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Comparison of binding interactions of dibromoflavonoids with transthyretin★✪

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The crystal structure of rat transthyretin (rTTR) complex with the dibromoflavone EMD21388 was determined to 2.3 Å resolution and refined to R = 0.203 and R_{free} = 0.288. Two different orientations of EMD21388, which differ in the channel penetration by 1.6 Å, were found in the A/C binding site of rTTR. The single ligand position observed in the B/D site is intermediate between the two positions found in the A/C site. The position of the dibromoflavone in the B/D site is similar to that reported for dibromoaurone in human TTR. The bromine atoms of EMD21388 form strong interactions in the P3 and P3′ pockets of rTTR. Due to the different molecular architectures of both ligands, dibromoflavone forms only one interaction with Lys-15 near the channel entrance, while direct interactions with the pair of Lys-15 were reported for dibromoaurone. The C3* methyl group of EMD21388 mediates the bridging interactions between two TTR subunits in the P2 pockets. The interactions of the O2* hydroxyl group of dibromoaurone with the Thr-119 side chain in the P3 pockets are not matched by similar interactions in EMD21388. Both these alternative interactions can explain the competitive binding of 3′,5′-dibromoflavonoids to transthyretin.

Flavones are a class of flavonoids with the phenyl substituent in position 2 of the benzo-γ-pyrone ring [1] (Fig. 1). The average diet in man contains about 1 g of different flavonoids [2] from fruits and vegetables. Flavones are also scavengers of free radicals, and therefore are of interest as antitumor and antioxidant agents [1–4]. Flavones also possess

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Abbreviation: TTR, transthyretin.

a broad spectrum of biological activity and inhibit many enzymes [1, 3–7]. The synthetic dibromoflavone EMD21388 has been shown to inhibit topoisomerase I thereby impairing the formation of topoisomerase–DNA complex and to act as an antiproliferative agent [8]. It is also a potent inhibitor of iodothyronine deiodinase [9] and competes strongly for the binding of thyroxine to transthyretin (TTR) with a binding affinity of 20 nM [10].

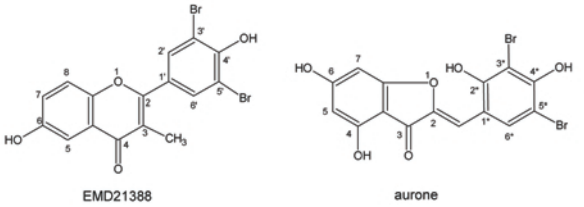

Figure 1. Schematic representation of EMD21388 and dibromoaurone with the numbering scheme.

Transthyretin is responsible for about 20% of thyroxine transport in the human circulatory system. The functional unit of TTR is a homotetramer with molecular mass of about 55 kDa. Each monomer of TTR is a β-barrel composed of 127 amino acids arranged into eight antiparallel β-strands forming two β-sheets and a short helical fragment [11]. The central channel of the tetramer contains two binding sites of T4 or its analogues. Each binding site consists of three halogen-binding pockets: the inner most P3, middle P2, and P1 pocket near the channel entrance [12]. Human TTR (hTTR) crystallizes in an orthorhombic lattice with a dimer in the asymmetric unit [11]. The twofold symmetry of the channel results in a statistical disorder of the ligand binding in the hTTR channel [12–15]. In contrast, rat transthyretin (rTTR), which has 85% sequence identity with hTTR, forms tetragonal crystals with the complete tetramer in the asymmetric unit [16] and no ligand disorder is observed. Therefore, detailed analysis of ligand interactions can be performed for the rTTR complexes. Structural data providing details of flavonoid binding in TTR have been reported for the hTTR-dibromoaurone complex [17] and preliminary data for the hTTR–EMD21388 complex [13].

The aim of this work was to compare the details of EMD21388 interactions in rat TTR with those reported for dibromoaurone in the hTTR complex and to define the mode of flavonoid interactions with rat transthyretin. Full details of human and rat TTR binding with EMD21388 will be reported at a later date [18].

