Chrysin directing an enhanced solubility through the formation of a supramolecular cyclodextrin-calixarene drug delivery system: a potential strategy in antifibrotic diabetes therapeutics

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Abstract

Background: Calixarene OTX008 and chrysin (CHR) are promising molecules for the treatment of fibrosis and diabetes complications, however, a suitable drug delivery system is required for their combined application due to their low solubility and bioavailability.

Research design and methods: Sulfobutylated β-cyclodextrin (SBECD) and its polymeric derivative were tested to solubilize CHR through the formation of a ternary complex in presence of calixarene OTX008. Phase-solubility studies revealed the solubility enhancement and complexation mechanisms, and dynamic light-scattering was used to measure the molecular association in the cyclodextrin-calixarene-CHR ternary system. Nuclear magnetic resonance, differential scanning calorimetry, and computational studies discovered the molecular interactions in the complex, while cellular studies tested the biocompatibility of the system.

Results: Using SBECD and OTX008 together proved more efficient in solubilizing CHR than using SBECD by itself. The ternary complex forms molecular associates in water, which contributes to the solubilization and delivery of both CHR and OTX008. Structural analysis identified non-covalent interactions involved in the formation of the complex, which was not cytotoxic in vitro under hyperglycemic conditions.

Conclusions: A novel ternary complex was developed for administering potential antifibrotic compounds in diabetes complications, with OTX008 serving as a pivotal structural and pharmacological element within the complex.

Keywords: Calixarene OTX008, chrysin, sulfobutylated β-cyclodextrin, ternary complex, solubilization mechanism, molecular simulation, fibrosis

1. Introduction

Calixarenes and cyclodextrins are cyclic molecules, with synthetic and natural origin respectively and they differ in their building blocks [1]. Calixarenes are composed of phenol units, linked by methylene bridges, and have a hydrophobic character, but they can be modified by the introduction of hydrophilic groups leading to water-soluble molecules [2]. Cyclodextrins (CDs) are water-soluble oligosaccharides containing glycosidic units, which are connected by α-1,4-glucosidic bonds [3].

Common property of these structures is that they are both able to form host-guest complexes with suitable guest molecules and by complexation improve the solubility and bioavailability of poorly soluble drugs [2,3]. Water soluble calixarenes, such as 4-sulphonic calix[n]arenes can solubilize testosterone in water, but it depends on the size of their ring [4]. Our earlier experiments showed, that β-cyclodextrins, including sulfobutylated β-cyclodextrin (SBECD) can improve the solubility and permeability of chrysin (CHR) [5].

CHR is a bioflavonoid, present in the honey and propolis and has a limited bioavailability, due to its poor water-solubility [6]. Recognized for its various therapeutic properties, CHR exhibits anti-inflammatory, anticancer, and antioxidant activities [7,8]. Recent studies have also highlighted its potential antifibrotic effects [9–11].

OTX008 interacts also with biomolecules such as proteins, nucleic acids and modulates the activity of the enzymes [12]. It has antitumor activity, downregulates the cancer cell proliferation and tumor angiogenesis [13]. Zucchetti et al. reported, that OTX008, which is a mimetic of the galectin-1 (Gal-1) targeting peptide, can inhibit endothelial cell functions, such as proliferation and motility [14]. OTX008 has recently been shown to inhibit galectin-1 (Gal-1) overproduction also in hyperglycemic conditions. Gal-1 is highly expressed in kidneys of type 1 and 2 diabetic mice and found to be a key fibrosis protein in diabetes. OTX008 inhibits and prevents accumulation of Gal-1 [15], revealing its potential to become a novel Gal-1 inhibitor therapeutic to target and treat fibrosis in diabetes.

Even if both OTX008 and CHR are promising molecules for the treatment of fibrosis acting on different targets they have limited absorption and poor water solubility and, thus a suitable drug delivery system (DDS) is required for their combined therapeutic application. The common formulation and delivery of the water insoluble flavonoid CHR and calixarene OTX008 has not been reported yet, therefore the aim of the research was to investigate their joint delivery by the following strategy.

While the molecular interaction between cyclodextrins and CHR is well-documented [5], a similar relationship of CHR with OTX008 has not been reported yet. The combined use of calixarenes and cyclodextrins to enhance solubility remains largely unexplored. Previously the aqueous solubility of niclosamide was improved by the combined application of 4-sulphonato-calix[6]arene and hydroxypropyl-β-cyclodextrin [16]. Given the small cavity and limited water solubility of OTX008, a weak interaction with CHR was anticipated. Consequently, our approach involved using SBECD to enhance OTX008 solubilization and its drug delivery potential. The polymerized SBECD (SBECDpolymer) was also involved in the solubilization tests to compare the effectiveness of the crosslinked polymer with SBECD monomers. Our aim was to characterize the CHR complexation ability of the macrocycles alone, and in combination to form a higher-ordered DDS as follows: characterization of the phase-solubility of binary systems of CHR with SBECD and SBECDpolymer, characterization of phase-solubility of ternary systems of CHR with OTX008+SBECD and OTX008+SBECDpolymer. According to their structure the formation of supramolecular complexes was expected by interactions between OTX008 and the anionic SBECD involving CHR via non-covalent bonds. For the investigation of this hypothesis the size distribution of the complexes in water was analyzed by dynamic light scattering (DLS). Chemical (NMR) and thermal (DSC) characterization of single components and their binary mixtures were performed to elucidate the interactions in the ternary mixture supported by molecular modelling.

The complexes underwent *in vitro* biocompatibility testing on embryonic rat cardiac H9c2 cells in normal and high glucose environments, laying the groundwork for potential medical applications.

2. Materials and methods

2.1.Materials

OTX008 (Calixarene 0118) was purchased from Selleck Chemicals GmbH. Sulfobutylated β-cyclodextrin sodium salt (SBECD) (DS~6) and the commercially available soluble sulfobutylated beta-cyclodextrin polymer (SBECDPolymer, 64.4 kDa) were the product of Cyclolab Ltd. (Budapest, Hungary). CHR (5,7-Dihydroxyflavone; CHR) was purchased from Alfa Aesar (by ThermoFisher Scientific, Kandel, Germany), and all other reagents are from Sigma. (Chemical structures of the molecules can be seen on Supplementary Figure 1.)

2.2.Methods

2.3.Solubilization studies

2.3.1. OTX008 solubilization with SBECD and SBECDPolymer (Binary systems)

7.3 m/m% SBECD and 9.6 m/m% SBECDPolymer solutions were prepared using ultrapure water (Millipore Direct-Q 5UV system, Merck Millipore, Burlington, MA, USA). OTX008 was dissolved in the cyclodextrin solutions at a 0.75 mg/ml final concentration to get OTX-SBECD and OTX-SBECDPolymer solutions.

