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Oxyresveratrol Supplementation In Hyper-Branched Cyclodextrin Based Nanosponges As Antiaging Enhancer in *Caenorhabditis Elegans*

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Abstract: 1) background: The desire to live longer lives demans novel strategies to perform this target. For that reason, in this work [1] the increase of the Caenorhabditis elegans (C.elegans) lifespan extension using hyper-branched cyclodextrin-based nanosponges (CD-NS) complexing oxyresveratrol (OXY) was evaluated. 2) Methods: The titration displacement of fluorescein was used to calculate the apparent complexation constant (K_F) between CD-NS and OXY. Moreover, PDE4 was expressed, purified and refolded in presence of cyclodextrins (CDs) to study its possible inhibition as pharmacological target of OXY. 3) Results: The effect of OXY on PDE4 displayed a competitive *in vitro* inhibition corroborated *in silico*. A maximum increase of the *in vivo* life expectancy of about 9.6% of using OXY/CD-NS complexes in comparison with the control was obtained without toxicity. 4) Conclusions: These results as a whole represent new opportunities to use OXY and CD-NS in lifespan products.

Keywords: lifespan; Caenorhabditis elegans; oxyresveratrol

1. Introduction

Increasing life expectancy is a common wish for much of humanity. While one of the easiest way to fulfill this wish would be by dietary restriction (DR) [2], a pharmacological approach would be of greater interest to avoid over-strict diets. In this respect, stilbenes have been proposed to act as modulators of the pathway [3]. Stilbenes are a well-known family of bioactive compounds which presents generally several bioactivities such as anticancer, antioxidant, antimicrobial or photoprotective [4].

One on the animal models most commonly used for testing drugs in health promoting and anti-aging tests is Caenorhabditis elegans (C.elegans) because of its transparent, small size, well-annotated genome, which has many tissues similar to those of animals and a rapid life cycle [5]. Its capacity to extend the lifespan depends on sir-2.1/SIRT1-dependent signaling and DAF-16/FOXO thought insulin and IGF-1 signaling (IIS) pathway or oxidative stress protection [6–9]

In this respect, one of the enzyme family that regulates this are phosphodiesterases (PDE) [3,10], which comprise a group of enzymes that degrade the phosphodiester bond in the secondary messenger molecules cAMP and cGMP to from AMP and GMP. They regulate the localization, duration, and amplitude of cyclic nucleotide signaling within subcellular domains. PDEs are

therefore important regulators of signal transduction mediated by these second messenger molecules.

On the other hand, although stilbenes are interesting molecules, its hydrophobicity and low stability [11–14] make their administration difficult. It was therefore though that using cyclodextrins (CD) and hyper-branched cyclodextrin based nanosponges (CD-NSs) make the administration more stable [15,16]. Indeed, although oxyresveratrol (OXY) has good bioaccesibility [17], insoluble CD-NS has proved the capacity to improve it [18], which would make the administration more effective.

CDs are torus-shaped oligosaccharides made up of α-(1,4) linked glucose units, the most common CDs being α, β and γ-CD, which contain six, seven and eight glucose units, respectively, although semisynthetic CDs also exist [19,20]. They present the capacity to complex different molecules creating the commonly called inclusion complex [21,22]. However, when bioactive compounds are to be used as a pharmaceutical product, the release must be slow, and unfortunately, this is not a quality of CD complexes; However, the use of cyclodextrin-based nanosponges, as hyper-branched (CD-NSs) should be also evaluated [23,24]. CD-NSs are cross-linked polymer structures with a three-dimensional network with a crystalline and amorphous structure, spherical in shape and possessing good swelling properties [25].

Bearing the above in mind, the work was planned as follows: Firstly, a study of the complexation between OXY and hyper-branched CD-NSs was carried out. Secondly, the in vitro and in silico enzymatic activity of PDE4 and its inhibition by OXY was characterized. Finally, an in vivo lifespan extension of OXY in the presence of CDs and CD-NSs on C.elegans were studied.

