Transthyretin complexes with curcumin and bromo-estradiol: Evaluation of solubilizing multicomponent mixtures.

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TITLE RUNNING HEAD: TTR complexed with hydrophobic compounds

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Abstract

Crystallographic structure determination of protein-ligand complexes of transthyretin (TTR) have been hindered by the low affinity of many compounds that bind to the central cavity of the tetramer. Since crystallization trials are carried out at protein and ligand concentration that approach the millimolar range, low affinity is less of a problem than the poor solubility of many compounds that have been shown to inhibit amyloid fibril formation. To achieve complete occupancy in co-crystallization experiments, the minimal requirement is one ligand for each of the two sites within the TTR tetramer. Here we present a new strategy for the co-crystallization of TTR using high molecular weight polyethylene glycol instead of high ionic strength precipitants, with ligands solubilized in multicomponent mixtures of compounds. This strategy is applied to the crystallization of TTR complexes with curcumin and 16α-bromo-estradiol and report the crystal structures with these compounds and with the ferulic acid the result of curcumin degradation.

Introduction

Transthyretin (TTR) is so named because of its ability to transport the thyroid hormone thyroxine (T_4) and retinol-binding protein bound to retinol in serum and cerebrospinal fluid. It is secreted by the liver into the blood, by the choroid plexus into the cerebrospinal fluid and by the retinal pigment epithelium. TTR stabilizers have been studied by X-ray crystallography for the development of anti-amyloidogenic drugs. Many of the amyloid inhibitors have a hydrophobic character and micromolar affinity. The reported K_a value of wild-type TTR for T_4 is only 10.6×10^6/M. Patients that have inherited higher affinity transporters are subject to euthyroid hyperthyroxinemia.[1] Interesting targets for TTR amyloidosis drug development include several natural compounds. Polyphenols have received attention for their health benefits, including the capacity to stabilize TTR tetramer structure. The most studied compounds were resveratrol,[2] a component of red grapes, epigallocatechin-3-gallate from green tea,[3] genistein[4] and related isoflavones found in soy, and curcumin from *Curcuma longa*, a member of the ginger family.[5] Curcumin has been shown to be of therapeutic value in preventing the formation and extension of β-amyloid fibrils.[6] It has been reported that curcumin, can bind to TTR and displace ANS at pH 7.2.[7] At this pH curcumin is unstable, so the molecule that binds to TTR could be one of curcumin's degradation products rather than curcumin itself (Scheme 1).[8]

The methods used in structural studies of wild-type TTR, its amyloidogenic variants and their complexes have varied widely. Both co-crystallization and soaking of crystals have been exploited successfully. TTR crystallizes in the same polymorph under a wide range of crystallization conditions. High ionic strength precipitants such as 1.4 M sodium citrate,[9] or 2 M ammonium sulphate,[10] have been used for most crystallizations of wild-type transthyretin. Small molecular weight polyethylene glycol (PEG) has also been used in the crystallization of wild-type TTR and of the amyloidogenic V30M variant.[11] High molecular weight PEG (15% (w/v) PEG 2,000 monomethyl ether) has been used to obtain crystals of the I84S mutant of TTR in complex with either CHF5074 or diflunisal at acidic pH.[12]

In this work we evaluate the effectiveness of high molecular weight PEG, using conditions from a standard crystallization kit for the crystallization of wild-type TTR (Figure 1) and its co-crystallization with a large variety of compounds with a particular focus on hydrophobic ligands, to verify the effectiveness of multicomponent mixtures designed for co-crystallization of low affinity leads that could results from screening of a fragment library.[13]

Curcumin and 1,3,5(10)-estratrien-16α-bromo-3,17β-diol (16α-bromo-estradiol; Scheme 1), a derivative of

estriol with a bromine instead of a hydroxyl at the 16α position (Scheme 1) were chosen for the test study. The ability of curcumin to inhibit TTR amyloidogenesis has been reported,[7] although to date no structural data is available to describe its mode of binding in complex with TTR. Despite the multiple biological activities attributed to curcumin,[14] to date no structure has been deposited in the Protein Data Bank (wwPDB),[15] probably because of its poor water solubility have prevented crystallization studies. The possible binding of 17β-estradiol to TTR has not been analysed before although this steroid up-regulates TTR expression[16] providing protection against Alzheimer's disease by binding to β-amyloid oligomers.[17] The brominated form of estradiol was chosen because its binding can be uniquely identified by its electron rich substituent directly from the electron density maps.

Here we report in detail the solubilization of the ligands at high concentration, the co-crystallization, cryoprotection steps and discuss the results obtained by X-ray crystallography describing the interaction of each ligand with its target.

Methods

Crystallization Experiments.

CrysChem sitting drop vapour diffusion plates were used for setting up the crystallization experiments. Lyophilized human TTR (Calbiochem) was dissolved in 100 µL 0.02% (w/v) NaN_3 and dialysed against 0.1 M NaCl, 50 mM sodium acetate, pH 5.5, overnight. The drops consisting of 1 µL protein solution and 1 µL precipitant solution were added on the pedestal and equilibrated against 500 µL reservoir solution in a cooled incubator at 20°C. To adjust the crystallization conditions from one TTR complex to another standard conditions from the Stura Footprint Screen[18] (Molecular Dimensions Ltd., U.K.) were mixed together and used to set up just three trials. The reservoir solution for the three trial drops contained 70-80% of (30% PEG 4000, 0.2 M imidazole malate, pH 6.0) with 20-30% of (18% monomethyl PEG 2,000 (MPEG-2K) 0.1M sodium cacodylate, pH 6.5) or (12% MPEG-5K, 0.1 M sodium acetate, pH 5.5) or water. The use of "reverse screening" where standard "working solutions" are mixed together to achieve better accuracy in crystallization experiments has been previously described.[19] Whenever crystals were not obtained spontaneously, the drops were systematically streak seeded.[20] If crystals were not obtained at this stage in any of the three drops, the precipitating power of the reservoir solution was boosted by the addition of a solution composed 50 µL of 5 M NaCl. If after 3 hours crystals did not appear, the seeding was repeated. For more reluctant co-crystallization situations, a booster solution consisting of 50 µL of 5 M NaCl, 0.2 M glacial acetic acid was used repeatedly. After each intervention, the drops were immediately streak seeded. This procedure was followed to grow crystals of TTR in the presence of NaOH treated gallic acid.

Phase separation and precipitation.

Compounds were dissolved in DMSO, a sample drop was observed under the microscope for clarity. For inhibitors poorly soluble or insoluble in DMSO, a new inhibitor solution was prepared by dissolving the ligand in various DMSO/dioxane/ethylene glycol mixes.[13]

DMSO/dioxane solubilized ligands in complex with TTR were tested for compatibility with high salt co-crystallization. The screening was carried out under the microscope to check for phase separation, a common occurrence, whereby the ligand segregates away from the protein. Predominately, hydrophobic ligands prefer an organic rich/salt poor phase while the protein tends to concentrate in the salt rich phase (Figure 2). Phase

separation can be exploited in crystallization trials, but with caution since the ligand-protein stoichiometric ratios in either phase becomes an undefined parameter and there is a strong risk to produce only crystals of the ligand-free protein.

