PAMAM dendrimers and branched polyethyleneglycol (nanoparticles) prodrugs of \((-\beta\)-d-(2R, 4R)-dioxolane-thymine (DOT) and their anti-HIV activity

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The synthesis, characterization, anti-HIV activity and cytotoxicity of dendrimers of \((-\beta\)-d-(2R, 4R)-dioxolane-thymine (DOT) and polyethylene glycol (PEG)–DOT conjugates are described. Dendrimers in this study were polyamidoamine (PAMAM) generation 2.0, 3.0, 5.0 and 6.0, along with 8.0-branched PEG with a molecular weight of 40 kDa. DOT was attached to PAMAM dendrimers or branched PEG via ester or phosphate groups. Size exclusion chromatography was used to purify the dendrimers and PEG conjugates, which were characterized by NMR and MALDI–TOF mass spectrometry. The synthesized PAMAM dendrimers and PEG conjugates were evaluated for anti-HIV activity against HIV-1, in primary human peripheral blood mononuclear cells (PBMCs) and cytotoxicity in PBMCs, CEM and Vero cells. PAMAM dendrimers of DOT with ester linkages and particularly phosphate linkers showed an increase in anti-HIV potency in comparison with DOT alone (140- and 56-fold, respectively). Unfortunately, the PAMAM dendrimers also exhibited increased cytotoxicity. Anti-HIV activity of PEG–DOT conjugates was found to be lower than that of DOT.

Keywords: anti-HIV activity, dendrimers, dioxolane thymine, drug delivery, nanoparticles

Introduction

A nanoparticle drug-delivery system is a device in which a drug is attached to a carrier such as synthetic polymer, antibody or liposome. The parameters such as the site specificity and minimization of side effects can be altered via modifying the properties of the carriers because absorption and distribution of the drugs in such a system depends on the properties of the carriers (Patri et al., 2002; Jevprasesphant et al., 2003). Dendrimers are synthetic, highly branched, spherical, monodisperse macromolecules that have modifiable surface functionality as well as internal cavities (Jansen et al., 1994). These characteristics, along with water solubility, are some of the features that make them attractive for biological and drug-delivery applications (Esfand & Tomalia, 2001; Liu & Frechet, 1999). Among the family of dendrimers, those most investigated for drug delivery are the polyamidoamine (PAMAM) dendrimers because they are biocompatible, non-immunogenic, water-soluble and possess terminal-modifiable functional groups for binding various targeting or guest molecules. Previously, PAMAM dendrimer conjugates have been used for delivering drugs and DNA (Kojima et al., 2002; Lou et al., 2002; Bielinska et al., 1996; Kukowska-Latallo et al., 1996). Moreover, PAMAM dendrimers have been shown to exhibit minimum cytotoxicity up to generation 5.0 (Malik et al., 2000)

Due to the structure and surface characteristics of these nanoparticles, they are readily taken up by monocyes/macrophages of the reticuloendothelial system via phagocytosis (Schafer et al., 1992; von Briesen et al., 2002). However, according to the study of Schafer & Steffan (1994) phagocytosis is highly dependent on the type of material and the particle size. Nanoparticles made from polyhexyl-cyanoacrylate or human serum albumin with a diameter of 200 nm were found to be most suitable for targeting antiviral substances to macrophages. Bender et al. (1996) incorporated the lipophilic HIV protease inhibitor saquinavir into polyhexylcyanoacrylate nanoparticles and found that the nanoparticle conjugates showed more than a 10-fold increase in antiviral activity. Lobenberg et al. (1998) also reported that nanoparticles aided the delivery of zidovudine (AZT) in tissues containing a large number of macrophages. McCarthy et al. (2005) developed two dendrimer-based antiviral agents SPL2923 and SPL6195 for HIV. Both were based on the PAMAM branching unit of the same generation, but built up either from ammonia (SPL2923) or ethylenediamine cores (SPL6195).
Polyethylene glycol (PEG) has been the most widely used polymer for conjugation to peptides and protein-type drugs (Torchilin & Lukyanov, 2003). PEG has been used extensively because it extends the half-life of most proteins and results in a greatly increased plasma presence (Greenwald, 2001). Compared with linear PEG, which is distributed throughout the body with a larger volume, branched PEG is distributed with a smaller volume and is readily delivered to the liver and spleen. Moreover, branched PEG protein conjugates exhibit increased pH and thermal stability as well as greater stability towards proteolytic digestion (Caliceti et al., 1999; Monfardini et al., 1995; Calogerpoulou et al., 2003). The successful application of α-interferon pegylation by Schering-Plough (PEG-INTRON®, molecular weight of PEG 12 kDa daltons; Takacs et al., 1999) and Hoffman-LaRoche (Pegasys®; molecular weight of PEG 40 kDa; Bailon PS & Palleroni AV [1997] Interferon conjugates. European patent EP 809 969 A27) has now centred attention on the use of fewer strands of high molecular weight PEG with proteins.

