Supporting Information

The role of orthosteric building blocks of bitopic ligands for muscarinic M1 receptors

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Chemistry

1) **Data S1**. Syntheses and experimental data of target compounds.

General Procedure for the Synthesis of the Quinolone-Isoxo Derivatives **3-C4**, **3-C6**, **3-C8**, and 3**-C10**

To a solution of bromoalkyl 4-oxo-quinoline-3-carboxamides **C-C4**, **C-C6**, **C-C8**, and **C-C10** in acetonitrile (5 mL), 2 to 4 equivalents of isoxo-base and a catalytic amount of K_2CO_3 were added. The reaction was heated at 80 °C making use of microwave assistance. After completion of the reaction (4-48 h) controlled by TLC (CH_2Cl_2/MeOH = 85:15, R_f = 0.50 – 0.70), the mixture was cooled to room temperature. The solvent was distilled off. The so obtained solid was crystallized from acetonitrile, filtered and dried *in vacuo*. Compounds **3-C8** and **3-C10** were purified via column alumina chromatography using petroleum CH_2Cl_2/MeOH 9:1 as eluent system.

*N-(4-(1-benzyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxamido)butyl)-4-(isoxazol-3-yloxy)-N,N-dimethylbut-2-yn-1-aminium bromide **3-C4***

Beige solid; 15% yield; mp 101-104 °C; R_f = 0.55 (MeOH/NH_4NO_2 (0.2 M) = 3:2); ^{1}H NMR ($CDCl_3$): 1.86-1.73 (m, 4H, NH-CH_2-C**H$_2$**-C**H$_2$**), 3.43 (s, 6H, $^+$N(C**H$_3$**)$_2$), 3.55-3.53, (m, 2H, NH-C**H$_2$**), 3.72- 3.66 (m, 2H, C**H$_2$**-N$^+$), 4.87 (s, 2H, ≡C-C**H$_2$**-N$^+$), 4.95 (s, 2H, O-C**H$_2$**-C≡), 5.53 (s, 2H, C**H$_{2benzyl}$**), 6.03 (d, 2H, **H**-4$_{isoxo}$, *J*=1.8) 7.15- 7.14 (m, 2H, C**H**$_{phenyl}$), 7.46 - 7.36 (m, 5H, **H**-7, **H**-8, C**H**$_{phenyl}$), 8.12 (dd, 1H, **H**-5, *J* = 3.0, J_{HF} = 8.7), 8.18 (s, 1H, **H**-5 $_{isoxo}$, *J*=1.8), 8.95 (s, 1H, **H**-2), 10.09 (t, 1H, N**H**, *J* = 5.6). ^{13}C NMR ($CDCl_3$): 19.84, 26.58, 29.69, 37. 45, 50.68, 54.83, 57.18, 58.15, 63.74, 75.89, 86.86, 96.31, 112.01, 112.24, 119.26 (d, J_{CF} = 7.7), 121.50 (d, J_{CF} = 25.4), 123.19, 126.08, 128.83, 129.47, 129.71, 133.85, 135.84, 148.422, 158.70, 160.30, 165.85, 170.39, 176.58. MS (ESI) m/z [M]$^+$ Calcd for $C_{30}H_{32}FN_4O_4^+$: 531,24 Found: 530.7. LS-MS purity 96.58%.

6-(1-benzyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxamido)-N-(4-(isoxazol-3-yloxy)but-2-yn-1-yl)-N,N-dimethylhexan-1-aminium bromide ***3-C6***

Ochre solid; 28% yield; mp 96-103 °C; R_f = 0.74 (MeOH/NH_4NO_2 (0.2 M) = 3:2); ^{1}H NMR ($CDCl_3$): 1.48-1.42 (m, 4H, NH-CH_2-CH_2-C**H$_2$**-C**H$_2$**), 1.68-1.64 (m, 2H, NH-CH_2-C**H$_2$**), 1.74-1.76 (m, 2H, NH-CH_2-CH_2-CH_2-CH_2-C**H$_2$**), 3.48-3.45 (m, 8H, NH-C**H$_2$**, N^+(C**H$_3$**)$_2$), 3.66-3.72, (m, 4H, NH-C**H$_2$**, C**H$_2$**-N), 4.90 (s, 2H, ≡C-C**H$_2$**-N^+), 4.95 (s, 2H, O-C**H$_2$**-C≡), 5.50 (s, 2H, C**H$_2$**$_{benzyl}$), 6.03 (d, 2H, **H**-4$_{isoxo,}$ *J*=1.8), 7.13- 7.15 (m, 2H, C**H**$_{phenyl}$), 7.35-7.33 (m, 4H, **H**-8, C**H**$_{phenyl}$), 7.42 - 7.44 (m, 1H, **H**-7), 8.13 (dd, 1H, **H**-5, *J* = 3.0, J_{HF} = 8.8), 8.20 (d, 1H, **H**-5 $_{isoxo,}$ *J*=1.8), 8.93 (s, 1H, **H**-2), 9.96 (t, 1H, N**H**, *J* =5.6). ^{13}C NMR: ($CDCl_3$) 22.56, 25.61, 26.26, 29.16, 38.77, 50.63, 54.64, 57.15, 58.09, 64.10, 75.87, 86.79, 96.28, 111.93, 112.16, 119.26 (d, J_{CF} = 7.7), 121.61 (d, J_{CF} = 25.4), 126.06, 128.77, 129.42, 133.91, 135.83, 148.38, 158.63, 160.31, 164.72, 170.37, 176.00. MS (ESI) m/z $[M]^+$ Calcd for $C_{32}H_{35}FN_4O_4^+$: 559,27 Found: 559.5. LS-MS purity 96.86%.