Precipitation and crystallization of the ligand was monitored under the microscope to select the best solubilizing mix, aiming for a 10-30 mM ligand concentration. The solubilized ligand was mixed with the protein in a 1:10 ratio (0.3 μL ligand – 3 μL TTR). The ligand-protein solution was then added onto the pedestal of the CrysChem sitting drop plate, checked for the presence of precipitation or ligand crystallization, before and after, addition of the reservoir solution. After addition of the reservoir solution to the drop, different amounts of solubilizing mixes were added into reservoir to allow for proper equilibration, since the mixes are hygroscopic. This precaution prevents drop expansion.[21]

Ligand solubilization and pH.

The experiments with curcumin were carried out at different pH because this ligand exists in multiple tautomeric forms. It has been suggested that curcumin-TTR binding is pH dependent because of its keto-enol tautomerization.[7] Irrespective of biochemical considerations, pH is an important parameter for ligand solubilization that should be considered in combination with the use of the multicomponent solubilizing mixes.

Crystallization of 16α-bromo-estradiol-TTR complex

The TTR-16α-bromo-estradiol complex was prepared from TTR at 5 mg/ml and 10 mM 16α-bromo-estradiol dissolved in DMSO, in a volumetric ratio of 6:1. Crystals were obtained from 70% of working solution A1 (WSA1: 30% PEG-4K, 0.2 M imidazole malate, pH 6.0) and 30% of either working solution B1 (WSB1: 18% MPEG-2K, 0.1 M sodium cacodylate, pH 6.5) or B2 (WSB2: 12% MPEG-5K, 0.1 M sodium acetate, pH 5.5). The two solutions were thoroughly mixed because of the high viscosity. To stimulate crystal growth, 0.05 ml of a booster solution (5 M NaCl, 0.2 M glacial acetic acid) was added to the 0.5 ml reservoir. The drops were immediately streak seeded. A single boost was sufficient to grow large crystals for X-ray diffraction.

The experiment was repeated with the same concentration of 16α-bromo-estradiol dissolved in dioxane. The reservoir solution contained 83% WSA1 and 17% of dioxane. This test showed that dioxane acts as a co-precipitant. The working solution A1 was replaced A2 with a lower PEG concentration (WSA2: 8% PEG-4K, 0.2 M imidazole malate, pH 6.0) keeping the dioxane at 17%. Crystals grew spontaneously. Other combinations of working solutions were sampled to improve crystal quality and explore a wider range of

crystallization space. These included reservoir solution composed of 58% WSA1 with 25% WSB2 and 17% dioxane with streak seeding. The reservoir for the ammonium sulphate conditions used consisted of 83% working solution C1 (WSC1: 2.0 M ammonium sulphate, 0.15 M sodium citrate, pH 5.5) and 17% dioxane. Crystals nucleated spontaneously. The reservoir solution for the low molecular weight PEG tests, contained 50% of working solution D1 (WSD1: 42% PEG-600, 0.2 M imidazole malate, pH 5.5) with 50% dioxane. The drop required streak seeding. Crystals were also obtained with higher concentrations of dioxane in the reservoir (40% WSD1 and 60% dioxane). The drop was streak seeded.

Turbidimetric assays for 16α-bromo-estradiol were performed following the procedure previously described[10] with the same wild-type TTR purchased from Calbiochem as in the crystallization trials.

Crystallization of TTR with Curcumin

Curcumin was dissolved in DMSO to make a 30 mM solution. For crystallization protein-ligand solutions were mixed with TTR at 5 mg/ml and curcumin at 10 and 1 mM. The experiments were carried out at pH >7.2 since the interaction of curcumin with TTR curcumin may be pH dependent. In a study of the displacement of ANS by curcumin it was found a decrease in fluorescence at pH 7.2 but not pH 4.3, the pH used for the turbidimetric assays.[7] All the various crystallization precipitants were tried. Curcumin degrades at alkaline pH.[22] To prepare a sample of degraded curcumin, 50 μL of the 30 mM DMSO solubilized compound was treated with NaOH for 30 min at 37 °C. The degradation of the curcumin was monitored by following the change in color from orange-red to pale brown. Crystallization trials were set up with a solution of TTR at 5 mg/ml and the degraded ligand in a volumetric ratio of 10:1.

Cryoprotectant composition and ligand exchange.

Soaking and ligand exchange experiments differ from the standard procedure for cryoprotection only in the time span in which the crystals remain in the cryosolution and the presence of high ligand concentration. The ligand is first dissolved in DMSO, DMSO/dioxane solution, typically 30 - 50 mM, 0.5 - 1 μL of the ligand solution is added to the 6 μL prepared final cryosolution and mixed thoroughly. After ensuring that the ligand does not precipitate or crystallize, the protein crystal is added and soaked for periods that range from 5 - 30 minutes. Because TTR ligands have affinities in the high micromolar range, to avoid the possibility of the ligand washing out of the crystals during the brief cryoprotection step, the final cryosolution always contained

ligand.

The ligand solubilization multi-component mixes[13] were also used during the cryoprotectant soaking step prepared in the same manner as previously described for CryoProtX (Molecular Dimensions, Ltd).[23] In brief: 40 µL from the solubilization multi-component mixes, 10 µL of buffer at 1 M, and 50 µL of the precipitant at a concentration twice that of the crystallization precipitant were mixed together to obtain 100 µL solution. A 6 µL drop of the final cryosolution was placed on a micro-bridge in an XRL plate with water in the reservoir to maintain moisture. The crystals were harvested from the sitting drop plates with a cryo-loop and immediately bathed in the solution on the micro-bridge, swished around to remove the mother liquor around the crystal. The crystals were then retrieved from the cryoprotectant solution with the same cryo-loop and plunged in liquid nitrogen in ESRF pucks which are then stored in a Dewar.

Data Collection and Structure Determination.

About 200 samples were tested at synchrotron facilities, namely at the ESRF (beam lines ID23-1 and ID23-2) in Grenoble and on beam line Proxima-2 at the Soleil storage ring in Saclay. Data processing was carried out using the automated system available at the synchrotron facilities[24] or on the laboratory computers using XDS[25] with the "xdsme" script. The structures were solved by rigid body refinement using REFMAC5[26] starting with a TTR model without inhibitor. The electron density maps were viewed in COOT[27] and the inhibitor built using the monomer library sketcher from CCP4 program suite[28] was placed in the difference electron density. The structure was subjected to at least three cycles of rebuilding and refinement with REFMAC5[26] and phenix.refine.[29]

Results

Crystallization

Crystals of TTR without any ligand were obtained from various conditions from the Stura Footprint Screen[18] (Molecular Dimensions, Ltd.). These include 2.0 M ammonium sulfate, 0.15 M sodium citrate, pH 5.5; 45% (w/v) MPEG-2K, 0.1 M sodium acetate, pH 5.5; 30% (v/v) PEG-4K, 0.2 M imidazole malate, pH 6.0 and 22.5% (w/v) MPEG-5K, 0.1 M ammonium acetate, pH 4.5 (Figure 1). Crystallization experiments of TTR-ligand complexes gave similar results but with reduced nucleation. In some cases, ligand precipitation was observed when the DMSO solubilized ligand was added in the volumetric ratio 1:10 to the protein at 10 mg/ml. The crystallized or precipitated ligand redissolved on storage or as a result of dilution with the reservoir solution when the vapor diffusion experiment was set up. Dioxane/DMSO mixtures were used for ligand solubilization when the precipitated ligand persisted. As an alternative, the ligand was solubilized in one of the solubilizing cryomixes (SM1-6).[13] As expected the cryomixes reduced nucleation due to the presence of glycerol and other diols.[21] These compounds may act directly on the protein by reducing its propensity to form water mediated lattice interactions. By increasing the protein concentration the nucleation and growth parameters can be recovered. In vapor diffusion experiments, the diols also act indirectly because of their hygroscopicity if there is a higher diol concentration in the drops compared to the reservoir. The addition of the booster solution, consisting of NaCl and acetic acid, to the reservoir after the initial equilibration increases the effective vapor withdrawing power of the reservoir and acts to lower the pH. The disequilibrium in the protein-precipitant drops was effectively countered by this approach when it was applied to drops that had remained clear during the entire crystallization process.

