The crystal structure of transthyretin in complex with diethylstilbestrol: a promising template for the design of amyloid inhibitors.

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Running Title: The interaction of DES with TTR
SUMMARY

Transthyretin (TTR) is a homotetrameric plasma protein that, in conditions yet not completely understood, may aggregate forming the fibrillar material associated with TTR amyloidosis. A number of reported experiments indicate that dissociation of the TTR tetramer occurs prior to fibril formation and therefore studies aiming at the discovery of compounds that stabilize the protein quaternary structure, thereby acting as amyloid inhibitors, are being performed. The ability of diethylstilbestrol (DES) to act as a competitive inhibitor for the thyroid hormone binding to TTR, indicated a possible stabilizing effect of DES upon binding.

Here we report the crystallographic study of DES binding to TTR. The structural data reveals two different binding modes, both located in the T4 binding channel. In both cases DES binds deeply in the channel and establishes interactions with the equivalent molecule present in the adjacent binding site. The most remarkable features of DES interaction with TTR are its hydrophobic interactions within the protein halogen binding pockets, where its ethyl groups are snugly fitted, and the hydrogen bonds established at the centre of the tetramer with Ser117.

Experiments concerning amyloid formation in vitro suggest that DES is effectively an amyloid inhibitor in acid mediated fibrillogenesis and may be used for the design of more powerful drugs. The present study gave us further insight in the molecular mechanism by which DES competes with thyroid hormone binding to TTR and highlights key interactions between DES and TTR that oppose amyloid formation.
INTRODUCTION

Amyloidoses are protein-misfolding diseases characterised by the conversion of a soluble protein into insoluble fibrils with a β-sheet secondary structure that deposit extracellularly. Transthyretin (TTR)\(^1\) is associated with two of such diseases, the non-mutated form is found in amyloid deposits of patients suffering from Senile Systemic Amyloidosis (SSA), whereas a large number of variants are linked to the neurodegenerative disease Familial Amyloidotic Polyneuropathy (FAP) (1). It is a 54 kDa protein that transports in plasma and cerebrospinal fluid, thyroxine (T4) and the retinol binding protein-retinol complex in plasma (2,3). Additionally, TTR is also known to bind the products of thyroxine metabolism as well as a variety of pharmacological agents such as penicillin, salicylate, cardioactive agents and steroids (4).

The X-ray crystallographic structure for TTR was determined by C.Blake and collaborators (1978) revealing a tetramer, with molecular 222 symmetry, composed of identical subunits assembled around a central channel where are located two T4-binding sites (5). Each monomer has a β-sandwich structure formed by two β-sheets, composed of strands DAGH and CBEF. A small helical fragment connects strands E and F. While strands DAGH mould the channel surface, the others CBEF define the external surface of monomer in the tetrameric structure. Two monomers linked by hydrogen bonds between strands FF’ and HH’ form one dimer. The tetramer assembly involves two dimers linked by hydrogen bonds and hydrophobic contacts between loop AB from one dimer and strand H from the other dimer. The three-dimensional structure of the complex between TTR and retinol binding protein (RBP) has been determined,

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\(^1\) Abbreviations: TTR, transthyretin; SSA, Senile Systemic Amyloidosis; FAP, Familial Amyloidotic Polyneuropathy; T4, thyroxine; RBP, retinol binding protein, DES, diethylstilbestrol; DMSO, dimethylsulfoxide; BM-I, binding mode I; BM-II, binding mode II, ThT, Thioflavin T
showing that RBP binds at the surface and does not interfere with T4 binding (3). The protein T4-binding site was also fully described (6). There are two equivalent T4 binding domains, each of them comprising three symmetry related sets of pockets in which the hormone halogen atoms accommodate: a innermost P3 pocket, a hydrophobic central P2 pocket and the outer-most P1 pocket that is positioned in the channel entry near the charged Lys-15 and Glu-54 residues. All pockets present a core for hydrophobic interactions, while extra hydrogen bond donors and acceptors are made available by conformational changes of the TTR side chains. Although both binding sites are sterically identical, the binding affinity is two orders of magnitude higher for the binding of the first T4 molecule, \( K_a1=10^8 \text{ M}^{-1} / K_a2=10^6 \text{ M}^{-1} \), and therefore a negative cooperativity mechanism has been proposed (7,8).

Despite the overwhelming amount of information collected about TTR-related amyloid diseases, there is still no effective and non-invasive treatment for FAP or SSA. The single mutations associated with FAP point to a destabilization of TTR, accelerating the dissociation of the folded tetramer into monomeric amyloidogenic intermediates (9,10,11). In fact, tetramer dissociation is probably a crucial step in the molecular mechanism that leads to amyloidosis. In vitro studies have shown that binding of the natural ligand T4 to the TTR central hydrophobic channel stabilizes the tetramer and consequently reduces amyloid fibril formation (12). In the past few years, considerable effort has been directed to the discovery of small molecules that could prevent TTR dissociation by binding in the protein central channel (13,14). Actually, stabilization of the native tetrameric fold, upon high affinity binding of a small compound, is considered to be a potential therapeutic strategy to SSA and FAP variants (15,16).