We previously reported (–)-β-D-(2R,4R)-dioxolane-thymine (DOT) indicating anti-HIV activity against most of the nucleoside-resistant HIV-1 mutants (Chong & Chu 2004; Chu et al., 2005) and we conceived of enhancing its potency by improving drug delivery to the target cells by using PAMAM dendrimers and branched PEG. Herein, we report the synthesis, characterization, anti-HIV activity and cytotoxicity of dendrimers of DOT and PEG–DOT conjugates.

Materials and methods

Chemistry
DOT was synthesized as previously described (Chu et al., 1991). PAMAM dendrimers were purchased from Dendritic NanoTechnologies Inc. (Mount Pleasant, MI, USA). PEG (eight-branched polyethylene glycol with molecular weight of 40 kDa) was purchased from Shearwater Polymers Inc. All other chemicals were obtained from Sigma Chemical Company (St Louis, MO, USA). Size exclusion chromatography was performed on Sephadex G50 or G75 (Sigma-Aldrich, St. Louis, MO, USA). 1H NMR spectra were recorded on a Varian Inova 500 (Varian NMR systems, Palo Alto, CA, USA) using VNMRC6-1 with Me4Si as an internal standard. Matrix assisted laser desorption ionization time of flight (MALDI–TOF) mass spectra were recorded on Hewlett Packard–MALDI instrument (Bruker Daltonics Inc, Billerica, MA, USA) using gentisic acid as a matrix. The percent DOT functionalization of dendrimers or PEG were determined by ultraviolet (UV) absorption at 265 nm on a Beckman Coulter DU 640 spectrophotometer (Beckman Coulter Inc, Fullerton, CA, USA).

Figure 1. Synthesis of dendrimer–DOT conjugates with ester and phosphate linkers

Reagents and conditions: (a) DOT, DIPC, DMAP, DMF. (b) (i) Diphenyl phosphite, pyridine, DMSO; (ii) DOT, pyridine. (c) CCl4/H2O/N-methylmor-
pholine/pyridine/MeCN (2.5/1.0/1.0/6.0/1.0, v/v), DMSO. (i) and (ii) represent different steps in a particular reaction. DIPC, disopropylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; DMSO, dimethyl sulfoxide; DOT, (–)-β-D-(2R,4R)-dioxolane-thymine.
Synthesis of conjugates

