A novel mitotropic oligolysine nanocarrier: Targeted delivery of covalently bound D-Luciferin to cell mitochondria

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Abstract

New and emerging therapeutic approaches focus on the targeted delivery of therapeutic agents to cell mitochondria with high specificity. Herein we present a novel mitotropic nanocarrier based on an oligolysine scaffold by addition of two triphenylphosphonium cations per oligomer. Although the parent oligolysine failed to enter healthy cells, the triphenylphosphonium modified carrier, with or without α-Luciferin, attached as cargo molecule, demonstrated striking mitochondrial specificity. Furthermore, the oligolysine bound α-Luciferin exhibited chemiluminescence, of lower intensity than free α-Luciferin, yet of remarkably longer steady-state temporal profile.

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1. Introduction

Mitochondrial dysfunction, and mtDNA mutations have been implicated as origins of numerous pathologies, spanning from neurodegenerative and neuromuscular diseases (DiMauro, 2004), obesity and diabetes (Reardon et al., 1992), inherited mitochondrial diseases (DiMauro, 2004) and enhancement of reperfusion injury following ischemia (Adlam et al., 2005, 2008). Consequently, the need of delivering therapeutic agents or mtDNA to cell mitochondria with high specificity has emerged.

The simplest strategy for mitochondrial targeting involves direct chemical conjugation of the selected bioactive molecules with appropriate mitotropic ligands. In this context, cell/mitochondria penetrating peptides (Del Caizo and Payne, 2003; Horton et al., 2008), the lipophilic triphenylphosphonium cation (TPP) (Adlam et al., 2005; Cocheme et al., 2007; Dessolin et al., 2002; Lei et al., 2010; Murphy and Smith, 2007; Smith et al., 2004; Tauskela, 2007) and the guanidinium group (Fernandez-Carneado et al., 2005; Sibrian-Vazquez et al., 2008) have successfully been applied to target their conjugated cargo-compounds to mitochondria. This synthetic approach is restrictive however, as it has to be de novo repeated for individual therapeutic agents, while it is strictly applicable to molecules able to retain their therapeutic properties following chemical attachment. In cases of unforeseen drug-property modification and inactivation due to ligand conjugation, alternative strategies have to be employed, e.g. the use of nanocarriers functionalized with the appropriate targeting ligands (Trojan-horse approach). This latter alternative is particularly attractive since mitochondria-specific nanovehicles can be exploited as generic platforms for loading a diversity of therapeutic agents while they can also be potentially employed for mtDNA complexation. The development of such mitotropic vehicles is currently the focus of intensive research (Armstrong, 2007; Cuchelkar et al., 2008; Duchen, 2004; Horobin et al., 2007; Mukhopadhyay and Weiner, 2007; Weissig et al., 2004; Yamada and Harashima, 2008) spearheading the field of subcellular organelle drug targeting (Bodapati et al., 2008; Huang et al., 2007; Lim, 2007), a new research frontier in drug delivery. The mitotropic nanocarrier approach has so far been focused on development of mitochrondriotropic liposomes (Bodapati et al., 2005, 2008; Weissig et al., 2006) through introduction of TPP on the liposomal surfaces which were subsequently loaded with selected drugs either in their aqueous cores or lipid bilayers.

In the present communication, we introduced TPP to the primary amino groups of biodegradable oligo(l-lysine) (Collet et al., 2010; Cottet et al., 2007; Tsogas et al., 2007), P1, through a lipophilic spacer, transforming it into a versatile nanocarrier, P1-TPP, commissioned to target cell mitochondria. We further demonstrated the ability of this mitotropic carrier to deliver cargo to cell mitochondria by covalently attaching to it α-Luciferin, the substrate of the firefly enzyme luciferase. Delivery of α-Luciferin to cell mitochondria is particularly pertinent to our ongoing work on Bioluminescence Activated Destruction (BLADe) (Theodossiou et al., 2003); however among others, one very significant implementation, could be the in-situ, live measurement of ATP changes in mitochondrial-luciferase expressing
cells. DU145 human prostate cancer cells were used throughout the present work.

2. Materials and methods

Experimental details concerning the materials used, synthesis of mitotopic oligomers, cytotoxicity of the oligomers, confocal microscopy and luciferase enzymatic assays can be found in the Supporting Information.

