PAMAM dendrimer-azithromycin conjugate nanodevices for the treatment of Chlamydia trachomatis infections

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Abstract

Chlamydia trachomatis is an important bacterial pathogen known to be etiological in genital infections, as well as several serious disease sequelae, including inflammatory arthritis. Chlamydiae can persist in infection, making treatment with antibiotics such as azithromycin (AZ) a challenge. The authors explore the use of neutral generation-4 polyamidoamine (PAMAM) dendrimers as intracellular drug-delivery vehicles into chlamydial inclusions. Azithromycin was successfully conjugated with the dendrimers, and the conjugate (D-AZ) released \(\sim 90\%\) of the drug over 16 hours. The conjugate readily entered both the Chlamydia-infected HEp-2 cells and the chlamydial inclusions. The conjugate was significantly better than free drug in preventing productive infections in the cells when added at the time of infection, and better in reducing the size and number of inclusions when added either 24 hours or 48 hours post infection. These studies show that dendrimers can deliver drugs efficiently to growing intracellular C. trachomatis, even if the organism is in the persistent form.

From the Clinical Editor: In this report, the use of polyamidoamine dendrimers as intracellular drug-delivery vehicles into chlamydial inclusions is investigated. This method results in efficient intracellular delivery of azithromycin to address chlamydia infection. © 2011 Elsevier Inc. All rights reserved.

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Urogenital infections with the obligate intracellular bacterial pathogen Chlamydia trachomatis are a major problem in the United States (US). Indeed, all new such genital infections must be reported to the Centers for Disease Control (CDC) from all 50 states and the District of Columbia. Information from the CDC indicates that 1 million or more new infections are reported each year in the US, giving a stable population of Chlamydia-infected individuals in the US in excess of 6 million.\textsuperscript{1} Importantly, it is well established that genital chlamydial infections can and often do engender serious sequelae, including severe reproductive problems in women, inflammatory arthritis in both genders, and others.\textsuperscript{2,3}

Studies from many groups have led to progress in understanding the molecular and cellular biology of C. trachomatis and in defining its pathogenic interaction with its host cells in the reproductive system, synovium and elsewhere.\textsuperscript{4,5} One important aspect of that understanding concerns the biology of the unusual biphasic developmental cycle of the organism. The cycle is initiated when elementary bodies (EBs), the extracellular form of the organism, attach to a target host cell. Once bound, EBs are brought into membrane-bound inclusion in the host-cell cytoplasm, and each EB undergoes a development process resulting in production of reticulate bodies (RBs), which undergo cell division. Near the termination of the cycle, most RBs dedifferentiate back to EBs, and these are released by host-cell lysis or exocytosis.\textsuperscript{6} In HeLa or HEp-2 cells, the cycle takes \(\sim 50\) hours. However, under some circumstances, including those relevant to infections at sites of chlamydial dissemination, the developmental cycle can be arrested at a late point, obviating production and release of new EBs. This state of arrested development under
certain growth conditions and/or within certain host-cell types is referred to as persistence, and the arrest in the cycle that engenders this state is transcriptionally governed. It is clear at this point that in Chlamydia-induced arthritis, fallopian tube occlusion and other sequelae, the organism resides in the affected tissue in the persistent state, and organisms in this infection state are surprisingly refractory to antimicrobial treatment.

Azithromycin (AZ), a second generation macrolide antibiotic, has been shown to be highly effective for the treatment of many important human bacterial infections. This drug also has anti-inflammatory and immunosuppressive activities. Another important feature of AZ is that it penetrates well into target cells, including those infected with chlamydiae, delivering antibiotic to the site of infection. For all these reasons, azithromycin has been utilized in the treatment of both acute and chronic Chlamydia-induced conditions.