Curcumin-TTR co-crystals.

Crystal of TTR with curcumin were obtained with 5 mg/ml TTR with 1 mM curcumin in DMSO. The reservoir consisted of 45% MPEG-2K, 0.1 M sodium cacodylate, pH 7.5. Crystals nucleated spontaneously and the lightly tinted crystals indicate that curcumin might be bound to TTR in the lattice (Figure 2E). Curcumin can decompose rapidly when exposed to sunlight or incubated in neutral-basic pH conditions at 37 °C, to yield trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal, ferulic aldehyde, ferulic acid, feruloyl methane or vanillin.[8] Crystals were obtained under a large range of conditions, without particular precaution to

prevent degradation during crystallization, to allow the binding of either curcumin or any of its degradation to TTR. The deep orange color of certain crystals obtained suggests that the lattice may contain the intact basic form of curcumin.[22] By analyzing the crystallographic data, the structures should be able to reveal which product is the effective ligand of TTR.

Analysis of the TTR-curcumin crystals

The ability to achieve high concentrations of curcumin in an aqueous solution has allowed us to determine the fate of this natural occurring compound in crystallization trials with TTR at pH 7.4, at lower and higher pH under a wide range of conditions. A total of seventeen crystals grown under different conditions were soaked in cryoprotectant with curcumin and others without. Data was collected from all of these and the electron density for each compared. There are variations from crystal to crystal. Without the addition of curcumin during the cryosoak, the degradation products predominate. The co-crystallization experiments have been complemented by soaking studies inspired by studies in solution that show the displacement of ANS by curcumin by monitoring the decrease in fluorescence that occurs at pH 7.2 but not pH 4.3.[7] The results obtained show that: (1) intact curcumin binds to TTR; (2) degradation products such as either ferulic acid or feruloylmethane might be found in older co-crystals (Figure 3A&B). (3) Ligand exchange can be achieved rapidly in the crystalline state (Figure 3C&D). These results are reported below.

Structure analysis of the TTR-curcumin/ferulic acid crystals

Six co-crystals obtained in the presence of curcumin, were briefly transferred to a cryosolution without any ligand. The data set showing the highest degree of degradation was deposited to the PDB (code: 4PME; Table 1). The elapse time between setting up the crystals and flash freezing was 20 days at pH 7.5. The electron density maps for all six crystals have been analysed. This was sufficient for some ligand degradation to occur. Analysis of the X-ray data show that the crystals belong to the orthorhombic space group $P2_12_12$ with cell parameters a = 43.0 Å, b = 84.7 Å, c = 64.1 Å with a dimer in the asymmetric unit. The typical TTR tetramer is preserved and the protein is mostly ordered although many residues have alternative side chain orientations and certain stretches adopt alternative backbone conformations. Small backbone shifts are found in the 90-93 and notable disorder characterizes the 97-104 stretch with probably more than two alternative backbone conformations. Only two are modeled in PDB entry 4PME since the electron density does not allow a clear

interpretation of the region. The curcumin ligand stretches from Ser^{117} towards the exit of the ligand binding tunnel past Lys^{15} and beyond Val^{121} and Thr^{123} all the way to Pro^{24}. Binding site B is clearly occupied by ferulic acid, a curcumin degradation product. The acidic group of this ligand positioned 2.7 and 3.0 Å from Lys^{15} (Figure 4). A second molecule of ferulic acid in a dual conformation might be bound in proximity of Arg^{21}, a residue which forms part of the binding site of epigallocatechin gallate.[3]

Structure analysis of the TTR-curcumin crystals

Co-crystals obtained in the presence of curcumin, were briefly transferred to a cryosolution containing 1.5 mM curcumin to replenish the degraded ligand (PDB code: 4PMF; Table 1). The data analysis show that the crystals belong to the orthorhombic space group $P2_12_12$ with cell parameters a = 43.4 Å, b = 85.5 Å, c = 64.2 Å with a dimer in the asymmetric unit. The crystals soaked in extra curcumin are similar with cell parameters with a small, 1 Å variation in the *b* cell parameter (b = 85.5 *vs.* 84.7 Å) superimposition of the two structures shows that there is a small variation in the tetrameric assembly. The molecules that constitute the tetramer are not perfectly identical with dual side chain and backbone. The problem of weak ligand affinity common to all TTR ligand poses a new problem in the interpretation of the position of curcumin, as a choice must be made at the branch point in the density, whether to place the extension of the molecule towards the top or the bottom (Figure 3B top; cyan). This problem does not affect ferulic acid (Figure 3B top; magenta) that remains centered in the cavity, although the density is still the superposition of the ligand and its symmetric so that ligand occupancy cannot exceed 0.5. This superposition of a non-symmetric ligand with its symmetry related creates an ambiguity in determining position of the ligand. The ambiguity ceases after the branch point, but each branch can be only half occupied with correspondingly weaker electron density (Figure 3B top; cyan). Water molecules fill the void in the unoccupied branch. As a result of degradation, the electron density for the ligand is weakened while that for the water molecules is strengthened. The electron density map, for the curcumin soaked crystal (Figure 3B bottom; PDB entry 4PMF) shows gaps in the electron density at a position that suggests that curcumin might be replaced to some extent by its breakdown product, vanillin. The presence of this degradation product is confirmed by analyzing other crystal structures obtained from crystals grown with other strategies. The positioning of the curcumin in other complexes grown at different pH is similar, but not identical. Positional variations could also explain why overall the electron density for the curcumin remains disappointing.

Structure Analysis of the 16α-bromo-estradiol-TTR complex

Crystals of the TTR-16α-bromo-estradiol complex were obtained in all the crystallization trials tested. Different strategies were tested to dissolve this ligand: DMSO, DMSO/dioxane mix, dioxane and cryosolutions SM(1-6) only. The drops do not appear and remain clear in all crystal growth experiments with ligands solubilized in DMSO alone (Figure 2A shows ligand crystallization; see C and D for phase separation) but require DMSO as part of the mixture. In the crystallization tests in which dioxane is used for ligand solubilization, crystals grow spontaneously without streak seeding. This confirms that in the crystallization process dioxane acts as a solvent for the ligand and as a co-precipitant agent for TTR.

Irrespectively of the precipitant used in the crystallization, crystals of the TTR tetramer have a strong tendency to grow in the orthorhombic space group $P2_12_12$ with cell parameters a = 43.2±1.8 Å, b = 85.4±2.4 Å, c = 64.4±2.5 Å with a dimer in the asymmetric unit. Crystals of the 16α-bromo-estradiol are within these parameters a = 43.2 Å, b = 86.1 Å, c = 63.4 Å (PDB entry: 4PM1; Table 1). Data has been collected to 1.23 Å resolution and refined to R_{value}=17.7% R_{free}=21.1%. The ligand lies on the two-fold crystallographic Z axis that positions molecule A in front of molecule A' and molecule B in front of B' in the cavity created by the formation of the tetramer (Figure 5A). The ligand bound to molecule A is not positioned in the same manner as that bound to molecule B (Figure 5B). The electron density for the 16α-bromo-estradiol is easily interpreted because of the electron-rich bromine in the 16α-position on the estradiol that defines uniquely how the steroid should be positioned. The bromine atom is bound in the halogen-binding pocket between Thr^{119} and Ser^{117} at a distance compatible with the formation of halogen or X-bonds[29] to these two residues.