3. Results and discussion

Following synthesis of P1-TPP summarized in Scheme I, we evaluated the cytotoxicities of both P1 and P1-TPP with standard XTT assays at two time-points, i.e. 0 h and 24 h after 3-h incubation with the oligomers. The toxicities of P1 and P1-TPP against media only controls are presented in Fig. 1A and B respectively, versus oligomer concentration. From these data it is evident that both P1 and P1-TPP were relatively subtoxic for concentrations up to 40 μM (survival ≥ 70%) at both assay time-points (0 h and 24 h) while the concentration of 100 μM was significantly toxic for P1 (survival ~ 30%) but not for P1-TPP (survival ≥ 65%). It can therefore be deduced that attachment of TPP to the P1 primary amines reduced the toxicity of the parent oligolysine, which is an additional benefit for P1-TPP.

In order to assess the subcellular localization patterns of the parent polymer P1 and of TPP functionalized derivative P1-TPP we employed laser scanning confocal fluorescence microscopy on live DU145 human prostate cancer cells using the fluorescein labeled P1-FITC and P1-TPP-FITC. Representative confocal microscopy images are shown in Fig. 1C. From these it is evident that while the parent polymer P1-FITC failed to enter live, healthy cells (data not shown), P1-TPP-FITC efficiently co-localized with mitotracker red in cell mitochondria (green + red = yellow). These results unequivocally demonstrate that functionalization with TPP induced efficient cell internalization, with specific subcellular localization to cell mitochondria, proving our working hypothesis.

Following these encouraging results, we next attempted to specifically deliver a cargo compound to cell mitochondria. For this purpose we decided to covalently tether α-Luciferin to our mitotropic oligolysine nanocarrier P1-TPP, thus producing P1-TPP-luc (Scheme I).

Our first priority was to probe whether α-Luciferin remained active, despite its conjugation to the oligolysine nanocarrier. For this reason an in vitro chemiluminescence assay with purified firefly luciferase enzyme (Leach and Webster, 1986) was employed, modified and adapted to our specific experimental requirements.

The results of the assay are shown in Fig. 2A from which it is evident that α-Luciferin induced chemiluminescence with initial intensity two orders of magnitude higher than that of P1-TPP-luc. However, even approximately 46 h later, P1-TPP-luc chemiluminescence intensity remained comparable to its initial (zero-time) values, whereas α-Luciferin chemiluminescence had subsided to background values. Upon interaction with bovine serum albumin (BSA), either in the assay mix or later in cell media serum, P1-TPP-luc formed nanoparticles. We measured the hydrodynamic radii of these nanoparticles by dynamic light scattering (DLS) and found them to be approximately 180 nm (polydispersity index: 0.55). Furthermore, by repeating the luminescence

Scheme I. Reagents and conditions: Synthesis of (10-carboxydecyl) triphenylphosphonium bromide: (a) triphenylphosphine (1 equiv.), 11-bromoundecanoic acid (1.05 equiv.), dry acetonitrile, reflux for 18 h, 90% yield. Synthesis of P1-TPP: (b) P1 (1 equiv.), (10-carboxydecyl) triphenylphosphonium bromide (2.5 equiv.), HBTU (4 equiv.), HOBr (4 equiv.), triethylamine (4 equiv.), dry DMF, overnight, room temperature, 75% yield. Synthesis of P1-TPP-luc: (c) P1-TPP (1 equiv.), α-Luciferin (1.1 equiv.), HBTU (2 equiv.), HOBr (2 equiv.), DIPEA (2 equiv.), dry DMF, overnight, room temperature, 73% yield. Synthesis of P1-TPP-FITC and P1-TPP-luc-FITC: (d) P1-TPP or P1-TPP-luc (1 equiv.), FITC (1.1 equiv.), dry DMF, 24 h, room temperature, 85% and 87% yield, respectively.
assays for P1-TPP-luc without BSA we found that although the initial chemiluminescence intensity doubled, the kinetic decrease was very rapid, much faster than even that of free D-Luciferin potassium salt (data not shown). We therefore hypothesize that P1-TPP-luc nanoparticles formed in the presence of either BSA or other serum proteins, constitute a D-Luciferin controlled release system ensuring a long-lasting luminescence steady-state. In addition, experiments on ATP dose dependence for free D-Luciferin (100–400 nM) revealed that upon doubling the ATP concentration each time, the chemiluminescence doubled and quadrupled proportionally to ATP dose. On the other hand, in replica experiments employing P1-TPP bound D-Luciferin, upon doubling the ATP concentration (100–400 nM), the chemiluminescence only increased by ~16% each time. These results indicate that the P1-TPP-luc nanoparticle system is stable as it no longer consumes ATP. This further suggests that only the released D-Luciferin is responsible for ATP consumption and the observed chemiluminescence intensity.