EXPERIMENTAL

Rat TTR was incubated for 24 h with an excess of EMD21388 and crystallized at 293(1) K using the hanging drop vapor diffusion method from 55–65% ammonium sulfate, 0.1 M acetate buffer, pH 5.0. The rat TTR-EMD21388 complex crystallizes in the tetragonal space group $P4_32_12$ and is isomorphous with apo rTTR [16].

The apo rTTR model [16] was used for the initial phasing. The parameter and topology files for the flavone molecule were prepared based on its crystal structure [19] and AM1 quantum chemical calculations [20]. The X-ray diffraction data were collected to 2.3 Å and divided into working (95%) and test (5%) sub-sets. The refinement was carried out with the X-PLOR [21] and CNS [22] programs with the maximum likelihood (ML) target function and bulk solvent correction applied. The ML σ_A-weighted electron density maps were calculated and the model was verified with the graphics program O [23]. The occupancies of the ligand were refined with CNS to 0.29 and 0.25 for the two orientations found in the A/C site and 0.40 for the single orientation in the B/D site. The final refinement performed with 12–2.3 Å resolution data resulted in R = 0.203 and R_{free} = 0.288 for 15537 and 793 reflections, respectively. The relatively high value of R_{free} is a consequence of the lack of the N-terminal amino acids and the averaged

conformation of the flexible loops around residue 100 of all TTR monomers. The Ramachandran plot prepared with PROCHECK [24] revealed 86.4% of amino acids positioned in the most favored regions and the remaining 13.6% of residues in the additional allowed regions. The structure has been deposited with Protein Data Bank (PDB code: 1KGJ). A summary of the structure refinement is presented in Table 1.

RESULTS AND DISCUSSION

The electron density revealed that in the A/C site EMD21388 is bound in two orientations (with a refined occupancy of 0.29 and 0.25 for Fl-129 and Fl-128, respectively) (Fig. 2). The flavone Fl-129 is bound closer to the tetramer center, while Fl-128 is shifted towards the channel entrance. Their relative orientations might be described as a rotation around the non-crystallographic twofold symmetry axis, combined with a 1.6 Å shift along the channel axis. In the B/D site, the ligand (Fl-328) is found in a single orientation with an occupancy of 0.40 (Fig. 3). The position of Fl-328 in the B/D site is intermediate between those found for the flavone in the A/C site (Fig. 4). Despite these orientation differences, all bromine atoms are positioned in both the P3 and P3′ pockets of each binding site, whereas the benzo-γ-pyrone ring of the flavone occupies the P1 pockets.

The most important binding interactions of EMD21388 are formed in the inner-most pockets P3. Bromine atoms of Fl-129 interact with polar groups of Ser-117 and Thr-119 (Table 2). Similar interactions for the dibromophenolic ring of Fl-328 were detected in the B/D site. Hydrogen bonds of the phenolic hydroxyl of Fl-129 and Fl-328 with the Ser-117 side chain are observed. Similar orientations of both Fl-129 and Fl-328 results in an analogous network of polar contacts formed to the surrounding amino acids in the P3 and P1 pockets. The observed 1.6 Å shift of the Fl-128 molecule towards the channel entrance (Fig. 4) prevents such polar interactions in the P3 and P3′ pockets. Instead, the dibro-

Table. 1. Data collection and refinement statistics for the rTTR–EMD21388 complex

Space group	$P4_32_12$
Cell parameters (Å)	a = b = 82.545 c = 161.741
Reflections measured (% of possible)	23846 (92.8)
Resolution range used in refinement	12.0–2.3
Reflections used ($F > 2.5\sigma(F)$)	16330
R factor (15537 reflections)	0.203
R_{free} (793 reflections)	0.288
Protein atoms	3732
Water molecules	186
B factor (Wilson) ($Å^2$)	6.85
B factor (average) ($Å^2$)	13.81
RMS deviations from ideality:	
Bond distances (Å)	0.008
Angles (°)	1.296
Residues in different regions of Ramachandran plot	
most favored regions (%)	86.4
additionally allowed regions (%)	13.6
Positional error estimated from Luzzati plot (Å)	0.29

