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Research Paper

Intracellular delivery of dendrimer triamcinolone acetonide conjugates into microglial and human retinal pigment epithelial cells

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ABSTRACT

Triamcinolone acetonide (TA) is a potent, intermediate-acting, steroid that has anti-inflammatory and anti-angiogenic activity. Intravitreal administration of TA has been used for diabetic macular edema, proliferative diabetic retinopathy and exudative age-related macular degeneration (AMD). However, the hydrophobicity, lack of solubility, and the side effects limit its effectiveness in the treatment of retinal diseases. In this study, we explore a PAMAM dendrimer-TA conjugate (D-TA) as a potential strategy to improve intracellular delivery and efficacy of TA to target cells. The conjugates were prepared with a high drug payload (~21%) and were readily soluble in saline. Compared to free TA, D-TA demonstrated a significantly improved toxicity profile in two important target [microglial and human retinal pigment epithelium (RPE)] cells. The D-TA was ~100-fold more effective than free TA in its anti-inflammatory activity (measured in microglia), and in suppressing VEGF production (in hypoxic RPE cells). Dendrimer-based delivery may improve the efficacy of TA towards both its key targets of inflammation and VEGF production, with significant clinical implications.

1. Introduction

Synthetic corticosteroids such as triamcinolone acetonide (TA) have both anti-inflammatory and anti-angiogenic activity, and are therefore potentially viable therapeutic options for treating various inflammatory and neovascular ocular diseases [1,2]. TA is an FDA-approved glucocorticosteroid administered intravitreally for the treatment of diabetic macular edema, macular edema associated with retinal vein occlusion [3], proliferative diabetic retinopathy [4], uveitis [5,6], sympathetic ophthalmia, and used as off-label drug for some forms of age-related macular degeneration (AMD) [7–9]. It has also been used for post-operative retinal surgery related inflammation [10]. A combination therapy of intravitreal TA (IVTA) along with photodynamic therapy (PDT) showed beneficial clinical outcomes with better visual acuity than PDT alone and reduced number PDT sessions required [11,12]. However, IVTA is often associated with side effects including elevation of intraocular pressure (IOP), cataractogenesis, photoreceptor cell death and retinal toxicity [13].

Solubilizing hydrophobic drugs such as TA is a challenge. Increasing the solubility of TA can lead to an increase in bioavailability, permeation through ocular tissue, and intracellular transport. If better drug solubility is achieved, it may result in prolonged efficacy at significantly lower concentration thereby reducing the chances of drug toxicity [14,15]. Following, intravitreal administration, TA forms epi-retinal crystals in the vitreous humor due to its lack of solubility [16], prolonging the therapeutic effect of TA, but may also cause side effects [16]. Drug aggregation and precipitation can lead to visual obscurity, unequal distribution, mechanical damage and local toxicity to the retinal tissue [16,17]. To address insolubility and sedimentation issues, TA formulations with benzyl alcohol or benzalkonium chlorides as vehicle preservatives were commercialized but resulted in vehicle-mediated toxicity and sterile-endophthalmitis [18,19].

PAMAM dendrimers are a class of well-defined, hyperbranched polymeric nanocarriers that are being investigated for ocular drug and gene delivery [20,21]. Their favorable properties such as small size, multivalency and water solubility can provide significant opportunities for many biologically unstable, hydrophobic drugs [22,23]. For example, dendrimer encapsulation of anti-glaucoma

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drugs resulted in better efficacy and also increased the drug uptake in corneal cell layers [15,24]. We have previously reported that, intravitreal administration of dendrimer fluocinolone acetonide (D-FA) conjugates selectively co-localized in activated microglial cells and provided sustained neuroprotection for a period of 30 days at a 40-fold lower dose compared to non-erodible controlled release implant for FA, in a rat RCS model of retinitis pigmentosa [25]. Upon systemic administration, dendrimer *N*-acetyl cysteine conjugates (D-NAC) targeted activated microglia in the brains of newborn rabbits with cerebral palsy, and significantly improved motor function, and reduced neurological injury [26,27]. We reported dendrimer-TA based gene delivery platform for improved delivery of genes and enhanced transfection in human retinal pigment epithelial cells [28]. Such targeting and enhanced permeation is desirable for intracellular delivery of corticosteroids to activated microglia that are located in deep layers of retina and RPE cells that have low permeability due to their tight junctions.

Despite its drawbacks, several pre-clinical and clinical studies have reported the multimodal therapeutic effects of TA [29]. For instance, TA demonstrated anti-inflammatory effect and inhibited microglial activation by suppressing the release of inflammatory cytokines and nitric oxide (NO) [30,31]. TA reduced VEGF expression in retinal pigment epithelium (RPE) cultures that were subjected to oxidative stress. Similar results were achieved in vivo with significant reduction of neovascularization in many clinically relevant animal models [10,4,32,33]. TA improves blood retinal barrier (BRB) health by enhancing the expression of adhesion molecules in endothelial cells [34,35], and significantly suppresses the blood vessel formation in choroidal endothelial cells [36]. These studies underscore the potential of TA as a drug, which can be further enhanced through superior intracellular delivery D-TA conjugates may be a viable formulation for targeted intracellular delivery of TA.

In this study, we investigate the ability of PAMAM dendrimers to deliver TA into activated microglia and human RPE cells, thus increasing its therapeutic effect. We synthesize, characterize and evaluate the efficacy of dendrimer-TA conjugates, relating to its anti-inflammatory and anti-angiogenic activity. D-TA improved the solubility of TA with reduced toxicity, improved cellular uptake and intracellular trafficking.

