed as per the manufacturer's instructions.

2.8 Time-dependent drug addition kinetics experiments

SUP-T1 cells ($0.4x10^6$/ml) with 100% viability were seeded in RPMI 1640, 0.1% FBS on four 12-well plates. Cells were then infected with HIV-$1_{93IN101}$and 5μM concentration of active curcumin analogues CA-23, 24, 25, and 26 were added at different time intervals of HIV-1 life cycle (0 to 72hrs) during 72 h time course of HIV-1 infection. The cells were then incubated for 2hr at 37°C in a 5% CO_2 incubator. after of every drug addition. Then cells were pelleted at 350 x *g* for 10 min, the supernatant was discarded, and cells were washed with RPMI 1640 containing 10% FBS. The cells were resuspended in fresh complete medium and were incubated for a further 96 h. The supernatants were then collected and analyzed using a p24 antigen capture assay kit (Advanced Bioscience Laboratories, Kensington, MD, USA). The extent of infection in the absence of test compound was considered to be equivalent to 0% inhibition. AZT, raltagravir and saquinavir

was employed as positive controls for HIV-1 RT, Integrase and Protease respectively.

2.9 Molecular docking of curcumin and curcumin analogues with HIV-1 RT, Integrase and Protease enzymes

Docking studies of curcumin and active analogues were performed using GOLD. The rotational flexibility of receptor hydrogens and ligand conformational flexibility are explored by genetic algorithm in GOLD. In GOLD docking was carried out using the wizard with default parameters population size (100); selection- pressure (1.1); number of operations (10,000); number of islands (1); niche size (2); and operator weights for migrate (0), mutate (100), and crossover (100) were applied. The active site with a 20 Å radius sphere was defined by selecting an active site residue of protein. Binding pockets of the proteins were explored in the CastP server and the site was selected based on its area and volume. It was checked with the active sites reported for binding of the existing inhibitors. A residue was selected and Docking was performed, if the necessary residues for interactions were not seen the binding pocket was changed. Default Genetic Algorithm settings were used for all calculations and a set of 10 solutions were saved for each ligand. GOLD was used by a GoldScore fitness function.

Gold Score is a molecular mechanism like function and has been optimized for the calculation of binding positions of ligand. It takes into account four terms:

$$\text{Fitness} = S_{(hb_{ext})} + 1.3750 * S_{(vdw_{ext})} + S_{(hb_{int})} + 1.0000 * S_{(int)}$$

$$S_{(int)} = S_{(vdw_{int})} + S_{(tors)}$$

Where S_{hb_ext} is the protein-ligand hydrogen bonding and S_{vdw_ext} are the van der

waals interactions between protein and ligand. S_{hb_int} are the intra molecular hydrophobic interactions whereas S_{vdw_int} is the contribution due to intra molecular strain in the ligand.

2.10 HIV-1 enzymatic Assays:

2.10.1 HIV-1RT assay

HIV-1 RT Assay Kit was used to test the active curcumin analogues anti HIV-RT activity. The assay was performed according to the manufacturer's instructions.

2.10.2HIV-1 Integrase assay:

The XpressBio HIV-1 Integrase Assay Kit was used to test the active curcumin analogues anti HIV-1 Integrase activity. The assay was performed according to the manufacturer's instructions.

2.10.3 HIV-1 Protease assay:

The SensoLyte® 490 HIV-1 Protease Assay Kit was used to test the active curcumin analogues anti HIV-1 protease activity. The assay was performed according to the manufacturer's instructions.

2.11 Statistical analysis

Statistical analyses were performed using SPSS Statistics 16 (SPSS,Chicago, IL). Data are presented as mean standard deviation. Differences between groups were evaluated using Students's t test or one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis. All experiments were repeated three times, unless otherwise specified. Values of P0.05were considered being statistically significant.

CHAPTER III:

Development of curcumin loaded apotransferrin nanoparticles as a potent anti HIV-1agent

3.1 Introduction

Curcumin [diferuloylmethane, or 1, 7-bis-(4-hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dion] is obtained from the rhizome of turmeric (*Curcuma longa*). Small Phase I clinical trials have established that curcumin is safe in humans even at high doses (12 g/day) but exhibits limited bioavailability due to poor absorption, rapid metabolism, and rapid systemic elimination (Anand et al., 2007). Almost the entire dose of orally administered curcumin is excreted in the feces (Sharma et al., 2004; Sharma et al., 2001). At high doses the plasma may contain nM concentrations of the parent compound, and glucuronide and sulfate conjugates (Cheng et al., 2001; Garcea et al., 2004). *In vitro* and animal studies point toward potential efficacy of curcumin in a wide variety of human diseases (Maheshwari et al., 2006). Enhanced bioavailability could bring this natural product to the forefront of promising therapeutic agents (Anand et al., 2007).

Numerous approaches have been adopted in an effort to improve the bioavailability of curcumin. These include use of adjuvants such as piperine that interfere with glucuronidation and a variety of novel formulations including liposomal, nanoparticle, and phospholipid complexes (Padhye et al., 2010). Structural analogues of curcumin have also been investigated. Nanoparticle formulations appear especially attractive, since encapsulating materials prevent access to curcumin by metabolising enzymes while, in most cases, providing excellent water solubility. Typical nanoparticle sizes are likewise appropriate for cellular uptake. Although many of these nanoparticle formulations have exhibited some degree of success, especially in terms of improved intracellular localization, none has proved sufficiently attractive for further development.

Building on the general advantages of nanoparticle formulations, we have sought to further enhance their cellular uptake by targeting specific cell-surface receptors that trigger endocytosis. HIV-1–infected cells are known to expresses transferrin receptors (Savarino et al., 1999), which bind transferrin or apotransferrin and transport it into the cell. In my present study curcumin-loaded apotransferrin nnanoparticles (nano-curc) have been formulated, in this formulation apotransferrin serve as the encapsulating material as well as target-guided vehicle. These curcumin loaded nano spheres were then assessed for their efficiency of cellular uptake and cytotoxicity in T-cells (SupT1).PBMC, Macrophages (U-937) and VK2/E6E7 (Human vaginal epithelial cell line) and studied it efficacy to inhibit HIV-1 replication in SupT1, PBMC's cells. The nano-curcumin formulation was further evaluated for its broad spectrum anti HIV-1 activity against several strains of HIV-1. The results clearly highlight the advantage of this delivery system over direct soluble curcumin administration.

To determine the mechanism of nano-curcumin anti-HIV activity in T-cells, expression of selected human genes viz.,topoII-α, TopoII-β, cox2 and TNF-α was carried out.

3.2 Results

3.2.1 Preparation and characterization of curcumin-loaded apotransferrin nanoparticles

Curcumin-containing Apotransferrin nanoparticles were prepared using sol-oil chemistry as described in materials and methods section. Transmission electron microscopy (TEM) analysis showed that the particles were nearly uniform in size and spherical in shape. This technique also confirmed the increase in diameter of loaded particles (Fig. 3.2.1A). The size of pure apotransferrin nanoparticles as assessed by scanning electron microscopy (SEM) ranged from 45–55 nm, increasing to 55–70 nm after curcumin loading (Fig. 3.2.1B). The surface morphological analysis of particles using atomic force microscopy (AFM) showed significant projections, which might contribute to the molecular recognition of particle by the receptor (Fig. 3.2.1C).

Fig 3.2.1

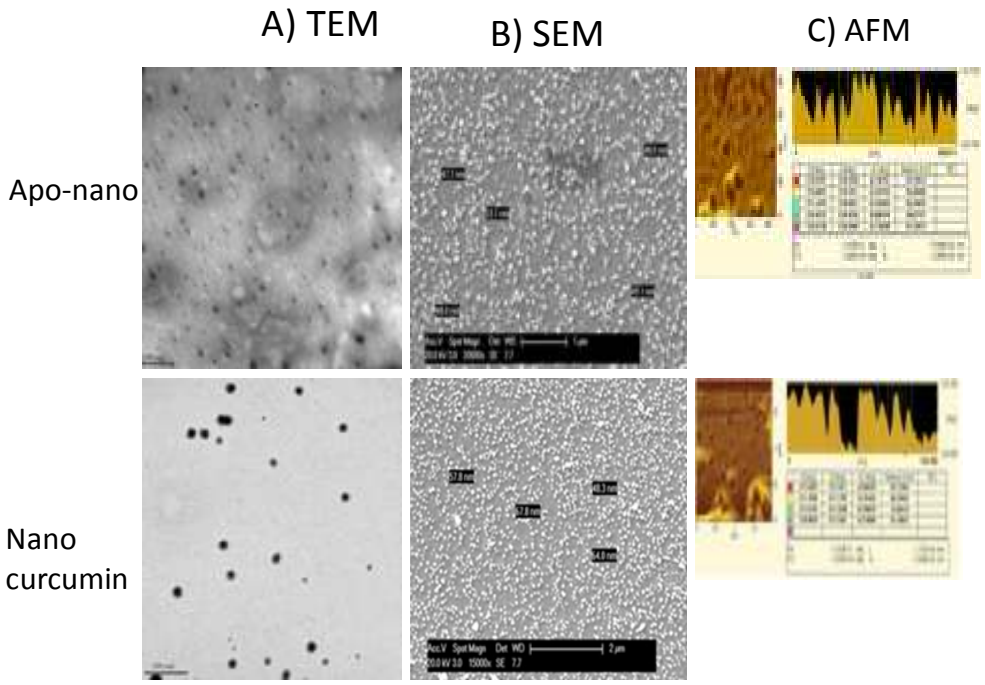


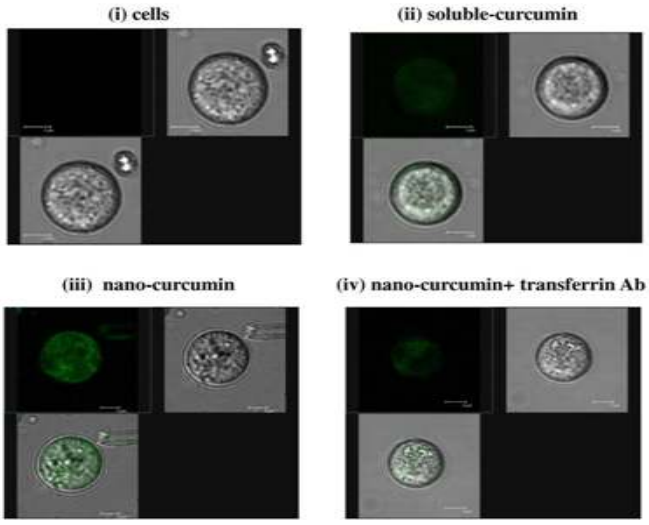
Fig 3.2.1: Preparation of curcumin-loaded apotransferrin nanoparticles: curcumin loading increases size of apotransferrin nanoparticles. The preparations of curcumin-loaded Apotransferrin nanoparticles (nano-curcumin; left) and Apotransferrin nanoparticles without curcumin (nano-Apotransferrin; right) were examined by A) TEM B) SEM and C) AFM as indicated

3.2.2Cellular uptake of curcumin following nano-curcumin administration

Cellular uptake of curcumin upon incubation with nanocurcumin was monitored by confocal microscopic analysis of the compound's intrinsic green fluorescence. Intracellular localization of curcumin was enhanced in nano-curcumin treated cells compared to those treated with soluble-curcumin (Fig. 3.2.1 A.ii, iii), indicating that apotransferrin encapsulation significantly increases cellular uptake of curcumin. The curcumin localization in overall population of SUPT1 cells was given in Fig. 3.2.1 A. To determine whether the enhanced uptake of Apotransferrin encapsulated curcumin requires interaction with the transferring receptor, we incubated the cells concurrently with nano-curcumin and antibody to the human transferrin receptor. The observed decrease in intracellular curcumin fluorescence (Fig. 3.2.1 A.IV) suggests that nano-curcumin uptake results from endocytosis mediated by the transferrin receptor. Similar results were seen when intracellular curcumin accumulation was quantified fluorimetrically in experiments conducted with SUPT1 and stimulated PBMCs (Fig. 3.2.2.B). These results confirm that cellular uptake of curcumin is significantly enhanced by Apotransferrin encapsulation and that this improved uptake is mediated by the transferring receptor in both SUPT1 (Fig. 3.2.2.B i) and PBMCs (Fig. 3.2.2.B ii).

Fig 3.2.2

A)


(i) cells
(ii) soluble-curcumin
(iii) nano-curcumin
(iv) nano-curcumin+ transferrin Ab

B)

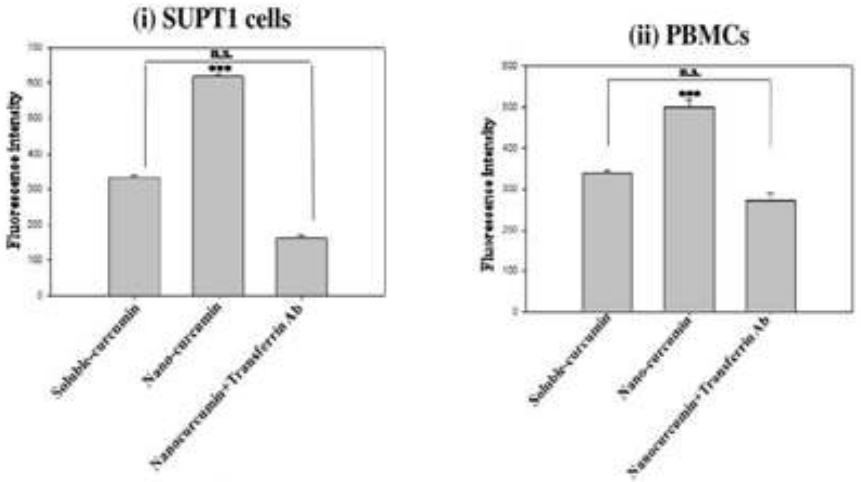

(i) SUPT1 cells
(ii) PBMCs
Fluorescence Intensity
Soluble-curcumin
Nano-curcumin
Nanocurcumin+Transferrin Ab

Fig 3.2.2: Cellular uptake of curcumin following nano-curcumin administration

Nanoparticle formulation increases curcumin uptake, which is inhibited by transferrin receptor blockade. A) SUP-T1 cells were incubated for 1 h with curcumin formulations as indicated, then examined by confocal microscopy. (i) Cells without curcumin; (ii) 1 μM solcurcumin; (iii) 1 μM nano-curcumin; or (iv) 1μM nano-curcumin in the presence of transferrin receptor antibody (100 ng/ml). Each panel contains three images: fluorescence, bright field and merged. B) SUP-T1 cells (i) or stimulated PBMCs (ii) were incubated for 1 h with curcumin formulations, after which intrinsic fluorescence of intracellular curcumin was determined quantitatively by fluorometric analysis. Cells were treated with 5 mMsolcurcumin, 5 μM nano-curcumin, or 5 μM nano-curcumin in the presence of antibodies to the transferrin receptor (TrR-Ab; 100 ng/ml). All the valuesare normalized to that obtained from SUPT1 cells

3.2.3: Cellular retention of curcumin following nano-curcumin administration

Cellular uptake and elimination of curcumin upon treatment with sol- or nano-curcumin (1 and 10 μM) was assessed by confocal microscopic analysis at different time points (1, 2, 4 and 6 h respectively)(figs3.2.3.1-4) Soluble-curcumin was taken up quickly by the cells, peaking at 2 h, but was rapidly eliminated out and was essentially gone by 4 h (Figs. 3.2.3.1-4). Nano-curcumin, by contrast was both taken up and released more slowly with a peak at 4 h and, 50% of the drug still present at 6 h (Figs. 3.2.3.1-4). Further, the results confirmed that nano-curcumin exhibits a time-dependent intracellular localization of curcumin (in SUPT1, PBMCs, VK2/E6/E7 and U-937 cells) that is stable for almost the full 6 h. In contrast, soluble-curcumin is taken up rapidly, especially at the higher concentration but disappeared from the cells quickly. This gradual and stable uptake of nano-curcumin is characteristic of receptor mediated transport. Moreover, it is important to note that uptake at 2 h is similar for the 1 and 10 μM concentrations of nanocurcumin (Figs.3.2.3.1-4) while uptake is significantly higher for 10 μM than for 1 μM nano-curcumin at 6 h. These results indicate that cells retain curcumin for longer periods following treatment with nano-curcumin than with sol-curcumin.

Fig: 3.2.3.1:

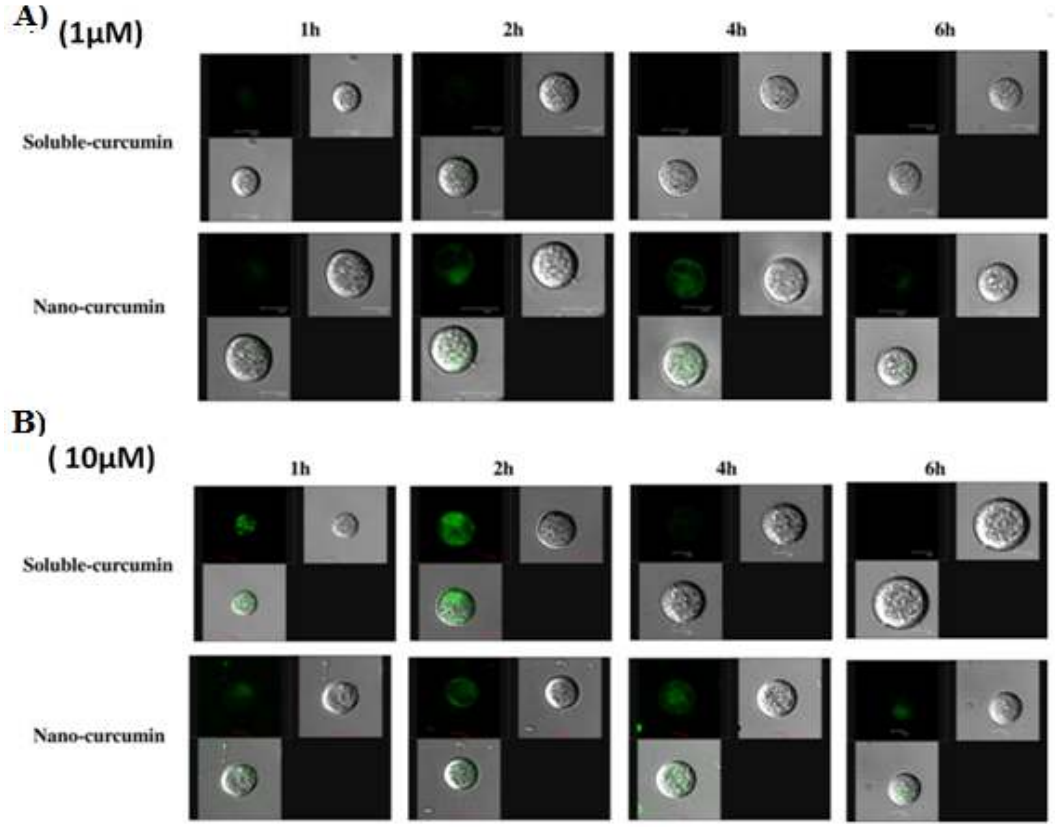


Fig: 3.2.3.1: Analysis of cellular retention of curcumin following nano-curcumin administration in SupT1 cells: Nanoparticle formulation exhibits increased cellular retention in SUP-T1 cells. Cells were incubated with 1 μM (Panel A) and 10 μM (Panel B) sol curcumin and nano-curcumin and examined by confocal microscopy at time points of 1, 2, 4 and 6 h. Each panel contains three images: fluorescence, bright field and merged.

Fig: 3.2.3.2:

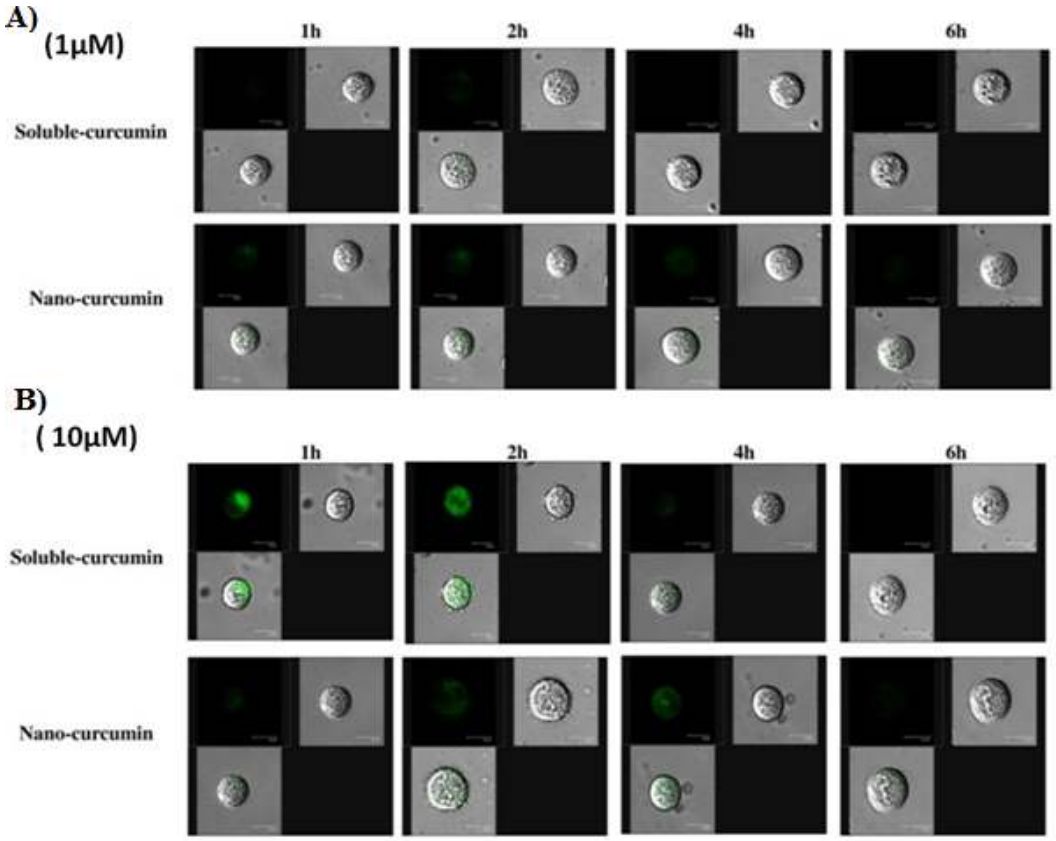


Fig: 3.2.3.2: Analysis of cellular retention of curcumin following nano-curcumin administration in PBMCs : Nanoparticle formulation exhibits increased cellular retention in PBMCs cells. Cells were incubated with 1 μM (Panel A) and 10 μM (Panel B) sol curcumin and nano-curcumin and examined by confocal microscopy at time points of 1, 2, 4 and 6 h. Each panel contains three images: fluorescence, bright field and merged

Fig: 3.2.3.3:

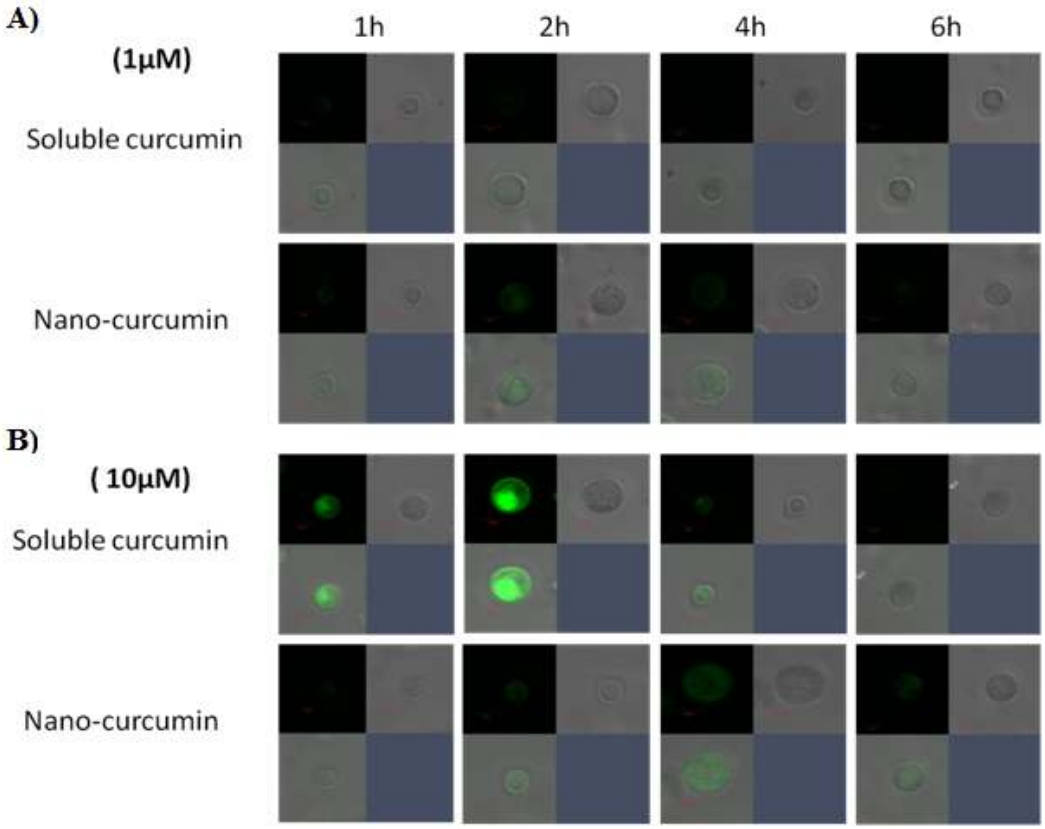


Fig: 3.2.3.3: Analysis of cellular retention of curcumin following nano-curcumin administration in VK2-E6/E7 cells : Nanoparticle formulation exhibits increased cellular retention in VK2/E6E7 cells. Cells were incubated with 1 μM (Panel C) and 10 μM (Panel D) sol curcumin and nano-curcumin and examined by confocal microscopy at time points of 1, 2, 4 and 6 h. Each panel contains three images: fluorescence, bright field and merged

Fig: 3.2.3.4

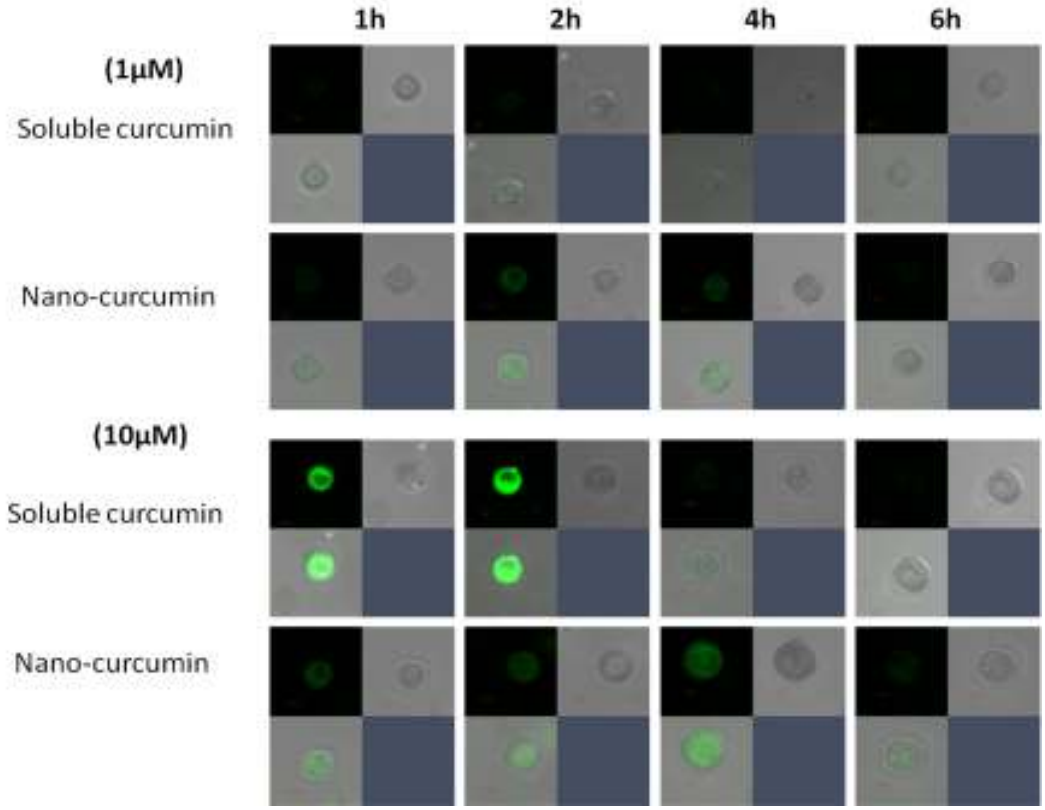


Fig: 3.2.3.4: **Analysis of cellular retention of curcumin following nano-curcumin administration in U937 cells :**Nanoparticle formulation exhibits increased cellular retention in U-937cells. Cells were incubated with 1 μM (Panel E) and 10 μM (Panel F) sol curcumin and nano-curcumin and examined by confocal microscopy at time points of 1, 2, 4 and 6 h. Each panel contains three images: fluorescence, bright field and merged

3.2.4: Cytotoxicity of nano-curcumin mediated delivery

To determine the relative cytotoxicity of curcumin in the nanocurcumin and sol-curcumin formulations, SUPT1 cells (Fig. **3.2.3**A) or stimulated PBMCs (Fig. **3.2.3**B) were incubated with increasing concentrations (1, 5, 10, 25, 50 and 100 μM) of the two formulations and cell survival was estimated by MTT assay. Both sol- and nano-curcumin formulations were found non-toxic at very low concentrations (1, 5 and 10 μM). However, at 25 μM concentration of sol-curcumin was extremely cytotoxic (almost 80%) while nano-curcumin at the same concentration was significantly less cytotoxic (Fig. **3.2.3**). The GI50 of sol-curcumin is 15.6 μM, while that of nano-curcumin it is 32.5 μM in SUPT1 cells. In stimulated PBMCs, the GI50 is 18 μM for sol-curcumin and 38mM for nano-curcumin. The low cytotoxicity of nano-curcumin highlights the observation that direct sol-curcumin administration is lethal to cells at concentrations above 10 μM. notably decreased cytotoxicity of Apotransferrin encapsulated formulation occurred despite increased cellular uptake and sustained retention.

