European Journal of Pharmaceutics and Biopharmaceutics 122 (2018) 176–185

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

Doxorubicin synergism and resistance reversal in human neuroblastoma BE(2)C cell lines: An in vitro study with dextran-catechin nanohybrids

Orazio Vittorio[a,b,1], Marion Le Grand[a,b,1], Sami A. Makharza[c], Manuela Curcio[d], Paola Tucci[d], Francesca Iemma[d], Fiore Pasquale Nicoletta[d], Silke Hampel[e], Giuseppe Cirillo[d,*]

[a] Children's Cancer Institute, Lowy Cancer Research Centre, University of New South Wales, Sydney, Australia
[b] Australian Centre for NanoMedicine, ARC Centre of Excellence in Convergent Bio-Nano Science and Technology, University of New South Wales, NSW, Sydney, Australia
[c] College of Pharmacy and Medical Sciences, Hebron University, Hebron, Palestine
[d] Department of Pharmacy Health and Nutritional Science, University of Calabria, 87036 Rende (CS), Italy
[e] Leibniz Institute of Solid State and Material Research Dresden, 01171 Dresden, Germany

ARTICLE INFO

Keywords:
Dextran-catechin conjugate
Hybrid nanocarrier
Doxorubicin
Neuroblastoma
Multidrug resistance
Synergism

ABSTRACT

Hybrid nanocarrier consisting in nanographene oxide coated by a dextran-catechin conjugate was proposed in the efforts to find more efficient Neuroblastoma treatment with Doxorubicin chemotherapy. The dextran-catechin conjugate was prepared by immobilized laccase catalysis and its peculiar reducing ability exploited for the synthesis of the hybrid carrier. Raman spectra and DSC thermograms were recorded to check the physicochemical properties of the nanohybrid, while DLS measurements, SEM, TEM, and AFM microscopy allowed the determination of its morphological and dimensional features. A pH dependent Doxorubicin release was observed, with 30 and 75% doxorubicin released at pH 7.4 and 5.0, respectively. Viability assays on parental BE(2)C and resistant BE(2)C/ADR cell lines proved that the high anticancer activity of dextran-catechin conjugate (IC_{50} 19.9 ± 0.6 and 18.4 ± 0.7 μg mL^{-1}) was retained upon formation of the nanohybrids (IC_{50} 24.8 ± 0.7 and 22.9 ± 1 μg mL^{-1}). Combination therapy showed a synergistic activity between doxorubicin and either bioconjugate or nanocarrier on BE(2)C. More interestingly, on BE(2)C/ADR we recorded both the reversion of doxorubicin resistance mechanism as a consequence of decreased P-gp expression (Western Blot analysis) and a synergistic effect on cell viability, confirming the proposed nanohybrid as a very promising starting point for further research in neuroblastoma treatment.

1. Introduction

Neuroblastoma is the third most common type of extracranial solid cancer in the pediatric population after leukemia and brain tumors, accounting for 15% of pediatric oncologic deaths [1]. The 5-year event-free survival for high-risk Neuroblastoma patients treated with surgery, radiation therapy and consolidation therapy with bone marrow transplantation after myeloablative doses of chemotherapy is 30% [2]. Recently, immunotherapy was found to increase overall survival when combined with maintenance therapy but proved to be ineffective in patients with recurrent or refractory bulky disease [3]. Moreover, due to the toxic side effects of chemotherapy, survivors of Neuroblastoma frequently have lifelong health issues from the therapy they received as a child [4,5]. Apart from intrinsic toxicity, the therapeutic efficacy of commonly employed therapeutics agents for Neuroblastoma treatments (e.g. vinca alkaloids, platinum drugs, and anthracyclines) is often hampered by the poor bioavailability of the drug at the site of action [6–9]. This is attributed to both the unfavorable pharmacokinetic profiles and the development of resistance at the cell levels [10–12], with the possibility to reduce the systemic dosages and reverse drug resistance being crucial to provide an effective therapy [13].

Multidrug resistance (MDR) is the major obstacle of chemotherapy in Neuroblastoma treatment, especially for Doxorubicin (DOX) therapy [14]. The identified resistance mechanism consists in the overexpression of P-glycoprotein (P-gp), an adenosine 5-triphosphate (ATP)-binding cassette (ABC) transporters pumping the anticancer drug out of tumor cells, resulting in failure of chemotherapy due to low intracellular drug concentrations [15].