*Synthesis of dendrimer–DOT conjugates with ester linkers (1–4).* Dendrimer–DOT conjugates with ester linkers were synthesized via the procedure shown in Figure 1. 1,6-Diaminohexane core PAMAM dendrimers with succinamic acid surface functionalities (1 mmol of succinamic acid), DOT (6 mmol), 4-(dimethylamino) pyridine (DMAP; 2 mmol) and diisopropylcarbodiimide (DIPC; 2 mmol) were suspended in 10 ml of dimethylformamide (DMF) and stirred at room temperature for 24 h. Dichloromethane (10 ml) was added to the reaction mixture and stirred for 30 min. Then water (10 ml) was added and stirred for 20 min. The water layer was separated and concentrated under reduced pressure. The residue was purified by size exclusion chromatography (Sephadex G50 for G2.0A, G3.0A and Sephadex G75 column for G5.0A, G6.0A; 100% dH2O) to give the desired product. Yields of G2.0A-DOT 1, G3.0A-DOT 2, G5.0A-DOT 3 and G6.0A-DOT 4 were

**Table 1. MALDI–TOF MS analysis and loading rates of dendrimer–DOT (1–7) and PEG–DOT conjugates (8 and 11)**

<table>
<thead>
<tr>
<th>Number</th>
<th>Dendrimers of PEG</th>
<th>Surface group number</th>
<th>Weight of dendrimers</th>
<th>Linkers</th>
<th>Theoretical weight*</th>
<th>MALDI</th>
<th>Average DOT number</th>
<th>Loading rate †</th>
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<td>Phosphate</td>
<td>42,500</td>
<td>39,045</td>
<td>8</td>
<td>100</td>
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</table>

* Dendrimer molecular weight plus the total (–)-β-D-(2R,4R)-dioxolane-thymine (DOT) molecular weight calculated from ultraviolet measurement.
† Loading rate is the ratio of the attached average DOT number to surface group number. MALDI–TOF, matrix assisted laser desorption ionization time of flight; PEG, polyethylene glycol.

**Figure 2. Dendrimer–DOT conjugates (1–7) and PEG–DOT conjugates (8 and 11)**

DOT, (–)-β-D-(2R,4R)-dioxolane-thymine; PEG, polyethylene glycol.
95, 92, 84 and 81%, respectively. $^1$H NMR for G2.0A-DOT 1 (MeOH-d$_4$): $\delta$ 1.91 (s, 5-Me), 2.40 (m, CH$_2$COO), 2.41–2.91(br, PAMAM), 3.83 (m, H5$'$), 4.28 (m, H2$'$), 5.03 (s, H4$'$), 6.37 (m, H1$'$), 7.91 (s, H6). The characterization of the conjugates is shown in Table 1 and Figure 2. (G2.0A, G3.0A, G5.0A and G6.0A are generation 2.0, 3.0, 5.0 and 6.0 1,6-diaminohexane core poly [amidoamine] dendrimer with succinamic acid surface groups).

**Synthesis of dendrimer–DOT conjugates with phosphate linkers (5–7).** Dendrimer–DOT conjugates with phosphate linkers were synthesized via the H-phosphonate chemistry as shown in Figure 1. A solution of 1,6-diaminohexane core PAMAM dendrimer with tris(hydroxymethyl)aminomethane surface functionalities (1 mmol of hydroxyl in dimethyl sulphoxide [DMSO], 3 ml) were added to a solution of diphenyl phosphite (2.5 mmol in DMSO, 3 ml) and pyridine (3 ml) over a period of 1 h at room temperature. The mixture was stirred for 20 min, DOT (3 mmol) was added and the mixture was stirred again at room temperature for 2 h. The solvent was removed under reduced pressure and the residue was purified by size exclusion chromatography (Sephadex; deionized water as eluent) to give the dendrimer–DOT conjugate intermediates with H-phosphonate linkers. The intermediates were dissolved in DMSO and a mixture of CCl$_4$/H$_2$O/N-methylmorpholine/pyridine/CH$_3$CN (2.5/1.0/1.0/6.0/1.0, v/v) was added and stirred for 20 min at room temperature. The solvents were removed under reduced pressure and the residue was purified (Sephadex; 100% dH$_2$O) to yield the dendrimer–DOT conjugates with phosphate linkers (5 to 7). Yields of G2.0H-DOT 5, G3.0H-DOT 6 and G5.0H-DOT 7 were 84, 81 and 72%, respectively. G2.0H-DOT 5: $^{31}$P NMR (MeOH-d$_4$): $-3.17$; $^1$H NMR (MeOH-d$_4$): $\delta$ 1.91 (s, 5-Me), 2.35–2.90 (br, PAMAM), 3.85 (m, H5$'$), 4.25 (m, H2$'$), 5.07 (s, H4$'$), 6.38 (m, H1$'$), 7.61 (s, H6). (G2.0H, G3.0H and G5.0H are generation 2.0, 3.0 and 5.0 1,6-diaminohexane core poly [amidoamine] dendrimer with tris [hydroxymethyl] aminomethane surface groups).