Diethylstilbestrol (DES) is a synthetic estrogen that was found to be a potent competitive inhibitor for thyroid hormone binding to TTR (17). This result suggested to us that it might act as
an amyloid inhibitor. It contains two hydrophobic phenyl groups with hydroxyl substituents that are connected by an ethylene group, a molecular structure that is similar to the structure of Resveratrol, a compound that has been described as an inhibitor of fibrilogenesis (13) (Fig.1). In order to obtain structural information about TTR/DES binding mode and find out essential features of the ligand that are responsible for the affinity of the compound to TTR, the structure of TTR-DES complex was determined by X-ray crystallography. In addition, the ability of DES to inhibit fibril formation, in vitro, was assayed.

EXPERIMENTAL PROCEDURES

Protein complex preparation and crystallization

Recombinant TTR-WT was expressed in Escherichia coli and isolated and purified as reported previously (18). Diethylstilbestrol (DES) (Sigma, St.Louis, MO, USA) was dissolved in dimethysulfoxide (DMSO) at 50 mg/mL. The protein sample was dialyzed against 10 mM HEPES buffer (pH 7.5), concentrated to 21 mg/mL and incubated for 24h at 4°C with a 10-fold molar excess of DES. The TTR-DES solution was mixed 1:1 with the reservoir solution (2.4 M ammonium sulfate, 7% glycerol and 0.2 M sodium acetate, pH 5.4), and equilibrated against the latter solution by the hanging drop vapor diffusion method. Both orthorhombic and monoclinic crystals were obtained at 14°C, after three months. Crystals were sequentially transferred to solutions similar to the reservoir solution, but with increasing concentrations of the cryoprotectant glycerol (10-25%), and subsequently flash frozen in liquid nitrogen.
Data collection and processing

Diffraction data sets were collected from two crystals using synchrotron radiation at beamline ID14-EH3 of the European Synchrotron Radiation Facility (ESRF), in Grenoble. Diffraction data was measured at 100 K using a MARCCD detector. Determination of the crystal orientation and integration of the reflections was performed with MOSFLM (19). The data were scaled and reduced using the programs SCALA and TRUNCATE (20). Two different crystal forms were observed for the TTR-DES complex, an orthorhombic (TTR-DES1) and a monoclinic form (TTR-DES2), which diffracted to 1.8 Å and 1.85 Å resolution, respectively. The TTR-DES1 crystal belongs to space group P2₁2₁2 with lattice parameters a = 42.3 Å, b = 85.4 Å, c = 63.4 Å and two monomers in the asymmetric unit. TTR-DES2 complex crystallized in space group C2 with four monomers in the asymmetric unit and unit cell dimensions a = 74.3 Å, b = 97.0 Å, c = 81.7 Å and β = 107.9°. The Matthews coefficient is 2.1Å³Da⁻¹ for TTR-DES1, indicating a solvent content of approximately 42%, whereas in the case of TTR-DES2 the Matthews coefficient is 2.6 Å³Da⁻¹ corresponding to a solvent content of nearly 52%.

Structure solution

The program AMoRe (21) was used for computing the molecular replacement solutions for both TTR-DES complexes, using the atomic coordinates of human TTR T119M variant (PDB accession number 1F86), refined at 1.1 Å resolution (22). Calculations including data from 15Å to 3.5Å resolution yielded correct solutions for both the rotation and translation functions. After rigid body fitting the correlation coefficient for the rendered solution of TTR-DES1 was 70.5, while for TTR-DES2 was 63.1 and the corresponding R-factors were 0.34 and 0.36, respectively.
Model Building and crystallographic refinement

In order to reduce systematic errors during refinement, the molecular replacement solutions were improved by a preliminary step of rigid body refinement, which was performed with CNS (23). This program was then used for simulated annealing and subsequent positional and individual temperature factor refinement of the models. The refinement cycles included 95% of the data, while a random sample of 5% of the reflections was used for R_free calculation. After each cycle of automated refinement, the model was inspected and manually adjusted to the computed σ_A-weighted (2Fo – Fc) and (Fo – Fc)-type electron-density maps using the graphic program Turbo-FRODO (24) on a SGI graphic workstation. Water molecules were added manually at the positions of positive peaks (>3σ) on the difference Fourier maps, when good hydrogen bond geometry was available. Several cycles of crystallographic refinement iterated with manual model building were performed until the protein models were completely fitted to the Fourier maps. The difference electron density maps clearly showed, on both binding pockets of the TTR tetramer, positive electron density. The atomic coordinates of the DES molecule taken from the HIC-UP database (25) were used to build a model of the ligand into the density. At that point, refinement of the TTR/DES complexes proceeded using ARP/WARP v.6.0 (26), which makes use of the CCP4 program REFMAC5 (27). After the last round of refinement, the ligand and its symmetry related positions were in good agreement with difference electron density maps. All residues of both complexes were in the allowed regions of the Ramachandran plot, as calculated with PROCHECK (28). The structure was further validated with the program WHAT_CHECK (29). The overall statistics for data collection and refinement are given on Table 1. The coordinates for TTR-DES1 and TTR-DES2 crystallographic models as well as the correspondent structure factors have been deposited at the Brookhaven Protein Databank under accession codes
1TT6 and 1TZ8, respectively.

