Synthesis of Dual AChE /5-HT4 Receptors Multi-Target Directed Ligands.

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Supporting Information

Experimental section

1. General

Melting points were determined on a Kofler melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin Elmer BX FT-IR apparatus using KBr pellets. The ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were obtained on a Jeol Lambda 400 spectrometer using DMSO-d*6* or $CDCl_3$ as solvent and TMS as internal standard. The chemical shifts (δ) are reported in ppm, and the coupling constants are in Hertz. Reactions were monitored by LC/MS and stopped when all starting material had disappeared. LC/MS (ESI) analyses were realised with a Waters alliance 2695 as separating module using the following gradient: A (95%)/B (5%) to A (5%)/B (95%) in 10 min. This ratio was hold for 3 min before return to initial conditions in 1 min. Initial conditions were then maintained for 5 min (A: H_2O, B: CH_3CN; each containing HCOOH: 0.1%; Column: C18 Xterra MSC118/2.1_50 mm). MS detection was performed with a Micromass ZMD 2000 by positive ESI. High-resolution mass spectra (EIMS) were obtained using a Jeol JMS GCMate spectrometer. For compounds **5a**, **5b**, **4b** and **15b**, high- resolution mass spectra were performed at 70 eV by electronic impact. Reactions were monitored by thin-layer chromatography (TLC) using 0.2 mm Polygram Sil silica gel G/UV 254 precoated plates with visualisation by irradiation with a short-wavelength UV light. Silica gel flash chromatography was performed using 63-200 mM Kieselgel Merck 60 silica gel. The microwave reactions were performed using a biotage initiator microwave oven using 2-5 mL sealed vials.

2. Chemistry

2.1. Reaction of deprotection

5-[(Piperidin-4-yl)methyloxy]pyrrolo[1,2-*a*]thieno[2,3*e*] pyrazine (5a).

General procedure: 420 mg (0.11 mmol) of compound **4a** are stirred in 4 mL of a binary solution of CH_2Cl_2/CF_3COOH (1/1) at room temperature. After 1 hour, the mixture is neutralised with a saturated solution of $NaHCO_3$. The organic phase is separated and washed with a saturated solution of natrium hydrogenocarbonate and twice with water. The organic phase is then dried over $MgSO_4$ and concentrated in vacuum to afford the product **5a** as a beige solid. Yield: 85%. MP: 118°C. IR (cm^{-1}): 3427.8, 2918.4, 2849.9, 1635.8, 1520.4, 1493.7, 1423.5, 1359.0, 1335.8, 1302.0, 1263.4, 1117.7, 735.0, 617.5. HREIMS (m/z $[M+H]^+$): 288.1171 (calc: 288.1158). ^{1}H NMR ($CDCl_3$) δ: 7.40 (d, *J* = 2.9 Hz, 1H), 7.26 (d, *J* = 4.9 Hz, 1H), 7.03 (d, *J* = 4.9 Hz, 1H), 6.92 (d, *J* = 3.9 Hz, 1H), 6.78 (m, 1H), 4.36 (d, *J* = 6.8 Hz, 2H, CH_2O), 3.16 (m, 2H, 2 CHN), 2.69 (m, 2H, 2 CHN), 2.06 (m, 1H, CH), 1.89 (m, 2H, 2 CH), 1,37 (m, 2H, 2 CH). ^{13}C NMR ($CDCl_3$) δ: 155.6, 136.1, 124.5, 124.3, 119.5, 115.6, 114.5, 113.2, 104.3, 70.6, 46.3 (2C), 36.1, 30.3 (2C). LC-MS (ESI): t_R=7.57min; m/z $[M+H]^+$: 288.15.

5-[(Piperidin-4-yl)methyloxy]-1-methylpyrrolo[1,2-*a*]thieno [3,2-*e*]pyrazine (5b).

Using the same procedure described for **5a** and starting from a solution of 127 mg (0.32 mmol) of **4b** in 4 mL of a binary solution of CH_2Cl_2/CF_3COOH (1/1), 87 mg of **5b** were obtained as a beige solid. Yield: 92%. MP: 102°C. IR (cm^{-1}): 3410.7, 3097.3, 2936.0, 2851.1, 1587.1, 1555.6, 1529.4, 1516.1, 1487.4, 1430.1, 1351.4, 1299.9, 1165.7, 1127.8, 715.8. HREIMS (m/z $[M+H]^+$): 302.1327 (calc: 302.1326). ^{1}H NMR ($CDCl_3$) δ: 7.87 (s, 1H), 6.91 (d, *J* = 3.9 Hz, 1H), 6.77 (s, 1H), 6.74 (m, 1H), 4.34 (d, *J* = 5.8 Hz, 2H, CH_2O), 3.19 (m, 2H, 2 CHN), 2.71 (m, 2H, 2 CHN), 2.67 (s, 3H, CH_3), 2.04 (m, 1H, CH), 1.89 (m, 2H, 2 CH), 1,40 (m, 2H, 2 CH). ^{13}C NMR ($CDCl_3$) δ: 154.6, 140.2, 125.2, 122.8, 119.6, 115.2, 114.9, 112.2, 103.6, 70.5, 51.9, 46.0 (2C), 35.8, 29.7 (2C), 17.2. LC-MS (ESI): t_R=7.89min; m/z $[M+H]^+$: 302.18.

2.2. Reaction of *O*-Arylation

5-[1-*t*-Butyloxycarbonyl(piperidin-4-yl)methyloxy]pyrrolo [1,2-*a*]thieno[2,3-*e*]pyrazine (4a).

General procedure: At 0°C, 262 mg of NaH 60% (6.56 mmol, 4.5 eq) are added to a solution of 470 mg of *N*-BOC-piperidin-4-yl methanol (2.19 mmol, 1.5 eq) in 5 mL of DMF. The mixture is stirred at room temperature for 45 minutes. 470 mg of **3a** (1.46 mmol, 1 eq) are then added and the mixture is stirred until the starting material has disappeared (2 h). 50 mL of water are added at 0°C to neutralise the excess of NaH and the solution is extracted with CH_2Cl_2 (3 X 40 mL). The organic phase is then washed with water (5 X 50 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The crude product is purified by SiO_2 column chromatography (preparation: CH_2Cl_2, gradient: CH_2Cl_2 then CH_2Cl_2/AcOEt (8/2)). Yield: 83%. MP: 128°C. IR (cm^{-1}): 3115.6, 3060.9, 2976.0, 2933.7, 2866.3, 1697.2, 1582.6, 1502.0, 1493.6, 1422.1, 1352.2, 1305.8, 1245.6, 1166.1, 1143.4, 1089.9, 980.0, 731.9. HRMS (m/z): 387.16183 (calc: 386.16164). ^{1}H NMR ($CDCl_3$) δ: 7.41 (s, 1H), 7.25 (d, *J* = 5.9 Hz, 1H), 7.04 (d, *J* = 5.9 Hz, 1H, $H_{2'}$), 6,91 (d, *J* = 3.9 Hz, 1H), 6.79 (m, 1H), 4.38 (d, *J* = 5.9 Hz, 2H, CH_2O), 4.15 (m, 2H, 2 CHN), 2.78 (m, 2H, 2 CHN), 2.08 (m, 1H, CH), 1.87 (m, 2H, 2 CH), 1.47 (s, 9H, 3 CH_3), 1.36 (m, 2H, 2 CH). ^{13}C NMR ($CDCl_3$) δ: 155.4, 154.8, 136.1; 124.4, 124.3, 119.4, 115.7, 114.6, 113.2, 104.3, 79.3, 69.9 (2C), 43.6, 35.8 (2C), 28.9; 28.4 (3C). LC-MS (ESI): t_R=13.57min; m/z $[M+H]^+$: 388.43.