2. Experiments

2.1. Materials

β-Cyclodextrin (β-CD) was purchased from Roquette (France). Hydroxypropyl-beta- (HPβ-CD) was purchased from Carbosynth (Berkshire, UK. DS 5.5). Oxyresveratrol (OXY, CID 5281717) was purchased from TCI Europe and used as received. The remaining chemicals were purchased from Sigma-Aldrich (Madrid, Spain). The samples were stored in darkness

2.2. Equipment and Experimental Procedure

2.2.1. Preparation of hyper-branched nanosponges

Hyper-branched water-soluble β-CD nanosponge was prepared as reported [26]. The white powder was dried and ground in a mortar, obtaining 1.8 g and preserved in darkness and dry conditions.

2.2.2. Fluorescein as displacement signal of CD-NS/OXY complex

To verify the complexation of Fluorescein by CD-NS, the fluorescence of 25 μM of fluorescein (ex 494 em 521) was monitored at increasing CD-NS quantities using a Shimazdu RF-6000 spectrofluorimeter (Shimadzu, Kyoto, Japan) equipped with thermostatically controlled cells to obtain its fluorescence spectra. Excitation and emission bandwidths were both set at 5 nm. The displacement was carried out studying the effect of increasing OXY (0, 5, 10, 15, 20, 22, 24, 28, 30, 40, 50 and 80 μM) concentration on a 25 μM of fluorescein mixed with 200 ppm CD-NS. All samples were prepared in water with the exception of fluorescein (acetone). The apparent complexation constant (KFapp) of OXY/CD-NS was calculated using the equations developed by Selvidge & Eftink in 1986 for CD/ligands interactions [27]. The average molecular weight of CD-NS [26] was used to obtain the concentration of polymer and its apparent complexation constant (KFapp, here called K1) with fluorescein using Benesi-Hildebrand plot [28] as intrinsic average constant. After that, the following algebraic solution was applied to obtain the OXY/CD-NS apparent constant (KFapp, here called K2):

$$K_2 = \frac{[CD.NS]_0 - \frac{v}{K_1(1-v)} - v[Fluorescein]_0}{\frac{v}{K_1(1-v)}([OXY]_0 - [CD-NS]_0 + v[Fluorescein]_0 + \frac{v}{K_1(1-v)})} \quad (1)$$

where [CD-NS]0, [Fluorescein]0 and [OXY]0 are the initial concentration of each molecule and v is the fraction of fluorescein bound to CD-NS (calculated as reported [27]).

2.2.3. PDE4 expression, purification and refolding

The expression was carried out as reported [29]. After expression, cells were lysed by sonication in 5 pulses of 15 s in a Branson Digital sonifier (Branson Ultrasonic Corporation, Connecticut, USA) and centrifuged at 8000 xg for 30 min at 4ºC. The resulting pellet was dissolved in 0.3 M NaCl, 6 M Urea, 1 % Triton X-100, 0.05 M phosphate buffer pH 7.4 with 0.5 mM benzamidine for 30 min at 500 rpm and 20 ºC in a thermomixer comfort (Eppendorf). It was centrifuged at 8000 xg for 30 min at 4ºC and the remaining pellet was dissolved in 0.3 M NaCl, 25 mM ZnSO4, 2.5 % Triton X-100, 0.05 M phosphate buffer pH 7.4 with 0.5 mM benzamidine for 150 min at 500 rpm and 20 ºC in a thermomixer comfort (Eppendorf). The sample was centrifuged at 8000 g for 30 min at 4ºC to remove pellet. The supernatant was incubated [30] adding 2 mM β-CD (15 min at 35 ºC) and after β-CD until 4 mM (15 min at 35 ºC). The final solution was concentrated using Amicon Ultra15 50KDa until 100 µL. A 10% SDS-PAGE was carried out to check purify and the molecular weight of the final protein with the EZ-Run™ Pre-stained Rec Protein ladder (Fisher). The concentration of PDE4 was determined by the Bradford assay (Biorad) using bovine serum albumin as standard.

