Journal of Controlled Release 159 (2012) 290–301

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Demonstrating the importance of polymer-conjugate conformation in solution on its therapeutic output: Diethylstilbestrol (DES)-polyacetals as prostate cancer treatment

Vanessa Giménez [a], Craig James [b], Ana Armiñán [a], Ralf Schweins [c], Alison Paul [b], María J. Vicent [a,*]

[a] Polymer Therapeutics Lab., Centro de Investigación Príncipe Felipe (CIPF), Av. Autopista del Saler 16, 46012 Valencia, Spain
[b] School of Chemistry, Cardiff University, Main Building, Park Place, Cardiff CF10 3AT, UK
[c] Institute Laue-Langevin, BP 156, 6, rue Jules Horowitz, 38042 Grenoble Cedex 9, France

ARTICLE INFO

Article history:
Received 4 September 2011
Accepted 24 December 2011
Available online 31 December 2011

Keywords:
Polymer therapeutics
Polyacetals
pH-dependent degradation
Solution conformation
SANS
Prostate cancer

ABSTRACT

The design of improved polymeric carriers to be used in the next generation of polymer therapeutics is an ongoing challenge. Biodegradable systems present potential advantages regarding safety benefit apart from the possibility to use higher molecular weight (Mw) carriers allowing PK optimization, by exploiting the enhanced permeability and retention (EPR)-mediated tumor targeting. Within this context, we previously designed pH-responsive polyacetalic systems, *tert*-polymers, where a drug with the adequate diol-functionality was incorporated within the polymer mainchain. The synthetic, non-steroidal estrogen, diethylstilboestrol (DES) clinically used for the treatment of advanced prostate cancer was chosen as drug. In order to improve the properties of this *tert*-polymer, novel polyacetalic systems as *block*-co-polymers, with more defined structure have been obtained. This second generation polyacetals allowed higher drug capacity than the *tert*-polymer, a biphasic DES release profile at acidic pH and due to its controlled amphiphilic character readily formed micelle-like structures in solution. These features result in an enhancement of conjugate therapeutic value in selected prostate cancer cell models. Exhaustive physico-chemical characterization focusing on nanoconjugate solution behavior and using advanced techniques, such as, pulsed-gradient spin-echo NMR (PGSE-NMR) and small-angle neutron scattering (SANS), has been carried out in order to demonstrate this hypothesis. Clear evidence of significantly different conformation in solution has been obtained for both polyacetals. These results demonstrate that an adequate control on molecular or supramolecular conformation in solution with polymer therapeutics is crucial in order to achieve the desired therapeutic output.

1. Introduction

The development of better polymeric carriers is an ongoing challenge to achieve the next generation of polymer therapeutics [1-3]. Biopersistent carriers as polyethylenglycol (PEG) or N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers can present disadvantages if chronic parenteral administration and/or high doses are required as there is the potential to generate 'lysosomal storage disease' syndrome. Preclinical evidence of intracellular vacuolation with certain PEG-protein conjugates is raising awareness of the potential advantage of biodegradable polymers regarding safety benefit apart from the possibility to use higher molecular weight (Mw) carriers allowing PK optimization, by enhancing the enhanced permeability and retention (EPR)-mediated tumor targeting [2,4,5]. Therefore, biodegradable polymers such as dextrins [6,7], polypeptides [4,8,9], polyesters [10,11] or polyacetals [12,13] could be considered as promising candidates to be used as carriers for targeted drug delivery.

To allow use of polymers of higher Mw, a family of hydrolytically labile water-soluble polyacetals was developed [13]. These can be functionalized to allow side-chain conjugation to a drug payload such as doxorubicin (Dox) [12]. These polyacetals show a clear pH-dependent degradation being relatively stable at pH 7.4 but degrade significantly faster at the acidic pH that is encountered in endosomes and lysosomes. *In vitro* and *in vivo* studies confirmed that the polyacetals are not toxic, they are not taken up extensively by the liver or spleen, and are also long circulating [13]. Moreover, the polyacetal–Dox conjugate (Mw 86 kDa) displayed significantly prolonged plasma circulation time and enhanced tumor accumulation compared to the HPMA copolymer-Dox conjugate (CF28068, known as PK1, Mw 30 kDa) in Phase II clinical trials [12,14].