**Fibril formation and quantitative Thioflavin (ThT) fluorescence assay**

Fibrils were produced by acidification and incubation during 72h at 37°C of solutions containing TTR at the average physiologic concentration (3.6µM). This assay is based in previously described protocols (30,31). Briefly, series of solutions (247.5 µL) containing 7.2µM TTR in 10 mM HEPES buffer, pH 7.5, 100 mM KCl were prepared. Then, 2.5 µL of concentrated stocks (0.72 mM and 1.44mM) of inhibitor (DES or Resveratrol) dissolved in DMSO were added to the TTR solution to achieve the final desired concentrations of inhibitor. DMSO (2.5µL) was added to TTR in order to determine the amount of fibril formation without the inhibitors. The samples were pre-incubated during 30min (37°C) and then were diluted 1:1 with 100 mM Na-Acetate buffer (pH 4.2) containing 100 mM KCl, to yield solutions with 3.6µM TTR at pH 4.4 and different concentrations of inhibitor (0, 3.6 µM and 7.2 µM). Triplicates were set for each concentration of DES. After incubation at 37°C for further 72h, the suspensions were centrifuged at 14000g for 30 min. The pellet was carefully resuspended in 250 µL of a solution containing 50mM phosphate buffer (pH 7.5), 100mM KCl and 10 µM ThT. Fluorescence measurements were recorded in a SPECTRAmax Microplate Spectrofluorometer at 25°C. The suspensions were excited at 440 nm, and the emission intensity at 482 nm, which is characteristic of ThT bound to amyloid fibrils (32), was recorded to measure the amount of fibrillar aggregates present in solution. In order to control the effect of the inhibitors over the ThT fluorescence experiments, 1.25 µL of the inhibitor solution (1.44mM) were added to the suspensions previously incubated without inhibitor. The suspensions were mixed and then excited at 440 nm, being the emission intensity at 482 nm recorded.
RESULTS

Refinement of the crystal structures

Two different crystal forms for the TTR-DES complex were found on the same crystallization drop. The orthorhombic crystal form TTR-DES1, diffracted to 1.8 Å, while diffraction data could be collected to 1.85 Å for the monoclinic crystal, here referred as TTR-DES2. Both structures were refined to crystallographic \( R_{\text{factor}} \) of 18.7% and 19.5% and \( R_{\text{free}} \) of 21.8% and 22.1% for TTR-DES1 and TTR-DES2 respectively. The final models display good stereochemistry and the r.m.s. deviations values for all C\( \alpha \) bond lengths and angle-bonded distances are presented on Table 1. The main-chain dihedral angles for all residues, in both complexes, are on the allowed positions of the Ramachandram plot, which was calculated with PROCHECK (28). In addition to the ligand, glycerol molecules were found in the electron density maps, in close proximity to the binding site. The hydroxyl groups from glycerol are hydrogen bonded to several amino acid side chains, namely Ser112, Ser115, Ser117, and to the main chain carbonyl group of Leu110. A total of 145 and 322 water molecules were positioned, within proper hydrogen bonding distances, for TTR-DES1 and TTR-DES2, respectively. An excess of DES was used during the preparation of the complex and therefore full ligand saturation was considered in all binding sites. In both structure complexes, two symmetry-related ligand positions arise in each binding site due to the two-fold crystallographic symmetry axis that bisects the binding channel. However, in the asymmetric unit of the monoclinic form, one of the binding sites has no two-fold symmetry and the initial orientation of DES was much clear in the electron density maps. Accordingly, the binding of DES where the two-fold symmetry axis was
present was described with a 50% statistical disorder and therefore half occupancy was assigned to those molecules, while full occupancy was assigned for the molecule in the other binding site. The final models for both complexes included not only ligand molecules, but also molecules that were added in the buffers used for complex preparations or crystallization (Table 1). The nine N-terminal residues were not defined in the electron density maps and are thus omitted in the final models of both TTR variants. Similarly, the three terminal residues 125-127 are missing for both models, with exception of Pro125 in monomer C of TTR-DES2. In addition, atoms from the side-chains of a few residues, which were disordered in the final electron density map, were thus excluded from the final model. Alternative conformations were modelled for some residues of both complexes and were refined with the ARP/WARP procedure constraining the sum of both conformations to occupancy of 1.0 (Table 1).