**Synthesis of PEG–DOT conjugates with an ester linker (8).** The strategy adopted for the synthesizing PEG–DOT conjugates with ester linkers is shown in Figure 3. DOT (456 mg, 2 mmol) dissolved in dichloromethane (10 ml) was added slowly (1 h) to a solution of succinic anhydride (200 mg, 2 mmol) and DMAP (122 mg, 1 mmol) in dichloromethane (30 ml). The

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**Figure 3. Synthesis of PEG–DOT conjugates with ester and phosphate linkers**

Reagents and conditions: (a) DIPC, DMAP, DCM, rt, 24 h. (b) (i) 1,2,4-triazole, Et$_3$N, THF; (ii) DOT, N-methylimidazole, THF. (c) PEG, N-methylimidazole, rt. (d) (i) 0.5N NAOH, THF, (ii) HCl. (i) and (ii) represent different steps in a particular reaction. DCM, dichloromethane; DIPC, diisopropylcarbodiimide; DMAP, 4(dimethylamino) pyridine; DOT, (-)-β-D-(2R,4R)-dioxolane-thymine; PEG, polyethyleneglycol; rt, room temperature THF, tetrahydrofuran.
mixture was stirred for 6 h and concentrated under reduced pressure. The residue was purified by flash chromatography (Silica 40-63 μm; 2% MeOH:MeCl₂) to give the intermediate DOT. PEG (800 mg, 0.02 mmol), DMAP (12.2 mg, 0.1 mmol) and DIPC (12.6 mg, 0.1 mmol) were added to succinyl DOT (31.2 mg, 0.1 mmol, in MeCl₂ 30 ml) and stirred for 24 h at room temperature. The solvent was removed under reduced pressure and the residue was dissolved in water (20 ml) and the insoluble compounds were removed by filtration. The filtrate was purified (Sephadex G75; 100% dH₂O) to yield compound 8 (92% yield). ¹H NMR (MeOH-d₄): δ 1.91 (s, 5-Me), 2.58 (m, CH₃COO), 3.57–3.64 (br, PEG), 4.21 (m, H5'), 4.35 (m, H2'), 5.28 (s, H4'), 6.40 (m, H1'), 7.61 (s, H6).

**Synthesis of PEG–DOT conjugates with a phosphate linker (11).** The PEG–DOT conjugates with phosphate linkers were synthesized as shown in Figure 3. 1,2,4-Triazole (110 mg, 1.6 mmol) and triethylamine (162 mg, 1.6 mmol) were added to a solution of 2-chlorophenyl dichlorophosphate (196 mg, 0.8 mmol) in THF (2 ml), and then stirred for 2 h at room temperature. The solvent was removed under reduced pressure and the residue was dissolved in water (20 ml). The solution was filtered and the filtrate was concentrated under reduced pressure. The residual solid was purified by size exclusion chromatography (Sephadex G75; 100% dH₂O) to furnish compound 9. Conjugate 9 was dissolved in THF and 0.5 N NaOH was added at 0°C. The mixture was heated to 50°C, stirred for 1.5 h, neutralized with dilute HCl at 0°C, and concentrated and purified (Sephadex G75; 100% dH₂O) to give the conjugate 10 in 77% yield. ³¹P NMR (MeOH-d₄): δ –6.61; ¹H NMR (MeOH-d₄): δ 1.90 (s, 5-Me), 3.55–3.67 (br, PEG), 4.16 (m, H5'), 4.30 (m, H2'), 5.16 (s, H4'), 6.37 (m, H1'), 7.32 (s, H6).