Fig: 3.2.4

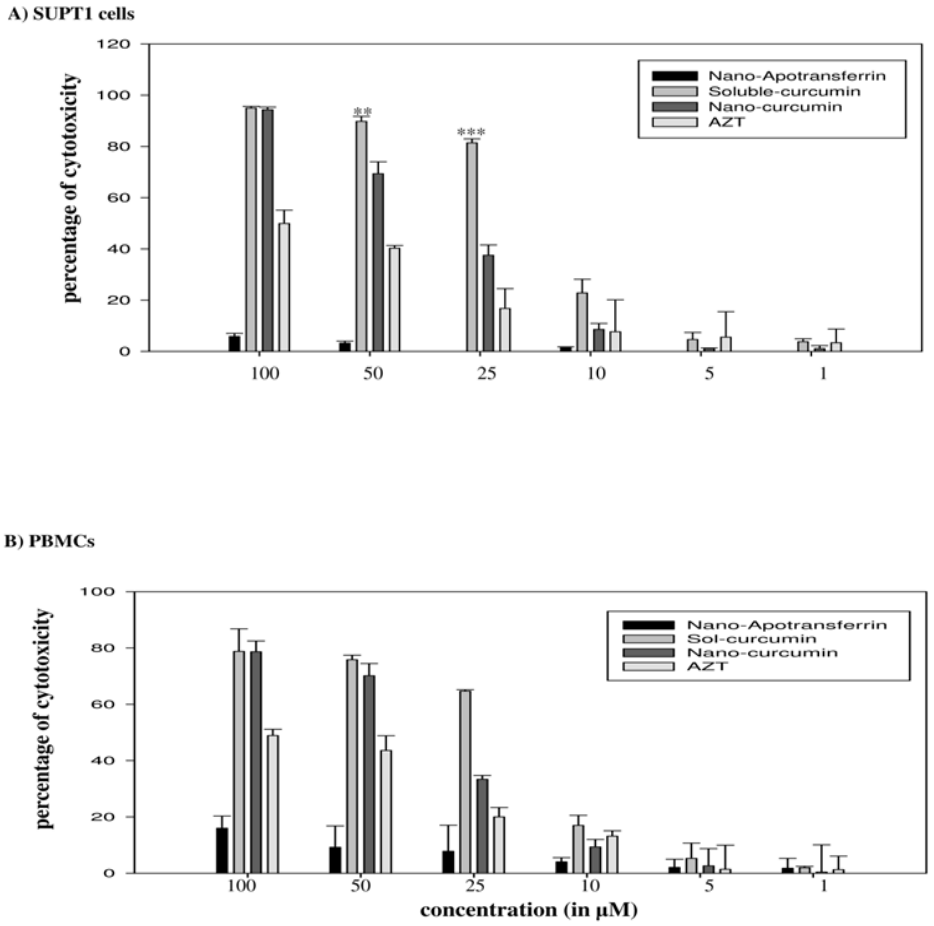


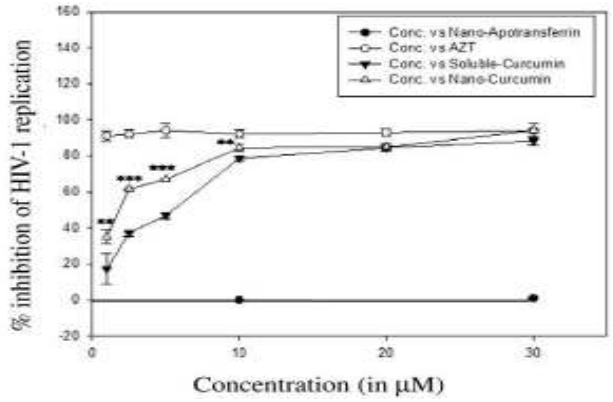
Fig 3.2.4: Cytotoxicity of nano-curcumin mediated delivery: Nanoparticle formulation decreases curcumin cytotoxicity. SUPT1 cells (Panel A) or stimulated PBMCs (Panel B) were exposed to increasing concentrations (1, 5, 10, 25, 50 and 100 µM) of sol-curcumin, nano-curcumin, azidothymidine (AZT) or nano-apotransferrin (10, 50 and 100 mg) for 16 h, after which cell viability was determined by MTT assay. PBMCs were cultured in the presence of IL-2 (20 IU/ml). Cell viability in the absence of drug was defined as 0% cytotoxicity.

3.2.5: HIV-1 neutralizing activity of nano-curcumin

SUPT1 cells or stimulated PBMCs were infected with HIV- $1_{93IN101}$ in the presence of increasing concentrations (1, 2.5, 5, 10, 20 and 30μM) of nano-curcumin or sol-curcumin. Nanocurcumin inhibited HIV-1 replication in a dose-dependent manner (Figs. **3.2.4.1** A and B). The IC50 of nano-curcumin is 1.75 μM, while that of sol-curcumin it is 5.1 μM in SUPT1 cells.In stimulated PBMCs, it is 5.1 μM for sol-curcumin and 2.4μM for nano-curcumin. These data suggest that nano-curcumin is almost three-fold more potent than sol-curcumin. About 80% inhibition of HIV-1 replication was estimated at 10 μM nanocurcumin (Fig. **3.2.4.1** A and B), a concentration at which its cytotoxicity is less than 10%. Although sol-curcumin exhibits a 75% inhibition of viral replication at this concentration, its cytotoxicity is 20%. The HIV-1 neutralizing activity of nanocurcumin is significantly reduced by antibody to human transferring receptor in both SUPT1 cells (Fig. **3.2.4.2** A) and PBMCs (Fig. **3.2.4.2**B) confirming that the superior ability of nano-curcumin to inhibit HIV-1 infection is dependent on cellular uptake mediated by the transferrin receptor.

Fig: 3.2.5.1

A)



B)

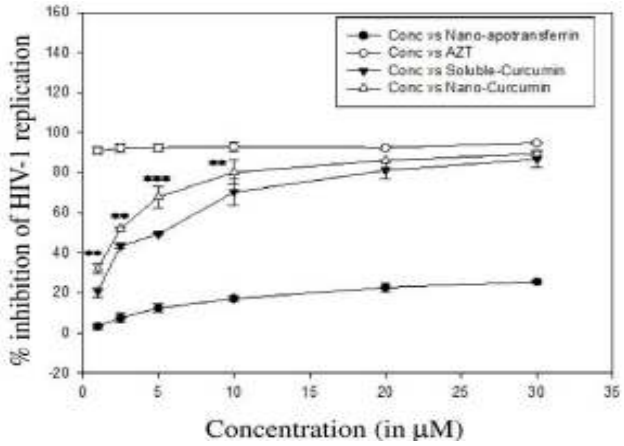
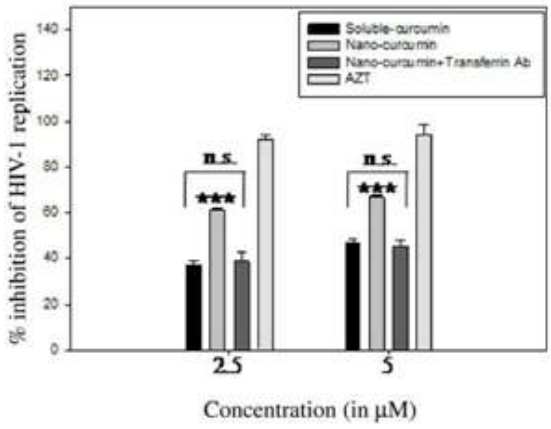


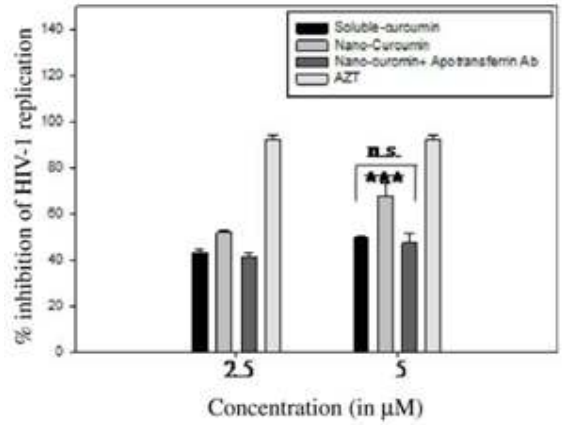
Fig 3.2.5.1: HIV-1 neutralizing activity of nano-curcumin: Nano-curcumin more effectively inhibits HIV-1 replication through a mechanism dependent on transferrin receptor. A & C) SUPT1 cells (Panel A) or stimulated PBMCs (Panel B) were challenged for 2 h with HIV-193IN101 (1ng p24/ml) in the presence of increasing concentrations (1, 2.5, 5, 10, 20 and 30 µM) of sol-curcumin, nano-curcumin, or nano-apotransferrin (10 and 50 mg). They were then incubated for a further 96 h, after which viral replication was measured by p24 antigen capture assay. In this experiment viral replication in the absence of drug was defined as 0% inhibition; Azidothymidine (AZT) was employed as a positive control.

Fig: 3.2.5.2

A)



B)



3.2.5.2: HIV-1 neutralizing activity of nano-curcumin in presence of transferrin receptor antibodies: A)SUP-T1 cells or B) stimulated PBMCs were challenged for 2 h with HIV-193IN101 in the presence of 2.5 or 5.0 µM concentrations of sol-curcumin, nano-curcumin or nano-curcumin in the presence of transferrin receptor antibody (100 ng/ml). After 96 h incubation,viral replication was measured by p24 antigen capture assay. In both these experiments, viral replication in the absence of drug was defined as 0% inhibition; Azidothymidine (AZT) was employed as a positive control.

3.2.6: Wide range of HIV-1 neutralizing activity of curcumin and nano-curcumin:

SUPT1 infected with different HIV- 1 strains in the presence of increasing concentrations (100μM, 1, 5 and 10μM) of nano-curcumin or sol-curcumin. The results showes that the nanocurcumin significantly inhibited four HIV-1 strains (HIV-1-$_{IIIB, 94UG103, IN98-026, 93IN101)}$ replication and pIND-c1 molecular clone of HIV-193IN101 replication (fig 3.2.6.1) more efficiently than soluble. The IC-50 values for soluble curcumin were 5, 6, 5 and 4.8 μM respectively, where as for nanocurcumin it was 2.5, 5, 2.8and 1.8μM. Also shows moderate inhibition against four HIV-1strains namely $_{KNH-1135, 93RW024, 9US667 and IN93-905}$, at 5uM concentration the soluble curcumin showed 43, 40, 21, and 26%of inhibition respectively where as this activity was enhanced to 50, 48, 30 and 35 % in presence of nanocurcumin. While HIV-1 strain $_{IN980-21}$found to have lower sensitivity to nanocurcumin as well as soluble curcumin (Fig 3.2.6.2).

Fig: 3.2.6.1

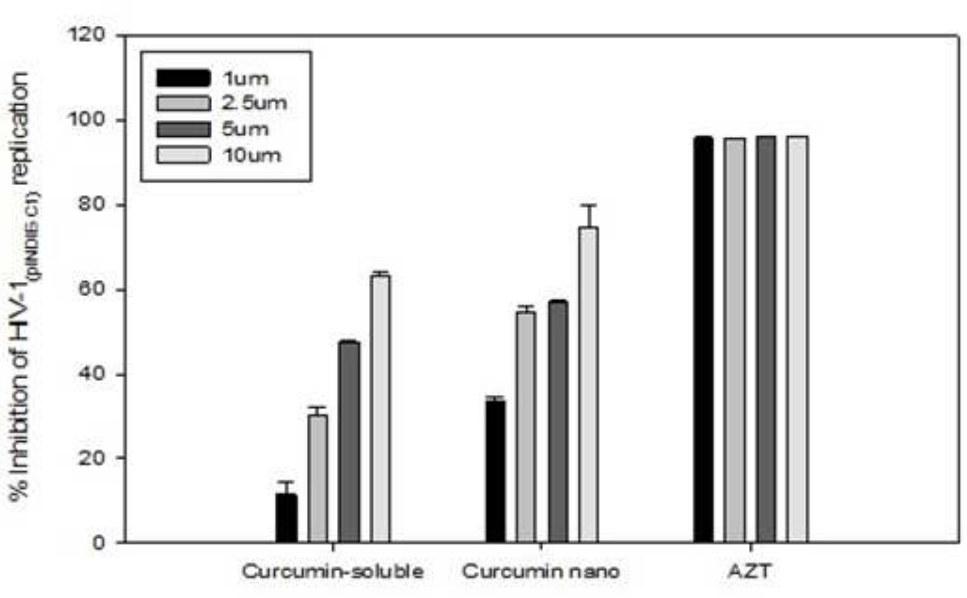
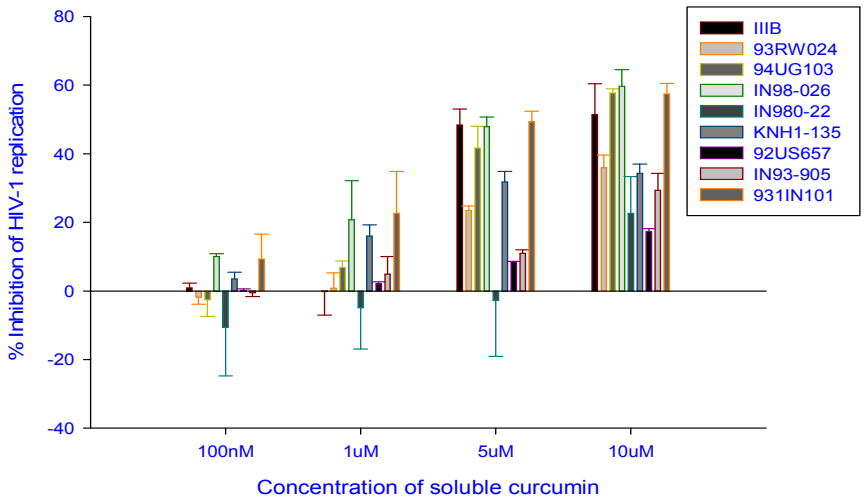


Fig 3.2.6.1: HIV-1 pINDE-c1 neutralizing activity of nano-curcumin:SUPT1 cells were challenged for 2 h with HIV-$_{pINDIE-C1}$ (1ngp24/ml) in the presence of increasing concentrations (1, 2.5, 5 and 10μM) of sol-curcumin, nano-curcumin. They were then incubated for a further 96 h, after which viral replication was measured by p24 antigen capture assay. In both these experiments, viral replication in the absence of drug was defined as 0%inhibition; AZT was employed as a positive control.

Fig: 3.2.6.2

A)



B)

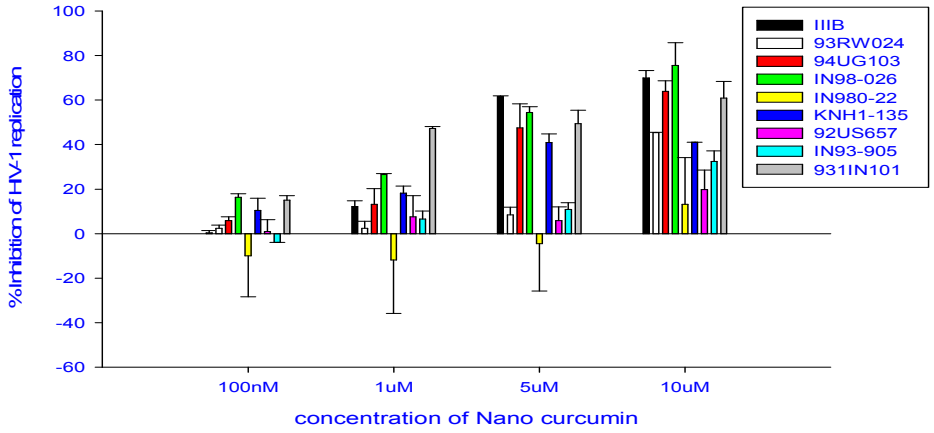


Fig 3.2.6.2: Wide range of HIV-1 neutralizing activity of nano-curcumin

Nano-curcumin (B) more effectively inhibits a panel of HIV-1 strains replication than soluble curcumin (A), SUPT1 cells were challenged for 2 h with different HIV-1 strains (1 mg p24/ml) in the presence of increasing concentrations (100nM,1, 5, and 10 µM) of sol-curcumin,They were then incubated for a further 96 h, after which viral replication was measured by p24 antigen capture assay

Fig: 3.2.6.3

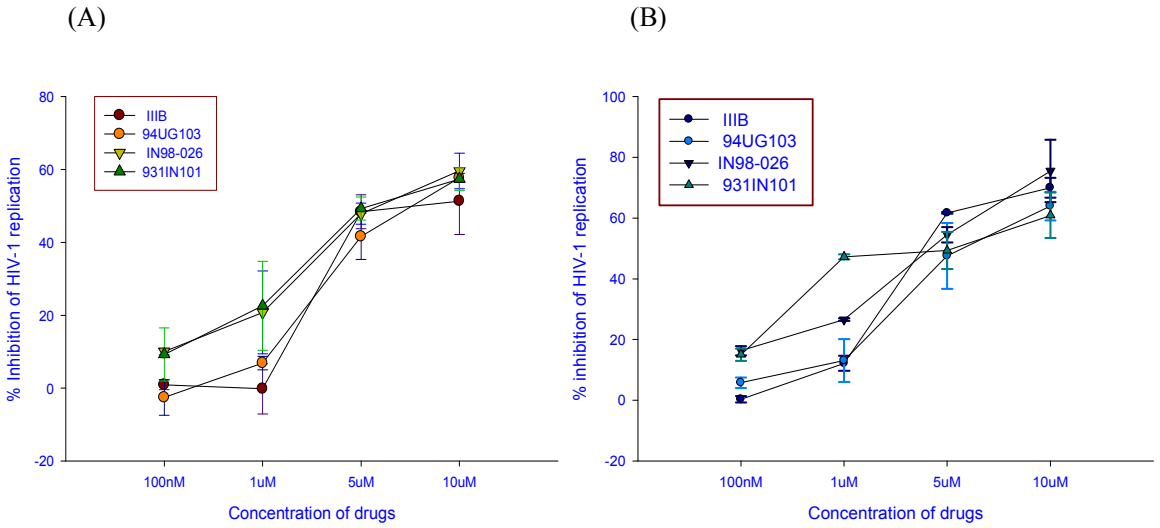


Fig 3.2.6.3: Wide range of HIV-1 neutralizing activity of curcumin and nano-curcumin

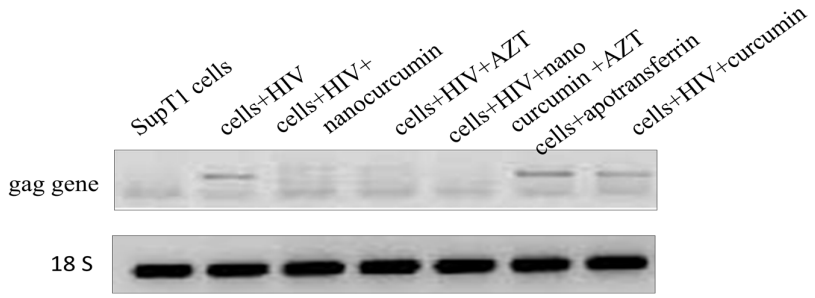
Nano-curcumin (B) more effectively inhibits a panel of HIV-1 strains replication than soluble curcumin (A), SUPT1 cells were challenged for 2 h with different HIV-1 strains(1 mg p24/ml) in the presence of increasing concentrations (100nM,1, 5, and 10 μM) of sol-curcumin, They were then incubated for a further 96 h, after which viral replication was measured by p24 antigen capture assay.

3.2.7: Effect of nano-curcumin on the expression of topoisomerase-IIα and proviral DNA synthesis

To determine the mechanism of nano-curcumin's anti-HIV activity in T-cells, expression of the human topoisomerase-IIα gene was monitored. SUP-T1 cells were acutely infected with HIV-$1_{93IN101}$ in the presence of 5 μM nano-curcumin, sol-curcumin or appropriate controls. Semi-quantitative and real-time PCR of topoisomerase-IIα expression demonstrated that this gene was up regulated on HIV -1 infection but was prominently down-regulated by nano-curcumin treatment (Fig. 3.2.7.2). This down-regulation was not observed when the cells were treated with sol-curcumin. In a different experiment, it was found that nano-curcumin significantly inhibited proviral DNA synthesis, monitored by gag gene expression (Fig. 3.2.7.1). Sol-curcumin had no effect on synthesis of proviral DNA.

Fig: 3.2.7.1

A)



B)

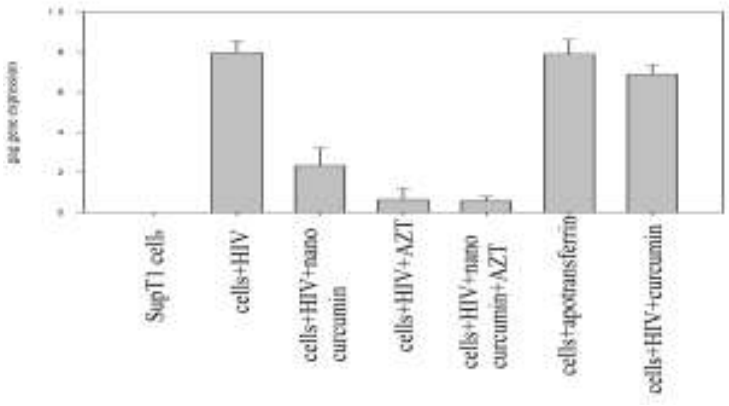
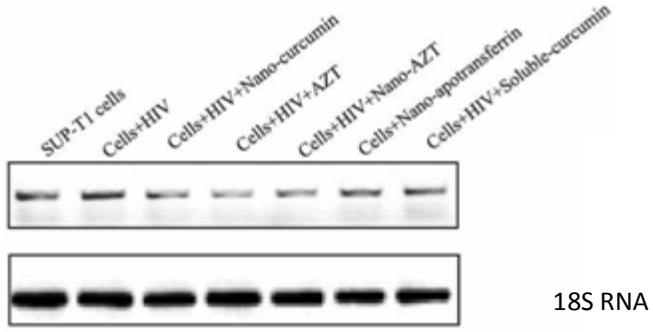


Fig3.2.7.1: Effect of nano-curcumin on the expression of proviral DNA synthesis: Inhibition of HIV-1 replication by nano-curcumin is due to abolished viral cDNA synthesis and/or altered topology. SUPT1 cells were challenged for 4 h with HIV-193IN101 in the presence of 5μM of sol-curcumin, nano-curcumin or nano-apotransferrin. Quantity of viral cDNA synthesized was shown by both (A) semi-quantitative and (B) real-time PCR using gag-specific primers. Template from normal SUP-T1 cells was used as negative control. Azidothymidine (AZT) was employed as a positive control and 18S was used as an internal control in both experiments.

Fig: 3.2.7.2

A)



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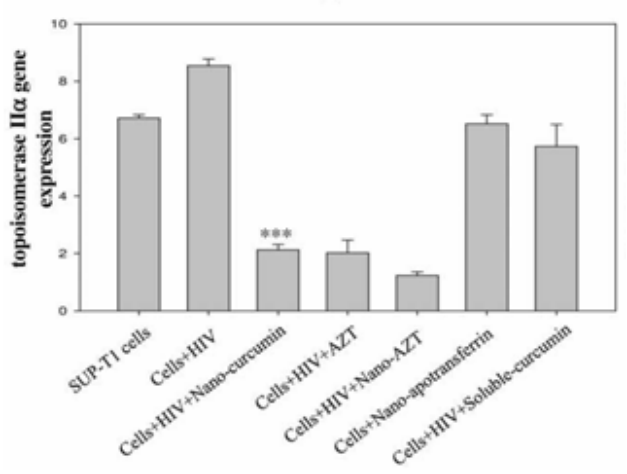


Fig 3.2.7.2: Effect of nano-curcumin on the expression of topoisomerase-IIα: Inhibition of HIV-1 replication by nano-curcumin is due to abolished viral cDNA synthesis and/or altered topology. SUPT1 cells were challenged for 4 h with HIV-193IN101 in the presence of 5μM of sol-curcumin, nano-curcumin or nano-apotransferrin. Quantity of viral cDNA synthesized was shown by both (A) semi-quantitative and (B) real-time PCR using gag-specific primers. Template from normal SUP-T1 cells was used as negative control. Azidothymidine (AZT) was employed as a positive control and 18S was used as an internal control in both experiments.

3.2.8: Effect of Nano-curcumin on HIV-1 induced inflammatory response in SupT1 cells:

HIV-1 infection enhanced expression of topoisomerase-IIβ, IL-1β and COX-2. Treatment of infected cells with nano-curcumin significantly inhibited expression of IL-1b and COX-2 (Fig. 3.2.8) as well as topoisomerase IIα (Fig. 3.2.7.1), but had no effect on the expression of topoisomerase IIβ or TNF-α (Fig. 3.2.8A). The results are further confirmed by estimation of IL-1β (Fig. 3.2.8B), COX-2 (Fig. 3.2.8.C) and TNF-α (Fig. 3.2.8.D) levels in infected cells. These results suggest that nano-curcumin effectively blocks HIV-1-mediated inflammatory responses and thus affects viral cDNA synthesis.

Fig: 3.2.8

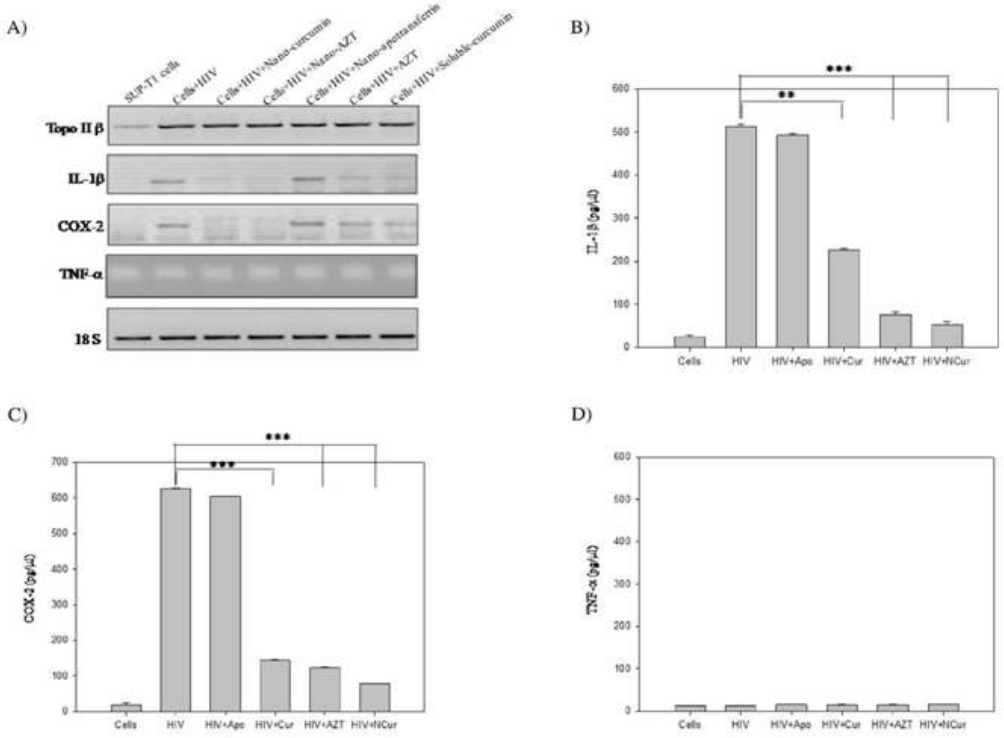


Fig3.2.7: effect of Nano-curcumin on HIV-1 induced inflammatory response in SupT1 cells: SUPT1 cells were challenged for 4 h with HIV-$_{193IN101}$ in the presence of 5 μM of sol-curcumin, nano-curcumin or nano-Apotransferrin. The expression of topoisomerase-IIβ IL-1β, TNF-α and COX-2 was determined by semi-quantitative-PCR. Template from normal SUP-T1 cells was used as negative control. 18S was used as an internal control in both experiments. IL-1b (Panel B), COX-2 (Panel C) and TNF-a (Panel D) were estimated using commercial kits as described in the methods section.

Discussion:

We show that curcumin-loaded apotransferrin forms spherical nanoparticles consistently in the size range 55-70 nm, which is in accord with the National Nanotechnology Initiative's definition of "nanomaterials". Other reported formulations have typically been larger in size (usually 100-200 nm) [Li L, Braiteh FS,(2005),Swamy KV, et al. (2010)], although Bisht*et al*. reported a polymeric nanoparticle formulation with a size range similar to ours. The spherical shape and surface characteristics of the nanoparticles, as shown by our TEM study, probably play a critical role in the cellular uptake and release characteristics displayed by this curcumin formulation *in vitro*. These particles are efficiently transported into cells through endocytosis mediated by the transferrin receptor [Kondapi,et al], with the curcumin then being released intra cellularly. Although a curcumin derivative, EF24, has been chemically linked to an endocytosis-inducing peptide [Sun A, (2006)], our nano-curcumin is to the best of our knowledge the first formulation in which cellular uptake of curcumin itself is enhanced by receptor-mediated endocytosis. The nano-curcumin formulation is especially attractive because of its simple preparation protocol that does not require either complex equipment or expensive reagents. Moreover, this delivery system is highly target-specific. The simplicity of the preparation would also minimize the possibility of potentially toxic reagents being carried over into formulations for future *in vivo* studies.

Curcumin is naturally fluorescent in the visible green spectrum, thus allowing it to be located and quantified within cells. Cells treated with both sol- and nano-curcumin displayed green fluorescence, confirming successful intracellular delivery of curcumin. The greater cellular uptake and retention exhibited by nano-curcumin, as demonstrated here, addresses the problem of curcumin bioavailability. The ability of nanoparticle preparations to increase cellular uptake of curcumin has also been demonstrated by other groups [Ratul Kumar (2010),Bisht S (2007)]. Since cytotoxicity is of prime concern for cellular assays, we performed our uptake and retention studies at nano-curcumin concentrations that are non-toxic to cells. Further studies showed that, despite greater intracellular concentrations,

nano-curcumin exhibited much less cytotoxicity than equivalent doses of sol-curcumin. Observed higher bioavailbility of curcumin when delivered in nanoformulation in Sup T1, U937 and VK2 vaginal epithelial cells clearly points out the ability of nanoformulation in delivery of curcumin to these cells which are present in vaginal epithelium, thus this formulation can as attractive microbicidal vehicle Nanocurcumin will be safe even at higher concentrations and thus has an advantage over AZT and curcumin even at higher concentrations of 12g/day known to be well tolerated in humans. On the other hand, use of higher dosage of AZT causes myopathy, cardiomyopathy and hepatotoxicity associated with mitochondrial DNA depletion. As a component of HAART (highly active antiretroviral theraphy) AZT causes cytopenias and lipodystrophy [OlanoJP, SinnwellTM, Deveaud C, Mhiri C, Moh R]. Thus such as those observed in the case of AZT, adverse events are not expected in the case of curcumin. The present study addresses the major problem of metabolic instability and nano-curcumin even at lower concentrations is shown to provide a highly stable and retarded intra-cellular release. The concentration limitation will reduce the cytotoxicity, while retarded release allows longer half-life.Studies showing that curcumin has anti-viral properties are few but reliable [Barthelemy S, Li CJ,Sui Z]. We now show that nano-curcumin, but not sol-curcumin, has high anti-HIV activity against wide spectrum of HIV-1 isolates namely HIV-$1_{IIIB,}$ HIV-$1_{94UG103,}$ HIV-$1_{IN98-026,}$HIV-$1_{KNH1-135,}$ HIV-$1_{93IN101,}$ HIV-$1_{93RW024,}$ HIV-1_{9US667} and HIV-$1_{IN93-905}$, while lowers sensitivity was observed with HIV-$1_{IN980-21}$. Nano-curcumin drastically decreased expression levels of topoisomerase IIα and inhibited proviral DNA synthesis. The high levels of intracellular curcumin achieved through nano-curcumin administration may account for this previously unreported observation. Curcumin has been previously demonstrated to inhibit HIV activation and replication [Barthelemy S, Li CJ,Sui Z, Taher MM]. Mechanisms involved include inhibition of both HIV protease [Sui Z] and integrase[Mazumder A]. Curcumin also inhibits Tat-mediated transactivation of the HIV long terminal repeat, which is essential for activation of latent virus [Barthelemy S, Li CJ]. Another mechanism may involve inhibition of the virus's ability to up regulate the host enzyme topoisomerase II [Kondapi AK], which

is required at the earliest stages of virus replication. Repression of topoisomerase using an inhibitor [Li CJ]or antisense nucleotides [Bouille´ P]results in impaired HIV-1 replication. Additionally, Topoisomerase II naturally recognizing DNA topological intermediates such as DNA curvature, flexibility, rigidity and distortion greatly influence the HIV integration event [Pruss D]. Moreover, curcumin has been shown to form a complex with DNA and topoisomerase, producing DNA breaks and blocking their repair, much as etoposide does [Martin-Cordero C]. Therefore, decrease in topoisomerase IIα, COX-2 and IL-1βlevels could predictably alter the HIV-1-mediated inflammatory response, which may in turn affect the topological reorganization of cellular DNA promoted by topoisomerase IIα. These effects on DNA organization might then prevent the HIV-1 integration event. This explains the inhibition of proviral DNA synthesis, monitored through *gag* gene expression, and the consequent blockage of HIV-1 replication observed in the current study.

CHAPTER IV:

Evaluation of anti-HIV activity of soluble and nanoformulation of curcumin analogues.

