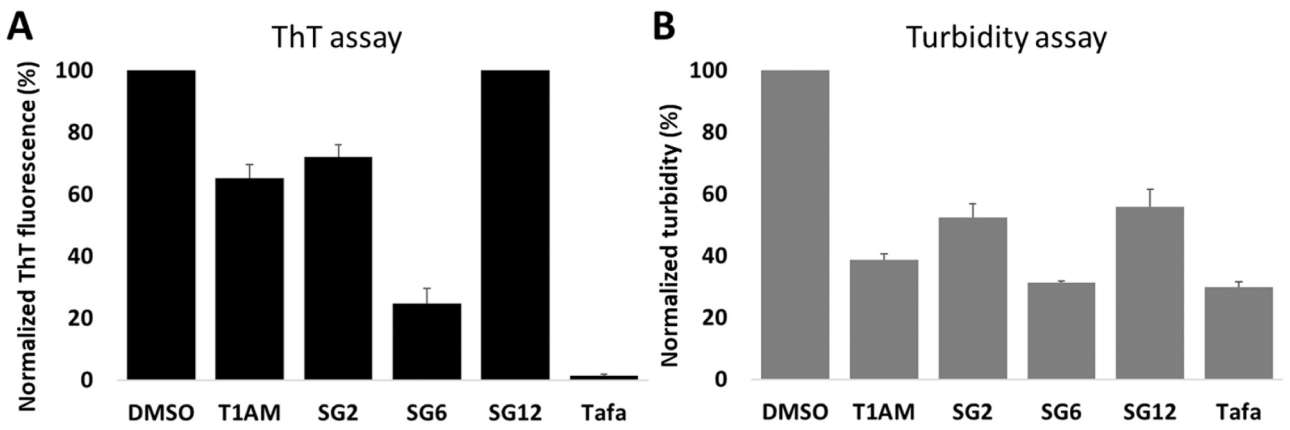

Fig. 3. TTR aggregation assay results in the presence/absence of 5-fold T1AM and its analogs. The results without any ligand (DMSO) or with 5-fold tafamidis (Tafa) were included for comparison. TTR aggregation was induced by mildly acidic conditions (the concentration of TTR was 32 μM) and was subsequently quantified using thioflavin-T (ThT) fluorescence (A) and turbidity (B) measurements after 3-day incubation at 37 °C (the daily measurement results of ThT fluorescence are shown in Fig. S5). The measurements were conducted in triplicate, and the standard deviations from those were denoted as error bars in the figure.