The TTR-DES1 complex

The TTR-DES1 complex crystallized in the orthorombic space group P2_12_12 as most of the reported structures of human TTR and TTR complexes (5,6,9,13,22,33,34). There are two monomers per asymmetric unit, designated by A and B, which form a dimer that associates with another dimer related by a crystallographic two-fold axis, forming a tetramer with two-fold symmetry along the axis of the protein binding channel. Therefore, each binding site consists of a pair of symmetry related monomers, A/A’ or B/B’ (prime specifies a symmetry related monomer or residue), and the observed electron density for the DES molecule is an average of the two symmetry related positions (Fig. 2A). The protein-drug observed interactions were the same for both binding sites. DES is located in the T4 binding channel. However, it is positioned nearly 4 Å deeper, towards the centre of the tetramer, compared to T4 (35). As already
mentioned, in the binding of T4 to the protein, three sets of halogen pockets were reported as establishing important and close contacts between TTR and the hormone. Our molecular model shows that DES distal phenyl group establishes hydrophobic contacts with the surrounding residues Leu110, Leu110’, and Ser117, Ser117’ (Fig. 2A). The ligand also induces a conformational shift of the side chain of Ser117, which has double conformation (Fig. 3A), one of the conformations being responsible for favourable interactions between the protein and the inhibitor. In fact, DES p-hydroxyl group establishes hydrogen bonds to the Oγ atoms of both Ser117, bridging the two dimers and contributing for an overall stabilization of the native tetrameric fold. The second conformation of Ser117 may also contribute to stabilize the tetramer since it forms intersubunit hydrogen bonds between the hydroxyls of the Ser117 from adjacent monomers in the same dimer. This extra intersubunit interaction was also observed in the TTR Thr119Met variant (22), which is known to have a protective effect over the amyloidogenic Val30Met TTR mutation probably due to a more stable native state(31,36). Although DES does not strongly interact with Thr119, an amino acid whose side chain is in the binding channel, the water molecule hydrogen bonded to Thr119 in the TTR crystal structures is displaced upon DES binding. As a result, Thr119 undergoes a conformational change that makes accessible its hydroxyl group to a new hydrogen bond with an ordered water molecule that connects the two symmetry related dimers through the interaction with the main-chain carbonyl group of Asp18’ (Fig. 3C). Additional non-polar contacts are observed for the ethyl groups of DES, which fit tightly in the central binding pockets P2 and P2’, between the side chains of Ala108, Leu17, Leu110 and Thr119. Near the channel entrance, the proximal aromatic ring is oriented between the side chains of Leu17, Ala108 and Lys15 taking advantage of hydrophobic and van der Waals contacts with these residues and mediating interactions between adjacent subunits. In addition,
the p-hydroxyl group participates in hydrogen bonding interactions with the ε-ammonium groups of Lys15, contributing for DES binding affinity to TTR and probably averting the repulsion that may occur between the two positively charged Lys15 from the adjacent dimers (37). Hereafter, we will refer to this mode of binding as binding mode I (BM-I) (Fig 2A).

The TTR-DES2 crystal structure reveals two different binding modes

The monoclinic form of the TTR-DES complex belongs to space group C2, which was previously observed for the Leu55Pro TTR variant and recently for the double mutant TTR-Ala108Tyr/Leu110Glu (38,39). These two structures are characterized by the loss of the β-strand D as result of a shift in the position of residues Glu54/Leu55/His56, which inhibits the formation of hydrogen bonds between chains A and D. On the contrary, the β-strand D is present in our structure and presents a conformation similar to those observed in the structures determined in the P2₁2₁2 space group.

The TTR-DES2 crystal contains a novel organization of the asymmetric unit, which contains four monomers: two monomers (A and C) form a complete binding site, while monomers E and F form one dimer. The two-fold crystallographic axis generates the tetramer AA’-CC’ and an E’F’ dimer that upon a translation along the Z axis forms the EF-E’F’ tetramer (Fig. 4). In this crystallographic structure we observe two binding sites, formed by monomers E/E’ and F/F’ with two-fold symmetry and consequently, the observed ligand electron density represents an average of two symmetry related DES molecules with half occupancy each. In contrast, the ligand positioned in the A/C binding site has no symmetrical distribution of the electron density. In fact, the AA’-CC’ tetramer has dimers composed of monomers related by crystallographic symmetry, which is a feature that had been only observed for the monoclinic crystal structure of
the double mutant TTR-Ala108Tyr/Leu110Glu (39). In this monoclinic crystal form of the complex, TTR-DES2, DES exhibits two binding modes: the BM-I mode, as observed in the orthorhombic crystal structure, and BM-II mode characterized by an even deeper penetration of DES in the TTR binding channel, towards the centre of the molecule.