5-[1-*t*-Butyloxycarbonyl(piperidin-4-yl)methyloxy]-1-methyl pyrrolo[1,2-*a*]thieno[3,2-*e*]pyrazine (4b).

Using the same procedure described for **4a** and starting with the formation of the alkoxide from 200 mg of *N*-BOC-piperidin-4-yl methanol (0.93 mmol, 1.3 eq) and 112 mg (2.79 mmol, 3.9 eq) of NaH 60% in 3 mL of DMF followed by the *O*-arylation on 159 mg of the compound **3b** (0.71 mmol, 1 eq), 252 mg of **4b** were obtained as a beige solid (purification: SiO_2, preparation: CH_2Cl_2, eluent: CH_2Cl_2 then CH_2Cl_2/AcOEt (8/2)). Yield: 68%. MP: 112°C. IR (cm^{-1}): 2975.4, 2945.3, 2926.1, 2869.0, 1699.0, 1587.9, 1531.8, 1517.1, 1488.0, 1461.5, 1428.8, 1346.9, 1300.7, 1246.5, 1162.1, 1144.4, 1126.5, 1085.6, 979.6, 723.7, 707.7. HREIMS (m/z $[M+H]^+$): 402.1851 (calc: 402.1862). ^{1}H NMR ($CDCl_3$) δ: 7.88 (s, 1H), 6.90 (d, *J* = 3.9 Hz, 1H), 6.78 (s, 1H), 6.74 (m, 1H), 4.36 (d, *J* = 5.8 Hz, 2H, CH_2O), 4.13 (m, 2H, 2 CHN), 2.68 (s, 3H, CH_3), 2.77 (m, 2H, 2 CHN), 2.68 (s, 3H, CH_3), 2.06 (m, 1H, CH), 1.86 (m, 2H, 2 CH), 1.47 (s, 9H, 3 CH_3), 1.33 (m, 2H, 2 CH). ^{13}C NMR ($CDCl_3$) δ: 154.8, 154.4, 140.1, 125.2, 122.8, 119.6, 115.2, 114.9, 112.2, 103.5, 79.3, 70.0 (2C), 43.6, 35.8 (2C), 28.8; 28.4 (3C), 17.2. LC-MS (ESI): t_R=14.44min; m/z $[M+H]^+$: 402.24.

5-[(1-(2-Fluorobenzyl)piperidin-4-yl)methyloxy]pyrrolo[1,2-*a*]thieno[2,3-*e*]pyrazine (16a).

Using the same procedure described for **4a** and starting with the formation of the alkoxide at 80°C for 1 h from 450 mg of (1-(2-fluorobenzyl)piperidin-4-yl)methanol (1.70 mmol, 1.5 eq) and 204 mg of NaH 60% (5.09 mmol, 4.5 eq) in 10 mL of toluene followed by the *O*-arylation on 235 mg of compound **3a** (1.13 mmol, 1 eq) at 80°C overnight, 135 mg of **16a** were obtained as a yellow oil (purification: SiO_2, preparation: cyclohexane, gradient: from cyclohexane to cyclohexane/AcOEt (1/1)). Yield: 30%. IR (cm^{-1}): 2934.6, 1585.0, 1521.5, 1493.2, 1422.5, 1357.5; 1335.6, 1225.7, 1150.1, 982.0, 752.8, 732.3. HRMS (m/z): 395.14757 (calc: 395.14674). ^{1}H NMR ($CDCl_3$) δ: 7.40 (m, 2H), 7.25 (m, 2H), 7.11 (m, 1H), 7.03 (m, 1H), 7.03 (d, *J* = 5.9 Hz, 1H), 6.90 (d, *J* = 3.9 Hz, 1H), 6.78 (m, 1H), 4.36 (d, *J* = 6.8 Hz, 2H, CH_2O), 3.61 (s, 2H, CH_2N), 2.97 (m, 2H, 2 CHN), 2.10 (m, 2H, 2 CHN), 1.89 (m, 3H), 1.47 (m, 2H, 2 CH). ^{13}C NMR ($CDCl_3$) δ: 161.4 (d, *J* = 246.2 Hz), 155.6, 136.2, 131.6 (d, *J* = 59.0 Hz), 128.6 (d, *J* = 8.3 Hz), 124.9 (d, *J* = 14.9 Hz), 124.5, 124.3, 119.5, 115.6, 115.2 (d, *J* = 22.3 Hz), 114.5, 113.2, 104.3, 70.3, 55.6, 53.1 (2C), 35.5, 29.1 (2C). LC-MS (ESI): t_R=8.47min; m/z $[M+H]^+$: 396.48.

5-[(1-(4-Methoxybenzyl)piperidin-4-yl)methyloxy]pyrrolo [1,2-*a*]thieno[2,3-*e*]pyrazine (17a).

Using the same procedure described for **4a** and starting with the formation of the alkoxide at 80°C for 1 h from 1 g of (1-(4-methoxybenzyl)piperidin-4-yl)methanol (4.26 mmol, 1.5 eq) and 306 mg of NaH 60% (12.77mmol, 4.5 eq) in 30 mL of toluene followed by the *O*-arylation on 590 mg of compound **3a** (2.84 mmol, 1 eq) at 80°C overnight, 382 mg of **17a** were obtained as a brown solid (purification: SiO_2, preparation: CH_2Cl_2, gradient: from CH_2Cl_2 to CH_2Cl_2/AcOEt (2/3)). Yield: 33%. MP: 128°C. IR (cm^{-1}): 2925.1, 2794.0, 2754.1, 1609.3, 1581.8, 1515.9, 1493.3, 1419.8, 1331.9, 1301.7, 1243.0, 1146.1, 979.5, 828.4, 730.1, 637.3. HRMS (m/z): 407.16859 (calc: 407.16673). ^{1}H NMR ($CDCl_3$) δ: 7.39 (s, 1H), 7.24 (m, 3H), 7.03 (d, *J* = 5.8 Hz, 1H), 6.91 (d, *J* = 3.8 Hz, 1H), 6.86 (d, *J* = 8.8 Hz, 2H), 6.78 (m, 1H), 4.36 (d, *J* = 6.8 Hz, 2H, CH_2O), 3.80 (s, 3H, CH_3O), 3.46 (s, 2H, CH_2N), 2.93 (m, 2H, 2 CHN), 2.03 - 1.85 (m, 5H), 1.47 (m, 2H, 2 CH). ^{13}C NMR ($CDCl_3$) δ: 158.6, 155.6, 136.2, 130.4, 130.4 (2C), 124.5, 124.3, 119.5, 115.6, 114.5, 113.5 (2C), 113.2, 104.3, 70.4, 62.8, 55.2, 53.2 (2C), 35.7, 29.1 (2C). LC-MS (ESI): t_R=8.69min; m/z $[M+H]^+$: 408.24.

5-[(1-Benzylpiperidin-4-yl)oxy]pyrrolo[1,2-*a*]thieno[2,3-*e*] pyrazine (18a).