2.3.2. CHR solubilization in OTX008-SBECD and OTX008-SBECD Polymer solutions (Ternary systems)

CHR was added in excess to the OTX-SBECD and OTX-SBECDPolymer solutions prepared by the method 2.3.1. and shaken for 72 hours at room temperature in closed vials. After incubation the samples were centrifuged at 11000 rpm for 10 minutes, the clear supernatant was removed and the dissolved CHR concentration was measured by UV spectrophotometer (Shimadzu UV-1900) at 270 nm. CHR solubilization was also performed in 7.3 m/m% SBECD and 9.6 m/m% SBEβCDPolymer solutions without OTX008 with the same incubation and sample preparation.

The clear CHR solutions were frozen at −110 °C, and the samples were lyophilized using a ScanVac CoolSafe freeze dryer (Labogene, Allerød, Denmark). The complexes were stored at −20 °C until used in further experiments.

2.3.3. Phase-solubility study

7.3 m/m% SBECD solution, 9.6 m/m% SBECDPolymer solution, OTX-SBECD and OTX-SBECDPolymer solutions were prepared and diluted in 96-well plates with ultrapure water. Excess amount of CHR was added to each well, plates were sealed with plate sealer and were shaken for 72 hours at room temperature. Samples were filtered with a MultiScreen Solvinert 96 Well Filter Plate (pore size 0.45 µm, PTFE, Merck Millipore Ltd. Tullagreen, Ireland) using a MultiScreen Resist vacuum manifold (EMD Millipore corporation, Burlington MA, USA) and the clear supernatants were placed on a Greiner UV-Star® 96 well plate. Samples were measured at 270 nm by a Thermo Fisher Multiskan Go (Thermo Fisher, Waltham, MA, USA) microplate reader.

2.3.4. pH-dependent CHR solubility determination

7.3 m/m% SBECD solutions were prepared in sodium acetate - acetic acid buffer with different pH values (pH= 3.65; 5.75; 7.45; 9.95) and placed into a 96 plate. Excess amount of CHR was added to the wells, the plate was sealed and the CHR solubility was determined as described in 2.3.3.

In another experimental setup the pH of the 7.3 m/m% SBECD and OTX-SBECD solutions were set to 8.64 and 3.65 with 0.2 M sodium acetate solution and 0.1 M HCl respectively and CHR solubilization test was performed as described earlier.

2.4. Size distribution measurement of the cyclodextrin complexes with dynamic light scattering (DLS)

The size distribution of particles in 7.3 m/m% SBECD solution, 9.6 m/m% SBECDPolymer solution, OTX-SBECD and OTX-SBECDPolymer solutions with the complexed CHR after the phase solubility test or without CHR was determined by a Malvern Nano-ZS Zetasizer (Malvern Instruments, Malvern, UK).

2.5. Nuclear Magnetic Resonance (NMR) studies

^{1}H and NOESY NMR characterisation of CHR, SBECD, OTX008, binary systems CHR-SBECD, OTX008-SBECD, and ternary system CHR-OTX008-SBECD were performed using a Bruker Ascend 400 MHz spectromenter with a BBFO probe, and the spectra recorded at ambient temperature in dimethylsulphoxide (DMSO-d_6) and/or deuterated water (D_2O) depending on the solubility of the materials (Typical solutions of 10 mg/mL). Lyofilised mixtures CHR-SBECD, OTX008-SBECD, and CHR-OTX008-SBECD were used as described 2.3.1 and 2.3.2. The solvent peaks were referenced to 2.5 ppm (DMSO-d_6) and 4.7 ppm (HDO, H_2O). Peak multiplicities were described as follows: singlet (s), multiplet (m), and broad (br).

2.6. Differential Scanning Calorimetry (DSC) studies

The melting temperature (T_m) and degradation temperature (T_d) of all components and their binary and ternary mixtures were determined by Differential Scanning Calorimetry (DSC; Mettler Toledo, DSC 30 STAR System) at the heating rate of 10 ^{o}C min-1 and under an inert N_2 atmosphere.

2.7. Computational studies

All calculations were undertaken using the Orca 4.2.1 software package [17]. Initial geometry optimization for CHR, OTX008 and SBECD and all interaction variants were conducted with GFN2-xtb semi-empirical quantum mechanical method [18]. The method has been shown to be robust for large scale calculations (up to a few thousand atoms) of organic, organometallic and biochemical systems. The inclusion of the D4 dispersion term makes GFN2-xtb ideal for studying structures involving non-covalent interactions [19].

To gain a more accurate energetic understanding of the molecular interactions the structures were re-optimized with unconstrained Density Functional Theory (DFT) calculations, using the RI-PBE(D3)/def2-svp level of theory [20–23], with subsequent solvated single point energy calculations at the RI-PBE(D3)/def2-tzvp level of theory [20–23] with the continuum solvent polarized method (cpcm), [24] simulating a water environment (epsilon = 80.3). Analytical frequency calculations on the gas phase geometries were performed to account for enthalpic and entropic contributions to the free energy term, as well as to confirm all intermediates are true minima on the potential energy surface. Due to the scaling limitations of DFT, and focus on the molecular interactions, the SBECD molecule was approximated by a single SBE arm when calculating the binding free energy for these interactions. A concentration-induced free-energy shift of $R\,T\ln V_M$=1.89 kcal mol^{-1} (V_M: molar volume of an ideal gas, T=298 K) has been included to account from the shift from gas (1 atm) to solution (1 mol dm^{-3}) phase.

2.8. H9c2 cell culture

Embryonic rat cardiac H9c2 (2-1) cells (ECACC, United Kingdom) were cultured in Dulbecco's modified Eagle's medium (DMEM; AU-L0101Aurogene, Italy), containing 5.5 mM d-glucose and supplemented with 10% heat inactivated fetal bovine serum (FBS; AU-S181H Aurogene, Italy), 1%

L-Glutamine (L-Glu; AU-X0550 Aurogene, Italy) and 1% penicillin/streptomycin solution (P/S; AU-L0022 Aurogene, Italy), at 37°C under an atmosphere of 5% CO2.

Reached an 80% confluence, H9c2 cells were trypsinized, seeded at a specific cell density for each assay and then exposed to NG, high glucose (HG; 33 mM d-glucose) or NG + 27.5 mM mannitol (M; as osmotic control) for 48 hours [25]. Cells were then treated for 6 days [26] in NG or HG medium with the following substances:

- CHR 0.399 mg/ml (CHR), dissolved in NaCl;
- SBECD 7.3 m/m%, dissolved in NaCl;
- SBECDPolymer 9,6 m/m%, dissolved in NaCl;
- Binary system SBECD+0.095 mg/ml CHR (SBECD+CHR), dissolved in NaCl;
- Binary system SBECDPolymer+0.0836 mg/ml CHR (SBECDPolymer+CHR), dissolved in NaCl;
- DMSO 2.5% as vehicle of OTX008;
- OTX008 (0.75-1.25-2.50 μM);
- Binary system OTX008 (2.5-1.25-0.75 μM)-SBECD (OTX008-SBECD), dissolved in NaCl;
- Binary system OTX008 (2.5-1.25-0.75 μM)-SBECDPolymer (OTX008-SBECDPolymer), dissolved in NaCl;
- Ternary system CHR (0.324 mg/ml)-OTX008 (2.5-1.25-0.75 μM)-SBECD (CHR-OTX008-SBECD), dissolved in NaCl;
- Ternary system CHR (0.399 mg/ml)-OTX008 (2.5-1.25-0.75 μM)-SBECDPolymer (CHR-OTX008-SBECDPolymer), dissolved in NaCl.