4.1 Introduction

Development of potent curcumin analogues against HIV-1:

Many antiretroviral drugs are currently available to treat human immunodeficiency virus type 1 (HIV-1) infection. Because of the emergence of viral strains with multidrug resistance (MDR), however, new anti-HIV-1 drugs operating with different inhibitory mechanisms are required. Viral enzymes such as reverse transcriptase (RT), protease and integrase (IN), gp41, and co-receptors are the main targets for novel antiretroviral drugs that are under development. Several natural products from plants belonging to a wide range of different structural classes, *e.g.* flavonoids, coumarins, tannins, lignans, terpenes, alkaloids, anthraquinones, and polysaccharides have been shown to be active as against RT, Integrase and protease activity. One of such natural molecules is curcumin, it has been studied extensively as a therapeutic agent in vitro and in cell culture and found that it has a potential against a wide range of diseases, such as cardiovascular diseases, neurological diseases, lung diseases, cancer, liver diseases, metabolic diseases, autoimmune diseases, and several other inflammatory diseases, anti bacterial anti viral properties including anti HIV-1 activity. However, these remarkable pleiotropic activities were ascribed to curcumin from its indicative molecular structure and chemistry, as well as its capability to influence many signaling molecules. Even though it is a potential drug molecule against several ailments but its clinical usefulness was limited because of several limitations. Therefore the compound has been formulated and administered using different drug delivery systems such as liposomes, micelles, polysaccharides, phospholipid complexes and nanoparticles that can overcome the limitation of bioavailability to some extent. In order to overcome low

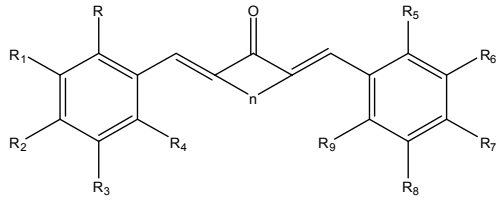
bioavailability and rapid metabolism of curcumin until now have been met with limited success. Hence, several studies were done to look for new synthetic curcumin analogs for better activity and to overcome the drawbacks of curcumin. In this search many of its structurally isomeric and naturally occurring derivatives were investigated and found some of them analogues consist even better pharmacological behavior than curcumin. (magano et al. 1997; Oyama et al. 1998; Nkamura et al. 1998; Rao et al. 1982; Ali et al. 1995; Masuda et al. 1993; Flynn et al. 19911.

The curcumin analogues sodium curcuminate and sodium phenateare found to be more anti-inflammatory than curcumin [Ghat&&Basu 19721. Some curcumin analogues have been reported to show better anticancer properties and a good antibiotic without side effects. The curcumin analogue dehydrozingerone is known to have anti-tumor activity [Motohashi et al. 19981. Dernethoxy curcumin is a better contain better tumor growth inhibitory activity than curcumin. Hence, number of research groups has taken the curcumin as a starting point to develop a wide variety of curcumin analogues against wide variety of ailments.

In order to develop potent drug candidates against HIV-1, in our laboratory a total of 28 curcumin analogues were synthesized using curcumin as a starting point. In my present study I have studied the anti HIV-1 of these curcumin analogues in Sup-T1 cells using HIV-1$_{(93INHIV-1)}$ strain and found four molecules found have strong anti-HIV-1 activity than curcumin, further we investigated the anti-HIV activity of **active curcumin analogues** against a panel of clinical isolates of various subtypes to study their broad spectrum of anti HIV-1 activity .the study was conducted using soluble and apotransferrin nanoformulation of active curcumin analogues.

Table: 4.1 Curcumin analogues screened for anti-HIV-1 activity

Scheme-1products



When n =3 (no of carbon atoms)

CA-no	R	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9
1	H	H	OH	OCH_3	H	H	H	OH	OCH_3	H
3	OH	H	H	Br	H	OH	H	H	Br	H
4	OCH_3	H	OCH_3	H	OCH_3	OCH_3	H	OCH_3	H	OCH_3
5	OCH_3	H	OCH_3	OCH_3	H	OCH_3	H	OCH_3	OCH_3	H
6	H	H	H	H	H	H	H	H	H	H
7	H	H	Cl	H	H	H	H	Cl	H	H
8	H	H	OCH_3	H	H	H	H	OCH_3	H	H
9	H	H	OH	H	H	H	H	OH	H	H
15	OCH_3	H	H	OCH_3	H	OCH_3	H	H	OCH_3	H
16	H	H	$N(CH3)_2$	H	H	H	H	$N(CH3)_2$	H	H

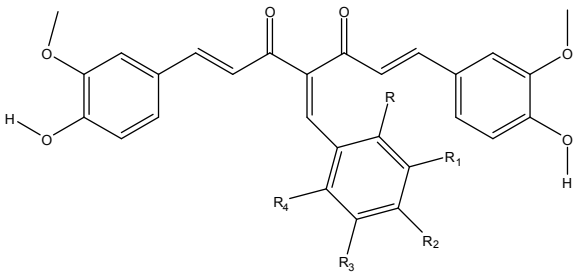
When n =2 (no of carbon atoms)

CA-no	R	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9
10	OCH_3	H	OCH_3	H	OCH_3	OCH_3	H	OCH_3	H	OCH_3
11	OCH_3	H	OCH_3	OCH_3	H	OCH_3	H	OCH_3	OCH_3	H
12	H	H	OCH3	H	H	H	H	OCH3	H	H
13	OH	H	H	Br	H	OH	H	H	Br	H
17	OCH_3	H	H	OCH_3	H	OCH_3	H	H	OCH_3	H
18	H	H	H	H	H	H	H	H	H	H
19	NO_2	H	H	H	H	NO_2	H	H	H	H

When n =0 (no of carbon atoms)

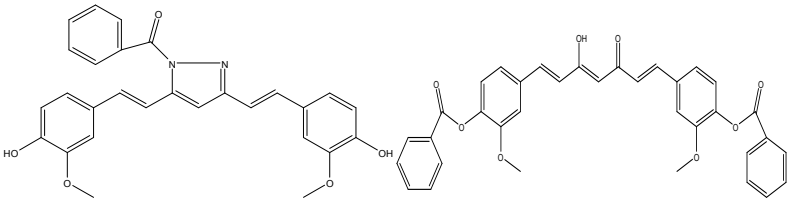
CA-no	R	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9
2	H	OCH_3	OH	H	H	H	OCH_3	OH	H	H
14	H	H	Cl	H	H	H	H	Cl	H	H

Scheme-2 products



CA-no	R	R_1	R_2	R_3	R_4
20	H	Cl	H	H	H
21	OCH_3	H	OCH_3	H	OCH_3
22	H	H	OH	OCH_3	H
23	OH	H	H	Br	H
24	OCH_3	H	OCH_3	H	OCH_3
25	OCH_3	H	OCH_3	OCH_3	H
26	H	H	H	H	H

Scheme-3 products



CA-27 CA-28

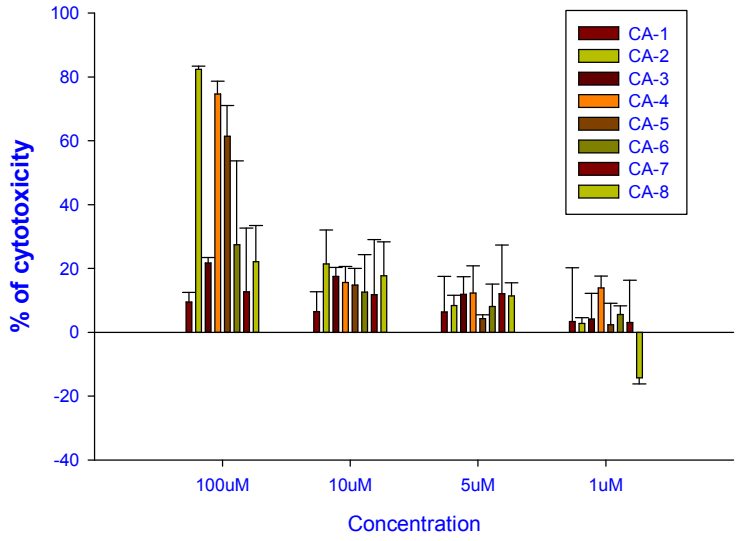
Results: 4.2

4.2.1 Study of cytotoxicity and anti HIV-1 activity of Curcumin analogue

To determine the relative cytotoxicity of curcumin analogues SUPT1 cells were incubated with increasing concentrations (1, 5, 10, and 100 μM) of the curcumin analogues and cell survival was estimated by MTT assay. In the first panel all the drugs were found non-toxic at very low concentrations (1, 5 and 10 μM). However, at 100 μM concentration drug 2, 4, 5 have showed 80, 60 and 50% of cytotoxicity respectively. In the 2nd and 3rd panel all of them have showed less toxicity and at 100uM concentration drugs 16,15,14,13,and 12 were showed 60,80, 50,55,and 40% of toxicity respectively(fig-2.1)

Fig4.2.1.1

A)



B)

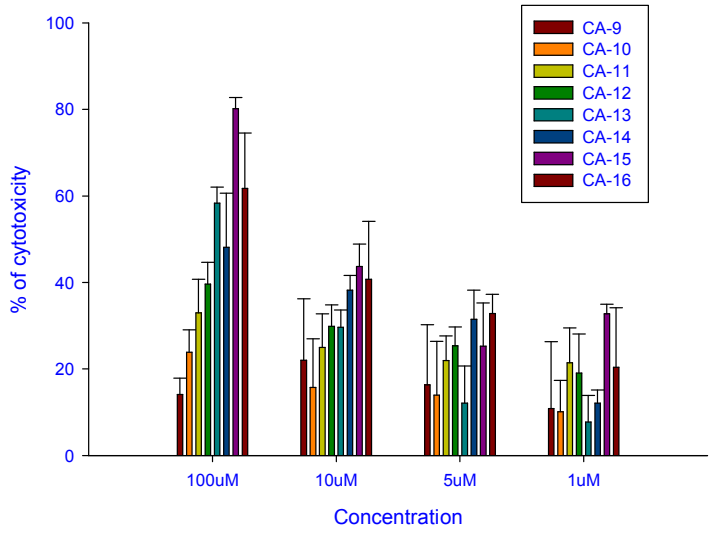
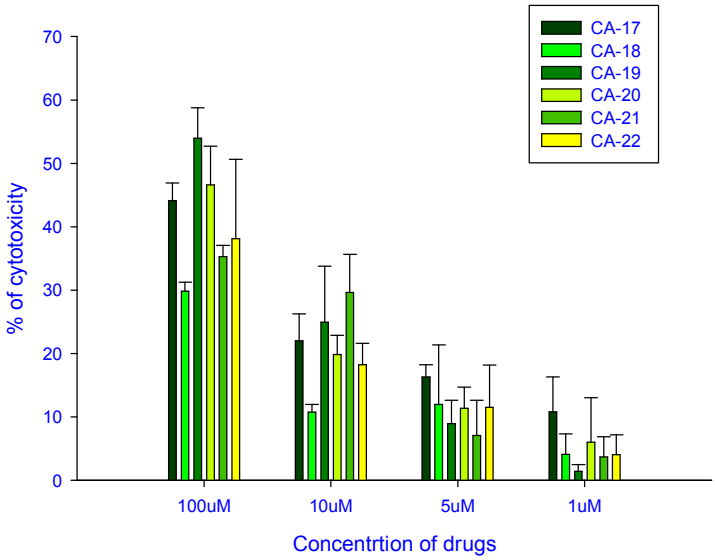


Fig 4.2.1.1 Cytotoxicity profile of curcumin analogues 1-16 in Sup-T1 cells :

Cytotoxicity profile of curcumin analogues in SupT1 cells: SupT1 cells were exposed to increasing concentrations (1, 5, 10, and100µM) of sol-curcumin analogues 1-8(Panel A) and 9-16(Panel B) for 16 h, after which cell viability was determined by MTT assay. Cell viability in the absence of drug was defined as 0% cytotoxicity.

Fig 4.2.1.2

A)



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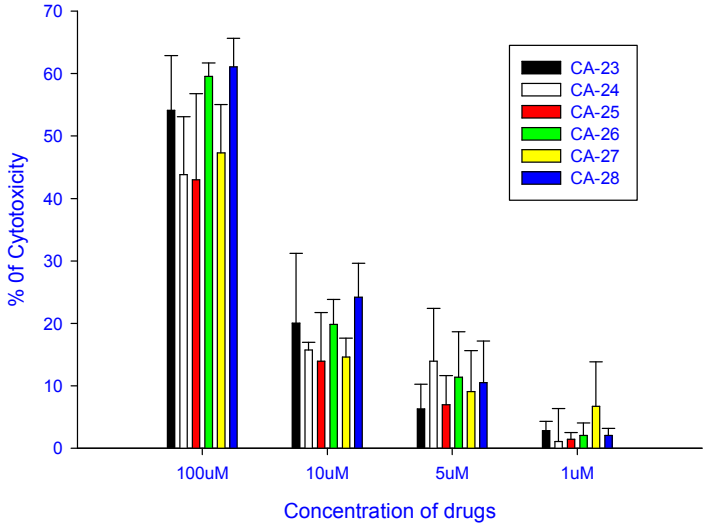


Fig 4.2.1.2 Cytotoxicity profile of curcumin analogues (drugs 17-28) in Sup-T1 cells: Cytotoxicity profile of curcumin analogues in SupT1 cells: SupT1 cells were exposed to increasing concentrations (1, 5, 10, and100µM) of sol-curcumin analogues 17-22(Panel A) and 23-28(Panel B) for 16 h, after which cell viability was determined by MTT assay. Cell viability in the absence of drug was defined as 0% cytotoxicity.

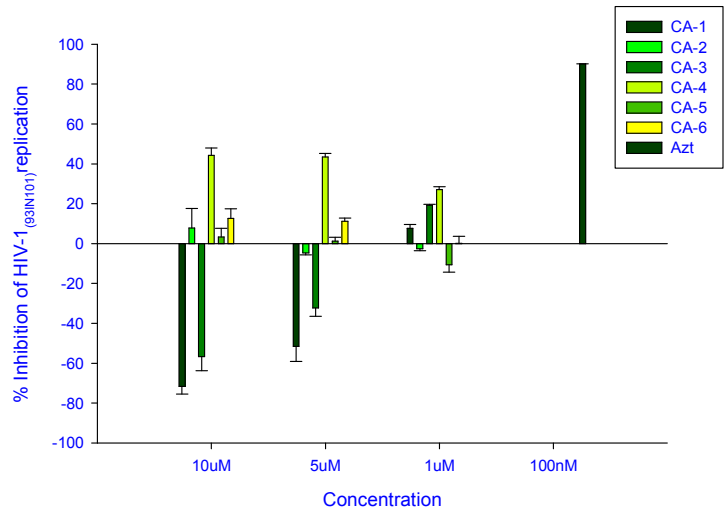
4.2.2: Anti HIV-1 activity of curcumin analogues

0.2 million SUPT1 cells were infected with 1ng/ml of HIV- $1_{93IN1019}$ in the presence of increasing concentrations (100nM, 1,5, and10uM) of curcumin analogues 1-6(Fig 4.2.2.1A) 7-15(fig4.2.2.1B) , 16-22(fig 4.2.2.2A) and 22-28(fig 4.2.2.2B) . After the cells were incubated for 96 hours the viral replication was measured by quantifying $_{P}$-24 viral core protein. Drugs 19, 20 have showed similar activity like curcumin where as the drugs 23-26 has showed improved activity then curcumin.

IC-50 values of CA23, CA24, CA25 and CA26 against HIV-1 were found to be 1.48, 1.6, 1.1 and 1.43μM respectively where as the IC-50 of curcumin was 4.8 μM (Fig: 4.2.2.3)

Fig-4.2.2.1

A)



B)

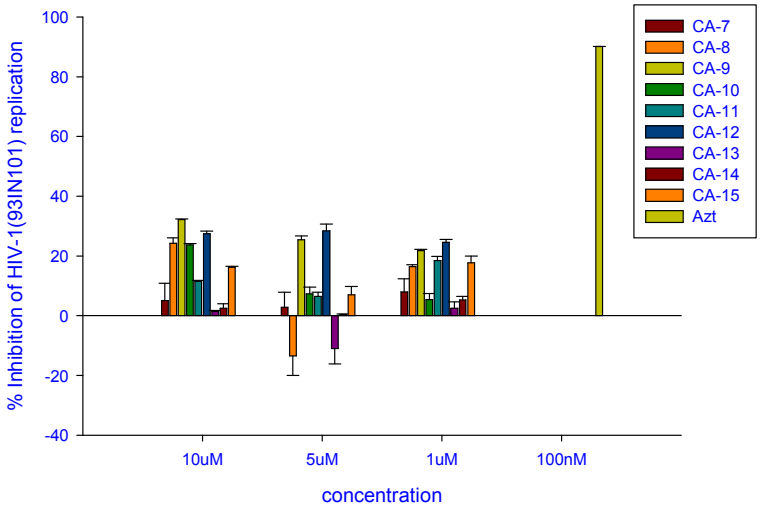
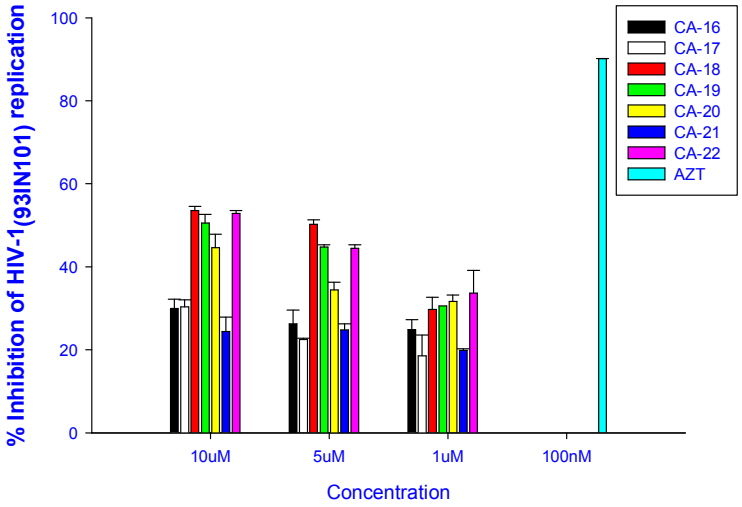


Fig 4.2.2.1 Anti HIV-1 activity of curcumin analogues 7-15 in Sup-T1 cells:

SUPT1 cells were challenged for 2 h with HIV-$1_{(93IN101)}$ (1ng p24/ml) in the presence of increasing concentrations (100nm, 1, 5 and 10µM) curcumin analogues 1A, 1B, 1C. They were then incubated for a further 96 h, after which viral replication was measured by p24 antigen capture assay. In both these experiments, viral replication in the absence of drug was defined as 0%inhibition; curcumin and AZT was employed as

Fig-4.2.2.2

A)



B)

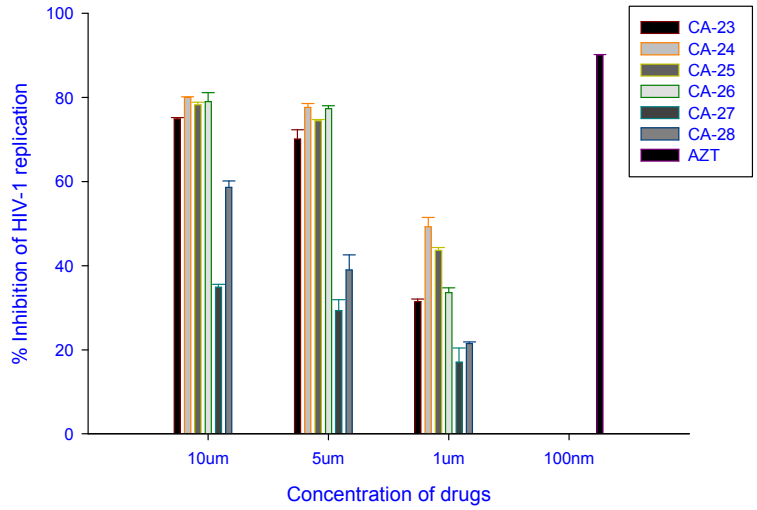
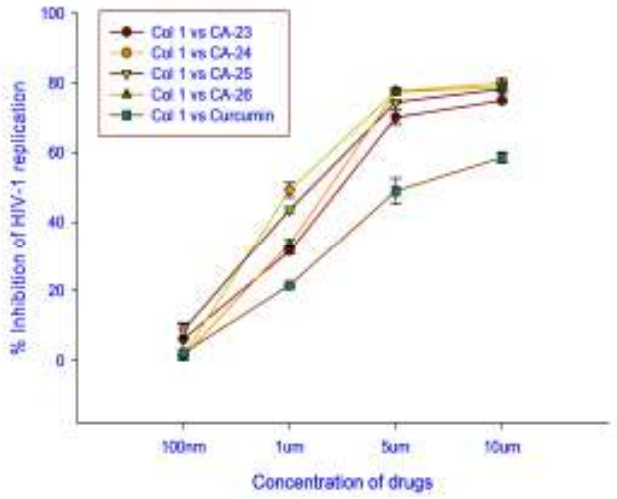


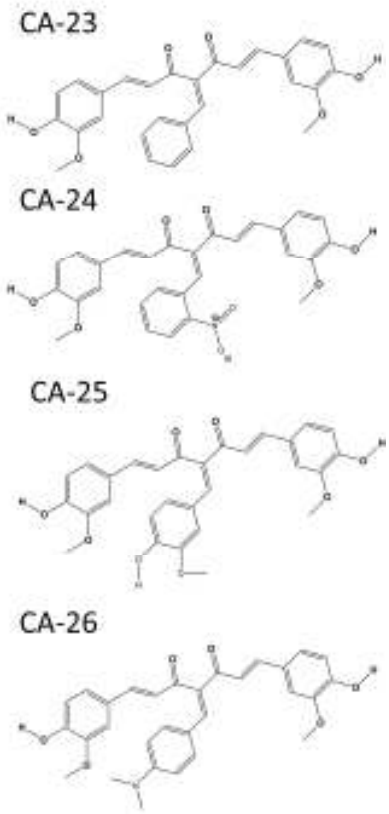
Fig 4.2.2.2 Anti HIV-1 activity of curcumin analogues 23-28 in Sup-T1 cells

SUPT1 cells were challenged for 2 h with HIV-$1_{(93IN101)}$ (1ng p24/ml) in the presence of increasing concentrations (100nm, 1, 5 and 10µM) curcumin analogues 23-28. They were then incubated for a further 96 h, after which viral replication was measured by p24 antigen capture assay. In both these experiments, viral replication in the absence of drug was defined as 0%inhibition; curcumin and AZT was employed as a positive control.

Fig-4.2.2.3



Drug	IC-50
Curcumin	5.1µM
CA-23	1.48µM
CA-24	1.6µM
CA25	1.1µM
CA26	1.43µM



Anti HIV-1 activity and IC-50 values of curcumin and active curcumin analogues:

Panel-A shows comparison of antiHIV-1 activity of active curcumin analogues and curcumin, panel B show structures of active curcumin analogues and panel C indicates the IC 50 values of curcumin and active curcumin analogues

4.3 anti-HIV-1 activity of nano-active curcumin analogues:

4.3.1: Preparation and characterization of apotransferrin nanoparticles of active curcumin analogues

Curcumin-containing Apotransferrin nanoparticles were prepared using sol-oil chemistry as described in materials and methods section. The results indicateall the particles are in uniform in size and spherical in shape. The size of pure Apotransferrin nanoparticles as assessed by scanning electron microscopy (SEM) ranged from 45–55 nm, increasing to 55–70 nm after curcumin loading (Fig.4.3.1). The surface morphological analysis of particles using atomic force microscopy (AFM) showed significant projections, which might contribute to the molecular recognition of particle by the receptor (Fig. 4.3.1).

Fig 4.3.1:

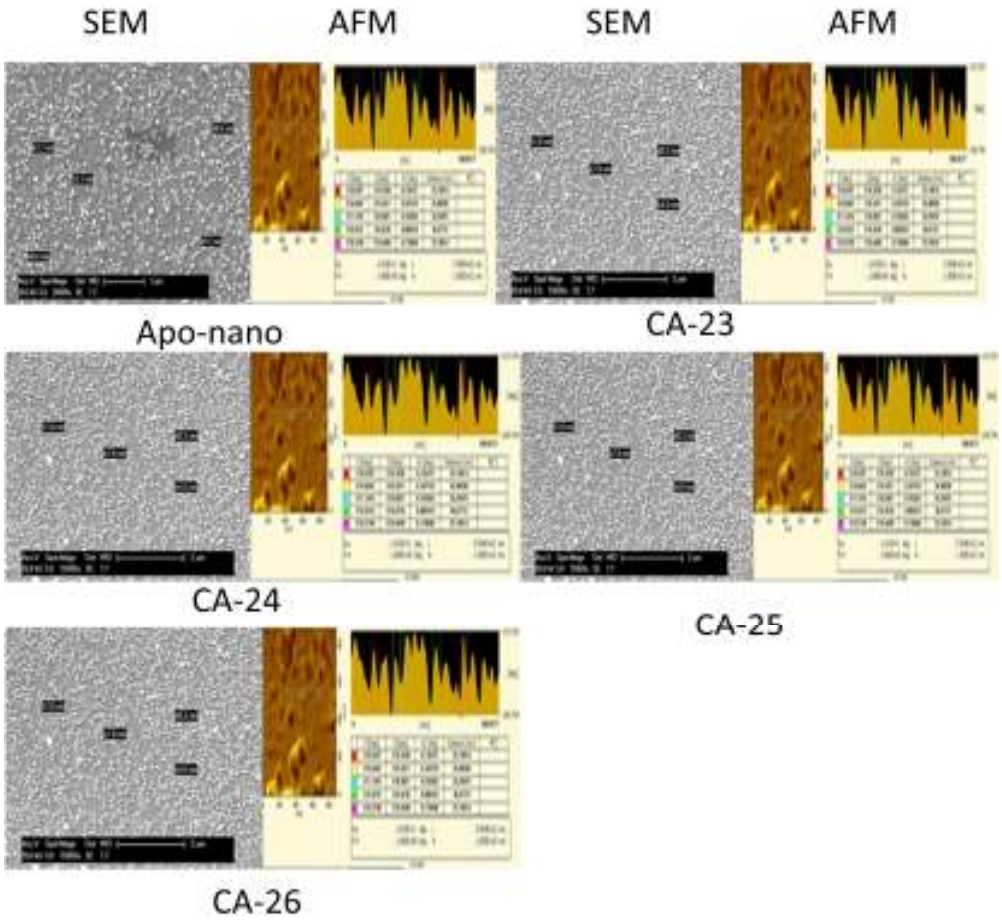


Fig 4.3.1 Characterization of apotransferrin nanoparticles ofactive curcumin analogues: Up on curcumin analogues loading increase the size of apotransferrin nanoparticles. The curcumin analogues (23, 23,25,and 26) loaded Apotransferrin nanoparticles(CA-23-26) and Apotransferrin nanoparticles without curcumin (Apo-nano) were examined by SEM and AFM as indicated

4.3.2 Broad spectrum anti-HIV-1 activity of soluble and nano-active curcumin analogues.

Considering the broad genetic diversity of HIV-1 existing in the world, we further investigated the anti-HIV activity of **active curcumin analogues** against a panel of clinical isolates of various subtypes, These isolates represent HIV-1 group M subtypes A (93RW024, 94UG103, KNH1-135), B(92US657,HIV-1-IIIB), C(98IN026, 93IN89022, 93IN905 ,93IN101HIV-1) and a molecular clone pINDIE-C1. The anti HIV-1 activity of curcumin analogues CA-23, 24, 25, 26 and curcumin were tested against the HIV-1 isolates in soluble and nano-formulations at their respective IC-50 concentrations that is at 1.48µM, 1.6 µM, 1.1 µM, 1.43 µM and 4.8 µM for CA-23, 24, 25, 26 and curcumin respectively. The results indicated all curcumin analogues exhibited significant inhibitory activities against HIV-1 isolates HIV-IIIB, 93RW024, 94UG103, IN98026. 47, 46 47 and 49.5% of inhibition of HIV-$_{IIIB}$ replication was observed in presence of soluble curcumin analogues CA-23, 24, 25 and 26 where as the nano curcumin analogues showed 68, 60,65and 63% of enhanced activity. 38, 48, 47.5 and 40% of inhibition of HIV-1 $_{93RW024}$ was observed with soluble curcumin analogues CA-23, 24, 25 and 26 respectively (fig 4.3.2.1), whereas the same nano curcumin analogues showed 59, 58, 59.2and 55% of inhibition. And 46, 49, 43, and 37%of HIV-1 $_{(94UG103)}$ replication was inhibited with soluble curcumin analogues CA-23, 24, 25 and 26 respectively and the activity was enhanced to 61, 60, 55, and 50% in presence of nanoformulation respectively(fig 4.3.2.1). In case of HIV-1$_{(IN98026)}$ CA-23, 24, 25 and 26 were showed 45, 48, 33, and 34% of inhibition and in presence of nano analogues the activity was enhanced to 52, 57, 52, and 37%. CA23, CA25 and CA26 were showed significant inhibition of HIV-1$_{92US657}$ replication (40-45% of inhibition was observed) where as CA25 showed 35% inhibitory activity against HIV-1$_{IN980-22}$.

And all the nano formulations have showed 10-20% of enhanced antiviral activity than soluble formulations (fig 4.3.2.1). However the antiviral activity found in soluble and nano **active curcumin analogues** against these isolates were approximately 3-fold more potent than the curcumin.

Fig 4.3.2.1

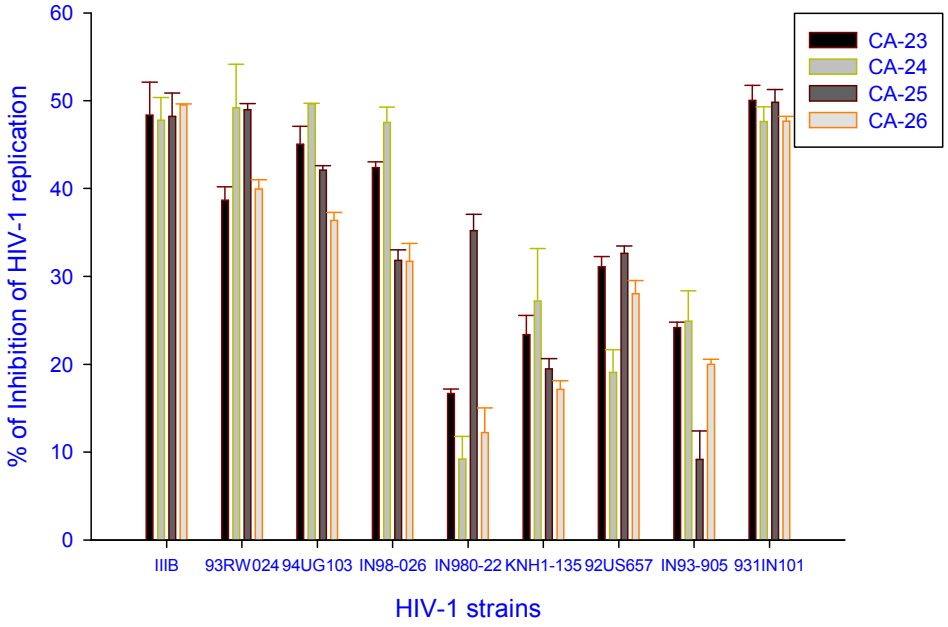


Fig-4.3.2.1 antiHIV-1 activity of soluble curcumin analogues against wide range of HIV-1 strains: SUPT1 cells were challenged for 2 h with different HIV-1 strains(1 ng p24/ml) in the presence their respective IC-50 concentrations of soluble analogues(CA-23, 24, 25, 26). They were then incubated for a further 96 h, after which viral replication was measured by p24 antigen capture assay

Fig 4.3.2.2

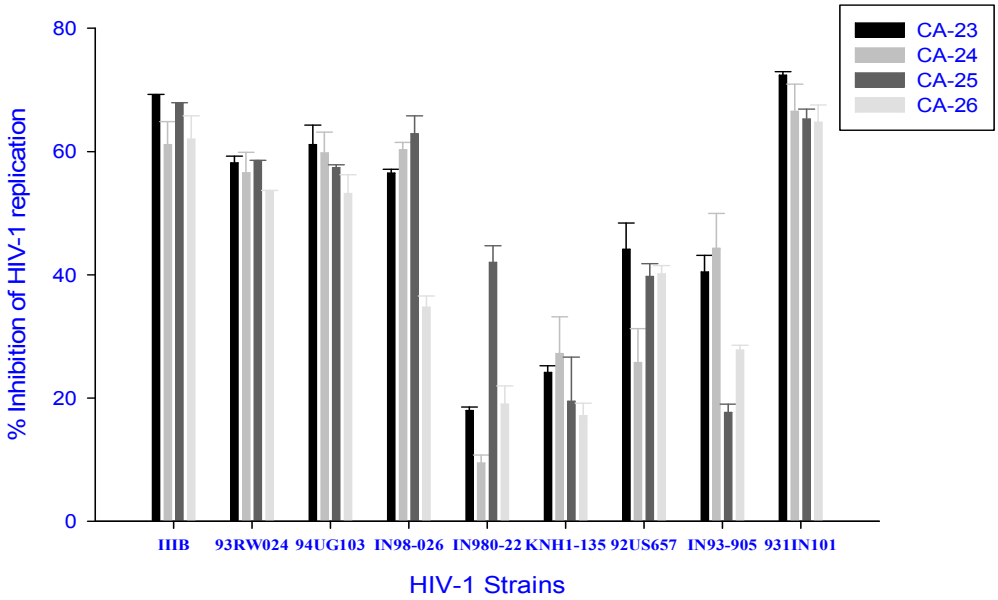


Fig-4.3.2.2 antiHIV-1 activity of nano active curcumin analogues against wide range of HIV-1 strains: SUPT1 cells were challenged for 2 h with different HIV-1 strains (1 ng p24/ml) in the presence their respective IC-50 concentrations of nano curcumin analogues (CA-23, 24, 25, 26). They were then incubated for a further 96 h, after which viral replication was measured by p24 antigen capture assay

Discussion

Twenty eight constrained curcumin structural analogs with various functional groups attached to their aromatic rings were analyzed for their anti HIV-1 activity in SupT1 cells. Cytotoxicity studies show curcumin and its novel analogs have less cytotoxicity at lower concentrations(10 μM) however at higher concentrations (100 μM) some compounds including curcumin exhibits moderate toxic behavior but were significantly different among each other.

