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Structure-activity relationships of flurbiprofen analogues as stabilizers of the amyloidogenic protein transthyretin

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Abstract

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3 The inherent amyloidogenic potential of wild type transthyretin (TTR) is enhanced by a large number of point
4 mutations, which destabilize the TTR tetramer, thereby promoting its disassembly and pathological aggregation
5 responsible for TTR-related amyloidosis. TTR stabilizers are able to interact with the thyroxine-binding sites of TTR,
6 stabilizing its tetrameric native state and inhibiting amyloidogenesis. Herein, we report on *in vitro*, *ex vivo* and X-ray
7 analyses to assess the TTR structural stabilization by analogues of flurbiprofen, a non-steroidal anti-inflammatory drug
8 (NSAID). Overall, considering together binding selectivity and protective effects on TTR native structure by
9 flurbiprofen analogues in the presence of plasma proteins, as determined by Western Blot, the aforementioned
10 properties of analyzed compounds appear to be better (CHF5075 and CHF4802) or similar (CHF4795) or worse
11 (CHF5074, also known as CSP-1103) as compared to those of diflunisal, used as a reference TTR stabilizer. Molecular
12 details of the determinants affecting the interactions of CHF5075, CHF4802 and CHF4795 with wild type TTR and of
13 CHF5074 with the amyloidogenic A25T TTR variant have been elucidated by X-ray analysis. Distinct interactions with
14 TTR appear to characterize flurbiprofen analogues and the NSAID diflunisal and its analogues as TTR stabilizers.
15 Relationships between stabilizing effect on TTR by flurbiprofen analogues determined experimentally and molecular
16 details of their interactions with TTR have been established, providing the rationale for their protective effects on the
17 native protein structure.
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31 **Keywords:** Amyloidogenic proteins; Transthyretin; Amyloidogenesis inhibitors; Transthyretin stabilizers; Ligand
32 binding design.
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1. Introduction

The amyloidoses are particularly relevant diseases, caused by pathological aggregation in tissues of several amyloidogenic peptides and proteins, among which transthyretin (TTR) represents a notable example (Chiti and Dobson, 2017). TTR is a homotetramer of about 55 kDa involved in the transport in extracellular fluids (such as blood, cerebrospinal fluid (CSF) and eye vitreous body) of thyroxine (T4) and in the co-transport of vitamin A, by forming a macromolecular complex with plasma retinol-binding protein (RBP4) (Wojtczak et al., 1996; Zanotti and Berni, 2004). Structurally, the TTR tetramer is a dimer of dimers, in which the four monomers associate in a highly symmetrical arrangement, characterized by 222 symmetry. A long channel coincident with one of the twofold symmetry axes, transverses the whole oligomeric protein and harbors two T4 binding sites at the dimer-dimer interface. Each pair of twofold-related monomers forms a funnel-shaped hormone-binding site, which is lined by three sets of halogen binding pockets (HBPs): an outer binding subsite (HBP 1 and HBP 1'), an inner binding subsite (HBP 3 and HBP 3'), and an intervening interface (HBP 2 and HBP 2') (Wojtczak et al., 1996). HBPs represent hydrophobic pockets, especially HBP 2 and HBP 2' and HBP 3 and HBP 3', wherein the iodine atoms of bound T4 are accommodated (Wojtczak et al., 1996).

Both wild type (wt) human TTR and a number of its amyloidogenic mutant forms have been associated with TTR-related amyloidosis (ATTR), which is believed to be caused by the destabilization of the TTR tetrameric structure (Hurshman Babbes et al., 2008; Cendron et al., 2009) and its rate-limiting dissociation into monomers, which undergo misfolding and misassembly (Connelly et al., 2010, and references therein). The typical deposition of cross- β -sheet amyloid fibrils in the case of ATTR is mainly found in peripheral nerves and the heart. While wt TTR may give rise in the elderly to cardiomyopathy, an under-recognized disease formerly called senile systemic amyloidosis (SSA) (Galant et al., 2017), a variety of genetic TTR variants are involved in the more aggressive hereditary TTR amyloidoses, in which the peripheral sensorymotor and autonomic nervous system (formerly called FAP, familial amyloidotic polyneuropathy) and the heart (formerly called FAC, familial amyloidotic cardiomyopathy) are mainly affected (Connelly et al., 2010). The two relevant Val30Met and Val122Ile mutations, which affect the most ATTR patients, are typically responsible for polyneuropathy and cardiomyopathy, respectively (Sekijima, 2015). In addition, some highly destabilizing mutations are responsible for familial leptomenigeal or oculo-leptomeningeal amyloidoses, which are associated with manifestations involving the central nervous system and the eye (Sekijima, 2015).

TTR ligands, designated TTR stabilizers, are able to stabilize the TTR tetramer and to inhibit its disassembly by occupying the T4 binding sites, and establishing interactions at the dimer-dimer interface that bridge the couple of subunits that form each binding site (Bulawa et al., 2012; Sant'Anna et al., 2016; Verona et al., 2017; Miller et al., 2018). Among them, tafamidis, which has been approved in many countries for the

Abbreviations used: TTR, transthyretin; RBP4, plasma retinol-binding protein; ATTR, TTR-related amyloidosis; wt TTR, wild type TTR; T4, thyroxine; CSF, cerebrospinal fluid; NSAID, non-steroidal anti-inflammatory drug; HBP, halogen binding pocket; FAP, familial amyloidotic polyneuropathy; FAC, familial amyloidotic cardiomyopathy; SD, standard deviation; PDB, Protein Data Bank.

1 therapy of ATTR polyneuropathy, was found to delay disease progression in patients at early stages of ATTR
2 polyneuropathy (Waddington Cruz et al., 2016) and cardiomyopathy (Sultan et al., 2017). The non-steroidal anti-
3 inflammatory drug (NSAID) diflunisal, a generic anti-inflammatory FDA-approved drug, was also found to be effective
4 in slowing neurological impairment in patients affected by TTR polyneuropathy (Berk et al., 2013). However, its long-
5 term therapeutic use is limited by its possible adverse effects (Kristen et al., 2019).
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8 Several analogues of the NSAID flurbiprofen have been synthesized with the aim of obtaining inhibitors of A β -
9 secretion in Alzheimer's disease (Peretto et al., 2005). One of these flurbiprofen analogues, CHF5074, also known as
10 CSP-1103, turned out to specifically interact with the T4 binding sites of TTR and to fully prevent an acidic-pH induced
11 conformational change in the amyloidogenic I84S TTR variant (Zanotti et al., 2013). Regarding its *in vivo* anti-
12 amyloidogenic potential, its ability to stabilize TTR in double humanized mice carrying human TTR and RBP (Mu et al.,
13 2015) and in healthy human subjects (Qiang et al., 2017) was reported. Despite being a derivative of the NSAID
14 flurbiprofen, CHF5074 exhibited a markedly reduced cyclooxygenase inhibitory activity (Imbimbo et al., 2007), and
15 was found to reduce in humans biomarkers of neuroinflammation (Ross et al., 2013). Herein, we report on a
16 comparative *in vitro* and *ex vivo* study of the anti-amyloidogenic potential of CHF5074 and of its as-yet-
17 uncharacterized close analogues CHF5075, CHF4802 and CHF4795 as TTR stabilizers, and on X-ray analysis of the
18 molecular details of their interactions with TTR leading to TTR structural stabilization.
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27 **2. Materials and Methods**