Dendrimers are emerging as a new class of nanoscale drug-delivery vehicles due to their well-defined structure, tailored or tailorable surface properties, and ability to deliver drugs intracellularly. They have been used as therapeutics for arthritis, and as effective drug delivery vehicles for small molecules can be encapsulated/complexed or covalently attached to the dendrimer through their functional groups. An important aspect is that the linking chemistry between the dendrimer and the drug molecule can be used to tailor the drug-release profile. The most commonly used covalent bonds between the drug and the dendrimer are ester and amide bonds. Ester bonds are amenable for hydrolytic or enzymatic cleavage. Recently, disulfide linkages have been used for conjugation of drug to dendrimer; these bonds can be cleaved by glutathione (GSH) intracellularly for release of the drug. It has been shown that poly(amideamine)(PAMAM) dendrimers, even without targeting ligands, localize in cells associated with inflammation, and neuroinflammation. This makes them valuable carriers for drugs to treat diseases that include chlamydial arthritis.

In this article we describe the synthesis and characterization of the hydroxyl-terminated generation four PAMAM dendrimer-AZ conjugate nanodevices (D-AZ), and we provide evaluation of their activity in vitro on C. trachomatis-infected cells in culture. We use generation-4, -OH terminated “neutral” dendrimers because of the favorable cytotoxicity profiles in comparison with higher generation, and amine-terminated PAMAM dendrimers. The hydroxy end-functionality also facilitates “ester-linking” chemistry more easily for eventual drug release. To our knowledge this is the first report of the use of dendrimers to deliver AZ to Chlamydia-infected cells, which has implications in the treatment of diseases engendered by the dissemination of C. trachomatis to sites other than that of the primary infection.

Methods

Materials

G4 hydroxyl-terminated PAMAM dendrimer (G4-OH) in methanolic solution was purchased from Dendritech, Inc. (Midland, Michigan). Benzotriazol-1-yl-oxytriptyridinephosphonium hexafluorophosphate (PyBOP), N,N′-Dicyclohexylcarbodiimide (DCC), 4-(dimethylamino)pyridine (DMAP), 6-(Fmoc-amino)caproic acid, piperidine, glutaric acid and N,N-dimethylacetamide (DMA) were purchased from Sigma-Aldrich Chemical Co., (St. Louis, Missouri). Triethylamine (TEA) and disopropylethylamine (DIEA) were purchased from Thermo Fisher Scientific, Inc. (Pittsburgh, Pennsylvania). Trifluoroacetic acid (TFA), dimethylformamide (DMF), and dimethyl sulfoxide (DMSO) were purchased from EMDS Chemicals (Gibbstown, New Jersey). Fluorescein isothiocyanate (FITC) was purchased from Alfa Aesar (Ward Hill, Massachusetts). Azithromycin was obtained from Baxter Healthcare Corporation in the form of an injectable formulation (Deerfield, Illinois). The anhydrous solvents DMSO, DMF, acetonitrile (ACN), tetrahydrofuran (THF) and dichloromethane (DCM) were purchased from Acros Organics (Morris Plains, New Jersey). All other solvents and chemicals were purchased from Fisher Scientific and were used without further purification. Regenerated cellulose (RC) dialysis membrane with molecular weight cut-off of 1000 Da was obtained from Spectrum Laboratories, Inc., (Rancho Dominguez, California).

Materials characterization

All reactions were carried out under nitrogen conditions. Thin-layer chromatography (TLC) was performed on silica gel GF254 plates (Whatman, Piscataway, New Jersey), and the spots were visualized with UV light and 2% H2SO4 in EtOH. NMR spectra were recorded on a Varian INOVA 400 spectrometer, using commercially available deuterated solvents. Proton chemical shifts are reported in ppm (δ) and tetramethylsilane (TMS) used for internal standard. Coupling constants (J) are reported in hertz (Hz). Mass spectra were recorded on Waters Micromass ZQ spectrometer (Waters Corp., Milford, Massachusetts). MALDI-TOF spectra were recorded on a Bruker Ultraflex system equipped with a pulsed nitrogen laser (337 nm), operating in positive ion reflector mode, using 19 kV acceleration voltage and a matrix of 2,5-dihydroxybenzoic acid.