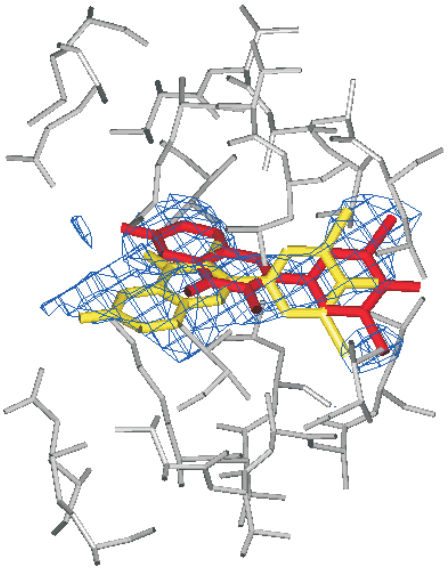

Figure 2. Electron density SA omit map (1σ) for the A/C site.

The EMD21388 ligand in the major population is shown in red (Fl129) and the minor population in yellow (Fl128).

mophenolic ring of Fl-128 forms hydrophobic interactions with Ala-108 and Ala-508 in the P2 pockets. This difference in the binding interactions explains why this orientation is less populated than the other two described above. In the A/C site Fl-129 forms direct interactions with Lys-15 *via* the O6 hydroxyl group (Fl-129 O6...Lys-15 NZ 3.36 Å). Due to differences in the ligand position, such interactions of Fl-128 are mediated by the 872OH2 water molecule and direct interactions between Lys-15 and the Fl-128 O4 carbonyl group are formed. In the B/D site, an interaction between Fl-328 O4 carbonyl oxygen and Lys-215 NZ is also observed. However, it is bridged by the 765OH2 water molecule. The O6 hydroxyl group of Fl-328 does not interact with either Lys-215 or Lys-615 because of their side chain conformations are different than those found in the A/C site.

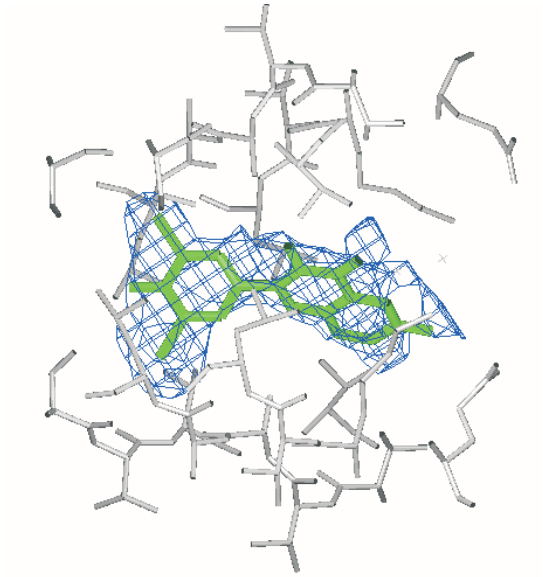

Figure 3. Electron density SA omit map (1σ) for the B/D site.

The ligand in the single population is shown in green (Fl328) and the surrounding protein residues in gray.

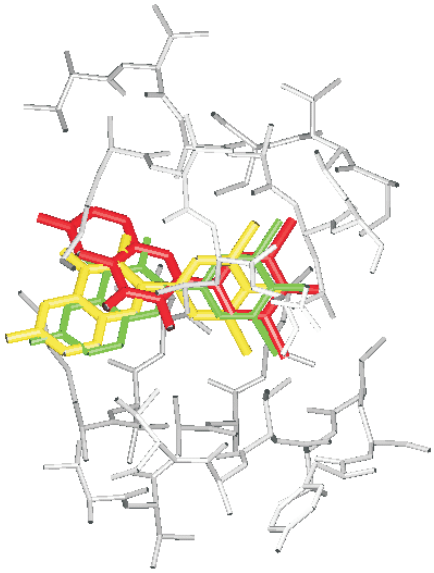

Figure 4. Superposition of the B/D site on the A/C site.