**Virology**

**Antiviral assays.** The procedures for the antiviral assays in human peripheral blood mononuclear cells (PBMCs) have been published previously (Schinazi et al., 1988, 1990). In summary, PBMCs were isolated by Ficoll-Hypaque discontinuous gradient centrifugation of whole blood samples (obtained from the Atlanta Red Cross, Atlanta, GA, USA) from healthy seronegative donors. Cells were stimulated with phytohaemagglutinin A (3 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) for 2–3 days prior to use. HIV-1, obtained from the Centers for Disease Control and Prevention (Atlanta, GA, USA) was used as the standard reference virus for the antiviral assays. Infections were done in bulk for 1 h, either with 100 TCID₅₀ /1 × 10⁵ cells for a flask (T25) assay or with 200 TCID₅₀ /2 × 10⁴ cells/well for a 24-well plate assay. Previous studies indicated that this was the optimum virus concentration in order to obtain good replication on day 5 when the virus is harvested from the supernatant. Cells were added to a flask or plate containing a 10-fold serial dilution of the test compound. Assay medium was RPMI-1640 supplemented with heat-inactivated 16% fetal bovine serum, 1.6 mM 1-glutamine, 80 IU/ml penicillin, 80 μg/ml streptomycin, 0.0008% DEAE-Dextran, 0.045% sodium bicarbonate and 26 IU/ml recombinant interleukin-2 (Chiron Corp., Emeryville, CA, USA). AZT was used as the positive control for the assay. Uninfected PBMCs were grown in parallel at equivalent cell concentrations as a control. The cell cultures were maintained in a humidified 5% CO₂ air at 37°C for 5 days, and supernatants were collected for reverse transcriptase activity. A 1-ml quantity of each supernatant was centrifuged at 9,740g for 2 h to pellet the virus. The pellet was solubilized with vortexing in 100 μl of virus solubilization buffer (0.5% Triton X-100, 0.8 M NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol and 0.05 M Tris, pH 7.8). Each sample (10 μl) was added to 75 μl of reverse transcriptase (RT) reaction mixture (0.06 M Tris, pH 7.8, 0.012 M MgCl₂, 0.006 M dithiothreitol, 0.006 mg/ml poly (rA) oligo (dT)₁₂–₁₈, 96 μg/ml dATP and 1 μM of 0.08 μCi/ml ³²P-thymidine-5′-triphosphate; Perkin Elmer, Boston, MA, USA) and incubated at 37°C for 2 h. The reaction was stopped by the addition of 100 μl of 10% trichloroacetic acid containing 0.05% sodium perophosphate. The acid-insoluble product was harvested onto filter paper using a Packard Harvester (Meriden, CT, USA), and RT activity was read on a Packard Direct Beta Counter (Meriden). RT results were expressed in counts per minute (CPM) per millilitre. The antiviral 50% effective concentration (EC₅₀) and 90% effective concentration (EC₉₀) was determined from the concentration–response curve using the median effect method (Belenkii & Schinazi, 1994).

