Inhibitors of Ketohexokinase. Discovery of Pyrimidinopyrimidines with Specific Substitution that Complements the ATP-Binding Site

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Supporting Information

(11 pages)

General Chemical Procedures. All reagents and solvents were obtained from commercial suppliers and used without further purification. For the synthesis of **47**, we used *N*-Boc-2,6-diazaspiro[3.3]heptane that was obtained from Prof. Erick Carriera, as a generous gift (Burkhard, J.; Carreira, E. M. *Org. Lett.* **2008**, *10*, 3525–3526). Solvents were routinely dried over 4A molecular sieves prior to use and reactions were conducted under Argon. New compounds were purified by reverse-phase preparative HPLC (as described below), generally isolated as trifluoroacetate salts, and characterized by electrospray (ESI) MS and ^{1}H NMR (300 or 400 MHz). Purities were judged by reverse-phase analytical HPLC and found to be as >95% (as described below). ^{1}H NMR spectra were recorded on a Bruker AC 300B (300 MHz) or a Bruker AM-400 (400 MHz) spectrometer with an appropriate deuterated solvent (usually CD_3OD) and Me_4Si as an internal standard (s = singlet, d = doublet, m= multiplet, t = triplet, br = broad). ESI-MS data were obtained on a Micromass mass spectrometer or an Agilent HPLC mass spectrometer. Preparative reverse-phase HPLC separations were performed on a Gilson HPLC by using a Phenomenex Kromasil 100A C18 column (25 cm x 50 mm; or 10 cm x 21.2 mm) with a gradient of MeCN/water/0.2% CF_3CO_2H. Analytical HPLC separations were performed in reverse-phase mode on a Supelco ABZ+Plus column (5 cm x 2.1 mm) or a YMC J'Sphere H80 S4 column (5 cm x 2 mm) with detection at 220 nm and 254 nm on a Hewlett Packard Series 1100 instrument. The normal gradients used were 10–90% or 20-90% MeCN/water/0.1% CF_3CO_2H over 6 min. All test compounds were at least 95% pure by reverse-phase analytical HPLC, exhibited the expected molecular ion by ES-MS, and possessed the expected ^{1}H NMR spectrum. Elemental microanalyses were performed by QTI, Inc. The amount of water was determined by Karl-Fischer (KF) analysis.

General Chemical Synthesis. Trichloride **III** (see Scheme 1) was stirred in dry THF or acetone with ice-bath cooling while an excess (2–3 mol equiv) of the R1 amine was added, and stirring was continued at 0–5°C. The reaction was followed by HPLC until completion, whence the solution was evaporated in vacuo to give an oil. This material may be used as is, or dissolved in CH_2Cl_2, washed (once with saturated $NaHCO_3$, three times with water, once with brine), dried (Na_2SO_4), and evaporated in vacuo to give the crude dichloride. The dichloride material is usually pure enough for further use.

The dichloride was combined with the R2 amine (2–5 mol equiv) in dry THF (with *i*-Pr_2EtN being added when the amine was used as an acid-addition salt) at ambient temperature, with progress followed by HPLC. After several hours, the reaction was concentrated to a residue, redissolved in CH_2Cl_2 or $CHCl_3$, washed (once with saturated $NaHCO_3$, several times with water, once with brine), dried (Na_2SO_4), and evaporated in vacuo to afford the crude monochloride. This material was usually clean and the crude monochloride advanced to the next step.

The monochloride was combined with the R3 amine (3–5 mol equiv) in THF at ambient temperature and stirred for several hours. When the reaction was complete (HPLC), the solvent was evaporated in vacuo to a residue, which was redissolved in CH_2Cl_2, washed (once with saturated $NaHCO_3$, several times with water, once with brine), dried (Na_2SO_4), and evaporated in vacuo to afford the crude product. (When a Boc protecting group was present, it could be removed at this point by stirring in 1:1 CH_2Cl_2/CF_3CO_2H at 0–5°C for several hours.) The crude product was purified by using preparative reverse-phase HPLC and evaporated in vacuo to give the trifluoroacetate salt. This salt was dissolved in water/CF_3CO_2H and lyophilized to an amorphous solid trifluoroacetate, which was suitable for chemical analysis and biological testing. Hydrochloride salts were also used when compounds were scaled up, e.g., in the case of **8** (vide infra).