The above results represent indefatigable evidence that D-Luciferin, covalently attached to P1-TPP, is still an active substrate for firefly luciferase. We, therefore, have every reason to believe that P1-TPP-luc will interact with intracellularly expressed, mitochondrial-tagged luciferase.

Subsequently, P1-TPP-luc was assayed for its cytotoxicity. The procedure was identical to that employed for P1 and P1-TPP (vide supra), albeit the concentration spectrum this time was 0 (media only controls)–80 μM P1-TPP-luc. Again the XTT assays were performed at two time-points, i.e. immediately after the 3-h incubation (0 h) and 24 h later (24 h). The results are summarized in Fig. 2B; from these data it is evident that P1-TPP-luc was relatively subtoxic for concentrations up to 50 μM (80% ≥ survival ≥ 65%) in the two assay time-points (0 h and 24 h) respectively, while for higher concentrations the cytotoxicity gradually increased (57% ≥ survival ≥ 45% at 60 μM and 42% ≥ survival ≥ 30% at 80 μM).

In order to verify successful D-Luciferin delivery to cell mitochondria by P1-TPP-luc we employed live cell confocal fluorescence imaging. The procedure employed was identical to the one described earlier for P1-FITC and P1-TPP-FITC. Representative confocal microscopy images for P1-TPP-luc-FITC are shown in Fig. 2C. From these images it is apparent that P1-TPP-luc-FITC (green fluorescence, left panel) efficiently co-localized with MitoTracker® Red CMXRos (red fluorescence, middle panel) in cell mitochondria as shown by their overlay image (yellow = green + red, right panel).

Fig. 1. Cytotoxicity in DU145 cells, as demonstrated by standard XTT assays performed following 3-h incubation with: (A) non-functionalized oligolysine, P1, (B) TPP-functionalized oligolysine, P1-TPP. The XTT assays were performed at two time points: immediately after incubation (grey bars) or 24 h later (white bars). (C) Representative confocal images of DU145 cells: (left) Triphenylphosphonium functionalized, FITC labeled oligolysine, P1-TPP-FITC, 40 μM, 3-h incubation; (middle) mitotracker red, 250 nM, 15 min incubation and (right) overlay image showing co-localization of green and red fluorescence in yellow. Live cell imaging; green channel (λex = 488 nm, λem = 522 nm); red channel (λex = 568 nm, λem ≥ 585 nm).
Evidently, P1-TPP-luc delivers its covalently bound cargo to cell mitochondria with high efficiency. We replicated P1-TPP-luc-FITC confocal imaging for various polymer concentrations; at 40 μM, 3 h incubation there was no clear mitochondrial localization, while at 60 μM, 2 h incubation the mitochondrial delivery was comparable to that of 50 μM P1-TPP-luc-FITC, 3-h incubation (data not shown). Given, however, the enhanced cytotoxicity of 60 μM P1-TPP-luc-FITC, we inferred that 50 μM was the optimal concentration for successful luciferin delivery. Moreover, there is a slight discrepancy between the optimal concentration of P1-TPP-FITC (40 μM) and P1-TPP-luc-FITC (50 μM). We believe that this divergence can be attributed to a minor charge modification due to D-Luciferin covalent bonding and/or the molecular weight difference of the carrier after additional attachment of one luciferin molecule.

The results of the present study constitute proof of principle that TPP functionalization can specifically direct molecules attached to an oligolysine vehicle to cell mitochondria. We intend to translate this work to higher generation polylysine dendrigrafts, and other dendritic polymers which possess nanocavities for the efficient encapsulation of compounds or can potentially be used for engineered mtDNA complexation and incorporation to the target mitochondrial genome.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.mito.2011.08.004.

References