**Cytotoxicity assays.** The compounds were evaluated for their potential toxic effects on uninfected PHA-stimulated human PBMCs, CEM (T-lymphoblastoid cell line obtained from American Type Culture Collection, Rockville, MD, USA) and Vero (African green monkey kidney) cells. Log phase Vero, CEM and PHA-stimulated human PBMCs were seeded at a density of 5 × 10⁴, 2.5 × 10⁴ and 5 × 10⁴ cells/well, respectively. All of the cells were plated in 96-well cell culture plates containing 10-fold serial dilutions of the test drug. The cultures were incubated for 2, 3 and 4 days for Vero, CEM and PBMCs, respectively, in humidified 5% CO₂ air at 37°C. At the end of
incubation, MTT tetrazolium dye solution (cell titre 96; Promega, Madison, WI, USA) was added to each well and incubated overnight. The reaction was stopped with stop solubilization solution (Promega). The plates were incubated for 5 h to ensure that the formazan crystals were dissolved. The plates were read at 570 nm using an ELISA plate reader (Model EL 312e; Bio-Tek Instruments, Inc., Winooski, VT, USA). The 50% inhibition concentration (IC₅₀) was determined from the concentration–response curve using the median effect method (Stuyver et al., 2002; Belen'kii & Schinazi, 1994).

Results

Chemistry
Four different generations of dendrimers, G2.0, G3.0, G5.0 and G6.0, were employed as nanoparticle carriers of DOT. The synthesis of dendrimer–DOT conjugates with ester linkers (1, 2, 3 and 4) are shown in Figure 1. 1,6-Diaminohexane core PAMAM dendrimers having succinic acid surface groups were allowed to react with the 5′-hydroxyl group of DOT to afford dendrimer–DOT conjugates. Though the reaction is not complex, it does have its complications. Steric effects limit the number of DOT molecules that can be attached to the dendrimers. By varying reaction conditions it was evident that a DOT concentration of 5 equivalents/acid gave a maximum number of dendrimer–DOT conjugations. However, not all the acid groups on the surface of the dendrimers were attached to the DOT due to steric effects. For example, the G5.0 dendrimer attached only 50 DOT molecules, even though it has 128 surface functionalities. The attached DOT number per dendrimer varies according to the dendrimer's surface group number (see Table 1). Size exclusion chromatography was used to purify the dendrimers. Yields of these reactions were found to be excellent, where conjugates 1, 2, 3 and 4 were isolated in 95, 92, 84 and 81% yields, respectively.

For the synthesis of dendrimer–DOT conjugates with phosphate linkers (5, 6 and 7) (Figure 1), the H-phosphonate approach was used (Stawinski 1992; Xiao et al., 2003). Two equivalents of diphenyl phosphate in pyridine were added slowly to the solution of dendrimers in DMSO/pyridine (2/1, v/v), which greatly reduced the formation of H-phosphonates of dendrimers. Oxidation of H-phosphonates by a mixture of CCl₄/H₂O/N-methylmorpholine/pyridine/CH₃CN (2.5/1.0/1.0/6.0/1.0, v/v) yielded the phosphate (Mohe et al., 2003).

Synthesis of PEG–DOT conjugates with ester linkers (8) is shown in Figure 3. 5′-Succinyl–DOT was separately prepared and conjugate 8 was synthesized by the reaction of branched PEG with a large excess of 5′-succinyl–DOT, DIPC and DMAP. Size exclusion chromatography (Sephadex G75) was used to purify compound 8 (92% yield). PEG–DOT conjugates with phosphate linkers (11) were synthesized according to Figure 3. Briefly, a 5′-(2-chlorophenylchlorophosphate) derivative of DOT 9 was prepared first, without purification, and allowed to react with PEG in the presence of N-methylimidazole to give conjugate 10. Conjugate 10 was purified (Sephadex G75), hydrolysed with a dilute NaOH solution and purified (Sephadex G75) to provide compound 11.