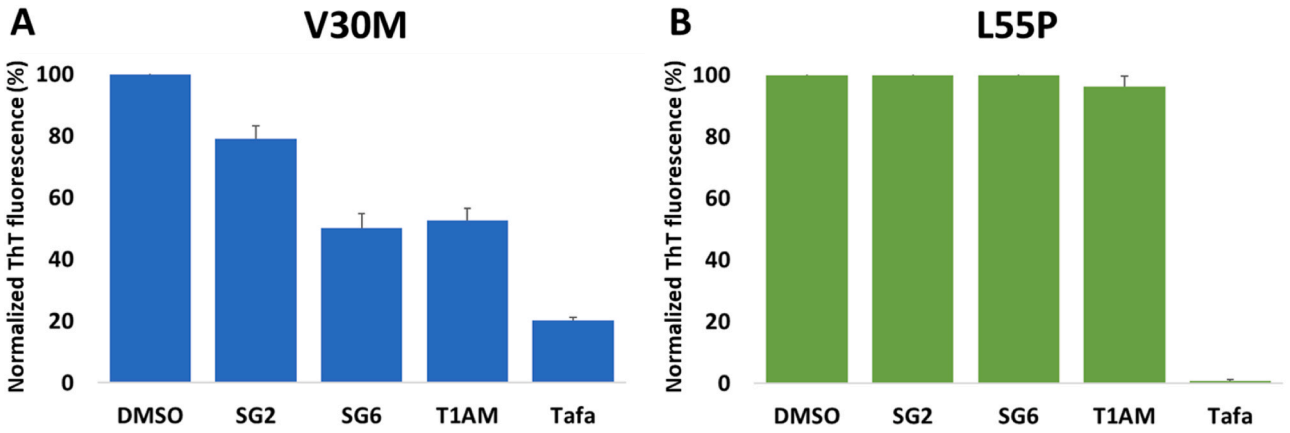

Fig. 4. Aggregation assay results of two pathogenic TTR variants, V30M (A) and L55P (B), in the presence/absence of 5-fold T1AM and its analogs. The results without any ligand (DMSO) or with 5-fold tafamidis (Tafa) were included for comparison. TTR aggregation was induced by mildly acidic conditions (the concentration of TTR was 32 μM) and was subsequently quantified using thioflavin-T (ThT) fluorescence measurements after 3-day incubation at 37 °C (the daily measurement results of ThT fluorescence are shown in Fig. S7). The measurements were conducted in triplicate, and the standard deviations from those were denoted as error bars in the figure.

monitored using ThT fluorescence and turbidity measurements (Fig. 3 and S5). As a reference molecule, we examined the effect of tafamidis, and we only focused on the data obtained with 5-fold ligands, because the results obtained in the presence of 1-fold T1AM analogs showed less distinguishable effects on TTR aggregation (Fig. S6). The measurement results indicated that among T1AM analogs, SG6 was the most effective inhibitor of TTR aggregation, while the effect of SG12 was negligible in ThT assays. This observation is consistent with the NMR results, where the binding interaction between TTR and SG6 was stronger than the others, whereas SG12 caused minimal perturbations to the NMR signals of TTR. In contrast, the relatively weak inhibitory effects of SG2 on TTR aggregation were somewhat inconsistent with the NMR results because the NMR signal perturbations by SG2 were comparable with those by SG6.

In addition, we repeated the same aggregation assay with two pathogenic variants of TTR, V30M and L55P, which exhibited more amyloidogenic propensities than WT [39,40]. Upon treatment of 5-fold T1AM analogs to V30M TTR, TTR aggregation was again slowed down significantly; the inhibitory effect of SG6 was the most pronounced among T1AM analogs (Fig. 4A and S7A). In contrast, T1AM analogs could not cause any noticeable effect on the aggregation of L55P TTR (Fig. 4B and S7B).

3.3. Molecular docking simulation of TTR with T1AM analogs

To investigate the details of the interaction between TTR and T1AM analogs at atomic resolution, we conducted molecular docking simulations and compared the resultant complex models. The binding of T4 with TTR includes the ionic bridge between K15 (TTR) and the carboxylic acid (T4) and the polar interaction between E54 (TTR) and the protonated amine (T4) [14]. Therefore, we aimed to investigate the interaction of the single amine (for T1AM, SG2, and SG12) or carboxylate (for SG6) moiety of T1AM analogs in the TTR binding site using molecular docking simulation, comparing results to the pose of tafamidis (Fig. 5) [21].

Overall, the docking models provided a detailed view of the interactions between TTR and T1AM analogs (Fig. 5, gray). First, although the docking model predicted that both SG2 and SG12 interact similarly in the TTR-binding site, the longer spacer of SG2 allows a stronger interaction with E54 of TTR (Fig. 5AB, gray). The aniline moiety of SG2 and SG12 lies in the inner region of TTR, towards S115, S117, and T119. In contrast, the molecular docking results for T1AM were completely different; a reversed pose of T1AM was suggested, thus placing the phenolic OH group near K15 (Fig. 5C, gray). This interaction is common and frequent within ligands crystallized with TTR [41,42] and is