Among 28 molecules screened for anti HIV-1 activity in SupT1 cells using 93IN101 HIV-1 Indian isolate, we found CA23, CA24, CA25 and CA26 were active and showed excellent antiHIV-1 activity than curcumin. The IC-50 values of CA23, CA24, CA25 and CA26 were found to be 1.48, 1.6, 1.1 and 1.43μM respectively where as the IC-50 of curcumin was 4.8 μM which indicates strong anti HIV-1 activity of active curcumin analogues than curcumin.

The broad spectrum antiHIV-1 activity of CA23, CA24, CA25 and CA26 shows all the active **curcumin analogues** displays strongest antiviral activity against HIV-IIIB, 93RW024, 94UG103, IN98026, 93IN101HIV-1 and CA23, CA25 and CA26 were effective against 92US657 and CA25 showed inhibitory activity against IN980-22 HIV-1 strains. This broad spectrum HIV-1 neutralization activity of curcumin analogues suggests their strong potency against HIV-1.

in summary, several curcumin analogues were synthesized from curcumin among CA23, CA24, CA25 and CA26 were found to have strong inhibitory activity against several globally circulating HIV-1isolates in SupT1 cells and this suggest their excellent anti HIV-1activity than curcumin and this molecules provide exciting possibilities of developing curcumin analogs selective towards anti-HIV-1 therapeutics giving hope of finding use of Curcumin or its analogs in the clinics for

the prevention or treatment of HIV-1. Further investigation is warranted to test anti HIV-1 activity in more detail in different cell types and with more isolates of HIV-1.

CHAPTER V:

Characterization of inhibitory activity anti HIV active curcumin analogues against reverse transcriptase, protease and integrase.

5.1 Introduction

HIV-1 life cycle comprises of several steps, which can be targeted by anti-HIV-1 drugs. These steps include entry, reverse transcription, integration, transcription, assembly and budding. In the first step the virus enters a cell and the virion is gets uncoated to liberate reverse transcription complex from the virion core into the cytoplasm. The viral RNA is reverse-transcribed to DNA by reverse transcriptase (RT) which is encoded by virus. Once the reverse transcription is complete the proviral DNA translocates in to the cell nucleus and there it is integrated into the genetic material of the cell by a second virally encoded enzyme called the integrase (IN).After successful integration of viral DNA in to the host DNA the activation of the host cell takes place that results in the transcription of the viral DNA into messenger RNA which is then translated into viral poly-proteins. The HIV protease (PR) the third virally encoded enzyme is required in this step to cleave a viral poly-protein precursor into individual mature proteins. For development of novel drug candidates against the HIV-1 understanding the chemical mechanism of these HIV-1 enzymes is very important. Extensive research on curcumin past several years has indicated that it has therapeutic potential against a wide range of diseases including HIV-1.The pleiotropic activities of curcumin were attributed from its complex molecular structure and chemistry as well as its potential to influence several signaling molecules.

The molecular effect of curcumin on HIV-1 was understood using several in-vitro and molecular docking studies the results suggest that the curcumin was shown to have a potential inhibitory effect on viral enzymes integrase (IN) and protease (PR).In another study the inhibitory affect of curcumin was reported against

purified HIV-1 IN, these results indicates the curcumin has potential in interacting with the catalytic core of the IN enzyme (Mazumder et al, 1994)

In the objective II it was demonstrated that four analogues of curcumin viz.CA-23, 24, 25, and 26 molecules showed higher activity against HIV-1 compared to curcumin. To understand the molecular targets of these active curcumin analogues in HIV-1 life cycle and their inhibitory effect on HIV-1 RT, integrase, and Protease, has been studied in this objective.

Results:

5.2Time-dependent drug addition kinetics of active curcumin analogues

To understand the effect of curcumin analogues on HIV-1 and the time in the HIV-1 life cycle at which the active curcumin analogues show plays their inhibitory activity, time-of-drug addition assay was performed in SupT1 cells by infecting HIV-$1_{93IN101}$ strain. The active curcumin analogues were added at different time points during 0 to 72 hour time course of HIV-1 infection in SupT1 cells. The reciprocal time frame associated with each retroviral step is elucidated based on the time period of sensitivity to the drug that inhibits that particular replication step of HIV-1. The inhibition by a particular drug is absent after the completion of the respective step known to be a target of that drug. For instance AZT blocks HIV-1 replication by inhibiting the Reverse transcriptase activity, hence inhibition of HIV-1 by AZT is not found if the drug is added after 5 h of infection. The HIV-1 replication was quantified irrespective of the time of drug addition by P-24 assay after 96 h of post infection. The inhibitory activity of drugs was assessed by employing AZT, Raltegravir and saquinavir the inhibitors of RT, Integrase and Protease of HIV-1 respectively as positive controls.

The first 10 hours of the timed drug addition was augmented to examine the inhibition of reverse transcription and integration of HIV-1 and the result showed all the active curcumin analogues exhibited 75-80% of inhibition when added up to 5hrs of time point, then 60-65% was found to be present when added during 5 to 10 hr, whereas only 40-50% inhibitory activity was exhibited when added from 10 hours to 72 hr of time point(fig5.2).While curcumin showed 50% of inhibition up to 10 hr of time point after that it was decreased to below 30% and this activity was

found to be up to 60 hr of time point suggesting active curcumin analogues poses strong HIV-1inhibitory activity against early replication events of HIV-1than curcumin. All the curcumin analogues showed similar inhibitory activity to that of positive controls of HIV-1 RT, Integrase and protease suggesting their ability to inhibit HIV-1 effectively.

Fig 5.1

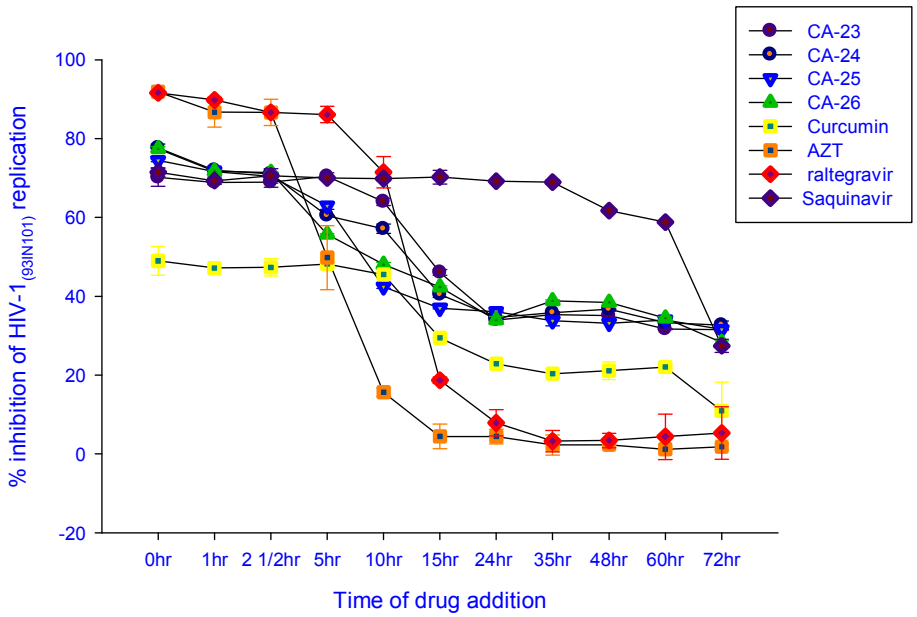


Fig 5.1 time-dependent drug addition kinetics of active curcumin analogues: Active curcumin analogues CA-23, 24, 25, and 26 were added at different time intervals of HIV-1 life cycle during 72 h time course of HIV-1 infection in SupT1 cells. The first 10 hours of the time drug addition was magnified to examine the inhibition of HIV-1 reverse transcription and integration process.AZT, raltegravir and saquinavir was employed as positive controls for HIV-1 RT, Integrase and Protease respectively. After 96 hr of post infection of HIV-1 in all samples the virus production was measured by P-24 assay.

5.2.: Docking of curcumin derivatives with HIV-1reverse transcriptase, integrase and protease

The time of drug addition experiment results showed that all the active curcumin analogues were effective at all stages of viral replication this indicates that the active analogues could inhibits HIV-1 replication by affecting reverse transcription, integration and viral protein synthesis. Hence, further we performed *in silico* docking studies to understand molecular effect on HIV-1 enzyme proteins. Docking studies of curcumin and active analogues were performed by binding to distinct binding motif on HIV-1 RT, IN and Proteases of HIV-1 by using GOLD software as mentioned in methodology.The results indicate that all the analogs designed show higher binding affinity to all the three targets in comparison with curcumin (fig: 5.2.1-3). It could be owing to the substitutions on the ring and the respective interactions. For the reverse transcriptase (fig: 3.2.1) the presence of an electronegative group decreases the affinity for binding resulting in lower fitness score. Presence of carbonyl groups increases the affinity as in the molecule CA-23 resulting in strong hydrogen bond interactions, the absence of which reduced the fitness scores slightly as in molecule curcumin. In case of Integrase(fig:5.2.2) the affinity profile owing to the substitutions is similar to that of reverse transcriptase, however presence of carbonyl groups rendered the molecules with lesser affinity when compared the one without it as in the case of molecule CA-25. In protease (fig 5.2.3) the analogs were seen to possess very good affinity and the site of binding was as in the case of the existing drug Saqueonavir. The substituent's were seen to be contributing to the hydrogen bonding and presence of aromatic amino acids at the binding pocket also ensures π-cation interactions resulting in high

binding affinity. A slight decrease in comparison with other molecules was seen in case of molecule CA-25 which could be due to the conformation of the molecule resulting the formation of a weak hydrogen bond.

Fig 5.2.1

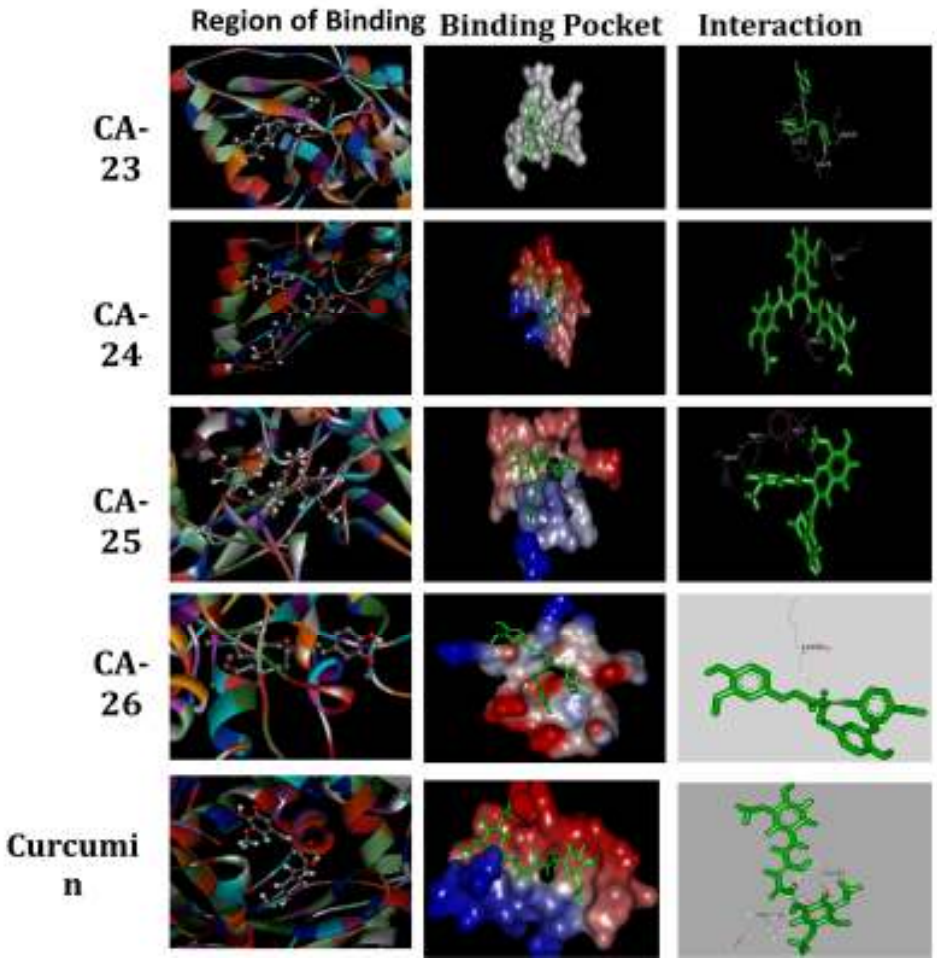


Fig 5.2.1 Docking studies of curcumin analogues with HIV-1 reverse transcriptase (RT) : Docking studies were performed using GOLD, The HIV-1 RT protein secondary structure coordinates were taken from RCSB PDB (2ykm). Docking was performed as mentioned in methodology; GOLD was used by a GoldScore fitness function.

Fig 5.3.2

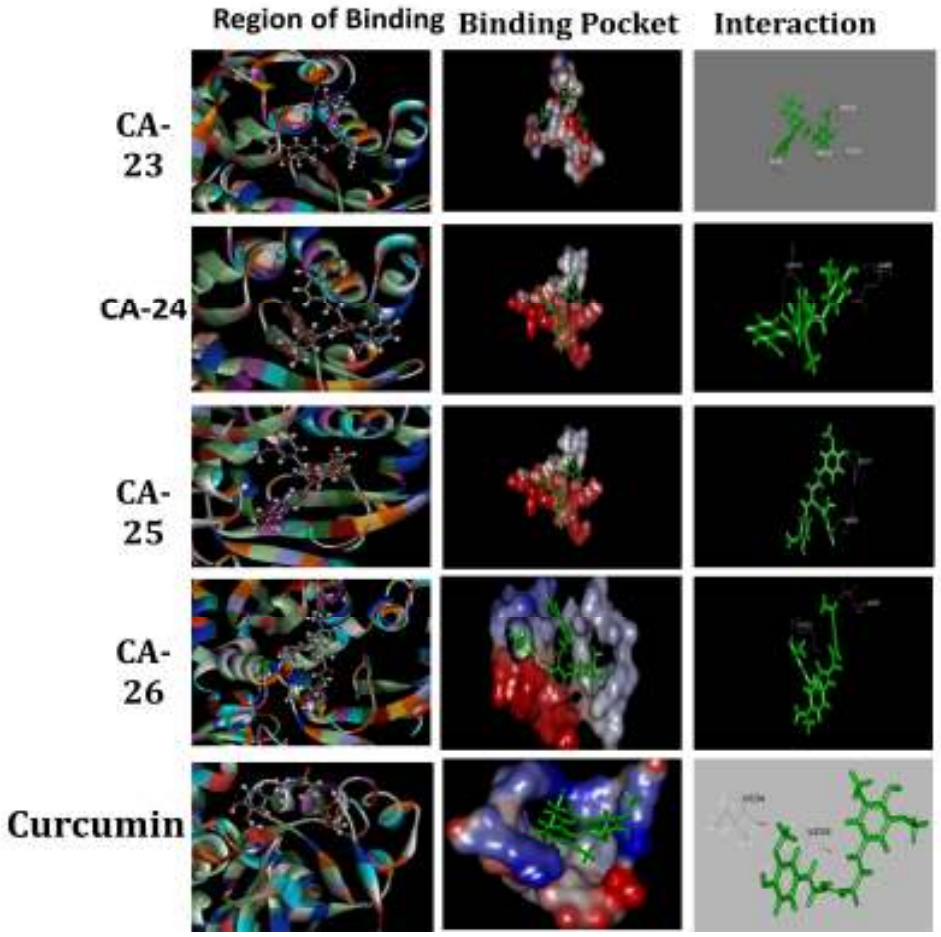


Fig 5.3.2 Docking studies of curcumin analogues with HIV-1 Integrase (IN) : Docking studies were performed using GOLD, The HIV-1 integrase protein secondary structure coordinates were taken from RCSB PDB (1EX4). Docking was performed as mentioned in methodology; GOLD was used by a Gold Score fitness function.

Fig 5.2.3

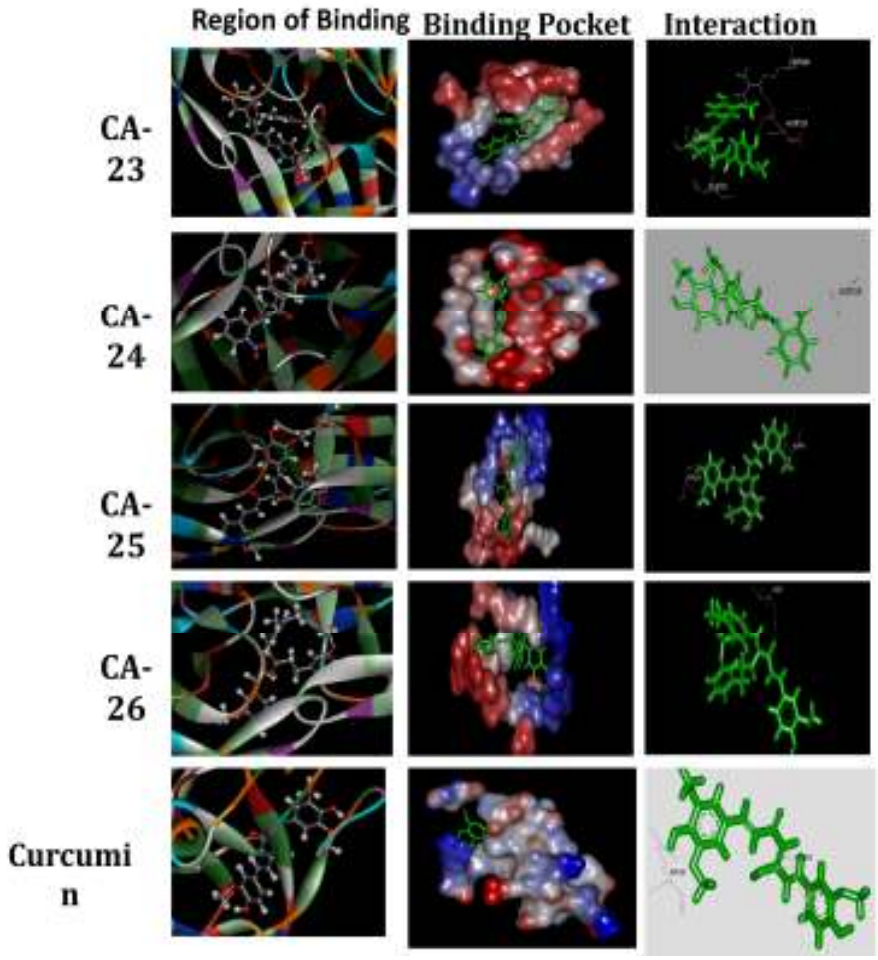


Fig 5.2.3 Docking studies of curcumin analogues with HIV-1 protease (PR): Docking studies were performed using GOLD, The HIV-1 protease protein secondary structure coordinates were taken from RCSB PDB (3WSJ). Docking was performed as mentioned in methodology; GOLD was used by a GoldScore fitness function.

Table: 5.1

	CA-23	CA-24	CA-25	CA-26	Curcumin
Reverse Transcriptase	**56.15**	**35.97**	**42.59**	**41.08**	**32.51**
Integrase	**43.22**	**41.66**	**46.97**	**39.32**	**36.57**
Protease	**50.53**	**48.85**	**45.81**	**46.16**	**43.55**

Gold fitness: The binding affinity of curcumin and its analogs of the curcumin and curcumin analogues with 1)Reverse Transcriptase(RT) 2)Integrase 3)Protease, The analogs designed show higher binding affinity to all the three targets in comparison with curcumin.

5.3: In-vitro analysis of effect active curcumin analogues on HIV-1 reverse transcriptase, integrase and protease enzymes

The time of drug addition and Insilco docking results suggest that the active curcumin analogues blocks the HIV-1 replication by inhibiting all stages of HIV-1 life cycle, and forming stable interactions with HIV-1 RT, integrase and protease than curcumin, Hence, to understand the effect of active curcumin analogues on HIV-1 RT, Integrase and Protease, further we performed in vitro enzymatic assays using commercially available kits for HIV-1 RT, integrase and protease.

The activity of HIV reverse transcriptase in presence of active curcumin analogues CA-23, 24, 25 and 26 results indicates that the CA-23, 24, and 26 was showed 40% of inhibitory activity of at 5µM concentration where as CA-25 found to have less activity at this concentrations (fig:3.3.1).The HIV-1 integrase results shows 40% inhibition was found in presence of CA-23, 24, 25 at 10µM concentration and CA-26 showed less activity (fig:3.3.2). The HIV-1 protease activity results indicates that CA- 26 found to have 40% inhibition at 5µM concentration where as CA-23,24 showed at this inhibition at 10µM and CA-25 showed have less activity(fig:3.3.3). In all three enzymatic experiments curcumin showed less activity than active curcumin analogues and a dose dependent of inhibition was observed. Nevirapine, $NaNH_3$, saquinovir was employed as control inhibitors for RT, integrase and protease of HIV-1respectively.

Fig 5.3.1:

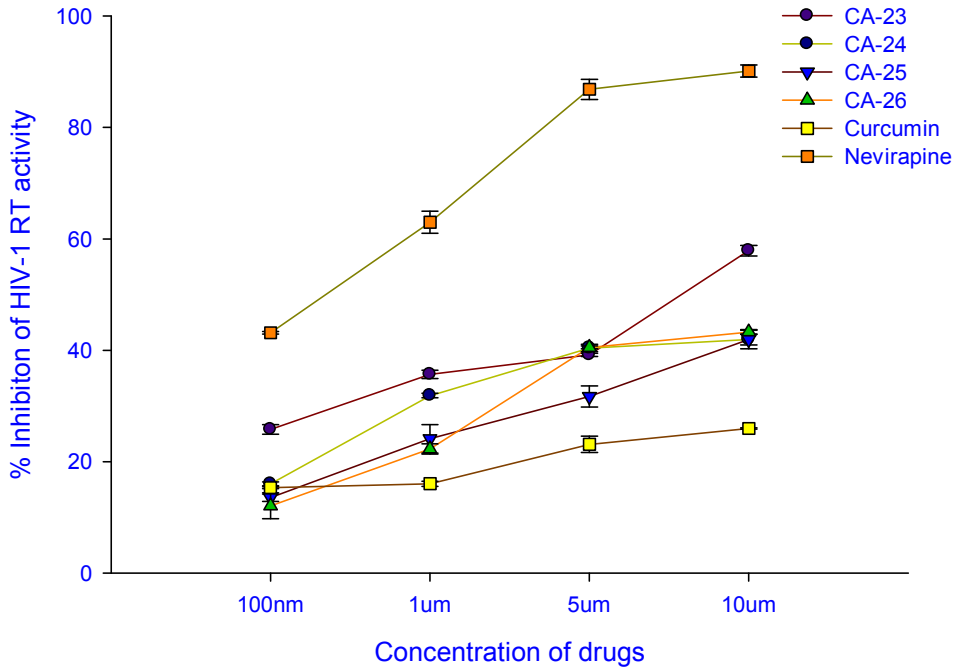


Fig 5.3.1: Inhibitory effect of active analogues on RT: Inhibitory activity of active curcumin analogues CA-23, 24, 25 and 26 on HIV reverse transcriptase. All active molecules shows a dose dependent inhibition in HIV RT activity where as curcumin doesn't shows significant activity on HIV-1 RT and the activity was compared with the reference control nevirapine. Values shown as mean ± SEM of three independent experiments.

Fig 5.3.2:

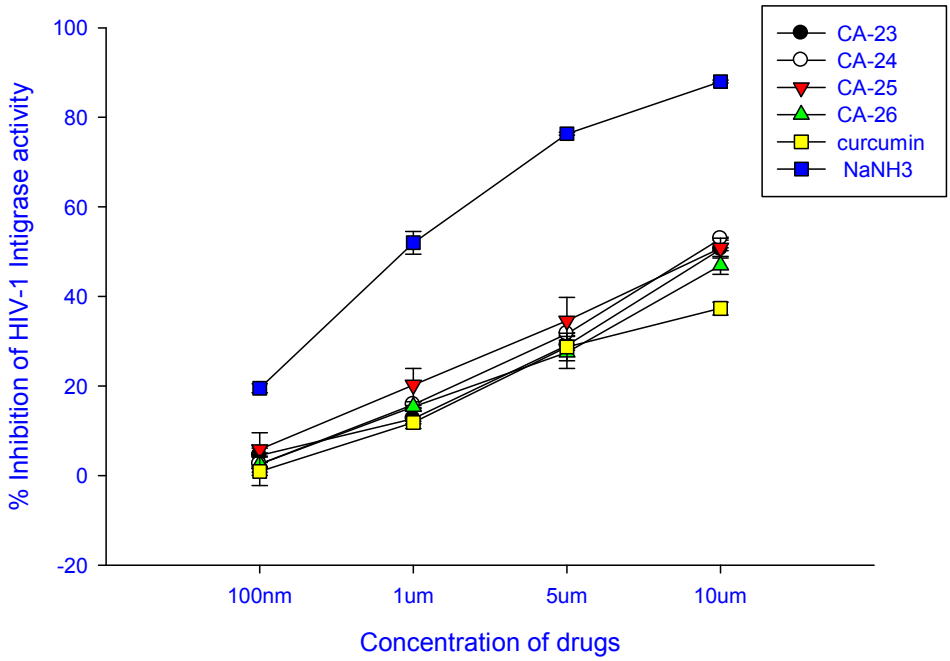


Fig 5.3.2: Inhibitory effect of active analogues on HIV-1 Integrase: Inhibitory activity of active curcumin analogues CA-23, 24, 25 and 26 on HIV-1 Integrase. All active molecules shows a dose dependent inhibition of HIV Integrase and strong inhibitory activity was observed when compared with curcumin and the activity was compared with the reference control $NaNH_3$. Values shown as mean ± SEM of three independent experiments

Fig 5.3.3:

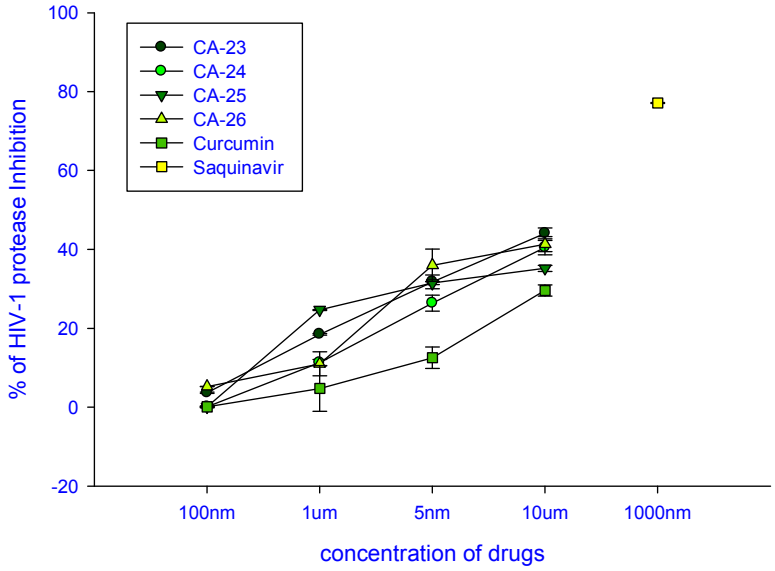


Fig 5.3.3: Inhibitory effect of active analogues on HIV-1 Protease: Inhibitory activity of active curcumin analogues CA-23, 24, 25 and 26 on HIV reverse transcriptase. All active molecules shows a dose dependent inhibition in HIV RT activity where as curcumin doesn't shows significant activity on HIV-1 RT and the activity was compared with the reference control Saquinavir. Values shown as mean ± SEM of three independent experiments

Discussion

The anti-HIV-1 effect of curcumin has been demonstrated in several studies by the molecular docking and several other studies and showed curcumin blocks HIV-1 replication by inhibiting enzymatic action of integrase and proteases. In our earlier study we reported anti HIV-1 activity of curcumin analogues CA23, CA24, CA25 and CA26 against several HIV-1 isolates in SupT1 cells. Here we demonstrated the molecular action of CA23, CA24, CA25 and CA26 on HIV-1 replication using Time-dependent drug addition kinetics experiment, molecular docking and enzymatic experiment of HIV-1 RT, Integrase and Protease

Using AZT, raltagravir and saquinavir the specific inhibitors of RT, integrase and protease of HIV-1 a time-of-drug addition experiment was performed by adding curcumin analogues CA23, CA24, CA25 and CA26 at different time points of HIV-1 replication from zero to 72 hr of time period, 70 % of HIV-1 inhibition was observed when curcumin analogues was added at early time points up to 5hrs; where as 47% of inhibition was observed in presence of curcumin. After 5hr of time point the inhibition was decreased to 45-50% for curcumin analogues and it was retained up to 72 hrs times point where as for curcumin the activity was decreased to 25-30% and this was found to be up to 60 hrs of time point only. This suggest strong anti HIV-1 activity of curcumin analogues than curcumin; Since curcumin analogues blocks viral replication up to 5hrs effectively with a time course that closely resembled AZT a drug which prevents reverse transcription of HIV-1 sRNA to dsDNA by selectively inhibiting the HIV-1 reverse transcriptase enzyme [43] and raltagravir that inhibits integration of viral proviral DNA in the host genome. After that the inhibition was decreased and this activity closely resembled to saquinavir a protease inhibitor, these results suggesting that the active curcumin analogues could

have effect on HIV-1 by inhibiting enzymatic action of HIV-1 Reverse transcriptase, Integrase and Protease which are crucial for viral replication. To understand more about the effect of curcumin analogues on these HIV-1 proteins we performed molecular docking studies and the results showed that the all the CAs interacting with RT, IN and PR of HIV-1 and the docking score found to be higher when compared to curcumin. The enzymatic activity of HIV-1 RT, IN and PR in presence of curcumin and curcumin analogues have demonstrated that curcumin analogues could able to inhibit the enzymatic action of all the three HIV-1 proteins and nearly 40% of inhibition was observed at 5 μM concentration. Whereas curcumin showed 15-25% of inhibition on HIV-1 Integrase and Protease .These results indicates that HIV-1 RT, Integrase and Protease enzymes are some of the targets for curcumin analoguesCA23, CA24, CA25 and CA26that contribute to their inhibition to HIV-1 replication. Further studies are required to identify other targets contribute to their strong antiHIV-1 activity over curcumin.