Liquid chromatography

HPLC characterization was carried out using a Waters instrument (Waters Corp.) equipped with two pumps, an autosampler and dual UV detector interfaced to Breeze software. The UV absorbance was detected at 210 nm for the characterization and release studies of the conjugates. The mobile phase used was Water/Acetonitrile with 0.14% TFA. Mobile phases were freshly prepared, filtered and degassed prior to use. A symmetry 300 C18 HPLC Column (5 μm particle size, 25 cm × 4.6 mm length × 1.D.) equipped with a guard column was used. The gradient method used for analysis was (90:10) water/acetonitrile to (10:90) in 20 minutes; the flow rate was 1 ml/minute. Release studies were performed in 95% 0.1 M PBS...
buffer solution (Ph = 7.4) and 5% EtOH (by volume). The conjugate was added to 3 ml of preheated release media. All samples contained 2.5 mg/ml AZ conjugate, and were stirred continuously and maintained at 37°C. At appropriate time intervals, samples were withdrawn and immediately analyzed by RP-HPLC to determine the AZ concentrations. Release experiments were performed in triplicate.

**Preparation of azithromycin free base**

AZ formulation (500 mg/vial) was dissolved (20 ml) in DI water in a round-bottom flask. The reaction mixture was cooled to 0 °C and the pH of the solution was adjusted to 9.5 by adding 0.1 N NaOH. The AZ free base was extracted with dichloromethane (2 × 50 ml), and the DCM layer was washed with 20 ml of brine, and dried over Na2SO4. Solvent was evaporated under reduced pressure to get AZ free base (480 mg, > 95%). 1H NMR confirmed the formation of free base.

**Synthesis of AZ glutaric acid linker (AZ-Glutaric acid, 4)**

To a stirred solution of intermediate 2 (225 mg, 0.014 mmol) dissolved in 20 ml of DMF, DIEA (0.14 mmol, 18 mg) was added under N2. Next, 500 μl triethylamine was added dissolved in 5 ml DMF (membrane MW cutoff 1000 Da) for 24 hours with successive changes of solvent. Solvent was evaporated under reduced pressure and subjected to high vacuum overnight, which produced a dark yellow gummy material designated D-AZ (5, 28 mg). 1H NMR (DMSO-d6): δ 0.78 (t, 3H), 0.84-0.86 (d, 3H), 0.95 (t, 6H), 1.01 (s, 3H), 1.06-1.17 (m, 14H), 1.25-1.40 (m, 1H), 1.48-1.54 (m, 2H), 1.62-1.65 (d, 1H), 1.73-1.79 (m, 1H), 1.87-1.90 (m, 2H), 2.22 (s, 3H), 2.30 (s, 6H), 2.38-2.42 (m, 1H), 2.46 (s, 1H), 2.49-2.50 (m, 2H), 2.52-2.55 (m, 1H), 2.64-2.71 (m, 1H), 2.90-2.92 (d, 1H), 3.05-3.09 (m, 1H), 3.42-3.44 (d, 1H), 3.49-3.51 (d, 1H), 3.65-3.69 (m, 1H), 4.03-4.09 (m, 1H), 4.14 (d, 1H), 4.20 (bs, 1H), 4.32-4.41 (m, 3H), 4.72-4.76 (dd, 1H), 4.83-4.84 (d, 1H).