1-((8-(1-benzyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxamido)octyl)dimethyl-l4-azanyl)-4-(isoxazol-3-yloxy)but-2-yn-1-ylium bromide **3-*C8***

Ochre solid; 30% yield; mp 113-116 °C; R_f = 0.67 (MeOH/NH_4NO_2 (0.2 M) = 3:2); ^{1}H NMR ($CDCl_3$): 1.25-1.55 (m, 8H, NH-CH_2-CH_2-C**H$_2$**-C**H$_2$**-C**H$_2$**-C**H$_2$**), 1.64-1.71 (m, 4H, NH-CH_2-C**H$_2$**, C**H$_2$**-CH_2-N^+), 3.42 (s, 6H,N^+(C**H**$_3$)$_2$), 3.50-3.54 (m, 4H, NH-C**H$_2$**, C**H$_2$**-N^+), 4.84 (s, 2H, $^+$N-C**H$_2$**-C≡), 4.95 (s, 2H, O-C**H$_2$**-C≡), 5.50 (s, 2H, C**H$_2$**$_{benzyl}$), 6.03 (d, 2H, **H**-4$_{isoxo,}$ *J*=1.8), 7.13- 7.15 (m, 2H, C**H**$_{phenyl}$), 7.33-7.44 (m, 5H, **H**-7, **H**-8, C**H**$_{phenyl}$), 8.13-8.15 (m, 1H, **H**-5), 8.19 (s, 1H, **H**-5$_{isoxo}$), 8.92 (s, 1H, **H**-2), 9.94 (t, 1H, N**H**, *J* =5.6). ^{13}C NMR: ($CDCl_3$) 22.64, 24.06, 25.85, 26.64, 28.65, 28.67, 29.41, 39.01, 50.76, 53.66, 57.10, 58.11, 64.41, 75.72, 86.99, 96.31, 110.69, 111.77, 119.22 (d, J_{CF} = 8.1), 121.76 (d, J_{CF} = 23.4), 123.19, 126.07, 128.78, 129.43, 129.71, 129.85, 133.94, 148.35, 148.35, 155.69, 160.28, 164.64, 170.38, 176.05. MS (ESI) m/z $[M]^+$ Calcd for $C_{34}H_{39}FN_4O_4^+$: 587,30 Found: 587.6. LS-MS purity 95.04%.

10-(1-Benzyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxamido)-N,N,N-trimethyldecan-1-aminium bromide ***3-C10***

Ochre solid; 30% yield; mp 108-110 °C; R_f = 0.71 (MeOH/NH_4NO_2 (0.2 M) = 3:2); ^{1}H NMR ($CDCl_3$): 1.22-1.41 (m, 12H, NH-CH_2-CH_2-C**H₂**-C**H₂**-C**H₂**-C**H₂**-C**H₂**-C**H₂**-C**H₂**), 1.61-1.68 (m, 4H, NH-CH_2-C**H₂**, C**H₂**-CH_2-N^+), 3.44 (s, 6H,N^+(C**H**$_3$)$_2$), 3.46-3.58 (m, 4H, NH-C**H₂**, C**H₂**-N^+), 4.86 (s, 2H, $^+$N-C**H₂**-C≡), 4.95 (s, 2H, O-C**H₂**-C≡), 5.49 (s, 2H, C**H₂**$_{benzyl}$), 6.03 (d, 2H, **H**-4$_{isoxo,}$ *J*=1.8), 7.13- 7.15 (m, 2H, C**H**$_{phenyl}$), 7.33-7.44 (m, 5H, **H**-7, **H**-8, C**H**$_{phenyl}$), 8.13-8.15 (m, 1H, **H**-5), 8.17 (s, 1H, **H**-5$_{isoxo}$), 8.93 (s, 1H, **H**-2), 9.92 (t, 1H, N**H**, *J* =5.5). ^{13}C NMR: ($CDCl_3$) 22.76, 25.97,26.97, 28.93, 29.11, 29.54, 39.27, 50.71, 54.76, 57.13, 58.07, 64.40, 75.70, 86.91, 96.28, 111.97, 112.20, 119.17 (d, J_{CF} = 8.0), 121.4 (d, J_{CF} = 25.1), 126.05, 128.75, 129.40, 129.84, 129.92, 133.96, 135.82, 148.82, 158.59, 160.23, 161.06, 164.56, 170.37, 176.0. MS (ESI) m/z $[M]^+$ Calcd for $C_{36}H_{44}FN_4O_4^+$: 615.33. Found:615.15. LC-MC purity 98.17%.

General Procedure for the Synthesis of the Quinolone-Oxotremorine-M Derivatives **4-C4, 4-C6, 4-C8, and 4-C10**

The reaction was performed by combining bromoalkyl 4-oxo-quinoline-3-carboxamides **C-C4**, **C-C6**, **C-C8**, and **C-C10**, 2 to 5 equivalents of oxotremorineM-base and a catalytic amount of K_2CO_3 in acetonitrile (3 mL) using the microwave irradiation (80 °C). The reaction was completed after 4-24h and cooled to room temperature. TLC (CH_2Cl_2/MeOH = 85:15, R_f = 0.20 – 0.35). The solvent was evaporated under reduced pressure. The final compounds were purified by recrystallization in acetonitrile or by alumina chromatography, when crystallization was not feasible (CH_2Cl_2/MeOH 100:0→90:10 as eluent system).