The major population of dibromoflavone in the A/C site is shown in red (Fl129), the minor one in yellow (Fl128) and the ligand in the single orientation found in the B/D site is shown in green (Fl328).

In all three ligand orientations the most important van der Waals interactions are formed in the P2 pockets between the C3* methyl group and Ala-108 CB and Leu-217 CD1. These interactions bridge the two TTR monomers constituting the binding site. Therefore, the presence of this methyl group of the ligand seems to be crucial for the effective EMD21388 binding and stabilization of the TTR tetramer.

Table 2. Interactions of EMD21388 with transthyretin in the rTTR–EMD21388 complex

A/C binding site Fl128(sof = 0.25) (Å)		A/C binding site Fl129(sof = 0.29) (Å)		B/D binding site Fl328(sof = 0.40) (Å)	
Br3..A108CB	3.64	Br3..T119OG1	2.90	Br3..T319OG1	3.16
Br3..A108C	3.47	Br3..S117CB	3.44	Br3..S317CB	3.41
Br3..A108O	3.36	Br3..S117C	3.50	Br3..S317O	3.42
		Br3..S1170	3.41	Br3..S317OG	3.46
Br5..A508O	3.30	Br5..S517OG	3.12	Br5..S717O	3.35
Br5..A508C	3.47	Br5..S517C	3.66		
Br5..L510N	3.54	Br5..T518N	3.57		
		Br5..T519N	3.38		
O4..K15NZ	3.02			O4'..S317OG	3.38
O4..761OH2	3.13	O4'..S117OG	3.76	O4..765OH2	3.26
O6..K415NZ	4.85	O4'..S517OG	3.17	O6..K215NZ	4.92
O6..872OH2	2.92	O6..K15NZ	3.36	O6..K615NZ	4.48
872OH2..K415NZ	3.37	O6..761OH2	2.87	K215NZ..765OH2	2.78
872OH2.E454OE1	2.98	K15NZ..761OH2	3.39	K615NZ..754OH2	3.24
761OH2..K15NZ	3.39	K15NZ..734OH2	3.16	765OH2..754OH2	2.94
C3*..A508CB	3.48	C3*..A108CB	3.28	C3*..A708CB	3.19
C3*..L17CD1	3.63	C3*..L417CD1	3.14	C3*..L217CD1	3.12

The forward binding of aurone in two different orientations has been described for each binding site of the hTTR–aurone complex [17]. Both orientations differ in the penetration of the binding site by 7 Å. In one of them, the aurone is bound close to the channel entrance and the model contains only the dibromophenolic ring of the ligand. Therefore this orientation is omitted from further discussion. The position of the second ligand is similar to that of Fl329 of this structure, with the bromine atom positions of both flavones nearly identical (Fig. 5).

In the TTR complexes of dibromoaurone [17] and dibromoflavone [13], the Ser-117 serines form hydrogen bonds with hydroxyl group O4' or O4* and bromine atoms of the ligands. However, the additional hydroxyl group in 2* position of dibromoaurone, not found in EMD21388, forms polar interactions with Thr-119 (Table 3) [17]. In the rTTR–EMD-21388 complex, the dibromoflavone does not form similar interactions. This results in significant differences in the side chain conformation of Thr-119 between the two complexes. Also significant differences in the net-

