Lamivudine-conjugated and efavirenz-loaded G2 dendrimers: Novel anti-retroviral nano drug delivery systems

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Abstract
Infection with human immunodeficiency virus (HIV)-1 causes immunological disorders and death worldwide which needs to be further assisted by novel anti-retroviral drug delivery systems. Consequently, finding newer anti-retroviral pharmaceuticals by using biocompatible, biodegradable nanomaterials comprising a nanoparticle as core and a therapeutic agent is of high global interest. In this experiment, a second generation of a negatively charged nano-biopolymer linear globular G2 dendrimer was carefully conjugated and loaded with well-known anti-HIV drugs lamivudine and efavirenz, respectively. They were characterised by a variety of analytical methods such as Zetasizer, Fourier-transform infrared spectroscopy, elemental analysis and liquid chromatography-mass spectroscopy. Additionally, conjugated lamivudine and loaded efavirenz with globular PEGylated G2 dendrimer were tested on an HEK293 T cell infected by single-cycle replicable HIV-1 virion and evaluated using XTT test and HIV-1 P24 protein load. The results showed that lamivudine-conjugated G2 significantly decreased retroviral activity without any cell toxicity. This effect was more or less observed by efavirenz-loaded G2. These nano-constructs are strongly suggested for further in vivo anti-HIV assays.

1 | INTRODUCTION

Human immunodeficiency virus (HIV) has brought about high rates of mortality, as much as a war fatality, since its start in 1981. Among the populations infected by HIV infection, HIV-1 subtype is a common cause of death [1–7]. There are enormous investigations on HIV/AIDS in different databases such as PubMed, National Institute of Health (NIH) and ISI web of Knowledge, which are not coherent. The absence of an effective treatment or an operative vaccine put HIV/AIDS in the category of impressive medical problems of our era. Anti-retroviral medical care such as highly active anti-retroviral therapy is still the basic pillar for HIV treatment [3–10]. In recent years, the development of investigations and applications in the field of nano-science has been improved [8]. Brilliant nanoparticles such as gold and silver nanoparticles are considered the most indicative of nano therapeutic purposes [9, 10]. Diverse nano particulate-based anti-retroviral applications, for example, gold nanoparticles, chitosan-based or poly (lactic-co-glycolic acid) (PLGA)-based even dendrimeric structures have been part of recent research studies. Their pharmaceutical advantages are that they are money saving, they blend effortlessly, have simple functionalisation, biocompatibility/biodegradability as well as an innate non-poisonous quality [9, 11–14]. Nanoparticle-based drug delivery systems may provide an opportunity to facilitate the eradication of viral load by delivering anti-viral drugs for therapeutic purposes (such as HIV-infected cells and viral reservoirs) [15–17].

Dendrimers were one of the nano-sized polymeric families of the nanoparticles. Based on their diverse molecular weights, generations and surface charges, they are divided into three main groups: positively charged (PAMAM), negatively charged (anionic linear globular: G2) and neutralised. The important and useful features of dendrimers are the presence of suitable space inside them, which is a good position to embed various hydrophobic and hydrophilic drug molecules. Also, the presence of numerous well-known functional groups at the surface of these spherical particles converts them into suitable carriers to bind various types of molecules or different ligands. In this way, they can help with targeting medications [18]. Negatively charged dendrimers, due to

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their unique properties and surface cellular adsorption, showed less cellular toxicity as well as higher effectiveness in lower molecular weight (G2 generation) compared to other groups [19].

Lamivudine is an effective anti-HIV pharmaceutical that is used for therapeutic purposes or prevention of HIV/AIDS [1]. Lamivudine was also successfully applied to treat other viral diseases such as the chronic Hepatitis B virus, while other applications were not favoured [20]. The effectiveness of lamivudine against both types of HIV, HIV-1 and HIV-2 was discussed in the literature [20]. Such anti-HIV pharmaceutical is generally employed in combination therapy with other anti-HIV drugs such as zidovudine and or abacavir [21]. Lamivudine is included as part of post-exposure prevention in persons who might be infected with HIV. Lamivudine is administered orally, with pharmaceutical dosage in the form of tablet or capsule [1]. Efavirenz, described as benzoxazinone derivative (\(2H\)-3,1-Benzoxazin-2-one,6-chloro-4-(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-(4S)), shows its anti-HIV activity by non-competitive binding to a hydrophobic pocket at the HIV reverse transcriptase, which provokes its conformational changes and leads to diminished enzyme activity. This drug does not need to be phosphorylated inside cells and makes a single daily dose viable. The application of nanoparticles as drug delivery vehicles is in the centre of attention for the studies on their effectiveness in anti-HIV drug delivery such as lamivudine, zidovudine etc. Concurrent use of nanoparticles like gold or chitosan or PLGA has been significantly increased. Such effect may decrease undesirable side effects such as cell toxicity as well as inefficient delivery of drugs into the infected cells [4–8]. Considering the desirable properties of negatively charged second generation PEGylated dendrimer, such as low cost of synthesis and water solubility characteristics, we designed for the first time to conjugate such dendrimeric structures to lamivudine and efavirenz. We characterised their structure and assessed their inhibitory effects on an in vitro model of HIV as a novel anti-retroviral cell delivery system.