28 *2.1 Materials*

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34 Recombinant human wt TTR and its mutant form A25T were prepared and quantified essentially as described
35 (Pasquato et al., 2007). Diflunisal and *trans*-resveratrol were from Sigma–Aldrich, and tafamidis was from Carbosynth.
36 Flurbiprofen and its analogues CHF5075, CHF4795, CHF4802 and CHF5074 were supplied by Chiesi Farmaceutici,
37 Parma, Italy.
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42 *2.2 Fluorometric competitive binding assay*

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45 Fluorescence binding experiments were carried out in 50 mM sodium phosphate buffer, 150 mM NaCl, pH
46 7.4, at 20 °C, using a PerkinElmer Life Sciences LS-50B spectrofluorometer. The fluorescence emission intensity of the
47 TTR ligand, and at the same time fluorescent probe, *trans*-resveratrol, which is nearly negligible for the unbound
48 compound in solution, increases substantially upon binding to TTR (Florio et al., 2015). Fluorescence measurements
49 were carried out to analyse the ability of flurbiprofen and its analogues CHF5074, CHF5075, CHF4802 and CHF4795,
50 dissolved in DMSO, to displace TTR-bound *trans*-resveratrol, by monitoring its fluorescence intensity decrease. The
51 evaluation of the ability of TTR synthetic ligands to compete with TTR-bound resveratrol (TTR 7.5 μ M, 1:1 *trans*-
52 resveratrol to TTR ratio) was based on the decrease in fluorescence emission intensity of TTR-bound *trans*-resveratrol
53 at 390 nm (excitation at 320 nm) as a function of competitor concentration.
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2.3 Inhibition of TTR fibrillogenesis at moderately acidic pH

In vitro TTR aggregation at moderately acidic pH was monitored by following the increase in turbidity, estimated spectrophotometrically at 400nm, as described (Miller et al., 2004). 7.2 μ M wt TTR was preincubated with 1 molar equivalent of flurbiprofen, CHF5074, CHF5075, CHF4795, CHF4802, diflunisal and tafamidis, dissolved in DMSO, or with DMSO used as negative control, at neutral pH (10 mM sodium phosphate buffer, pH7, 100mM KCl, 1mM EDTA) for 3 h at room temperature, prior to incubation at acidic pH upon addition of an equal volume of 100mM sodium acetate, 100 mM KCl, 1 mM EDTA, pH 4.2 (final pH 4.3), at 37°C, to promote protein aggregation.

2.4 Western Blot analysis

The ability of TTR stabilizers to selectively stabilize human plasma TTR was assayed for TTR present in the human plasma, in the presence of 4 M urea, by means of a Western Blot procedure carried out according to the protocol described by Nilsson et al. (2016), with some modifications, as follows. Aliquots of human plasma were diluted 16 times in 40 mM sodium phosphate buffer, 150 mM NaCl, pH 7.4, and supplemented with 15 and 30 μ M flurbiprofen, CHF5074, CHF5075, CHF4795, CHF4802, diflunisal and tafamidis dissolved in DMSO. One more plasma sample supplemented with DMSO was used as a negative control. After 2 hour incubation at 20°C, an equal volume of 8 M urea was added to each sample to obtain a final 4 M urea concentration (final ligand concentrations: 7.5 and 15 μ M). The incubation was prolonged for 18 hours at 20°C, followed by non-denaturing SDS-PAGE, using Tris-Glycine buffer containing 0,025% SDS in the running buffer and 0.2% SDS in the loading buffer. SDS at these concentrations does not denature TTR tetramers but does prevent re-association of monomers (Nilsson et al., 2016). The blotting step was accomplished by means of a Trans-Blot SD transfer apparatus (BIO-RAD), and the membrane after the blotting step was incubated overnight in blocking buffer containing 5% Skim Milk, at 25°C. Immunodetection of TTR monomers was performed by employing rabbit anti-human TTR polyclonal Ab (Dako) as primary Ab, and anti-rabbit Ab labeled with Dylight 680 (SERACARE) as secondary Ab. Western Blot images were recorded by using an Odyssey Image System (LI-COR). The employed rabbit anti-TTR antibody does not appear to specifically discriminate between the native fraction of tetrameric TTR from other protein aggregates, and as a consequence only the change in TTR monomers, which reflects the level of tetrameric TTR dissociation upon denaturation, could be accurately monitored.

2.5 TTR crystallization, data collection and structure determination

Crystals of wt TTR in complex with CHF5075, CHF4795 and CHF4802 and of the amyloidogenic A25T TTR mutant form in complex with CHF5074 were prepared by co-crystallization by using the hanging-drop vapor diffusion method essentially as described (Florio et al., 2015). TTR (5 mg/ml) in 20 mM sodium phosphate buffer, pH 7, was incubated with a 4-fold molar excess of each flurbiprofen analogue solubilized

1 in DMSO. Drops (1,5 μ l) were formed by mixing equal volumes of the solution containing TTR-ligand
2 complexes and of the reservoir/precipitant solution (2.2 M ammonium sulfate, 0.1M KCl, 30 mM sodium
3 phosphate, pH 7.0). Single crystals were obtained in about one week of incubation at room
4 temperature. Frames with an oscillation of 0.1° each were collected at the ID23-1 beamline of European
5 Synchrotron Radiation Facility (ESRF, Grenoble, France). All the crystals belong to the space group $P2_12_12_1$,
6 with one dimer in the asymmetric unit. Datasets were processed with the software XDS (Kabsch, 2010) and
7 scaled with Scala (Evans, 2006) contained in the CCP4 suite (Winn et al., 2011). In this space group the
8 native tetrameric structure is generated through the crystallographic twofold axes. The structure was
9 determined by molecular replacement using Phaser (McCoy et al., 2007) as implemented in the CCP4 suite
10 (Potterton et al., 2004). The structure was refined using Refmac5 (Adams et al., 2013). In the last cycles,
11 TLS refinement was applied. Map visualization and manual adjustment of the models were performed using
12 the Coot graphic interface (Emsley and Cowtan, 2004). Statistics on data collection and refinement are
13 reported in **Table 1**.