Three independent experiments were done, each performed in triplicates (N = 9).

2.9. Cell viability assay

H9c2 cells were plated at a density of 1 x 10^4 cells per well in 96-well plates [27], exposed to NG or HG medium for 48 hours and then treated as above described.

At the end of the stimulation period, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) solution (1:10 in culture medium, 300 µl/well) was added to each well, incubated for 4 h at 37°C and then removed. Each well was then washed for 20 min with isopropanol-HCl 0.2 N. Optical density (OD) values were measured at 570 nm using a 96-well plate reader (iMark, Bio-Rad Laboratories, Italy) [25].

2.10. Statistical analysis

The results are reported as mean ± standard deviation (SD). Statistical significance was determined using one-way Analysis of Variance (ANOVA) followed by Tukey's comparison test. A P-value less than 0.05 was considered significant to reject the null hypothesis.

3. Results

3.1. OTX008 solubilization with SBECD and SBECDPolymer

OTX008 is poorly soluble in water. To achieve the desired, therapeutically relevant 0.75 mg/ml OTX008 concentration, SBECD and SBECDPolymer were used as solubilizers. SBECD was more effective solubilizer, 7.3 m/m% SBECD and 9.6 m/m% SBECDPolymer solutions were able to

dissolve OTX008 at 0.75 mg/ml in water, thus these concentrations were applied in further experiments.

3.2. CHR solubilization in OTX008-SBECD and SBECDPolymer solutions

CHR is insoluble in water, however its solubility can be improved by cyclodextrins as we described earlier [5]. Here we tested 7.3 m/m% SBECD and 9.6 m/m% SBECDPolymer solutions with or without 0.75 mg/ml OTX008 and found, that in the presence of OTX008 CHR solubility increased significantly ($p<0.0001$), compared to the only cyclodextrin-containing solutions (Supplementary Figure 2.).

The clear solutions were lyophilized, and the solid products were dissolved in 0.9 m/m% NaCl solutions. The lyophilized products dissolved completely in the saline solution, which were further suitable for *in vitro/in vivo* experiments.

3.3. Phase-solubility study

In the phase-solubility study serial dilutions of SBECD, SBECDPolymer, OTX008-SBECD and OTX008-SBECDPolymer solutions were used to solubilize CHR. There was a linear relationship between CHR solubility and SBECD or SBECDPolymer concentrations, however OTX008-SBECD and OTX008-SBECDPolymer showed a different behavior. Both OTX008-SBECD and OTX008-SBECDPolymer had non-linear, positively skewed curves, showing, that association of the complexes occurred in the solution (Figure 1.). It should be noted, that higher SBECDPolymer concentration was required to dissolve the same amount of OTX008.

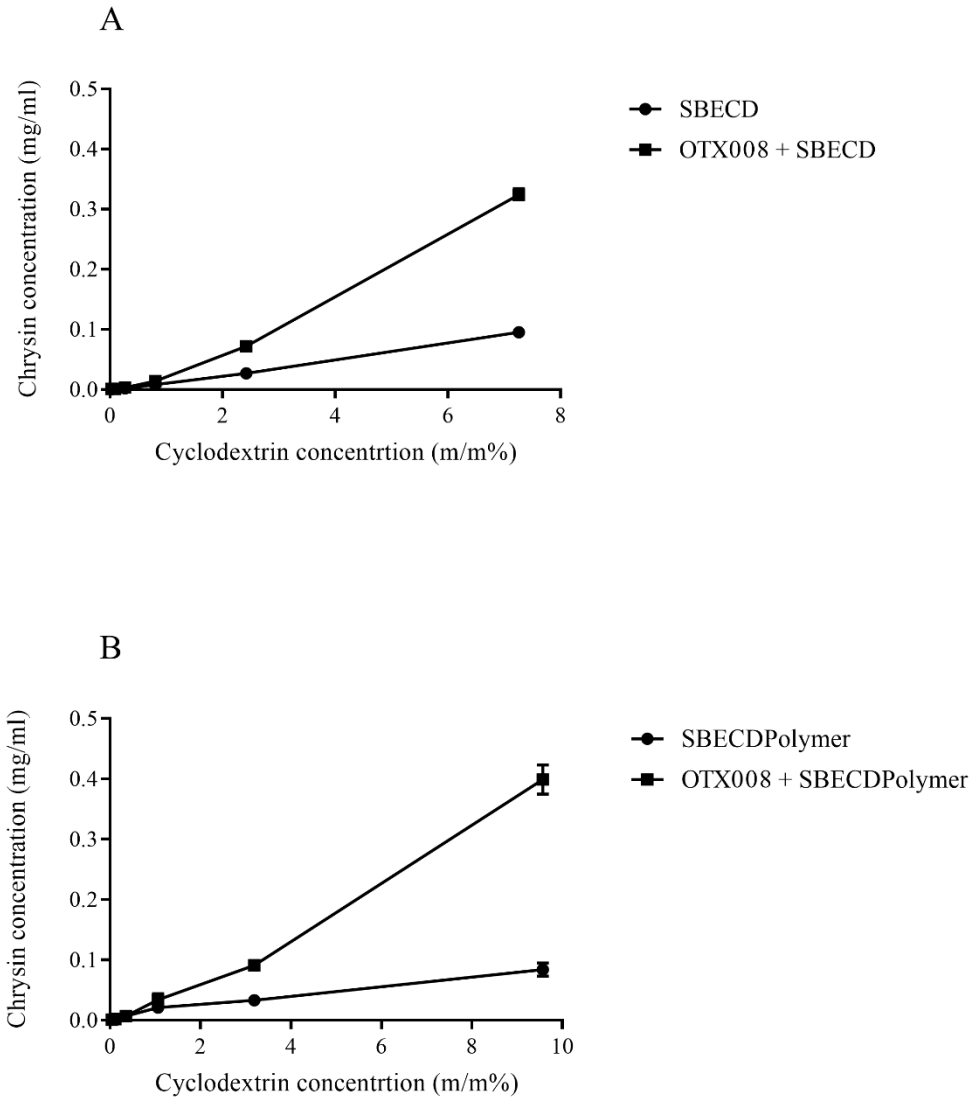

Figure 1. Phase-solubility curves of CHR in SBECD, OTX008-SBECD (A), SBECDpolymer, and OTX008-SBECDpolymer (B) solutions. OTX008 concentration was 0.75 mg/ml in the solutions with the highest cyclodextrin concentrations at 7.3 m/m% SBECD and 9.6 m/m% SBECDpolymer. (CHR-Chrysin, SBECD- Sulfobutylated β-cyclodextrin sodium salt, SBECDPolymer- sulfobutylated beta-cyclodextrin polymer)

3.4. Size distribution measurement of the cyclodextrin complexes with dynamic light scattering (DLS)

In solution, at a 7.3 m/m% concentration SBECD showed associated molecules in DLS measurements (351.8 nm). The size of the aggregated particles increased when SBECD is mixed with OTX008 (1002 nm) and became significantly higher after complexation of CHR in the ternary CHR-OTX008-SBECD complex (2698 nm).