Turbidimetric assays with 16α-bromo-estradiol, not fully dissolved in DMSO/water, following the procedure previously described[10] showed a 40% inhibition for this ligand.

Ligand exchange in co-crystals

Crystals of the TTR-16α-bromo-estradiol complex were soaked in 1.5 mM curcumin in an experiment equivalent to that used to replenish degraded curcumin. The resultant electron density obtained from the processed data can be interpreted only by a superimposition of 16α-bromo-estradiol and curcumin (Figure 3C&D). The ability to achieve high ligand concentrations in the SM1-6 solutions[13] allows for the exchange of 16α-bromo-estradiol by curcumin.

Evaluation of the solubilizing solutions as cryoprotectants

All the solubilizing solutions SM1-6[13] were tested as cryoprotectants on more than one hundred samples without problems of crystals dissolving and without the formation of ice rings or shadows on any of the X-ray diffraction images. Data sets collected included several of the colored co-crystals obtained in the presence of NaOH treated gallic acid (Figure 2F). The electron density maps from these were carefully inspected, but none showed the presence of any ligand in the main ligand binding tunnel or elsewhere. The color clearly indicates the presence of a ligand, but no specific site could be identified.

Discussion

The main biotechnological problem addressed in this work is that of low affinity and poor water solubility that poses greater difficulties for the resolution of crystals structures compared to biological tests where it is possible to work at lower ligand concentrations. By using a set of mixed solvents that do not denature proteins but efficiently solubilize the ligands we have obtained the first crystal structure of a protein complex with curcumin. The main contribution is given by combining dioxane and DMSO which allows for greater overall concentrations of organic solvents that permissible with DMSO alone without disrupting the integrity of the protein fold.[30] Dioxane complements DMSO, but does not substitute for DMSO.[13] Curcumin is completely soluble in DMSO and not in dioxane, but the combination of the two solvents has allowed the preparation of protein-ligand solutions suitable for crystallization. In the steroid-curcumin exchange experiment, the effect of the addition of other components from the diol family that belong to a different selectivity classes comes into play. Both ligands need to be soluble in the soaking solution for the exchange to take place rapidly. The ability to exchange ligands is important when crystals grow better with one ligands than with another one. The reason why curcumin was soaked into the larger crystals of 16α-bromo-estradiol rather than the other way round, was to ensure that even when large crystals are used exchange still takes place readily.

We exploited the color of curcumin to guide the crystallization looking for conditions where the crystals are richer in color compared to the background color of the surrounding solution. We obtained suitable conditions with both curcumin and the alkali treated gallic acid (Figure 2E-F). However, most TTR crystals grown in the presence of curcumin did not show color more intense than the background of the surrounding mother liquor, probably because of the pH. Curcumin is a yellow powder that can change color in aqueous solutions at different pH. At very low pH (<1) curcumin is predominantly protonated (H_4A^+) giving a red color, in the pH range 1-7 the color turns to yellow characteristic of the neutral form (H_3A) while at basic pH (>7.5) the aqueous solution displays an orange red color corresponding to the HA^- and HA^{2-} forms.[22] The pH at which curcumin binds to TTR[7] is also the pH where it starts to degrade.[31] Since the degradation products of curcumin do not maintain the color, the color of the crystals can give an indication to which ligand is preferred by the protein. Although the soaking experiments show that intact curcumin can bind, crystals obtained without the soaking step indicate also the presence and probable predominance of the uncolored ferulic acid after one or more days of storage of the crystals in a cooled incubator at 20°C. Even the crystals that were replenished with

curcumin for 20 minutes, the ligand electron density remains at a low level. This is typical for many ligands bound to TTR and cannot be used to evaluate curcumin degradation. However, the lack of continuity at a certain level of electron density in the TTR-curcumin map is suspect and could suggests that degradation fragments as small as vanillin are produced.[31]

From purely drug design considerations, both the TTR-16α-bromo estradiol and curcumin complex crystal structures add to our current knowledge. Curcumin because of the extension of the binding site and 16α-bromo estradiol because the bromine at this halogen binding site is rare but not unique. Compounds with bromines have been reported in complex with TTR in several crystal structures (PDB ID: 2QGD;[32][33] 3P3S;[33] 3CN1, 3CN2, 3CN3, 3CN4;[34] 3ESP;[35] 4F18[36]). However, in all these cases the bromine atoms are in proximity of Lys^{15} while the bromine in the case of 16α-bromo-estradiol is positioned between Thr^{119} and Ser^{117}. This halogen binding pocket is used by by iodine in thyroxine[37] (Figure 5D&H) and by chlorine in several structures (Figure 5C&G). In the TTR complex with 3',5'-dibromo-2',4,4',6-tetrahydroxyaurone, the bromine atoms can occupy either the halogen pocket close Lys^{15} or the same pocket used by 16α-bromo-estradiol between Thr^{119} and Ser^{117} also compatible with the formation of an X-bond[38].

The methodology developed in the course of this work with the ability to achieve higher water solubility for a large number low affinity hydrophobic ligands should help make the transition for a lead to potentially useful molecules not only for TTR, but also for other proteins. Although we have currently applied the strategy of the mixed solvents only to co-crystallization and crystal soaking, there is a need to adapt the procedure for other biological applications.

In the case of TTR, we obtained only a 40% inhibition in the turbidimetric assay[10] with 16α-bromo estradiol, lower than for many other compounds. The result is consistent with the X-ray results and encouraging given that the steroid was not sufficiently solubilized in DMSO/water to allow TTR to be fully saturated by the steroid. This shows a need to apply the methodology of protein compatible mixed solvents[13] to other biological procedures, such as turbidimetric tests. The turbidimetric tests measure acid-induced amyloidosis[2]. Curcumin failed to prevent the acid induced aggregation of TTR despite its high affinity binding and stabilization of the TTR tetramer, because curcumin binding is pH dependent.[7] In our solubilization studies with curcumin, we found that as we varied the pH from acidic to basic, not only did we get color changes, we were also better able to solubilize the ligand. Among the various pH variation tests carried out, TTR crystals grown with curcumin at pH 7.5 were transferred briefly to a cryoprotectant solution

at pH 5.5. Even a short lived pH change could have allowed the ligand to escape into the crystal's solvent channels, but curcumin was retained at the TTR binding site. Low aqueous solubility can affect the availability of ligand for binding, but it can also prevent the ligand from escaping into the solvent, in which, it is poorly soluble. Curcumin binding to TTR could be partly influenced by its pH-dependent solubility.

To conclude, the use of biologically compatible mixed solvents has expanded the range of projects that can be tackled by X-ray crystallography. A much wider range of biological applications may benefit from a similar approach.