**Synthesis of PAMAM G4-OH AZ conjugate (D-AZ, 5)**

To a stirred solution of AZ-glutamic acid (4, 100 mg, 0.115 mmol) in a 50 ml round-bottom flask in dry DMSO (10 ml), DCC (24 mg, 0.115 mmol) and DMAP (14 mg, 0.115 mmol) were added under N2. Next, 500 μl triethylamine was added to the reaction mixture. After 1 hour of stirring, generation-4 hydroxyl-terminated PAMAM dendrimer (D4-OH, 16.5 mg, 0.0015 mmol) was added dissolved in 5 ml DMF, and the mixture was stirred for 48 hours with additional DCC added after 24 hours. The reaction mixture was purified by dialysis using dialysis membrane (MW cutoff 1000 Da) with DMF. The reaction mixture was evaporated under reduced pressure and kept under high vacuum to produce a white fluffy material designated D-AZ (5, 28 mg). 1H NMR (DMSO-d6): δ 0.78 (t, 3H), 0.84-0.86 (d, 3H), 0.95 (t, 6H), 1.01 (s, 3H), 1.06-1.17 (m, 14H), 1.25-1.40 (m, 1H), 1.48-1.54 (m, 2H), 1.62-1.65 (d, 1H), 1.73-1.79 (m, 1H), 1.87-2.08 (m, 6H, linker protons and AZ protons), 2.20-3.70 (dendrimer protons and AZ protons), 4.03-4.09 (m, 1H), 4.14 (d, 1H), 4.20 (bs, 1H), 4.32-4.41 (m, 3H), 4.72-4.76 (dd, 1H), 4.83-4.84 (d, 1H), 8.00 (bs, amide protons of dendrimers).

**Growth of C. trachomatis and host cells**

*C. trachomatis* serovar K (strain UW-31) was used throughout this study. Stock inocula of chlamydial EB were prepared from growth in the human bronchial epithelial cell line designated HEP-2, using standard methods, and stored at -80°C for all experiments. Host cells were grown in Iscove’s modified Dulbecco’s medium (Gibco, Grand Island, New York) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, Georgia), 0.2 μg of gentamicin per ml (Gibco, Invitrogen, China) and 2 mM L-glutamine (Sigma, St. Louis, Missouri). Cells were maintained at 37 °C in 5% CO2.

**Infection of HEP-2 cells with C trachomatis**

HEP-2 cells were grown to semiconfluent monolayer in chamber slides (0.5 × 10⁶ cells/chamber, Nunc, Rochester New York) and infected at 10 EB/cell (MOI = 10) in infection media (Iscove’s modified Dulbecco’s medium supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, Georgia), 0.2 μg/ml of cyclohexamide (Sigma). Infected cells and uninfected control cultures were incubated for another 24 or 48 hours. For studies of persistently infected cells, confluent HEP-2 cell monolayers were infected at a MOI = 10, and 2 hours later,
Penicillin G (100U/ml) was added to induce persistent infection. Cells were pulsed 24–48 hours later with dendrimer constructs (see below).

Visualization of chlamydial inclusions

In some experiments, chlamydial inclusions within the host HEp-2 cell cytoplasm were detected by staining with Chlamydia-specific antibodies. Briefly, infected HEp-2 cells in chamber slides were fixed with 95% methanol for 5 minutes, after which they were stained with FITC-labeled mouse monoclonal antibodies specific for chlamydial lipopolysaccharide (Pathfinder,™ BioRad, Redmond, Washington). In other experiments, at 24 hours pi, infected HEp-2 cell monolayers were pulsed with FITC-conjugated G4 dendrimer (D-FITC) for 1 hour at a concentration of 10 μg per FITC basis in infection media. Cells were then either wet mount coverslipped, or counterstained with Evans blue and mounted with coverslips after MeOH fixation. Cells were observed at 1000× magnification on a real time microscope (Riveal, Quorum Technologies Guelph, Canada), formerly produced by Richardson Technologies, Toronto, Canada), or under 400× magnification on an epifluorescent microscope (Nikon E600), respectively.