In the case of SBECDPolymer, the biggest aggregates were detected in OTX008-SBECDPolymer (5021 nm), while the highest intensity ratio (67.8%) of large aggregates (945.6 nm) can be found in the presence of CHR and OTX008 (Supplementary Figure 3. and Table 1.) Even if the complex polymeric structure of the SBECDPolymer sample makes the interpretation of the results more difficult, the increase of size and ratio of larger populations was seen and reveal the interaction of CHR and OTX008 with the polymer.

Table 1. Size distribution of SBECD, SBECDPolymer, binary and their ternary complexes.

	Peak 1.		Peak 2.		Peak 3.	
	Size	Intensity %	Size	Intensity %	Size	Intensity %
SBECD	351.8 nm	100 %	-	-	-	-
CHR-SBECD	1.1 nm	20.8 %	94.83 nm	38.1%	3764 nm	41.1%
OTX008-SBECD	1.5 nm	13.2%	1002 nm	86.8%	-	-
CHR-OTX008-SBECD	1.2 nm	24.8%	2698 nm	75.2%	-	-
SBECDPolymer	44.35 nm	16.1%	214.6 nm	12.6%	1546 nm	59.3%
CHR-SBECDPolymer	49.3 nm	14.1 %	699.7 nm	32.4%	4695 nm	53.5%
OTX008-SBECDPolymer	36.95 nm	19.1%	233.8 nm	29.1%	5021 nm	38.3%
CHR-OTX008-SBECDPolymer	20.93 nm	10.5%	84.43 nm	14.1%	945.6 nm	67.8%

(CHR-Chrysin, SBECD- Sulfobutylated β-cyclodextrin sodium salt, SBECDPolymer- sulfobutylated beta-cyclodextrin polymer)

3.5.pH-dependent CHR solubility determination

The pH-dependent solubility of CHR in presence or absence of OTX008 was tested. Increasing the pH in the presence of 7.3 m/m% SBECD slightly increased the solubility of CHR (Supplementary Figure 4.).

The pH of 7.3 m/m% SBECD solution was 6.23. Increasing the pH of SBECD solution with sodium acetate to pH=8.64 slightly increased CHR solubility, while acidification of the OTX008-SBECD solution significantly decreased the solubility of CHR, similar to the value measured in SBECD solution. However, in the presence of OTX008 plus SBECD or SBECDPolymer, in alkaline conditions the solubility of CHR increased significantly by 3-4 times. OTX008-SBECD and OTX008-SBECDPolymer solutions had pH= 9.36 and 9.14 in purified water respectively.

Changing the pH from 3.65 to 9.95 caused only a twofold improvement of CHR's solubility in the presence of SBECD. Both OTX008 and alkalinity are necessary for the significant solubility improvement with SBECD / SBECDPolymer. In the presence of OTX008 and SBECD the pH change to 9.36 resulted a fourfold increase in the solubility of CHR. The ternary complexes of CHR, OTX008 and SBECD / SBECDPolymer are formed, which were reflected in the size increment of the molecular associations. The molecular interactions of OTX008 + SBECD / SBECDPolymer and the formation a ternary complex are necessary for significant solubility enhancement of CHR.

Even if the crosslinked SBECDpolymer seemed to be a suitable solubilizer of CHR, the results showed, that it has less ability to solubilize OTX008. According to the DLS results SBECDPolymer has multimodal molecular size distribution. The chemical and thermal studies for further

understanding of the molecular interactions between the three molecules are concentrated in the CHR+OTX008+SBECD ternary mixture.

3.6. NMR studies

To elucidate the reasons behind the enhanced solubility of CHR in presence of both OTX008 and SBECD, 1 H and NOESY NMR spectra of the ternary mixture CHR-OTX008-SBECD, binary mixtures CHR-SBECD and OTX008-SBECD and single components CHR, OTX008 and SBECD were recorded. The ideal deuterated solvent for the spectrometric analysis of all components was DMSO-d_6 while D_2O used as best representative of physiological conditions and the evaluation of potential interactions between the components. The spectra of clear solutions of CHR (Figure 2A.) and OTX008 (Supplementary Figure 5.) were recorded in DMSO-d_6 as from their insolubility in D_2O. The SBECD was examined in both deuterated solvents and showed the strongest and predominant response to the applied magnetic field when in mixtures as it can be seen in Supplementary Figure 6 and 7, and as expected from its high concentration in the solution.

3.6.1. Binary mixture of CHR-SBECD

When CHR is mixed with SBECD (0.01 mol/mol, CHR/SBECD) in DMSO-d_6 (Supplementary Figure 8.) or D_2O (Supplementary Figure 9.), the 1H NMR spectra of the mixture results identical to pure SBECD confirming the high preference of CHR molecules accommodated inside the toroid cavity of the cyclodextrin structure. This is in accordance with the formation of large aggregates observed in DLS experiments and confirmed by observing no free CHR in NOESY spectra of the binary mixture (Supplementary Figure 10 and 10a.)

3.6.2. ***Binary mixture of OTX008-SBECD***

The spectra of the mixture reveal only the presence of SBECD in DMSO-d_6 (Supplementary Figure 11 and 12.), this can be associated to the molar ratio of the two components used for the preparation of the binary mixture (0.02 mol/mol, OTX008/SBECD). Interestingly, as from simulations described in the next paragraph the SBECD-OTX008 interaction is not favorable (+0.9 kcal mol^{-1}).

3.6.3. Ternary mixture CHR-OTX008-SBECD

When CHR is mixed with SBECD (0.03 mol/mol, CHR/SBECD) and OTX008 (1.6 mol/mol, CHR/OTX008) in DMSO-d_6 (Supplementary Figure 13, 13a-c and 14.) or D_2O (Supplementary Figure 15.), the 1H NMR spectra of the mixture show~~s~~ some differences in protons chemical shifts when compared to those observed for the single components of the mixture. In DMSO-d_6 spectra, the signals attributed to the two OHs of the CHR molecule bonded to carbon atoms in 5 and 7 position are seen very weak or even not seen at all (Figure 2A.). This can be due to the molar ratio of the CHR in the ternary mixture or because are involved in the non-bonded interaction that keeps the supramolecular structure together.

No changes observed for protons bonded to carbon atoms in positions 10 to 10’ suggesting that the potential interaction of the CHR with the other two components of the ternary mixture is involving the opposite part of the molecule; small chemical shifts to higher magnetic field, 0.02 ppm, 0.05 ppm and 0.03 ppm respectively for protons H-3, H-8 and H-6 indicating interaction of the condensed part of the CHR (Figure 2A and Supplementary Figure 13b and 13c.). The shape of the last 3 protons is broader that could imply electrostatic interactions through hydrogen bonding with the two other components (OTX008 and SBECD) partners which contain several of oxygen and/or nitrogen atoms.