Using the same procedure described for **4a** and starting with the formation of the alkoxide at room temperature from 150 mg of 1-benzyl-4-hydroxypiperidine (0.78 mmol, 1.5 eq) and 94 mg of NaH 60% (2.36 mmol, 4.5 eq) in 5 mL of DMF for 45 min followed by the *O*-arylation on 109 mg of compound **3a** (0.52 mmol, 1 eq), 108 mg of **18a** were obtained as a brown oil (purification: SiO_2, preparation: CH_2Cl_2 + Et_3N (2%), eluent: CH_2Cl_2 then CH_2Cl_2/AcOEt (9/1)). Yield: 57%. IR (cm^{-1}): 2925.2, 2854.2, 2804.7, 1582.0, 1518.1, 1492.5, 1420.1, 1357.5, 1335.4, 1151.6, 1035.4, 733.0, 699.9. HRMS (m/z): 363.14052 (calc: 363.14115). ^{1}H NMR ($CDCl_3$) δ: 7.39-7.28 (m, 6H), 7.23 (d, *J* = 5.9 Hz, 1H), 7.02 (d, *J* = 5.9 Hz, 1H), 6.91 (d, *J* = 2.9 Hz, 1H), 6.78 (dd, *J* = 3.9 Hz, *J* = 2.9 Hz, 1H), 5.42-5.36 (m, 1H, CH-O), 3.57 (s, 2H, CH_2N), 2.79-2.75 (m, 2H, 2 CHN), 2.44 - 2.40 (m, 2H, 2 CHN), 2.15 - 2.09 (m, 2H, 2 CH), 1.99 - 1.91 (m, 2H, 2 CH). ^{13}C NMR ($CDCl_3$) δ: 154.6, 138.4, 136.3, 129.1 (2C), 128.2 (2C), 127.0, 124.5, 124.1, 119.8, 115.5, 114.4, 113.1, 104.3, 70.6, 63.1 (2C), 50.7, 30.9 (2C). LC-MS (ESI): t_R=8.22min; m/z $[M+H]^+$: 363.86.

5-[(1-Benzylpiperidin-4-yl)ethyloxy]pyrrolo[1,2-*a*]thieno[2,3-*e*]pyrazine (19a).

Using the same procedure described for **4a** and starting with the formation of the alkoxide at room temperature from 128 mg of 1-benzyl-4-ethyloxypiperidine (0.58 mmol, 1.5 eq) and 70 mg of NaH 60% (1.75 mmol, 4.5 eq) in 5 mL of DMF for 45 min followed by the *O*-arylation on 81 mg of compound **3a** (0.39 mmol, 1 eq) at room temperature for 2 h, 52 mg of **19a** were obtained as a colourless oil (purification: SiO_2, preparation: CH_2Cl_2 + Et_3N (2%), eluent: CH_2Cl_2 then CH_2Cl_2/AcOEt (9/1)). Yield: 34%. IR (cm^{-1}): 2920.4, 1520.9, 1493.7, 1422.1, 1363.2, 1335.3, 1150.5, 974.3, 732.9, 699.8. HRMS (m/z): 391.17130 (calc: 391.17182). H NMR ($CDCl_3$) δ: 7.40 (d, J = 2.9 Hz, 1H), 7.31 (m, 3H), 7.25 (m, 3H), 7.03 (d, J = 5.8 Hz, 1H), 6.90 (d, J = 2.9 Hz, 1H), 6.78 (m, 1H), 4.54 (t, J = 6.8 Hz, 2H, CH_2O), 3.49 (s, 2H, 2 CHN), 2.89 (m, 2H, 2 CHN), 1.95 (m, 2H, 2 CH), 1.82 (qd, J = 6.8 Hz, 2H, CH_2), 1.78 (m, 2H, 2 CH), 1.56 (m, 1H, CH), 1,37 (m, 2H, 2 CH). ^{13}C NMR ($CDCl_3$) δ: 155.5, 137.8, 136.2, 129.4 (2C), 128.2 (2C), 127.1, 124.5, 124.3, 119.6, 115.6, 114.5, 113.1, 104.3, 64.0, 63.3, 53.7 (2C), 35.4, 32.9, 32.1 (2C). LC-MS (ESI): t_R=8.75min; m/z $[M+H]^+$: 392.13.

2.3. Reaction of *N*-alkylation

5-[(1-(3-Fluorobenzyl)piperidin-4-yl)methyloxy]pyrrolo[1,2-*a*]thieno[2,3-*e*]pyrazine (6a).

General procedure: To a solution of 64 mg (0.22 mmol) of **5a** in DMF (5 mL) are added 40 mg (0.29 mmol, 1.3 eq) of K_2CO_3, 37 mg (0.22 mmol, 1 eq) of KI and 32 µL (0.27 mmol, 1.2 eq) of 3-fluorobenzyl chloride. The mixture is then warmed to 110°C for 1 hour. After cooling, the resulting solution is diluted in water and extracted with CH_2Cl_2 (3 X 50 mL). The organic phase is then washed with water (5 X 60 mL), dried over $MgSO_4$ and evaporated in vacuo. The crude product is purified by flash chromatography (SiO_2, preparation: CH_2Cl_2 + Et_3N (2%), eluent: CH_2Cl_2) to obtain 72 mg of compound **6a** as a yellow oil. Yield: 82%. IR (cm^{-1}): 2933.1, 2881.9, 2801.1, 2759.2, 1587.8, 1521.8, 1493.7, 1422.8, 1335.7; 1300.7; 1150.2, 983.4; 732.3. HRMS (m/z): 395.14627 (calc: 395.14674). ^{1}H NMR ($CDCl_3$) δ: 7.40 (s, 1H), 7.26 (m, 2H), 7.09 (m, 2H), 7.03 (d, J = 5.9 Hz, 1H), 6.93 (m, 2H), 6.78 (m, 1H), 4.37 (d, J = 5.9 Hz, 2H, CH_2O), 3.51 (s, 2H, CH_2), 2.93 (m, 2H, 2 CHN), 2.06 (m, 2H, 2 CHN), 1.89 (m, 3H), 1.69 (m, 2H, 2 CH), 1.49 (m, 2H, 2 CH). ^{13}C NMR ($CDCl_3$) δ: 162.9 (d, ^1J = 245.3 Hz), 155.6, 141.3 (d, ^3J = 7.4 Hz), 136.2, 129.5 (d, ^3J = 8.3 Hz), 124.5 (d, ^{4}J = 2.5 Hz), 124.5, 124.3, 119.5, 115.8 (d, ^2J = 21.5 Hz), 115.6, 114.5, 113.7 (d, ^2J = 21.5 Hz), 113.2, 104.3, 70.3, 62.8, 53.4 (2C), 35.6, 29.1 (2C). LC-MS (ESI): t_R=8.57min; m/z $[M+H]^+$: 396.16.

5-[(1-(4-Fluorobenzyl)piperidin-4-yl)methyloxy]pyrrolo[1,2-*a*]thieno[2,3-*e*]pyrazine (7a).