Dose response for drug efficacy

Infected HEp-2 cell monolayers were pulsed at 24 hours (t = 24) or 48 hours (t = 48) post-infection (pi) with AZ+CA (AZ citrate salt), AZ-CA (salt free form of AZ), or D-AZ in infection media while keeping equal concentrations of AZ in each treatment. Cells were then further incubated for an additional 24 hours. Similar treatments with free citric acid (CA) and PAMAM dendrimer (D) were done as controls. On termination, slides were fixed, inclusions stained as above, and the inclusion numbers for each treatment were calculated from triplicate wells based on the number of antibody-labeled inclusions counted in triplicate wells observed under a Nikon, Eclipse E600 microscope (Nikon Inc., Melville, New York) at 200× magnification. Inclusion sizes, measured by inclusion area (observed under 400× magnification), were determined from 10 representative microscopic fields using ImagePro software (Media Cybernetics, Bethesda, Maryland) on similarly treated cells in chamber slides.

Statistical analysis and graphical representation of data

All relevant statistical analyses were performed using SigmaStat software (SPSS Inc, Chicago, Illinois) and graphically represented using SigmaPlot software (SPSS). Treatment groups were compared using paired t-test or Mann-Whitney Rank Sum test. Group variations were described as mean ± SEM. Groups with P < 0.05 were considered significantly different.

Results

Chemistry and reactivities of azithromycin

AZ is a derivative of erythromycin (first-generation antibiotic), formed by insertion of a methyl-substituted nitrogen at the 9α-position of the lactone ring, and reduction of the ketone functional group to methylene to create a 15-membered lactone ring. AZ belongs to the azalide class of antibiotic and is therefore a second-generation antibiotic. Like erythromycin, it has 10 chiral centers in the lactone ring and glycosylated in 3- and 5- positions by desosamine and cladinose sugars, respectively. Addition of second basic nitrogen to the structure prevents degradation of the drug and increases antibacterial activity against gram-positive and gram-negative bacteria, and against some atypical pathogens. AZ has 5 hydroxyl groups in 6-, 11- and 12- positions in the macrolide ring, the 2′- position in cladinose, and the 4′′- position in the desosamine moiety. The hydroxyl groups differ in their reactivities depending on position, and the order of reactivity has been established by acetylation. Acetylation occurs first at the 2′- position due to the vicinity of a tertiary amine, which acts as an autocatalyst. When one molar equivalent of acetic anhydride is used for acetylation, 2′-monoacetate is the only product formed without any catalyst. Deacetylation can be achieved by quenching the reaction with methanol; that deacetylation also is a function of a tertiary amine group at the adjacent carbon. This might be the key reason for drug release from the D-AZ conjugate, because this group may enable autocatalytic hydrolysis. The second and third reactive hydroxyl groups are at 4′′- and 11- positions, respectively. Due to the tertiary carbon center, the 6- and 12-hydroxyl groups are least reactive.

Synthesis of PAMAM G4-OH-FITC (D-FITC, 3)

We reported previously the synthesis of the bifunctional dendrimer having a Boc-protected amine group. We used a linker with protecting groups that have a basic pH deprotection mechanism, so that the linkers are “stable” even upon deprotection. We reacted 6-(Fmoc-amino)caproic acid with generation-4 PAMAM hydroxyl-functionality dendrimers under coupling reaction conditions to get intermediate 1 (Figure 1, A). We used PyBOP as coupling reagent and DIEA as base for better yield. The characteristic peak at 3.97 ppm in NMR confirmed the formation of an ester bond between PAMAM G4-OH and linker (Figure S1). Three multiplets correspond to aliphatic protons of linker between 1.2–1.5 ppm, and three multiplets correspond to aromatic protons of linker between 7.2–7.4 ppm, further confirming formation of the intermediate. The linker loading to the dendrimer surface was estimated using proton NMR, by comparing amide protons of the dendrimer and aromatic protons of the linker, which suggested that 8 molecules of linker were reacted per dendrimer. Deprotection of Fmoc groups with piperidine/DMF (2:8) mixture was carried out at room temperature. The absence of aromatic protons in the linker from 7.2–7.4 ppm in NMR confirms formation of intermediate 2 (Figure S2). The presence of aliphatic proton peaks between 1.2–1.5 ppm in NMR confirmed that linker with free amine has been attached to the dendrimer. After deprotection, we verified (using 1H NMR) that 8 molecules of linker were still present on the surface of the dendrimer. Finally, we reacted FITC with intermediate 2 under basic conditions to produce FITC-labeled PAMAM dendrimer with hydroxy groups on the surface (D-FITC). We generated a stable isothiocyanate bond between dendrimer and FITC. A characteristic peak (multiplet) at 3.90 ppm in NMR corresponds to aliphatic protons of CH2NHCS,
confirming formation of the product (Figure S3). The presence of aromatic proton peaks at 6.5 ppm of FITC in NMR spectra further confirms formation of D-FITC (3). In the reaction, we used 3 mole equivalents of FITC, per bifunctional dendrimer molecule. The same numbers of FITC molecules are estimated to be present in the final conjugate, suggesting an efficient reaction. The weight percentage of FITC in the dendrimer is 6–7%.