In the same spectrum (Supplementary Figure 13.), OTX008 protons bonded to nitrogen atoms (NH) are subjected to a small chemical shift to a higher magnetic field from 8.32 to 8.29 ppm (0.003 ppm). Interestingly, a larger low filed effect from 6.65 ppm to 6.71-6.59 ppm of OTX008 aromatic protons indicated that the upper aromatic cup shape of OTX008 is distorted by the 3 members interaction (Supplementary Figure 13c.). This is also supported by the broadness of the resulted signal centered at 6.70 ppm. The above observations indicated that the CHR molecules are electrostatically bonded to carbonyl groups of the OTX008 side arms and are causing this distortion (confirmed by simulations). No changes on the chemical shifts of the SBECD protons (Supplementary Figure 13.).

Three signals attributed to CHR aromatic protons H-3, H-8 and H-6 at 6.60 ppm, 6.55 ppm and 6.22 ppm respectively are appeared in the NOESY spectra of the ternary mixture confirming that any interaction of CHR with the system is involving the phenolic aromatic ring (Supplementary Figures 14-15.).

Some small differences (0.05 ppm) in the chemical shifts of the aromatic protons of CHR were observed in spectra recorded in D_2O when the binary CHR-SBECD and the ternary mixture were compared (Figure 2.). Those peaks were broader indicating the complex new situation in the macrostructure. The NOESY spectra also confirm the presence of free CHR molecules not involved on the holding together the CHR-OTX008-SBECD macrostructure (Figure 2.).

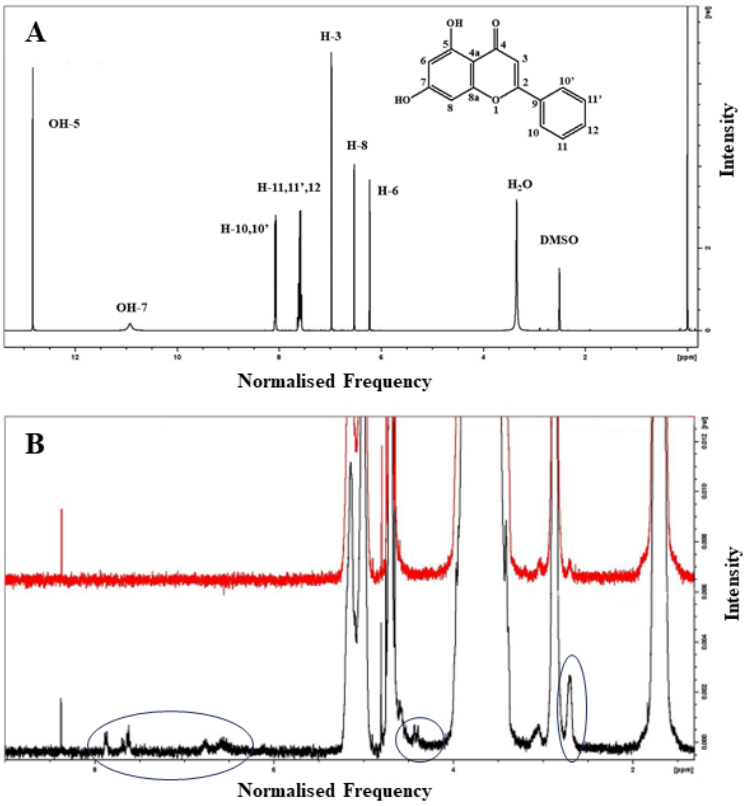

Figure 2. (A) Full 1H NMR spectrum of CHR in DMSO-d_6 with assigned peaks. (B) 1H NMR spectra of CHR-OTX008-SBECD mixture (Black line) and CHR-SBECD mixture (Red line) in D_2O showing the presence of CHR and OTX008 in the ternary structure (framed areas), area 9.0-1.8 ppm. (CHR-Chrysin, SBECD- Sulfobutylated β-cyclodextrin sodium salt, DMSO-Dimethyl sulfoxide)

3.7. Thermal analysis

The thermal analysis of all components and their binary and ternary mixtures revealed that properties of all components were affected when mixed (Supplementary Table 1. and Supplementary Figures 16-17.). In the ternary mixture, all components degrade together with a peak at 300 ^{o}C indicating the formation of a unique macrostructure while those CHR molecules accomodated in the free volume of the macrostructure kept their thermal identity with melting point at 290 ^{o}C.

3.8. Computational studies on CHR-OTX008-SBECD interactions

Initial supramolecular structures for the interactions between CHR, OTX008 and SBECD were optimized with the GFN2-xtb quantum semi-empirical method. This method has been previously shown to produce good quality geometries for a range of species, from small organics to polymers.

To gain a better understanding of the relevant interactions, binding free energies have been calculated with DFT calculations (RI-PBE_D3BJ/def2-tvzp level of theory). All starting geometries were taken from the optimized GFN2-xtb structures.

The calculated geometries of optimized binding interactions between all molecular components (OTX008, CHR, SBE and CD) are shown in Figure 3. with calculated free energies in reported in Table 2.

Given the size and conformation flexibility of the SBECD molecule, full DFT calculations would prove computational too expensive while also have a significant level of uncertainty with the results. A model system for the binding interaction between SBECD, CHR and OTX008 was chosen to focus on the SO_3^- interactions as these showed to the preferential in the GFN2-xtb optimization. The GFN2-xtb optimization interactions between SBECD and CHR, and OTX008 and SBECD are also presented in Supplementary Figure 18.