2. Materials and methods

2.1. Chemicals and reagents

Hydroxyl- and amine-functionalized ethylenediamine core generation four PAMAM dendrimers (G4-OH; diagnostic grade; 64 end-groups) were purchased from Dendritech Inc. (Midland, MI, USA). Triamcinolone acetonide (TA), glutaric anhydride, piperidine, *N,N'*-diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), anhydrous dimethylformamide (DMF), dimethylacetamide (DMA) and 6-(Fmoc-amino) caproic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) was purchased from Bachem Americas Inc. (Torrance, CA, USA). Cy5-mono-NHS ester was purchased from Amersham Biosciences-GE Healthcare (Pittsburgh, PA, USA). ACS grade DMF, dichloromethane (DCM), diethylether, hexane, ethyl acetate, HPLC grade water, acetonitrile, and methanol were obtained from Fisher Scientific and used as received for dialysis, purification and column chromatography. Dialysis membrane (MW cut-off 1000 Da) was obtained from Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA).

The reactions were carried out under nitrogen. Thin-layer chromatography (TLC) was performed on silica gel GF_{254} plates (Whatman, Piscataway, NJ), and the spots were visualized with UV light. Proton NMR spectra of the final conjugates as well as intermediates were recorded on a Bruker (500 MHz) spectrometer using commercially available DMSO-d_6 solvent. Proton chemical shifts were reported in ppm (δ) and tetramethylsilane (TMS) used as internal standard. All data were processed using ACD/NMR processor software (Academic Edition).

2.2. Synthesis of dendrimer conjugates

2.2.1. Synthesis of triamcinolone acetonide-21-glutarate (TA-linker, 1)

Triamcinolone acetonide-21-glutarate (TA-Linker, 1) was synthesized using a previously established procedure [28]. A detailed synthesis description is provided as a part of supplementary information.

2.2.2. Synthesis of dendrimer-triamcinolone acetonide conjugates (D-TA, **2**)

TA-21-glutarate (TA-Linker, 139.8 mg, 0.255 mmol) was dissolved in anhydrous DMF (5 mL) in a 50 mL round bottomed flask under nitrogen, to which PyBOP (266.2 mg, 0.516 mmol) dissolved in DMF (5 mL) and DIEA (200 μL) was added, and the reaction mixture was allowed to stir for 1 h in an ice bath. PAMAM G4-OH (238.8 mg, 0.017 mmol) dissolved in anhydrous DMF (10 mL) was added drop wise to the reaction mixture above, and stirred for 48 h under nitrogen. The mixture of solvents was evaporated at 25 °C under vacuum. The crude product was redissolved in DMF (20 mL) and subjected to dialysis in DMF (membrane MW cutoff = 1 kDa) for 24 h, where the solvent was changed at least 4 times. The obtained solution was evaporated under reduced pressure at room temperature, followed by high vacuum overnight, to produce an off-white semi-solid dendrimer-triamcinolone conjugate (D-TA, 470.9 mg). The resultant semi solid product was dissolved in ice cold DI water and dialyzed against DI water (membrane MWCO = 1 kDa) at 4 °C for 5 h by changing the water every hour to remove traces of DMF. The resultant water layer was lyophilized to get fluffy white powder of D-TA (402.3 mg) (Fig. S1-B). The D-TA conjugates were characterized by ^{1}H NMR for drug loading and reverse-phase HPLC for purity. ^{1}H NMR (DMSO-d_6) δ 0.82 (s, —CH_3 protons of TA), 1.14 (s, —CH_3 protons of TA), 1.34 (s, —CH_3 protons of TA), 1.49 (s, —CH_3 protons of TA), 1.53–2.05 (m, —C*H* protons of TA, —CH_2 protons of linker), 2.20 (brs, —CH_2 protons of G4-OH), 2.31–2.48 (m, —C*H* protons of TA, —CH_2 protons of linker, —CH_2 protons of G4-OH), 2.64 (bs, —CH_2 protons of G4-OH), 3.09–3.11 (t, —CH_2 protons of G4-OH), 3.28 (m, —CH_2 protons of G4-OH), 3.38–3.40 (t, —CH_2 protons of G4-OH), 3.58 (s, —C*H* protons of TA), 4.00–4.02 (m, CH_2OC=O protons, G4-OH), 4.20 (brs, —C*H* protons of TA), 4.73–6.01 (singlets and doublets, —C*H* protons of TA, OH protons of G4-OH), 6.22–7.31 (two doublets, aromatic protons of TA), 7.79–8.07 (m, amide protons of G4-OH).

2.2.3. Synthesis of intermediate dendrimer conjugates

The synthesis protocols for the intermediate conjugates D-OH-NHFmoc (**3**), Fmoc-functionalized intermediate D-TA (**4**) and NH_2-D-TA (**5**) are provided as part of supplementary information.

2.2.4. Synthesis of Cy5-labeled dendrimer-triamcinolone acetonide conjugates (Cy5-D-TA, 6)

The NH_2-D-TA (**5**), (25 mg, 0.0013 mmol) was dissolved in 1 mL of borate buffer (pH 9.0) at room temperature. The reaction mixture was cooled to 0 °C, and Cy5 mono NHS ester (2.18 mg, 0.0027 mmol) dissolved in 1 ml of DMF was added. *N*-Hydroxysuccinimide (1.58 mg, 0.013 mmol) dissolved in 500 μL of DMF was added to reaction. The reaction mixture was stirred overnight at room temperature. The crude product was dissolved