European Journal of Pharmaceutical Sciences 50 (2013) 149–158

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutical Sciences

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

Hsp70 silencing with siRNA in nanocarriers enhances cancer cell death induced by the inhibitor of Hsp90

Mirela Matokanovic [a,c], Karmela Barisic [a], Jelena Filipovic-Grcic [b], Dusica Maysinger [c,*]

[a] Department of Medical Biochemistry and Hematology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Domagojeva 2, HR-10000 Zagreb, Croatia
[b] Department of Pharmaceutics, Faculty of Pharmacy and Biochemistry, University of Zagreb, Domagojeva 2, HR-10000 Zagreb, Croatia
[c] Department of Pharmacology and Therapeutics, McGill University, Montreal, QC, Canada H3G 1Y6

ARTICLE INFO

Article history:
Received 28 December 2012
Received in revised form 18 March 2013
Accepted 1 April 2013
Available online 10 April 2013

Keywords:
Cancer
Hsp70
Chitosan
siRNA nanocarriers
Celastrol
Tumor spheroids

ABSTRACT

Inducers of heat shock protein 70 (Hsp70) commonly promote cancer cell viability whereas inhibitors of Hsp90 reduce it. The anticancer agent celastrol, interferes with signal transduction pathways involving these heat shock proteins. The objective of this *in vitro* study was to silence inducible Hsp70 and to promote celastrol-induced tumor cell death. Hsp70 siRNA loaded chitosan-TPP carriers were prepared by ionic gelation and characterized by photon correlation spectroscopy and asymmetric flow field-flow fractionation combined with dynamic light scattering. Viability of human leukemia and glioblastoma cells and Hsp70 silencing was determined following treatment with chitosan-TPP-Hsp70 siRNA particles. The results showed that silencing of Hsp70 by chitosan-TPP-Hsp70 siRNA treatment significantly reduced cell viability, and enhanced antiproliferative effects of celastrol in leukemia and glioblastoma cells. In glioblastoma spheroids, higher concentrations of celastrol and Hsp70 siRNA in chitosan-TPP nanocarriers were necessary to induce cell death.

1. Introduction

Mechanisms of cancer cell resistance towards chemotherapeutics are poorly understood, and increasing the efficiency of anticancer treatments by interfering with tumor defense mechanisms is a major objective of cancer biology research (Hanahan and Weinberg, 2000, 2011). Functional studies indicate that tumor resistance towards apoptosis and possibly other types of cell death can be reduced by diminishing the activity of the heat shock protein (HSP) family members (Beere et al., 2000; Clemons et al., 2005; Goloudina et al., 2012; Portt et al., 2011; Yang et al., 2012). The HSPs are a group of stress proteins which can be classified according to their molecular weight into several sub-families: HSP110, HSP90, HSP70, HSP60, and the small HSPs (Goloudina et al., 2012).

Members of the HSP90 sub-family have been extensively studied and a large number of drugs based on heat shock protein 90 (Hsp90) inhibition are now in preclinical and clinical development for treatment of cancer (Biamonte et al., 2010; Travers et al., 2012). A number of studies, stimulated by an increasing interest in the use of natural compounds for anticancer treatment, have determined that many of them are Hsp90 inhibitors (Janin, 2010). Celastrol, a compound extracted from the root bark of Trypterygium wilfordii, was found to down-regulate Hsp90s' ability to bind ATP and to disrupt the interaction of Hsp90 with co-chaperone Cdc37 (Peng et al., 2010). Given Hsp90's ability to provide protection against cell death in tumor cells (Whitesell and Lindquist, 2005), celastrol's ability to disturb this chaperon function was considered as a likely explanation of why celastrol could be beneficial in anticancer treatment. However, Hsp90 inhibition was found to subsequently upregulate the expression of the members of another HSP protein sub-family, HSP70 (Mou et al., 2011). This proved to be unfortunate. The HSP70 sub-family, which consists of at least 13 different members, is one of the largest HSP sub-families, and its members have been found to promote cancer cell growth by providing protection against both extrinsic and intrinsic apoptotic stimuli (Beere, 2005; Gotoh et al., 2004). Moreover, in RNA interference (RNAi) studies of human cancer cells of various origin, depletion of Hsc70 (the major constitutively expressed protein of the HSP70 family) and of inducible Hsp70 (the major inducible protein of the HSP70 family, also known as Hsp72 or HSPA1A), increased the extent of apoptosis compared to the apoptosis depletion of these proteins caused in healthy cells of equivalent origin (Powers et al., 2008).