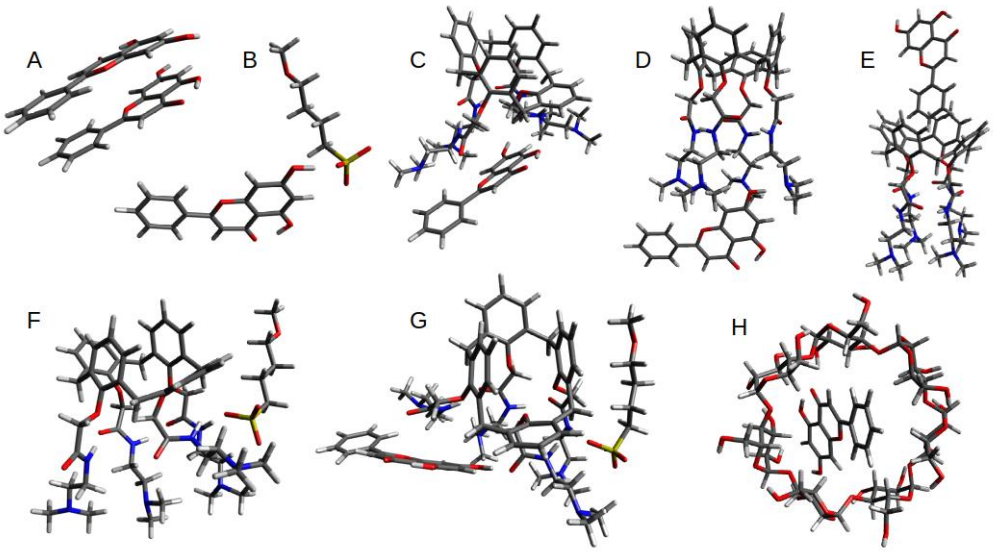

Figure 3. The figure shows the DFT optimized structures evaluated in this study, see Table 2 for calculated binding energies. Structure A shows the preferential CHR-CHR dimer alignment, with a binding energy of -14.0 kcal mol^{-1}, alternative stacked orientations were within 2 kcal mol^{-1}, suggesting multiple alignment possibilities. The hydrogen bonded dimer adducted was calculated to be 9 kcal mol^{-1} less favorable. Structure B highlights the CHR-SBE interaction, with a strong hydrogen bond between the -OH group of the CHR molecule and SO_3^- group of the SBE arm. Structures C-E show the possible interactions between CHR and OTX008; with hydrogen bonding between the CHR hydroxyl group and the carbonyl on the OTX008 unit being preferential. Structure F shows the most favored interaction between the amine groups of the OTX008 unit and the SBE arm. Structure G highlights the tertiary adduct involving CHR-OTX008-SBE, the structure shows a cooperative effect where the OTX008-SBE interaction (R-NH---SO3--R) facilitates the OTX008-CHR interaction (R-CO---HO-R). This compensates for the increased entropic contribution of the tertiary complex. Structure H shows the host-guest binding of the CHR in the CD cavity.

Table 2. Binding free energies for each interaction. See Figure 3. for the structures.

Structure	**Interaction**	**G_{solv} kcal/mol**
A	CHR dimer	-14.0
B	SBECD-CHR (arm)	-6.2
C	OTX008-CHR (carbonyl)	-12.6
D	OTX008-CHR (amine)	-10.8
E	OTX008-CHR (calixarene)	-2.7
F	SBECD-OTX008	+0.9
G	SBECD-OTX008-CHR	-8.6
H	SBECD-CHR (cavity)	-4.5

(CHR- Chrysin, SBECD- Sulfobutylated β-cyclodextrin sodium salt)

The relative binding energies (Table 2.) clearly highlight the preferential binding of the OTX008-CHR interaction via the carbonyl of the amide arms. There is also a reasonably strong interaction between the SBECD and CHR molecules both with the arms and the cyclodextrin cavity of SBECD. Interestingly the SBECD-OTX008 interaction is not favorable (+0.9 kcal mol^{-1}). This agrees with the reduction in binding energy on the combination of SBECD -OTX008-CHR compared to the initial OTX008-CHR interaction. The formation of CHR dimers is also preferential.

3.9. Cell viability

H9c2 cell viability was not decreased in NG or HG medium by the exposition to CHR, SBECD, SBECDPolymer, SBECD+CHR, SBECDPolymer+CHR, DMSO or M. Moreover, all the doses of OTX008 tested (2.5-1.25-0.75 μM) alone and in combination with SBECD or SBECDPolymer (OTX008-SBECD and OTX008SBECDPolymer) and CHR-SBECD or CHR-SBECDPolymer (CHR-OTX008-SBECD and CHR-OTX008-SBECDPolymer) did not reduce cell viability in NG conditions (Figure 4A.). These doses were not toxic also when added to HG medium (Figure 4B.). On the contrary, they lead to a significant improvement in cell viability on H9c2 cells exposed to HG. Particularly, OTX008 alone, OTX008-SBECD and OTX008-SBECDPolymer significantly increased cell viability at all 3 doses analyzed (Figure 4C, D, E.). CHR-OTX008-SBECD and CHR-OTX008-SBECDPolymer were effective starting from 1.25 μM OTX008 (Figure 4D.). Interestingly, OTX008-SBECD, OTX008-SBECDPolymer or CHR-SBECD-OTX008 lead to the highest increase in cell viability at the maximum dose of OTX008 (2.5 μM) (Figure 4E.).