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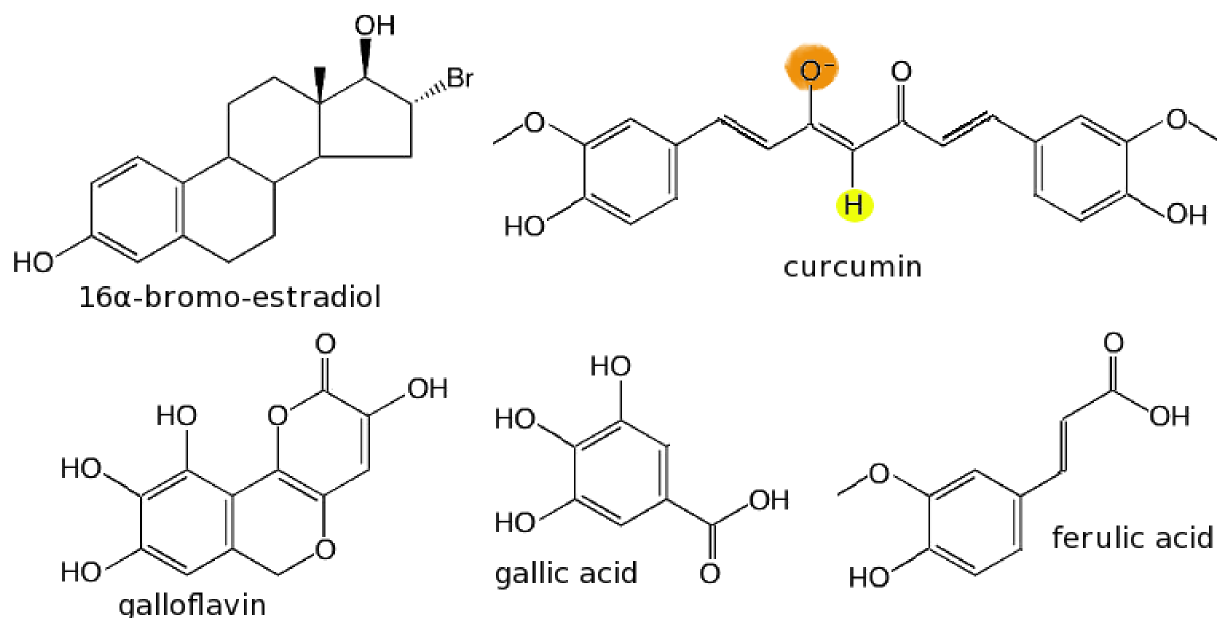
TABLE 1

Statistics for data collection, processing and refinement on TTR-ligand complexes

PDB code	4PM1	4PME	4PMF
Ligand(s)	**·bromo-estradiol**	**Curcumin / Ferulic acid**	**Curcumin**
Crystallization	70% PEG_4D 30% PEG_3A	45% MPEG 2,000 0.1 M sodium cacodylate, pH 7.5.	40% MPEG 2,000, 1% dioxane 0.1 M sodium cacodylate, pH 7.5.
Cryoprotectant	CM29*, 12.5% MPEG 5K, 25% MPEG 550, PCTP# buffer (50% A / 50% B) with 1 mM steroid.	SM5*, 25% MPEG 5K, 100 mM AAB# buffer 40% A / 60% B) [pH 7.2]	SM5*, 25% MPEG 5K, 100 mM AAB# buffer 20% A / 80% B) [pH 8.2] with 1.5 mM curcumin.
Data Collection			
Source	ESRF ID23-1	ESRF ID23-1	ESRF ID23-1
wavelength (Å)	0.8726	0.9793	0.9793
Space group	$P2_12_12$	$P2_12_12$	$P2_12_12$
Unit-cell parameters (Å)	43.2 86.1 63.4	43.0, 84.7, 64.1	43.2, 85.5, 64.2
Molec./asym.	2	2	2
Resolution (Å)	1.23	1.26	1.35
R_{merge}(%)	11.8	5.5	6.5
$R_{p.i.m.}$(%)	11.1	5.2	6.1
Mean $I/\sigma(I)$	11.7	8.19	18.1
Completeness (%)	98.0	99.7	99.9
Multiplicity	9.32	8.84	8.95
Refinement			
Resolution (Å)	1.23	1.26	1.35
No. of reflections	68063	63061	52990
R_{work} (%)	17.7	18.1	17.6
R_{free} (%)	21.1	20.7	20.9
R.m.s. deviations			
Bond lengths (Å)	0.007	0.007	0.007
Bond angles (°)	1.304	1.178	1.258
Ramachandran&			
favoured (%)	99	97	97
outliers (#)	2	0	1

*Cryoprotection: CM29: 25 % diethylene glycol, 12.5 % MPD, 37.5 % 2,3-butanediol, 12.5 % 1,4-dioxane. SM5: 25% dioxane, 25% DMSO, 12.5% ethylene glycol, 12.5% 1,2-propanediol, 12.5% glycerol, 12.5% 2,3-butanediol (companion paper)

#Buffers are at 100mM. AAB: sodium acetate, ADA, bicine (component A pH 4; B pH 9); PCTP: sodium propionate, sodium cacodylate, Bis-Tris-propane(component A pH 4; B pH 9.5)[39].



SCHEME 1

Chemical structure of hydrophobic compounds used in the solubilization tests and crystallization studies. Steroid binding to TTR has not been demonstrated. 16α-bromoestradiol is a suitable compound for crystallization-based screening because of its electron-rich substituent. Galloflavin, produced by treating gallic acid with NaOH is dark in color, gives TTR crystals that are darker than the surrounding mother liquor, an indication of accumulation of the compound in the lattice (not necessarily at a well defined site). Curcumin is a colored compound that degrades to uncolored ferulic acid at pH higher than 7.5. Rarely TTR crystals grown in the presence of curcumin are colored. Curcumin complexes with proteins have not yet been reported, either because of poor solubility in an acqueous environment or because of chemical instability of the compound. A diverse set of crystallization trials were carried out without ligand purification to allow TTR to select among the intact ligand or degradation products, the ones with the best affinity under the different scenarios.