Using the same procedure described for **6a** and starting from a mixture of 88 mg (0.31 mmol) of **5a**, 49 µL of 4-fluorobenzyl chloride (0.37 mmol, 1.2 eq), 51 mg of KI (0.31 mmol, 1 eq), 55 mg of K_2CO_3 (0.40 mmol, 1.3 eq) and 5 mL of DMF, 100 mg of **7a** were obtained as yellow solid (purification: SiO_2, preparation: CH_2Cl_2 + Et_3N (2%), eluent: CH_2Cl_2). Yield: 82%. MP: 116°C. IR (cm^{-1}): 2937.8, 2796.6, 2758.4, 1603.0, 1583.9, 1521.3, 1508.4, 1494.7, 1422.7, 1335.8, 1301.1, 1220.3, 1152.4, 982.6, 836.9, 732.7. HRMS (m/z): 395.14674 (calc: 395.14554). ^{1}H NMR ($CDCl_3$) δ: 7.40 (d, J = 2.9 Hz, 1H), 7.28 (m, 3H), 7.01 (m, 3H), 6.92 (d, J = 2.9 Hz, 1H), 6.78 (m, 1H), 4.37 (d, J = 5.7 Hz, 2H, CH_2O), 3.49 (s, 2H, CH_2N), 2.92 (m, 2H, 2 CHN), 1.95 (m, 5H), 1.48 (m, 2H, 2 CH). ^{13}C NMR ($CDCl_3$) δ: 161.2 (d, ^1J = 243.7 Hz), 155.5, 136.2, 134.1, 130.6 (d, ^3J = 7.4 Hz, 2C), 124.4, 124.3, 119.5, 115.6, 114.9 (d, ^2J = 21.5 Hz, 2C), 114.5, 113.1, 104.3, 70.3, 62.6, 53.2 (2C), 35.6, 29.1 (2C). LC-MS (ESI): t_R=8.62min; m/z $[M+H]^+$: 396.06.

5-[(1-(Pyridin-4-yl)piperidin-4-yl)methyloxy]pyrrolo[1,2-*a*] thieno[2,3-*e*]pyrazine (8a).

Using the same procedure described for **6a** and starting from a mixture of 69 mg (0.24 mmol) of **5a**, 47 mg of 4-chloromethylpyridine hydrochloride (0.29 mmol, 1.2 eq), 76 mg of K_2CO_3 (0.55 mmol, 2.3 eq), 40 mg of KI (0.24 mmol, 1 eq) and 5 mL of DMF, 54 mg of **8a** were obtained as a beige solid (purification: SiO_2, preparation: cyclohexane + Et_3N (2%), eluent: cyclohexane/AcOEt (4/1)). Yield: 59%. MP: 110°C. IR (cm^{-1}): 2936.6, 2800.7, 2761.6, 1601.7, 1582.9, 1521.2, 1493.8, 1421.8, 1300.0, 1149.6, 982.9, 732.5. HRMS (m/z): 378.15045 (calc: 378.15141). ^{1}H NMR ($CDCl_3$) δ: 8.54 (d, J = 4.9 Hz, 2H, 2 CH), 7.41 (s, 1H), 7.28 (d, J = 4.9 Hz, 2H, 2 CH), 7.26 (d, J = 4.9 Hz, 1H), 7.04 (d, J = 4.9 Hz, 1H), 6.90 (d, J = 3.9 Hz, 1H), 6.78 (m, 1H), 4.38 (d, J = 6.8 Hz, 2H, CH_2O), 3.52 (s, 2H, CH_2), 2.91 (m, 2H, 2 CHN), 2.09 (m, 2H, 2 CHN), 1.94 (m, 3H), 1,49 (m, 2H, 2 CH). ^{13}C NMR ($CDCl_3$) δ: 155.5, 149.7, 148.0, 136.2, 124.4 (2C), 124.3, 123.8 (2C), 119.5, 115.7, 114.5, 113.2, 104.3, 70.2, 62.1, 53.5 (2C), 35.5, 29.1 (2C). LC-MS (ESI): t_R = 7.18min; m/z $[M+H]^+$: 379.19.

5-[(1-(Methylbenzyl)piperidin-4-yl)methyloxy]pyrrolo[1,2-*a*] thieno[2,3-*e*]pyrazine (9a).

Using the same procedure described for **6a** and starting from a mixture of 78 mg (0.27 mmol) of **5a,** 45 µL of (1-bromoethyl)benzene (0.33 mmol, 1.2 eq), 49 mg of K_2CO_3 (0.55 mmol, 2.3 eq), and 5 mL of DMF, 70 mg of **9a** were obtained as a colourless oil (purification: SiO_2, preparation: cyclohexane + Et_3N (2%), eluent: cyclohexane/AcOEt (1/1)). Yield: 66%. IR (cm^{-1}): 2928.1, 2797.8, 1697.2, 1521.2, 1493.5, 1422.3, 1356.2, 1334.2, 1300.4, 1149.9, 731.9, 701.4. HRMS (m/z): 391.17099 (calc: 391.17182). ^{1}H NMR ($CDCl_3$) δ: 7.39 (s, 1H), 7.31 (m, 4H), 7.02 (d, J = 5.9 Hz, 1H), 6,89 (d, J = 3.9 Hz, 1H), 6.77 (m, 1H), 4.34 (d, J = 5.9 Hz, 2H, CH_2O), 3.45 (qd, J = 6.8 Hz, 1H, CH), 3.11 (m, 1H, 1 CHN), 2.87 (m, 1H, 1 CHN), 2.05 (m, 1H, CH), 1.99 – 1.48 (m, 6H), 1.40 (d, J = 6.8 Hz, 3H, CH_3). ^{13}C NMR ($CDCl_3$) δ: 155.6, 143.7, 136.2, 128.1 (2C), 127.7 (2C), 126.7, 124.5, 124.3, 119.5, 115.6, 114.5, 113.1, 104.3, 70.4, 64.9; 50.5, 50.3, 35.8, 29.4, 29.3, 26.9. LC-MS (ESI): t_R=8.58min; m/z $[M+H]^+$: 392.19.

5-[(1-(Ethylphenyl)piperidin-4-yl)methyloxy]pyrrolo[1,2-*a*] thieno[2,3-*e*]pyrazine (10a).

Using the same procedure described for **6a** and starting from a mixture of 68 mg (0.24 mmol) of **5a**, 26 µL of phenethyl chloride (0.28 mmol, 1.2 eq), 39 mg of KI (0.24 mmol, 1 eq), 43 mg of K_2CO_3 (0.31 mmol, 1.3 eq) and 5 mL of DMF, 37 mg of **10a** were obtained as a colourless oil (purification: SiO_2, preparation: CH_2Cl_2, gradient: from CH_2Cl_2 to CH_2Cl_2/AcOEt (1/4)). Yield: 37%. IR (cm^{-1}): 2931.7, 2852.9, 1670.5, 1583.8, 1521.5, 1494.0, 1422.7, 1301.0, 1150.8, 981.3, 732.4, 700.2. HRMS (m/z): 391.17182 (calc: 391.17182). ^1H NMR ($CDCl_3$) δ: 7.41 (d, *J* = 3.0 Hz, 1H), 7.26 (m, 6H), 7.04 (d, *J* = 5.8 Hz, 1H), 6.93 (d, *J* = 2.9 Hz, 1H), 6.79 (m, 1H), 4.39 (d, *J* = 5.8 Hz, 2H, CH_2O), 3.09 (m, 2H, 2 CHN), 2.85 (m, 2H, 2 CHN), 2.62 (m, 2H), 2.09 (m, 2H), 1.95 (m, 3H, 3 CH), 1.55 (m, 2H, 2 CH). ^{13}C NMR ($CDCl_3$) δ: 155.6, 140.5, 136.2, 128.7 (2C), 128.4 (2C), 126.0, 124.5, 124.4, 119.5, 115.7, 114.5, 113.2, 104.3, 70.3, 61.0, 53.5 (2C), 35.7, 33.8, 29.2 (2C). LC-MS (ESI): t_R=8.73min; m/z $[M+H]^+$: 392.13.