2 | MATERIALS AND METHODS

All combinations for the arrangement of integrated nanosoluble including Sulfo-NHS, dimethyl sulfoxide (DMSO), N, N'-Dicyclohexylcarbodiimide (DCC), citrus extract, silver and polyethylene glycol (PEG) 600 were purchased from Merck, Germany. The pS Pax2, pmzNL4-3 or pMD2.G plasmids were set up for transfection individually. For sub-atomic cell tests, HEK293 cells (human embryonic kidney) were used and cultured in a Dulbecco’s modified Eagle’s medium (DMEM) essential medium and foetal bovin serum (FBS).

2.1 Synthesis of G2 anionic linear globular dendrimer

At first, two generations of dendrimers (G1, G2) were prepared according to the reported method [22]. Briefly, diacid poly (ethylene glycol) was chlorinated using thionyl chloride (SOCl2) and reacted with citric acid as the monomer to generate G1. Then, the G1 was coupled with citric acid to produce G2 with DCC in the pyridine medium. Subsequently, to purify the synthesised G2 dendrimer, it was run on the Sephadex G-75 column (Merk). The G2 solution previously deposited solid impurities such as DCC on filter paper and was transferred to the column and the purified solution was collected. This step was repeated and all the impurities were removed and the pure G2 dendrimer soluble in water was lyophilised. The G2 dendrimer structure was approved by verification tests such as Fourier-transform infrared spectroscopy (FTIR) and liquid chromatography–mass spectrometry (LC-MS) [22].

2.2 Preparation of drugs (lamivudine and efavirenz)-G2 dendrimer combinations

Due to the significantly increased tendency of lamivudine molecules in the oxygen atoms on the surface of the G2 dendrimer, the reaction and conjugation between lamivudine and dendrimer was possible using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC), which belongs to a carbodiimide family and sulfo-NHS/CaCl2 as catalysts and water resistance agents as well. In exceptional conditions using isolation and light protection, the reactant molecules mixture was stirred for at least one week. Then, the conjugated nanodrug was separated from other free un-reactant molecules such as those of lamivudine, EDC and sulfo-NHS/CaCl2 by dialysis using 500–1000 KD cut off (Spectrum Company). During the analyses the final product (liquid or lyophilised) was lastingly protected from light and air. The structure of the product was assessed by FTIR, mass spectroscopy and 1H nuclear magnetic resonance spectrum. It was also compared with G2 dendrimer data from previous publications. The size and charge of the nanoconjugate was assessed as well. The same procedure was performed for the combination of efavirenz-G2 dendrimer nanocomposite. However, all the evaluations confirmed the loading of drug in the dendrimer and not its conjugation.

2.3 Stability assessment of the combinations

The study of drug (lamivudine and efavirenz) release was carried out by dialysis with 500–1000 KD cut off. A certain amount of each drug combination with the G2 dendrimer (20 mg) was placed in a separate dialysis bag in a 50 ml PBS pH = 7.4 on a magnetic stirrer at room temperature. Finally, the concentration of the drug was measured at 254 nm.

2.4 Biological studies

2.4.1 Transfection of the plasmids into the HEK293 T cell line

Three plasmids, pS Pax2, PmzNL4-3 and pMD2.G, were used to produce HIV-1 single cycle replication (SCR) virions
They were responsible for encoding the HIV-1 Gag-Pro-Pol protein, production of proviruses without reverse transcriptase enzyme and vesicular stomatitis virus G glycoprotein (VSVG), respectively [25, 26]. The HEK293 T-cell line was cultured in a 6-well plate in DMEM, Sigma with FBS (15%), penicillin (100 U/ml), streptomycin (100 lg/ml), and glutamine (2 mmol/L) until it reached the number of approximately 1.2 × 10^6 cell/well. In a separate tube, 3 mg of plasmid mixture was dissolved in 150 μL incomplete culture medium. Then, 40 μL Lipofectin® reagent was added and incubated at room temperature for 10 min then mixed with 1 ml of complete medium. Then, the cell culture medium was replaced with 1 ml of this medium drop by drop. Next, the plate was incubated at 37°C, 5% CO₂. The supernatants were collected at 24, 48 and 72 h after incubation. The solutions were mixed and centrifuged for 2 h at 60,000 g 4°C. The resultant pellet virions were dissolved in appropriate volumes of RPMI 1640 through delicate blending overnight at 4°C to stop any incidental infection. Finally, they were evaluated by the P24 ELISA test (HIV p24 ELISA, BioMerieux, Capture P24 ELISA) and stored at −70°C for further utilisation [27].