In this crystal structure and concerning the BM-I mode, the proximal p-hydroxyl group of DES interacts not only with ε-N Lys15 group from one monomer but also with ε-N Lys15, from the other monomer, mediated by a water molecule (Fig. 2B). The later interaction further stabilizes DES in the AC binding pocket. A water molecule, just at the centre of the tetramer, was also observed in this binding mode. Interestingly, it forms a hydrogen bond with the distal hydroxyl groups of the two DES molecules, mediating in this manner an interaction that might promote simultaneous occupation of both binding sites (Fig.3A and Fig.4A). Half occupancy was assigned to this water molecule since it is on a crystallographic two-fold symmetry axis. The molecule present on the E-E’ binding cavity, which we refer to as BM-II mode, was found to be about 2 Å deeper than the observed DES position in BM-I mode. Its p-hydroxyl group was nearly positioned in the region occupied by water molecule present in BM-I, establishing a weak hydrogen bond (3.5 Å) with the hydroxyl group from the neighbouring DES molecule, present in the F-F’ binding site (Fig. 3B and 4B).

The protein-DES interactions in the F-F’ binding site were equivalent to those described in BM-I, apart from the small deviations due to subtle differences in the geometry of the binding channels (Fig 2A and 4B). On the other hand, as was mentioned above, a second binding mode (BM-II) was found for the E-E’ binding site (Fig 2C). As a result of the deeper penetration of DES, Ser117 has single conformation that establishes simultaneous hydrogen bonds with DES and Ser117 residue from the neighbouring monomer. These contacts together with the previously
described interaction between both DES molecules generate a network of H-bond interactions at
the centre of the tetramer, which extends the monomer-monomer and dimer-dimer contacts (Fig
3B). The P3 pocket, as described for T4 binding, is more relevant in this binding mode. The
distal phenyl group of DES participates in a series of hydrophobic contacts with Leu110,
Leu110’, Ser117 and Ser117’, while the ethyl groups fit within the P3 and its symmetry related
pocket P3’, establishing several contacts with the hydrophobic groups of Ala108, Ala109,
Leu110, Thr119 and Ser117. As in BM-I, Thr119 undergoes a ligand-induced conformational
change, leading to a new intersubunit contact (Thr119 OH…H2O…OC Ala18). The hydroxyl
group of DES, which is closer to the channel entry, is unable to establish any polar interaction
with the protein side chains since it is too far from the side chain of Lys15. The proximal
aromatic ring is found among the side chains of Ala108, Leu17 and Thr119 that line the middle
P2 and P2’ pockets.

The main differences between the two binding modes arise from the shift in the position of DES
along the channel. In BM-II mode, DES forms more extensive interactions with TTR in the P3
and P3’innermost pockets where DES fits its ethyl groups, whereas in BM-I mode these groups
are at the middle P2 and P2’ pockets. On the other hand, DES, in BM-II, cannot participate in H-
bonding at the channel entry, while it does so in BM-I mode.

*The crystallographic packing in the monoclinic and the orthorhombic forms of the TTR:DES
complex*

The two crystallographic structures of the TTR-DES complex show very similar polypeptide-
chain conformation, displaying only a significant difference between the FG-loop of the TTR-
DES2 monomers and the same loop in one of the TTR-DES1 monomers. In fact, this loop shows
different conformation in the monomers that form the asymmetric unit of TTR-DES1. Other TTR structures, solved in P2_12_12 space group, quite often show differences in the FG-loops from independent monomers and it was proposed that this occurs due to clashes with residues from symmetry related monomers present in the surrounding area (5,33,34). In the monoclinic form of the complex, TTR-DES2, the novel crystallographic packing allows the same path of the FG-loop for all the monomers in the asymmetric unit cell. An alignment of the individual monomers from other monoclinic structures of wild-type and variant TTRs (8,38,39) also reveals similarity between the FG-loops of all monomers and this fold is identical to the present in monomer A of the orthorhombic TTR structures.