Conclusions

Conclusion of study

Current strategy for clinical management of HIV-1infected patients depends heavily on life-long antiretroviral therapy (ART)/ called Highly Active Anti-Retroviral Therapy (HAART).HAART regimens have become the standard of care for AIDS patients in developed countries (Palella et al, 1998; Matthews et al, 2004). This therapy increased the life expectancy for AIDS patient and established much more effective than monotherapies. Even though HAART is effective in many cases but has several limitations. It has provided clinical improvement in majority of the patients but fails to eradicate the virus from treated patients (Saag et al, 1999). The prolonged usage of ARV drugs leads to cytotoxicity, emergence of drug resistant variants, and weak immune responses towards viral antigens.Therefore additional therapeutic approaches and novel drugs targeting HIV-1 infection are of utmost importance.

The natural product curcumin has showed multiple therapeutic usages including anti-HIV-1 activity, even though it is potent molecule but its clinical usefulness was limited because of low bioavailability, rapid systemic eliminations and rapid degradation etc.. Therefore we aim to improve the bioavailability of curcumin using apotransferrin nanoparticles drug delivery system, which works through transferrin receptor mediated endocytosis.

In the objective one we have encapsulated the curcumin in apotransferrin nanoparticles and prepared apo-nanocurcumin formulation and performed cell culture assays in order to address the major limitations of curcumin. The nano-curcumin showed enhanced bioavailability than soluble curcumin and greater retention time of curcumin was observed when delivered in nanoformulation in Sup T1, U937 and VK2 vaginalthan soluble curcumin. Further studies showed that, despite greater intracellular concentrations, nano-curcumin exhibited much less cytotoxicity than equivalent doses of sol-curcumin. Nano-curcumin show

significant improvement in anti-HIV-1 activity over soluble curcumin against spectrum of virus strains.

In the second objective, various curcumin derivatives were evaluated for antiviral activity, and identified four anaologues viz.,CA-23, 24, 25 and 26 with higher anti HIV-1 activity compared to curcumin against a spectrum of HIV-1 strains.Further, in enhance bioavailability of these four molecules, apotransferrin nanoparticles formulations were preparedusing four molecules by encapsulating active curcumin analogues and studied their antiviral activity. The results showed an improved antiviral activity than soluble drugs.

In objective 3, a comprehensive analysis was performed to study molecular activity of four active curcumin analogues during HIV-1 replication events. The results of time of drug addition studies showed that the curcumin analogues are active when they are added at all post-infection time pointsduring HIV-1 replication. Analysis of interaction of analogues to HIV-1 coded enzymes using docking suggested that**the active curcumin analogues**are interacting with the binding sites of HIV-1 reverse transcriptase, integrase and proteaseand found to have higher binding affinity than curcumin. These results were confirmed by assessment of inhibitory activity of these analogues on the enzymatic activity four enzyme. The results of these studies indeed confirmed that the active curcumin analogues were effective in inhibiting the enzymatic activity of HIV-1 reverse transcriptase, integrase and protease when compared with curcumin.

In summary, this study identified four active analogues of curcumin that have affinity to three enzymes coded by HIV viz. reverse transcriptase, integrase and protease. Further, an apotransferrin nanoparticle based delivery system has been developed for enhanced bioavailability with decreased toxicity.

References

Alimonti, J. B., T. B. Ball, and K. R. Fowke. 2003. Mechanisms of CD4+ T lymphocyte cell death in human immunodeficiency virus infection and AIDS. J. Gen. Virol. 84: 1649-1661.

Alkhatib, G., C. Combadiere, C. C. Broder, Y. Feng, P. E. Kennedy, P. M. Murphy, and E. A. Berger. 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science 272: 1955-1958.

Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB (2007) Bioavailability of curcumin: problems and promises. Mol Pharm 4: 807–818.

Babu PS, Srinivasan K (1997) Hypolipidemic action of curcumin, the active principle of turmeric (Curcuma longa) in streptozotocin induced diabetic rats. Mol Cell Biochem 166: 169–175.

Baier, M., A. Werner, N. Bannert, K. Metzner, and R. Kurth. 1995. HIV suppression by interleukin-16. Nature 378: 563.

Banda, N. K., J. Bernier, D. K. Kurahara, R. Kurrle, N. Haigwood, R. P. Sekaly, and T. H. Finkel. 1992. Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. J. Exp. Med. 176: 1099-1106.

Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier. 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 220: 868-871.

Barthelemy S, Vergnes L, Moynier M, Guyot D, Labidalle S, et al. (1998) Curcumin and curcumin derivatives inhibit Tat-mediated transactivation of type 1 human immunodeficiency virus long terminal repeat. Res Virol 149: 43–52.

Benson, C. A. 2006. Structured treatment interruptions--new findings. Top. HIV.Med. 14: 107-111.

Berson, J. F., D. Long, B. J. Doranz, J. Rucker, F. R. Jirik, and R. W. Doms. 1996. A seven-transmembrane domain receptor involved in fusion and entry of T-cell-tropic human immunodeficiency virus type 1 strains. J. Virol. 70: 6288-6295.

Billich, A. 2003. S-1360 Shionogi-GlaxoSmithKline. Curr. Opin. Investig. Drugs 4: 206-209.

Bisht S, Feldmann G, Soni S, Ravi R, Karikar C, et al. (2007) Polymeric

nanoparticle-encapsulated curcumin (‘‘nanocurcumin’’): a novel strategy for human cancer therapy. J Nanobiotech 5: 3.

Bouille´ P, Subra F, Mouscadet JF, Auclair C (1999) Antisense-mediated repression of DNA topoisomerase II expression leads to an impairment of HIV-1 replicative cycle. J Mol Biol 285: 945–954.

Boyd, M. R., K. R. Gustafson, J. B. Mc Mahon, R. H. Shoemaker, B. R. O'Keefe, T. Mori, R. J. Gulakowski, L. Wu, M. I. Rivera, C. M. Laurencot, M. J. Currens, J. H. Cardellina, R. W. Buckheit, Jr., P. L. Nara, L. K. Pannell, R. C. Sowder, and L. E. Henderson. 1997. Discovery of cyanovirin-N, a novel human immunodeficiency virus-inactivating protein that binds viral surface envelope glycoprotein gp120: potential applications to microbicide development. Antimicrob. Agents Chemother. 41: 1521-1530.

Brenchley, J. M., T. W. Schacker, L. E. Ruff, D. A. Price, J. H. Taylor, G. J. Beilman, P. L. Nguyen, A. Khoruts, M. Larson, A. T. Haase, and D. C. Douek. 2004. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. J. Exp. Med. 200:749-759.

Brinkman, K., J. A. Smeitink, J. A. Romijn, and P. Reiss. 1999. Mitochondrial toxicity induced by nucleoside-analogue reverse-transcriptase inhibitors is a key factor in the pathogenesis of antiretroviral-therapy-related lipodystrophy. Lancet 354: 1112-1115.

Brinkman, K., S. Vrouenraets, R. Kauffmann, H. Weigel, and J. Frissen. 2000. Treatment of nucleoside reverse transcriptase inhibitor-induced lactic acidosis. AIDS 14: 2801-2802.

Brother, M. B., H. K. Chang, J. Lisziewicz, D. Su, L. C. Murty, and B. Ensoli. 1996. Block of Tat-mediated transactivation of tumor necrosis factor beta gene expression by polymeric-TAR decoys. Virology 222: 252-256.

Bryant, M. and L. Ratner. 1990. Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. Proc. Natl. Acad. Sci. U.S.A. 87: 523-527.

Buckheit, R. W., Jr., E. L. White, V. Fliakas-Boltz, J. Russell, T. L. Stup, T. L. Kinjerski, M. C. Osterling, A. Weigand, and J. P. Bader. 1999. Unique anti-human immunodeficiency virus activities of the nonnucleoside reverse transcriptase inhibitors calanolide A, costatolide, and dihydrocostatolide. Antimicrob. Agents Chemother. 43: 1827-1834.

Bukrinsky, M. I., S. Haggerty, M. P. Dempsey, N. Sharova, A. Adzhubel, L. Spitz, P. Lewis, D. Goldfarb, M. Emerman, and M. Stevenson. 1993. A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. Nature 365: 666-669.

Butler, M. S. 2005. Natural products to drugs: natural product derived compounds in clinical trials. Nat. Prod. Rep. 22: 162-195.

Camaur, D. and D. Trono. 1996. Characterization of human immunodeficiency virus type 1 Vif particle incorporation. J. Virol. 70: 6106-6111.

Camerini, D. and B. Seed. 1990. A CD4 domain important for HIV-mediated syncytium formation lies outside the virus binding site. Cell 60: 747-754.

Chakravarti, R. and V. Sahai. 2004. Compactin- a review. Appl. Microbiol. Biotechnol. 64: 618-624.

Chan, D. C., D. Fass, J. M. Berger, and P. S. Kim. 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell 89: 263-273.

Chan, D. C, and P. S. Kim. 1998. HIV entry and its inhibition. Cell 93:681-684. Chen, H. and A. Engelman. 1998. The barrier-to-autointegration protein is a host factor for HIV type 1 integration. Proc. Natl. Acad. Sci. U.S.A. 95: 15270-15274.

Chapman, T. M, and C. M. Perry. 2004. Everolimus. Drugs 64:861-872.

Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, et al. (2001) Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res 21: 2895–2900.

Cherepanov, P., G. Maertens, P. Proost, B. Devreese, J. Van Beeumen, Y. Engelborghs, E. De Clercq, and Z. Debyser. 2003. HIV-1 integrase forms stable tetramers and associates with LEDGF/p75 protein in human cells. J. Biol. Chem. 278: 372-381.

Chin, Y. W., M. J. Balunas, H. B. Chai, and A. D. Kinghorn. 2006. Drug discovery from natural sources. AAPS. J. 8: E239-E253.

Choe, H., M. Farzan, Y. Sun, N. Sullivan, B. Rollins, P. D. Ponath, L. Wu, C. R. Mackay, G. LaRosa, W. Newman, N. Gerard, C. Gerard, and J. Sodroski. 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell 85: 1135-1148.

Chokekijchai, S., E. Kojima, S. Anderson, M. Nomizu, M. Tanaka, M. Machida, T. Date, K. Toyota, S. Ishida, and K. Watanabe. 1995. NP-06: a novel anti-human immunodeficiency virus polypeptide produced by a Streptomyces species. Antimicrob. Agents Chemother. 39: 2345-2347.

Chun, T. W., R. T. Davey, Jr., D. Engel, H. C. Lane, and A. S. Fauci. 1999. Re-emergence of HIV after stopping therapy. Nature 401: 874-875.

Chun, T. W., D. Engel, M. M. Berrey, T. Shea, L. Corey, and A. S. Fauci. 1998. Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. Proc. Natl. Acad. Sci. U.S.A. 95: 8869-8873.

Chun, T. W., D. Engel, S. B. Mizell, C. W. Hallahan, M. Fischette, S. Park, R. T. Davey, Jr., M. Dybul, J. A. Kovacs, J. A. Metcalf, J. M. Mican, M. M. Berrey, L. Corey, H. C. Lane, and A. S. Fauci. 1999. Effect of interleukin-2 on the pool oflatently infected, resting CD4+ T cells in HIV-1-infected patients receiving highly active anti-retroviral therapy. Nat. Med. 5: 651-655.

Ciuffi, A., M. Llano, E. Poeschla, C. Hoffmann, J. Leipzig, P. Shinn, J. R. Ecker, and F. Bushman. 2005. A role for LEDGF/p75 in targeting HIV DNA integration. Nat. Med. 11: 1287-1289.

Cirla, A. and J. Mann. 2003. Combretastatins: from natural products to drug discovery. Nat. Prod. Rep. 20: 558-564.

Clavel, F., D. Guetard, F. Brun-Vezinet, S. Chamaret, M. A. Rey, M. O. Santos-Ferreira, A. G. Laurent, C. Dauguet, C. Katlama, C, and Rouzioux. 1986. Isolation of a new human retrovirus from West African patients with AIDS. Science 233: 343-346.*

Cocchi, F., A. L. DeVico, A. Garzino-Demo, A. Cara, R. C. Gallo, and P. Lusso. 1996. The V3 domain of the HIV-1 gp120 envelope glycoprotein is critical for chemokine-mediated blockade of infection. Nat. Med. 2: 1244-1247.

Coffin, J., A. Haase, J. A. Levy, L. Montagnier, S. Oroszlan, N. Teich, H. Temin, K. Toyoshima, H. Varmus, and P. Vogt. 1986. Human immunodeficiency viruses. Science 232: 697.

Cohen, E. A., G. Dehni, J. G. Sodroski, and W. A. Haseltine. 1990. Human immunodeficiency virus vpr product is a virion-associated regulatory protein. J. Virol. 64: 3097-3099.

Cohen, J. 1998. Exploring how to get at and eradicate hidden HIV. Science 279: 1854-1855.

Condra, J. H., W. A. Schleif, O. M. Blahy, L. J. Gabryelski, D. J. Graham, J. C. Quintero, A. Rhodes, H. L. Robbins, E. Roth, and M. Shivaprakash. 1995. In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature 374: 569-571.

Cos, P., L. Maes, D. Vanden Berghe, N. Hermans, L. Pieters, and A. Vlietinck. 2004. Plant substances as anti-HIV agents selected according to their putative mechanism of action. J. Nat. Prod. 67: 284-293.

Cullen, B. R. 1998. Retroviruses as model systems for the study of nuclear RNA export pathways. Virology 249: 203-210.

Curran, M. P. and G. M. Keating. 2005. Mycophenolate sodium delayed release: prevention of renal transplant rejection. Drugs 65: 799-805.

Dandekar, D. H., K. N. Ganesh, and D. Mitra. 2004. HIV-1 Tat directly binds to NFkappaB enhancer sequence: role in viral and cellular gene expression. Nucleic Acids Res. 32: 1270-1278.

Daniel, R., J. G. Greger, R. A. Katz, K. D. Taganov, X. Wu, J. C. Kappes, and A. M. Skalka. 2004. Evidence that stable retroviral transduction and cell survival following DNA integration depend on components of the nonhomologous end joining repair pathway. J. Virol. 78: 8573-8581.

Darkes, M. J. and G. L. Plosker. 2002. Cefditoren pivoxil. Drugs 62:319-336.

De la Asuncion, J. G., M. L. del Olmo, J. Sastre, A. Millan, A. Pellin, F. V. Pallardo, and J. Vina. 1998. AZT treatment induces molecular and ultrastructural oxidative damage to muscle mitochondria. Prevention by antioxidant vitamins. J. Clin. Invest. 102: 4-9.

Deleu, D., Y. Hanssens, and M. G. Northway. 2004. Subcutaneous apomorphine : an evidence-based review of its use in Parkinson's disease. Drugs Aging 21: 687-709.

De Noronha, C. M., M. P. Sherman, H. W. Lin, M. V. Cavrois, R. D. Moir, R. D. Goldman, and W. C. Greene. 2001. Dynamic disruptions in nuclear envelope architecture and integrity induced by HIV-1 Vpr. Science 294: 1105-1108.

Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. Di Marzio, S. Marmon, R. E. Sutton, C. M. Hill, C. B. Davis, S. C. Peiper, T. J. Schall, D. R. Littman, and N. R. Landau. 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature 381: 661-666.

Deodhar SD, Sethi R, Srimal RC (1980) Preliminary study on antirheumatic activity of curcumin (diferuloyl methane). Ind J Med Res 71: 632–634.

Deveaud C, Beauvoit B, Hagry S, Galinier A, Carriere A, et al. (2005) Site specific alterations of adipose tissue mitochondria in 39-azido-39-deoxythymidine (AZT)-treated rats: An early stage in lipodystrophy. Biochem Pharmacol 70: 90–101.

Dewick PM. *Medicinal Natural Products: A Biosynthetic Approach.* 2nd ed. Chichester, UK: John Wiley & Sons; 2002.

Doms, R. W. and D. Trono. 2000. The plasma membrane as a combat zone in the HIV battlefield. Genes Dev. 14: 2677-2688.

Dragic, T., V. Litwin, G. P. Allaway, S. R. Martin, Y. Huang, K. A. Nagashima, C. Cayanan, P. J. Maddon, R. A. Koup, J. P. Moore, and W. A. Paxton. 1996. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature 381: 667-673.

Eich, E., H. Pertz, M. Kaloga, J. Schulz, M. R. Fesen, A. Mazumder, and Y. Pommier. 1996. (-)-Arctigenin as a lead structure for inhibitors of human immunodeficiency virus type-1 integrase. J. Med. Chem. 39: 86-95.

Ensoli, B., G. Barillari, S. Z. Salahuddin, R. C. Gallo, and F. Wong-Staal. 1990. Tat protein of HIV-1 stimulates growth of cells derived from Kaposi's sarcoma lesions of AIDS patients. Nature 345: 84-86.

Fauci, A. S. 1988. The human immunodeficiency virus: infectivity and mechanisms of pathogenesis. Science 239: 617-622.

Feng, Y., C. C. Broder, P. E. Kennedy, and E. A. Berger. 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science 272: 872-877.

Fenton, C., G. M. Keating, and M. P. Curran. 2004. Daptomycin. Drugs 64: 445-455.

Finkel, T. H., G. Tudor-Williams, N. K. Banda, M. F. Cotton, T. Curiel, C. Monks, T. W. Baba, R. M. Ruprecht, and A. Kupfer. 1995. Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV-and SIV-infected lymph nodes. Nat. Med. 1: 129-134.

Finzi, D., J. Blankson, J. D. Siliciano, J. B. Margolick, K. Chadwick, T. Pierson, K. Smith, J. Lisziewicz, F. Lori, C. Flexner, T. C. Quinn, R. E. Chaisson, E. Rosenberg, B. Walker, S. Gange, J. Gallant, and R. F. Siliciano. 1999. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat. Med. 5: 512-517.

Franke, E. K., H. E. Yuan, and J. Luban. 1994. Specific incorporation of cyclophilin A into HIV-1 virions. Nature 372: 359-362.

Frankel, A. D. and J. A. Young. 1998. HIV-1: fifteen proteins and an RNA. Annu. Rev. Biochem. 67: 1-25.

Fujioka, T., Y. Kashiwada, R. E. Kilkuskie, L. M. Cosentino, L. M. Ballas, J. B. Jiang, W. P. Janzen, I. S. Chen, and K. H. Lee. 1994. Anti-AIDS agents, 11. Betulinic acid and platanic acid as anti-HIV principles from Syzigium claviflorum, and the anti-HIV activity of structurally related triterpenoids. J. Nat. Prod. 57: 243-247.

Gallay, P., T. Hope, D. Chin, and D. Trono. 1997. HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. Proc. Natl. Acad. Sci. U.S.A. 94: 9825-9830.

Gallay, P., S. Swingler, J. Song, F. Bushman, and D. Trono. 1995. HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. Cell 83: 569-576.

Gallo, R. C., S. Z. Salahuddin, M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Palker, R. Redfield, J. Oleske and B. Safai. 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science 224: 500-503.

Garber, M. E., T. P. Mayall, E. M. Suess, J. Meisenhelder, N. E. Thompson, and K. A. Jones. 2000. CDK9 autophosphorylation regulates high-affinity binding of the human immunodeficiency virus type 1 tat-P-TEFb complex to TAR RNA. Mol. Cell Biol. 20: 6958-6969.

Garcea G, Jones DJ, Singh R, Dennison AR, Farmer PB, et al. (2004) Detection of curcumin and its metabolites in hepatic tissue and portal blood of patients following oral administration. Br J Cancer 90: 1011–1015.

Geijtenbeek, T. B., D. S. Kwon, R. Torensma, S. J. van Vliet, G. C. van Duijnhoven, J. Middel, I. L. Cornelissen, H. S. Nottet, V. N. KewalRamani, D. R. Littman, C. G. Figdor, and Y. van Kooyk. 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell 100: 587-597.

Giles, F., E. Estey, and S. O'Brien. 2003. Gemtuzumab ozogamicin in the treatment of acute myeloid leukemia. Cancer 98: 2095-2104.

Goh, W. C., M. E. Rogel, C. M. Kinsey, S. F. Michael, P. N. Fultz, M. A. Nowak, B. H. Hahn, and M. Emerman. 1998. HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo. Nat. Med. 4: 65-71.

Gonzalez, M. E. and L. Carrasco. 2001. Human immunodeficiency virus type 1 VPU protein affects Sindbis virus glycoprotein processing and enhances membrane permeabilization. Virology 279: 201-209.

Gottlieb, M. S., R. Schroff, H. M. Schanker, J. D. Weisman, P. T. Fan, R. A. Wolf,

and A. Saxon. 1981. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. N. Engl. J. Med. 305: 1425-1431.

Greene, W. C. and B. M. Peterlin. 2002. Charting HIV's remarkable voyage through the cell: Basic science as a passport to future therapy. Nat. Med. 8: 673-680.

Gulick, R. M. 2003. New antiretroviral drugs. Clin. Microbiol. Infect. 9: 186-193.

Han, Y., M. Wind-Rotolo, H. C. Yang, J. D. Siliciano, and R. F. Siliciano. 2007. Experimental approaches to the study of HIV-1 latency. Nat. Rev. Microbiol. 5: 95-106.

Harouse, J. M., S. Bhat, S. L. Spitalnik, M. Laughlin, K. Stefano, D. H. Silberberg, and F. Gonzalez-Scarano. 1991. Inhibition of entry of HIV-1 in neural cell lines by antibodies against galactosyl ceramide. Science 253: 320-323.

Harris, R. S. and M. T. Liddament. 2004. Retroviral restriction by APOBEC proteins. Nat. Rev. Immunol. 4: 868-877.

Harrison, G. P. and A. M. Lever. 1992. The human immunodeficiency virus type 1 packaging signal and major splice donor region have a conserved stable secondary structure. J. Virol. 66: 4144-4153.

Hatziioannou, T., D. Perez-Caballero, A. Yang, S. Cowan, and P. D. Bieniasz. 2004. Retrovirus resistance factors Ref1 and Lv1 are species-specific variants of TRIM5alpha. Proc. Natl. Acad. Sci. U.S.A. 101: 10774-10779.

Heinzinger, N. K., M. I. Bukinsky, S. A. Haggerty, A. M. Ragland, V. Kewalramani, M. A. Lee, H. E. Gendelman, L. Ratner, M. Stevenson, and M. Emerman. 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc. Natl. Acad. Sci. U.S.A. 91: 7311-7315.

Higuchi, H., K. Mori, A. Kato, T. Ohkuma, T. Endo, H. Kaji, and A. Kaji. 1991. Antiretroviral activities of anthraquinones and their inhibitory effects on reverse transcriptase. Antiviral Res. 15: 205-216.

Ho, D. D. 1995a. Time to hit HIV, early and hard. N. Engl. J. Med. 333: 450-451.

Ho, D. D. 1998. Toward HIV eradication or remission: the tasks ahead. Science 280: 1866-1867.

Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz. 1995b. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 373: 123-126.

Hoff, R. and J. McNamara. 1999. Therapeutic vaccines for preventing AIDS: their use with HAART. Lancet 353: 1723-1724.

Hoglund, S., A. Ohagen, K. Lawrence, and D. Gabuzda. 1994. Role of vif during packing of the core of HIV-1. Virology 201: 349-355.

Howes, M. J., N. S. Perry, and P. J. Houghton. 2003. Plants with traditional uses and activities, relevant to the management of Alzheimer's disease and other cognitive disorders. Phytother. Res. 17: 1-18.

Jacque, J. M. and M. Stevenson. 2006. The inner-nuclear-envelope protein emerin regulates HIV-1 infectivity. Nature 441: 641-645.

Jarvis, B., D. P. Figgitt, and L. J. Scott. 2004. Micafungin. Drugs 64: 969-982.

Johnson, A. A., A. S. Ray, J. Hanes, Z. Suo, J. M. Colacino, K. S. Anderson, and K. A. Johnson. 2001. Toxicity of antiviral nucleoside analogs and the human mitochondrial DNA polymerase. J. Biol. Chem. 276: 40847-40857.

Joseph, A. M., J. S. Ladha, M. Mojamdar, and D. Mitra. 2003. Human immunodeficiency virus-1 Nef protein interacts with Tat and enhances HIV-1 gene expression. FEBS Lett. 548: 37-42.

Jowett, J. B., V. Planelles, B. Poon, N. P. Shah, M. L. Chen, and I. S. Chen. 1995. The human immunodeficiency virus type 1 vpr gene arrests infected T cells in the G2 + M phase of the cell cycle. J. Virol. 69: 6304-6313.

Kalpana, G. V., S. Marmon, W. Wang, G. R. Crabtree, and S. P. Goff. 1994. Binding
and stimulation of HIV-1 integrase by a human homolog of yeas transcription
factor SNF5. Science 266: 2002-2006.

Kamsteeg, M., T. Rutherford, E. Sapi, B. Hanczaruk, S. Shahabi, M. Flick, D. Brown, and G. Mor. 2003. Phenoxodiol, an isoflavone analog induces apoptosis in chemoresistant ovarian cancer cells. Oncogene 22: 2611-2620.

Kao, S. Y., A. F. Calman, P. A. Luciw, and B. M. Peterlin. 1987. Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. Nature 330: 489-493.

Karpas, A., G. W. Fleet, R. A. Dwek, S. Petursson, S. K. Namgoong, N. G. Ramsden, G. S. Jacob, and T. W. Rademacher. 1988. Aminosugar derivatives as potential anti-human immunodeficiency virus agents. Proc. Natl. Acad. Sci. U.S.A. 85: 9229-9233.

Keating, G. M. 2005. Exenatide. Drugs 65: 1681-1692.

Kedei, N., D. J. Lundberg, A. Toth, P. Welburn, S. H. Garfield, and P. M. Blumberg. 2004. Characterization of the interaction of ingenol 3-angelate with protein kinase C. Cancer Res. 64: 3243-3255.

Kim, S. Y., R. Byrn, J. Groopman, and D. Baltimore. 1989. Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. J.Virol. 63: 3708-3713.

Kiso Y, Suzuki Y, Watanabe N, Oshima Y, Hikino H (1983) Antihepatotoxic Principles of Curcuma longa Rhizomes1. Planta Medica 49: 185–187.

Klatzmann, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J. C. Gluckman, and L. Montagnier. 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature 312: 767-768.

Kondapi AK, Satyanarayana N, Saikrishna AD (2006) A study of the topoisomerase II activity in HIV-1 replication using the ferrocene derivatives as probes. Arch Biochem Biophys 450: 123–132.

Korber, B., M. Muldoon, J. Theiler, F. Gao, R. Gupta, A. Lapedes, B. H. Hahn, S. Wolinsky, and T. Bhattacharya. 2000. Timing the ancestor of the HIV-1 pandemic strains. Science 288: 1789-1796.

Koumis, T. and S. Samuel. 2005. Tiotropium bromide: a new long-acting bronchodilator for the treatment of chronic obstructive pulmonary disease. Clin. Ther. 27: 377-392.

Kuiken, C. L., V. V. Lukashov, E. Baan, J. Dekker, J. A. Leunissen, and J. Goudsmit. 1996. Evidence for limited within-person evolution of the V3 domain of the HIV-1 envelope in the amsterdam population. AIDS 10: 31-37.

Kumar, M. and D. Mitra. 2005. Heat shock protein 40 is necessary for human immunodeficiency virus-1 Nef-mediated enhancement of viral gene expression and replication. J. Biol. Chem. 280: 40041-40050.

Kuttan R, Bhanumathy P, Nirmala K, George MC (1985) Potential anticancer activity of turmeric (Curcuma longa). Cancer Lett 29: 197–202.

Kwon, D. S., G. Gregorio, N. Bitton, W. A. Hendrickson, and D. R. Littman. 2002. DC-SIGN-mediated internalization of HIV is required for trans-enhancement of T cell infection. Immunity. 16: 135-144.

Lama, J., A. Mangasarian, and D. Trono. 1999. Cell-surface expression of CD4 reduces HIV-1 infectivity by blocking Env incorporation in a Nef- and Vpu-inhibitable manner. Curr. Biol. 9: 622-631.

Landau, N. R., M. Warton, and D. R. Littman. 1988. The envelope glycoprotein of the human immunodeficiency virus binds to the immunoglobulin-like domain of CD4. Nature 334: 159-162.

Lapadat-Tapolsky, M., H. De Rocquigny, D. Van Gent, B. Roques, R. Plasterk, and J. L. Darlix. 1993. Interactions between HIV-1 nucleocapsid protein and viral DNA may have important functions in the viral life cycle. Nucleic Acids Res. 21: 831-839.

Lee-Huang, S., P. L. Huang, H. C. Chen, P. L. Huang, A. Bourinbaiar, H. I. Huang, and H. F. Kung. 1995. Anti-HIV and anti-tumor activities of recombinant MAP30 from bitter melon. Gene 161: 151-156.

Letvin, N. L. 2006. Progress and obstacles in the development of an AIDS vaccine. Nat. Rev. Immunol. 6: 930-939.

Levy, J. A., A. D. Hoffman, S. M. Kramer, J. A. Landis, J. M. Shimabukuro, and L. S. Oshiro. 1984. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. Science 225: 840-842.

Levy, Y. 2005. Therapeutic HIV vaccines: an update. Curr. HIV/AIDS Rep. 2: 5-9.

Lewis, W. 2000. Cardiomyopathy in AIDS: a pathophysiological perspective. Prog. Cardiovasc. Dis. 43: 151-170.

Lewis, W. and M. C. Dalakas. 1995. Mitochondrial toxicity of antiviral drugs. Nat. Med. 1: 417-422.

Lewis, W., B. J. Day, and W. C. Copeland. 2003. Mitochondrial toxicity of NRTI antiviral drugs: an integrated cellular perspective. Nat. Rev. Drug Discov. 2: 812-822.

Li CJ, Zhang LJ, Dezube BJ, Crumpacker CS, Pardee AB (1993) Three inhibitors of type 1 human immunodeficiency virus long terminal repeat-directed gene expression and virus replication. Proc Natl Acad Sci USA 90: 1839–1842.

Li L, Braiteh FS, Kurzrock R (2005) Liposome-encapsulated curcumin: in vitro and in vivo effects on proliferation, apoptosis, signaling, and angiogenesis. Cancer 104: 1322–1331. *

Lim, S. E. and W. C. Copeland. 2001. Differential incorporation and removal of antiviral deoxynucleotides by human DNA polymerase gamma. J. Biol. Chem. 276: 23616-23623.

Lin, Y. M., H. Anderson, M. T. Flavin, Y. H. Pai, E. Mata-Greenwood, T. Pengsuparp, J. M. Pezzuto, R. F. Schinazi, S. H. Hughes, and F. C. Chen. 1997. In vitro anti-HIV activity of biflavonoids isolated from Rhus

succedanea and Garcinia multiflora. J. Nat. Prod. 60: 884-888.

Little, S. J., S. Holte, J. P. Routy, E. S. Daar, M. Markowitz, A. C. Collier, R. A. Koup, J. W. Mellors, E. Connick, B. Conway, M. Kilby, L. Wang, J. M. Whitcomb, N. S. Hellmann, and D. D. Richman. 2002. Antiretroviral-drug resistance among patients recently infected with HIV. N. Engl. J. Med. 347: 385-394.

Lori, F., M. G. Lewis, J. Xu, G. Varga, D. E. Zinn, Jr., C. Crabbs, W. Wagner, J. Greenhouse, P. Silvera, J. Yalley-Ogunro, C. Tinelli, and J. Lisziewicz. 2000. Control of SIV rebound through structured treatment interruptions during early infection. Science 290: 1591-1593.