Synthesis of PAMAM G4-OH-AZ (D-AZ, 5)

Because AZ lacks an appropriate reactive functional group, it is difficult to conjugate with the G4-OH PAMAM dendrimer directly. The schematic presentation of the synthesis of dendrimer-AZ (D-AZ, 5) is shown in Figure 1, B. To obtain a reactive acid functionality, we reacted AZ with glutaric acid under coupling reaction conditions using DCC as coupling reagent, and DMAP and triethylamine as bases. A multiplet at 2.0 ppm in NMR represents methylene groups of glutaric acid and confirms the formation of the intermediate 4 (Figure S4). This intermediate was further reacted with the hydroxy group of the PAMAM dendrimer under coupling reaction conditions to produce the therapeutic nanodevice, Dendrimer-AZ (D-AZ, 5). The conjugate was characterized by proton NMR, MALDI-TOF mass, and reverse phase HPLC. Multiplets between 0.8–1.3 ppm corresponding to methyl protons of AZ confirm the formation of the final conjugate 5 (Figure S5 and S6). Comparing the amidic proton of the dendrimer with aliphatic protons of AZ between 4.0–5.0 ppm, which further confirm the product, we conclude that 6.6 molecules were tagged per molecule of dendrimer. We also detected comparable loading of AZ by comparing amidic protons of the dendrimer with the protons of methyl groups of the drug between 0.8–1.3 ppm. In HPLC chromatograms, the elution times of free AZ (11.5 minutes), free dendrimer (8 minutes), and the D-AZ conjugate (16.5) are distinct, showing that the conjugate is pure, with no measureable amount of free AZ or dendrimer (Figure S7). A molecular weight peak at 18,984 Da in MALDI-TOF of the final conjugate suggests that 6.1 molecules reacted to the surface of the dendrimer (Figure S8, panel B). For the comparison, the mass spectrum of G4-OH dendrimer is shown in Supplementary Figure S8 (panel A). This represents a drug payload in the conjugate of ~16% by weight (based on proton NMR and MALDI TOF mass), which is relatively high for a polymer-drug conjugate. The conjugate is soluble in PBS (~1 mg/ml) and saline solution in contrast with the free drug.

