Research Article

Synthesis, Characterization and Hepatoprotective Activity of Neem Gold Nanoparticles for Improved Efficacy and Sustained Drug Release Profile of Azidothymidine

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ABSTRACT

HIV is a retrovirus which belongs to the family of lentiviruses. From the beginning of the HIV epidemic almost 75 million people have been infected and about 36 million people have died of HIV. Globally, 35.3 million people were living with HIV at the end of 2012. Gold nanoparticles *(A. indica*-GNPs) were synthesized from leaf extract of *Azadirachta indica* by microwave assistance. Surface chemistry of monodispersed A.*indica* GNPs (30-40nm) was explored as natural linkers for attachment of AZT and verified by infra-red spectroscopy. *A.indica GNP-AZT* nanoconjugate was found to exhibit 94.6% drug loading capacity and followed 1st order of release kinetics at physiological pH. Hepato-protective property of *A.indica* GNPs was studied by cytokine analysis on an *in vitro* model of primary co-culture of rat hepatocyte-kupffer cells by ELISA. *A. indica* GNPs demonstrated upregulation of Interleukin (IL)-10 and downregulation of Tumor necrosis factor (TNF)-α with controlled IL-6 secretion in lipopolysaccharide (LPS) induced co-cultures; thereby persuading optimal interplay of pro and anti-inflammatory cytokines and rendered hepato-protectivity. Quantification of p24 antigen done by ELISA for assessing anti-viral activity suggested a 20% increment ($P<0.05$) in anti-viral efficacy of AZT when conjugated to *A. indica* GNPs. Thus biogenic A.indica GNPs can be looked upon as an effective candidate to improvise the treatment strategies against HIV infection.

Keywords: Hepatoprotective, Neem Gold Nanoparticles, Hepatocyte-Kupffer cell co-culture model, IL-6, IL-10, TNF-α, Azidothymidine.

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) of humans is caused by human immunodeficiency viruses (HIV) types 1 and 2 (HIV-1 and HIV-2). HIV is a retrovirus which belongs to the family of lentiviruses. AIDS is a condition in humans where immune system begins to fail which leads to life-threatening opportunistic infections or malignancies associated with the progressive failure of the immune system.[1,2] From the beginning of the HIV epidemic almost 75 million people have been infected and about 36 million people have died of HIV. Globally, 35.3 million people were living with HIV at the end of 2012.[3] Anti-retroviral therapy (ART) is the mainstay for HIV treatment.[4] Currently, nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) anti-HIV class of drugs are exercised in developing countries. First line ART drugs like Azidothymidine (AZT) are effective in treating patient with HIV however, this AZT has some significant drawbacks such as reduced bioavailability, poor permeability, rapid clearance and undesirable side effects like severe (sometimes fatal) liver and blood problems.[5] Recent studies proved that engineered nano-structures can contribute significantly for the development of current antiretroviral therapy.[6] Over the decade, metallic nanoparticles are seen to span in different fields of biomedical sciences like drug delivery.[7] Among metal nanoparticles, gold nanoparticles are the most ventured nanostructures in biological studies.[8] Gold nanoparticles (GNP) are favourable for their facile synthesis, ease of functionalization, biocompatibility and inherent non-toxicity. The unique chemical and physical properties of GNP provide versatility in delivery methods and tunability of surface properties.[9,10] A range of eco-friendly approaches such as plant mediated biosynthesis of nanoparticles has gained special impetus owing to its simplicity, rapid rate of synthesis, high stability and ease of surface decoration.[11] In recent years, numerous plants such as neem, Aloe vera etc.[12,13] Plants like neem are documented for various medicinal properties like anti-viral, anti-bacterial, anti-oxidant and immune-modulatory activities.[14,15]

In this study, we aim to synthesize nanoparticles using *Azadirachta indica* plants extract where we are expecting to impart *Azadirachta indica* qualities onto the nanoparticles and improve their efficacy.

MATERIALS AND METHODS

Reagents

Chloroauric acid, Azidothymidine and all other chemicals were purchased from Sigma Aldrich, USA. Anti-viral activity was assessed using zeptometrix RETRO-TEK HIV-1 P24 antigen ELISA kit. Kupffer cells (KC) and hepatocytes for liver inflammation *in vitro* model were purchased from Life Technologies. HIV-1_{Ba-L} virus was provided by NIH AIDS reference reagent program. All experiments were performed in nano-pure water (18 MΩ). Glassware for nanoparticles synthesis were washed prior starting

the experiments with Aqua regia to remove traces of metal contaminant.