22 2.6 Accession numbers

25 Atomic coordinates and structure factors of TTR in complex with flurbiprofen analogues have been deposited
26 at the Protein Data Bank (PDB) with PDB ID: TTR-CHF5075, PDB ID: **6R67**; TTR-CHF4802, PDB ID: **6R66**;
27 TTR-CHF4795, PDB ID: **6R68**; A25T TTR-CHF5074, PDB ID: **6R61**.

31 3. Results and Discussion

35 3.1 Comparative analysis of the interactions of flurpirofen analogues with TTR in competitive binding 36 assays.

40 Fluorescence titrations were carried out to analyze the competition between the fluorescent *trans*-
41 resveratrol molecule and increasing concentrations of flurbiprofen and its analogues CHF5074, CHF5075,
42 CHF4802 and CHF4795 (**Fig. 1A**), for the binding to a preferential T4 binding site of wt TTR, according to
43 (Florio et al., 2015). Based on such competitive binding assays, flurbiprofen exhibited a very low binding
44 affinity for TTR relative to that of *trans*-resveratrol, while all four flurbiprofen analogues could efficiently
45 displace TTR-bound *trans*-resveratrol (**Fig. 1B**). These results indicate that the presence of substituents
46 (both Cl atoms and methyl groups) in the phenyl ring of flurbiprofen most distant from the carboxyl group is
47 a necessary requirement in order to induce a significantly higher binding affinity for TTR, which appears to
48 be relatively similar for the four analogues, in comparison with the precursor flurbiprofen molecule. A
49 comparative analysis of ligand binding affinities could not be extended to include diflunisal and tafamidis as
50 reference TTR stabilizers. In fact, while flurbiprofen and its derivatives are devoid of any fluorescence in the
51 emission region of TTR-bound *trans*-resveratrol, diflunisal and tafamidis possess intrinsic fluorescence
52 emission in such region, so that reliable displacement titrations for the latter compounds could not be
53 performed. It should be pointed out that relatively similar binding affinities of TTR ligands do not necessarily

1 reflect a similar ability to structurally stabilize TTR. In fact, the stabilizing effects of ligands on TTR can be
2 mainly attributed to their ability to non-covalently bridge the two subunits that form the T4 binding cavity
3 (Bulawa et al., 2012; Sant'Anna et al., 2016; Verona et al., 2017; Miller et al., 2018).
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6 *3.2 Comparative analysis of the ability of flurbiprofen analogues to inhibit TTR fibrillogenesis at moderately* 7 *acidic pH and to selectively stabilize TTR in the presence of plasma proteins.* 8 9

10 The inhibition of TTR aggregation by flurbiprofen, its analogues, diflunisal and tafamidis at
11 moderately acidic pH (pH 4.3) was estimated by monitoring the increase in turbidity of protein solutions
12 monitored at three different incubation times (**Fig. 1 C**). A very weak inhibition was observed for
13 flurbiprofen, consistent with the very weak interaction of this compound with TTR (**Fig. 1 B**). A relatively
14 weak inhibition was observed for diflunisal and CHF5074, while the strongest inhibition was found for
15 tafamidis and the flurbiprofen analogues CHF4802, CHF 5075 and CHF4795. However, more informative
16 data regarding the ability of fibrillogenesis inhibitors to selectively stabilize TTR in the presence of plasma
17 proteins were obtained by using the Western Blot methodology described In the Materials and Methods
18 section. In fact, this methodology allowed us to evaluate the ability of ligands to interact selectively with TTR
19 in the presence of the large amount of the other plasma proteins, especially serum albumin, and at the same
20 time to provide evidence for their stabilizing effect on the tetrameric native structure of TTR under partially
21 denaturing conditions. When flurbiprofen analogues were tested for their ability to selectively stabilize
22 plasma TTR in the presence of 4 M urea, the relative amount of TTR monomer was assumed to be
23 representative of the fraction of denatured protein present in the sample (Nilsson et al., 2016). Binding
24 selectivity along with protective effect on TTR native structure in diluted plasma by flurbiprofen
25 analogues, was found to be better (CHF5075 and CHF4802), or similar (CHF4795) or worse (CHF5074) as
26 compared to that of the TTR stabilizer diflunisal, and worse for all compounds, including diflunisal, in
27 comparison with tafamidis (**Fig. 1D**). Our *ex vivo* results are consistent with the previous observation of a
28 higher stabilizing effect on TTR in the human plasma of diflunisal in comparison with CHF5074 under
29 partially denaturing conditions, at acidic pH (Qiang et al., 2017).
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44 *3.3 Crystal structure of A25T TTR in complex with CHF5074* 45 46

47 X-ray analyses have been conducted to elucidate molecular details of the interactions of CHF5074
48 with the A25T TTR mutant form, which is responsible for familial leptomeningeal amyloidosis (Sekijima,
49 2015). Crystals of A25T TTR in complex with CHF5074 diffracted to a maximum of 1.47 Å resolution (**Table**
50 **1**). The protein moiety in the crystal structure of the TTR-ligand complex is very similar to that of the
51 uncomplexed human wt TTR (Hornberg et al., 2000, PDB ID: 1F41) and to the crystal structure of the
52 uncomplexed TTR-A25T mutant form (Azevedo et al., 2011, PDB ID: 3TFB): the r.m.s.d. between equivalent
53 Ca atoms are 0.76 Å, 0.70 Å, respectively. The only significant difference is present in the loop 98-104 of
54 TTR monomers, which is generally a flexible region in all TTR structures and far from the ligand cavity. Two
55 sulfate molecules are visible in the area between the two long loops formed by the terminal and the initial
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1 part of strands B and C, but in a region far from the binding site of the ligand. The ligand is bound inside
2 the two TTR binding cavities in the 'forward' binding mode, i.e. with the dichloro-phenyl ring pointing
3 towards the innermost part of the cavity and the carboxyl group towards the solvent, as in the case of the
4 CHF5074-wt TTR complex (Zanotti et al., 2013, PDB ID: 4I85): the two structures present a r.m.s.d.
5 between equivalent Ca atoms of 0.68 Å.
6

7 As in all the other cases of binding of ligands to the central channel of human TTR, the molecular
8 (and crystallographic) symmetry of the channel implies the presence of two possible orientations for the
9 ligand within each binding site, giving rise to an intrinsic disorder in its electron density. Ligand occupancies
10 refined to final values of 0.54 and 0.50 for sites A and B, respectively: given the twofold symmetry of each
11 site, this corresponds to an occupancy close to 100% (sites A and B are different based on the environment
12 of the two TTR monomers present in the crystallographic dimer; the convention for the distinction of the two
13 binding sites (Cianci et al., 2015) is in agreement with the large majority of coordinate data sets of TTR in
14 the PDB). The quality of the ligand fitting supported by the real-space R value (RSR) of 0.16/0.19 and by the
15 real-space correlation coefficient (RSCC) of 0.87/0.92 (**Table 1**). The two parameters indicate a good fitting
16 of the ligand when their values are, respectively, below 0.4 and above 0.80 (Smart et al., 2018). The
17 interactions of CHF5074 with wt TTR and the A25T TTR mutant form are essentially identical, a consequence
18 of the fact that the A25T mutation does not perturb the TTR structure. The lack of significant conformational
19 changes in the structure of amyloidogenic mutant forms of TTR in comparison with wt TTR (Schormann et
20 al., 1998) further suggests that complexes of wt TTR with TTR stabilizers represent reliable models of
21 ligand-TTR interactions also concerning TTR mutant forms.
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33 *3.4 Crystal structures of wt TTR in complex with CHF5075, CHF4795 and CHF4802*