The percent DOT functionalization of the dendrimers and PEG was determined by measuring the fluorescence absorption at 265 nm in water. When the ester group was used as the linker, the measurement suggests that an average of 5-, 12-, 50- and 105-DOT units were incorporated into G2.0A, G3.0A, G5.0A and G6.0A dendrimers with a percent incorporation of 31, 37, 39 and 40%, respectively (Table 1). The low percent incorporation of DOT to the dendrimers was ascribed to the steric hindrance of the dendrimers used. When phosphate groups were used as linkers, an average of 4, 10 and 45 DOT units were incorporated into G2.0H, G3.0H and G5.0H dendrimers with a percent incorporation of 25, 31 and 35%, respectively. The decreased percent incorporation with phosphate linkers compared with ester linkers may be related to the reactivity of the phosphate group as well as the steric hindrance of the dendrimer. The percent incorporation of DOT to PEG was 100% for both ester and phosphate linkers. The high efficiency of loading was possible because of the linear nature of PEG.

Although the accurate characterization of high molecular weight compounds can be difficult, both the dendrimer–DOT conjugates 1 to 7 and PEG–DOT conjugates 8 and 11 proved to be amenable to characterization by ¹H NMR and MALDI–TOF mass spectrometry. From the ¹H NMR integrated ratio of the signals at 2.38–2.45 ppm and at 1.92 ppm, which correspond to the methylene protons next to the carbonyl groups of the dendrimers and the methyl protons in DOT, respectively, average DOT conjugates were estimated and it was found that for G2.0H-DOT (5) and G3.0-DOT (2,6), NMR data agrees with the value derived from the UV measurement. However, for G5.0-DOT (3,7) and G6.0-DOT (4), the average numbers by ¹H NMR experiments did not agree with the value from the measurement of UV. The molecular weights of dendrimer–DOT and PEG–DOT conjugates were evaluated by MALDI–TOF and the results are shown in Table 1. In all cases, the measured molecular weights of conjugates by MALDI–TOF were somewhat different from the theoretical values. This is not surprising when considering the high molecular nature of these conjugates and the variability of DOT conjugation. The high molecular weight of these conjugates may allow them to form many salts while in the mass spectrometer, which means many mass-to-charge...
DOT was attached to PEG through a phosphate group, although it was not cytotoxic. Conjugate antiviral activity (compounds DOT with phosphate linkers have exhibited a greater activity than DOT) with succinamic acid surface groups that can be easily hydrolyzed and used (Bodanszky & Kwei, 1978). PAMAM dendrimers of succinamic acid surface groups that can be easily hydrolyzed have exhibited greater anti-HIV activity while the toxicity was also significantly increased. Compound 4 didn’t show anti-HIV activity up to 10 µM, which may be due to its high molecular weight. The high molecular weight of compounds 1–3 may be attributed to less stable succinamic acid surface groups that can be easily hydrolyzed (Bodanszky & Kwei, 1978). PAMAM dendrimers of DOT with phosphate linkers have exhibited a greater antiviral activity (compounds 5, 6 and 7 were 140-, 5- and 56-fold, respectively) in comparison with DOT. Unfortunately, the cytotoxicity of the PAMAM–DOT conjugates was also found to be high. Compound 5 didn’t show anti-HIV activity up to 10 µM, which may be due to its high molecular weight. The high molecular weight of compounds 1–3 may be attributed to less stable succinamic acid surface groups that can be easily hydrolyzed (Bodanszky & Kwei, 1978). PAMAM dendrimers of DOT with phosphate linkers have exhibited a greater antiviral activity (compounds 5, 6 and 7 were 140-, 5- and 56-fold, respectively) in comparison with DOT.

### Table 2. Anti-HIV activity and cytotoxicity of PAMAM–DOT and PEG–DOT conjugates 1–7 and PEG–DOT conjugates 8 and 11

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<th>Selectivity Index IC50/EC50 in PBMCs</th>
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<tr>
<td>6</td>
<td>0.11 ±0.09</td>
<td>0.57 ±0.06</td>
</tr>
<tr>
<td>7</td>
<td>&lt;0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>8</td>
<td>4.9</td>
<td>33.5</td>
</tr>
<tr>
<td>11</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>DOT</td>
<td>0.56 ±0.5</td>
<td>2.8 ±1.2</td>
</tr>
<tr>
<td>AZT</td>
<td>0.007 ±0.0047</td>
<td>0.04 ±0.02</td>
</tr>
</tbody>
</table>