5-[(1-(Propylphenyl)piperidin-4-yl)methyloxy]pyrrolo[1,2-*a*] thieno[2,3-*e*]pyrazine (11a).

Using the same procedure described for **6a** and starting from a mixture of 68 mg (0.24 mmol) of **5a**, 26 µL of methylcyclopropyl chloride (0.28 mmol, 1.2 eq), 39 mg of KI (0.24 mmol, 1 eq), 43 mg of K_2CO_3 (0.31 mmol, 1.3 eq) and 5 mL of DMF, 37 mg of **11a** were obtained as a colourless oil (purification: SiO_2, preparation: CH_2Cl_2, gradient: from CH_2Cl_2 to CH_2Cl_2/AcOEt (1/4)). Yield: 46%. IR (cm^{-1}): 2937.0, 1521.2, 1494.0, 1422.3, 1354.6, 1334.9, 1150.4, 982.4, 732.2, 700.0. HRMS (m/z): 405.18691 (calc: 405.18746). ^1H NMR ($CDCl_3$) δ: 7.40 (s, 1H), 7.27 (m, 2H), 7.18 (m, 4H), 7.03 (d, *J* = 5.9 Hz, 1H), 6.92 (d, *J* = 3.9 Hz, 1H), 6.78 (m, 1H), 4.37 (d, *J* = 6.8 Hz, 2H, CH_2O), 3.99 (m, 2H, 2 CHN), 2.64 (t, *J* = 7.8 Hz, 2H), 2.40 (m, 2H, 2 CHN), 1.94 (m, 7H), 1.50 (m, 2H, 2 CH). ^{13}C NMR ($CDCl_3$) δ: 155.6, 142.2, 136.2, 128.4 (2C), 128.3 (2C), 125.7, 124.5, 124.4, 119.5, 115.6, 114.5, 113.2, 104.3, 70.3, 58.5, 53.5 (2C), 35.7, 33.9, 29.1 (2C), 28.9. LC-MS (ESI): t_R=8.87min; m/z $[M+H]^+$: 405.05.

5-[(1-Methylcyclopropylpiperidin-4-yl)methyloxy]pyrrolo [1,2-*a*]thieno[2,3-*e*]pyrazine (12a).

Using the same procedure described for **6a** and starting from a mixture of 68 mg (0.24 mmol) of **5a**, 26 µL of methylcyclopropyl chloride (0.28 mmol, 1.2 eq), 39 mg of KI (0.24 mmol, 1 eq), 43 mg of K_2CO_3 (0.31 mmol, 1.3 eq) and 5 mL of DMF, 37 mg of **12a** were obtained as a colourless oil (purification: SiO_2, preparation: CH_2Cl_2 + Et_3N (2%), eluent: CH_2Cl_2). Yield: 46%. IR (cm^{-1}): 2932.9, 2771.9, 1521.5, 1494.0, 1422.8, 1357.4, 1333.9, 1300.4, 1151.5, 984.0, 732.1, 706.8. HRMS (m/z): 341.15547 (calc: 341.15616). ^1H NMR ($CDCl_3$) δ: 7.40 (m, 1H), 7.26 (d, *J* = 5.8 Hz, 1H), 7.03 (d, *J* = 5.8 Hz, 1H), 6.92 (d, *J* = 3.9 Hz, 1H), 6.78 (dd, *J* = 3.9 Hz, *J* = 2.0 Hz, 1H), 4.38 (d, *J* = 5.8 Hz, 2H, CH_2O), 3.16 (m, 2H, 2 CHN), 2.30 (d, *J* = 5.9 Hz, 2H, CH_2N), 2.07-1.91 (m, 5H), 1.44 (m, 2H, 2 CH), 0.92 (m, 1H), 0.53 (m, 2H, CH_2), 0.12 (m, 2H, CH_2). ^{13}C NMR ($CDCl_3$) δ: 155.5, 136.1, 124.4, 124.3, 119.5, 115.6, 114.5, 113.2, 104.3, 70.1, 63.8, 53.2 (2C), 35.4, 28.6 (2C), 7.9, 4.1 (2C). LC-MS (ESI): t_R=8.18min; m/z $[M+H]^+$: 342.13.

5-[(1-Methylcyclobutylpiperidin-4-yl)methyloxy]pyrrolo[1,2-*a*]thieno[2,3-*e*]pyrazine (13a).

Using the same procedure described for **6a** and starting from a mixture of 71 mg (0.25 mmol) of **5a**, 33 µL of methylcyclobutyl chloride (0.30 mmol, 1.2 eq), 44 mg of K_2CO_3 (0.32 mmol, 1.3 eq) and 4 mL of DMF, 21 mg of **13a** were obtained as a colourless oil (purification: Al_2O_3, preparation: CH_2Cl_2, eluent: CH_2Cl_2 then CH_2Cl_2/AcOEt (9/1)). Yield: 24%. IR (cm^{-1}): 2929.9, 2853.8, 2799.1, 2760.6, 1521.5, 1493.8, 1422.2, 1334.6, 1300.3, 1150.4, 982.3, 731.6. HRMS (m/z): 355.17182 (calc: 355.117137). ^1H NMR ($CDCl_3$) δ: 7.40 (d, *J* = 3.9 Hz, 1H), 7.26 (m, 1H), 7.03 (d, *J* = 5.8 Hz, 1H), 6.91 (d, *J* = 3.9 Hz, 1H), 6.78 (m, 1H), 4.35 (d, *J* = 5.9 Hz, 2H, CH_2O), 2.93 (m, 2H, 2 CHN), 2.56 (m, 1H, CH), 2.43 (d, *J* = 5.9 Hz, 2H, CH_2N), 2.09-1.67 (m, 11H), 1.49 (m, 2H, 2 CH). ^{13}C NMR ($CDCl_3$) δ: 155.1, 136.0, 124.4, 124.3, 119.3, 115.8, 114.7, 113.3, 104.3, 70.4, 65.7, 53.5 (2C), 35.5, 34.1, 29.7, 29.0, 28.3 (2C), 18.8. LC-MS (ESI): t_R=8.46min; m/z $[M+H]^+$: 356.28.

5-[(1-Methylcyclopentylpiperidin-4-yl)methyloxy]pyrrolo[1,2-*a*]thieno[2,3-*e*]pyrazine (14a).