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36 The crystal structures of wt TTR in complex with the flurbiprofen analogues CHF4795, CHF5075 and
37 CHF4802 have been solved at 1.30 Å – 1.45 Å resolution (**Table 1**). The electron densities are quite well
38 defined for the protein moieties, which are virtually identical among them and to those of the TTR-CHF5074
39 (Zanotti et al., 2013, PDB ID: 4I85) and TTR-flurbiprofen (Klabunde et al., 2000, PDB ID: 1DVT) complexes.
40 The r.m.s.d. of the equivalent C α atoms are: CHF5075/flurbiprofen 0.785 Å and CHF5075/CHF5074 0.482 Å;
41 CHF4795/flurbiprofen 0.745 Å and CHF4795/CHF5074 0.501 Å; CHF4802/flurbiprofen 0.088 Å and
42 CHF4802/CHF5074 0.202 Å. The TTR-bound ligands are all positioned in the 'forward' binding mode (**Fig.**
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49 The electron density of the flurbiprofen derivative molecules is well defined for the inner and the
50 central rings, whilst it is poorly visible for the hydrophilic carboxylate group moiety, due to the twofold
51 symmetry of the binding site that generates a double conformation of this flexible part of the ligand.
52 Occupancies for each ligand pairs refined to the following values: CHF4795 0.47/0.46; CHF4802 0.54/0.54;
53 CHF5075 0.47/0.45, indicating a similar occupancy close to 100% for both A and B sites of TTR (**Fig. 2A**). It
54 should be pointed out that CHF5075, CHF4795 and CHF5082, as well as CHF5074 (Zanotti et al., 2013, PDB
55 ID: 4I85), penetrate less deeply (by about 1.5 Å) in the inner portion of the T4-binding cavity as compared
56 to flurbiprofen (Klabunde et al., 2000, PDB ID: 1DVT) (**Fig. 3A**). This effect is due to the steric hindrance of
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1 the substituents present in the inner ring in flurbiprofen analogues. The lack of such substituents in the
2 flurbiprofen molecule allows for its deeper penetration in the binding cavities, whilst at the same time the
3 lack of interacting substituents markedly lowers its binding affinity and stabilizing effect on TTR, as revealed
4 in the competitive binding (**Fig. 1B**), fibrillogenesis inhibition (**Fig. 1C**) and Western Blot (**Fig. 1D**) assays,
5 respectively.
6

7 The position of the inner 3',5'-dichloro substituted phenyl ring of TTR-bound CHF5075 and CHF4795
8 is virtually superimposable on the same moiety of bound tafamidis (**Fig. 3B**) (Bulawa et al., 2013, PDB ID:
9 3TCT). Owing to this superposition, the two flurbiprofen analogues establish the same hydrophobic
10 interactions established by tafamidis with HBP3 and HBP3' (Bulawa et al., 2013). The two methyl groups of
11 CHF4802, which occupy approximately the same positions of the Cl atoms present in the TTR-bound
12 CHF5075 (**Fig. 2B**), can contribute, to a similar extent, to the stabilization of the TTR structure (**Fig. 1C**),
13 participating in hydrophobic interactions. In this respect, it must be considered that in all the structures, the
14 position of the inner and central phenyl rings is sandwiched in between the hydrophobic interactions of
15 Leu17, Ala108, Leu110 and Thr119, belonging to HBP2 and HBP3, and, correspondingly, of the same
16 residues belonging to HBP2' and HBP3', thereby contributing to the stabilization of the TTR tetramer. Also in
17 the TTR-CHF5074 complex (Zanotti et al., 2013, PDB ID: 4I85) the main interactions of the ligand take place
18 with HBP3 and HBP3', but in this case only the 3'-chloro substituent of the inner phenyl ring interacts
19 strongly with the HBP3 region of one of the two monomers of the T4 binding cavity. In fact, the adjacent 4'-
20 chloro substituent of CHF5074 is located in the center of the cavity, and differently from the case of 3',5'-
21 dichloro substituents, its stabilizing interactions are expected to be significantly weaker owing to the longer
22 distances from HBP3 and HBP3' (**Fig. 2B**).
23

24 In a previous study, structure-activity relationships of diflunisal, which exhibits both forward and
25 reverse binding modes, and its analogues have also been investigated (Adamski-Wemer et al., 2004). The
26 3',5'-dichloro substituted phenyl ring of the compounds CHF5075 and CHF4795 overlaps one of the two
27 phenyl rings of diflunisal (PDB ID: 3D2T), in both forward and reverse binding mode (**Fig. 4A**), highlighting
28 its interactions with the hydrophobic pocket formed by residues Ala108, Leu110, and Thr119. Interestingly,
29 in the case of the reverse binding mode of diflunisal, the interaction with Thr119 is enforced by a hydrogen
30 bond established with the meta-carboxyl group on the inner phenyl ring, which virtually overlaps the meta-
31 chlorine in the two aforementioned flurbiprofen analogues. However, in this last case the interaction with
32 Lys15 is lost, whereas such interaction is conserved in all the flurbiprofen analogues analyzed in this study.
33 In the case of the diflunisal analogue 2'-4'-dichloro-4-hydroxy-1,1'-biphenyl-3-carboxylic acid (PDB ID:
34 2B77), in which two fluorine atoms are substituted by chlorines in one of the aryl rings, a hydrogen bond is
35 established between the hydrophilic aryl ring and Ser117, and a reverse binding mode is uniquely found
36 (**Fig. 4 B**). The diflunisal analogue 3',5'-difluorobiphenyl-4-carboxylic acid (PDB ID: 2B9A) is rather similar to
37 the aforementioned CHF5075 and CHF4795 (two fluorines replace at the same positions the two chlorines in
38 one of the aryl ring), and, nevertheless, it still uniquely exhibits a reverse binding mode (**Fig. 4 C**). It is
39 tempting to speculate that the forward binding mode of flurbiprofen analogues is favored by the bridging of
40 the carboxyl group to the fluoro-phenyl ring by either a cyclopropyl or a methyl group, leading to an
41 effective interaction with Lys15 (**Fig. 2**).
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1 With regard to the fluoro substituent present in the central phenyl ring of flurbiprofen analogues
2 (**Fig. 2B**), it may participate in hydrophobic interactions, mainly with HBP2. However, only one of the two
3 monomers at the dimer-dimer interface can be affected by this interaction, and for this reason we expect
4 that its contribution to the stabilization of the TTR tetramer is rather limited, consistent with the very low
5 protective effect of flurbiprofen itself. Finally, the negatively charged carboxylate group interacts with Lys15
6 or Lys15' (HBP1 or HBP1'); despite that, this area of each ligand is not very well visible, owing to the
7 crystallographic twofold axis running through the central cavity of TTR and the same happens for the side
8 chain of Lys15 (**Fig. 3B**), but probably some flexibility of the ligand is also present.
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14 **4. Conclusions**

