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Polymer-doxycycline conjugates as fibril disrupters: An approach towards the treatment of a rare amyloidotic disease

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ABSTRACT

The term amyloidosis describes neurological diseases where an abnormal protein is misfolded and accumulated as deposits in organs and tissues, known as amyloid, disrupting their normal function. In the most common familial amyloid polyneuropathy (FAP), transthyretin (TTR) displays this role primarily affecting the peripheral nervous system (PNS). Advanced stages of this inherited rare amyloidosis, present as fibril deposits that are responsible for disease progression. In order to stop disease progression, herein we designed an efficient family of nanoconjugates as fibril disrupters. These polymer conjugates are based on doxycycline (doxy), already in phase II trials for Alzheimer's disease, covalently linked to poly-L-glutamic acid (PGA). The conjugates were rationally designed, looking at drug loading and drug release rate by adequate linker design, always considering the physiological conditions at the molecular target site. Conjugation of doxycycline exhibited greater potential towards TTR fibril disaggregation in vitro compared to the parent drug. Exhaustive physico-chemical evaluation of these polymer-drug conjugates concluded that drug release was unnecessary for activity, highlighting the importance of an appropriate linker. Furthermore, biodistribution studies through optical imaging (OI) and the use of radiolabelled polymer-drug conjugates demonstrated conjugate safety profile and renal clearance route of the selected PGA-doxy candidate, settling the adequacy of our conjugate for future in vivo evaluation. Furthermore, preliminary studies in an FAP in vivo model at early stages of disease development showed non-organ toxicity evidences. This nanosized-system raises a promising treatment for advanced stages of this rare amyloidotic disease, and also presents a starting point for possible application within other amyloidosis-related diseases, such as Alzheimer's disease.

1. Introduction

Polymer therapeutics (PT) can be underlined as the most successful first generation nanomedicines after last year's report on the US Top 10 selling drugs that include the polymeric drug glatiramer acetate for multiple sclerosis (*Copaxone*®, Teva Pharm; $3.7 billion), and the polymer conjugate pegfilgrastim for the treatment of neutropenia (*Neulasta*®, Amgen; $3.6 billion) [1,2]. These complex, multicomponent constructs present the therapeutic agent covalently linked to a water-soluble polymer vehicle, in contrast to other drug delivery systems [3,4]. In order to achieve a second generation of polymer conjugates defined strategies are pursued [4–7] including, the use of improved polymer structures [8,9], exhaustive physico-chemical characterisation through leading techniques [10,11], implementation of polymer-based combination therapies [7] and rational conjugate design towards novel molecular targets in areas such as regenerative medicine and chronic and debilitating diseases [6,12–14], which will further progress this platform technology.

Focusing on the later strategy, one of these potential targets within our ageing society are neurodegenerative disorders, including amyloidosis. In these diseases an abnormal protein is misfolded and accumulated as deposits in organs and tissues (known as *amyloid*) disrupting their normal function. Amongst the different types of amyloidosis, Alzheimer's (AD) and Parkinson's (PD) diseases are renowned examples affecting the central nervous system (CNS). Familial amyloidotic polyneuropathies (FAP) also constitute a group of inherited amyloidosis and the most common is caused by mutation of a protein called transthyretin (TTR). A mutation in this protein promotes its aggregation, resulting in fibril formation. These extracellular amyloid deposits occur in several organs and tissues, mainly in the peripheral nervous system (PNS) [15,16], promoting organ failure. TTR has also been proposed to trigger neurodegeneration through engagement with the Receptor of Advanced Glycation End

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products (RAGE receptor), leading to neuronal toxicity and death [17]. The ligand-RAGE receptor pathway has been involved in a wide spectrum of human conditions and illnesses, including neurodegeneration and ageing [18]. In FAP, blockade of the RAGE signal transduction constitutes another key strategy for therapy. Our work on the PEGylation of a peptide capable of inhibiting RAGE receptor interaction with TTR prefibrillar aggregates has already resulted in a promising opportunity for FAP therapy in early stages of the disease [19].