Methodology

Synthesis of biogenic GNP (*A.indica GNP*)

All the procedures were conducted in accordance with guidelines under animal protocols approved by the Institutional Animal Ethics Committee (Ethics Committee Approval No. HITRT/IEC/12/2011 dated 24th January 2011). *A. indica* plant leaf extract was used for synthesis of gold nanoparticles. For preparing the plant extract the leaves were crushed in distilled water, centrifuged and the supernatant was filtered using Whatman filter paper to obtain a clear solution. The extract was stored at 4°C till further use. A stock solution of 50,000 ppm chloroauric acid ($HAuCl_4$) was prepared and diluted as per pre-requisite for the experiment. For synthesis of *A. indica* GNP, a concentration of 100 ppm gold salt was added to the solution of diluted plant extract from the stock solution at inherent pH (5.7) and heated by microwave assistance. Spectroscopic measurements were executed using a dual beam Varian Cary® UV-Vis spectrophotometer. Morphological features of the synthesized *A. indica GNP* were studied by Transmission electron microscope (TEM) (Zeiss Microimaging GmbH, Germany) and nanoparticles tracking analysis software. Elemental analysis was carried by energy dispersive spectroscopy (EDS). For comparative cytokine analysis chemically determined gold nanoparticles (cGNP) were synthesized by standard Turkevich citrate reduction method.[16]

Cytotoxicity studies

Biocompatibility of *A. indica GNPs* for therapeutic access was inspected by standard tissue culture based colorimetric method. Briefly, the effects of neem extract, *A. indica GNP* and cGNP were studied on Vero cell line using MTT formazan assay. Vero cell lines were maintained in MEM medium supplemented with 10% (v/v) Fetal bovine serum at 37°C in 5% CO_2. Cells were seeded into 96 flat bottom well plates at concentration of $4x10^5$cells/ml. After 24hr, the medium was changed and different dilutions of above mentioned test compounds were added into wells respectively. After overnight incubation the cells were washed once with PBS and incubated with MTT dye (5mg/ml) for 4hrs at 37°C. After 4hrs of incubation, Dimethyl sulfoxide (DMSO) was added to each well and read at 570nm using a microplate reader (Biotek India Ltd.). Transition of colour change from yellow to purple is directly proportional to cellular mitochondrial metabolism and cell viability. Percentage cell viability was determined by following equation:

$$\text{Percent (\%) Cell Viability} = \frac{S - N}{P - N} \times 100 \quad \ldots\ldots\ldots\ldots\ldots (1)$$

Where, S = Sample reading, N= Negative control (cell with DMSO) reading and P = Positive control (only cells) reading.

Hepato-protective studies

Cytokine secretion and intercellular communication between Kupffer cells (KCs) and Hepatocytes (HCs) similar to *in vivo* conditions is seen best when direct co-culturing of both the cells is done; as suggested in various *in vitro* models. [17] Thus hepato-protective potential of the nanoparticles was tested on direct co-culture of primary HCs and KCs cells of rat *in vitro*.

Co-culturing of rat hepatocytes and rat Kupffer cells

Primary Sprague Dawley rat Kupffer cells and hepatocytes were commercially obtained from Invitrogen™. Kupffer cells were seeded with half the density of hepatocytes and cultured for 48hr prior to all experiments. The Hepatocyte/Kupffer cell co-culture was plated as per manufacturers' instructions.

Lipopolysaccharide based inflammation & interaction of co-cultures with nanoparticles

Kupffer cell activation was induced by treating co-cultures with LPS (1µg/ml) for 24 hrs. Following treatment, all cultures were treated with different dilutions (1, 1:10, 1:100) of nanoparticles- (*A.indica GNP* and cGNP) and later analyzed for cytokine production (i.e. TNF-alpha (TNF-α), Interleukin–6 (IL-6) and Interleukin-10 (IL-10)) by ELISA. Aliquots of the cell culture supernatant were drawn at a time frame of 24hours and 48 hours after induction with nanoparticles. Parallel to this assay, the cell control (co-culture without LPS induction), as well as LPS control (LPS induced cultures without any test compounds) were also monitored and supernatant was aliquoted at the above mentioned time points. Cytokine estimation was performed using Rat Cytokine Quantitation kits (manufactured by M/s. Diaclone, France). The assay protocol was carried out as per the manufacturers' instructions.