*N-(4-(1-benzyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxamido)butyl)-N,N-dimethyl-4-(2-oxopyrrolidin-1-yl)but-2-yn-1-aminium bromide 4-****C4***

Beige solid; 35% yield; mp 172-174 °C; R_f = 0.20 (MeOH/CH_2Cl_2 = 95:5); ^{1}H NMR (CD_3OD): 1.71-1.78 (m, 2H, NH-CH_2-C**H₂**),1.89-1.92 (m, 2H, NH-CH_2-CH_2-C**H₂**), 2.02-2.09 (m, 2H, **H**-4$_{oxo}$), 2.36 (t, 2H, **H**-

3_{oxo}, *J*=8.1), 3.18 (s, 6H, $N^+(C\mathbf{H_3})_2$), 3.49-3.56 (m, 6H, N^+-C**H**$_2$, **H**-$5_{oxo,}$ NH-C**H**$_2$), 4.24 (s, 2H, **H**-6_{oxo}), 4.35 (s, 2H, **H**-9_{oxo}), 5.73 (s, 2H, C**H**$_{2benzyl}$), 7.24-7.26 (d, 2H, C**H**$_{phenyl}$), 7.32-7.39 (m, 3H, C**H**$_{phenyl}$), 7.54 (ddd, 1H, **H**-8, *J*=3.0, *J*=7.7, *J*=9.4), 7.83 (dd, 1H, **H**-7, *J*=4.3, *J*=9.4), 8.08 (dd, 1H, **H**-5 *J*=3.0, *J*=9.0), 9.03 (s, 1H, **H**-2).^{13}C NMR: (CD_3OD) 19.11, 21.62, 27.98, 31.90, 33.45, 39.49, 48.43, 51.60 55.70, 58.99, 65.45, 72.79, 88.62, 112.47 (d, J_{CF} =23.4), 122.41 (d, J_{CF} =8.2), 123.19 (d, J_{CF} =25.7), 128.14, 129.91, 130.73, 131.25, 136.80, 138.02, 150.54, 163.17, 167.54, 177.91, 206.43. MS (ESI) m/z $[M]^+$ Calcd for $C_{31}H_{36}FN_4O_3^+$: 531.28. Found: 531.10. HPLC purity 98.73%.

((6-(1-benzyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxamido)hexyl)(methyl)(4-(2-oxopyrrolidin-1-yl)but-2-yn-1-yl)-l4-azanyl)methylium bromide ***4-C6***

Beige solid; 39% yield; mp 181-182 °C; R_f = 0.22 ($MeOH/CH_2Cl_2$ = 95:5); ^{1}H NMR (CD_3OD): 1.48-1.55 (m, 2H, NH-CH_2-C**H**$_2$),1.69-1.73 (m, 2H, NH-CH_2-CH_2-C**H**$_2$), 1.82-1.86 (m, 2H, NH-CH_2-CH_2-C**H**$_2$), 2.04-2.08 (m, 2H, **H**-4_{oxo}), 2.36 (t, 2H, **H**-3_{oxo}, *J*=8.1), 3.16 (s, 6H, $N^+(C\mathbf{H_3})_2$), 3.42-3.47 (m, 2H, N^+-C**H**$_2$), 3.49 (t, 2H, NH-C**H**$_2$, *J*=6.8), 3.53 (t, 2H, **H**-5_{oxo}, *J*=7.1), 4.24 (s, 2H, **H**-6_{oxo}), 4.34 (t, 2H, **H**-9_{oxo}, *J*=1.7), 5.72 (s, 2H, C**H**$_{2benzyl}$), 7.24-7.26 (m, 2H, C**H**$_{phenyl}$), 7.29-7.39 (m, 3H, C**H**$_{phenyl}$), 7.50-7.55 (m, 1H, **H**-8), 7.82 (dd, 1H, **H**-7, *J*=4.2, *J*=9.4), 8.07 (dd, 1H, **H**-5 *J*=3.0, *J*=9.0), 9.02 (s, 1H, **H**-2).^{13}C NMR: (CD_3OD) 18.69, 23.50, 26.87, 27.45, 30.21, 30.74, 31.48, 39.88, 48.32, 51.18, 55.15, 58.61, 65.41, 72.41, 88.10, 112.02 (d, J_{CF} =22.1), 121.97 (d, J_{CF} =8.1), 122.73 (d, J_{CF} =25.3), 127.70, 129.53, 130.30, 130.80, 136.38, 150.06, 162.71, 166.84, 177.46 (d, J_{CF} =17.5), 205.73. MS (ESI) m/z $[M]^+$ Calcd for $C_{33}H_{39}FN_4O_3^+$: 558.30. Found: 559.15. HPLC purity 98.05%.

1-benzyl-N-(8-(dimethyl(4-(2-oxopyrrolidin-1-yl)but-2-yn-1-yl)-l4-azanyl)octyl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxamide ***4-C8***

Beige solid; 16% yield; mp 174-178 °C; R_f = 0.27 ($MeOH/CH_2Cl_2$ = 95:5); ^{1}H NMR (CD_3OD): 1.29-1.31 (m, 4H, NH-CH_2-CH_2-CH_2-C**H**$_2$-C**H**$_2$), 1.43-1.46 (m, 4H, NH-CH_2-CH_2-C**H**$_2$-CH_2-CH_2-C**H**$_2$), 1.67-1.68 (m,

2H, NH-CH_2-C**H**$_2$, C**H**$_2$-CH_2-N^+), 2.05-2.11 (m, 2H, **H**-4 $_{oxo}$), 2.37 (t, 2H, **H**-3_{oxo}, *J*=8.1), 3.13 (s, 6H,$N^+(CH_3)_2$), 3.38-3.42 (m, 2H, N^+-C**H**$_2$), 3.47 (t, 2H, NH-C**H**$_2$, *J*=6.7), 3.53 (t, 2H, **H**-5_{oxo}, *J*=7.1), 4.23 (s, 2H, **H**-6_{oxo}), 4.32 (s, 2H, **H**-9 $_{oxo}$), 5.71 (s, 2H, C**H**$_{2benzyl}$), 7.25-7.27 (m, 2H, C**H**$_{phenyl}$), 7.31-7.38 (m, 3H, C**H**$_{phenyl}$), 7.50-7.55 (m, 1H, **H**-8), 7.81 (dd, 1H, **H**-7, *J*=4.1, *J*=9.5), 8.07 (dd, 1H, **H**-5 *J*=2.8, *J*=9.5), 9.01 (s, 1H, **H**-2).^{13}C NMR: (CD_3OD) 18.28, 23.13, 25.57, 26.76, 27.51, 29.60, 30.05, 31.04, 32.57, 39.67, 47.82, 50.67, 54.65, 58.19, 65.06, 71.95, 87.66, 111.61 (d, J_{CF} =23.2), 121.4, 121.53 (d, J_{CF} =7.4), 122.42 (d, J_{CF} =25.8), 127.00, 127.26, 129.10, 129.87, 135.97, 149.65, 162.26, 166.37, 176.46 (d, J_{CF} =115.4), 206.50. MS (ESI) m/z $[M]^+$ Calcd for $C_{35}H_{44}FN_4O_3^+$: 587,34. Found: 587.20. HPLC purity 98.17%.