Using the same procedure described for **6a** and starting from a mixture of 67 mg (0.23 mmol) of **5a**, 36 µL of methylcyclopentyl iodide (0.28 mmol, 1.2 eq), 42 mg of K_2CO_3 (0.30 mmol, 1.3 eq) and 5 mL of DMF, 55 mg of **14a** were obtained as a colourless oil (purification: SiO_2, preparation: CH_2Cl_2 + Et_3N (2%), eluent: CH_2Cl_2 then CH_2Cl_2/AcOEt (9/1)). Yield: 64%. IR (cm^{-1}): 2941.4, 2864.1, (1651.1), 1521.3, 1493.8, 1422.2, 1335.1, 1300.3, 1151.4, 980.9, 732.0. HRMS (m/z): 369.18883 (calc: 369.18746). ^1H NMR ($CDCl_3$) δ: 7.40 (d, *J* = 2.9 Hz, 1H), 7.26 (d, *J* = 5.8 Hz, 1H), 7.03 (d, *J* = 5.8 Hz, 1H), 6.92 (d, *J* = 5.8 Hz, 1H), 6.78 (m, 1H), 4.36 (d, *J* = 6.8 Hz, 2H, CH_2O), 2.98 (m, 2H, 2 CHN), 2.30 (d, *J* = 5.8 Hz, 2H, CH_2N), 2.12-1.49 (m, 14H), 1.20 (m, 2H, CH_2). ^{13}C NMR ($CDCl_3$) δ: 155.6, 136.2, 124.5, 124.3, 119.5, 115.6, 114.3, 113.2, 104.3, 70.4, 65.1, 53.8 (2C), 37.4, 35.7, 31.7 (2C), 29.0 (2C), 25.2 (2C). LC-MS (ESI): t_R=8.55min; m/z $[M+H]^+$: 370.26.

5-[(1-Methylcyclopentylpiperidin-4-yl)methyloxy]-1-methyl pyrrolo[1,2-*a*]thieno[3,2-*e*]pyrazine (14b).

Using the same procedure described for **6a** and starting from a mixture of 180 mg (0.60 mmol) of **5b**, 93 µL of methylcyclopentyl iodide (0.72 mmol, 1.2 eq), 107 mg of K_2CO_3 (0.78 mmol, 1.3 eq) and 6 mL of DMF, 105 mg of **14b** were obtained as a colourless oil (purification: SiO_2, preparation: CH_2Cl_2 + Et_3N (2%), eluent: CH_2Cl_2 then CH_2Cl_2/AcOEt (9/1)). Yield: 46%. IR (cm^{-1}): 2942.4, 2863.9, 1582.9, 1587.6, 1529.9, 1516.4, 1488.2, 1462.7, 1428.7, 1349.2, 1301.2, 1230.2, 1165.7, 1127.3, 980.5, 718.0 HRMS (m/z): 383.20246 (calc: 383.20246). ^1H NMR ($CDCl_3$) δ: 7.88 (d, *J* = 2.9 Hz, 1H), 6.92 (d, *J* = 3.9 Hz, 1H), 6.76 (s, 1H), 6.73 (m, 1H), 4.35 (d, *J* = 6.8 Hz,

2H, CH_2O), 2.97 (m, 2H, 2 CHN), 2.67 (s, 3H, CH_3), 2.28 (d, *J* = 7.8 Hz, 2H, CH_2N), 2.11-1.45 (m, 14H), 1.19 (m, 2H, CH_2). ^{13}C NMR ($CDCl_3$) δ: 154.7, 140.3, 125.2, 122.8, 119.7, 115.1, 114.9, 112.2, 103.6, 70.6, 65.1, 53.8 (2C), 37.5, 35.7, 31.7 (2C), 29.1, 25.2 (2C), 17.3. LC-MS (ESI): t_R=9.02min; m/z $[M+H]^+$: 384.00.

5-[(1-Methylcyclohexylpiperidin-4-yl)methyloxy]pyrrolo[1,2-*a*]thieno[2,3-*e*]pyrazine (15a).
Using the same procedure described for **6a** and starting from a mixture of 108 mg (0.38 mmol) of **5a**, 63 µL of (bromomethyl)cyclohexane (0.45 mmol, 1.2 eq), 68 mg of K_2CO_3 (0.49 mmol, 1.3 eq) and 5 mL of DMF, 80 mg of **15a** were obtained as a colourless oil (purification on a spot 2 apparatus, column: EVF SiO_2, gradient: from CH_2Cl_2 to CH_2Cl_2/AcOEt (2/8)). Yield: 55%. IR (cm^{-1}): 2920.7, 2848.8, 1583.1, 1521.3, 1493.7, 1422.2, 1334.7, 1150.8, 983.6, 731.6, 705.1. HRMS (m/z): 383.20349 (calc: 383.20312). ^1H NMR ($CDCl_3$) δ: 7.41 (d, *J* = 3.9 Hz, 1H), 7.27 (d, *J* = 5.8 Hz, 1H), 7.03 (d, *J* = 5.8 Hz, 1H), 6.92 (d, *J* = 3.9 Hz, 1H), 6.78 (m, 1H), 4.36 (d, *J* = 6.8 Hz, 2H, CH_2O), 2.92 (m, 2H, 2 CHN), 2.12 (d, *J* = 6.8 Hz, 2H, CH_2N), 1.95-1.17 (m, 16H), 0.87 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 155.6, 136.2, 124.5, 124.4, 119.5, 115.6, 114.5, 113.1, 104.3, 70.4, 66.2, 54.0 (2C), 35.8, 35.2, 32.1 (2C), 29.1 (2C), 26.8, 26.2 (2C). LC-MS (ESI): t_R=8.70min; m/z $[M+H]^+$: 384.25.

5-[(1-Methylcyclohexylpiperidin-4-yl)methyloxy]-1-methyl pyrrolo[1,2-*a*]thieno[3,2-*e*]pyrazine (15b).
Using the same procedure described for **6a** and starting from a mixture of 81 mg (0.27 mmol) of **5b**, 45 µL of (bromomethyl)cyclohexane (0.32 mmol, 1.2 eq), 48 mg of K_2CO_3 (0.35 mmol, 1.3 eq) and 4 mL of DMF, 50 mg of **15b** were obtained as a colourless oil (purification: SiO_2, preparation: CH_2Cl_2 + Et_3N (2%); eluent: CH_2Cl_2 then CH_2Cl_2/AcOEt (9/1)). Yield: 47%. IR (cm^{-1}): 2921.5, 2850.1, 1587.5, 1516.1, 1488.1, 1464.3, 1428.4, 1347.6, 1165.5, 1127.0, 983.6, 717.9. HREIMS (m/z $[M+H]^+$): 398.2266 (calc: 398.2252). ^1H NMR ($CDCl_3$) δ: 7.87 (d, *J* = 2.9 Hz, 1H), 6.91 (d, *J* = 2.9 Hz, 1H), 6.76 (s, 1H), 6.73 (m, 1H), 4.34 (d, *J* = 6.8 Hz, 2H, CH_2O), 2.90 (m, 2H, 2 CHN), 2.67 (s, 3H, CH_3), 2.11 (d, *J* = 6.8 Hz, 1H, CH_2N), 1.93-1.13 (m, 16H), 0.87 (m, 2H). ^{13}C NMR ($CDCl_3$) δ: 154.7, 140.3, 125.2, 122.8, 119.8, 115.1, 114.9, 112.2, 103.6, 70.6, 66.2, 54.0 (2C), 35.8, 35.2, 32.1 (2C), 29.1 (2C), 26.8, 26.2 (2C), 17.3. LC-MS (ESI): t_R=9.32min; m/z $[M+H]^+$: 398.02.