*In human peripheral blood mononuclear cells (PBMCs) unless otherwise indicated. Based on triplicate assays. Where available, the standard deviation is provided based on triplicates. †Mole concentration of (−)-β-D-(2R,4R)-dioxolane-thymine (DOT) instead of dendrimer or polyethylene glycol (PEG). AZT, zidovudine; EC50, 50% effective concentration; EC90, 90% effective concentration; IC50, 50% inhibition concentration.

**Anti-HIV activity**

The anti-HIV activity and cytotoxicity of the PAMAM dendrimers and the PEG conjugates are presented in Table 2. The PAMAM dendrimer conjugates of DOT linked with an ester moiety exhibited high anti-HIV activity. Compounds 1 and 2 showed fourfold more potent anti-HIV activity than that of DOT without increased cytotoxicity. Compound 3 showed 13-fold more anti-HIV activity while the toxicity was also significantly increased. Compound 4 didn’t show anti-HIV activity up to 10 µM, which may be due to its high molecular weight. The poor antiviral activity of 8 and 11 may be due to the inability of these esterases to release the parent compound (DOT). These results indicate that the antiviral activity is influenced not only by the delivery of the prodrg, but also by the stability of the conjugates. Ishiyama et al. (1997) also supports the fact that, while designing the prodrugs it is important to focus not only the delivery of the produgs into the cell membrane, but also on their reversion to the parent compounds in the cytoplasm or at the target cells.

**Discussion**

DOT was reported from our laboratories and showed anti-HIV activity against most of the nucleoside-resistant HIV-1 mutants. We conducted molecular modeling studies of DOT and found that the dioxolane moiety plays a significant role in stabilizing the binding between the mutant HIV-1 RT and the nucleoside triphosphate (Chu et al., 2005). However, DOT is only modestly potent in vitro (EC50, 0.56 μM) as an anti-HIV agent and it would be desirable to develop more potent derivatives. There could be a number of reasons for the modest anti-HIV activity of DOT, which may include a poor intracellular penetration, a low initial phosphorylation by kinase or fast degradation of the triphosphate form. Thus, the present study was undertaken to devise an effective drug delivery system to increase the potency of DOT by improving drug delivery to the target cells by using...
PAMAM dendrimers and branched PEG. For this study, we selected generation 2.0, 3.0, 5.0 and 6.0, 1,6-diaminohexane core PAMAM dendrimers and 8-branched PEG with a molecular weight of 40 kDa as DOT carriers. Ester groups or phosphate groups were used as the linkers between DOT and dendrimers or PEG. These linkers are important for the activation of DOT because they undergo cleavage by plasma enzymes to release the parent drug. The synthesized dendrimers and conjugates were evaluated for their anti-HIV-1 activity in primary human PBMCs as well as cytotoxicity.

In conclusion, we have synthesized several dendrimers of DOT and PEG-DOT conjugates as nucleoside delivery systems. Dendrimers of DOT with ester, particularly the phosphate linker, exhibited potent anti-HIV activity (56- to 140-fold increase in comparison with the parent compound DOT), although cytotoxicity was also increased. The PEG-DOT conjugates (8,11) have not shown any significant anti-HIV activity. From the synthesized dendrimer-DOT conjugates (1–4), compound 1 exhibited the highest anti-HIV-1 activity without enhancement of cytotoxicity. Similarly, for dendrimer-DOT conjugates of the phosphate linker series (5–7), compound 5 was found to be the most potent. These preliminary results suggest that generation 2.0 dendrimers with phosphate linkers may be useful as the prodrugs of DOT. Further biological studies are necessary to access the full potential of these prodrugs.

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References


Anti-HIV activity of dendrimer–DOT and PEG–DOT conjugates