2.5 | Evaluation of the nanodrugs’ effectiveness on cell viability and HIV replication

The anti-viral impacts of various concentrations were additionally tested. HEK-293 T cells were cultured in 96 well plates (5 × 10^3 cell/well). The SCR HIV virions and 600 ng P24 were added to each well. The nanodrugs and controls (drugs and vehicle) were added in different concentrations from 100 pM to 10 μM in triplicate. After 5 h, the wells were washed with 10% DMEM/FBS twice. After that, nanodrugs and controls were added again. The supernatants were collected after 72 h of incubation. To evaluate the cellular proliferation and CC₅₀ of the drugs, the XTT assay (Roche) was applied according to the manufacturer’s instruction. The XTT solution was added to each well (50 μL), and the plate was incubated at 37°C for 4 h. Then, cell absorptions (O.D) were read by an ELISA reader in 450 nm. Finally, P24 concentration of the supernatants was determined by an ELISA assay [27].

3 | RESULTS

3.1 | Lamivudine-conjugated and efavirenz-loaded G2 dendrimer characterisation

The process of G2 synthesis and lamivudine-G2 conjugation is shown in Figure 1. It shows two lamivudine molecules attached to different parts of the G2 dendrimer.

Comparison of the dynamic size of the dendrimer, the lamivudine-conjugated and efavirenz-loaded G2 dendrimer, obtained from a dynamic light scattering analysis showed 91 nm, 158 nm and 144 nm, respectively. Furthermore, these conjugations and loadings caused to change the zeta potential from −22 mV to −5.4 charge for the lamivudine-conjugated G2 dendrimer and −7.2 mV for the efavirenz-loaded one (Figure 2, Figure 3).

FTIR spectroscopy was used to study the surface functional groups of the lamivudine-G2 conjugate in the area of 400-4000 cm⁻¹. Figure 4 shows the FTIR spectrum of the lamivudine conjugated to the G2 dendrimer. The peaks at 2878 cm⁻¹ and 3413 cm⁻¹ are related to the aliphatic CH and–OH respectively. The presence of an amine group was confirmed by the appearance of a peak at 3573 cm⁻¹. The C = O peak that appeared at 1727 cm⁻¹ is related to the amide bond. Furthermore, the peak at 1439 cm⁻¹ is a dual bond of lamivudine structure.

The FTIR spectrum related to the loading of the efavirenz in G2 dendrimer is shown in Figure 5. The peaks 666 and 755 cm⁻¹ are related to the halogen elements (fluoride and chloride) in the efavirenz structure. The peak 1434 cm⁻¹ is related to the C = C of efavirenz. The peaks from 1728 cm⁻¹ are related to the C = O functional group. The widening of the peak at 3455 cm⁻¹ is related to the dendrimer OH groups. Also, the peak of 1954 cm⁻¹ is related to the triple bond in the efavirenz drug. Furthermore, the map elemental analysis of these two formulations confirmed the loading and conjugation of drugs to the dendrimer (Figure 6, Figure 7).

For the careful endorsement of the lamivudine conjugation to G2, LC/MS was applied. The contrasted extraordinary fracture designs in the lamivudine-G2 dendrimer confirmed the conjugation process. As shown in Figure 8, which highlighted the LC-MS of the lamivudine conjugated to the G2 dendrimer, the 657 m/z peak actually confirms the conjugation of lamivudine to the G2 dendrimer. Also, the 952 m/z peak represents a lamivudine molecule that is attached to a part of the G2 dendrimer.

3.2 | Stability assessment

No significant release of lamivudine or efavirenz was observed during 6 and 12 h of evaluation at any pH or medium. These findings indicated an acceptable stability for such nanoparticle-based pharmaceutical delivery approach. After 24 h a slight release, about 7%, was found, which is only related to the dendrimer instability at pH 4 in the DMSO matrix.