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17 Our results provide insights into the structure-activity relationships of flurbiprofen and its analogues
18 as TTR stabilizers. The presence of substituents, both Cl atoms and methyl groups, in the flurbiprofen phenyl
19 ring most distant from the carboxyl group is a necessary requirement in order to induce significantly high
20 affinity and stabilizing effect upon binding to the T4-binding sites of TTR. Despite the favorable properties
21 already reported for the flurbiprofen analogue CHF5074 with respect to its anti-amyloidogenic potential
22 (Zanotti et al, 2013; Mu et al., 2015; Qiang et al., 2017), we have found that a very limited change in this
23 molecule, consisting in the minimal displacement of a single Cl atom in the aforementioned phenyl ring,
24 leads to a substantial increase in the ability to stabilize TTR. In particular, in the TTR-CHF5074 complex a
25 weak bridging effect can be afforded by the apical Cl atom in the innermost phenyl ring, whilst in the
26 complex of TTR with the very similar CHF5075 and CHF4795 both Cl atoms in meta position effectively
27 bridge the two symmetric monomers that line the T4-binding cavity, consistent with their higher protective
28 effect on TTR as compared to CHF5074 (**Fig. 1D**).
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37 As reported above, in addition to its stabilizing properties on the native TTR structure, CHF5074 is
38 reported to reduce the biomarkers of neuro-inflammation (Ross et al., 2013), a property possibly beneficial
39 to counteract neuro-inflammatory and degenerative effects associated with TTR amyloid deposition in
40 leptomeningeal and oculo-leptomeningeal ATTR, for which a therapy is lacking to date (Kristen et al., 2019).
41 Presumably, its very close analogues analysed in this study may also afford an anti-inflammatory activity in
42 the central nervous system, while exhibiting a stabilising effect on the native TTR structure significantly
43 higher than that of CHF5074.
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56

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FIGURE CAPTIONS

Fig. 1. A, Chemical structures of TTR stabilizers: flurbiprofen, its analogues CHF5074, CHF5075, CHF4795 and CHF4802, tafamidis and diflunisal. **B, Fluorometric competitive binding assay.** Competitive fluorometric titrations (excitation at 320 nm, and emission at 390 nm) for the binding of flurbiprofen and its analogues CHF5074, CHF5075, CHF4802 and CHF4795 to TTR were performed by monitoring the displacement of the TTR-bound fluorescent probe *trans*-resveratrol. **C, Inhibition of TTR fibrillogenesis by flurbiprofen analogues.** Flurbiprofen, its analogues CHF5074, CHF5075, CHF4795 and CHF4802, diflunisal and tafamidis were analyzed for their ability to inhibit TTR aggregation at moderately acidic pH (pH 4.3) at three different incubation times (28, 68 and 99 hours). **D, Binding selectivity and structural stabilization of human plasma TTR by flurbiprofen analogues.** Flurbiprofen, its analogues CHF5074, CHF5075, CHF4795 and CHF4802, diflunisal and tafamidis were analyzed for their ability to selectively stabilize TTR in diluted human plasma at two different ligand concentrations (7.5 and 15 μ M), in the presence of 4 M urea, by means of Western Blot. The intensities of the gel bands at these ligand concentrations above the histograms are representative of the degree of dissociation of the TTR tetramer, upon denaturation, in the presence of each analyzed ligand. The percentages of TTR dissociation, normalized to 100% for TTR dissociation in the absence of ligands, represent the mean values (SD) of three independent replicates for each ligand.

Fig. 2. A, Fo-Fc electron density maps (blue), counteracted at 3.0 σ , showing the interface between the two monomers that line each flurbiprofen analogue (CHF5075, CHF4802, CHF5074, CHF4795) bound in the two cavities of the TTR tetramer. The protein is represented as cartoon, with the amino acids involved in the interactions as sticks. The ligand lays on the twofold axis and the two aromatic rings of the ligands fit quite well the electron densities, whilst the carboxylate region is less ordered. **B, Representations of the T4 binding cavities occupied by flurbiprofen** (Klabunde et al., 2000, PDB ID: 1DVT), **tafamidis** (Bulawa et al., 2012, PDB ID: 3TCT), and **diflunisal, in both forward and reverse binding modes** (Adamski-Wermer et al., 2004, PDB ID: 3D2T). The bound molecules are shown as sticks and the main interactions between each molecule and the residues Ser117, Leu110, Ala108, Leu17, Thr 106 and Lys15 in HBP1, HBP2, and HBP3 (Wojtczak et al., 1996) are highlighted.

Fig. 3: Close-up views of the superpositions of CHF5075 (cyan) with flurbiprofen (A) and tafamidis (B) bound in the T4 binding cavities of TTR. It is shown the displacement of TTR-bound flurbiprofen (orange) as compared to TTR-bound CHF5075 (cyan) (A) and the very similar positions of the innermost phenyl ring with the 3',5'-dichloro substituents of both tafamidis (magenta) and CHF5075 (cyan) (B).

Fig. 4: Close-up views of the superpositions of TTR-bound CHF5075 (cyan) with diflunisal (green) (A), which exhibits both forward and reverse binding modes, and with its analogues

2'-4'-dichloro-4-hydroxy-1,1'-biphenyl-3-carboxylic acid (lilac) (B) and 3',5'-difluorobiphenyl-4-carboxylic acid (pink) (C), which uniquely exhibit the reverse binding mode.

