Supporting Information

Dissecting the allosteric FXR modulation: A chemical biology approach using guggulsterone as chemical tool

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Table of Contents

Molecular Dynamics Results

The molecular dynamics (MDs) trajectories were analysed using the Desmond tools and taking into account two parameters: the backbone root mean square deviation (RMSD) and the interaction energy between ligand and protein (for the **1** and **4** holo systems). The former was functional to check the protein stability, the latter was important to evaluate the effect of the mutations in the S2/S3 sites. In Figure S1 are reported the RMSD of all the MDs regarding the apo- (Figure S1a) and holo- forms of FXR (Figure S1b/c/d). It is important to note that all the mutations displayed a light incidence on the apo forms system stability, producing movements in line with the wild type (WT) recorded one, without any particular jump indicating a loss of secondary structure elements (Figure S1a) or heavy conformational rearrangements. The same check has been done for the holo forms and interestingly the RMSD regarding the T396E MD of compound **1** (Figure S1b) highlighted a different trend with respect to the Y270E and T396F ones. In particular, the impact of this mutation is higher than the other for the FXR complexes with guggulsterone (GS), with an increase of the RMSD values during the whole 100ns trajectory (orange line).

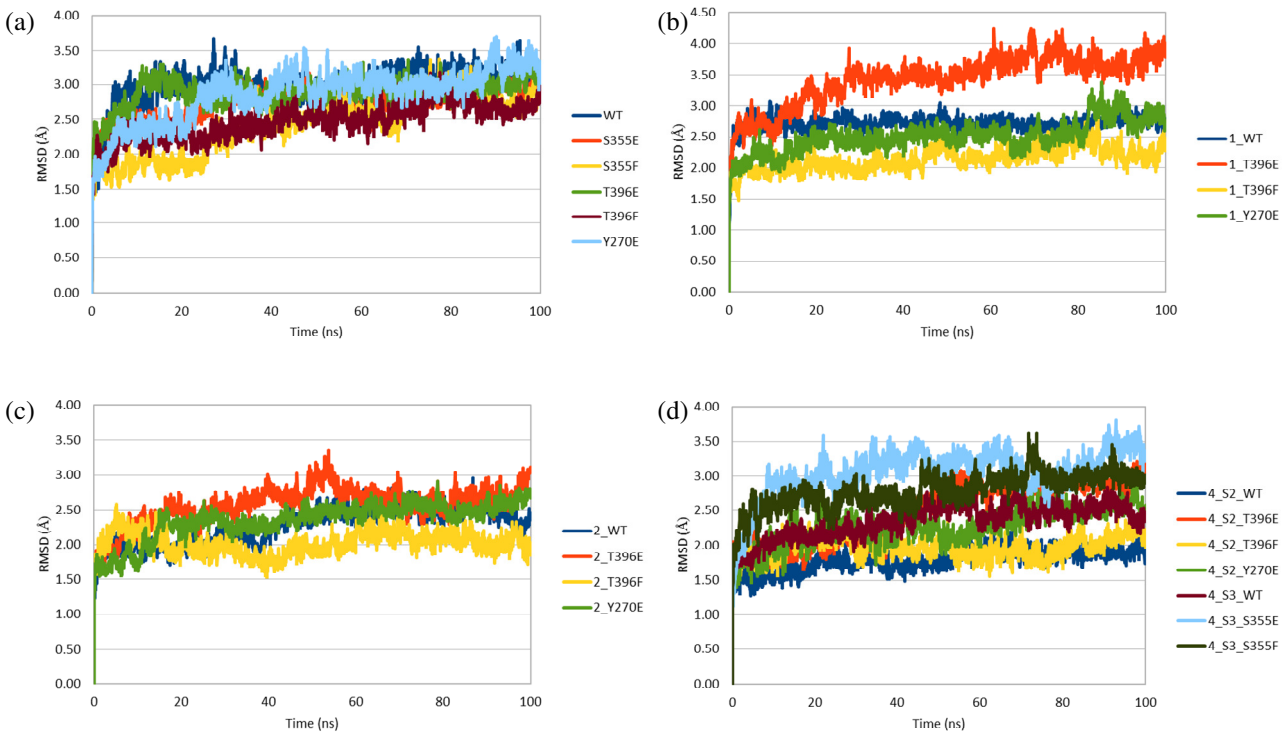

Figure S1. Backbone RMSD results of the WT and mutated FXR apo proteins (a) and in complex with ligand **1** (b), **2** (c) and **4** (d). For all the complex systems the comparison with the WT forms is always reported, moreover for compound **4** the results are including both the S2 and S3 poses as starting structure.