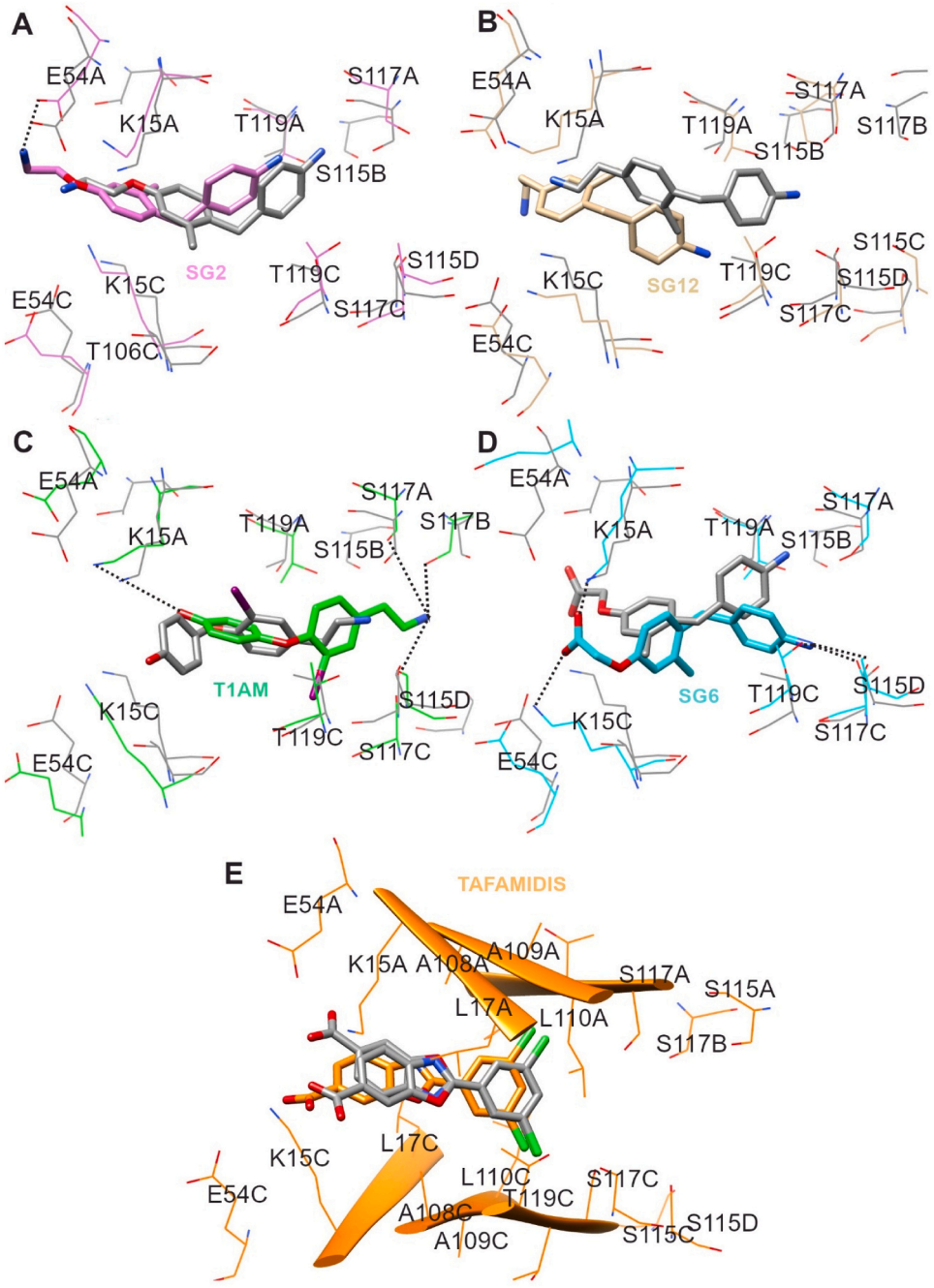

Fig. 5. Superposition of the docking results (gray), and the average structure of MD simulation of SG2 (A; pink), SG12 (B; tun), T1AM (C; green), SG6 (D; cyan) and tafamidis (E; orange). The chain (A, B, C, or D) from which each residue originates is indicated along with the residue information.

preferred over the unusual interaction between the amine and E54. The amine chain occupies the serine-rich region, engaging S117 of both chains A and C, whereas the same locus was accompanied by the aniline group of SG compounds. Docking of SG6 (Fig. 5D, gray) is, as expected, in the classical forward mode, guaranteeing the ionic bond between carboxylate and K15 and the polar interaction between the aniline moiety and S117. The binding mode described here for T1AM analogs is very similar to the classical arrangement of other kinetic stabilizers of TTR, such as tafamidis (Fig. 5E, gray) [21]: a strong ionic binding anchors the carboxylate to K15, in the outer region of the binding site, and many lipophilic interactions stabilize the ligand in the central part of the pocket, which is composed by three parallel sheets (residues 15–17, 108–110 and 117–119 for both specular chains of interfaced dimers). The two chlorines protrude towards the inner polar pocket, rich in serines (S115 and S117) belonging to all neighboring monomers, and strongly involved in the stabilization of the TTR tetramer [43].