To study the function of antiapoptotic proteins with RNAi, liposome based delivery systems have been mainly used. With the progress of nanotechnology, the approaches to creating

Abbreviations: HSP, heat shock protein family; Hsp, heat shock protein; RNAi, RNA interference; siRNA, small interfering RNA.

* Corresponding author.
E-mail address: dusica.maysinger@mcgill.ca (D. Maysinger).

0928-0987/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ejps.2013.04.001

particles appropriate for entrapping siRNA began to take account of non-lipid polymers, including chitosan (Malmo et al., 2012). Chitosan, made from the chitin of marine anthropods and insects, is a linear, random copolymer of D-glucosamines (deacetylated units) and N-acetyl-D-glucosamines (acetylated units) linked through glycosidic bonds (Baldrick, 2010; Tan et al., 2009; Zhang et al., 2010). The stability of chitosan-based delivery systems is ensured by strong electrostatic interactions between highly positively charged chitosan amino groups and negatively charged nucleotides (Bhattarai et al., 2010; Katas and Alpar, 2006; Malmo et al., 2012). More importantly, chitosan was reported as non-toxic, biocompatible, biodegradable (Baldrick, 2010) and due to its muco-adhesiveness (de la Fuente et al., 2010), appropriate for nonparenteral administration (de Jesús Valle et al., 2008).

The objective of the present study was to incorporate Hsp70 siRNA into chitosan-TPP nanocarriers and to test the efficiency of this system in Hsp70 silencing. Following reduction in Hsp70 expression, the antiproliferative effects of Hsp70 silencing in the presence or absence of the Hsp90 inhibitor, celastrol, were explored. In order to test how effectively the simultaneous inhibition of Hsp70 and Hsp90 acted as a general treatment approach for different human tumors, in this study we used tumor cells originating from the blood and from the brain. In order to take into account the complexity of three dimensional growth of tumors *in vivo*, Hsp70/Hsp90 inhibition was also tested in human glioblastoma spheroids.

2. Materials and methods

2.1. Materials

Ultrapure chitosan glutamate (Protasan UP G113) with approximate molecular weight <200 kDa and 75–90% deacetylation was purchased from NovaMatrix (Sandvika, Norway). Celastrol was purchased from PI&PI Technologies Inc. (Taihe, Baiyun, China). All cell culture media and reagents were purchased from Life Technologies Inc. (ON, Canada). All other reagents were purchased from Sigma Aldrich (ON, Canada), unless stated otherwise.

2.2. Chitosan-TPP-siRNA nanoparticles preparation

Chitosan-TPP nanoparticles were produced based on modified protocol for ionic gelation of tripolyphosphate (TPP) with chitosan glutamate described elsewhere (Katas and Alpar, 2006). Briefly, for the association of small interfering RNA (siRNA) with the chitosan and TPP, 15 μl of siRNA (1.33 μg/μl) in double distilled water was added to the TPP solution (0.2 ml, 0.84 mg/ml, sterilized through 0.45 μm filter) before adding this drop-wise to the chitosan solution (0.5 ml, 2 mg/ml prepared in 0.1 M sodium acetate buffer, pH 4.5) under constant magnetic stirring at room temperature. Nanoparticles were spontaneously obtained during stirring and were incubated at room temperature for 30 min before further analysis or cell transfection.