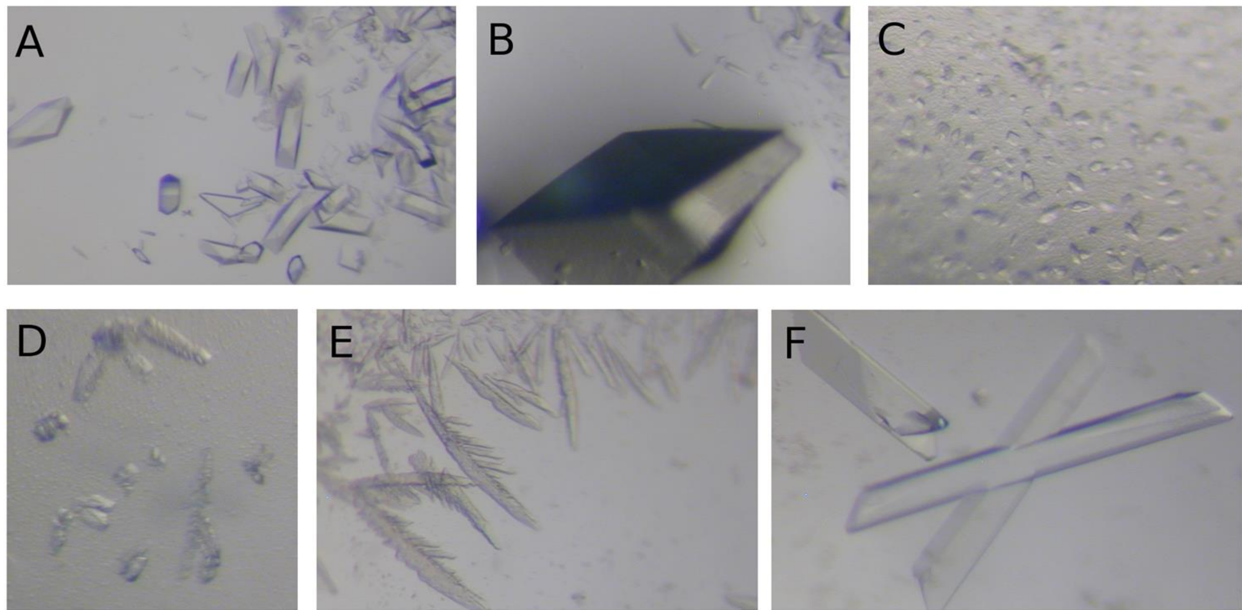


FIGURE 1

Crystals of wild-type human transthyretin (TTR) obtained under various crystallization conditions from the Stura footprint (Molecular Dimensions, Ltd) in the absence of any ligand. **(a)** Crystals obtained from 33% PEG-600, 0.2 M imidazole malate, pH 5.5. **(b)** Crystals from 2 M ammonium sulphate, 0.15 M sodium citrate, pH 5.5. **(c)** Shower of crystals from 1.5 M sodium citrate 10 mM sodium borate, pH 8.5. Larger crystals can be obtained by lowering the precipitant concentration. **(d)** Crystal grow in small attached blocks from 27% PEG 10,000, 0.1 M ammonium acetate, pH 4.5 **(e)** Feather-like crystals grow from 60% monomethyl PEG 550, 0.1 M HEPES, pH 8.2.**(f)** Large prismatic crystals are obtained from 36% monomethyl PEG 5,000, 0.1 M sodium acetate, pH 5.5.

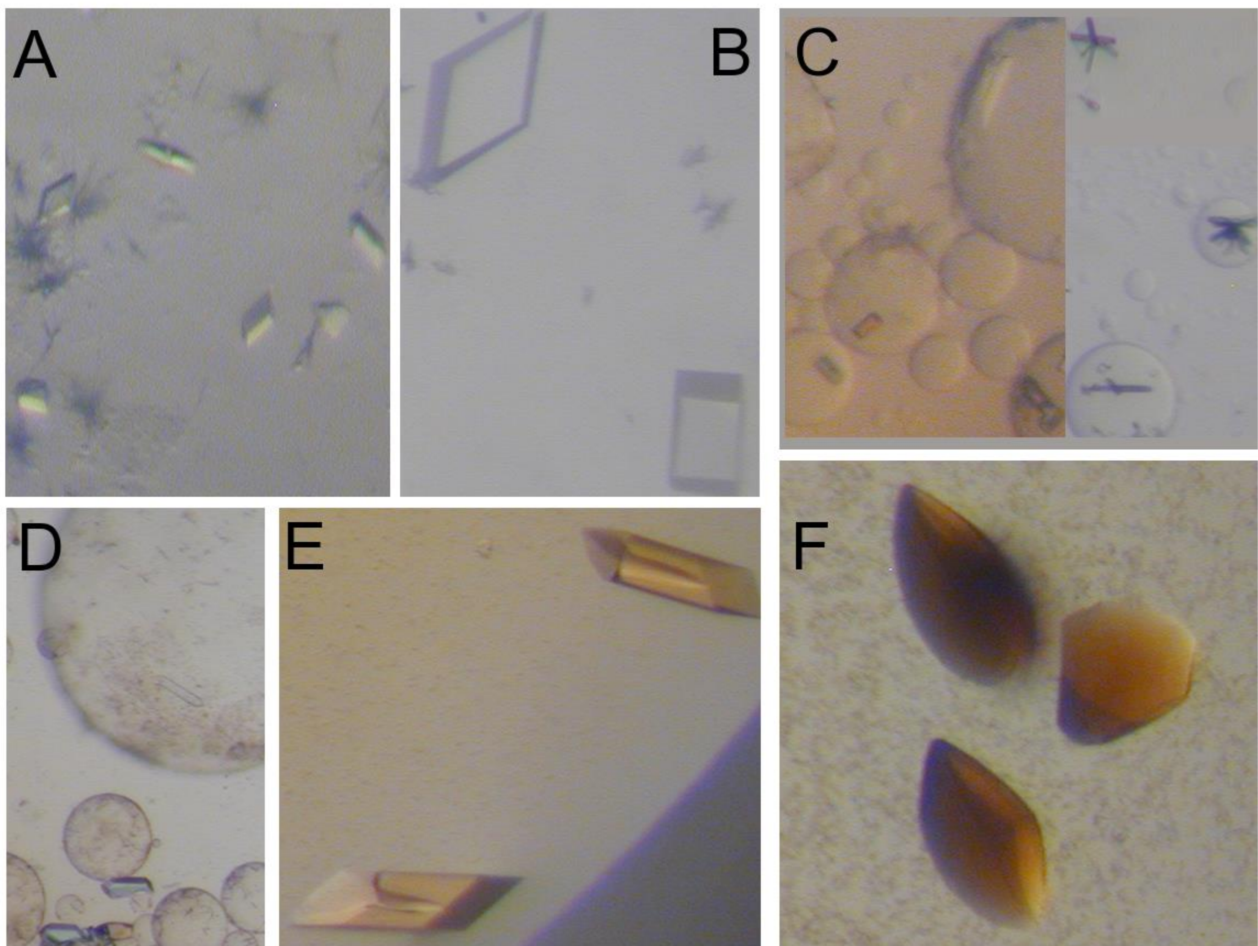


FIGURE 2

Photomicrographs of crystals of wild-type human transthyretin (TTR) grown in the presence of various ligands. **(a)** Crystals of the TTR-16α-bromo-estradiol complex. The steroid is well solubilized in the crystallization as the drop is set up, but it crystallizes as the drop shrinks to the final volume during vapor diffusion equilibration and crystallization.**(b)** Crystals of the TTR-16α-bromo-estradiol complex. The crystals are grown with a lower concentration of PEG. PEG can participate in the solubilization of the steroid, but the small change in concentration has almost no influence on ligand crystallization.**(c)** Steroid crystals grow inside solvent droplets in a crystallization trial with sodium citrate. **(d)** Phase separation in high salt crystallization conditions. Ligand crystals grow inside the less polar solvent while TTR crystals grow outside the droplets. The color is obtained using cross-polarizers. **(e)** Crystals of TTR grown in the presence of curcumin at pH 7.4 show an orange color denser than the background solution. **(f)** Crystals grown in the presence of NaOH treated gallic acid, show a deep brown color.