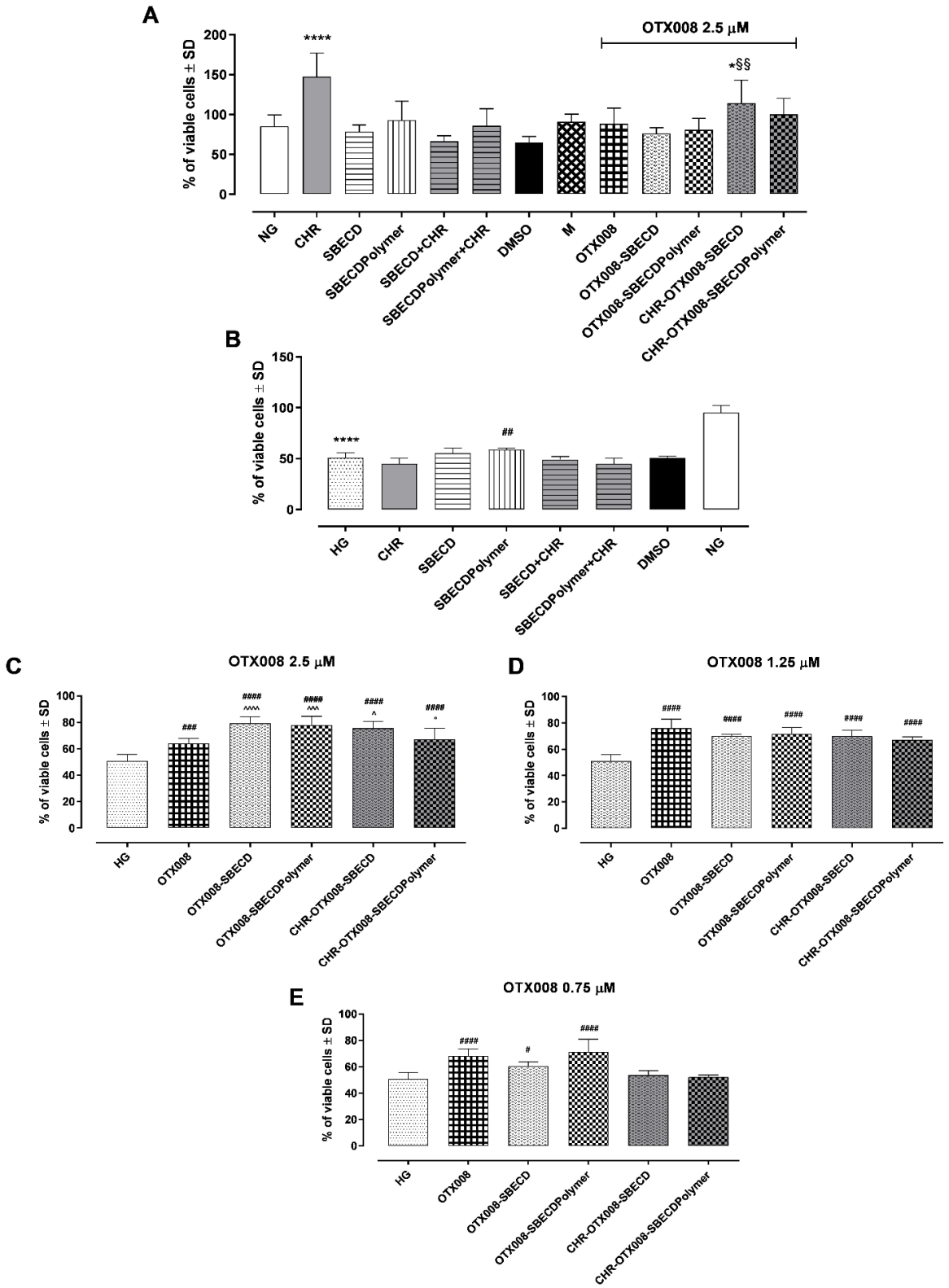

Figure 4: H9c2 cell viability after the treatment with the compounds in NG (A) and HG (B) conditions (where NG group is reported as control). In NG cells, OTX008 was tested at the maximum dose of 2.5 μM. In HG cells, OTX008 was tested at the doses of 2.5-1.25-0.75 μM (C, D, E

respectively). Cell viability was determined by MTT assay and reported by % of cell viability ± SD. NG = 5.5 mM D-glucose; HG = 33 mM D-glucose; CHR = CHR 0.399 mg/ml; SBECD = Sulfobutylated -β-cyclodextrin 7.3 m/m%; SBECDPolymer = Sulfobutylated-β-cyclodextrin polymer 9,6 m/m%; SBECD+CHR = SBECD+0.095 mg/ml CHR; SBECDPolymer+CHR = SBECDPolymer+0.0836 mg/ml CHR; DMSO = dimethyl sulfoxide 2.5%; M = mannitol 27.5 mM; OTX008 = OTX008 (2.5-1.25-0.75 μM); OTX008-SBECD = OTX(2.5-1.25-0.75 μM)-SBECD; OTX008-SBECDPolymer = OTX(2.5-1.25-0.75 μM)-SBECDPolymer; CHR-OTX008-SBECD = CHR (0.324 mg/ml)-OTX(2.5-1.25-0.75 μM)-SBECD-; CHR-OTX008-SBECDPolymer = CHR (0.324 mg/ml)- OTX(2.5-1.25-0.75 μM)-SBECDPolymer. *P < 0.05, **** P < 0.0001 vs NG; # P < 0.05, ## P < 0.01, #### P < 0.0001 vs HG; ^ P < 0.05, ^^^ P < 0.001, ^^^^P < 0.0001 vs OTX008; °P < 0.05 vs OTX008-SBECDPolymer; §§ P < 0.01 vs OTX008-SBECD.

4. Discussion

Fibrosis, stemming from chronic hyperglycemia, impacts vascular structures, causing conditions like diabetic retinopathy and hepatic fibrosis [28]. This fibrogenic process involves inflammatory mediators, cytokines, and growth factors, especially TGF-β. This leads to increased extracellular matrix deposition. Past research on the flavonoid CHR in rodents showed its anti-fibrotic effects. CHR counteracts fibrosis by inhibiting hepatic cell activation via the TGF-β1/Smad pathway [29]. Additionally, CHR adjusts extracellular matrix dynamics and reduces collagen synthesis [11].

Recent studies highlight Gal-1 protein as a therapeutic target for diabetic fibrosis. Elevated Gal-1 levels were found in diabetic mice kidneys, contributing to kidney fibrogenesis [30,31]. Additionally, OTX008 has proven effective in preventing Gal-1 accumulation and countered the effects of TGF-β on ARPE-19 cells in high glucose situations [32].

We proposed here a combined use of CHR and OTX008 to address fibrosis, but there was a need for an suitable delivery mechanism to ensure their simultaneous solubilization and deployment.

SBECD was selected as an optimal carrier as a widely known, parenterally safe cyclodextrin derivative with excellent solubilization properties and polyanionic nature [33–35], CHR was successfully solubilized with SBECD earlier [5] while the interaction between SBECD and OTX008 was predicted based on their chemical structure. Indeed, the solubilization experiments and further chemico-physical analysis revealed the interaction among SBECD, OTX008 and CHR forming a ternary complex. At first, it was found that OTX008 could be dissolved by SBECD. Interestingly SBECDpolymer showed less efficacy, a higher SBECDpolymer concentration was required to dissolve the same amount of OTX008; DLS experiments revealed the polydispersity of the polymer and a mixture of aggregates with different sizes while the 3D polymeric structure in solution does not permit the chemical interactions of the components. Due to these findings, SBECDpolymer was excluded from further investigation through structure analysis and molecular simulation. Phase solubility tests of CHR clearly showed the difference between the solubilization mechanisms and the structure of the formed complexes in the binary CHR-SBECD and ternary CHR-OTX-SBECD complexes. The positively skewed curve of the ternary complexes points to the fact, that bigger molecular associates were formed and increased the solubility of CHR compared to SBECD or SBECDPolymer. This phenomenon is well-known in the case of cyclodextrins [36], however in calixarene-cyclodextrin macrostructures it is less studied. The solubility of CHR is pH-dependent, in the range of pH 6.20-9.40 the two OH groups of the molecule are ionizable and the two ionizations take place forming a dianion [37]. It is in accordance with our results, in the presence of SBECD increasing the pH from 3.65 to 9.95 resulted approximately a twofold increase in CHR solubility. SBECD-CHR host-guest interaction is favorable from computational calculations (-4.5 kcal mol^{-1}), supported also by the NMR data, with CHR accommodated inside the toroid cavity of the cyclodextrin ring. Molecular simulation revealed another interesting mechanism in the interaction of

CHR and SBECD. The OH groups of CHR chemically interacts with the sulfobutyl ether (SBE) pendant groups, which is not the conventional host-guest mechanism of cyclodextrins but can contribute to the solubilization of CHR. SBECD has an average of six SBE arms, thus it can amplify the number of interactions with CHR molecules, beside the host-guest interaction. The solubilization of OTX008 with SBECD resulted also an alkaline solution with a pH of 9.36, however increased the CHR solubility fourfold, showing, that the interactions with OTX008 was required for the significant CHR solubilization. In the ternary complex of CHR-OTX008-SBECD the NMR signals of CHR's OHs are significantly changed, confirming the involvement of OH groups in the formation of ternary complexes. The signals of the amide group and aromatic rings of OTX008 are also modified showing involvement on the interaction in the ternary complex. Indeed, the most preferential interactions are on the carbonyl and amine groups (-12.6 kcal mol^{-1} and -10.8 kcal mol^{-1} respectively) of OTX008 with CHR.