3.4. Molecular dynamics (MD) simulation of the TTR-ligand complexes

To further evaluate the stability of the complexes between TTR and T1AM analogs, we conducted MD simulations. After constraint relaxation (frame 40), the protein backbone reached a Cα RMSD stability of 2 Å in all complexes (Fig. 6). In the TTR-SG2 complex, (Fig. 6A) a shift of the RMSD plot is detected, due to the specular movement of the ligand between the two interfaced dimers, near frame 400; the RMSD of SG2 returned stable at an average value of 1.5 Å, like all the other ligands.

Ultimately, despite some system fluctuations, all compounds retained their binding disposition. The final structures of the MD

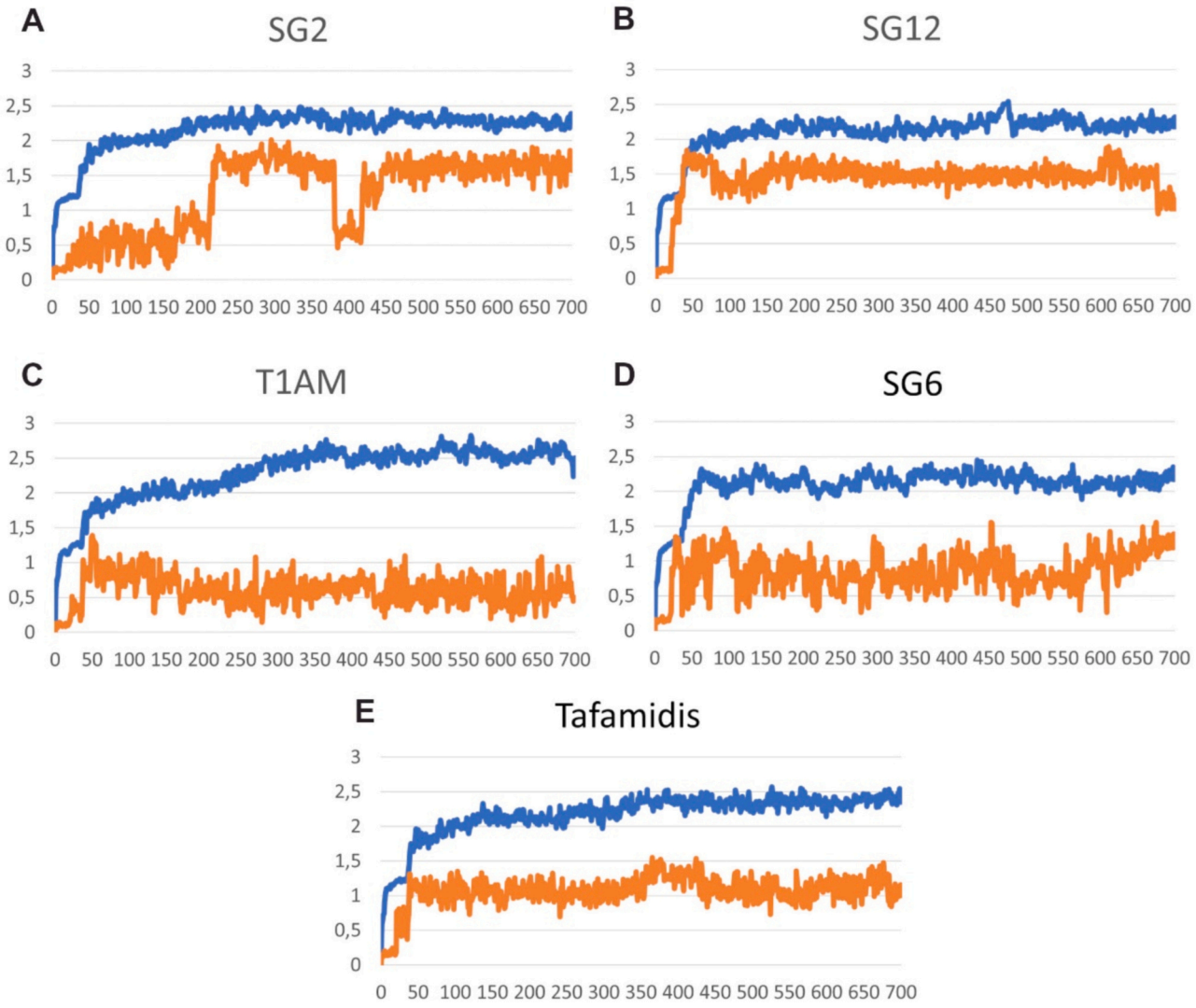

Fig. 6. RMSD of the TTR Cα atoms (blue line) and ligand heavy atoms (orange line) in molecular dynamics simulations of the TTR-ligand complexes: SG2-TTR (A), SG12-TTR (B), T1AM-TTR (C), SG6-TTR (D) and tafamidis-TTR (E).

simulations superimposed on the initial docking pose are shown in Fig. 5. It was evident from the model of SG12-TTR complex (Fig. 5B, tan) that K15 attempted to reach E54 and detach it from the protonated amine of the ligand. Not only the initial interaction with E54 failed, the interactions with serines in the inner pocket were also lost. The behavior of SG2 in complex with TTR was different (Fig. 5A, pink): although the initial interaction between the aminoethyloxy chains of SG2 and E54 was retained during the simulation, it failed to maintain stable interaction with S117 and T119, probably because the molecule length is not sufficient to reach the inner region when the protonated amine anchors E54. T1AM (Fig. 5C, green), in its inverted pose, maintained a polar interaction with K15, which appeared weaker than a hydrogen bond, because K15 was engaged in strong ionic bonding with E54. The ligand still seemed to engage in fruitful interactions with serine residues. The comparison with SG6 (Fig. 5D, cyan), showed how the usual ionic interaction with K15 of the carboxylate group of SG6 guarantees a better