((10-(1-benzyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxamido)decyl)(methyl)(4-(2-oxopyrrolidin-1-yl)but-2-yn-1-yl)-l4-azanyl)methylium bromide ***4-C10***

White solid; 27% yield; mp 169-172 °C; R_f = 0.33 ($MeOH/CH_2Cl_2$ = 85:15); ^{1}H NMR (CD_3OD): 1.39-1.49 (m, 12H, NH-CH_2-CH_2-C**H**$_2$-C**H**$_2$-C**H**$_2$-C**H**$_2$-C**H**$_2$-C**H**$_2$), 1.64-1.68 (m, 2H, NH-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-CH_2-C**H**$_2$), 1.77-1.79 (m, 2H, NH-CH_2-C**H**$_2$), 2.06-2.10 (m, 2H, **H**-4_{oxo}), 2.38 (t, 2H, **H**-3_{oxo}, J=8.1), 3.17 (s, 6H,$N^+(CH_3)_2$), 3.38-3.42 (m, 2H, N^+-C**H**$_2$), 3.46 (t, 2H, NH-C**H**$_2$, *J*=6.9), 3.55 (t, 2H, **H**-5_{oxo}, *J*=7.1), 4.24 (s, 2H, **H**-6_{oxo}), 4.33 (s, 2H, **H**-9 $_{oxo}$), 5.72 (s, 2H, C**H**$_{2benzyl}$), 7.24-7.26 (m, 2H, C**H**$_{phenyl}$), 7.29-7.39 (m, 3H, C**H**$_{phenyl}$), 7.53 (ddd, 1H, **H**-8, *J*=3.1. *J*=7.8, *J*=9.4), 7.81 (dd, 1H, **H**-7, *J*=3.0, *J*=9.0), 8.08 (dd, 1H, **H**-5 *J*=3.0, *J*=9.0), 9.02 (s, 1H, **H**-2).^{13}C NMR: (CD_3OD) 18.71, 23.63, 27.29, 28.11, 30.11, 30.32, 30.37, 30.45, 30.55, 34.49, 33.01, 40.18, 50.10, 51.12, 55.13, 58.63, 65.52, 72.39, 88.07, 112.05 (d, J_{CF} =24.4), 121.90, 121.9 (d, J_{CF} =8.1), 122.69 (d, J_{CF} =25.5), 127.67, 129.51, 130.29, 130.77 (d, J_{CF} =7.1), 136.39, 137.57, 150.07, 162.69, 166.77, 177.46, 208.90. MS (ESI) m/z $[M]^+$ Calcd for $C_{37}H_{47}FN_4O_3^+$: 614.36. Found: 615.25. HPLC purity 95.67%.

Pharmacology

2) **Data S2**. Maximum agonist effect and potency of bitopic compounds in Gα/PLC-β3 split-luciferase complementation assays.

Compound	Split-Luc Assay		
	E_{max}	pEC_{50}	n
1-C4	105.10 ± 2.45	7.40 ± 0.08	**3**
1-C6	71.23 ± 3.27	6.92 ± 0.13	**6**
1-C8	112.00 ± 2.39	8.09 ± 0.08	**3**
1-C10	62.59 ± 4.43	6.01 ± 0.16	**4**
iperoxo	104.60 ± 0.85	8.89 ± 0.04	**4**
2-C4	86.76 ± 1.69	5.79 ± 0.04	**3**
2-C6	57.10 ± 4.46	5.32 ± 0.12	**4**
2-C8	60.08 ± 1.53	6.30 ± 0.06	**3**
2-C10	45.76 ± 2.10	6.47 ± 0.11	**6**
ACh	96.91 ± 1.28	6.88 ± 0.02	**9**
3-C4	34.98 ± 1.25	6.35 ± 0.09	**3**
3-C6	80.36 ± 8.74	6.33 ± 0.27	**3**
3-C8	51.05 ± 1.43	6.74 ± 0.08	**3**
3-C10	54.69 ± 5.56	5.11 ± 0.17	**4**
Isoxo	69.01 ± 3.26	8.25 ± 0.20	**3**
4-C4	88.97 ± 2.16	6.76 ± 0.07	**3**
4-C6	55.86 ± 2.32	6.11 ± 0.11	**4**
4-C8	76.90 ± 1.92	7.31 ± 0.11	**3**
4-C10	50.95 ± 2.03	6.16 ± 0.13	**3**
Oxotremorine-M	71.82 ± 3.56	8.20 ± 0.18	**3**
5-C4	75.44 ± 0.94	6.08 ± 0.04	**3**
5-C6	103.80 ± 4.19	6.03 ± 0.09	**5**
5-C8	98.56 ± 1.56	7.69 ± 0.06	**3**
5-C10	87.51 ± 2.88	6.99 ± 0.10	**5**
CCh	97.78 ± 1.71	6.25 ± 0.04	**5**

TABLE S1. Maximum agonist effect and potency of bitopic compounds derived from concentration-response curves of Gα and PLC-β3 complementation displayed in Figures 1-2 of the main text. Data are expressed as the means ± S.E.M. of 3-6 independent experiments performed in triplicate.