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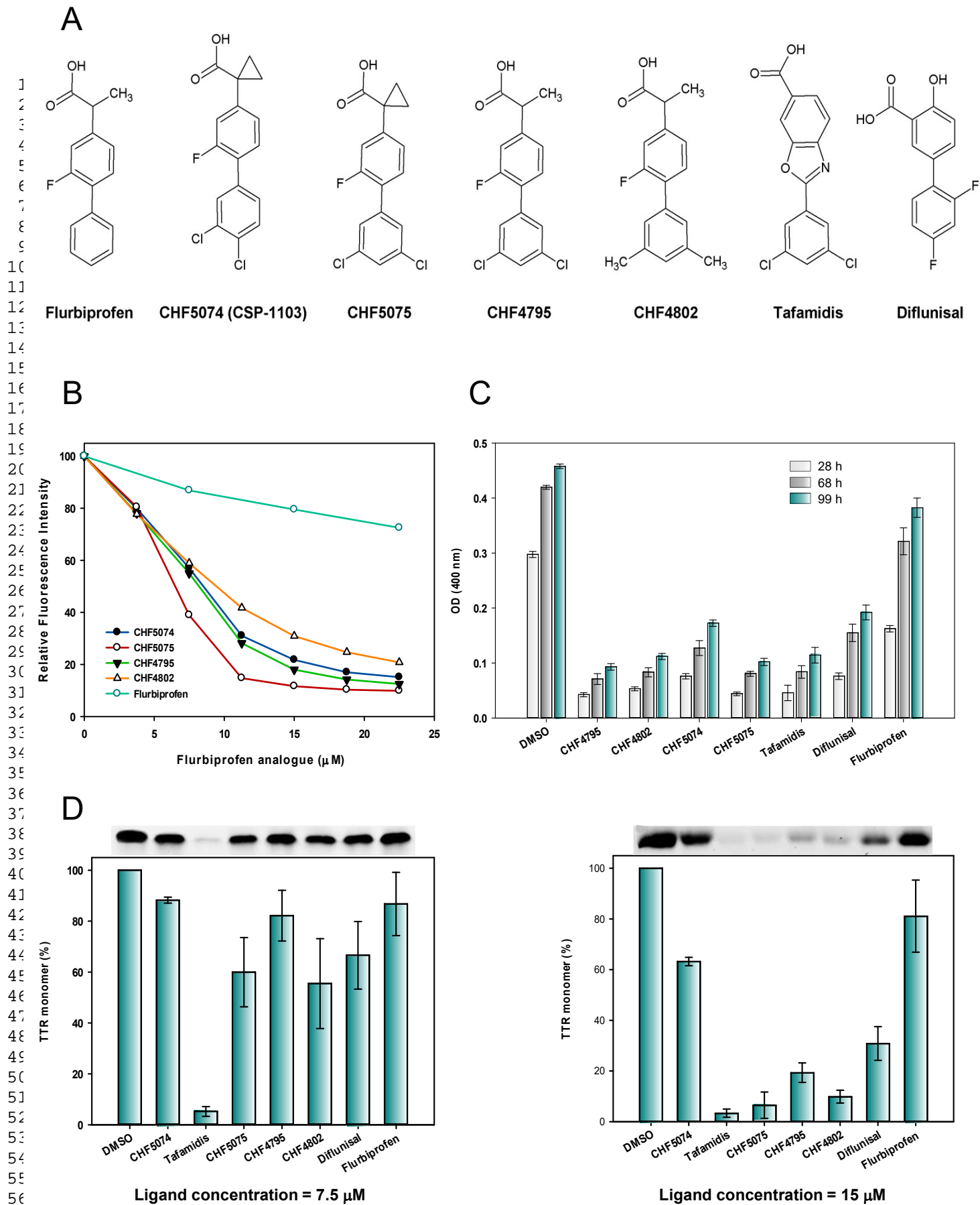
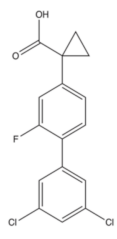
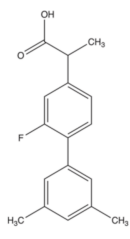


FIGURE 1

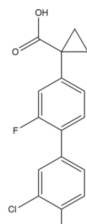
Figure



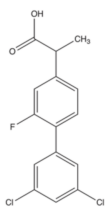
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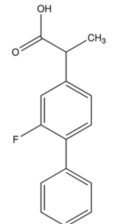
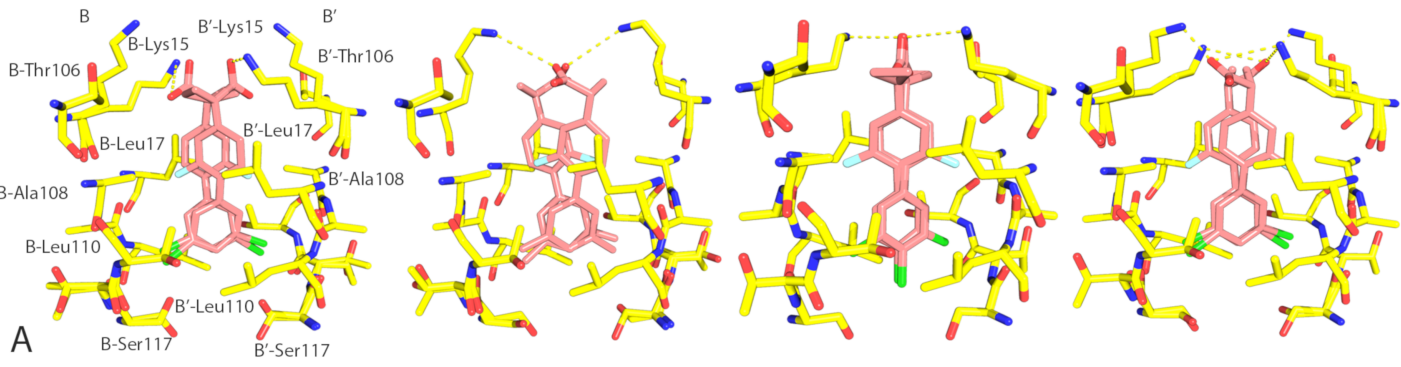
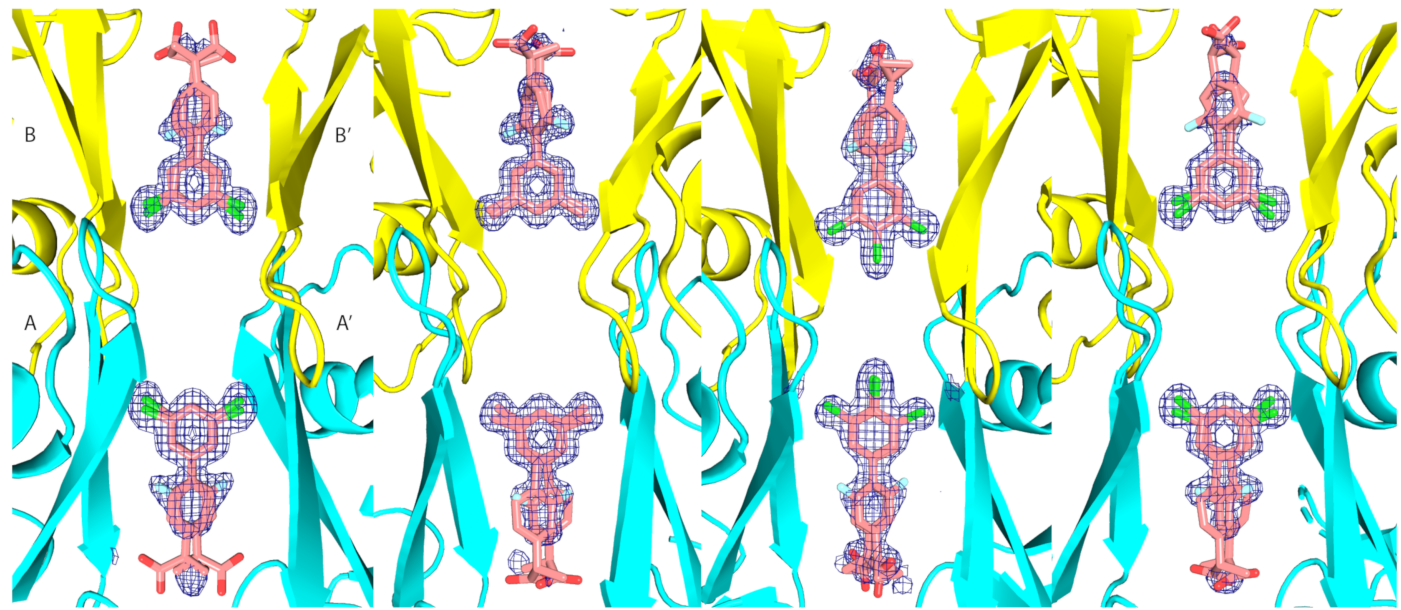
CHF4802