Lowe, D. M., A. Aitken, C. Bradley, G. K. Darby, B. A. Larder, K. L. Powell, D. J. Purifoy, M. Tisdale, and D. K. Stammers. 1988. HIV-1 reverse transcriptase: crystallization and analysis of domain structure by limited proteolysis. Biochemistry 27: 8884-8889.

Luster, A. D. 1998. Chemokines, chemotactic cytokines that mediate inflammation. N. Engl. J. Med. 338: 436-445.

Mahady GB, Pendland SL, Yun G, Lu ZZ (2002) Turmeric (Curcuma longa) and curcumin inhibit the growth of Helicobacter pylori, a group 1 carcinogen. Anticancer Res 22: 4179–4181.

Malim, M. H., J. Hauber, S. Y. Le, J. V. Maizel, and B. R. Cullen. 1989. The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature 338: 254-257.

Mandraju RK, Kondapi AK (2007) Regulation of Topoisomerase II a and b in uninfected and HIV-1 infected Neuroblastoma and Astrocytoma cells: Involvement of Distinct nordihydroguaretic acid sensitive inflammatory pathways. Arch Biochem Biophys 461: 40–49.

Martin-Cordero C, Lopez-Lazaro M, Galvez M, Ayuso MJ (2003) Curcumin as a DNA topoisomerase II poison. J Enzyme Inhib Med Chem 18: 505–509.

Martinez-Picado, J., M. P. DePasquale, N. Kartsonis, G. J. Hanna, J. Wong, D. Finzi, E. Rosenberg, H. F. Gunthard, L. Sutton, A. Savara, C. J. Petropoulos, N. Hellmann, B. D. Walker, D. D. Richman, R. Siliciano, and R. T. D'Aquila. 2000. Antiretroviral resistance during successful therapy of HIV type 1 infection. Proc. Natl. Acad. Sci. U.S.A. 97: 10948-10953.

Masur, H., M. A. Michelis, J. B. Greene, I. Onorato, R. A. Stouwe, R. S. Holzman, G. Wormser, L. Brettman, M. Lange, H. W. Murray, and S. Cunningham-Rundles. 1981. An outbreak of community-acquired Pneumocystis carinii pneumonia: initial manifestation of cellular immune dysfunction. N. Engl.

J. Med. 305: 1431-1438.

Matthews, T., M. Salgo, M. Greenberg, J. Chung, R. DeMasi, and D. Bolognesi. 2004. Enfuvirtide: the first therapy to inhibit the entry of HIV-1 into host CD4 lymphocytes. Nat. Rev. Drug Discov. 3: 215-225.

Mazumder A, Raghavan K, Weinstein J, Kohn KW, Pommier Y (1995) Inhibition of human immunodeficiency virus type-1 integrase by curcumin. Biochem Pharmacol 49: 1165–1170.

Mc Cutchan, F. E. 2000. Understanding the genetic diversity of HIV-1. AIDS 14 Suppl 3: S31-S44.

Mc Dougal, J. S., M. S. Kennedy, J. M. Sligh, S. P. Cort, A. Mawle, and J. K. Nicholson. 1986. Binding of HTLV-III/LAV to T4+ T cells by a complex of the 110K viral protein and the T4 molecule. Science 231: 382-385.

Mehandru, S., M. A. Poles, K. Tenner-Racz, A. Horowitz, A. Hurley, C. Hogan, D. Boden, P. Racz, and M. Markowitz. 2004. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. J. Exp. Med. 200: 761-770.

Miranda, L., J. Wolf, S. Pichuantes, R. Duke, and A. Franzusoff. 1996. Isolation of the human PC6 gene encoding the putative host protease for HIV-1 gp160 processing in CD4+ T lymphocytes. Proc. Natl. Acad. Sci. U.S.A. 93: 7695-7700.

Mitchell, G., D. W. Bartlett, T. E. Fraser, T. R. Hawkes, D. C. Holt, J. K. Townson, and R. A. Wichert. 2001. Mesotrione: a new selective herbicide for use in maize. Pest. Manag. Sci. 57: 120-128.

Moore, J. P., P. W. Parren, and D. R. Burton. 2001. Genetic subtypes, humoral immunity, and human immunodeficiency virus type 1 vaccine development. J. Virol. 75: 5721-5729.

Mukhtar, R. Y., J. Reid, and J. P. Reckless. 2005. Pitavastatin. Int. J. Clin. Pract. 59: 239-252

Mhiri C, Baudrimont M, Bonne G, Geny C, Degoul F, et al. (1991) Zidovudine myopathy: a distinctive disorder associated with mitochondrial dysfunction. Annu Neurol 29: 606–614.

Moh R, Danel C, Sorho S, Sauvageot D, Anzian A, et al. (2005) Haematological changes in adults recieving a zidovudine-containing HAART regimen in combination with cotrimoxazole in Cote d'lvoire. Antivir Ther 10: 615–624.

Nakamura, M., S. Kunimoto, Y. Takahashi, H. Naganawa, M. Sakaue, S. Inoue, T. Ohno, and T. Takeuchi. 1992. Inhibitory effects of polyethers on human immunodeficiency virus replication. Antimicrob. Agents Chemother. 36: 492-494.

Nakane, H., M. Arisawa, A. Fujita, S. Koshimura, and K. Ono. 1991. Inhibition of HIV-reverse transcriptase activity by some phloroglucinol derivatives. FEBS Lett. 286: 83-85.

Neville, M., F. Stutz, L. Lee, L. I. Davis, and M. Rosbash. 1997. The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export. Curr. Biol. 7: 767-775.

Nirmala C, Puvanakrishnan R (1996) Protective role of curcumin against isoproterenol induced myocardial infarction in rats. Mol Cell Biochem 159: 85–93.

Olano JP, Borucki MJ, Wen JW, Haque AK (1995) Massive hepatic steatosis and lactic acidosis in a patient with AIDS who was recieving zidovudine. Clin Infect Dis 21: 973–976.

Ogata, T., H. Higuchi, S. Mochida, H. Matsumoto, A. Kato, T. Endo, A. Kaji, and H. Kaji. 1992. HIV-1 reverse transcriptase inhibitor from Phyllanthus niruri. AIDS Res. Hum. Retroviruses 8: 1937-1944.

Ogg, G. S., X. Jin, S. Bonhoeffer, P. R. Dunbar, M. A. Nowak, S. Monard, J. P. Segal, Y. Cao, S. L. Rowland-Jones, V. Cerundolo, A. Hurley, M. Markowitz, D. D. Ho, D. F. Nixon, and A. J. McMichael. 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. Science 279: 2103-2106.

Ott, D. E., L. V. Coren, D. G. Johnson, B. P. Kane, R. C. Sowder, Y. D. Kim, R. J. Fisher, X. Z. Zhou, K. P. Lu, and L. E. Henderson. 2000. Actin-binding cellular proteins inside human immunodeficiency virus type 1. Virology 266: 42-51.

Ott, D. E., L. V. Coren, B. P. Kane, L. K. Busch, D. G. Johnson, R. C. Sowder, E. N. Chertova, L. O. Arthur, and L. E. Henderson. 1996. Cytoskeletal proteins inside human immunodeficiency virus type 1 virions. J. Virol. 70: 7734-7743.

Padhye S, Chavan D, Pandey S, Deshpande J, Swamy KV, et al. (2010) Perspectives on chemopreventive and therapeutic potential of curcumin analogs in medicinal chemistry. Mini Rev Med Chem 10: 372–387. *

Palella, F. J., Jr., K. M. Delaney, A. C. Moorman, M. O. Loveless, J. Fuhrer, G. A. Satten, D. J. Aschman, and S. D. Holmberg. 1998. Declining morbidity and mortality among patients with advanced human immunodeficiency virus

infection. HIV Outpatient Study Investigators. N. Engl. J. Med. 338: 853-860.

Pan, L. Z., A. Werner, and J. A. Levy. 1993. Detection of plasma viremia in human immunodeficiency virus-infected individuals at all clinical stages. J. Clin. Microbiol. 31: 283-288.

Pan, Y., B. Ma, and R. Nussinov. 2005. CD4 binding partially locks the bridging sheet in gp120 but leaves the beta 2/3 strands flexible. J. Mol. Biol. 350: 514-527.

Pando, M. A., L. M. Eyzaguirre, M. Segura, C. T. Bautista, R. Marone, A. Ceballos, S. M. Montano, J. L. Sanchez, M. Weissenbacher, M. M. Avila, and J. K. Carr. 2006. First report of an HIV-1 triple recombinant of subtypes B, C and F in Buenos Aires, Argentina. Retrovirology. 3: 59: 1-5.

Panganiban, A. T. and D. Fiore. 1988. Ordered interstrand and intrastrand DNA transfer during reverse transcription. Science 241: 1064-1069.

Pargellis, C. A., M. M. Morelock, E. T. Graham, P. Kinkade, S. Pav, K. Lubbe, D. Lamarre, and P. C. Anderson. 1994. Determination of kinetic rate constants for the binding of inhibitors to HIV-1 protease and for the association and dissociation of active homodimer. Biochemistry 33: 12527-12534.

Parissi, V., C. Calmels, V. R. De Soultrait, A. Caumont, M. Fournier, S. Chaignepain, and S. Litvak. 2001. Functional interactions of human immunodeficiency virus type 1 integrase with human and yeast HSP60. J. Virol. 75: 11344-11353.

, W., R. I. Connor, and N. R. Landau. 1993. Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of gag and mutational analysis. J. Virol. 67: 7229-7237.

Pereira, L. A., K. Bentley, A. Peeters, M. J. Churchill, and N. J. Deacon. 2000. A compilation of cellular transcription factor interactions with the HIV-1 LTR promoter. Nucleic Acids Res. 28: 663-668.

Perelson, A. S., P. Essunger, Y. Cao, M. Vesanen, A. Hurley, K. Saksela, M. Markowitz, and D. D. Ho. 1997. Decay characteristics of HIV-1-infected compartments during combination therapy. Nature 387: 188-191.

Perry, C. M. and T. Ibbotson. 2002. Biapenem. Drugs 62: 2221-2234.

Peterlin, B. M. and D. Trono. 2003. Hide, shield and strike back: how HIV-infected cells avoid immune eradication. Nat. Rev. Immunol. 3: 97-107.

Pitcher, C. J., C. Quittner, D. M. Peterson, M. Connors, R. A. Koup, V. C. Maino,

and L. J. Picker. 1999. HIV-1-specific CD4+ T cells are detectable in most individuals with active HIV-1 infection, but decline with prolonged viral suppression. Nat. Med. 5: 518-525.

Pommier, Y., A. A. Johnson, and C. Marchand. 2005. Integrase inhibitors to treat HIV/AIDS. Nat. Rev. Drug Discov. 4: 236-248.

Preston, B. D., B. J. Poiesz, and L. A. Loeb. 1988. Fidelity of HIV-1 reverse transcriptase. Science 242: 1168-1171.

Pruss D, Bushman FD, Wolffe AP (1994) Human immunodeficiency virus integrase directs integration to sites of severe DNA distortion within the nucleosome core. Proceedings of the National Academy of Sciences of the United States of America 91: 5913–5917.

Pruss D, Reeves R, Bushman FD, Wolffe AP (1994) The influence of DNA and nucleosome structure on integration events directed by HIV integrase. Journal of Biological Chemistry 269: 25031–25041.

Qian ZM, Li H, Sun H, Ho K (2002) Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. Pharmacol Rev 54: 561–587.

Qian-Cutrone, J., S. Huang, J. Trimble, H. Li, P. F. Lin, M. Alam, S. E. Klohr, and K. F. Kadow. 1996. Niruriside, a new HIV REV/RRE binding inhibitor from Phyllanthus niruri. J. Nat. Prod. 59: 196-199.

Ramratnam, B., J. E. Mittler, L. Zhang, D. Boden, A. Hurley, F. Fang, C. A. Macken, A. S. Perelson, M. Markowitz, and D. D. Ho. 2000. The decay of the latent reservoir of replication-competent HIV-1 is inversely correlated with the extent of residual viral replication during prolonged anti-retroviral therapy. Nat. Med. 6: 82-85.

Rasty, S., P. Thatikunta, J. Gordon, K. Khalili, S. Amini, and J. C. Glorioso. 1996. Human immunodeficiency virus tat gene transfer to the murine central nervous system using a replication-defective herpes simplex virus vector stimulates transforming growth factor beta 1 gene expression. Proc. Natl. Acad. Sci. U.S.A. 93: 6073-6078.

Rimando, A. M., J. M. Pezzuto, N. R. Farnsworth, T. Santisuk, V. Reutrakul, and K. Kawanishi. 1994. New lignans from Anogeissus acuminata with HIV-1 reverse transcriptase inhibitory activity. J. Nat. Prod. 57: 896-904.

Roberts, J. D., K. Bebenek, and T. A. Kunkel. 1988. The accuracy of reverse transcriptase from HIV-1. Science 242: 1171-1173.

Robertson, D. L., J. P. Anderson, J. A. Bradac, J. K. Carr, B. Foley, R. K. Funkhouser, F. Gao, B. H. Hahn, M. L. Kalish, C. Kuiken, G. H. Learn, T.

Leitner, F. McCutchan, S. Osmanov, M. Peeters, D. Pieniazek, M. Salminen, P. M. Sharp, S. Wolinsky, and B. Korber. 2000. HIV-1 nomenclature proposal. Science 288: 55-56.

Ross, T. M., A. E. Oran, and B. R. Cullen. 1999. Inhibition of HIV-1 progeny virion release by cell-surface CD4 is relieved by expression of the viral Nef protein. Curr. Biol. 9: 613-621.

Roy, S., U. Delling, C. H. Chen, C. A. Rosen, and N. Sonenberg. 1990. A bulge structure in HIV-1 TAR RNA is required for Tat binding and Tat-mediated trans-activation. Genes Dev. 4: 1365-1373.

Ratul Kumar D, Naresh K, Utpal B (2010) Encapsulation of curcumin in alginate-chitosan-pluronic composite nanoparticles for delivery to cancer cells. Nanomed: Nanotech, Biol Med 6: 153–160.

Saag, M. S. and J. M. Kilby. 1999. HIV-1 and HAART: a time to cure, a time to kill. Nat. Med. 5: 609-611.

Sader, H. S. and A. C. Gales. 2001. Emerging strategies in infectious diseases: new carbapenem and trinem antibacterial agents. Drugs 61: 553-564.

Sai Krishna AD, Mandraju RK, Kishore G, Kondapi AK (2009) An Efficient Targeted Drug Delivery through Apotransferrin Loaded Nanoparticles. PLoS One 4: e7240. *

Savarino A, Calosso L, Piragino A, Martini C, Gennero L, et al. (1999) Modulation of surface transferrin receptors in lymphoid cells de novo infected with human immunodeficiency virus type-1. Cell Biochem and Func 17: 47–55.

Schubert, U., S. Bour, A. V. Ferrer-Montiel, M. Montal, F. Maldarell, and K. Strebel. 1996. The two biological activities of human immunodeficiency virus type 1 Vpu protein involve two separable structural domains. J. Virol. 70: 809-819.

Schwartz, O., V. Marechal, S. Le Gall, F. Lemonnier, and J. M. Heard. 1996. Endocytosis of major histocompatibility complex class I molecules is induced by the HIV-1 Nef protein. Nat. Med. 2: 338-342.

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3: 1101–1108.

Sewell, A. K., D. A. Price, A. Oxenius, A. D. Kelleher, and R. E. Phillips. 2000. Cytotoxic T lymphocyte responses to human immunodeficiency virus: control and escape. Stem Cells 18: 230-244.

Sharma OP (1976) Antioxidant activity of curcumin and related compounds.

Biochem Pharmacol 25: 1811–1812.

Shoba G, Joy D, Joseph T, Majeed M, Rajendran R, et al. (1998) Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. Planta Medica 64: 353–356.

Sharp, M., S. Wolinsky, and B. Korber. 2000. HIV-1 nomenclature proposal. Science 288: 55-56.

Shaw, G. M., B. H. Hahn, S. K. Arya, J. E. Groopman, R. C. Gallo, and F. Wong-Staal. 1984. Molecular characterization of human T-cell leukemia (lymphotropic) virus type III in the acquired immune deficiency syndrome. Science 226: 1165-1171.

Sheehy, A. M., N. C. Gaddis, J. D. Choi, and M. H. Malim. 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature 418: 646-650.

Sinnwell TM, Sivakumar K, Soueidan S, Jay C, Frank JA, et al. (1995) Metabolic abnormalities in skeletal muscle of patients receiving zidovudine therapy observed by ^{31}P in vivo magnetic resonance spectroscopy. J Clin Invest 96: 126–131.

Siekevitz, M., S. F. Josephs, M. Dukovich, N. Peffer, F. Wong-Staal, and W. C. Greene. 1987. Activation of the HIV-1 LTR by T cell mitogens and the trans-activator protein of HTLV-I. Science 238: 1575-1578.

Smith SD, Shatsky M, Cohen PS, Warnke R, Link MP, et al. (1984) Monoclonal antibody and enzymatic profiles of human malignant T-lymphoid cells and derived cell lines. Cancer Research 44: 5657–5660.

Srimal RC, Dhawan BN (1973) Pharmacology of diferuloyl methane (curcumin), a non-steroidal anti-inflammatory agent. J Pharm Pharmacol 25: 447–452.

Srivastava R, Dikshit M, Srimal RC, Dhawan BN (1985) Anti-thrombotic effect of curcumin. Thrombosis Res 40: 413–417.

Srivastava, V., A. S. Negi, J. K. Kumar, M. M. Gupta, and S. P. Khanuja. 2005. Plant-based anticancer molecules: a chemical and biological profile of some important leads. Bioorg. Med. Chem. 13: 5892-5908.

Stellbrink, H. J., J. van Lunzen, M. Westby, E. O'Sullivan, C. Schneider, A. Adam, L. Weitner, B. Kuhlmann, C. Hoffmann, S. Fenske, P. S. Aries, O. Degen,

C. Eggers, H. Petersen, F. Haag, H. A. Horst, K. Dalhoff, C. Mocklinghoff, N. Cammack, K. Tenner-Racz, and P. Racz. 2002. Effects of interleukin-2 plus highly active antiretroviral therapy on HIV-1 replication and proviral DNA

(COSMIC trial). AIDS 16: 1479-1487.

Strebel, K., D. Daugherty, K. Clouse, D. Cohen, T. Folks, and M. A. Martin. 1987. The HIV 'A' (sor) gene product is essential for virus infectivity. Nature 328: 728-730.

Stremlau, M., C. M. Owens, M. J. Perron, M. Kiessling, P. Autissier, and J. Sodroski. 2004. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature 427: 848-853.

Sugiura, T., Y. Ariyoshi, S. Negoro, S. Nakamura, H. Ikegami, M. Takada, T. Yana, and M. Fukuoka. 2005. Phase I/II study of amrubicin, a novel 9-aminoanthracycline, in patients with advanced non-small-cell lung cancer. Invest New Drugs 23: 331-337.

Sui Z, Salto R, Li J, Craik C, Ortiz de Montellano PR (1993) Inhibition of the HIV-1 and HIV-2 proteases by curcumin and curcumin boron complexes. Bioorg Med Chem 1: 415–422.

Sun A, Shoji M, Lu YJ, Liotta DC, Snyder JP (2006) Synthesis of EF24-tripeptide chloromethyl ketone: a novel curcumin-related anticancer drug delivery system. J Med Chem 49: 3153–3158.*

Suntoke, T. R. and D. C. Chan. 2005. The fusion activity of HIV-1 gp41 depends on interhelical interactions. J. Biol. Chem. 280: 19852-19857.

Swingler, S., B. Brichacek, J. M. Jacque, C. Ulich, J. Zhou, and M. Stevenson. 2003. HIV-1 Nef intersects the macrophage CD40L signalling pathway to promote resting-cell infection. Nature 424: 213-219.

Tagboto, S. and S. Townson. 2001. Antiparasitic properties of medicinal plants and other naturally occurring products. Adv. Parasitol. 50: 199-295.

Taher MM, Lammering G, Hershey C, Valerie K (2003) Curcumin inhibits ultraviolet light induced human immunodeficiency virus gene expression. Mol Cell Biochem 254: 289–297.

Thorstensen K, Romslo I (1993) The transferrin receptor: its diagnostic value and its potential as therapeutic target. Scand J Clin Lab Inv Supplement 215: 113–120.

Tateyama, M., N. Oyaizu, T. W. McCloskey, S. Than, and S. Pahwa. 2000. CD4 T lymphocytes are primed to express Fas ligand by CD4 cross-linking and to contribute to CD8 T-cell apoptosis via Fas/FasL death signaling pathway. Blood 96: 195-202.

Thali, M., A. Bukovsky, E. Kondo, B. Rosenwirth, C. T. Walsh, J. Sodroski, and

H. G. Gottlinger. 1994. Functional association of cyclophilin A with HIV-1 virions. Nature 372: 363-365.

Turpin, J. A. 2003. The next generation of HIV/AIDS drugs: novel and developmental antiHIV drugs and targets. Expert. Rev. Anti. Infect. Ther. 1: 97-128. UNAIDS, W.H.O., 2006. Report on the Global AIDS Epidemic. UNAIDS Report. UNAIDS, Geneva, Switzerland.

Upendhar, G. Curcumin-Loaded Apotransferrin Nanoparticles Provide Efficient Cellular Uptake and Effectively Inhibit HIV-1 Replication *In Vitro*. PloS One August 2011 | Volume 6 | Issue 8 | e23388

Venkatesan N, Punithavathi D, Arumugam V (2000) Curcumin prevents adriamycin nephrotoxicity in rats. British J Pharmacol 129: 231–234.

Violot, S., S. S. Hong, D. Rakotobe, C. Petit, B. Gay, K. Moreau, G. Billaud, S. Priet, J. Sire, O. Schwartz, J. F. Mouscadet, and P. Boulanger. 2003. The human polycomb group EED protein interacts with the integrase of human immunodeficiency virus type 1. J. Virol. 77: 12507-12522.

von-Schwedler, U. K., M. Stuchell, B. Muller, D. M. Ward, H. Y. Chung, E. Morita, H. E. Wang, T. Davis, G. P. He, D. M. Cimbora, A. Scott, H. G.

Wang, W. K., M. Y. Chen, C. Y. Chuang, K. T. Jeang, and L. M. Huang. 2000. Molecular biology of human immunodeficiency virus type 1. J. Microbiol. Immunol. Infect. 33: 131-140.

Weekes, M. P., M. R. Wills, J. G. Sissons, and A. J. Carmichael. 2006. Large HIV-specific CD8 cytotoxic T-lymphocyte (CTL) clones reduce their overall size but maintain high frequencies of memory CTL following highly active antiretroviral therapy. Immunology 118: 25-38.

Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, and B. H. Hahn. 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature 373: 117-122.

Weinreb, N. J., J. A. Barranger, J. Charrow, G. A. Grabowski, H. J. Mankin, and P. Mistry. 2005. Guidance on the use of miglustat for treating patients with type 1 Gaucher disease. Am. J. Hematol. 80: 223-229.

Weissenhorn, W., A. Dessen, S. C. Harrison, J. J. Skehel, and D. C. Wiley. 1997. Atomic structure of the ectodomain from HIV-1 gp41. Nature 387: 426-430.

Weller, I. V. and I. G. Williams. 2001. ABC of AIDS. Antiretroviral drugs. BMJ 322: 1410-1412.

Willey, R. L., F. Maldarelli, M. A. Martin, and K. Strebel. 1992. Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. J. Virol. 66: 7193-7200.

Wong, J. K., M. Hezareh, H. F. Gunthard, D. V. Havlir, C. C. Ignacio, C. A. Spina, and D. D. Richman. 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. Science 278: 1291-1295.

Wu, Y. and J. W. Marsh. 2001. Selective transcription and modulation of resting T cell activity by preintegrated HIV DNA. Science 293: 1503-1506.

Xu, X. N. and G. Screaton. 2001. HIV-1 Nef: negative effector of Fas? Nat. Immunol. 2: 384-385.

Yang, X. W., J. Zhao, Y. X. Cui, X. H. Liu, C. M. Ma, M. Hattori, and L. H. Zhang. 1999. Anti-HIV-1 protease triterpenoid saponins from the seeds of Aesculus chinensis. J. Nat. Prod. 62: 1510-1513.

Yang, Y., B. Dong, P. R. Mittelstadt, H. Xiao, and J. D. Ashwell. 2002. HIV Tat binds Egr proteins and enhances Egr-dependent transactivation of the Fas ligand promoter. J. Biol. Chem. 277: 19482-19487.

Yoder, K. E. and F. D. Bushman. 2000. Repair of gaps in retroviral DNA integration intermediates. J. Virol. 74: 11191-11200.

Zhihua Sui,Rafael salto, Jia Li, Charles craik and Paul R, Ortiz de Montallano inhibition of HIV-1 and HIV-2 Proteases by curcumin and curcumin boran complexes , Bioorganic and medicinal chemistry, vol-1,No.6,pp 415-422,1993

Zapp, M. L. and M. R. Green. 1989. Sequence-specific RNA binding by the HIV-1 Rev protein. Nature 342: 714-716.

Zennou, V., C. Petit, D. Guetard, U. Nerhbass, L. Montagnier, and P. Charneau. 2000. HIV-1 genome nuclear import is mediated by a central DNA flap. Cell 101: 173-185.

Zhanel, G. G., M. Walters, A. Noreddin, L. M. Vercaigne, A. Wierzbowski, J. M. Embil, A. S. Gin, S. Douthwaite, and D. J. Hoban. 2002. The ketolides: a critical review. Drugs 62: 1771-1804.

Zhang, L., B. Ramratnam, K. Tenner-Racz, Y. He, M. Vesanen, S. Lewin, A. Talal, P. Racz, A. S. Perelson, B. T. Korber, M. Markowitz, and D. D. Ho. 1999. Quantifying residual HIV-1 replication in patients receiving combination antiretroviral therapy. N. Engl. J. Med. 340: 1605-1613.

Zhu, K., C. Dobard, and S. A. Chow. 2004. Requirement for integrase during reverse transcription of human immunodeficiency virus type 1 and the effect of cysteine mutations of integrase on its interactions with reverse transcriptase. J. Virol. 78: 5045-5055.

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Curcumin-Loaded Apotransferrin Nanoparticles Provide Efficient Cellular Uptake and Effectively Inhibit HIV-1 Replication *In Vitro*

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Abstract

Background: Curcumin (diferuloylmethane) shows significant activity across a wide spectrum of conditions, but its usefulness is rather limited because of its low bioavailability. Use of nanoparticle formulations to enhance curcumin bioavailability is an emerging area of research.

Methodology/Principal Findings: In the present study, curcumin-loaded apotransferrin nanoparticles (nano-curcumin) prepared by sol-oil chemistry and were characterized by electron and atomic force microscopy. Confocal studies and fluorimetric analysis revealed that these particles enter T cells through transferrin-mediated endocytosis. Nano-curcumin releases significant quantities of drug gradually over a fairly long period, ~50% of curcumin still remaining at 6 h of time. In contrast, intracellular soluble curcumin (sol-curcumin) reaches a maximum at 2 h followed by its complete elimination by 4 h. While sol-curcumin ($GI_{50} = 15.6$ μM) is twice more toxic than nano-curcumin ($GI_{50} = 32.5$ μM), nano-curcumin ($IC_{50} < 1.75$ μM) shows a higher anti-HIV activity compared to sol-curcumin ($IC_{50} = 5.1$ μM). Studies *in vitro* showed that nano-curcumin prominently inhibited the HIV-1 induced expression of Topo II α, IL-1β and COX-2, an effect not seen with sol-curcumin. Nano-curcumin did not affect the expression of Topoisomerase II β and TNF α. This point out that nano-curcumin affects the HIV-1 induced inflammatory responses through pathways downstream or independent of TNF α. Furthermore, nano-curcumin completely blocks the synthesis of viral cDNA in the gag region suggesting that the nano-curcumin mediated inhibition of HIV-1 replication is targeted to viral cDNA synthesis.

Conclusion: Curcumin-loaded apotransferrin nanoparticles are highly efficacious inhibitors of HIV-1 replication *in vitro* and promise a high potential for clinical usefulness.

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Introduction

Curcumin, (diferuloyl methane) is a polyphenol obtained from the rhizome of the herb *Curcuma longa* (turmeric). Curcumin has been shown to exhibit anti-oxidant [1], anti-inflammatory [2], anti-microbial [3] and anti-carcinogenic [4] activities. It also is hepato- and nephro-protective [5,6], suppresses thrombosis [7], protects against damage due to myocardial infarction [8] and exhibits hypo-lipidemic [9] and anti-rheumatic activities [10]. Various animal models and human studies have established that curcumin is extremely safe even at very high doses (12 g/day). In spite of its efficacy and safety, curcumin has not yet been utilized as a therapeutic agent due to its limited bioavailability, a result of poor absorption, high rate of metabolism and rapid systemic elimination [11]. Almost the entire dose of orally administered curcumin is excreted in the faeces. At high doses, the plasma contains nanomolar concentrations of the parent compound and glucuronide together with sulfate conjugates [12,13]. Enhanced bioavailability should bring this natural product to the forefront of promising therapeutic agents.

Numerous approaches were tried earlier that aimed at improving the bioavailability of curcumin. These include usage of adjuvants which can block metabolic pathways of curcumin [14] and encapsulation in liposomes or nanoparticles of various compositions [15,16]. Though these delivery systems are biocompatible, they mostly lack target specificity. In order to enhance specificity, many drug-loaded materials are conjugated with apotransferrin/transferrin proteins [17,18], which are abundantly expressed in actively proliferating cells. Encapsulation with these proteins enables preferential localization into target cells through receptor-mediated endocytosis [19]. This apotransferrin nanoparticle-drug delivery system also provides all the general advantages offered by nano-formulations such as appropriate size for cellular uptake, excellent water dispensability and improved intracellular localization. HIV-1 infected cells are known to express transferrin receptors, which bind

transferrin or apotransferrin and transport it into the cell [20]. These receptors could be targeted for ligand-mediated transport of curcumin into the infected cells. In the present study, we formulated curcumin-loaded apotransferrin nanoparticles (nano-curcumin) using a sol-oil technique. These curcumin loaded nanospheres were then assessed for their efficiency of cellular uptake and cytotoxicity in T-cells. The nano-curcumin formulation was further evaluated for its efficacy to inhibit HIV-1 replication. The results clearly highlight the advantage of this delivery system over direct soluble-curcumin administration.

Results

Preparation of curcumin-loaded apotransferrin nanoparticles

Curcumin-containing apotransferrin nanoparticles were prepared using sol-oil chemistry as described in materials and methods section. Transmission electron microscopy (TEM) analysis showed that the particles were nearly uniform in size and spherical in shape. This technique also confirmed the increase in diameter of loaded particles (Fig. 1A). The size of pure apotransferrin nanoparticles as assessed by scanning electron microscopy (SEM) ranged from 45–55 nm, increasing to 55–70 nm after curcumin loading (Fig. 1B). The surface morphological analysis of particles using atomic force microscopy (AFM) showed significant projections, which might contribute to the molecular recognition of particle by the receptor (Fig. 1C). The proteinaceous nature of nanoparticle surface was confirmed by their sensitivity to pH 5–6. Drug loading was 50% with 500 μg of curcumin/mg of protein upon complete saturation.

Cellular uptake of curcumin following nano-curcumin administration

Cellular uptake of curcumin upon incubation with nano-curcumin was monitored by confocal microscopic analysis of the compound's intrinsic green fluorescence. Intracellular localization of curcumin was enhanced in nano-curcumin treated cells compared to those treated with soluble-curcumin (Fig. 2A ii and iii), indicating that apotransferrin encapsulation significantly increases cellular uptake of curcumin. The curcumin localization in overall population of SUPT1 cells was given in Fig. S1. To determine whether the enhanced uptake of apotransferrin-encapsulated curcumin requires interaction with the transferrin receptor, we incubated the cells concurrently with nano-curcumin and antibody to the human transferrin receptor. The observed decrease in intracellular curcumin fluorescence (Fig. 2A iv) suggests that nano-curcumin uptake results from endocytosis mediated by the transferrin receptor. Similar results were seen when intracellular curcumin accumulation was quantified fluorimetrically in experiments conducted with SUPT1 and stimulated PBMCs (Fig. 2B). These results confirm that cellular uptake of curcumin is significantly enhanced by apotransferrin encapsulation and that this improved uptake is mediated by the transferrin receptor in both SUPT1 (Fig. 2B i) and PBMCs (Fig. 2B ii).