Synthesis of *A.indica GNP*-AZT conjugate

Here we report drug attachment at room temperature (RT, 22-24°C). AZT was added to 10ml of *A.indica GNP* (100mg/L) aqueous solution at a final concentration of 100µg/ml. The mixture was sealed and kept under continuous stirring at RT for 4 hours. The whole conjugate was characterized using UV-Vis spectrophotometry and Fourier transformed infrared spectroscopy (FTIR).

Percent Drug loading efficiency (%DLE)

The consequential *A. indica GNP*-AZT conjugate was purified using extensive dialysis against nano-pure water for 1 hours using a dialysis membrane tube (MW cut-off of 3000 Da) to remove and quantitate the excess amount of unbound AZT molecules. Drug loading efficiency onto *A.indica GNPs* was calculated using following equation:

$$\text{Drug loading efficiency} = \frac{\text{Total amount of drug loaded} - \text{Free drug}}{\text{Theoretical amount of drug loaded}} \times 100 \quad \ldots\ldots\ldots\ldots (2)$$

In vitro release of AZT

For drug release examination, 5 ml of *A. indica GNP*-AZT complex was sealed in a two different dialysis tubes and the entire system was kept at 37ºC in 50 ml phosphate buffer solution at pH 7.2 under mild stirring to comprehend drug release profile. To measure the drug release content, 3 ml of sample was periodically removed and replaced with an equivalent volume of the phosphate buffer solution. The amount of released AZT was analyzed with spectrophotometer at 266nm. The experiments were performed in triplicate for each of the samples.

Drug release kinetics

With an objective to design a nano-drug architecture with sustained drug release profile; that can ease dosage frequency and improvise overall HIV disease management, we relate the *in vitro* release data to various mathematical models to predict the drug release mechanism and kinetics. Correlation coefficient (R) was calculated for each release model and the best fit release model was selected with highest 'R' value.

Assessment of enhanced anti-HIV activity

The efficacy of our nano-conjugate was determined against R5 HIV-1_{Ba-L}. Activation of peripheral blood mononuclear cells (PHA-PBMCs) by phytohaemagglutinin (PHA) and interleukin-2 (IL-2) and Drug susceptibility assay to determine the anti-viral activity of the compounds was carried out as per standard protocols of division of AIDS National Institute of Allergy and Infectious Diseases, National Institutes of Health. [18,19,20]

Statistical analysis

All data were analyzed using the GraphPad Prism software (Version 3.0). Anti-viral experiment was repeated three times and the data were analyzed by two-tailed student's t-test. A P-value less than 0.05 considered statistically significant.

RESULTS AND DISCUSSION

Nanoparticle characterization

Conversion of Au^{+3} ions of chloroauric acid to gold nanoparticles (Au^{0}) by diluted neem leaf extract was followed by a transition of solution from greenish yellow to ruby red colour and displayed a sharp peak at 535nm (Fig 1a). Appearance of an intense peak at 535nm is typical of gold nanoparticles due to quantum confinement effect and results as a consequence of Surface Plasmon Resonance (SPR) exhibited by the oscillating electrons present on the surface of these nanoparticles which resonate upon interaction with impinged electromagnetic waves. [21,22] Mono-dispersivity and size of nanoparticles was determined by NANOSIGHT's nanoparticle tracking analysis (NTA); versions 2.3 build 0027 and showed majority of nanoparticles cluster (in green and red) in size range of 30-40nm with high amount of mono-dispersivity (Fig 1c).

