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Supporting information for article:

The structural study of mutation-induced inactivation of human muscarinic receptor M4

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**Table S1**  Supporting information RMSD values of the mutation-induced inactive M4 structure with other classical mAChRs structures

| State | mAChRs (PDB code) | ligand   | RMSD value (Å) |
|-------|-------------------|----------|----------------|
| Inactive | M1R(5CXV)   | tiotropium | 1.227          |
|        | M2R(3UON)     | QNB*     | 0.713          |
|        | M3R(4U15)     | tiotropium | 0.701          |
|        | M4R(5DSG)     | tiotropium | 0.699          |
| Active  | M1R(6OIJ)     | iperoxo  | 1.472          |
|        | M2R(6OIK)     | iperoxo  | 1.251          |

*R-(2)-3-quinuclidinyl benzilate.
Table S2  Supporting information $R_{free}/R_{work}$ values of the mutation-induced inactive M4 structure with three fatty acids from docking results

| HMDB ID | Compound name | Chemical structure | $R_{free}/R_{work}$ (%) |
|---------|---------------|--------------------|------------------------|
| None    | None          |                    | 26.42/23.14            |
| 0010212 | 17,18-EpETE   | ![17,18-EpETE](image) | 26.79/21.65            |
| 0034295 | Floionolic acid | ![Floionolic acid](image) | 26.45/21.72            |
| 0010217 | 5-oxo-ETE     | ![5-oxo-ETE](image)  | 26.70/21.63            |
**Figure S1**  Protein purification and crystal packing of the mutation-induced inactive M4 structure. (a) Analytical size-exclusion chromatography trace of purified mutation-induced M4 protein. (b) Crystal picture of M4 obtained in lipidic cubic phase. (c-d) Crystal packing and the overall structure of mutation-induced inactive M4. M4 and PGS are coloured in teal blue and orange, respectively. Six-point mutations in the crystallization construct are shown as pink spheres.
Figure S2  Residues in the rational designed ionic network are conserved in class A GPCRs. (a-c) TM2, TM3 and TM7 sequence conservation across 286 human class A GPCRs (including 81 orphan receptors and non-olfactory receptors). (d) The percentages of the polar amino acids in the conserved positions of 2×50 in TM2, 3×39 of TM3 and 7×49 in TM7.
Figure S3 Molecular docking and molecular dynamic simulation results of tiotropium using the mutation-induced inactive M4 structure. (a) The overall comparison with M4-tiotropium structure (PDB code 5DSG). (b) The interaction residues in the orthosteric binding pocket are similar except for side chains of W164$^{4.57}$, Y416$^{6.51}$ and Y439$^{7.39}$. 
Figure S4  Electron density maps for three different fatty acids. (a) The initial omit 2|F_o|-|F_c| map. (b-d) The refined 2|F_o|-|F_c| maps for 17,18-EpETE (b), Floionolic acid (c) and 5-oxo-ETE (d), after refinements.
Figure S5  Omit electron density maps for the mutation-induced M4 structure. (a) The initial omit 2|F_o|-|F_c| map (grey) for seven transmembrane domains (I, II, III, IV, V, VI and VII). Contoured at 2.0σ at 3.0 Å. (b-f) The omit 2|F_o|-|F_c| (blue) and |F_o|-|F_c| (green) maps for tyrosine lid (b), residues Trp6.48 (c), the R(R449)PxxY (Y453) motif (d), DRY motif (e), and ionic network residues in the mutation-induced M4 structure (f). The sidechains of the residues are selected for omit map generation. 2|F_o|-|F_c| omit maps contoured at 1.0σ at 3.0 Å, |F_o|-|F_c| omit maps contoured at 3.5σ at 3.0 Å.