Release studies

The drug release from the conjugate was analyzed in 95% PBS buffer - 5% ethanol solution. Ethanol was added to enhance solubility of free AZ and to make sure that the same medium would be used for both free AZ and D-AZ. To confirm that the drug release was due to hydrolysis of the ester linkage, a control experiment was performed, with the conjugate dissolved in DMSO no drug release was observed in DMSO over a 24-hour period, suggesting that complexation or encapsulation were not involved in the drug loading in the conjugate. In PBS, the conjugate released about 70% of its payload within the first 3 hours, with more than 90% released over a 16-hour period.
The initial release of AZ was relatively rapid in comparison with later stages of the release study due to continuous reduction in ester linkage concentration as the conjugate is cleaved. The release profile of the conjugate agrees well with ester linkages used to conjugate similar drugs, such as erythromycin to PAMAM dendrimer conjugates, whereas the rate of release is significantly faster in comparison with ester linkages used to attach hydrophobic drugs to PAMAM dendrimers without linkers. Perhaps this is due to the more accessible hydrolysis site provided by the linker. The stability of the ester linkage is affected by neighboring groups on the drug. The presence of the tertiary amine group adjacent to the ester link in D-AZ conjugate may enable effective hydrolysis. Thus, the ester linkage used in D-AZ conjugates is more susceptible to hydrolysis.

Cell uptake in vitro

Acute (Figure 3, A) and persistent chlamydial infections (Figure 3, C and Figure S9) were created using protocols described above. These images suggest that acute (panel A) and persistent (panel C) inclusions are formed. To examine whether PAMAM dendrimers are capable of entering into chlamydial inclusions within infected cells, C trachomatis-infected HEP-2 cell cultures were pulsed with FITC-labeled dendrimer (D-FITC) for 1 hour at 24–48 hours pi and observed by fluorescence microscopy. In Figure 3, panels A and C have only FITC-labeled
antichlamydial antibody, and no D-FITC, whereas panels B and D have only D-FITC. A similar staining pattern was seen with D-FITC in these cells, suggesting that D-FITC is present in acute (panel B) and persistent (panel D) inclusions. The labeled dendrimers clearly entered inclusions in both acutely- and persistently-infected HEp-2 cells; increased pulsing time increased D-FITC localization in inclusions somewhat, but also increased diffuse detection within the cytoplasm. The latter is obscured by Evans blue counterstaining. The dendrimer-associated fluorescence is concentrated in the inclusion in comparison with the other components of the host cell, suggesting that these dendrimers traffic to and localize selectively...
into the inclusion upon entering the host cells. This could lead to significant benefits, because dendrimers could deliver the antibiotics preferentially to the inclusions to be more effective than free antibiotic.

**Efficacy of dendrimer-azithromycin conjugate nanodevices (D-AZ) compared to free AZ**

Infected HEp-2 cells were treated with either free AZ (control) or D-AZ at equivalent AZ concentrations at either the time of infection (t = 0) or 24 hours pi (t = 24). D-AZ added at t = 0 (10.3 μg/ml of AZ) completely prevented infection of the host cells by *C. trachomatis* K serovar, as measured by the subsequent total absence of chlamydial inclusions (Figure 4, B). When D-AZ was added 24 hours pi, inclusion counts were reduced by more than 30%, and the inclusions were much smaller (Figure 4, C) in comparison with untreated control (Figure 4, A). The efficacy of the conjugate was better than that of free drug.

To enable a quantitative comparison of the efficacy of AZ in attenuating chlamydial growth or viability when delivered as a dendrimer conjugate versus delivery of free drug, *C. trachomatis*-infected HEp-2 cell cultures were pulsed with AZ+CA (AZ in the citrate form), AZ-CA (salt free form of AZ) or with D-AZ conjugates at varying concentrations of AZ at t = 0. Effects were determined at t = 24 hours pi, (Figure 5, A). At 48 hours pi (Figure 5, B), D-AZ performs significantly better than either AZ +CA or AZ-CA, not only in reducing number of inclusions (P < 0.001), but also in reducing inclusion sizes more effectively than did free AZ (P < 0.002). The improved performance of D-AZ is especially pronounced at lower drug doses (2 ng/ml). At 2 ng/ml, the conjugate reduces the area of inclusions by 50%, whereas the free drug does not show any measurable reduction. This suggests that the dendrimer may be transporting the drug and delivering it into inclusions faster than the free drug. This is consistent with the fact that dendrimer selectively localize in the inclusions (Figures 3 and 4), and release the drug readily (over a period of 16 hours, Figure 2). Given the rapidity of uptake of D-FITC into inclusions, it is likely the bulk of drug is released once D-AZ enters the inclusions. At higher doses, the amount of the drug used is much higher than the dose required for treatment, so that even a smaller fraction is sufficient.