Numerous compounds have been shown to inhibit the drug efflux function of P-gp and therefore, increase the intracellular concentration of chemotherapies with ultimate decrease in cellular resistance. Several drugs are known as P-gp inhibitors such as, cyclosporines, calmodulin

* Corresponding author.
E-mail address: giuseppe.cirillo@unical.it (G. Cirillo).
[1] These authors contributed equally.

https://doi.org/10.1016/j.ejpb.2017.11.005
Received 5 July 2017; Received in revised form 7 November 2017; Accepted 8 November 2017
Available online 10 November 2017
0939-6411/

inhibitors, indole alkaloids, coronary vasodilators, quinolines, hormones and calcium channel blockers [9]. However, no significant clinical output for any P-gp inhibitor can be declared so far. This is because these inhibitors have shown a low bioavailability within the tumor and strong side effects such as, immunosuppressive and cardiovascular effects.

In the efforts to find more targeted and less toxic therapies for Neuroblastoma, naturally occurring polyphenols, and catechins in particular, have shown great potential as both preventing and therapeutic drugs in ongoing researches and clinical trials [16–22]. Furthermore, they received a great deal of attention for cancer treatment by virtue of synergistic effects when associated with conventional anticancer drugs [23–25], and the ability to act as P-gp modulators with key benefits in overcoming MDR [26]. Polyphenols, indeed, were effectively employed in combination therapies based on DOX, to reverse drug resistance in in vitro and *in vivo* studies on different cancer types, including breast and liver [27–29]. Nevertheless, the clinical applicability of polyphenols is hindered by their low stability and bioavailability, and the conjugation with macromolecular systems was proved to be a valuable tool to solve this issue [30,31]. In our previous work, we developed a Dextran-Catechin conjugate (Dex-CT), possessing increased serum stability compared to free catechin (CT), and high anticancer activity in different cancer cells, including Neuroblastoma [32,33]. In this study, we demonstrate the efficiency of Dex-CT conjugate to synergize DOX combination therapy for Neuroblastoma treatment (BE(2)C cell) and reverse the P-gp resistance in BE(2)C/ADR cells, with the aim to highlight the underlying mechanism of resistance reversal. To maximize the efficacy of the therapeutic protocol in vitro, a further upgrading result was obtained by the development of a new DOX nanoformulation where the Dex-CT bioconjugate acted as functional coating element in the fabrication of a DOX hybrid nanocarrier. For this purpose, Nanographene oxide (NGO) was selected as starting material by virtue of its peculiar structural physic-chemical features, excellent biocompatibility and high drug loading capability [34]. By combining the peculiar features of NGO with the biological effects of Dex-CT conjugate, we expect to prepare a functional drug delivery system (Dex-CT-rGO), in which the anticancer activity is related to both the controlled release of DOX and the carrier itself.

2. Materials and methods

2.1. Synthesis of immobilized laccase

The biocatalyst was synthesized according to the literature [35,36]. In a typical procedure, Laccase from Trametes versicolor (25 mg, EC 1.10.3.2) and Acrylamide (534 mg) were dissolved in sodium citrate buffer solution (3.0 mL, 10^{-3} mol L^{-1}, pH 5.0) and, then Polyethylene Glycol Dimethacrylate750 (466 mg) as crosslinker and 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one (Irgacure 2959, 2.4%, with a maximum absorption at around 275 nm) as photoinitiator were added. The solution was poured in a polymerization cell consisting of two 10 × 10 cm^2 glass plates, separated with Teflon spacers (thickness 1.6 mm), brought together using binder clips. The polymerization was initiated by a high pressure mercury lamp (HPK 125, Philips, Amsterdam, Netherland, 10 mW cm^{-2}, wavelength 275 nm, irradiation time 10 min). Finally, the obtained hydrogel was extensively washed with water to remove unreacted species and dried for 12 h in an oven under vacuum at 40 °C.

Irgacure 2959 was from BASF, Ludwigshafen, Germany, all other chemicals from Sigma Chemical Co., Milan, Italy.