2.3. Physical characterization of chitosan-TPP-siRNA nanoparticles: particle size, polydispersity index and zeta potential

The average particle size and size distribution (polydispersity index) was determined by photon correlation spectroscopy (Zetasizer 3000 HS, Malvern Instruments, Malvern, UK). Measurements were performed at a scattering angle of 90° and at a temperature of 25 °C. The hydrodynamic diameter was calculated from the autocorrelation function of the intensity of light scattered from particles with the assumption that the particles had a spherical shape. The zeta potential was obtained by laser Doppler anemometry using a Zetasizer 3000 HS. For the measurements, samples were diluted with 10 mM NaCl solution and placed in an electrophoretic cell, where a potential of 150 mV was established.

2.4. Asymmetrical flow field-flow fractionation (AF4) for characterization of chitosan-TPP-siRNA nanoparticles

Asymmetric flow field-flow fractionation was performed using an AF4 system (AFx2000MT, Postnova Analytics, Landsberg, Germany) combined with an UV/Vis detector (SPD-20A, Postnova Analytics) for a set wavelength of 260 nm, a multiangle light scattering detector (MALS, Dawn 8+, Wyatt Technology), and a dynamic light scattering detector (Wyatt-QELS, Wyatt Technology). The scattering angle was kept at 106°. Complete principle of AF4 has been described elsewhere (Yohannes et al., 2011). Data collection and analysis were done using ASTRA version 5.3.4.20 (Wyatt Technology).

2.5. Determination of siRNA loading efficiency

The loading efficiency (LE) of siRNA entrapped into chitosan-TPP nanoparticles was calculated based on the absorbance (at 260 nm) of the free siRNA in the supernatant obtained after particle centrifugation (13000 *g*, 15 min). Supernatant recovered from unloaded chitosan-TPP nanoparticles (without siRNA) was used as a blank. LE (%) was calculated according to the following formula:

$$\mathrm{LE}(\%) = [(\text{total siRNA} - \text{free siRNA})/\text{total siRNA}] \times 100$$

2.6. Cell cultures and materials

Human leukemia cells (Jurkat, ATCC) and human breast carcinoma cell line (MCF7, ATCC) were cultured in RPMI 1640 medium containing 10% foetal bovine serum (FBS) and 1% penicillin–streptomycin (PS), while human glioblastoma cells (U251N, ATCC) and human hepatocellular carcinoma (HepG2, ATCC) were cultured in DMEM medium containing 10% FBS and 1% PS. During treatment and growth cells were maintained at 37 °C and 5% CO_2 in a humidified atmosphere.

2.7. Cell transfection and Hsp70 silencing

Cells were seeded 24 h prior to transfection in 96-well, 24-well or 6-well plates (Sarstedt) depending on the assay to be performed. For transfection cells were incubated with chitosan-TPP-siRNA nanoparticles in complete growth media at 37 °C and 5% CO_2 for indicated period of time. Cells treated with "empty" chitosan-TPP nanocarriers, were used as negative control. siRNA incorporated in HiPerFect (Qiagen) and HiPerFect alone were used for comparison with chitosan-TPP nanocarrier. Transfection of siRNA incorporated into HiPerFect was performed according to the manufacturer's protocol. siRNA designed for targeting mRNA transcribed from human *hsp*70-1 gene (NCBI reference sequence: NM_005345.5) with a sense sequence 5′-CGG UUU CUA CAU GCA GAG Att-3′ (siRNA ID: s6966, Life Technologies Inc.) and siRNA of scrambled sequence (Life Technologies Inc.) with no homology to any known human gene (confirmed by conducting NCBI BLAST query) were used.

2.8. Cell viability

Three different approaches were used to determine cell viability: (i) cell counting using trypan blue, (ii) labelling nuclei with Hoechst 33342 (fluorescent microscopy) and (iii) determination of mitochondrial metabolic activity (MTT). Cell viability was calculated as a percentage relative to the viability of cells incubated in