The crystallographic packing observed in the monoclinic and orthorhombic structures is present on Fig.5. The channel axis of both tetramers in the monoclinic form is perpendicular to the crystallographic z-axis while in the orthorhombic lattice, TTR-DES1, is parallel to the z-axis. The tetramers generated by the twofold symmetry in TTR-DES2 are oriented almost perpendicularly relative to each other, forming alternative layers in the crystal that are parallel to the (001) plane and contain only one type of tetramer (AA’-CC’ or EF-E’F’). Interestingly enough, this sublattice arrangement is also observed for the P2_1 crystals of wild-type TTR (8), which contains two independent tetramers in the asymmetric unit (pdb accession code 1ICT). In the orthorhombic form, the tetramers are organized in a more compact structure, with successive layers along the x-axis. The intermolecular space of each layer is filled with the tetramers from the next layer and there are interactions between the C-terminal segments of the α-helices from tetramers that are present in non-consecutive layers. In the TTR-DES2 crystal, both the C-terminal of α-helices and the FG-loops contact with the equivalent fragments, from monomers within the asymmetric unit and symmetry related monomers, forming a cluster of four α-helices.
and four FG loops that establishes important interlayer interactions between the two tetramers. This organization was previously described in the tetragonal lattice of rat TTR and in the wild-type TTR P21 crystals (8,40), however it is not observed in TTRDES1. Additionally, the crystal packing of the TTR-DES2 crystal involves contacts already described for other structures solved in monoclinic space group: contacts between the DE loops, namely hydrogen bonds between the amine groups of His56(E), Gly57(E) and the carboxyl oxygens of Glu62(F’) and hydrogen bonds between the amine group of Glu66(C) and the carbonyl group of Glu62(C’); the main chain oxygen atoms of Gly83(C’ and E) are also hydrogen bonded to the guanidium group of Arg21 (F and A respectively) and finally an hydrogen bond between the side-chains of A-Arg34 and C’-Ser46 that is a significant intralayer interaction in the AA’-CC’sublattice. All these packing interactions are absent from the TTR-DES1 complex.

Effect of DES on amyloid fibril formation

In order to test if DES had any effect as an inhibitor of TTR fibril formation, the amount of fibril formation was monitored in the presence and absence of DES and Resveratrol. Resveratrol is a molecule that shares the stilbene moiety with DES and was reported as an efficient inhibitor of acid-mediated fibril formation (13). Fibril formation was measured by ThT fluorescence given that emission at 482nm is characteristic of ThT bound to amyloid fibrils and is proportional to the amount of fibrils in solution (32). After confirming that there was no quenching of the ThT fluorescence spectra due to the presence of the inhibitors, the amount of fibril formation was calculated as the emission intensity from the protein samples with the inhibitors divided by the value obtained without inhibitor. The results are presented in figure 5 and show that DES at 7.2µM reduces the acid-induced fibril formation below 6% compared to the results in the
absence of inhibitor, being highly effective at this concentration. Unsurprisingly, the extent of fibril formation increases with a decrease in inhibitor concentration. However, DES is still a very good inhibitor at equimolar concentration (3.6µM), reducing the amount of fibril formation to nearly 13%. For both inhibitor concentrations, the results suggest that DES is a slightly more efficient inhibitor of TTR fibril formation than Resveratrol.

DISCUSSION

In this work we observed the binding of DES to TTR in two different modes and no significant variation was observed between the protein conformations on the correspondent binding sites. In fact, an alignment of Cα atoms from the monomers involved in different binding modes did not reveal any significant rms deviations for the residues close to the binding sites. The most relevant differences were observed in relation to the conformations of Ser117 and Lys15. The binding in alternative positions along the length of the TTR channel was also described for other small compounds such as flavones derivatives in human TTR and thyroxine in human and rat TTR (8, 41,42,43). The later complex shows in the channel centre a hydrogen-bonded network that rather resembles DES binding since it includes an interaction via a water molecule of one thyroxine molecule with Ser117, which then links both binding domains via a hydrogen bond with the adjacent Ser117.

DES is a potent competitive inhibitor for thyroid-hormone binding to TTR and this appears to result from the sum of several hydrophobic interactions mediated by the stilbene core and the ethyl groups, as well as hydrogen bonding between its hydroxyl groups and the side chains of Lys15 and Ser117. Studies concerning the binding of T4 to TTR lead to the proposal of a
succession of events, occurring after ligand binding in one site, that force the collapse of the newly occupied site and a consequent opening of the second one, which does not allow for a strong binding of a second T4 molecule (8). DES is less bulky than T4 and the rigidity conferred to the second binding site after the occupation of the first one (44) would not impair the ability of DES to enter the channel. In fact, taking into account the observed interaction between two bound DES molecules, it is reasonable to consider that after the binding of the first DES molecule, the second molecule entering the binding channel is attracted to a deeper position allowing for an interaction between the two DES molecules. Then, the interaction with the first DES molecule might favour the binding of the second one, which points for different mechanism of DES binding relative to the negative cooperativity observed for T4. Furthermore the hydrogen bonds established between the two molecules in the TTR channel will most probably lead a much more stable complex structure. In addition, it is imperative to notice that DES binding mediates several new intersubunit hydrophilic and hydrophobic interactions that will decrease the capacity of the protein to dissociate upon DES binding. In fact, compounds that bind tightly in the TTR binding channel should increase the energetic barrier associated with protein dissociation and a number of small molecules representative of very distinct structural families are emerging as inhibitors of TTR amyloid formation (13,14,45,46). Furthermore, reported data indicates that the cytotoxic species are oligomers, which occur prior to fibril formation (47). Considering that tetramer dissociation occurs prior to amyloid formation, molecules acting as tetramer stabilizers may be very important therapeutic agents. In fact, constructed TTR variants with the monomers or dimers linked by disulphide bridges showed the capacity for amyloid formation only after the addition of β-mercaptoethanol in sufficient amount to break those covalent links (48). Provided that the ability of DES for binding on the protein T4 binding
channel is linked to its capacity to stabilize the tetramer (15), DES is expected to be a good candidate to act as amyloid inhibitor since it links directly both dimers.