Cellular retention of curcumin following nano-curcumin administration

Cellular uptake and elimination of curcumin upon treatment with sol- or nano-curcumin (1 and 10 μM) was assessed by confocal microscopic analysis at different time points (1, 2, 4 and 6 h respectively). Soluble-curcumin was taken up quickly by the cells, peaking at 2 h, but was rapidly eliminated out and was essentially gone by 4 h (Figs. 3 and 4). Nano-curcumin, by contrast was both taken up and released more slowly with a peak at 4 h and ~50% of the drug still present at 6 h (Figs. 3 and 4). Further, the results confirmed that nano-cucumin exhibits a time-dependent intracellular localization of curcumin (in both SUPT1 cells PBMCs) that is stable for almost the full 6 h. In contrast, soluble-curcumin is taken up rapidly, especially at the higher concentration but disappeared from the cells quickly. This gradual and stable uptake of nano-curcumin is characteristic of receptor-mediated transport. Moreover, it is important to note that uptake at 2 h is similar for the 1 and 10 μM concentrations of nano-curcumin (Figs. 3 & 4 A and B) while uptake is significantly higher for 10 μM than for 1 μM nano-curcumin at 6 h (Figs. 3 & 4 A and B).

These results indicate that cells retain curcumin for longer periods following treatment with nano-curcumin than with sol-curcumin.

Cytotoxicity of nano-curcumin mediated delivery

To determine the relative cytotoxicity of curcumin in the nano-curcumin and sol-curcumin formulations, SUPT1 cells (Fig. 5A) or stimulated PBMCs (Fig. 5B) were incubated with increasing concentrations (1, 5, 10, 25, 50 and 100 μM) of the two formulations and cell survival was estimated by MTT assay. Both sol- and nano-curcumin formulations were found non-toxic at very low concentrations (1, 5 and 10 μM). However, 25 μM concentration of sol-curcumin was extremely cytotoxic (almost 80%) while nano-curcumin at the same concentration was significantly less cytotoxic (Fig. 5). The GI_{50} of sol-curcumin is 15.6 μM, while that of nano-curcumin it is 32.5 μM in SUPT1 cells. In stimulated PBMCs, the GI_{50} is 18 μM for sol-curcumin and 38 μM for nano-cucumin. The low cytotoxicity of nano-curcumin highlights the observation that direct sol-curcumin administration is lethal to cells at concentrations above 10 μM. Notably, decreased cytotoxicity of apotransferrin encapsulated formulation occurred despite increased cellular uptake and sustained retention.

HIV-1 neutralizing activity of nano-curcumin

SUPT1 cells or stimulated PBMCs were infected with HIV-$1_{93IN101}$ in the presence of increasing concentrations (1, 2.5, 5, 10, 20 and 30 μM) of nano-curcumin or sol-curcumin. Nano-curcumin inhibited HIV-1 replication in a dose-dependent manner (Figs. 6A and 6C). The IC_{50} of nano-curcumin is 1.75 μM, while that of sol-curcumin it is 5.1 μM in SUPT1 cells. In stimulated PBMCs, it is 5.1 μM for sol-curcumin and 2.4 μM for nano-curcumin. These data suggest that nano-cucumin is almost three-fold more potent than sol-curcumin. About 80% inhibition of HIV-1 replication was estimated at 10 μM nano-curcumin (Fig. 6A & C), a concentration at which its cytotoxicity is less than 10% (Figs. 5 A and B). Although sol-curcumin exhibits a 75% inhibition of viral replication at this concentration, its cytotoxicity is 20%. The HIV-1 neutralizing activity of nano-curcumin is significantly reduced by antibody to human transferrin receptor in both SUPT1 cells (Fig. 6B) and PBMCs (Fig. 6D), confirming that the superior ability of nano-curcumin to inhibit HIV-1 infection is dependent on cellular uptake mediated by the transferrin receptor.

Effect of nano-curcumin on the expression of *topoisomerase IIα* and proviral DNA synthesis

To determine the mechanism of nano-curcumin's anti-HIV activity in T-cells, we monitored expression of the human *topoisomerase IIα* gene. SUP-T1 cells were acutely infected with HIV-$1_{93IN101}$ in the presence of 5 μM nano-curcumin, sol-curcumin or appropriate

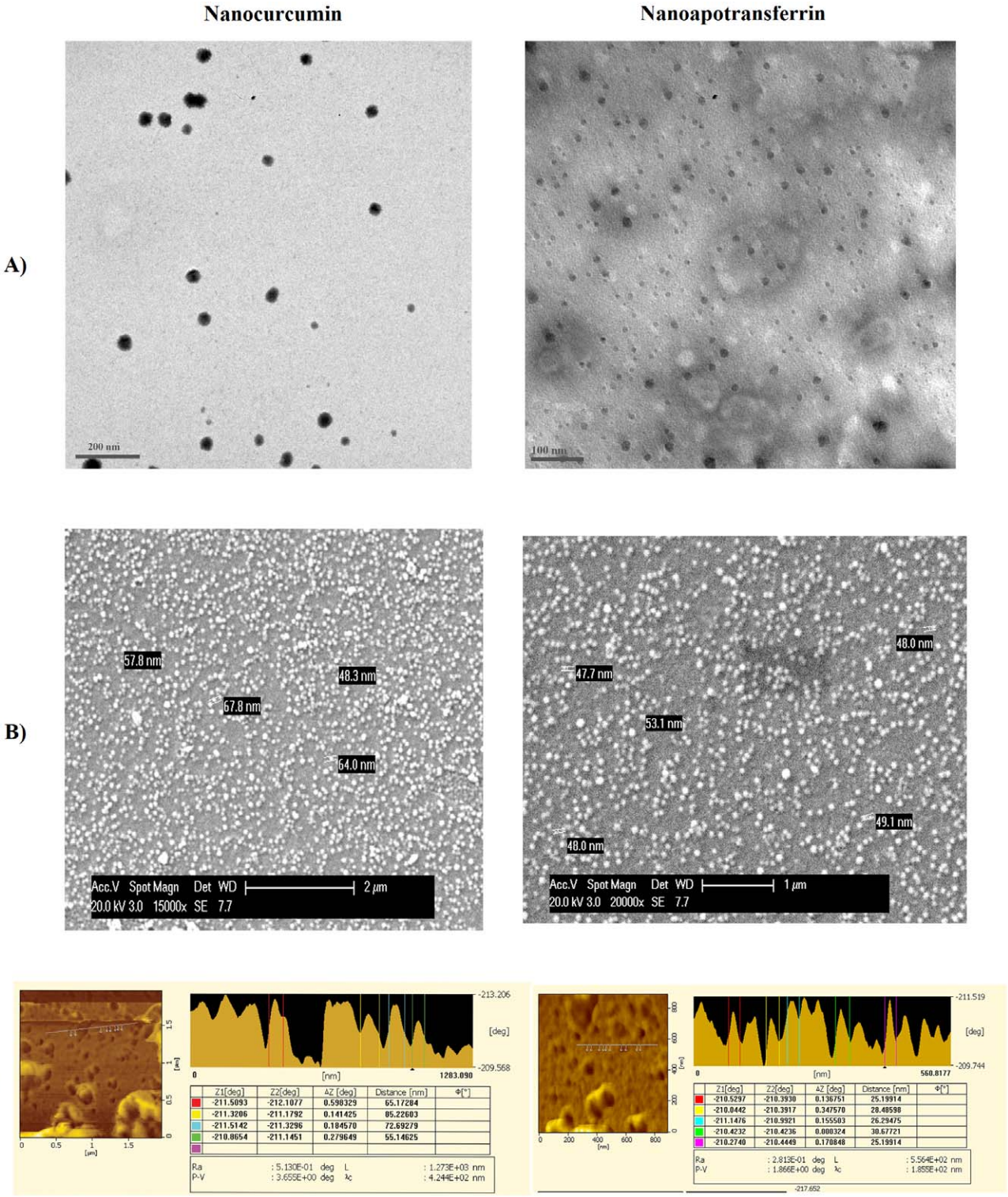

Figure 1. Curcumin loading increases size of apotransferrin nanoparticles. The preparations of curcumin-loaded apotransferrin nanoparticles (nano-curcumin; left) and apotransferrin nanoparticles without curcumin (nano-apotransferrin; right) were examined by A) TEM B) SEM and C) AFM as indicated.
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controls. Semi-quantitative and real-time PCR of *topoisomerase IIα* expression demonstrated that this gene was upregulated on HIV infection but was prominently down-regulated by nano-curcumin treatment (Fig. 7A). This down-regulation was not observed when the cells were treated with sol-curcumin. In a different experiment, it was found that nano-curcumin significantly inhibited proviral DNA synthesis, monitored by *gag* gene expression (Fig. 7B). Sol-curcumin had no effect on synthesis of proviral DNA.

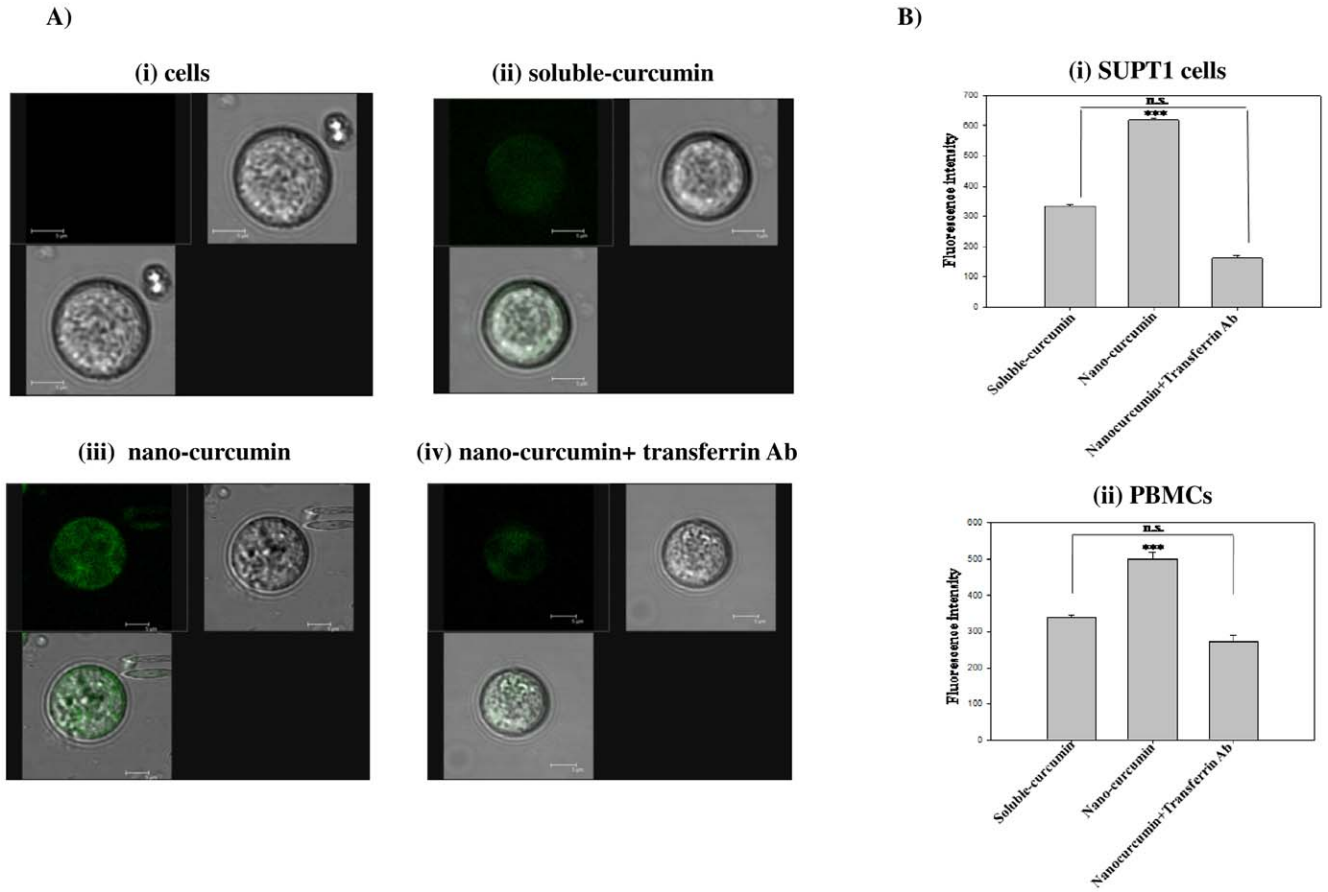

Figure 2. Nanoparticle formulation increases curcumin uptake, which is inhibited by transferrin receptor blockade. A) SUP-T1 cells were incubated for 1 h with curcumin formulations as indicated, then examined by confocal microscopy. (i) Cells without curcumin; (ii) 1 μM sol-curcumin; (iii) 1 μM nano-curcumin; or (iv) 1 μM nano-curcumin in the presence of transferrin receptor antibody (100 ng/ml). Each panel contains three images: fluorescence, bright field and merged. B) SUP-T1 cells (i) or stimulated PBMCs (ii) were incubated for 1 h with curcumin formulations, after which intrinsic fluorescence of intracellular curcumin was determined quantitatively by fluorometric analysis. Cells were treated with 5 μM sol-curcumin, 5 μM nano-curcumin, or 5 μM nano-curcumin in the presence of antibodies to the transferrin receptor (TrR-Ab; 100 ng/ml). All the values are normalized to that obtained from SUPT1 cells. Error bars indicate standard deviation (SD). ***, $P \leq 0.001$ compared to sol-curcumin; n.s.: non-significant.
doi:10.1371/journal.pone.0023388.g002

Nano-curcumin completely blocks HIV-1 induced inflammatory response

HIV-1 infection enhanced expression of *topoisomerase IIβ*, *IL-1β* and *COX-2*. Treatment of infected cells with nano-curcumin significantly inhibited expression of *IL-1β* and *COX-2* (Fig. 8) as well as *topoisomerase IIα* (Fig. 7), but had no effect on the expression of *topoisomerase IIβ* or *TNF-α* (Fig. 8). The results are further confirmed by estimation of IL-1β (Fig. 8B), COX-2 (Fig. 8C) and TNF-α (Fig. 8D) levels in infected cells. These results suggest that nano-curcumin effectively blocks HIV-1-mediated inflammatory responses [21] and thus affects viral cDNA synthesis.

Discussion

Results reported here show that curcumin-loaded apotransferrin forms spherical nanoparticles consistently in the size range 55–70 nm, which is in accord with the National Nanotechnology Initiative's definition of "nanomaterials". Other reported formulations have typically been larger in size (usually 100–200 nm) [15,22], although Bisht *et al.* reported a polymeric nanoparticle formulation with a size range similar to ours [23]. The spherical shape and surface characteristics of the nanoparticles, as shown by our TEM study, probably play a critical role in the cellular uptake and release characteristics displayed by this curcumin formulation *in vitro*. These particles are efficiently transported into cells through endocytosis mediated by the transferrin receptor [19], with the curcumin then being released intracellularly. Although a curcumin derivative, EF24, has been chemically linked to an endocytosis-inducing peptide [24], our nano-curcumin is to the best of our knowledge the first formulation in which cellular uptake of curcumin itself is enhanced by receptor-mediated endocytosis. The nano-curcumin formulation is especially attractive because of its simple preparation protocol that does not require either complex equipment or expensive reagents. Moreover, this delivery system is highly target-specific [19]. The simplicity of the preparation would also minimize the possibility of potentially toxic reagents being carried over into formulations for future *in vivo* studies.

Curcumin is naturally fluorescent in the visible green spectrum, thus allowing it to be located and quantified within cells. Cells treated with both sol- and nano-curcumin displayed green fluorescence, confirming successful intracellular delivery of curcumin. The greater cellular uptake and retention exhibited by nano-curcumin, as demonstrated here, addresses the problem of curcumin bioavailability. The ability of nanoparticle prepara-

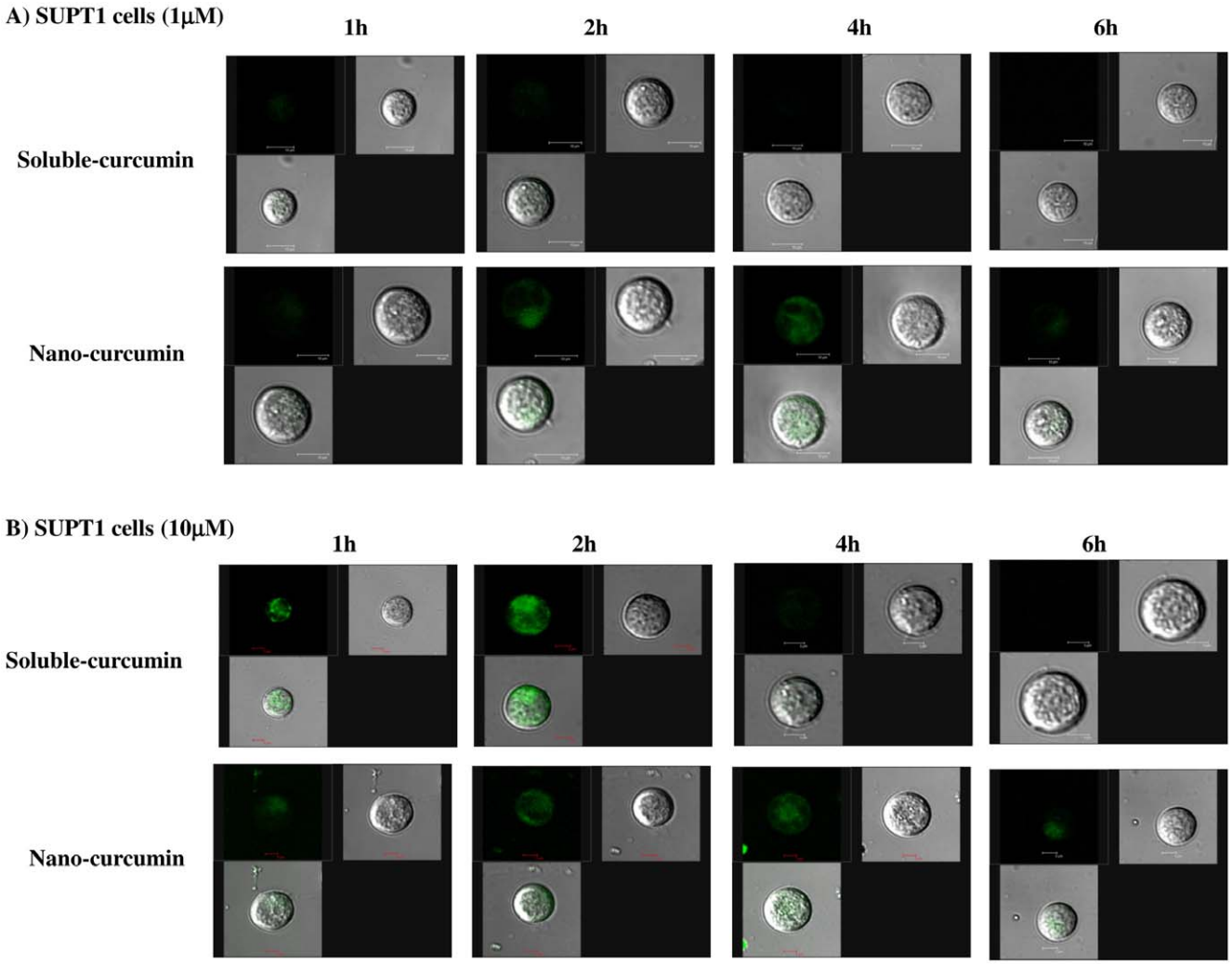

Figure 3. Nanoparticle formulation exhibits increased cellular retention in SUP-T1 cells. Cells were incubated with 1 μM (Panel A) and 10 μM (Panel B) sol-curcumin and nano-curcumin and examined by confocal microscopy at time points of 1, 2, 4 and 6 h. Each panel contains three images: fluorescence, bright field and merged.
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tions to increase cellular uptake of curcumin has also been demonstrated by other groups [22,23]. Since cytotoxicity is of prime concern for cellular assays, we performed our uptake and retention studies at nano-curcumin concentrations that are non-toxic to cells. Further studies showed that, despite greater intracellular concentrations, nano-curcumin exhibited much less cytotoxicity than equivalent doses of sol-curcumin.

Studies cited in the introduction demonstrate that curcumin is extremely safe and will be tolerated at dosages up to 12 g/day. This leads to the presumption that nano-curcumin will also be safe even at high concentrations, On the other hand, high-dosage AZT can cause myopathy, cardiomyopathy and hepatotoxicity associated with mitochondrial DNA depletion. As a component of HAART (highly active antiretroviral therapy) AZT causes cytopenias and lipodystrophy [25–29]. Similar dose-limiting adverse effects are not expected for curcumin. The present study addresses the major problem of metabolic instability and shows that nano-curcumin, even at relatively low concentrations provide a highly stable and retarded release, leading to prolonged intracellular accumulation. This prolonged exposure will reduce the needed concentration and thus the cytotoxicity, while retarded release allows longer half-life.

Studies showing that curcumin has anti-viral properties are few but reliable [30–32]. We now show that nano-curcumin, but not sol-curcumin, has high anti-HIV activity. Nano-curcumin drastically decreased expression levels of *topoisomerase IIα* and inhibited proviral DNA synthesis. The high levels of intracellular curcumin achieved through nano-curcumin administration may account for this previously unreported observation. Curcumin has been previously demonstrated to inhibit HIV activation and replication [30–33]. Mechanisms involved include inhibition of both HIV protease [32] and integrase [34]. Curcumin also inhibits Tat-mediated transactivation of the HIV long terminal repeat, which is essential for activation of latent virus [30,31]. Another mechanism may involve inhibition of the virus's ability to upregulate the host enzyme topoisomerase II [35], which is required at the earliest stages of virus replication. Repression of topoisomerases using an inhibitor [31] or antisense nucleotides [36] results in impaired HIV-1 replication. Additionally, Topoisomerase II naturally recognizing DNA topological intermediates such as DNA curvature, flexibility, rigidity and distortion greatly influence the HIV integration event [37,38]. Moreover, curcumin has been shown to form a complex with DNA and topoisomerase, producing DNA breaks and blocking their repair, much as etoposide

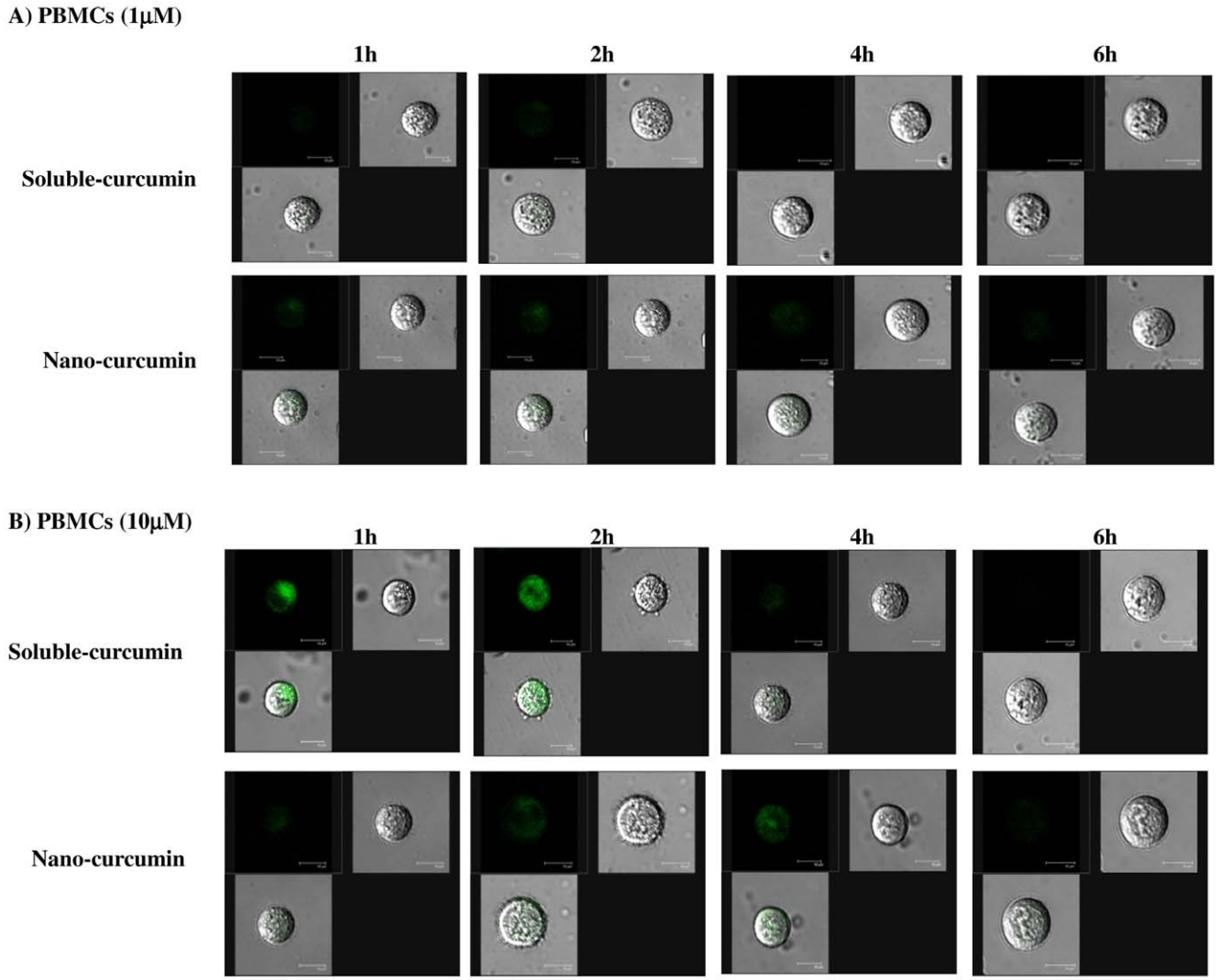

Figure 4. Nanoparticle formulation exhibits increased cellular retention in stimulated PBMCs. Cells were incubated with 1 μM (Panel A) and 10 μM (Panel B) sol-curcumin and nano-curcumin and examined by confocal microscopy at time points of 1, 2, 4 and 6 h. Each panel contains three images: fluorescence, bright field and merged.
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does [39]. Therefore, decrease in *topoisomerase IIα*, *COX-2* and *IL-1β* levels could predictably alter the HIV-1-mediated inflammatory response, which may in turn affect the topological reorganization of cellular DNA promoted by topoisomerase IIα. These effects on DNA organization might then prevent the HIV-1 integration event. This explains the inhibition of proviral DNA synthesis, monitored through *gag* gene expression, and the consequent blockage of HIV-1 replication observed in the current study.

In summary, the ability of curcumin to block HIV replication and activation at several stages is well established. However, clinical use has been hampered by the absence of an effective dosage regime. Curcumin-loaded apotransferrin nanoparticles, through their ability to target an endocytosis-promoting cellular receptor, increase cellular uptake of the drug and enhance its ability to inhibit HIV-1 replication while simultaneously reducing cytotoxicity. Further studies using relevant *in vivo* experimental models are required. Nevertheless, this formulation appears promising as a step toward clinical usage as a multivalent antiretroviral of this well-known but underutilized therapeutic agent.

Materials and Methods

Preparation of stimulated PBMCs

Human blood was obtained from a healthy donor as per the approval of the Institutional Ethics Committee, University of Hyderabad. Human Peripheral blood mononuclear cells (PBMC) were isolated from blood by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich) and the cells were cultured in RPMI 1640 medium (GIBCO-BRL) supplemented with 10% FBS. PBMCs were stimulated using 10 μg/ml phytohemagglutinin (PHA) (Sigma-Aldrich) for 2 days in the presence of IL-2 (20 IU/ml; Sigma-Aldrich) in 5% CO_2 at 37°C. Cells were washed after 2 days and continued in culture with IL-2 for another 24 h and were stored at −70°C till further use.

Nanoparticle preparation

Nanoparticles were prepared using a variation of the procedure described previously [19]. Ten mg of apotransferrin (Sigma-Aldrich) in 100 μl of phosphate-buffered saline (pH 7.4)

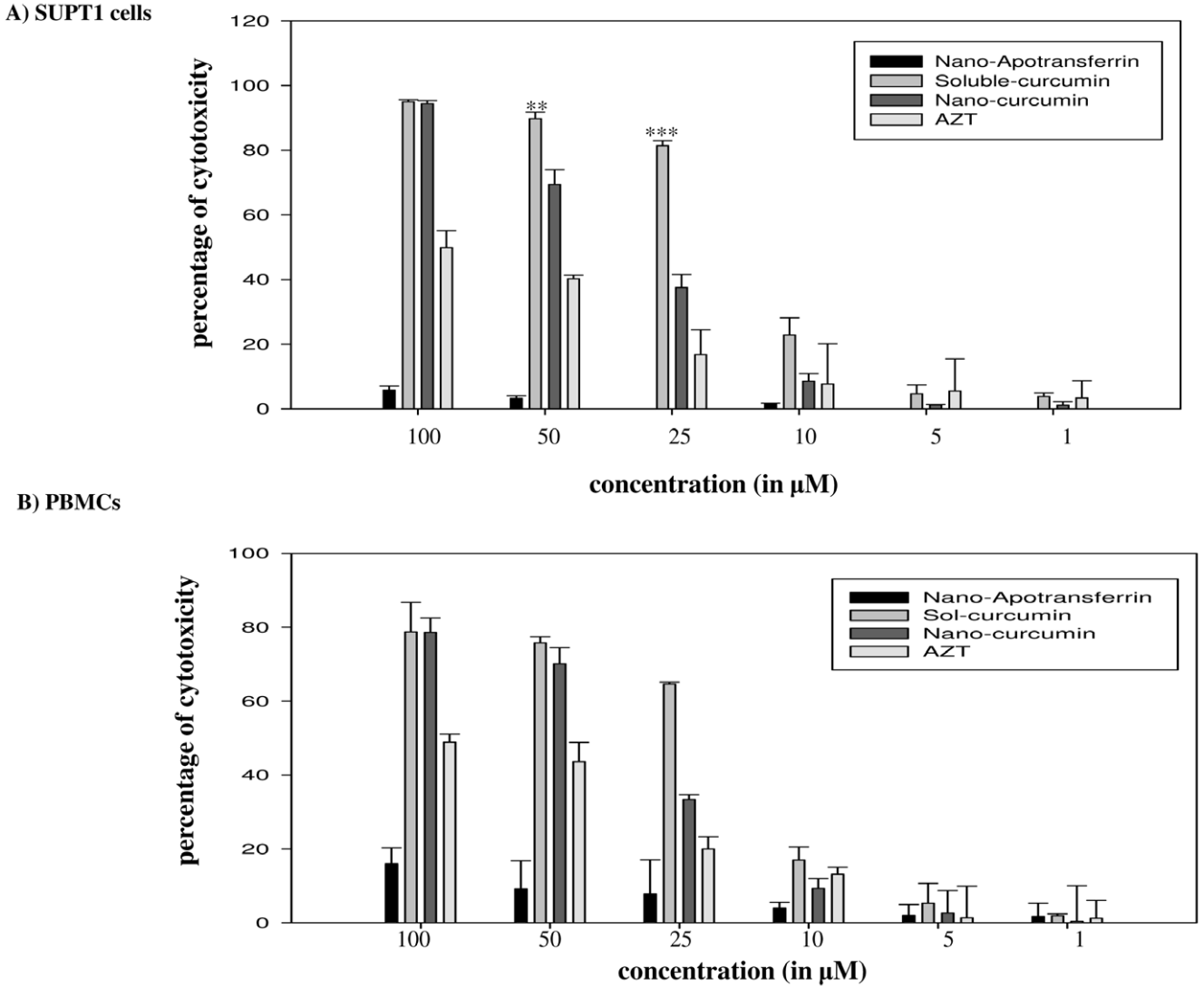

Figure 5. Nanoparticle formulation decreases curcumin cytotoxicity. SUPT1 cells (Panel A) or stimulated PBMCs (Panel B) were exposed to increasing concentrations (1, 5, 10, 25, 50 and 100 μM) of sol-curcumin, nano-curcumin, azidothymidine (AZT) or nano-apotransferrin (10, 50 and 100 μg) for 16 h, after which cell viability was determined by MTT assay. PBMCs were cultured in the presence of IL-2 (20 IU/ml). Cell viability in the absence of drug was defined as 0% cytotoxicity. Error bars indicate SD. ** $P \leq 0.01$, and ***, $P \leq 0.001$ compared to nano-curcumin. * indicates μg apotransferrin protein that carry equivalent molar concentration of the drug.
doi:10.1371/journal.pone.0023388.g005

was slowly mixed with 3.6 mg of curcumin (Sigma-Aldrich) in 100 μl DMSO (100 mM) and the mixture was incubated on ice for 5 min. The mixture of apotransferrin and curcumin was slowly added to 15 ml of olive oil at 4°C with continuous dispersion by gentle manual vortexing. The sample was sonicated 15 times at 4°C using a narrow stepped titanium probe of ultrasonic homogenizer (300V/T, Biologics Inc., Manassas, Virginia, USA). The sonication amplitude was 5 μm and the pulses were 30 sec long with an interval of 1 min between successive pulses. The resulting mixture was immediately frozen in liquid nitrogen for 10 min and was then transferred to ice and incubated for 4 h. The particles formed were pelleted by centrifugation at 6000 rpm for 10 min and the pellet was extensively washed with diethyl ether and dispersed in PBS. The particles' protein content was estimated by the Biuret method and the protein content was used to determine the amount of nanoparticles used for each experiment.