3.3 | Cytotoxic effects of nanodrugs on HEK293 T cells

The effect of original drugs and their combinations with the G2 dendrimer was studied on the HEK293 T-cell viability at different concentrations. They exhibited various cell survival effects. Regarding lamivudine, the nanodrug performed similar to the original drug at concentrations of 100 pM and 1 nM, while in the rest of the concentrations the nanodrug performed stronger than the original drug in terms of cell survival (Figure 9). With regards to efavirenz, most of the drug and nanodrug concentrations exhibited different cell survival effects. Of these concentrations, 10 and 100 μM were more effective
concentrations of the nanodrug. It should be noted that the original drug exhibited more remarkable cell survival than the nanodrug only at 10 and 100 nM concentrations (Figure 10).

In all these tests, the carrier, G2 dendrimer alone, was also tested in parallel and showed no toxicity effects on cell viability and concentration.

3.4 | Inhibition of HIV p24 production (replication assay)

The HIV replication was examined utilising VSVG pseudovirus. The SCR HIV-1 virions are only ready to replicate for 1 cycle. The drugs and nanodrugs were investigated in
terms of the ability to inhibit HIV viral replication in different concentrations. As shown in Figure 11, the nanodrug of lamivudine at 1, 10 and 100 nM concentrations was approximately 2, 2 and 5 times stronger in its inhibitory function on the HIV replication than lamivudine. The IC$_{50}$ of lamivudine and its conjugated form to the G2 dendrimer were 960 and 94 nM, respectively. The concentrations 100 pM, 1 and 10 nM were more effective in the nanodrug of efavirenz than that of the original drug. At higher concentrations, the drug and nanodrug exhibited similar anti-viral effects. The IC$_{50}$ of efavirenz and its loaded form in the G2 dendrimer were 8 nM and 900 pM, respectively (Figure 12). The carrier, G2 dendrimer alone, was unable to affect the viral replication.

4 | DISCUSSION

The main objectives of this study were to develop and evaluate the physico-chemical properties of conjugated and loaded nanodrugs of lamivudine and efavirenz with the PEG-citrate G2 dendrimer. We aimed at improving their water solubility,
stability, targeted delivery and fulfilment of their biological effects in terms of HIV replication inhibitory effect.

In most reports, PAMAM dendrimers, which show toxicity, have been used for a variety of purposes. This cytotoxicity is strongly associated with the functions of end-dendrimer groups. Positively charged groups, such as amines, show dose-dependent toxicity. To overcome this toxicity, the use of biocompatible materials or cell monomers of dendrimers has been suggested. The dendrimer studied in this report is not only completely biocompatible but also has a very high aqueous solubility. And due to its negative charge, it has no toxicity level for the cell and is completely soluble in water. Also, the presence of citric acid in the structure of this macromolecule can enter the citric acid cycle [28]. In this method, DCC was used as a crosslinker, which has much less toxicity, and the use of toxic substances such as...
dichloromethane and pyridine was eliminated. PEG also reacts easily with citric acid, which is considered as the nucleus in this dendrimer. The results of various characterisation analyzes confirmed the successful synthesis of this structure [29].

The results of the physico-chemical tests showed that lamivudine was conjugated to G2 but efavirenz was loaded into the G2 dendrimer. The XTT results showed substantial effects of nanodrugs on cell viability. This effect, compared to the original drugs, was significantly effective from 10 nM to 10 µM concentrations in the lamivudine conjugated to G2 but for the efavirenz loaded in G2, 10 and 100 µM concentrations showed significant effects (P < 0.05). Also, the results of the p24 antigen evaluation showed that the nanodrugs in both cases were more effective against HIV replication in a dose-dependent manner. In lower concentrations, the nanodrugs performed stronger than the original drugs. However, in higher concentrations they performed more or less similar to the original drugs. Even the IC_{50} of nanodrugs decreased considerably
compared to the original drugs (960 to 94 nM in lamivudine; 8 nM to 900 pM in efavirenz). In this study, the lamivudine nanodrug showed stronger inhibitory effects compared to similar concentrations of the original drug, due to the strong amide bond with G2. It was more effective than the efavirenz nanodrug at similar concentrations, which was loaded inside G2. This indicates the direct effect of drug linkage to the dendrimer in enhancing nanodrug effectiveness in viral inhibition and reducing cell cytotoxicity.

Different studies have compared different drug activities with the form of their nanodrugs. In 2004, saquinavir, the enzyme inhibitor of HIV-protease, was PEGylated with PEG molecules and influenced the virus-infected MT-2 cells. The results showed higher antiviral capacity of the PEGylated drug than the original drug. Its cytotoxicity was much lower than the drug itself. The PEGylated drugs by PEG molecules are slowly excreted by the renal system due to their increased volume, thus their half-life is increased [30].