The presented geometries and binding energies suggest that the experimentally enhanced entrapment of CHR with an OTX008-SBECD mixture is additive in nature and the higher solubility observed can be justified by a combination of these mechanisms, leading to a supramolecular structure. The SBECD is able to bind at least 6 CHR molecules (one on each arm) with the potential for multiple CHR per SO_3^- moiety. Subsequently the addition of OTX008 allows for 1:1 CHR-OTX008 adducts to be formed. The CHR is preferentially located within the amide arms (allowing for additional secondary interactions) and optimization an external amide interaction proves less stable, suggesting the 1:1 complex most favored.

The hypothesis here is that the OH groups of CHR molecules located in position 7 (Figure 2A.) are involved in hydrogen bonding with OTX008 molecules (CONH) and/or in interactions with the tails of SEBCD to produce a supramolecular structure; the higher solubilization of the CHR in the ternary structure is due to the cooperation of three effects: i) electrostatic bonding of CHR with OTX008 and SBECD molecules acting as bridge, ii) inclusion of CHR molecules in the SBECD toroid and

calixarene structures and iii) entrapment of CHR molecules in the free volume of the new supramolecular structure, facilitated by the strong CHR-CHR binding energy (Figure 5.). This high solubilization of CHR is strictly associated to this specific ternary system.

Based on the phase solubility data and DLS results large molecular associations are formed in the solution of OTX008 and SBECD (1002 nm, 86.8% intensity), which further increased after the solubilization of CHR (2698 nm, 75.2% intensity). Between the molecules, which forms the supramolecular associations free volume takes place [38,39] where CHR can be accumulated by the supramolecular structure. The special structure of the two association builder macrocycles OTX008 and SBECD helps the accumulation of CHR between macro chains or side groups. The structure supports the formation of the favored supramolecular carrier by the binding free energies, complexation, and free volumes.

An attempted scheme with the proposed supramolecular structure is reported in Figure 5.

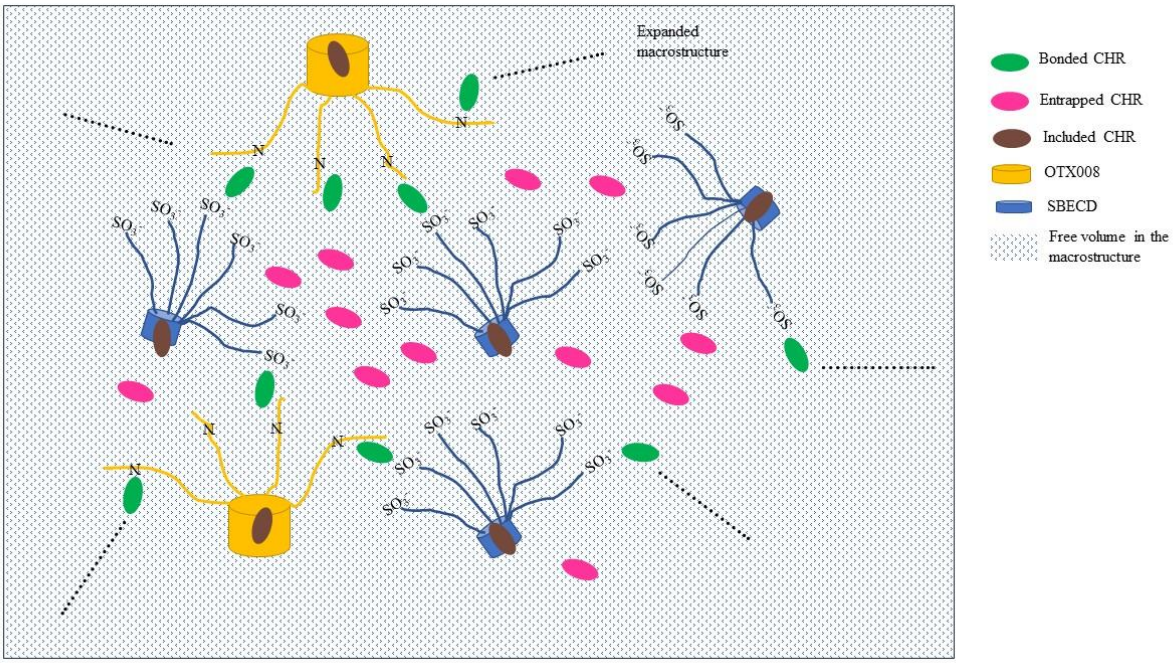

Figure 5. Schematic representation of CHR-OTX008-SBECD macrostructure with CHR molecules making the link between SBECD and OTX008 molecules (Green discs), dispersed in the free volume

created in the macrostructure (Purple discs) and trapped in the CD toroid or calixarene structure (Brown discs). The synergy of the three situations gives the enhancement of the CHR solubility in the ternary mixture. (CHR-Chrysin, SBECD- Sulfobutylated β-cyclodextrin sodium salt)

Finally, the biocompatibility of the complexes was tested on H9c2 (2-1) cells under normal and hyperglycemic conditions. The tested compounds alone, binary, and ternary complexes were not toxic on cells in NG or HG medium. Moreover, the binary and ternary complexes of OTX008 prevented the toxic effects of HG medium on H9c2 cells, especially at the maximum dose of OTX008 (2.5 μM) was the most effective. Building on our prior research that highlighted CHR's antifibrotic capabilities [11,29] and OTX008's intervention in the profibrotic Gal-1/TGF-β pathway in a hyperglycemic environment [30], this newly devised drug delivery system CHR-OTX008-SBECD emerges as a promising candidate for modulating fibrotic progression associated with chronic diabetes. Further detailed in vitro and in vivo studies are essential to discern the specific cellular and molecular mechanisms driving its therapeutic potential.

5. Conclusion

Addressing fibrosis under hyperglycemic conditions remains a significant challenge, necessitating innovative approaches and therapeutic agents. In this study, we introduce a new ternary formulation and application of two potential drugs with SBECD, each targeting distinct pharmacological pathways, within a Drug Delivery System (DDS) optimized for parenteral use. This marks the novel dual formulation of the calixarene derivative OTX008 and flavonoid CHR within a single DDS, where they uniquely and actively influence the structural formation of the resultant supramolecular ternary complex. Notably, OTX008 serves as a pivotal element of the ternary complex, both

structurally and pharmacologically. Our findings suggest that the tailored ternary complex presents a novel promising solution for the integrated treatment of fibrosis in hyperglycemic scenarios and can open a new chapter in drug delivery of poorly water-soluble drugs.

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7. **Conflict of interest:** The authors report there are no competing interests to declare.

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Analysis and interpretation of the data: Eleftheria Dossi, Alex Hamilton, Maria Consiglia Trotta, Ágnes Rusznyák, Judit Váradi, Ferenc Fenyvesi, Csilla Sajtos, Caterina Claudia Lepre, Michele D'Amico

Drafting of the paper: Anca Hermenean, Eleftheria Dossi, Ferenc Fenyvesi, Ágnes Rusznyák, Judit Váradi

Review and editing: Anca Hermenean, Eleftheria Dossi, Ferenc Fenyvesi

All authors agree to be accountable for all aspects of the work.

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