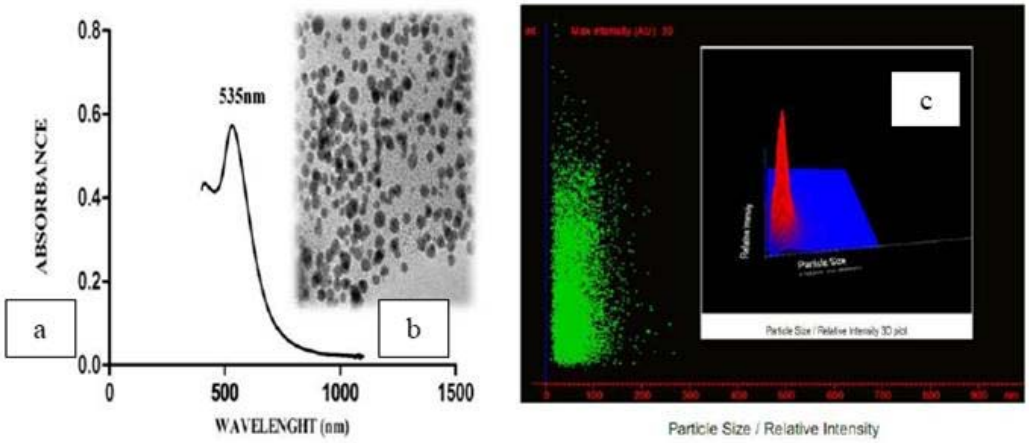

Figure 1: (a) Sharp UV-VIS absorption spectrum of GNPs synthesized using *A.indica* leaves at 535nm, (b) TEM images of biogenic GNP synthesized using *A.indica* leaf extract and (c) Evaluation of particle size vs. relative intensity by NTA analysis

TEM images further confirmed formation of spherical monodispersed nanoparticles in size range of 30-40 nm (Fig 1b) which is in accordance with UV-VIS spectroscopy and NTA interpretations. The time required for the colour change was less than a minute; indicating rapid synthesis of nanoparticles through dielectric loss resulting from conversion of radiation energy to thermal energy. Previous reported cases of synthesis of *A.indica GNP* from *A.indica* leaf extract displayed polydispersivity in size of nanoparticles and that these different sized nanoparticles had to be separated by centrifugation before employing them for therapeutic or diagnostic application.[23] Microwave assistance in our case provided faster and more uniform heating profile than conventional heating and resulted in fine monodispersed nanoparticles.

Cell Viability

cGNP showed a progressive decrease in cell survival with respect to increase in concentration with (50 percent cytotoxic concentration) CC_{50} value of 47.14 ± 1.07 ppm.

However, *A.indica* GNP were not found toxic to cells and displayed above 90% cell survival at all concentrations. Surface ingredients of *A.indica GNP* that are borrowed from *A. indica* might have some cell growth factors and promoters that may have attributed to such excellent biocompatibility to *A. indica* GNP (Fig 2a).

Hepato-protective studies

Increasing dilutions of chemically synthesized and *A.indica* GNPs were examined for their effect on cytokine profile (Fig 2b). For comparative cytokine kinetics we here present only one significant dilution of each test compounds i.e. 1:10. Co-cultures treated with biogenic nanoparticles (*A.indica GNPs*) showed significant rise in IL-6 levels in the first 24 hours of induction (47.6 pg/ml) as compared to the positive control induced with LPS (44.6 pg/ml). However, after 48 hours IL-6 levels remained steady in *A.indica GNP* treated cultures (46.8 pg/ml) as compared to the drastic elevated levels seen in LPS induced cultures (54.49 pg/ml). Citrate synthesized GNP did not display any substantial change in IL-6 concentration and remained fairly constant. Comparative analysis between both the test nanomolecules and positive control suggests that nanoparticles play a key role in controlling IL-6 levels. This can be clearly intrapolated form the latter half of the induction period, where both the nanoparticles does not allow IL-6 levels to elevate as compared to LPS induced cultures. Another interesting inference that sprouts out is the initial rise of IL-6 levels in *A. indica GNPs* treated co-cultures. This early rise in IL-6 levels may be associated with activation of anti-inflammatory cytokines like IL-10.