3) **Data S3**. Radioligand binding experiments: Methods and results

Membrane Preparation

Stably transfected CHO cells overexpressing the human M1 mAChR subtype were grown to a confluence of approx. 80%. Cells were harvested by gently scraping in ice-cold harvesting buffer (20 mM HEPES, 10 mM Na_2EDTA, pH= 7.4). The collected cells were homogenized twice on ice for 25

seconds at level 6 using a polytron homogenizer and the resulting cell fragments were centrifuged using the rotor JA25.50 at 40,000 g for 10 minutes (4 °C). The supernatant was aspirated, and the pellet resuspended in ice-cold storage buffer (20 mM HEPES, 0.1 mM Na_2EDTA, pH= 7.4). This step was repeated twice, and finally the pellets were resuspended in ice-cold assay buffer (10 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, pH= 7.4). Aliquots were filled in reaction tubes, frozen quickly in liquid nitrogen, and stored at -80 °C until use. The protein concentration was approximately 2 mg/ml, determined using the Pierce™ BCA Protein Assay Kit according to the manufacturer's instructions.

Radioligand Binding Experiments

Radioligand binding experiments using CHO-hM1 membranes were performed in round bottom 96-well microtiter plates applying 20 µg protein/well. Experiments were conducted at room temperature using [^{3}H]NMS as the radioactive probe (2 nM for [^{3}H]NMS dissociation and 0.2 nM for equilibrium binding experiments). The plate was incubated at room temperature using a microplate shaker (Titramax 101 Platform Shaker, Heidolph) with an incubation time ranging from 1 h for the dissociation to 24 h for the equilibrium experiments. Homologous competition assays were performed as controls to determine the affinity of NMS for M1 receptors (pK_D = 8.88±0.06, n=5).[1] Nonspecific binding was determined in the presence of an excess of atropine (10 µM). Binding reactions were terminated by rapid filtration through glass fiber mats previously soaked with a 1% polyethyleneimine solution in a cell harvester filtration device. After filtration, the filter mats were washed with ice-cold wash water, covered with melt-on scintillator sheets on a heating block, and the radioactivity was counted in a Perkin Elmer MicroBeta 2450 Microplate Counter. The inhibition constants K_i of receptors for the studied compounds were determined in competition experiments in which membranes were incubated in the presence of a constant concentration of 0.2 nM [^{3}H]NMS and increasing concentrations of the competitor. The incubation lasted 24 h to achieve full equilibrium. Two-point kinetic experiments with measurements of

[^{3}H]NMS binding at t = 0 and t = 20 min were performed. By means of two-point dissociation experiments,[2] the dissociation rate constant k_{-1} can be determined by measuring the specific binding just before the start of the dissociation reaction (t = 0, adding a competitive antagonist in excess) and at a predetermined time t during the dissociation process (t = 20 min), as described previously[2].