Currently in the clinics, there is not specific pharmacological therapy for this disease. Effective FAP treatment alternatives are mainly reduced to orthotropic liver transplantation (OLT), thus, suppressing the main source of mutant TTR [20,21]. Recently, Tafamidis (Vyndaquel®) has been approved by the EMEA and is in phase III in USA for TTR-FAP treatment in patients with stage 1 symptomatic polyneuropathy. Tafamidis is only preferential for treating early stage symptomatic FAP as a potent TTR stabiliser [22] maintaining neurologic function [23]. However, there is still an urgent need to encourage medical research on new strategies for the treatment of this degenerating disease mainly on advanced stages. Amongst the therapies under investigation in this line, disruption of amyloid fibrils is gaining importance in clinical trials [24–27], and the tetracycline doxycycline (doxy) is one of the most advanced compounds under evaluation. Doxy has already demonstrated to be the most effective compound at disaggregating TTR mature fibrils [28]. Cardoso et al. have proved that doxy acts as a TTR fibril disrupter in vitro as well as in vivo, with an additional decreasing of fibril associated standard markers [24,29,30]. The neuroprotector effect of this class of compounds, in addition to its own anti-microbacterial properties, was described in several other disease models including cerebral ischemia, spinal cord injury, PD, AD, Huntington's disease, amyotrophic lateral sclerosis and multiple sclerosis [17,31]. Consequently, breaking up TTR fibrils combined with a possible neuroprotective effect constitutes a promising target for therapeutic purposes against FAP and herein we aimed to address this possibility throughout rationally designed polymer-doxy conjugates that could enhance the already demonstrated benefits of doxy in the clinics.

Major goals of polymer conjugation enclose changes in the biodistribution in comparison to the parent drug, avoidance of undesired accumulation in non-affected organs and reduction of the administered dose and/or its frequency. In this study, a polypeptidic vehicle has been selected: poly-L-glutamic acid (PGA). Polyglutamates are highly biocompatible, biodegradable and multifunctional polymers, which have been successfully used alone or as building blocks in polymer-drug conjugates and polymeric micelles for various medical applications [8]. PGA degradability is triggered by cysteine proteases (particularly cathepsin B), which play a key role in the lysosomal degradation of this polymer [32]. In addition, PGA conjugated to paclitaxel (named as Opaxio™) is currently in Phase III for the treatment of various cancers including ovarian, prostate or head and neck carcinomas and was recently granted as orphan drug status for the treatment of glioblastoma in combination with radiotherapy [33]. In this work, we reported a novel application for this polypeptide: PGA-doxy conjugates as a future treatment for a rare amyloidotic disorder: FAP with possible extension to other amyloidosis-related diseases.

2. Materials and methods

2.1. Materials

Poly-L-glutamic acid (Mw 17800, Mw/Mn 1.2) was obtained from Polypeptide Therapeutic Solutions SL (PTS, Valencia, Spain). Z-Gly-Gly-OH (N-carbobenzyloxy-glycylglycine) was acquired from Bachem (Bubendorf, Switzerland). Triisopropylsilane (TIPS) was bought from VWR. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride (DMTMM·Cl) salt was synthesised according the protocol of Kunishima et al. [34] and the tetrafluoroborate salt (DMTMM·BF_4) as in the reference [35]. The Cy5.5 derivative (6-SIDCC) was provided by Mivenion GmbH (Berlin, Germany). DOTA derivative (2,2′,2″-(10-(4-((2-aminoethyl)amino)-1-carboxy-4-oxobutyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid) was purchased in CheMatech (Dijon, France). All other chemical reagents and solvents were purchased from Sigma Aldrich (Madrid, Spain).