interaction with respect to the similar docking pose of SG2. Fig. 5E shows the results obtained with tafamidis, which were used as a control and subjected to the same docking and dynamics procedure. Notably, the final structure of tafamidis overlaps well with the experimental pose, validating the suitability of our approaches.

To explore the role of the key residues in ligand stabilization and their correlation with inhibitory activity against TTR aggregation, we analyzed the MD trajectories in detail (Fig. 7). This helped us to identify the effect of fluctuations on interactions between the residues and the ligands over time, which were not discernible in Fig. 5. Residues involved in the interaction with a distance less than 5 Å from the ligand were analyzed, with a focus on two regions: the outer region, which usually binds with K15, and the inner region, which interacts with S115 and S117. Water molecules were also analyzed separately: the one closest (less than 3 Å apart) to the ligand end in the K15 region, and the other closest to the opposite end of the ligand in the inner region.

For SG2 (Fig. 7A), the involvement of many residues, particularly S117 and T119, is evident in more than half of the unconstrained simulation. In addition, E54 stably interact with the amine of SG2. The protonated amine group of K15 was located approximately 4.5 Å from the amine group of SG2 for the entire simulation, interacting with E54 and weakly with the ether oxygen atom of the SG2 linker (not reported in Fig. 5 for clarity). The first level of interactions is however with water, in both the outer and inner regions of the binding sites. This means that during the simulation the interaction of SG2 with E54, S117 and T119 was recurrently displaced by the solvent. Regarding SG12 (Fig. 7B), the initial interaction between SG12 and E54 loosened during the unconstrained simulation, as did the nearness of K15. Only fluctuating interactions between the aniline group and residues S117 and T119 (Fig. 7B; black and orange lines, respectively) were visible. In a large