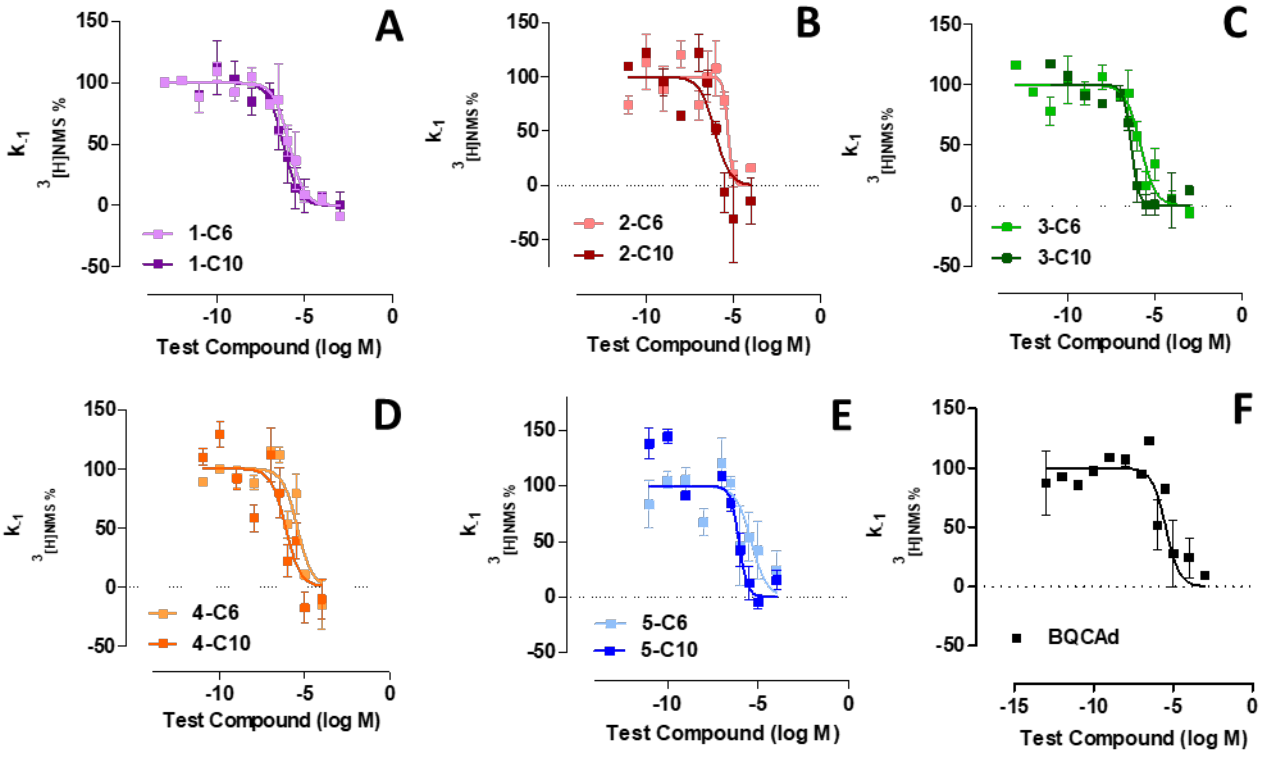

Figure S1. Allosteric effects of the bitopic ligands **1-Cn** (panel **A**), **2-Cn** (panel **B**), **3-Cn** (panel **C**), **4-Cn** (panel **D**), **5-Cn** (panel **E**) and their corresponding allosteric moiety BQCAd (Panel F) as reflected by the inhibition of [^{3}H]NMS dissociation from M1 receptors in CHO-hM1 membranes. The allosteric effect is expressed as the ratio of the rate of [^{3}H]NMS dissociation in the presence of compound relative to the rate of dissociation of [^{3}H]NMS in the absence of test compound expressed in percent k^{-1}. The inflection point represents the concentration that retards the radioligand dissociation by half and is therefore equal to the $IC_{50,diss}$ value. Data represent mean ± S.E.M. from three to four independent two-point kinetic experiments, conducted in quadruplicate.

Table S2. $pIC_{50,diss}$ values of bitopic ligands with a chain of 6 and 10 methylene units at M1R obtained from radioligand dissociation binding experiments.

Compound	Radioligand binding [3H]NMS Dissociation pIC_{50}	Compound	Radioligand binding [^{3}H]NMS Dissociation pIC_{50}
1-C6	5.89 ± 0.09	**1-C10**	6.22±0.08
2-C6	5.23 ± 0.16	**2-C10**	6.07±0.19
3-C6	5.81 ± 0.09	**3-C10**	6.34±0.08
4-C6	5.57 ± 0.09	**4-C10**	6.11±0.09
5-C6	5.43 ± 0.13	**5-C10**	6.12±0.14

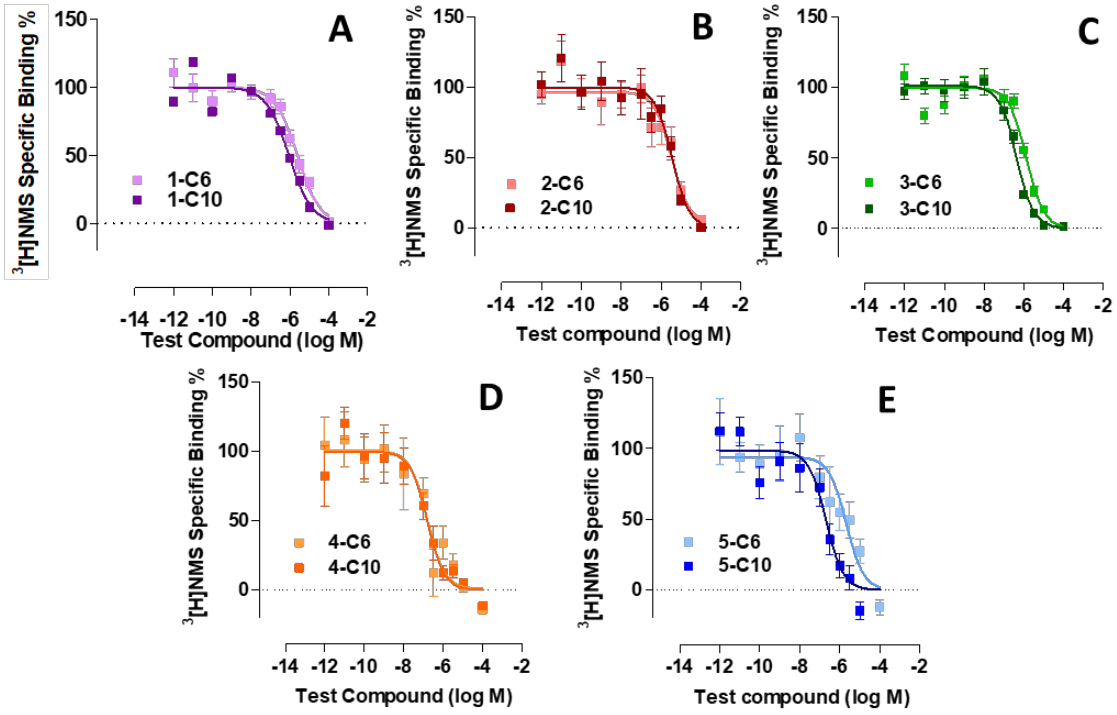

Figure S2. Effect of increasing concentrations of bitopic ligands **1-Cn** (panel **A**), **2-Cn** (panel **B**), **3-Cn** (panel **C**), **4-Cn** (panel **D**), **5-Cn** (panel **E**) on specific equilibrium binding of [^{3}H]NMS in CHO-hM1 membranes. Data represent mean ± S.E.M. from 3–5 independent experiments, conducted in quadruplicate.

Table S3. pIC50 and pKi values for bitopic ligands obtained from radioligand binding experiments.

Compound	Radioligand binding [^{3}H]NMS Equilibrium	Compound	Radioligand binding [^{3}H]NMS Equilibrium
1-C6	pKi 5.93 pIC_{50} 5.52±0.06	**1-C10**	pKi 6.34 pIC_{50} 5.59±0.06
2-C6	pKi 5.86 pIC_{50} 5.46±0.19	**2-C10**	pKi 5.82 pIC_{50} 5.45±0.18
3-C6	pKi 6.32 pIC_{50} 5.90±0.08	**3-C10**	pKi 6.75 pIC_{50} 6.34±0.08
4-C6	pKi 7.22 pIC_{50} 6.79±0.26	**4-C10**	pKi 7.20 pIC_{50} 6.79±0.18
5-C6	pKi 6.13 pIC_{50} 5.68±0.25	**5-C10**	pKi 6.69 pIC_{50} 6.68±0.17

4) Standards agonists and PAMs results in equilibrium and dissociation radioligand binding at M1R and their comparison with values reported in literature.