5-[(1-Benzylpiperidin-4-yl)amino]pyrrolo[1,2-*a*]thieno[2,3-*e*] pyrazine (20a).
In a microwave vial, 300 mg of compound **3a** (1.44 mmol) are dissolved in 3 mL of *N*-methylpyrrolidinone. 885 µL (4.33 mmol, 3 eq) of 4-amino-1-benzylpiperidine are added to the solution and the mixture is warmed under microwave irradiation at 210°C for 1 hour. The mixture is then diluted in water (50 mL) and extracted with dichloromethane (3 X 40 mL). The organic phase is then washed with water (3 X 50 mL), dried over $MgSO_4$ and evaporated under reduced pressure. The crude product is then purified by column chromatography on silica gel (preparation: CH_2Cl_2 + Et_3N (2%), eluent: CH_2Cl_2) to afford **20a** as a brown oil. Yield: 60%. MP: 95°C. IR (cm^{-1}): 3107.7, 3027.7, 2938.5, 2803.1, 2760.6, 1581.0, 1526.1, 1492.0, 1363.7, 1340.0, 1307.1, 1070.2, 734.5, 700.6. HRMS (m/z): 391.17130 (calc: 362.15650). ^1H NMR ($CDCl_3$) δ: 7.33-7.25 (m, 6H), 7.20 (d, *J* = 5.8 Hz, 1H), 6.96 (d, *J* = 5.8 Hz, 1H), 6.71 (m, 1H), 6.61 (m, 1H), 4.64 (d, *J* = 7.8 Hz, 1H, NH), 4.23 (m, 1H, 1 CHN), 3.54 (s, 2H, CH_2N), 2.87 (m, 2H, 2 CHN), 2.25 (m, 2H, 2 CHN), 2.15 (m, 2H, 2 CH), 1.60 (m, 2H, 2 CH). ^{13}C NMR ($CDCl_3$) δ: 148.4, 138.4, 138.2, 129.1 (2C), 128.2 (2C), 127.0, 124.5, 121.1, 119.4, 115.1, 114.3, 112.3, 101.1, 63.1, 52.4 (2C), 47.3, 32.6 (2C). LC-MS (ESI): t_R=6.49min; m/z $[M+H]^+$: 363.22.

1-Benzyl-*N*-(pyrrolo[1,2-*a*]thieno[2,3-*e*]pyrazin-5-yl) piperidine-4-carboxamide (21a).
Under Ar, in 2 mL of degased dioxane are successively added 104 mg (0.5 mmol) of compound **4a**, 131 mg of carboxamide (0.6 mmol, 1.2 eq), 405 mg of Cs_2CO_3 (1.25 mmol, 2.5 eq), 31 mg of BINAP (0.05 mmol, 10%) and 6 mg of $Pd(OAc)_2$ (0.025 mmol, 5%). The mixture is then warmed under Ar at 100°C. After 3h30, the result of the reaction is concentrated under reduced pressure. The residue is then dissolved in CH_2Cl_2, washed with water and purified by flash chromatography (SiO_2, gradient from CH_2Cl_2 to CH_2Cl_2/AcOEt (9/1)) to give **21a** as a brown solid. Yield: 79%. MP: 117°C. IR (cm^{-1}): 3416.7 (N-H), 2924.8, 2853.9, 1714.1, 1585.4, 1514.8, 1494.5, 1436.8, 1410.5, 1366.5, 1331.3, 1295.0, 736.9;, 699.9. HRMS (m/z): 390.15068 (calc: 390.15141). ^1H NMR ($CDCl_3$) δ: 7.81 (s, 1H, NH), 7.50 (s, 1H), 7.34 – 7.28 (m, 6H), 7.10 (d, *J* = 5.3 Hz, 1H), 6.94 (s, 1H), 6.91 (m, 1H), 3.53 (s, 2H, CH_2N), 2.99 (m, 2H, 2 CHN), 2.11 – 1.93 (m, 7H). ^{13}C NMR ($CDCl_3$) δ: 174.9, 142.9, 138.3, 136.4, 129.0 (2C), 128.2 (2C), 127.0, 126.5, 124.5, 120.7, 116.6, 114.8, 114.3, 106.3, 63.1, 53.0 (2C), 43.4, 28.7 (2C). LC-MS (ESI): t_R=7.05min; m/z $[M+H]^+$: 391.23.

3. *In vitro* tests of AChE and buChE biological activity
Inhibitory capacity of compounds on AChE and BuChe biological activity was evaluated through the use of the spectrometric method of Ellman.[1] Lyophilized electric eel AChE (Type III, Sigma Aldrich) or equine BuChE (Sigma Aldrich from equine serum) was dissolved in 0.2 M phosphate buffer pH 7.4 such as to have enzyme solutions stock with 2.5 units/mL enzyme activity. Acetyl- or butyrylthiocholine iodide (Sigma Aldrich) was used as a substrate of the enzymatic reaction and 5,5-dithiobis(2-nitrobenzoic) acid (DTNB, Sigma Aldrich) as a label for the measurement of cholinesterase activity. In the procedure, 1880 µL of 0.12 mg/mL DTNB dissolved in phosphate buffer pH 7.4 were mixed with 40 µL of test compound solution and 20 µL of enzyme stock solution were mixed. After 5 min of preincubation, 40 µL of 10 mM acetyl- or butyrylthiocholine iodide solution was added to the assay solution. The change in absorbance at 412 nm was recorded (AGILENT 8453 UV–VIS Spectroscopy System) during 10 min.

First screening of AChE and BuChE activity was carried out at a 10^{-5} M concentration of compounds under study. For the compounds with significant inhibition (≥ 80%) after 4 min of reaction, IC_{50} values were determined graphically from a 6-point inhibition curves using the Origin software, and expressed as IC_{50} ± SD. Donepezil (from Tocris) and tacrine (from Tocris) were used as control respectively.

4. Propidium competition assay

Propidium exhibits an increase in fluorescence on binding to AChE peripheral site, making it a useful probe for competitive ligand binding to the enzyme.[2] Fluorescence was measured in a Tecan Infinite M200 plate reader. Measurements were carried out in 200 µl solution volume, in 96-well plates. The buffer used was 1 mM Tris/HCl, pH 8.0, 5 U eeAChE which was incubated, for 15 min at 25°C, with a 150 µL 10^{-5}M solution of the compounds or donepezil (from Tocris) as control. One micromolar propidium iodide 50 µL solution was added 10 min before fluorescence measurement. The excitation wavelength was 535 nm, and that of emission, 595 nm. Each assay was repeated, at least, three different times.

5. Binding experiments

Binding to native 5-HT_4R from guinea pig was determined using the method of Grossman.[3] For membrane preparations male guinea pigs (300-350 g, Charles River) were subjected to euthanasia by cervical dislocation and decapitated. Brains were rapidly removed at 4°C and striatal regions carefully dissected and pooled. The tissues were then suspended in 10 volumes of HEPES buffer 50 mM pH 7.4 at 4°C. After homogenization at 4°C (Ultra-Turrax, maximal speed, 15 sec), and ultracentrifugation (23,000 x *g*, 60 min, 4°C), the pellet was resuspended in 10 volumes of HEPES buffer 50 mM pH 7.4 at 4°C in order to obtain a tissue concentration of about 100 mg protein/mL. The protein concentration was determined by the method of Lowry[4] using bovine serum albumin as standard.