The conjugation of drug to dendrimer causes to high drug payload which can quickly enter inside the cells and pinpoint in cytoplasm and/or nucleolus which caused to enhance the activity. This phenomenon has been observed in many drugs [31].

The study in 2016 showed conjugation of Chlorambucil with G2 dendrimer. It increased the effectiveness of treatment dramatically compared to the original drug (decreased IC_{50} from 141 μg/ml to 7.27 μg/ml in the conjugation form). This study affirmed the effectiveness of dendrimers that could be used as a potential candidate in the pharmaceutical industry [29].

Other research group in Pasteur Institute of Iran produced a new type of Lamivudine PEGylated to chitosan nanoparticles (LPC) and evaluated its anti-HIV effect and toxicity in different doses on HEK293 T cell using the same plasmids we used in our study. Among different doses, 0.1 mM (the lowest dose) showed the lowest toxicity with the highest anti-viral effect, which confirmed the improvement of drug functionality by PEGylation [27]. Vedha Hari and colleagues examined

\[ \text{FIGURE 9} \] The effect of Lamivudine and its conjugated form to G2 on the cell viability. Except for two concentrations of 100 pM and 1 nM with the similar function to the original drug, the remaining concentrations of Lamivudine nanodrug performed stronger than original drug in terms of cell survival.

\[ \text{FIGURE 10} \] The effect of efavirenz and its loaded form in G2 on the cell viability. Drug and nanodrug concentrations exhibited different cell survival effects. Original drug exhibited greater cell survival compared to the nanodrug only at 10 and 100 nM concentrations, however, 10 and 100 μM were more effective concentrations of nanodrug.
the efavirenz nanoparticles and reported a significant increase in its solubility. Efavirenz is an anti-HIV molecule with low solubility and a variable active effect (45%) at 800 mg dose. Analysing it with an infrared, colourimetric assay and scanning the nanoparticles showed the compatibility and stability of this nanodrug. The nanoparticles showed active effects twice as much as the original drug, which indicated an increase in cell solubility [32]. Nano-lacto efavirenz was prepared and tested. It showed reduced cell cytotoxicity and doubled the impact of nanodrug on viral suppression compared to efavirenz [33].

In a study at the University of Texas, scientists examined anti-HIV drug activity in conjugation with diamond nanoparticles on brain cells. These particles also showed improved activity. Although the results were acceptable, diamond is not economically justified for therapeutic use, as its stability was unclear in the study [34]. However, in our study we used a carrier which is economically acceptable for the pharmaceutical industry and the active effect of the produced drug is maintained.

In a study on methotrexate, the combination of methotrexate and dendrimer created a stable combination of this insoluble drug. When the drug was loaded in the dendrimer, it was immediately released in PBS buffer and performed similar to the original drug. But with a covalent bond, the complex was stable under the same condition. By methotrexate covalent conjugation, its cellular stable release caused the elimination of the receptor-producing cells, indicating targeted drug delivery [35].

In another study, a series of evaluations were conducted in vitro using G1 and G2 dendrimers in which PEG was the nucleus and citric acid was on the margin. Each of them had different sizes, charge and molecular weight. Cell toxicity was
evaluated using crystal violet, MTT and LDH assays. The mechanism of cell death was also investigated on the HT1080 cells. According to the results, no significant harmful effect was observed for dendrimers up to 0.5 mg/ml. Both apoptosis and necrosis were attributed to the death of the cells. The G1 exhibited more toxicity than G2. They stated that the potential for using these hybrid structures will be huge in various fields of nanomedicine [36]. Two conjugates of cisplatin with two generations of biodegradable anionic citric dendrimers (G1 and G2) which were prepared in aqueous solution were tested. Based on the in vitro results, the cisplatin-G2 conjugate was more effective against cancer cells compared to the cisplatin-G1 conjugate and cisplatin alone. Also, similar haemolytic behaviour was observed for both the conjugates and cisplatin. It was concluded that these conjugates with such high potential and low haemolytic activity were the right candidates as new and effective anti-tumour agents [37].

Considering the above, we noticed that nanodrugs conjugated with their carriers are more suitable candidates than original drugs and even the loaded drugs in the carrier. Therefore, the application of better carriers and the simultaneous use of several nanodrugs to produce effective and applicable medicines in order to reach doses that result in lower toxicity with more inhibitory power against HIV replication is of interest. This way the creation of resistant strains would be prevented.

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CONFLICT OF INTEREST
The authors declare they have no conflict of interest.

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