Table S4. Radioligand binding affinities of standard agonists and [3H]NMS dissociation rates expressed as p$IC_{50,diss}$. The inflection point represents the concentration that retards the radioligand dissociation by half and is therefore equal to the IC50 value. Graphs not shown.

Radioligand binding [^{3}H]NMS			
Compound	**Dissociation p$IC_{50,diss}$**	**Compound**	**Equilibrium**
BQCAd	5.47 ± 0.21 (Lit. 4.26 ± 1.32)[3]	**Iperoxo**	pKi 6.13 pIC_{50} 5.93 ± 0.06 (Lit. 5.67 ± 0.65)[3]
BQCA	5.32 ±0.14	**ACh**	(Lit. 4.32 - 4.76)[3,4]
		Isoxo	pKi 5.90 pIC_{50} 5.49 ± 0.10
		Oxo-M	pKi 6.13 pIC_{50} 5.74 ± 0.33 (Lit. 5.09 ± 0.13)[4]
		CCh	(Lit. 3.17 - 4.46)[4–6]

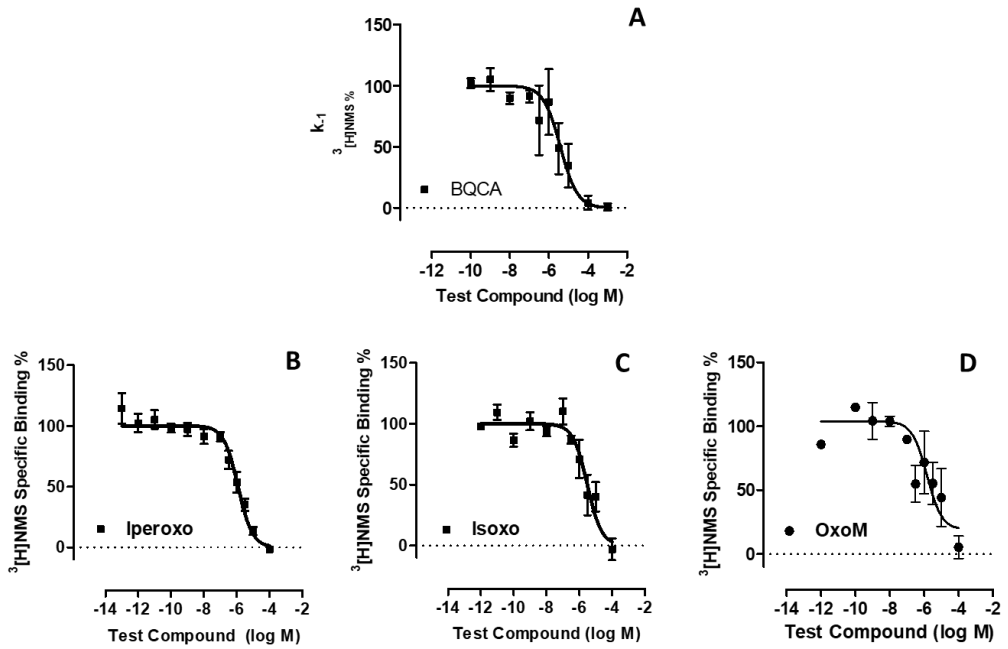

Figure S3.

A. Result for the BQCA allosteric modulator retarding [^{3}H]NMS radioligand dissociation (panel **A**) from M1 receptors in CHO-hM1 membranes. The allosteric effect is expressed as the ratio of the rate of [^{3}H]NMS dissociation in the presence of compound relative to the rate of dissociation of [^{3}H]NMS in the absence of test compound expressed in percent k^{-1}. The inflection point represents the concentration that retards the radioligand dissociation by half and is therefore equal to the $IC_{50,diss}$ value.

B, C, D. Effect of increasing concentrations of agonists (iperoxo (panel **B**), Isoxo (panel **C**), Oxo-M (panel **D**)) on specific equilibrium binding of [^{3}H]NMS in CHO-hM1 membranes. Data represent mean ± S.E.M. from 3–5 independent experiments, conducted in quadruplicate.

5) **Figure S4.** Dose-response evaluation of **n-C4** in G protein-activation assay.