2.2. Chemical data

All reactions requiring anhydrous conditions were performed under an argon or nitrogen atmosphere. For size exclusion chromatography (SEC) analysis an HPLC from Waters (Milford, USA) was used, equipped with an Autosampler 717, a photodiode array detector unit 2996 (model Z996), 3 pumps 515 and a multi lambda fluorescence detector (model 2475). For doxycycline detection, a LIChroCART® LiCrospher® 100 column (RP-18, 125 × 4 mm) was used. The flow rate was 1 mL/min, using an acetonitrile (ACN) gradient (0.1% TFA) in aqueous 0.1% TFA (method: t = 0 min B = 95%, t = 33 min B = 5%, t = 35 min B = 5%, t = 40 min B = 95%, λ = 273 nm). For the conjugates analysis, TSK-Gel Columns: G2500 and 3000 (30.0 cm × 7.8 mm; 6 μm) were used and an isocratic gradient with phosphate buffer salt (PBS) (0.1 M pH = 7.4, flow rate = 1 mL/min, 50 min, λ = 273 nm). For liquid chromatography–mass spectroscopy (LC–MS) analysis, the equipment used was an Acquity Ultra Performance LC (Waters) with a PDA detector and a Micromass ZQ Waters 400 LC Single quadrupole mass spectrometer, with a RP-18 (Kinetex column (2.6 μm, C18, 100 × 4.6 mm)) and H_2O/ACN (both 0.1% formic acid) as mobile phase. UV spectra were recorded on a Jasco V-530 UV/Vis spectrophotometer. Fluorescence was measured with a Victor^2 Wallac 1420 Multilabel HTS Counter Perkin Elmer (Northwolk, CT, US). Most of the polymer conjugates were purified by SEC in PD10 pre-packed columns (GE Healthcare, Buckinghamshire, UK) using water as eluent.

2.3. Synthesis of PGA-X-doxy conjugates

2.3.1. Ester linkage (PGA-COO-doxy) (Figs. 1, S3)

Carboxyl groups of PGA were previously activated with N-hydroxysuccinimide (NHS). PGA-NHS (average Mw_{unit} 179.6 Da) was dissolved in anhydrous dimethylformamide (DMF). Separately, the corresponding amount for the desired molar percentage of doxy (Mw 512.94 Da) (Table S1) was dissolved in the same conditions and added dropwise to the PGA-NHS solution. Next, a catalytic amount of N,N-dimethylaminopyridine (DMAP) was incorporated and pH was adjusted to 8 with di-isopropylethylamine (DIEA). Reaction was allowed to proceed protected from light for 16 h under agitation at room temperature (RT), after which DMF was evaporated under high vacuum. Non-reacted drug as well as organic soluble reaction subproducts were removed by washing the crude with a mixture of chloroform:acetone (4:1). The product containing fraction was placed on ice for 30 min before the solvent was removed by centrifugation and the resulting precipitate dried under vacuum. The sodium salt of the conjugate was obtained by dissolving the product in $NaHCO_3$ 1 M. This aqueous solution was purified by SEC (Sephadex™ G-25; H_2O as eluent). Fractions were analysed by HPLC/GPC techniques. Fractions containing the conjugate were mixed and lyophilised. Note: by varying doxycycline equivalents, different wt.% loadings were achieved (Table S2).

2.3.2. Amide linkage (PGA-CONH-doxy) (Fig. 1, S4)

For amide conjugates, several carboxyl activation methodologies were used (Supporting information). The most reproducible synthesis was achieved through the DMTMM activation procedure. Previously, the sodium salt form of PGA (0.66 mmol, Mw_{unit} 151 Da) was dissolved in H_2O i.e., for 30 mol% carboxyl activation and 15 mol% doxy loading: separately, doxy-NH_2 (0.2 mmol, Mw 557.53 Da) and DMTMM·Cl (0.2 mmol, Mw 276.77 Da) were dissolved in H_2O and then added to the PGA solution. The reaction was allowed to proceed at pH 8 for 24 h at RT and protected from light. Then, product was dried by lyophilisation