**Azithromycin-dendrimer conjugates do not induce chlamydial persistence but do target cells persistently infected with the organism**

Persistent chlamydial infections are a well-documented clinical problem in reactive arthritis and other human diseases resulting from dissemination of *C. trachomatis* from its site of primary infection in the genital tract. Current opinion holds that chlamydial persistence evolved as an adaptive response to the stress of intracellular existence, and we therefore examined whether treatment of infected cells with dendrimers would provide stress sufficient to induce persistent infection. *Chlamydia*-infected HEp-2 cell cultures were pulsed with dendrimers at 12 hours pi, and the cultures were harvested at 24 hours pi, at which time RNA/cDNA was prepared from them for transcript analysis. Persistently infecting chlamydiae show a transcript profile that is significantly different from that of organisms undergoing normal active infection. For example, in persistent chlamydiae, transcripts from the *fisK, fisW, omp1*, and other genes are severely attenuated in comparison with transcript levels in actively growing cells. Assays targeting these 3 genes in dendrimer-treated chlamydiae-infected HEp-2 cells showed no transcriptional attenuation from any of them compared to levels in control (i.e., nondendrimer-treated infected cells) cultures (Figure S10, panel A); host HEp-2 cells also showed no upregulation of expression for any of more than 90 genes assessed in these assays (Figure S10, panel B). Given these results and data provided above concerning treatment of acutely infected cells with D-AZ complexes, we next examined whether D-AZ conjugates could effectively enter persistently infected cells to alter persistent infection. As shown in Figure 3, FITC-dendrimers rapidly entered persistently infected HEp-2 cells. An important aspect is that when PenG-treated HEp-2 cells were pulsed with D-AZ, the sizes of inclusions were reduced significantly (P < 0.02 at 8 and 16 ng/ml compared to free AZ; both methods of treatment had significant effects in comparison with untreated cells) suggesting reduction in infectious load by D-AZ in comparison with free AZ (Figure 6). This shows that D-AZ can also be effective in attenuating persistent infections. Experiments are in progress testing effects of D-AZ on persistently infected monocytes.

**Discussion**

In this study, we examined the means by which AZ, an important antibiotic with efficacy against many gram-negative and gram-positive bacterial pathogens, can be conjugated with PAMAM dendrimers, for the treatment of chlamydiae within infected host cells. We further examined whether such
dendrimer-drug conjugates would show increased efficacy over free drug in killing or attenuating growth of *C. trachomatis* when that organism is in its intracellular growth state within a human epithelial cell type, HEP-2. The hydroxyl terminated (neutral) PAMAM dendrimer was conjugated with AZ with an esterase cleavable bond. AZ was modified with a suitable linker at 2′-position to get a reactive acid functionality to minimize the steric hindrance and increase the drug release. Over a period of 16 hours, 90% of the drug was released from the conjugate. The presence of tertiary amine in 2′-position of AZ is responsible for preferential release of the drug from the nanodevice. We demonstrated that dendrimers alone home easily into the infected cells and further, rapidly concentrate in the *Chlamydia*-containing inclusions within host cells. Entry of the dendrimers into infected cells/chlamydial inclusions does not provide sufficient stress to induce persistent infection. Importantly, dendrimer-drug conjugates displayed significantly higher antimicrobial efficacy in comparison with free drug, as judged by strongly attenuated inclusion numbers and smaller inclusion areas. Thus, conjugation of AZ with G4-PAMAM dendrimers produced as described here show promise as a new therapeutic means for treating genital and other chlamydial infections.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at doi:10.1016/j.nano.2011.04.008.

**References**