For radioligand binding studies, 600 µg of membrane were incubated in duplicate at 37°C for 30 min with [^{3}H]-GR113808 (Perkin Elmer), fixed concentration of compound and HEPES buffer 50 mM pH 7.4 at 37°C. Incubation was terminated by rapid vacuum filtration through 0.5% polyethylenimine-presoaked Whatman GF/B filters (Alpha Biotech) using a Brandel Cell Harvester. Filters were subsequently washed three times with 4 ml of HEPES buffer 50 mM pH 7.4 at 4°C.

The method was validated from saturation studies: 6 concentrations of [^{3}H]-GR113808 were used to give final concentrations of 0.02-0.8 nM, non-specific binding of [^{3}H]-GR113808 was defined in the presence of 30 µM serotonin to determine the K_d and the Bmax.

For competition studies, [^{3}H]-GR113808 was used to give a final concentration of 0.1 nM. Percentages of inhibition of the binding of [^{3}H]-GR113808 were obtained for concentrations of 10^{-6} and 10^{-8} M of the ligands tested. For some of these compounds, affinity constants were calculated from 5-point inhibition curves using the EBDA-Ligand software, and expressed as K_i ± SD.

6. Cyclic AMP radioimmunoassay

For measurement of intracellular cyclic AMP accumulation, stably transfected cells were grown to confluence and were incubated with serum-free medium 4 h before the beginning of the assay. Then, the cells were preincubated for 15 min with serum-free medium supplemented with 5 mM theophylline, 10 µM pargyline and 1 µM GR127935 in CHO cells to block the activity of endogenous 5-HT_{1B}R. 5-HT or other serotoninergic ligands were then added for an additional 15 min. The reaction was stopped by aspiration of the medium and addition of 500 µL of ice-cold ethanol. After 30 min incubation at room temperature, the ethanol fraction was collected and evaporated under vacuum. The pellet was reconstituted and cyclic AMP was quantified using a radioimmunoassay kit (cyclic AMP competitive radioimmunoassay, Immunotech, Marseille, France). Student's t-tests were performed using the QuickTTest software.

7. Membrane preparation and radioligand binding assays

Membrane preparation and radioligand binding assays were performed as previously described.[5] Briefly, cells grown at confluence were washed twice with phosphate-buffered saline (PBS) and centrifuged at 300 x *g* for 5 min. The resulting pellet was resuspended in 1 mL of ice-cold HEPES buffer (50 mM, pH 7.4) and centrifuged at 40,000 x *g* for 15 min at 48C. The final pellet containing intracellular membrane component as well as plasma membranes was resuspended in 1 mL HEPES buffer and protein concentrations were determined by the method of Bradford.[6] Radioligand binding assays were performed in 500 µL buffer (50 mM HEPES, pH 7.4) containing 20 µL of [^{3}H]-GR113808, 50 µg of membrane preparation and 20 µL of displacing drug. Saturation experiments were performed using [^{3}H]-GR113808 at nine concentrations ranging from 0.01 mM to 4 nM. Non-specific binding was measured in the presence of 10 µM ML10375 and subtracted from total binding to determine the affinity of [^{3}H]-GR113808 for its receptor (K_d, nM) and the total number of receptors (Bmax fmol mg^{-1} protein). At a concentration of [^{3}H]-GR113808 corresponding to K_d, the total radioactivity was >500 d.p.m. and non-specific binding <30% of total binding for the saturation experiment to be considered as valid. Competition assays were performed in the presence of nine concentrations of the displacing ligands (10^{-12}-10^{-4} M) and a concentration of [^{3}H]-GR113808 corresponding to its K_d for the receptor. Incubations were performed at 25°C for 30 min and the reaction was terminated by rapid filtration through Whatman GF/B filter paper using the Brandel model 48R cell harvester. Radioactivity was measured using a Beckman model LS 6500C liquid scintillation counter. Binding data were analysed by computer-assisted nonlinear regression analysis (Prism; GraphPad Software, San Diego, CA, U.S.A.).

8. X-Ray Crystallography

Single crystals of **17a** suitable for X-ray crystallographic analysis were obtained by slow evaporation from dichloromethane. Data for crystal structure analysis were collected at 150 K with a Bruker–Nonius Kappa CCD area detector diffractometer with graphite–monochromatised Mo K_α radiation (λ=0.71073 Å). The structure was solved using direct methods and refined by full-matrix least-squares analysis on F^2. Crystallographic data: Crystal size: 0.37×0.24×0.11mm. Formula $C_{23}H_{25}N_3O_2S$, formula weight 407.53, crystal system monoclinic, space group $P2_1/c$, a = 18.4455(5) Å, b = 5.8641(2) Å, c = 20.5004(6) Å, α = 90°, β = 114.067(1)°, γ = 90°, V = 2024.69(11) $Å^3$, Z = 4, calculated density = 1.337 g/cm^3, μ = 0.185 mm^{-1}, R_{int} = 0.037, R[F^2>2σ(F^2)] = 0.040, wR(F^2) = 0.095. Programme(s) used to solve structure: SHELXS–97. Programme(s) used to refine structure: SHELXL–97. Software used to prepare material for publication: SHELXL–97. Crystallographic data for compound **17a** have been deposited at the Cambridge Crystallographic Data Centre, CCDC N° 819449. Copies of this information may be obtained free of charge from the Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (+44-1223-336408; E-mail: deposit@ccdc.cam. ac.uk or http://www.ccdc.cam.ac.uk).

9. Computational Procedure.

9.1. 5HT_4R model

First, the sequence of the human 5-HT_4R was retrieved from the UniProt Knowledgebase (UniProtKB)[7] (ID: Q712M9_ HUMAN). The @tome server[8] (using screening methods like FUGUE,[9] SP3,[10] PSIBLAST,[11,12] HHSEARCH[13]) identified the β2 adrenergic receptor as the best 3D experimental template for the homology modelling of the 5-HT_4R (Sequence identity = 39%). The high-resolution (2.4 Å) crystal structure of the human β2 adrenergic receptor (β2AR)-T4 lysozyme fusion protein bound to the carazolol (PDB: 2RH1)[14] was used as the 3D template. The alignment between the two sequences was manually optimised. Disulphide bond: C93-C184 between the transmembrane helix 3 (TM3) and the extracellular loop (ECL2) was conserved. This alignment was used as the basis for the homology modelling with the Modeller software.[15] The resulting model was then evaluated by methods like verify3D[16] and Eval23D.[17]

9.2. AChE

The crystal structure of AChE from *Torpedo californica* /Donepezil (E2020)[18] complex (PDB file identificator 1EVE) was used as template to construct the complex models. All water molecules were deleted before the docking runs.

9.3. Docking

The docking of the compounds into the two targets was carried out with the GOLD programme (v5.0) using the default parameters.[19,20] This programme applies a genetic algorithm to explore conformational spaces and ligand binding modes and the ChemScore fitness function was used for both docking studies.

The binding site in the 5-HT_4R model was defined as a 10Å sphere centered on aspartic acid residue Asp_{100}. Since the mutagenesis studies showed that the interaction between the positively ionisable amine of ligands and Asp_{100} of 5-HT_4R is crucial for ligand binding, a hydrogen bond constraint between positively ionisable amine ligand and OD of Asp_{100} was used during the docking. Furthermore, we required special attention during the docking procedure to some amino acids in the binding site, which were kept flexible: Arg_{96}, Thr_{97}, Asp_{100}, Thr_{104}, Tyr_{192}, Ser_{197}, Trp_{272}, Asn_{279}, Trp_{294}, Tyr_{302}.

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