2.2. Synthesis of Dex-CT

Dex-CT was synthesized according to the literature [35]. Briefly, Dextran from Leuconostoc spp (310 mg, Mr ~6000) and Catechin (500 mg) were dissolved in tartaric acid (sodium) buffer solution (20 mL, 10^{-3} mol L^{-1}, pH 4.5)/ethanol mixture 95/5 by vol. Immobilized Laccase (500 mg, 0.23 U) was added to the reaction feed and the flask maintained at 50 °C under 70 rpm for 5 h under aired conditions. The resultant polymer solutions were introduced into dialysis tubes (of 6-27/32″ Medicell International LTD, MWCO: 3500–5000 Da) and dipped into a glass vessel containing distilled water at 20 °C for 48 h with eight changes of water. The absence of unreacted species in the conjugate was checked by High-Pressure Liquid Chromatography (HPLC) analysis after the purification step. The HPLC analysis conditions were: Jasco PU-2089 Plus liquid chromatography equipped with a Rheodyne 7725i injector (fitted with a 20 μL loop), a Jasco UV-2075 HPLC detector operating at 260 nm, Jasco-Borwin integrator (Jasco Europe s.r.l., Milan, Italy) and Tracer Excel 120 ODS-A column particle size 5 μm, 15 × 0.4 cm (Barcelona, Spain); mobile phase consisting of methanol/water/orthophosphoric acid (20/79.9/0.1 by vol) mixture (HPLC grade, Carlo Erba, Milan, Italy) at a flow rate of 1.0 mL min^{-1}.

The resulting solutions were frozen and dried with a freeze drier (Micro Modulyo, Edwards Lifesciences, USA) to afford vaporous solids.

All chemicals were from Sigma Chemical Co., Milan, Italy.

2.3. Preparation of NGO

NGO particles were synthesized by a modified hummers method [37]. Briefly, graphite (1.0 g 99.99%, 200 mesh) and NaCl (50.0 g) were ground for a few minutes using hand mortar and pestle. The ground graphite was suspended in distilled water to dissolve NaCl then removed by filtration. The collected graphite was mixed with H_2SO_4 (23 mL, 96% w/w) overnight (H_2SO_4 intercalating graphite layers). Afterward, intercalated graphite suspension was placed in an ice bath to preserve the reaction temperature below 5 °C (exothermic reaction). Subsequently, $KMnO_4$ (3.0 g) was added gradually with stirring to achieve graphite oxide. The resultant product was sonicated for 3 h and stirred for 30 min at 35 °C and 45 min at 50 °C respectively. Subsequently, distilled water (46 mL) was poured to the mixture and stirred for 45 min at 98–105 °C. Thereafter, the product was cooled down to room temperature (RT), and then distilled water (40 mL) and H_2O_2 (10 mL, 3%) were added to reduce the residual permanganate and manganese dioxide to soluble manganese sulfate. The suspension was filtrated and washed three times with HCl (5% w/w) and warm distilled water (40 °C) to remove unfavorable materials. The resulting material was cracked in water under optimized conditions using a horn-tipped ultrasonic probe (BANDELIN ultrasonic, max. power of 25–60%) at 28 W for 2 h, in order to reduce the dimensions (lateral width and thickness) of exfoliated GO sheets [38].

The NGO suspension was separated into different sizes via sucrose density gradient centrifugation [39]. Briery, gradient sucrose solutions (20–60% w/v) were dropped gently into the bottom of centrifuged tube with exact volume. On the top of 20% sucrose, NGO particles (335 mL) were added, the tube directly centrifuged under controlled conditions (5880*g* for 5 min) using Beckman Coulter, Allegra 64 R centrifuge. Along the tube, three visualized zones were produced - rinse thoroughly with distilled water to remove sucrose – and separated as uniform sizes (NGO 300 nm).

All chemicals were from Sigma Chemical Co, Milan, Italy.

2.4. Synthesis and characterization of Dex-CT-rGO

Dex-CT (100 mg) were mixed with a freshly prepared NGO solution (5 mg in 20 mL deionized water) and heated at 60 °C for 24 h. The resulting solutions were frozen and dried with a freeze drier (Micro Modulyo, Edwards Lifesciences, USA) to afford vaporous solids.

Scanning Electron Microscopy (SEM) images were obtained using a FEI, NOVA NanoSEM 200 with an acceleration voltage of 15 kV after depositing samples onto self-adhesive, conducting carbon tape (Plano GmbH, Wetzlar, Germany). Transmission Electron Microscopy (TEM) were obtained with a TEM Jeol 1400 Plus electron microscope,