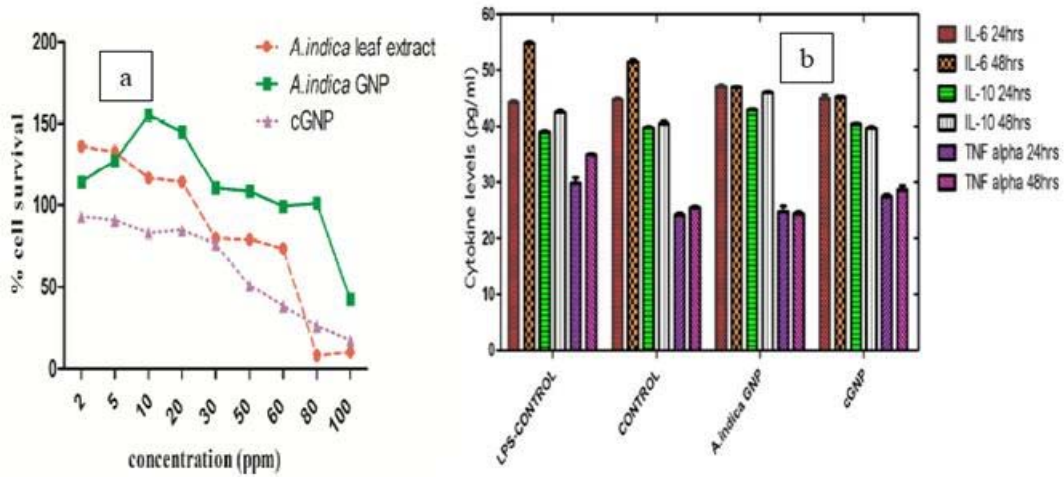

Figure 2: (a) Comparative effect of neem extract, *A.indica* GNPs and cGNPs on cell survival studied by MTT assay. The results are mean ± SD of two separate experiments done in duplicates. (b) Expression of cytokines i.e. IL-6, IL-10 and TNF-α by LPS induced rat Kupffer-hepatocyte co-culture interacted by gold nanoparticles respectively. The results are mean ± SD of three separate experiments done in duplicates.

A. indica GNP treated co-culture displayed marked increase in IL-10 (45.9 pg/ml) as compared to citrate GNP (39.9 pg/ml); clearly suggesting a hepato-protective property of *A.indica GNP* against the inflammation induced by LPS. As consistent with its purported role of proximal mediator of inflammation; the induction of TNF-α was very rapid in LPS positive control post 24 and 48 hours of induction. In contrast to positive control, co-cultures exposed to GNPs showed a diminution in the levels of this pro-inflammatory cytokine. However, a noteworthy down regulation of TNF-α was seen in *A.indica GNP* treated co-cultures. It can be assumed that the components of *A.indica* leaf extract along with an active participation in synthesis and stabilisation of GNP, might also impart anti-inflammatory features onto *A. indica GNPs*.

Synthesis of drug-nano conjugate

The primary investigation to understand interaction of drug with nanoparticles was done by analyzing the spectral shift of *A. indica GNPs* after interacting with the drug. The SPR of metal nanoparticles is very sensitive to any change in immediate environment of nanoparticles.[24] After the conjugation procedure, the *A. indica GNP* showed a dampening in their characteristic SPR peak with a prominent blue shift of 7nm (from 535nm to 528nm) indicating change in the surface chemistry of nanoparticles. Further examination to figure out probable interaction was executed by FTIR spectra. FTIR spectra of gold nanoparticles synthesized from leaf extract of *A.indica* revealed presence of various functional groups. A prominent peak at 1633 cm^{-1} suggests –NH bend, which can be speculated to be of amides and amines. Mild peaks at 2922 cm^{-1} and 2855 cm^{-1} correspond to functional groups like –SH, -OH and –CH, whereas a small but prominent peak at 2429 cm^{-1} and 1382 cm^{-1} propose –OH bend of carboxylic moieties. These functional groups may belong to proteins, aldehydes and various surfactants of neem extract that have been impregnated onto *A. indica GNPs* during synthesis. Anchoring of AZT with *A. indica GNP* was studied by comparing FTIR spectra of *A. indica GNP*, AZT and *A. indica GNP*-AZT conjugate.

Comparing these FTIR data highlighted three important peak characteristics: Disappearance of the azide peak (2106 cm^{-1}) of AZT from the conjugate spectra, peak shifts corresponding to –OH bend of carboxylic acid (1429 to 1431 cm^{-1} and 1382 to 1383 cm^{-1}) with increase in peak transmittance and persistence of peak 3910 cm^{-1} and 2922cm^{-1} corresponding to –OH and $–CH_3$ functional groups of AZT. Thus the interaction can be contemplated to occur between carboxylic acid and azide functional group of *A. indica GNP* and AZT respectively via covalent bonding. Persistence of 3910 cm^{-1} and 2922cm^{-1} peak in conjugate further clarify that –OH and $–CH_3$ functional groups of AZT did not participate in conjugation reaction.