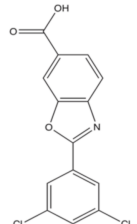
CHF5074 (CSP-1103)



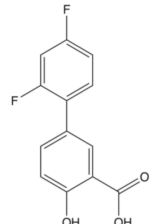
CHF4795



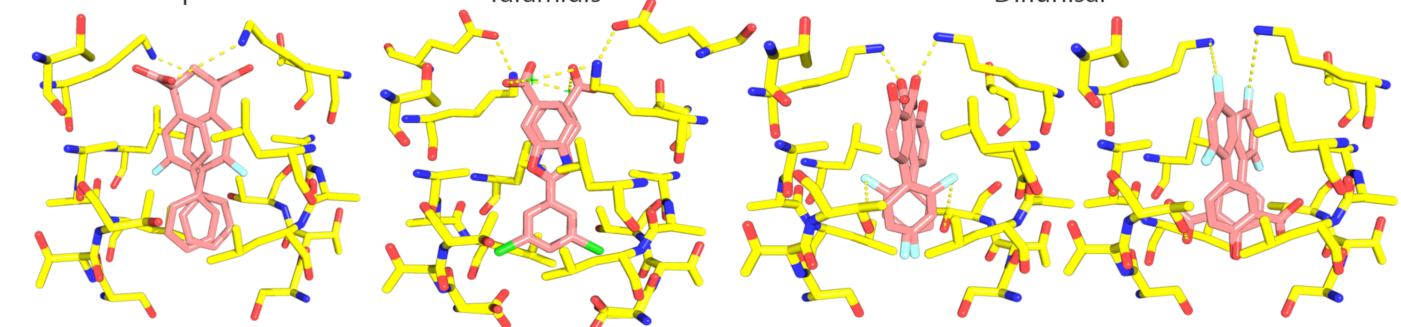
Flurbiprofen



Tafamidis



Diflunisal



B

FIGURE 2

Forward binding mode

Reverse binding mode

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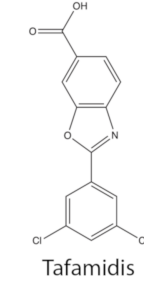
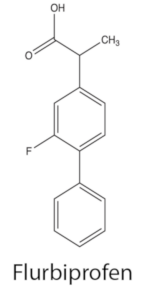
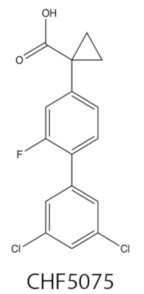
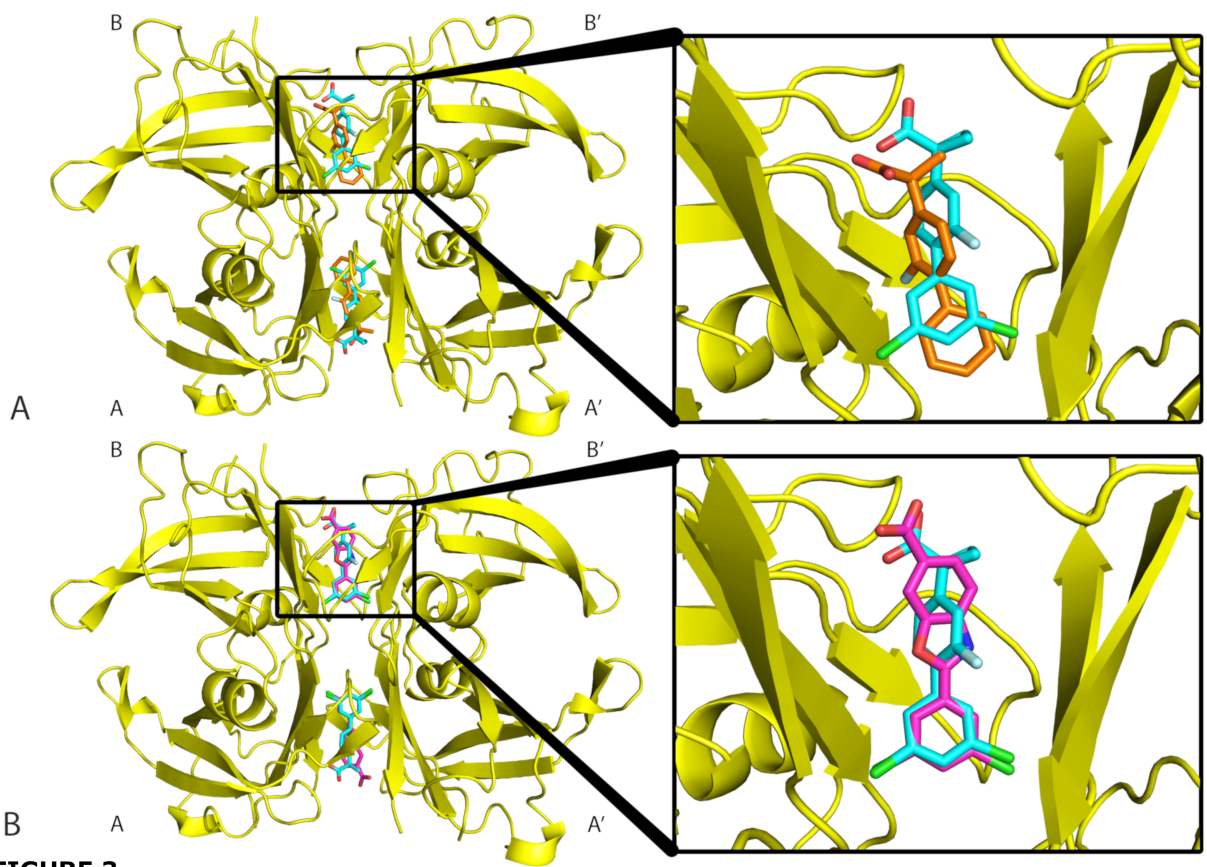