Characterization of nanoparticles

Structure and morphology of the nanoparticles were investigated using a scanning electron microscope (SEM, Philips FEI-XL 30 ESEM; FEI, Hillsboro, OR, USA) operated at 20 KV, transmission electron microscope (TEM, Techni) operated at 80 KV and atomic force microscope (AFM; SPM400). For SEM the particles were gold coated, the TEM sample was prepared by fixing the sample on 200 mesh type-B carbon coated copper grid (ICON) using 2% osmium tetroxide in 50 mM phosphate buffer followed by staining with phosphotungstic acid, and the AFM sample was spin coated on a glass cover slip. Manufacturer's instructions were followed for data collection, and analysis of particles.

Nanoparticle localization assay

SUPT1 cells obtained from the NIH-AIDS Reference and Reagents Program were used [40]. (1×10^6) SUPT1 cells or

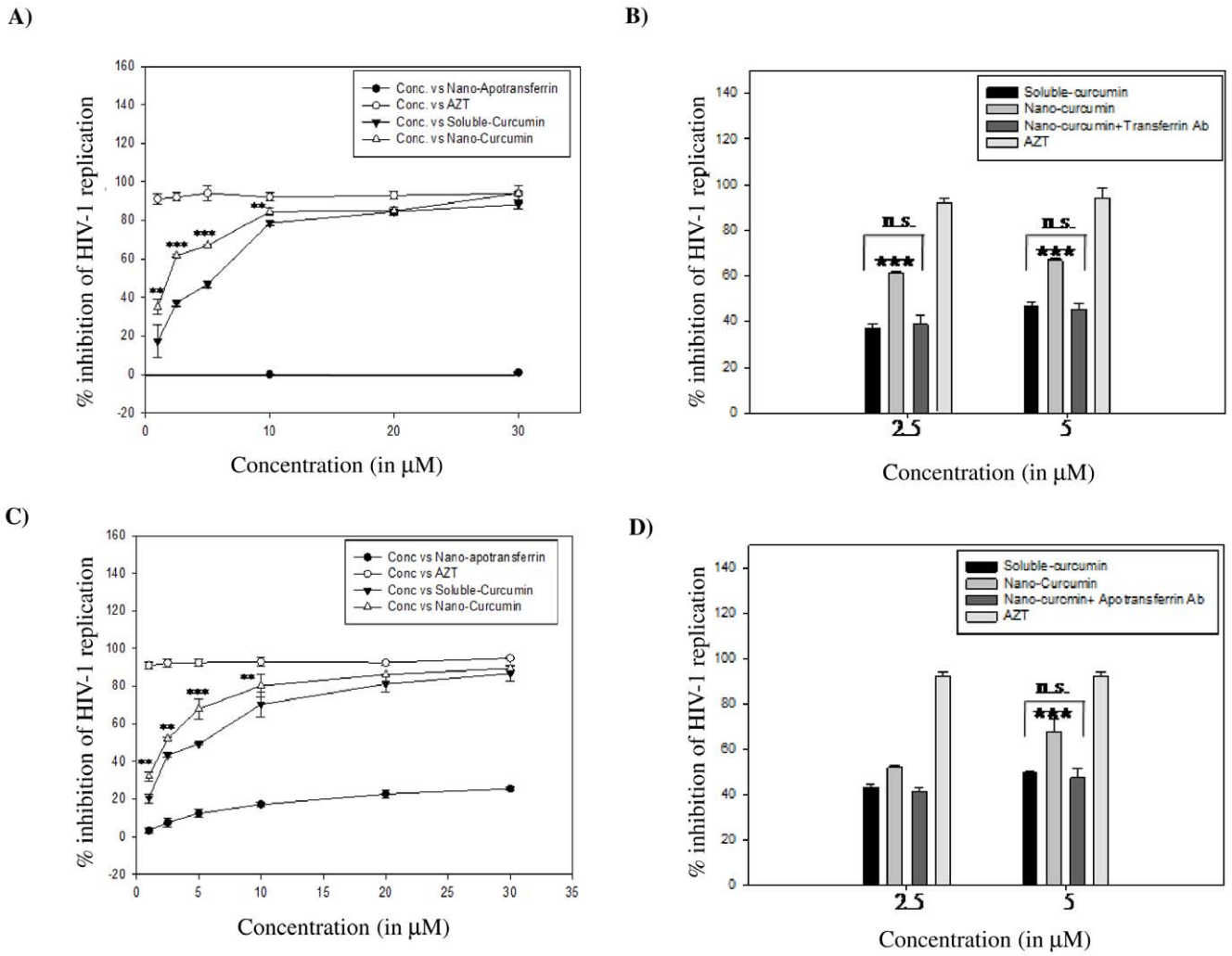

Figure 6. Nano-curcumin more effectively inhibits HIV-1 replication through a mechanism dependent on transferrin receptor. A & C) SUPT1 cells (Panel A) or stimulated PBMCs (Panel C) were challenged for 2 h with HIV-$1_{93IN101}$ (1 mg p24/ml) in the presence of increasing concentrations (1, 2.5, 5, 10, 20 and 30 μM) of sol-curcumin, nano-curcumin, or nano-apotransferrin (10 and 50 μg). They were then incubated for a further 96 h, after which viral replication was measured by p24 antigen capture assay. *indicates μg apotransferrin protein that carry equivalent molar concentration of the drug. B & D) SUP-T1 cells or stimulated PBMCs were challenged for 2 h with HIV-$1_{93IN101}$ in the presence of 2.5 or 5.0 μM concentrations of sol-curcumin, nano-curcumin or nano-curcumin in the presence of transferrin receptor antibody (100 ng/ml). After 96 h incubation, viral replication was measured by p24 antigen capture assay. In both these experiments, viral replication in the absence of drug was defined as 0% inhibition; Azidothymidine (AZT) was employed as a positive control. Error bars indicate SD. ** $P \leq 0.01$, and ***, $P \leq 0.001$ compared to sol-curcumin; n.s.: non-significant.
doi:10.1371/journal.pone.0023388.g006

stimulated PBMCs were seeded in 30 mm dishes (Corning Lifesciences) and treated with curcumin, either soluble or incorporated into nanoparticles at 1 and 10 μM concentrations and the cells were incubated at different time points (1, 2, 4 and 6 h). After incubation, the cells were washed thrice with phosphate-buffered saline (pH 7.4) and observed under the laser confocal microscope to analyse the amount of intracellular curcumin employing the intrinsic fluorescence of curcumin (λEx 458 nm and Em 530 nm).

Competition of transferrin receptor antibodies with the apotransferrin-drug nanoparticles

Cells (1×10^6) were incubated in serum-free medium for 60 min in a 12-well plate. Nano-curcumin or sol-curcumin (equivalent to 1 or 5 μM curcumin) was added to the cells in either the presence or absence of 400 ng/ml monoclonal anti-human transferrin receptor antibody (Calbiochem) and incubated for 2 h. After incubation, cells were washed thrice with phosphate-buffered saline (pH 7.4) and lysed by sonication in lysis buffer (0.1% Triton-X-100). The lysate was cleared by centrifugation at 12,000 rpm for 20 min at 4°C. Curcumin was quantified through its intrinsic fluorescent emission (λ Ex 458 nm and Em 530 nm) as measured by a fluorescence spectrometer (Shimadzu FL 2000; Shimadzu, Kyoto, Japan). In parallel, the cells were observed under a laser confocal microscope. Experiments using stimulated PBMCs were conducted in the presence of IL-2 (20 IU/ml).

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay

SUPT1 cells or stimulated PBMCs (0.2×10^6/well) were seeded in a 96-well plate and incubated at 37°C for 4 h in a 5% CO_2 incubator (Forma Scientific, Marietta, OH, USA). These cells

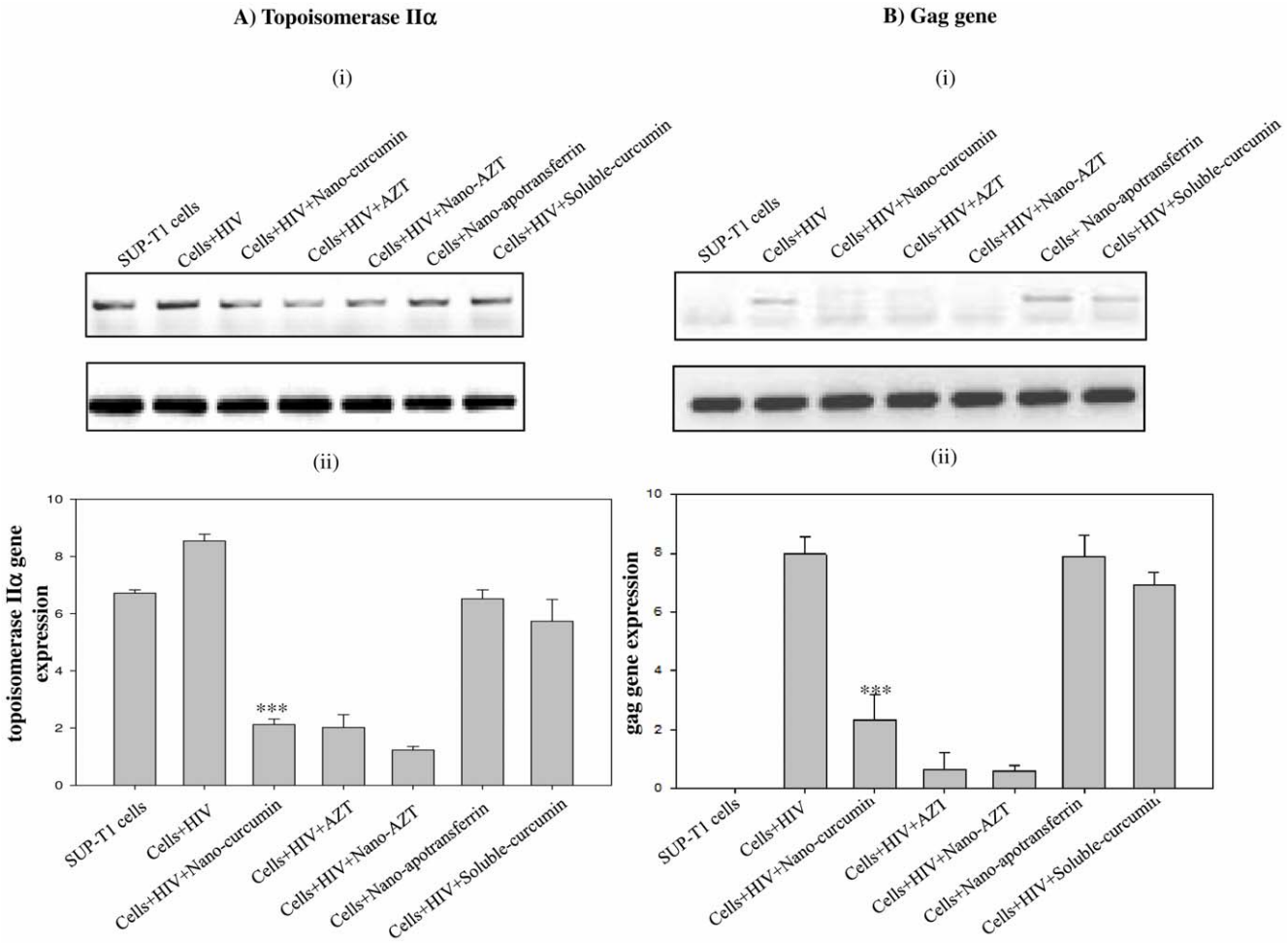

Figure 7. Inhibition of HIV-1 replication by nano-curcumin is due to abolished viral cDNA synthesis and/or altered topology. SUPT1 cells were challenged for 4 h with HIV-$1_{93IN101}$ in the presence of 5 μM of sol-curcumin, nano-curcumin or nano-apotransferrin. A) The expression of topoisomerase IIα was determined by (i) semi-quantitative and (ii) quantitative-PCR. B) Quantity of viral cDNA synthesized was shown by both (i) semi-quantitative and (ii) real-time PCR using *gag*-specific primers. Template from normal SUP-T1 cells was used as negative control. Azidothymidine (AZT) was employed as a positive control and 18S was used as an internal control in both experiments. Error bars indicate SD. ***, $P \leq 0.001$ compared to sol-curcumin.
doi:10.1371/journal.pone.0023388.g007

were treated with increasing concentrations of curcumin, either soluble or incorporated into nanoparticles (nano-curcumin was assessed by the amount of associated protein), and incubated for 16 h. The cells were pelleted at 1200 rpm for 10 min and resuspended in new medium. To this, 20 μl of 5 mg/ml MTT (Sigma-Aldrich) was added and incubated for 4 h. The cells were then pelleted at 1200 rpm for 20 minutes, the medium was removed, and the precipitate was dissolved in DMSO and read in an ELISA microplate reader at 570 nm.

HIV-1 neutralization assay

SUPT1 cells or stimulated PBMCs (0.4×10^6/ml) with 100% viability were seeded in RPMI 1640, 0.1% FBS on four 12-well plates. Increasing concentration of nano-curcumin, sol-curcumin, or nano-curcumin in the presence of anti-human transferrin receptor antibody (as indicated) were added to the cells which were then infected with HIV-$1_{93IN101}$ at a final virus concentration equivalent to 1 ng of p24 per ml. The infected cells were incubated for 2 h at 37°C in a 5% CO_2 incubator. The cells were then pelleted at 350× g for 10 min, the supernatant was discarded, and cells were washed with RPMI 1640 containing 10% FBS. The SUP-T1 cells were resuspended in fresh complete medium and were incubated for a further 96 h. PBMCs were incubated for 7 days. The supernatants were then collected and analyzed using a p24 antigen capture assay kit (Advanced Bioscience Laboratories, Kensington, MD, USA). The extent of infection in the absence of test compound was considered to be equivalent to 0% inhibition. Azidothymidine (AZT) was employed as a positive control. Experiments using stimulate PBMCs were conducted in the presence of IL-2 (20 IU/ml).

qPCR analysis of *topoisomerase IIα* and *gag* expression and semi-quantitative PCR analysis of *topoisomerase IIβ*, *COX-2*, *IL-1β* and *TNF-α* expression

Genomic DNA (to monitor *gag* expression) and total RNA were isolated from treated samples using suppliers' (Qiagen, GmbH, Germany & Sigma-Aldrich respectively) protocol. cDNAs were synthesized using Superscript™ III first strand synthesis system (Invitrogen, Carlsbad, CA, USA). Primers were designed based on the gene sequences available in PUBMED nucleotide database. Multiple alignment (wherever required) was performed using the ClustalW program. For each gene, sequences of the forward and reverse primers

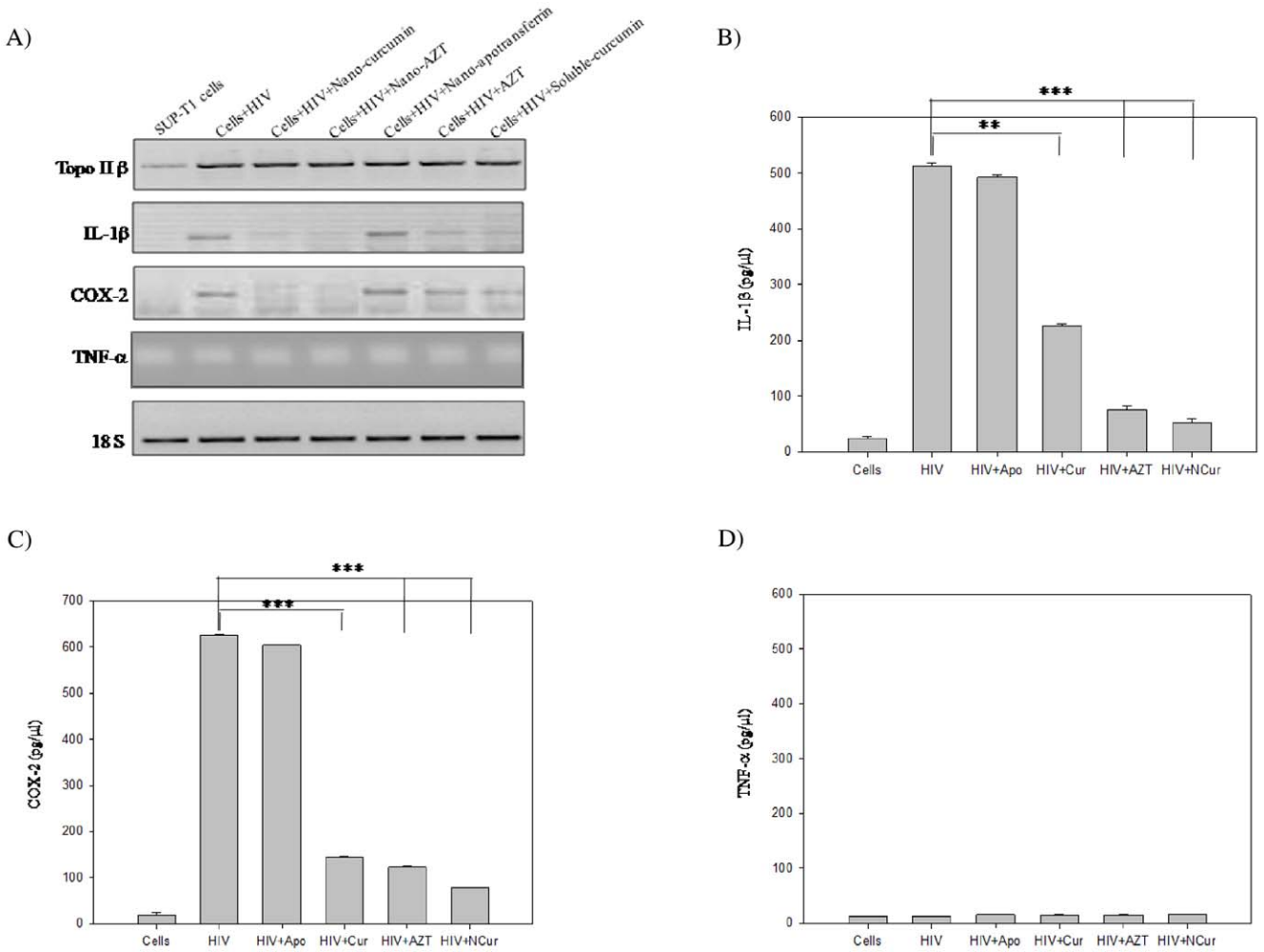

Figure 8. Action of nano-curcumin against virus-induced inflammatory response. A) SUPT1 cells were challenged for 4 h with HIV-$1_{93IN101}$ in the presence of 5 μM of sol-curcumin, nano-curcumin or nano-apotranferrin. The expression of topoisomerase IIβ, IL-1β, TNF-α and COX-2 was determined by semi-quantitative-PCR. Template from normal SUP-T1 cells was used as negative control. 18S was used as an internal control in both experiments. IL-1β (Panel B), COX-2 (Panel C) and TNF-α (Panel D) were estimated using commercial kits as described in the methods section. Error bars indicate SD. ***, $P \leq 0.001$ compared to nano-curcumin.
doi:10.1371/journal.pone.0023388.g008

used in each respective PCR, are as follows: *gag* Fwd: 5′ GCAGGGCCTATTGCACCAGGC 3′, *gag* Rev: 5′ GGCCAGGTCCTCCCACTCCC 3′; *topo IIα* Fwd: 5′ GGGTTCTTGAGCCCCTTCACGA 3′, *topo IIα* Rev: 5′ GTAGGTGTCTGGGCGGAGCAA 3′; 18S Fwd: 5′ GCTACCACATCCAAGGAAGGCAGC 3′, 18S Rev: 5′ CGGCTGCTGGCACCAGACTTG 3′; *topo IIβ* Fwd: 5′ GCCCAGTTGGCTGGCTCTGT 3′, *topo IIβ* Rev: 5′ GCATGGGATGAGGATCCAGGCC; *COX-2* Fwd: 5′ AACAGGAGCATCCTGAATGG 3′, *COX-2* Rev: 5′ GGTCAATGGAAGCCTGTGATG 3′; *IL-1β* Fwd: 5′ AGCTGATGGCCCTAAACAGA 3′, *IL-1β* Rev: 5′ TCTTTCAACACGCAGGACAG 3′; *TNF-α* Fwd: 5′ AGCCCATGTTGTAGCAAACC 3′, *TNF-α* Rev: 5′ CCAAAGTAGACCTGCCCAGA 3′. Real-time PCR was performed on an ABI Prism® 7500 fast thermal cycler (Applied Biosystems, Foster, CA, USA). Each sample was run in triplicate in a final volume of 25 μl containing 1 μl of template (1:10 dilution), 10 pmol of each primer and 12 μl of Power SYBR® Green PCR master mix (Applied Biosystems). The real-time PCR results were presented as change in expression relative to control using target gene C_T values normalized to that of 18S gene C_T values based on the comparative C_T method [41].

IL-1 β, PGE_2 and TNF α assays

Cells (1×10^5) were treated with either HIV, nano-curcumin, AZT, soluble-curcumin or apotransferrin for 4 h. Levels of IL-1β, PGE_2 and TNF-α in the culture media were quantified using ELISA kits (R&D Systems, Minneapolis, MN, USA). The protocol was followed as per the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using SPSS Statistics 16 (SPSS, Chicago, IL). Data are presented as mean ± S.D. Differences between groups were evaluated using Students's *t* test or one-way analysis of variance (ANOVA) with Tukey's post-hoc analysis. All experiments were repeated three times, unless otherwise specified. Values of $P<0.05$ were considered to be statistically significant.

Supporting Information

Figure S1 Data in Figure 2 presented in a population of cells to show overall curcumin localization in a population of cells.
(TIF)

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Author Contributions

Planned inflammatory studies: RCR. Conceived and designed anti-HIV, nanoparticle formulation, localization studies and Topoisomerase II experiments in the study: AKK. Performed the experiments: UG RKC GK. Analyzed the data: AKK RCR. Wrote the paper: AKK RCR.

References

1. Sharma OP (1976) Antioxidant activity of curcumin and related compounds. Biochem Pharmacol 25: 1811–1812.
2. Srimal RC, Dhawan BN (1973) Pharmacology of diferuloyl methane (curcumin), a non-steroidal anti-inflammatory agent. J Pharm Pharmacol 25: 447–452.
3. Mahady GB, Pendland SL, Yun G, Lu ZZ (2002) Turmeric (Curcuma longa) and curcumin inhibit the growth of Helicobacter pylori, a group 1 carcinogen. Anticancer Res 22: 4179–4181.
4. Kuttan R, Bhanumathy P, Nirmala K, George MC (1985) Potential anticancer activity of turmeric (Curcuma longa). Cancer Lett 29: 197–202.
5. Kiso Y, Suzuki Y, Watanabe N, Oshima Y, Hikino H (1983) Antihepatotoxic Principles of Curcuma longa Rhizomes1. Planta Medica 49: 185–187.
6. Venkatesan N, Punithavathi D, Arumugam V (2000) Curcumin prevents adriamycin nephrotoxicity in rats. British J Pharmacol 129: 231–234.
7. Srivastava R, Dikshit M, Srimal RC, Dhawan BN (1985) Anti-thrombotic effect of curcumin. Thrombosis Res 40: 413–417.
8. Nirmala C, Puvanakrishnan R (1996) Protective role of curcumin against isoproterenol induced myocardial infarction in rats. Mol Cell Biochem 159: 85–93.
9. Babu PS, Srinivasan K (1997) Hypolipidemic action of curcumin, the active principle of turmeric (Curcuma longa) in streptozotocin induced diabetic rats. Mol Cell Biochem 166: 169–175.
10. Deodhar SD, Sethi R, Srimal RC (1980) Preliminary study on antirheumatic activity of curcumin (diferuloyl methane). Ind J Med Res 71: 632–634.
11. Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB (2007) Bioavailability of curcumin: problems and promises. Mol Pharm 4: 807–818.
12. Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, et al. (2001) Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res 21: 2895–2900.
13. Garcea G, Jones DJ, Singh R, Dennison AR, Farmer PB, et al. (2004) Detection of curcumin and its metabolites in hepatic tissue and portal blood of patients following oral administration. Br J Cancer 90: 1011–1015.
14. Shoba G, Joy D, Joseph T, Majeed M, Rajendran R, et al. (1998) Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. Planta Medica 64: 353–356.
15. Li L, Braiteh FS, Kurzrock R (2005) Liposome-encapsulated curcumin: in vitro and in vivo effects on proliferation, apoptosis, signaling, and angiogenesis. Cancer 104: 1322–1331.
16. Padhye S, Chavan D, Pandey S, Deshpande J, Swamy KV, et al. (2010) Perspectives on chemopreventive and therapeutic potential of curcumin analogs in medicinal chemistry. Mini Rev Med Chem 10: 372–387.
17. Qian ZM, Li H, Sun H, Ho K (2002) Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. Pharmacol Rev 54: 561–587.
18. Thorstensen K, Romslo I (1993) The transferrin receptor: its diagnostic value and its potential as therapeutic target. Scand J Clin Lab Inv Supplement 215: 113–120.
19. Sai Krishna AD, Mandraju RK, Kishore G, Kondapi AK (2009) An Efficient Targeted Drug Delivery through Apotransferrin Loaded Nanoparticles. PLoS One 4: e7240.
20. Savarino A, Calosso L, Piragino A, Martini C, Gennero L, et al. (1999) Modulation of surface transferrin receptors in lymphoid cells de novo infected with human immunodeficiency virus type-1. Cell Biochem and Func 17: 47–55.
21. Mandraju RK, Kondapi AK (2007) Regulation of Topoisomerase II a and b in uninfected and HIV-1 infected Neuroblastoma and Astrocytoma cells: Involvement of Distinct nordihydroguaretic acid sensitive inflammatory pathways. Arch Biochem Biophys 461: 40–49.
22. Ratul Kumar D, Naresh K, Utpal B (2010) Encapsulation of curcumin in alginate-chitosan-pluronic composite nanoparticles for delivery to cancer cells. Nanomed: Nanotech, Biol Med 6: 153–160.
23. Bisht S, Feldmann G, Soni S, Ravi R, Karikar C, et al. (2007) Polymeric nanoparticle-encapsulated curcumin (''nanocurcumin''): a novel strategy for human cancer therapy. J Nanobiotech 5: 3.
24. Sun A, Shoji M, Lu YJ, Liotta DC, Snyder JP (2006) Synthesis of EF24-tripeptide chloromethyl ketone: a novel curcumin-related anticancer drug delivery system. J Med Chem 49: 3153–3158.
25. Olano JP, Borucki MJ, Wen JW, Haque AK (1995) Massive hepatic steatosis and lactic acidosis in a patient with AIDS who was recieving zidovudine. Clin Infect Dis 21: 973–976.
26. Sinnwell TM, Sivakumar K, Soueidan S, Jay C, Frank JA, et al. (1995) Metabolic abnormalities in skeletal muscle of patients receiving zidovudine therapy observed by ^{31}P *in vivo* magnetic resonance spectroscopy. J Clin Invest 96: 126–131.
27. Deveaud C, Beauvoit B, Hagry S, Galinier A, Carriere A, et al. (2005) Site specific alterations of adipose tissue mitochondria in 3′-azido-3′-deoxythymidine (AZT)-treated rats: An early stage in lipodystrophy. Biochem Pharmacol 70: 90–101.
28. Mhiri C, Baudrimont M, Bonne G, Geny C, Degoul F, et al. (1991) Zidovudine myopathy: a distinctive disorder associated with mitochondrial dysfunction. Annu Neurol 29: 606–614.
29. Moh R, Danel C, Sorho S, Sauvageot D, Anzian A, et al. (2005) Haematological changes in adults recieving a zidovudine-containing HAART regimen in combination with cotrimoxazole in *Cote d'Ivoire*. Antivir Ther 10: 615–624.
30. Barthelemy S, Vergnes L, Moynier M, Guyot D, Labidalle S, et al. (1998) Curcumin and curcumin derivatives inhibit Tat-mediated transactivation of type 1 human immunodeficiency virus long terminal repeat. Res Virol 149: 43–52.
31. Li CJ, Zhang LJ, Dezube BJ, Crumpacker CS, Pardee AB (1993) Three inhibitors of type 1 human immunodeficiency virus long terminal repeat-directed gene expression and virus replication. Proc Natl Acad Sci USA 90: 1839–1842.
32. Sui Z, Salto R, Li J, Craik C, Ortiz de Montellano PR (1993) Inhibition of the HIV-1 and HIV-2 proteases by curcumin and curcumin boron complexes. Bioorg Med Chem 1: 415–422.
33. Taher MM, Lammering G, Hershey C, Valerie K (2003) Curcumin inhibits ultraviolet light induced human immunodeficiency virus gene expression. Mol Cell Biochem 254: 289–297.
34. Mazumder A, Raghavan K, Weinstein J, Kohn KW, Pommier Y (1995) Inhibition of human immunodeficiency virus type-1 integrase by curcumin. Biochem Pharmacol 49: 1165–1170.
35. Kondapi AK, Satyanarayana N, Saikrishna AD (2006) A study of the topoisomerase II activity in HIV-1 replication using the ferrocene derivatives as probes. Arch Biochem Biophys 450: 123–132.
36. Bouillé P, Subra F, Mouscadet JF, Auclair C (1999) Antisense-mediated repression of DNA topoisomerase II expression leads to an impairment of HIV-1 replicative cycle. J Mol Biol 285: 945–954.
37. Pruss D, Bushman FD, Wolffe AP (1994) Human immunodeficiency virus integrase directs integration to sites of severe DNA distortion within the nucleosome core. Proceedings of the National Academy of Sciences of the United States of America 91: 5913–5917.
38. Pruss D, Reeves R, Bushman FD, Wolffe AP (1994) The influence of DNA and nucleosome structure on integration events directed by HIV integrase. Journal of Biological Chemistry 269: 25031–25041.
39. Martin-Cordero C, Lopez-Lazaro M, Galvez M, Ayuso MJ (2003) Curcumin as a DNA topoisomerase II poison. J Enzyme Inhib Med Chem 18: 505–509.
40. Smith SD, Shatsky M, Cohen PS, Warnke R, Link MP, et al. (1984) Monoclonal antibody and enzymatic profiles of human malignant T-lymphoid cells and derived cell lines. Cancer Research 44: 5657–5660.
41. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3: 1101–1108.