Polyacetals can be prepared by a mild polymerization method involving the reaction of diols with divinyl ethers [15]. To move a step further on this design we synthesized polyacetals incorporating a drug with bis-hydroxyl functionality into the polymer backbone [16]. Degradation of the polymer backbone in the acidic environment of the lysosome or the extracellular fluid of some tumors would then

* Corresponding author. Tel.: +34 963289681; fax: +34 963289701.
E-mail address: mjvicent@cipf.es (M.J. Vicent).

0168-3659/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2011.12.035

trigger drug release eliminating the need for a biodegradable linker. For this purpose, we used the *tert*-polymerization process developed for the synthesis of the functionalized polyacetals [13] in combination with the model drug diethylstilbestrol (DES) [16]. DES is a synthetic non-steroidal estrogen and its administration was a classic form of androgen deprivation therapy (ADT), standard approach to the treatment of advanced prostate cancer for more than 50 years. Its use, however, has been severely limited by a poor water solubility and wide ranging dose-related toxicities, mainly cardiovascular side effects and in particular thromboembolic events [17-20]. DES can be considered as an 'old' treatment, however, is taken renewed consideration as very recently has been demonstrated that low-dose DES is safe and effective in castrate-resistant prostate cancer (CRPC) patients when used before the initiation with chemotherapy [19]. Also, a combination of DES to chemotherapeutics such as docetaxel was found to produce a significant level of antitumor activity in patients with metastatic, androgen independent prostate cancer (AIPC) [21,22]. It is hypothesized that, apart from clearly reducing DES toxicity by means of the EPR-mediated tumor targeting [1,23], the conjugation of DES to polymeric carriers would more easily allow a low-dose clinical regime as a controlled release of the drug could be achieved for a prolonged period of time. Also, polymer multivalency would allow the synthesis of polymer-based combination conjugates that could better exploit the synergism observed already with, i.e. docetaxel [21,24]. Our previous research with DES-polyacetals already demonstrated that DES solubility can be greatly enhanced upon polymerization. And more interestingly, the conjugates underwent degradation that was clearly pH-dependent, with greater DES release at acidic pHs. Additionally, the active isomerism of the estrogen was maintained (*trans*-DES) [25] and the conjugates displayed enhanced *in vitro* cytotoxicity compared to free DES. These *tert*-DES polyacetals could therefore be defined as the first water-soluble anticancer polymeric drugs designed for acidic pH-triggered release where the drug is incorporated into the polymer mainchain [16]. Ratifying the utility of this synthetic strategy, another recent example has been reported using curcumin as a diol-functionalized anticancer drug. The polyacetal-based polycurcumins showed a clear antitumor effect *in vitro* and *in vivo* in ovarian cancer models [26].

The first synthesized *tert*-polymer had a drug content of ~4 wt.% and a polydispersity (Mw/Mn) around 1.8. The initial aim of the current study was to synthesize a second generation of DES-based polyacetals with improved properties, such as narrower Mw distributions and higher drug loading, and more importantly to study with these model systems, if slight structural modifications could significantly influence conjugate therapeutic output. These second generation polyacetals were obtained using a block-*co*-polymer methodology. *Tert*-DES and *block*-DES were then tested in selected prostate cancer cell models. In order to explain the differences encountered once biologically evaluated, an exhaustive characterization of the conformation in solution for both polyacetalic systems was carried out by means of different techniques, such as: transmission electron microscopy (TEM), dynamic light scattering (DLS), pulsed-gradient spin-echo NMR (PGSE-NMR) [27] and small-angle neutron scattering (SANS) [28] which have recently been used to good effect for understanding the solution conformation of polymer-conjugates [29-32].

2. Materials and methods

2.1. Materials and instruments

Tri(ethylene glycol) divinyl ether (TEGDVE), 2-amino-1,3-propanediol (Serinol), Fluorenylmethyloxycarbonyl chloride (Fmoc-Cl), Triethylamine and *N*,*N*-dimethylformamide (DMF) were from Fluka Chemika (Masserschmittstr, D). Dioxane, tetrahydrofuran anhydrous (THF) and toluene anhydrous were supplied from Aldrich (Dorset, UK). Before use, the THF was distilled from sodiumbenzophenone. Poly (ethylene glycol) (PEG), p-toluenesulfonic acid monohydrate (*p*-TSA), diethylstilboestrol (DES) were obtained from Aldrich and dried in a vacuum oven at 80 °C before use. Deuterated chloroform-d_1, DMSO-d_6 and D_2O were purchased from Deutero GmbH. DMSO-d_6 was dried and stored over molecular sieves (4 Å). Deuterated methanol MeOD (>99%-d) was from Sigma-Aldrich (UK), and used as received.