2.2.4. PDE4 activity assay

The assay of PDE4 activity was as previously described [31]. The effect of pH and Mg2SO4 concentration were studied changing their values in sample buffer. For Km, Vmax and kcat (product generated per enzyme and time) determination, a non-linear plot using Michaelis-Menten kinetic was used.

$$V = \frac{Vmax[S]}{K_m + [S]} = \frac{k_{cat}[E][S]}{K_m + [S]} \quad (2)$$

Where [S] is the substrate concentration and [E] the enzyme concentration.

For OXY inhibition assay, the I50 (value where the 50% of the enzyme is inhibited) was studied. To obtain Ki (the concentration required to produce half maximum inhibition), a conversion from I50 to Ki for competitive inhibition was applied [32].

$$K_i = \frac{I_{50}}{1 + \frac{[S]}{K_m}} \quad (3)$$

2.2.5. Molecular modeling and docking

The sequence reported by Genscript (deleting 70 aminoacids in N-terminal position) after optimization was uploaded to Swiss-Model [33] with default parameters using PDB ID 4WZI as template. The resulting protein was used to carry out the molecular docking experiments. The model and the ligand (cAMP. RSV or OXY, obtained from ZINC database) were uploaded to Swiss-Dock [34] with default parameters. The results were analyzed using Chimera (Version 1.9) and Pymol (version 1.9).

2.2.6. In vivo Lifespan assay in C.elegans

The protocol was carried out as reported [35] using lifespan machine [36]. The tested molecules were prepared to achieve the less DMSO possible concentration due to its intrinsic toxicity on C.elegans [37]. Only HPβ-CD samples were possible to use without DMSO. The remaining samples presented 1% DMSO due to OXY solubilization or DMSO remaining in the CD-NS after purification. As control, 1 % DMSO treatment were used in all assys with the exception of HPβ-CD assay. The samples were sterilized by filtration (CD or CD-NS) or autoclave (OXY). All the experiment plates were done in triplicate. The plates were closed and incubated for 20min at 20°C. Plates that present condensation were open under sterile conditions and the lids dried with disposable sterile wipers.

Closed lid plates were loaded into the scanners of the lifespan machine. The machine acquired an image of each loaded plate every hour for the duration of the experiment and the analysis detected the time of the death for each worm. The experiments were set at 25°C for 20 days.

2.2.7. The OXY apparent critical micellar concentration determination

The apparent critical micellar concentration (c.m.c) was carried out as reported [38] with slight modifications. Briefly, tubes with different OXY concentrations (5, 10, 15, 20, 25, 30, 35, 40, 60, 100 and 150 μM) using phosphate buffer (pH 6) were incubated in presence of 0.88 μM diphenyl-hexatriene (DPHT) for 10 minutes in darkness. The fluorescence signal (excitation 358, emission 430) of each one was obtained using a Portable Fluorometer Fluo-100 (AllSheng, China) with appropriate filters and compared with the signal of the lowest OXY concentration.

2.2.8. Data analysis

The experiments were carried out at least in triplicate. Graphical representations and enzymatic kinetic were made using SigmaPlot (Version 10.0). A t-test was applied using social science statistics (https://www.socscistatistics.com/) fixing the significance level at $P < 0.05$. Mathematical analysis of the obtained data in Lifespan Machine was performed using the online application for survival analysis OASIS 2 [39] with the Kaplan-Meier estimator, Boschloo's Test, Kolmogorov-Smirnov Test and Survival Time F-Test. Other mathematical operations were carried out using wxMaxima software (version 12.04.0).

3. Results and discussion

3.1. Fluorescein as a nanosensor for hyper-branched CD-NS complexation and applicable to OXY particles.

The first objective was to demonstrate that hyper-branched CD-NSs could complex OXY. For this, the first tried was to use the intrinsic OXY fluorescence to evaluate K_{Fapp} [40]; however, its low fluorescence signal generated several mistakes. For that reason, a host displacement with fluorescein was used, which is a well-studied CD complexed molecule [41,42]. The complexation of fluorescein quenches the fluorescence signal [41].The effect of CD-NS on fluorescein is showed in **figure** 1A where it can be seen that the fluorescein signal was decreased by CD-NS addition. No scattering was reported so the data suggest that the decrease in fluorescein signal was due to its complexation. This finding can be used to obtain a K_{Fapp} between fluorescein and CD-NS with a constant "K_1" of $5.6 \times 10^4 \pm 2.5 \times 10^3$ M^{-1}, $R^2 > 0.99$) using Benesi-Hildebrand plot [28].