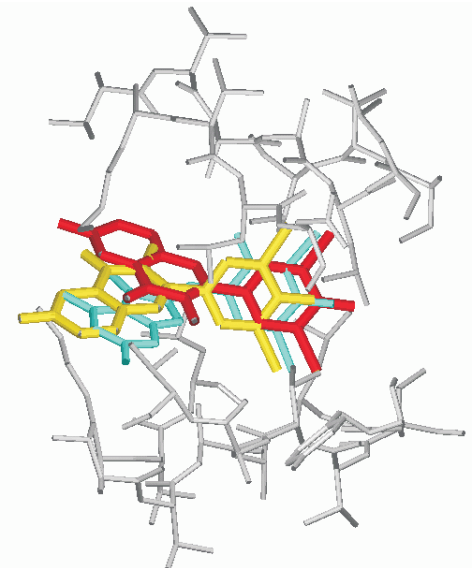

Figure 5. Superposition of the dibromoflavonoid complexes of TTR.

The EMD21388 dibromoflavone in the major population is displayed in red (Fl129), in the minor population in yellow (Fl128), and dibromoaurone in cyan.

Table 3. Interactions of aurone with transthyretin in the hTTR–aurone complex [17]

A/C binding site Fl9_527 (Å)		B/D binding site Fl9_725	
Br1...S517OG	3.17	Br1...S717OG	3.05
Br1...T519CG2	3.02	Br1...T719CG2	3.22
Br2...T519OG1	3.64	Br2...S317CB	3.64
Br2...T519CG2	3.19	Br2...S317OG	3.19
O4*...S117OG	3.36	Br2...T319CG2	3.44
O4*...S517OG	3.38	O4*...S317OG	3.48
O2*...T519OG1	2.57	O4*...S717OG	3.40
O41...K15NZ	3.17	O2*...T719OG1	3.47
O41...K415NZ	3.08	O41...K215NZ	3.52
		O41...K615NZ	2.68

work of binding interactions are observed in the P1 pockets. The dibromoaurone molecule forms direct hydrogen bonds with both Lys-15 and Lys-415 amino groups [17]. Such interactions are possible because of the additional CH bridge between the ring systems of dibromoaurone (Fig. 1). The lack of such a bridge in EMD21388 results in either a single direct interaction with Lys-15 (Fl-129) or an interaction mediated by water molecule (Fl-128). The difference in the ligand architecture is also reflected in the position of the dibromoaurone ring system closer by 2.3 Å to the channel surface than that of EMD21388. The ring system of dibromoaurone penetrates the cleft between Thr-106 and Lys-15 side chains, while EMD21388 is bound along the channel axis. However, the aurone molecule does not form any interactions bridging two TTR monomers with the hydrophobic interactions analogous to those described in the P2 pocket for the C3* methyl group of EMD21388. Therefore, the stabilizing effect of dibromoaurone seems to be smaller than for EMD21388.

CONCLUSIONS

In both binding sites of rTTR the EMD21388 ligand is bound in the forward mode with bromine atoms occupying the inner-most P3 pockets. In the A/C site of rTTR two orientations of the ligand were found. EMD21388 in the major population is bound along the channel axis, 1.6 Å deeper than the similarly oriented ligand of the minor population. In the B/D site, only a single ligand orientation was detected which corresponds to a position intermediate between the two populations reported for the A/C site. The interactions of the dibromophenolic ring formed in the P3 pockets by rTTR–EMD21388 are similar to those reported for the hTTR–dibromoaurone complex. The binding of the aurone in the P3 pockets TTR seems to be more effective because of the additional hydrogen bond of O2* hydroxyl with Thr-119, which has no equivalent in the EMD21388 complex. The interactions of both dibromoflavonoids with Lys-15 in the P1 pockets are similar. The EMD21388 flavone is positioned near the channel axis, while the benzopyrone ring system of the dibromoaurone ligand is bound in the cleft between Lys-15 and Thr-106, about 2.3 Å closer to the channel surface. However, EMD21388 forms bridging interactions between the TTR monomers *via* the C3* methyl group. Therefore, EMD21388 might better stabilize the TTR tetramer and prevent the amyloidogenic transformation, although its binding affinity is similar to that of dibromoaurone.

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