A similar trend can be observed for the same mutation (T396E) also for the MDs in complex with OCA (**2**, Figure S1c) and UPF-930 in S2 oriented starting pose (**4**, Figure S1d). Moreover, for the UPF-930 MDs in its S3 oriented pose, it is evident that both the mutations produced higher RMSD values with respect to the WT one (Figure S1d; cyan, dark green and dark red line respectively).

The subsequent analysis focused on the interaction energies developed by **1** and **4** in complex with FXR WT and mutated forms (Figure S2a and S2b, respectively). The GS displayed a stronger interaction in the T396E dynamic with respect to the WT and Y270E ones, reaching and maintaining a value of nearly 55 kcal/mol during the whole trajectory (orange line).

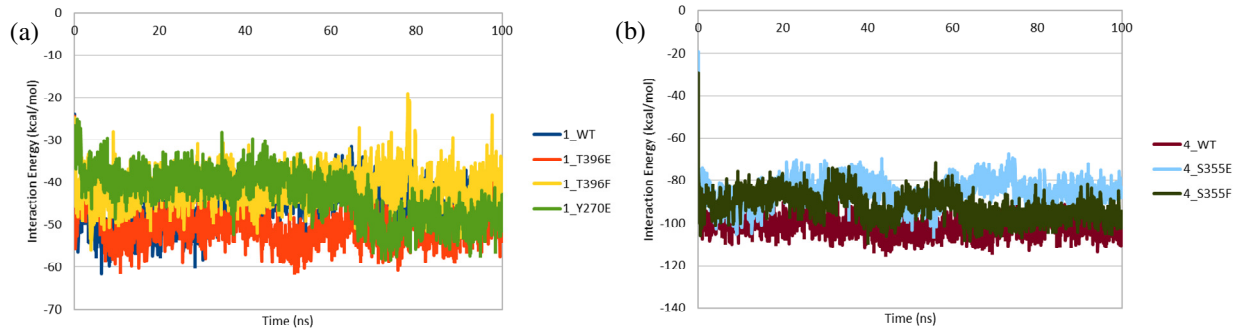

Figure S2. Protein-ligand interaction energies of the WT and mutated FXR proteins and in complex with **1** (a) and **4** (b).

In contrast, the S3 mutants destabilized the 4 interaction in that FXR binding site region, resulting in less favourable energies with respect to the WT (Figure S2b; dark green, cyan and dark red lines respectively).

In conclusion, these data indicated the T396E mutant as suitable to see an improved antagonizing effect by enforcing the **1** S2 binding. On the other hand, the S3 mutations resulted in a destabilizing effect of the **4** S3 binding coupled to higher backbone RMSD values (Figure S1d), thus suggesting to be useful point mutations to gain insights into this binding area.

Western blotting analysis

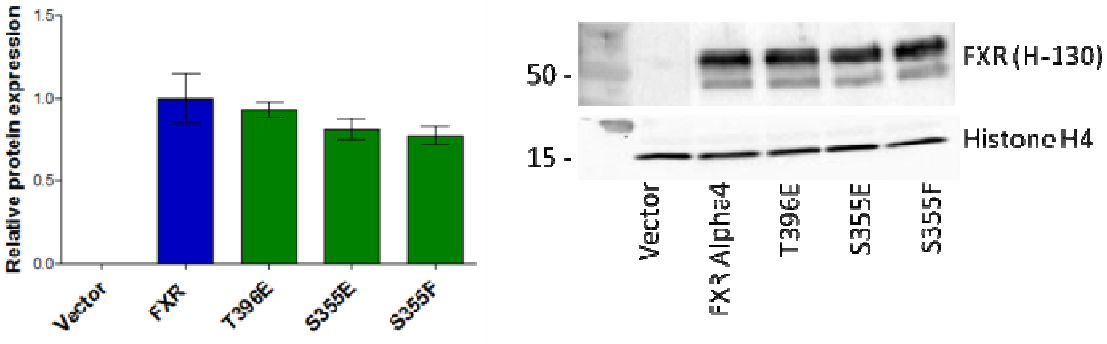

Figure S3. Comparable protein expression level among the strains expressing FXR wild type and its T396E, S355E and S355F mutants detected by western blotting analysis and respective graphical quantification.