At this point, the effect of OXY on fluorescein/CD-NS signal was studied to obtain its K_{Fapp} (K_2), using 45.5 μM (200 ppm) of CD-NS to check the K_F because it was the concentration where the asymptote started. **Figure** 1B shows that the fluorescein signal increased as a consequence of the entrance of OXY and the release of fluorescein. Using equation 1 at several OXY concentrations [27], the average K_2 value was found at $1.20 \times 10^5 M^{-1} \pm 1.23 \times 10^4$ M^{-1}. This supports the idea that CD-NS can complex OXY and lays the foundations to evaluate the K_{Fapp} using soluble NS.

3.2. In vitro and in silico PDE4 activity assay and inhibition

The next step was to check the activity of the pure refolded PDE4. As the protein was able to convert cAMP to AMP, the optimal pH and [Mg^{2+}] (the zinc ion is strongly linked to the catalytic center [43,44]) were studied before the enzymatic characterization (data not showed).

Figure 2A shows the effect of cAMP on PDE, showing a Michaelis-Menten plot in the optimal conditions (pH 7 and 7.5 mM Mg_2SO_4). The refolded protein gave a $K_{mapp} = 230 \pm 9$ μM, $V_{maxapp} = 3.3 \times 10^{-8} \pm 0.1 \times 10^{-8}$ mols/s/mg and $k_{catapp} = 1 \times 10^{-3} \pm 3 \times 10^{-5}$ s^{-1} ($R^2 \approx 0.98$) in a Michaelis Menten plot.

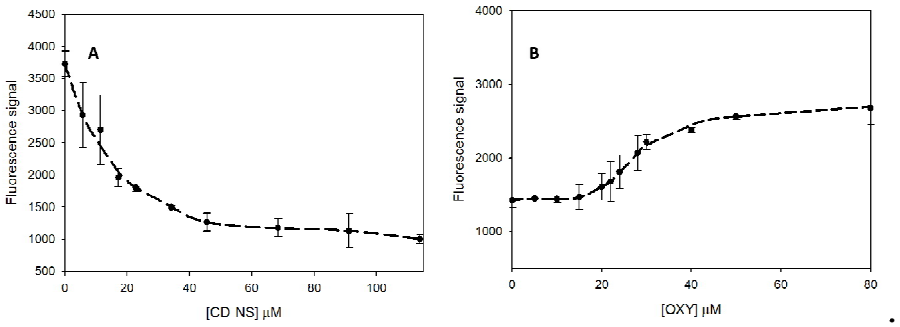

Figure 1. (A) Effect of CD-NS on fluorescein (25 µM) fluorescence signal (conditions: water at 25 ºC). (B) Effect of OXY (0, 5, 10, 15, 20, 22, 24, 28, 30, 40, 50 and 80 µM) concentration on fluorescein/CD-NS complex fluorescence signal (condition: water at 25 ºC).

The above data demonstrated that the refolded protein presented activity and perhaps also had the capacity to test the inhibitory profile against OXY. So, after characterization, the next step was to study the effect of OXY on the enzymatic activity (**figure** 2B). The I_{50} = 10 ± 0.6 µM suggests strong inhibition. Recently, it was reported that human PDE4 presents a resveratrol competitive inhibition [10]. For that reason, it is reasonable to assume the same inhibition for the protein. The calculation of K_i was carried out using Eq.3, with a K_{iapp} of 3.2 ± 0.16 µM, which pointed to the ability of OXY to inhibit PDE4 directly *in vitro*, suggesting that this enzyme might be affected after OXY supplementation.