2.2. Characterization

^{1}H- and ^{13}C-NMR spectra were obtained at 300 MHz using a FT-spectrometer from Bruker and analyzed using the Topspin software. Pulsed Gradient Spin-Echo (PGSE) NMR experiments were performed on a Bruker AMX360 NMR spectrometer using a stimulated echo-sequence as described elsewhere [33]. The configuration uses a 5 mm diffusion probe (Cryomagnet Systems, Indianapolis) and a Bruker gradient spectroscopy accessory unit. The polymer self-diffusion coefficients *D*s were obtained for solutions at 25 °C using methodology and analysis previously described [34].

For size exclusion chromatography (SEC) measurements the polymers were dried at 40 °C overnight in a vacuum oven and subsequently characterized by SEC at 25 °C with THF as the eluent at a flow rate of 0.8 mL min^{-1}. Prior to injection the samples were filtered (0.22 μm nylon membrane) and sonicated for 5 min. The SEC equipment consisted of a Waters 717 plus autosampler, a TSP Spectra Series P 100 pump triSEC-Viscotek, array, TDA™ 302 series system, including two Waters Styragel 7.8×300 mm Columns (HR3 and HR4) for THF/DMF and a Viscotek TDA 302 triple detector Array with refractive index (RI), Small Angle Light Scattering, Right Angle Light Scattering, viscosimeter and a model 2501 UV as detector. Mw/Mn and Mw of the polyacetals were determined using the software OmniSec 4.2. Calibration was achieved with well defined Poly(-methyl methacrylate) (PMMA)/THF standards (PolyCAL PMMA 65KDa Standard, d*n*/d*c* = 0.0854 mL/g; Mn = 61304 Mw = 64368 IV = 0.228), provided by Polymer Standards Service (PSS)/Mainz Germany.

MTT assay measurements were performed using a Victor^2 Wallac 1420 Multilabel HTS Counter Perkin Elmer plate reader (Northwolk, CT, USA). Live cell confocal fluorescence microscopy studies were carried out at the confocal microscopy service at CIPF (Valencia, Spain) and were performed using a Leica confocal microscope from Leica Microsystems GmbH (Wetzlar, D) equipped with a l-blue 63 oil immersion objective and handled with a TCS SP2 system, equipped with an acoustic optical beam splitter (AOBS). Excitation was with an argon laser (548, 476, 488, 496 and 514 nm) and blue diode (405 nm). Images were captured at an 8-bit gray scale and processed with LCS software Version 2.5.1347 (Leica, Germany) containing multicolor, macro and 3D components.

***DES-Polyacetal* (*tert*-DES) 1** was synthesized by *tert*-polymerization of vinyl ethers and alcohols in THF by optimization of the previously reported protocol [16]. Briefly, PEG (2 g, Mw = 4.000 g/mol, 0.5 mmol), *p*-TSA (0.003 g, 0.015 mmol) and DES (0.134 g, 0.5 mmol) (all previously dried for 24 h in a vacuum oven) were added to THF (6 mL, distilled over sodium) and stirred until dissolved. Next, TEGDVE (0.2 mL 1.07 mmol) was slowly added to the solution using a syringe to preserve anhydrous conditions. The reaction was stirred vigorously for 3 h in the dark at RT. Triethylamine was then added to neutralize the *p*-TSA catalyst (until pH 8) and then followed by rapid stirring for 30 min. The solution was then poured into a rapidly stirring hexane/diethyl ether mixture (4:1, 100 mL) cooled over an ice bath, and stirred for a further 30 min to fully precipitate polyacetal **1**. The product was isolated as a white powder by filtration and further dried in vacuum at RT. To ensure complete removal of *p*-TSA an extraction with $CHCl_3$ and saturated $NaHCO_3$ solution was carried out and afterwards the organic phases