The data presented herein shows that using acidic in vitro fibril formation conditions (pH 4.4, 72h, 37°C), DES is indeed an excellent inhibitor of amyloid formation, being even more efficient than Resveratrol. We compared the crystallographic structures of the complexes TTR/DES and TTR/Resveratrol (PDB accession code 1DVSH (13) in order to understand the structural reasons leading to a more efficient inhibition of fibril formation by DES. The ethyl groups only present in our structure bind at the same time either in the middle pockets P2/P2′ (BM-I) or the innermost pockets P3/P3′ (BM-II), certainly mediating extra interactions at the dimer-dimer interface. In contrast, Resveratrol is unable to bind deeply in any of these pockets. At the channel entry, DES is allowed to establish a direct polar interaction with Lys15, while a water molecule connects it to the Lys15 from the adjacent dimer. Instead, Resveratrol has two hydroxyl groups that mediate this dimer-dimer interaction through water molecules. However, this interaction was not persistently found in all DES binding sites since in BM-II mode, DES penetrates deeper towards the center of the molecule impairing any interactions at the channel entry. Nevertheless, if we consider the results from the fibril formation tests, DES binding has an overall stabilizing effect over the TTR quaternary structure that is somewhat superior to the effect of Resveratrol binding and this is no doubt due to the ethyl groups, forming two arms that bind strongly in the two dimers. Furthermore, DES has the unique feature of interaction between the molecules present in adjacent binding sites that contributes for the formation of a network of hydrogen bonds, which brings together the four monomers and most likely reduces their tendency to dissociate.
Although DES seems to be a good amyloid inhibitor, its use as therapeutic agent is hampered by its potent estrogen biological activity (49) as well as its ability for binding other proteins, such as the plasma sex-hormone binding globulin, the human estrogen receptor α and estrogen-related receptor γ (50,51,52). Nonetheless, DES is a promising template for the design of amyloid inhibitors and the structural information about the TTR-DES complex will be used to assist in the design of DES derivatives with increased affinity and selectivity to TTR.

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FIGURE LEGENDS

FIG. 1. The structures of compounds diethylstilbestrol and resveratrol

FIG. 2. Interactions of DES with TTR in the two crystal forms. The side chains of the protein residues that interact with the ligand are shown. Hydrogen bonds are represented as dashed lines. DES is shown in ball-and-stick representation. The $|F_o|-|F_c|$ omit map correspondent to the position of DES is contoured at 2.5σ (A) Binding Mode-I (BM-I) for the orthorhombic crystal. (B) BM-I present in the AC binding site of the monoclinic crystal. (C) In Binding Mode II (BM-II) a shift of about 2 Å towards the center of the channel is observed for the DES position.

FIG. 3. A close view of the interactions present in the TTR-DES complexes, resulting from ligand-induced conformational changes in the side chains of Ser117 and Thr119. (A) At the center of the binding channel, a double conformation is observed for Ser117 in BM-I. In one conformation the side chain of Ser117 interacts with the equivalent residue from the adjacent monomer, while in the other it forms hydrogen bonds with DES. For the monoclinic crystal, this set of connections is increased by the interaction mediated by a water molecule between the two DES molecules. (B) In the second binding mode (BM-II), Ser117 is in a single conformation and interacts simultaneously with DES and with the adjacent monomer. (C) In both cases, extra dimer-dimer interactions appear as a result of a conformational change in Thr119, which allows for an interaction, mediated by a water molecule between the hydroxyl group and the main-chain carbonyl group of Asp18.
FIG. 4. In the monoclinic TTR-DES2 crystal there are four monomers in the asymmetric unit (shown in blue). Two tetramers with different symmetry were observed. (A) The tetramer AA´-CC´(A´ and C´ are symmetry related monomers) has a water molecule at the center of the binding channel which mediates an interaction between the DES molecules present in both binding sites (B) The dimer EF is assembled in a tetramer EF-E´F´ with a two-fold axis running along the binding channel. Different binding modes are observed in the EE´ and FF´ binding sites (E´ and F´ are symmetry related monomers). In this case, a hydrogen bond is formed between the two DES molecules.