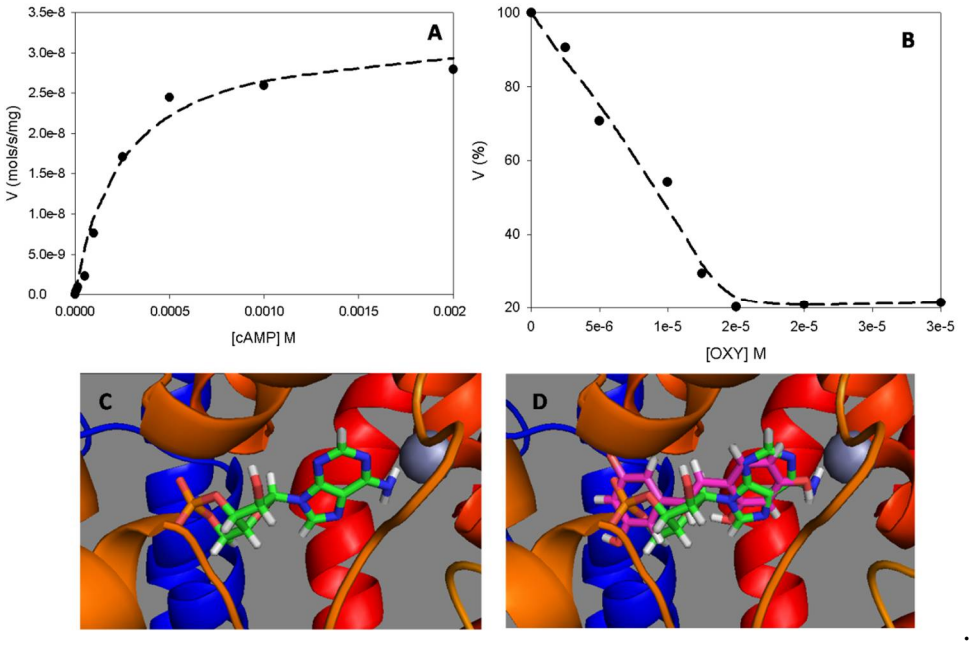

Figure 2. (A) Michaelis-Menten fit of the Effect of cAMP on PDE4 activity (sample buffer at 25 ºC). (B) Effect of OXY on PDE4 activity at 4 x 10^{-4} M of cAMP (sample buffer at 25 ºC). (C) Molecular docking of cAMP/PDE4, (D) Overlapping OXY and cAMP docking results. (E) Polar interactions of cAMP with PDE4 and (F) Polar interactions of OXY with PDE4.

To check the possibility of non-competitive interactions [45], a molecular docking was carried out. The molecular modeling of PDE4 also demonstrated that OXY enters the active site of PDE4 to inhibit the protein (**figure** 2C and D). Moreover, the docking score of cAMP (-8.18) and OXY (-8.56) would also justify the lower concentration of OXY than cAMP on PDE4.

3.5. In vivo lifespan on C.elegans

After demonstrating that PDE4 can be inhibited by OXY (suggesting that this pathway could be also affected), a lifespan study of the effect of OXY (alone or complexed) on *C.elegans* was carried out (**Figure** 3A). The results showed that OXY increases the average life expectancy around 6.5% ($P<0.05$) with maximum at 25 μM (**Figure** 3B). This was interesting, so the low maximum value of OXY concentration and possible explanations were studied. Our research group has demonstrated that stilbenes can form aggregates [46] at high concentrations, which may decrease their bioavailability. To demonstrate this effect on OXY, the apparent critical micellar concentration (c.m.c) was calculated. The c.m.c of 13 μM obtained suggested the possible aggregation of OXY (**Figure** 3C). This would explain the decrease in survival, although it was still better than the control above 25 μM.