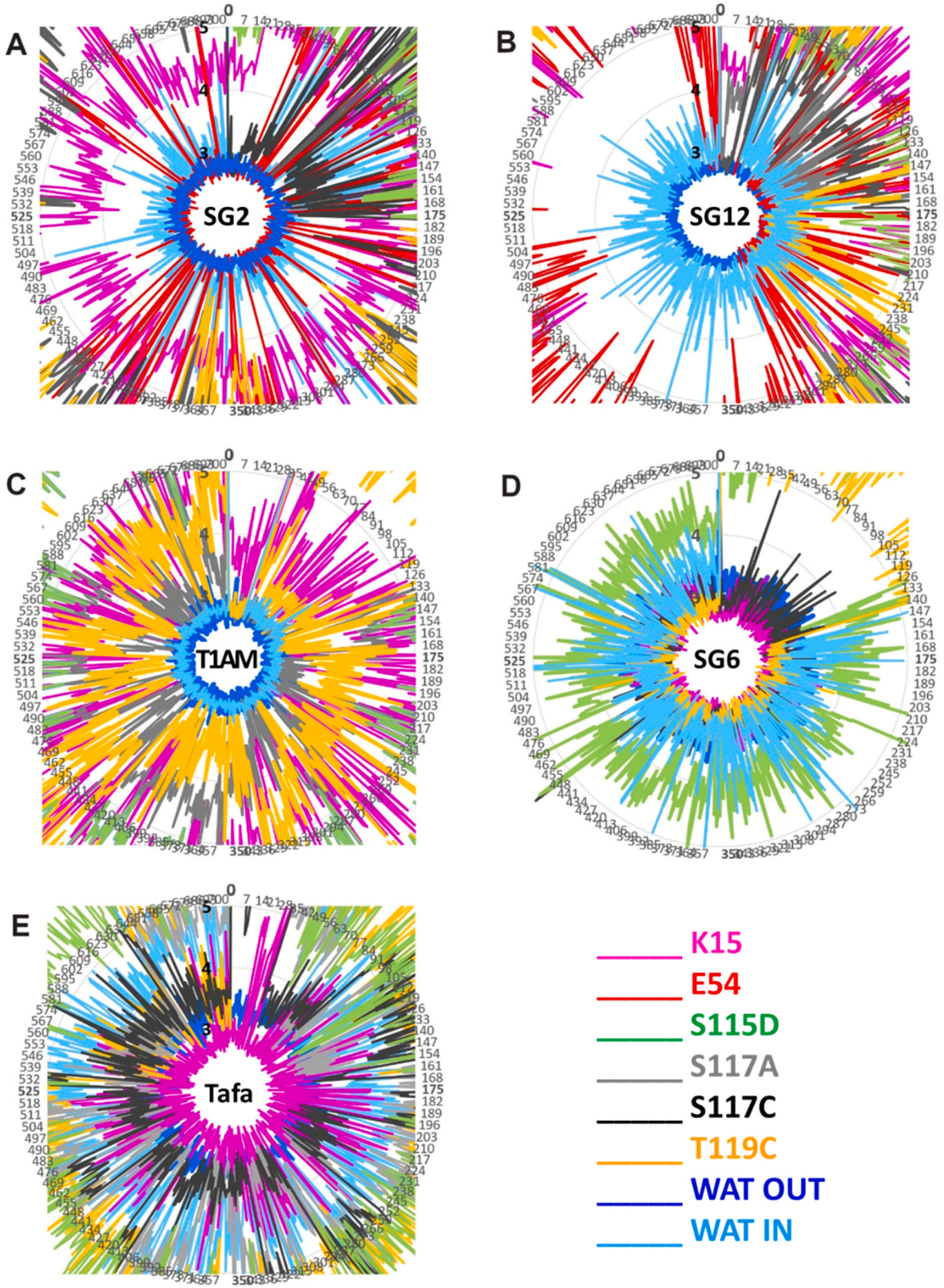

Fig. 7. Distances of the key residues of TTR from the ligand during molecular dynamics simulation of the TTR-ligand complexes. Only residues involved within distances less than 5 Å are reported: K15 (magenta), E54 (red), S115D (S115 from the chain D; green), S117A (gray), S117C (black), T119C (gold). For K15 and E54, due to the high flexibility, the closest value between chains A and C has been reported for clarity. Water molecules less than 3 Å from the protonated amine of SG2 and SG12, the phenolic hydroxyl of T1AM, and the carboxylate of SG6 and tafamidis are reported in blue (WAT OUT), whereas water molecules less than 3 Å from the aniline nitrogen of SG2, SG12 and SG6 and chlorines of tafamidis are reported in cyan (WAT IN).