%DLE and drug release kinetics

In the process of designing an efficient carrier system for drug delivery, it is imperative that the candidate carries high amount of drug payload. From equation 2, % DLE was found to be 94.6%. Such high amount of payload carrying capacity might be due to high surface to volume ratio of spherical *A. indica GNP*s, which makes them favourable candidates for drug delivery system. Kinetics of drug release was investigated by statistical model dependent method at physiological pH (7.2). Model suggesting highest regression co-efficient (R^2) was considered best fit for release profile. The log value of cumulative percent drug remaining to be released w.r.t. to time (Fig 3a). Whereas table 1, summarizes the r^2 values of each model. From the table 1, it can be concluded that AZT conjugated to *A.indica GNP*s follow 1st order release kinetics at pH 7.2. The amount of drug released would be constant and dependent upon the concentration of drug remaining in the nano-conjugate system.

Table 1: Summarized R^2 values of various models applied for drug release kinetics.

No.	Models	R^2 value at pH 7.2
1	Zero Order	0.761
2	First Order	0.81
3	Higuchi	0.76
4	Hixson-Crowel	0.78

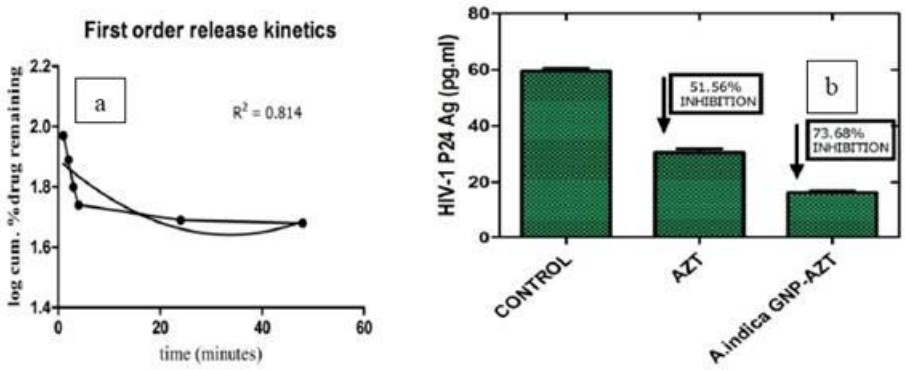

Figure 3: (a) Drug release profile of AZT onto *A.indica GNP*s following 1st order release kinetics and (b) Effect of *A. indica GNP*-AZT nano-conjugate and AZT alone on p24 production on HIV-1 infected PBMC.

Anti-HIV activity

The ultimate goal of our study was to evaluate if our compound showed an improvised anti-viral activity. PBMCs infected with HIV-1_{Ba-L} were challenged with *A. indica GNP*-AZT nano-conjugate and AZT of same concentration separately and analysed for the reduction in HIV p24 antigen production by ELISA. Comparative analysis (Fig 3b) revealed a noteworthy reduction in p24 antigen production in nano-conjugate (15.79 pg/ml) challenged culture as compared to p24 levels in cultures challenged with AZT (29.06 pg/ml) alone. A substantial 20% increase in activity was seen when AZT was fired with *A.indica GNP*s. The results obtained were found significant after applying two-tailed Students t' test with P value < 0.05. A protein and polypeptide rich surface chemistry of *A. indica* GNP might have increased cellular association of the nano-conjugate; enhancing their ability to deliver drugs more efficiently and thus increasing the overall efficacy of the therapy.

CONCLUSION

Biogenic GNPs synthesized from *A. indica* showed some remarkable attributes like ease of functionalization, high drug loading capacity with sustained release profile. Understanding the up-regulation of IL-10 and optimal control of IL-6 and TNF-α by *A.indica GNP*s, it can be hypothesized that these biogenic nanoparticles might inhibit nuclear factor kappa β (NFKβ) and mitogen activated protein kinase (MPPK); thereby persuading optimal interplay of pro and anti-inflammatory cytokines and rendering hepato-protectivity. These biogenic drug delivery vehicles therefore get an upper edge as compared to their chemical counterparts in performing multiple functions along with drug delivery. This became apparent when biogenic GNPs were found to augment efficacy of AZT along with holding the capacity to reduce its hepato-toxicity. Thus biogenic GNPs can be looked upon as a potent tool for modulating the pharmacokinetics of the incorporated molecule and

improvise the overall management of HIV/AIDS treatment.

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