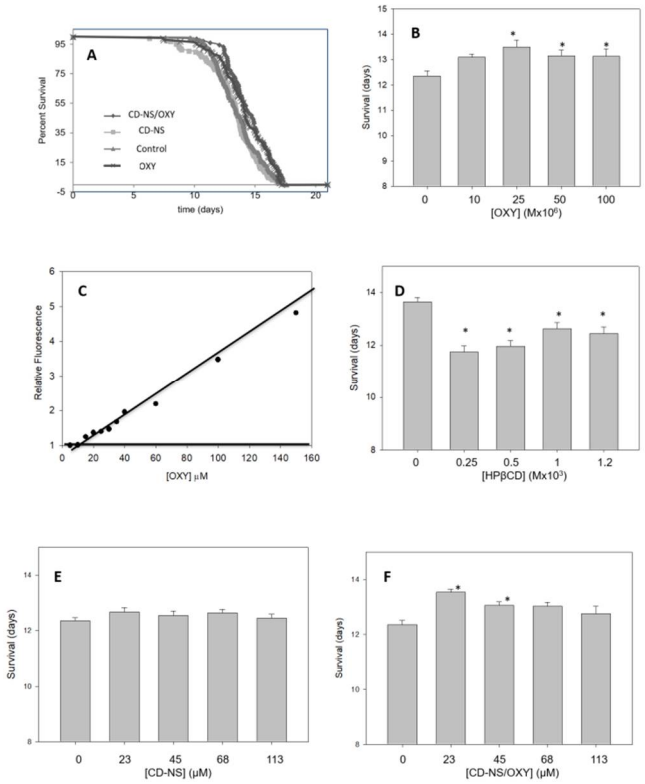

Figure 3. (A) Survival percentage of worms in the presence of 200 ppm CD-NS, 100 μM OXY, and a mixture of both. (B) Mean lifespan of C.elegans in presence of different OXY concentrations. (C) Effect of OXY on DPHT fluorescence signal. (D) Mean lifespan of C.elegans in presence of different HPβ-CD concentrations, (E) Mean lifespan of C.elegans in presence of different CD-NS concentrations. (F) Mean lifespan of C.elegans in presence of different CD-NS concentrations at 100 μM OXY (even the control). (* = $P<0.05$ related to control).

To increase OXY disaggregation and stability, its supplementation in the presence of HP-βCD, one of the lest toxic CD derivatives used also to treat the rare disease Niemann Pick type C [47,48], and hyper-branched CD-NS was considered as a good option. However, HP-βCD was found to be toxic ($P<0.05$, **figure** 3D) for *C.elegans*, the sequestration of essential compounds inside worms such as membrane cholesterol [49] perhaps affecting their life expectancy. Indeed, there are also known to be toxic concentrations for human intestine, but its elimination by urine or feces remove them totally to the system, while plate dishes are close systems preventing this point.

On the other hand, hyper-branched CD-NS was not being toxic since its polymeric nature would have a lower uptake capacity for *C.elegans*. Effectively, **figure** 3E showed that the polymer did not present any adverse effect on *C.elegans.* Furthermore, the effect of the polymer on *C.elegans* in presence of OXY increased life expectancy by around 9.6%, which is more than was possible with free OXY (6.5 %, **figure** 3F). The disaggregation effect of CD-NS complexes on OXY and the higher stability would increase the OXY bioavailability [15,24,46]. However, the greater the quantity of CD-NS, the lower effect, perhaps because OXY complexation is much more intense than the quantity released and *C.elegans* cannot use OXY.

4. Conclusions

In this work synthesized hyper-branched CDs polymers were used to supplement the OXY provided to *C.elegans*. The complexation of OXY by CD-NS was demonstrated by monitoring the displacement of fluorescein. On the other hand, *C.elegans* PDE4 was expressed, refolded and purified. Refolding, with CDs acting as molecular chaperons, was enough to characterize the enzyme activity and its inhibition by OXY, showing its potential role of the regulation of lifespan extension. The inhibition was also studied with molecular docking, showing the most probable interactions between OXY and PDE4. The complex of CD-NS/OXY increased life expectancy more than free OXY. The findings as a whole represent a new opportunity to use OXY as an ingredient of nutraceutical products focused on lifespan extension.

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