FIGURE 3

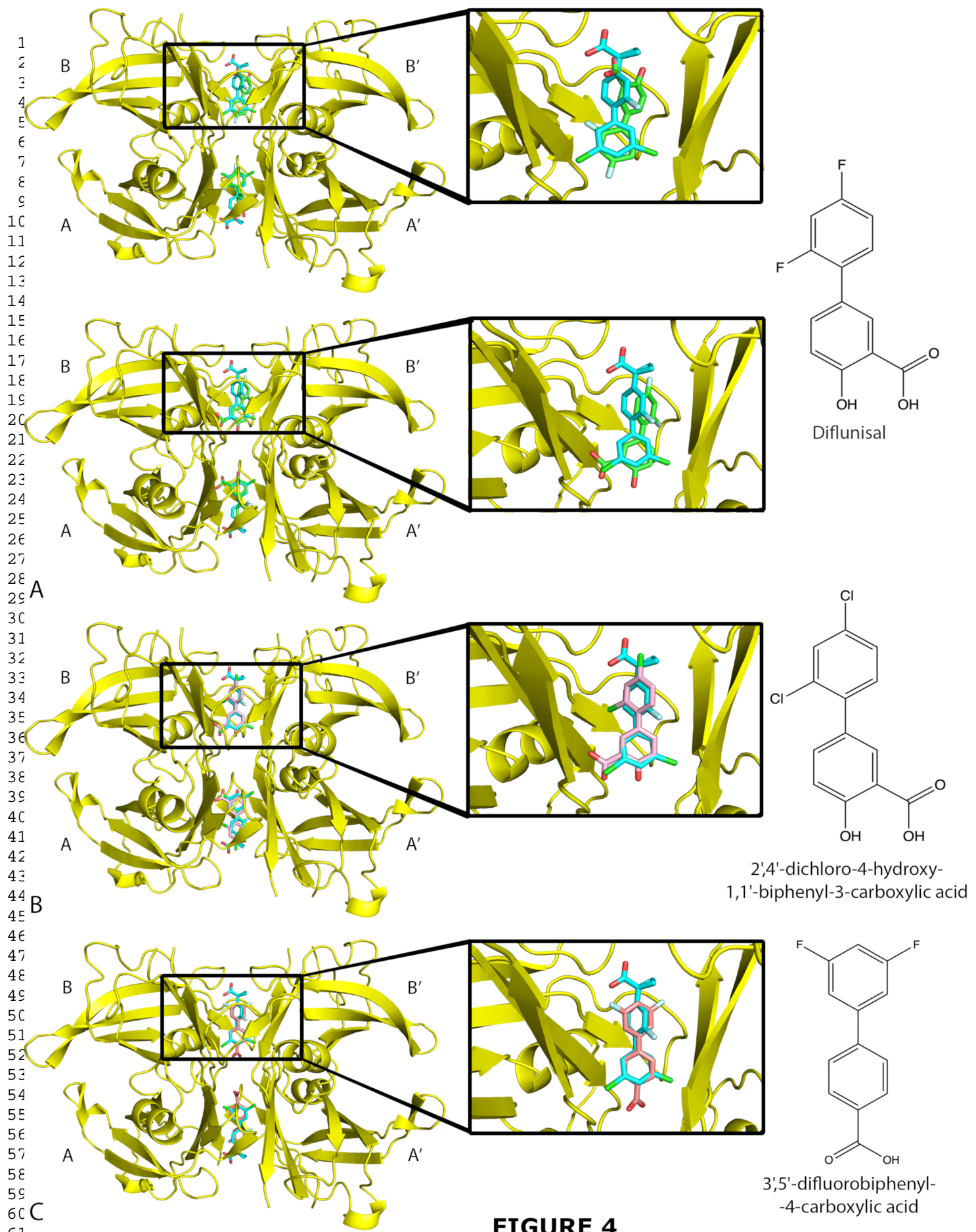


FIGURE 4

	TTR-CHF4795 (PDB ID: 6R68)	TTR-CHF5075 (PDB ID: 6R67)	TTR-CHF4802 (PDB ID: 6R66)	A25T TTR-CHF5074 (PDB ID: 6R6I)
Data collection				
Cell parameters (a, b, c, Å)	42.58; 85.07; 64.95	42.42; 84.99; 64.17	42.32; 84.96; 63.89	42.85; 85.81; 64.16
Wavelength (Å)	0.95372	0.95372	0.95372	0.97242
Resolution (Å)	64.95-1.45	42.45-1.30	42.32-1.30	64.16 – 1.47
Completeness (%)	100 (100)	99.9 (99.9)	100 (100)	99.8 (98.8)
Total number of reflections	533263	372262	707164	249643
Unique reflections	42661	57271	57493	40925
Multiplicity	12.5 (12.8)	6.5 (6.6)	12.3 (11.9)	6.1 (5.7)
Rmerge	0.090 (0.580)	0.040 (0.681)	0.081 (0.818)	0.043 (1.855)
Rpim	0.040 (0.250)	0.025 (0.423)	0.034 (0.359)	0.019 (0.859)
<I/sigma(I)>	21.8 (6.2)	18.7 (2.4)	20.5 (4.0)	17.6 (1.2)
Refinement				
Rcryst/Rfree	0.160/0.194	0.158/0.175	0.166/0.193	0.173 / 0.218
Protein atoms/solvent atoms	2083/303	2062/315	2081/360	1850/153
Geometry				
Ramachandran favored/outliers (%)	99.1/0.4	99.1/0.0	98.7/0.0	96.9 / 0.9
r.m.s.d. on bond length (Å), angles (°)	0.005/0.952	0.004/0.759	0.005/0.801	0.006 / 0.949
Ligand validation				
Real-space R value (RSR)	0.18(AA')/0.20(BB')	0.19(AA')/0.14(BB')	0.25(AA')/0.17(BB')	0.16(AA')/0.19(BB')
Real-space Correlation Coefficient (RSCC)	0.96(AA')/0.97(BB')	0.94(AA')/0.96(BB')	0.90(AA')/0.89(BB')	0.87(AA')/0.92(BB')

TABLE 1

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