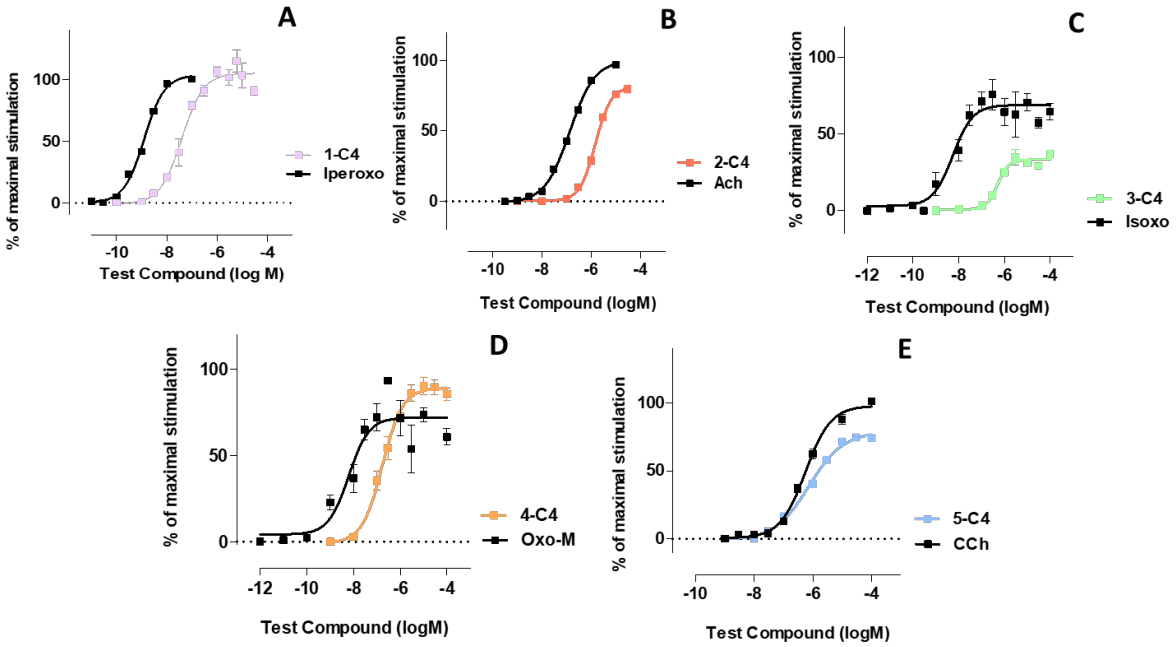

Figure S4. Gα/PLC-β3 split-luciferase interaction assay in HEK293T cells expressing the human muscarinic M1 receptors (hM1). Concentration-response-curves for the reference compound iperoxo (panel **A**), Ach (panel **B**), Isoxo (panel **C**), Oxo-M (panel **D**), Carbachol (CCh) (panel **E**), whose maximum stimulation was defined as 100% and for **n-C4** derivatives. Data represent means ± SEM of 3-6 experiments conducted in triplicate.

Figure S5. Binding mode comparison of dualsteric ligands at the M1 and the M2 receptor

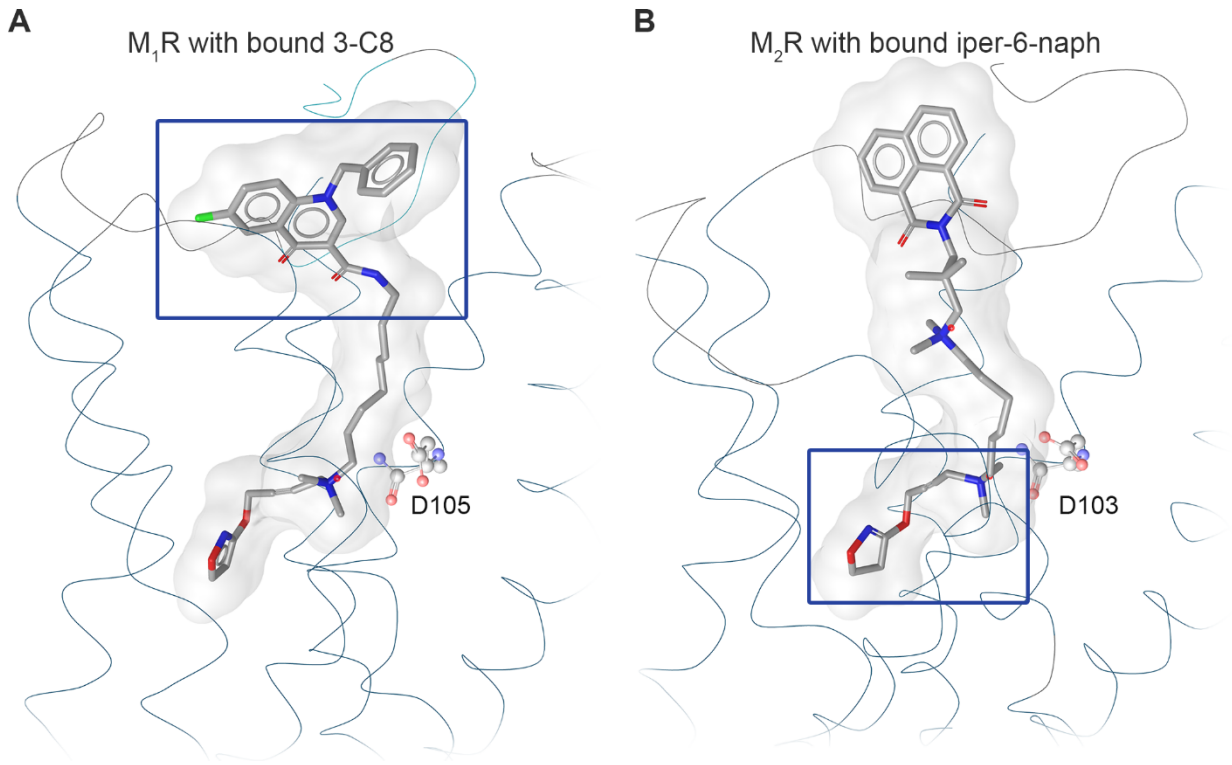

Figure S5. Comparison of proposed binding modes for bitopic (dualsteric) ligands at the M1 (A) and the M2 receptor (B). We suggest that the more specific building blocks (blue frames) control the orientation of the less specific moieties. BQCA and its derivatives have been previously shown to be highly selective for M1 receptors,[7,8] due to subtype specific allosteric vestibules. Although the highly conserved orthosteric binding pocket, iperoxo shows a ten-fold higher affinity to M2 compared to M1 receptors.[9] Due to the flexibility of the linker, the less specific part of the molecule can adopt an optimal position based on steric requirements and interaction possibilities. This allows for more structural variations of the less specific building block. While BQCA derived bitopic ligands haven't been shown to act via M2 receptors, we recently demonstrated that bitopic ligands of the phth- and naph series (e.g. iper-6-phth, or iper-6-naph) can function via M1 receptors.[10] M2 receptor figure was readapted from previous study.[11]

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