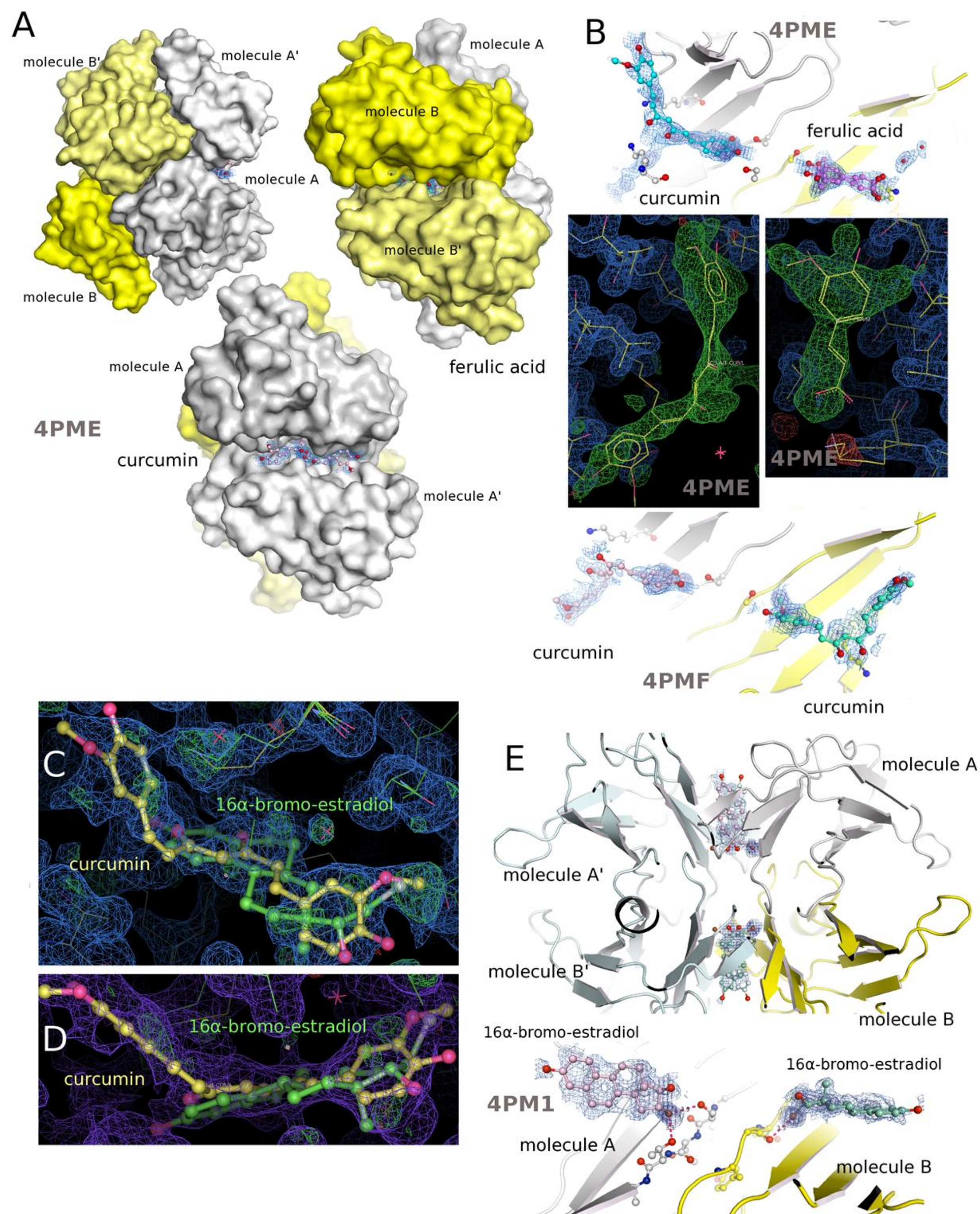


FIGURE 3

Electron density for the TTR-complexes with curcumin and 16α-bromo-estradiol. **(a)** Surface representation of the TTR tetramer shown with the ligand and its electron density sandwiched between two dimers. The three views show the tetramer arrangement with bound curcumin and ferulic acid. The asymmetric unit contains a dimer (A: gray, B: yellow). The second dimer (A': white, B': pale yellow) is generated through a two-fold crystallographic symmetry operation that places the symmetric of molecule A, molecule A'. The same is true for molecules B and B'. Curcumin extends beyond the classical binding site while its degradation product ferulic acid is hardly visible in the TTR surface representation. **(b)** Electron density for the two curcumin complexes. Degradation is evident (PDB id: 4PME: top) for crystals stored for several days. The amount of degradation varies from crystal to crystal. 4PME contains mainly curcumin degraded to ferulic acid in one site, while judging from the experimental electron density after refinement with occupancy for both ligands set at zero occupancy, the second site still contains traces of intact curcumin. Soaking with curcumin for 20 minutes during the cryopreservation step allows the exchange of ferulic acid and the replenishment of curcumin in the site (PDB id: 4PMF: bottom). **(c)** Experimental map shown in COOT[27] for crystals grown in the presence of 16α-bromo-estradiol and soaked during the cryopreservation step with curcumin. When refined with only curcumin show residual electron density for the steroid, suggesting an incomplete exchange during the short 20 minute soak as positive density peaks are visible for the bromine atom and the carbon at position 18. **(d)** If the refinement is carried out with only the bromo-estradiol, positive electron density peaks appear for positions characteristic of curcumin. **(e)** Positioning of 16α-bromo-estradiol within the TTR tetramer (top: PDB id: 4PM1). The bromine atom is bound in the halogen binding clearly identified by the higher electron density between Ser^{117} and Thr^{119}. The electron density for the oxygen and carbon atoms of the steroid are smeared out due to the superposition of the two symmetric ligands with each at occupancy=0.5 for a fully occupied ligand binding site.