FIG. 5. The crystallographic packing observed for both forms of the TTR-DES complex. (A) The packing diagram for TTR-DES2 shows alternative layers along the Z-axis formed by tetramers AA´-CC´ and EF-E´F´. Monomers A, C, E and F are colored blue, grey, red and green, respectively. (B) The orthorhombic crystal packing of TTR-DES1. Monomers A and B are colored blue and green respectively.

FIG. 6. Extent of TTR fibril formation in the presence of DES (white bars) and Resveratrol (grey bars). Fibrils were produced by acidification and monitored by the quantitative ThT fluorescence assay. The emission intensity in the absence of inhibitor was assigned to be 100%, and the amount of fibril formation for two concentrations of the inhibitor (3.6µM or 7.2µM) is represented in relation to the amount produced in the absence of the inhibitor.
### Table 1

Summary of crystallographic analysis and refinement statistics for both DES-TTR complexes

|                        | WT-DES1            | WT-DES2            |
|------------------------|--------------------|--------------------|
| Resolution Range (Å)   | 42.69-1.8          | 56.77-1.85         |
| Space Group            | P2₁,2,2            | C2                 |
| Unit Cell Dimensions   | a= 42.3 b=85.4 c=63.4  | a=74.3 b= 97.0 c= 81.7  |
|                        | α=β=γ= 90         | α=γ= 90 β=107.9   |
| Number of observations (unique) | 21979         | 46580              |
| Multiplicity (overall) | 4.6               | 3.0                |
| Rmerge<sup>a</sup> (overall/outer shell) | 6.2 / 35.0  | 6.4 / 26.7         |
| Completeness (%)       | 99.9 / 99.6       | 99.4 / 98.9        |
| I/σ(I) (overall/outer shell) | 9.3 / 2.1   | 9.8 / 2.5          |

#### Structure Refinement

R<sub>factor</sub>/<R<sub>free</sub> (%) | 18.7 / 21.8  | 19.5 / 22.1  |
N<sub>o</sub> of unique reflections (working/test set) | 21724 / 1120  | 46540 / 2314  |
Water molecules | 145  | 322  |
Additional small molecules | Glycerol/sulfate  | Glycerol/DMSO acetate/carbonate  |
Residues with alternate conformation (A,B,C,E and F refer to different monomers in the asymmetric unit) | Ser115A,Ser117A, Ser85B,Ser115B, Ser117B | Cys10A, Ser115A, Ser117A, Ser115C, Ser117C, Ser23E, Ile107E, Ser115E, Ser115F  |
Total number of atoms | 1909 | 3849  |
Number of Protein atoms | 1712 | 3437  |
Average overall B-factor (Å<sup>2</sup>) | 18.416  | 27.134  |
Average Protein B-factor (Å<sup>2</sup>) | 16.881  | 25.778  |
Average main-chain B-factor (Å<sup>2</sup>) | 16.259  | 25.134  |
Average side-chain B-factor (Å<sup>2</sup>) | 17.603  | 26.520  |
Average water B-factor (Å<sup>2</sup>) | 32.615  | 39.174  |
Average ligand B-factor (Å<sup>2</sup>) | 26.794  | 33.588  |
R.m.s. bonded B’s (Å<sup>2</sup>) | 1.289  | 1.418  |
R.m.s. deviations from ideal values
  Bonds (Å) | 0.008  | 0.011  |
  Angles (º) | 1.289  | 1.418  |
Ramachandran Plot statistics
  Most favoured region (%) | 91.5  | 91.8  |
  Additionally allowed region (%) | 8.5  | 7.7  |
  Generously allowed region (%) | 0  | 0.5  |

<sup>a</sup>R<sub>merge</sub> = ΣₙΣᵢ |Iᵢₙ - <Iᵢₙ> / ΣₙΣᵢ <Iᵢₙ>, where Iᵢₙ is the observed intensity of the ith measurement of reflection (h), including symmetry related ones, and <Iᵢₙ> is the mean intensity of the i observations of reflection h over all measurements of Iᵢₙ.

<sup>b</sup>R<sub>factor</sub> = Σ|Fₐ| - |Fₐ| / Σ |Fₐ| where |Fₐ| and |Fₐ| are observed and calculated structure factor amplitudes, respectively

<sup>c</sup>R<sub>free</sub> is the cross-validation R-factor computed for a randomly chosen subset of 5% of the total number of reflections, which were not used during refinement.
FIGURE 1

DES  Resveratrol
FIGURE 2
FIGURE 3
FIGURE 4
FIGURE 6
The crystal structure of transthyretin in complex with diethylstilbestrol: A promising template for the design of amyloid inhibitors
Eurico Morais-de-Sá, Pedro J. B. Pereira, Maria J. Saraiva and Ana M. Damas

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