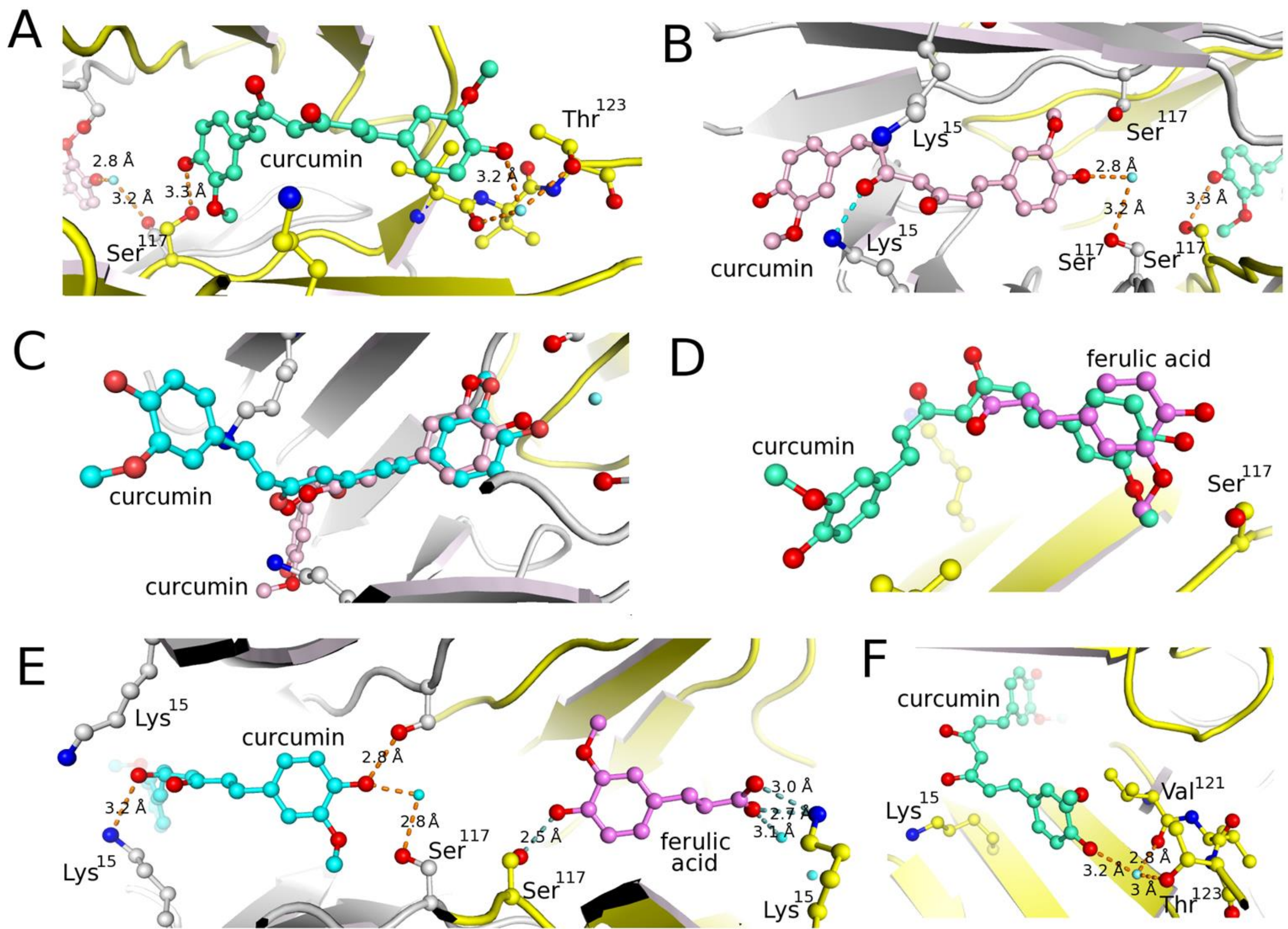


FIGURE 4

Schematic representation of the interactions between TTR and curcumin or ferulic acid. **(a)** In the structure replenished with curcumin (PDB id: 4PMF), the curcumin molecules bind to the two sites on TTR in a slightly different manner. The ligand interacts with Ser^{117}, Lys^{15} and *via* a water molecule to Val^{121} and Thr^{123}. **(b)** The interaction made by curcumin with Ser^{117} is variable. Within the second TTR binding site is it iteracts indirectly *via* a water molecule. **(c)** Small changes in positioning of curcumin are observed in the curcumin/ferulic acid complex (curcumin: cyan; PDB id: 4PME) compared to the curcumin replenished structure (pink; 4PMF). The positioning of the outer binding part of the curcumin molecule is ambiguous because the electron density is shared by the ligand and its symmetric. **(d)** There are minor variations between the positioning of the ferulic acid compared to curcumin. **(e)** The variations may be result of optimization of the binding for the two ligands. **(f)** Curcumin binding extends beyond the central cavity of the tetramer that has been well documented in other studies. Optimization of the binding with the 121-132 stretch of residues made by curcumin *via* a water molecule could lead to new more effective anti-amyloidigenic molecules able to interact directly.

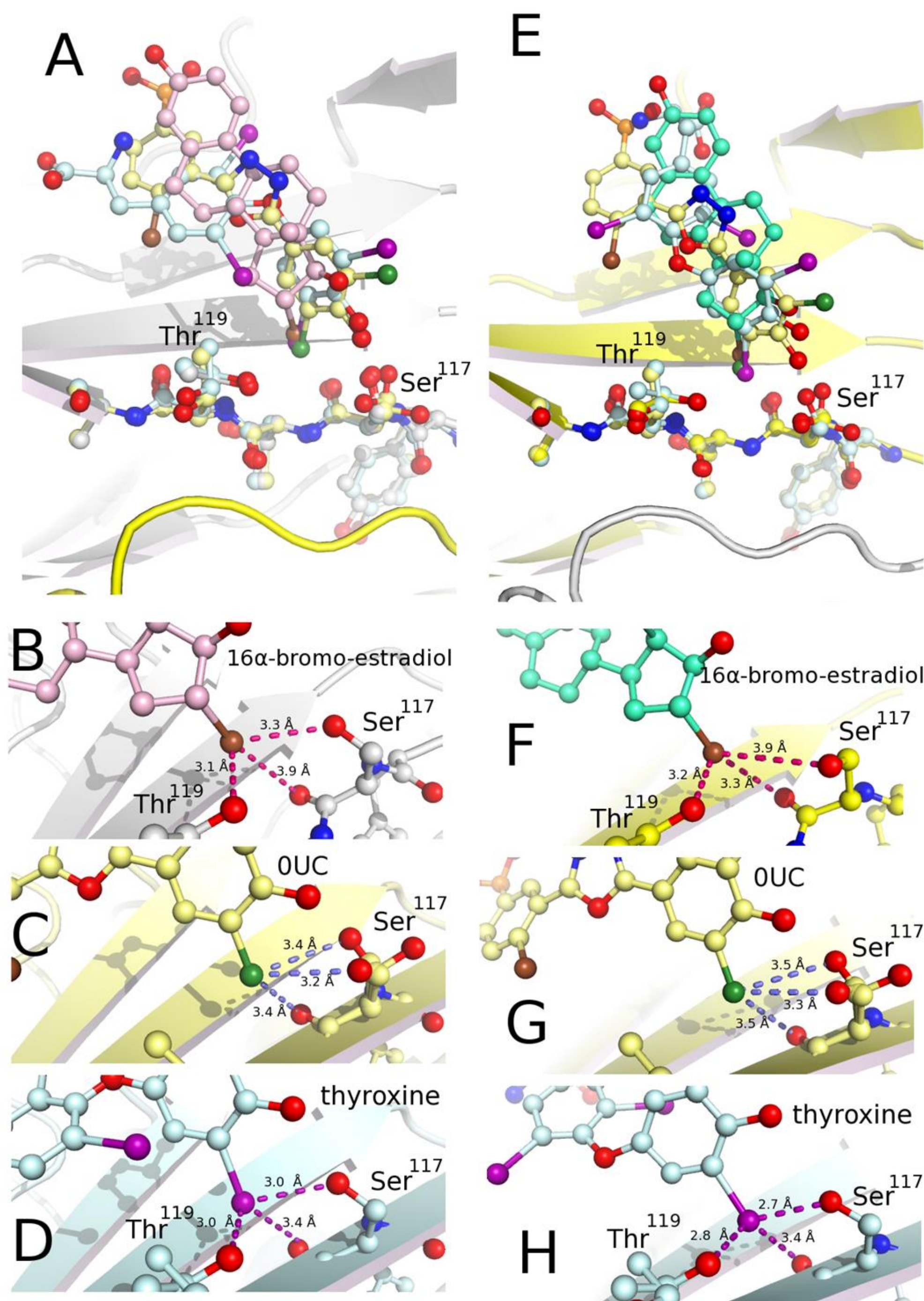


FIGURE5

Halogen atom positioning in the TTR-16α-bromo-estradiol complex (PDB-ID: 4PM1) compared to thyroxine (T4, PDB-ID: 3OZK[37]) and 4-bromo-3-(5-(3,5-dichloro-4-hydroxyphenyl)-1,3,4-oxadiazol-2-yl)-benzenesulfonamide (PDB-ID: 4FI8[36]). **(a)** The bromine atom of 16α-bromo-estradiol selects the position used by one of the iodine atoms of the natural ligand, thyroxine. **(b)** The interactions of the bromine atom resemble those of the natural ligand although the X-bond distances are longer. **(c)** The interaction pattern for the chlorine atom for ligand 0UC in 4FI8 does not interact with Thr^{119}. **(d)** Distances for T4 with Ser^{117} and Thr^{119} in molecule A. **(e)** Ligands establish different interaction with molecule B compared to molecule A. **(f)** In molecule B Ser^{117} adopts a different rotamer. This changes the interaction pattern with the bromine atom. **(g)** In molecule B, chlorine atom from ligand in 4FI8 is less well held compared to molecule A. **(h)** T4 established shorter interactions in molecule B compared to molecule A.