Representative Chemical Synthesis. Compound 8, N8-(Cyclopropylmethyl)-N4-[2-(methylthio)phenyl]-2-(1-piperazinyl)-pyrimido[5,4-*d*]pyrimidine-4,8-diamine. 5-Amino oratic acid **I** (27 g, 158 mmol) was combined with formamide (450 mL), heated at 170°C for 6 h, and then stirred at ambient temperature overnight. White solid was filtered, washed with fresh formamide, and dried at ambient temperature in vacuo for 16 h to afford **II** (22.2 g, 78%), which was used directly for chlorination. A portion of **II** (5.0 g, 27.8 mmol) was combined with phosphorus oxychloride (275 mL) and phosphorus pentachloride (25 g, 120 mmol) and refluxed for 16 h (ref 30 in main text). The reaction was cooled to 30°C and evaporated in vacuo to a viscous oil (10–15 mL), which was poured with vigorous stirring into ice water (500 mL). After 15 min a solid was filtered, washed with water, and dried in air to give tan solid **III** (3.07 g, 47%). The solid was purified via Soxhlet extraction with $CHCl_3$ and the solution was evaporated in vacuo to give a light yellow, powdery trichloride **III** (2.16 g), ESI-MS m/z 235 (MH^+), ^{1}H NMR ($CDCl_3$) δ 9.29 (s, 1H). Trichloride **III** (470 mg, 2.0 mmol) in THF (20 mL) was ice-bath cooled, stirred vigorously, and treated with 2-

methylthioaniline (556 mg, 4.0 mmol) in THF (2 mL), which was added dropwise over about 5 min. Analysis by HPLC at 20 min showed one major new product with the desired MS and no starting material **III** remaining. The reaction was evaporated in vacuo to afford a yellow, solid dichloride intermediate (1.06 g; some aniline present), which was used directly in the following step. A purified sample was a yellow solid with ESI-MS m/z 338 (MH^+); ^1H NMR ($CDCl_3$) δ 9.09 (s, 1H), 8.71 (dd, 1H, J = 1.2, 8.3 Hz), 7.63–7.60 (m, 1H), 7.49–7.44 (m 1H), 7.26–7.20 (m, 1H), 2.48 (s, 3H). The crude dichloride was stirred at ambient temperature in THF (40 mL), treated with cyclopropanemethylamine (710 mg, 10 mmol) in THF (2 mL), and stirred for 1 h. The reaction was evaporated in vacuo to an oil, which was dissolved in $CHCl_3$ and washed (once with saturated $NaHCO_3$, twice with water, once with brine), dried (Na_2SO_4), and evaporated in vacuo to give crude monochloride (800 mg), ESI-MS m/z 373 (MH^+), ^1H NMR ($CDCl_3$) δ 8.72–8.68 (m, 1H), 8.60 (s, 1H), 7.62–7.55 (m, 1H), 7.50–7.45 (m, 1H), 7.20–7.15 (m, 1H), 3.50–3.45 (m, 2H), 2.49 (s, 3H), 1.20–1.10 (m, 1H), 0.70–0.60 (m, 2H), 0.40–0.30 (m, 2H). This intermediate was pure enough (HPLC) to advance to the final step. The monochloride (ca. 2 mmol) and piperazine (860 mg, 10 mmol) were combined in THF (20 mL) and stirred at ambient temperature for 5 h. The reaction was evaporated in vacuo to give an oil, which was dissolved in CH_2Cl_2, washed (once with saturated $NaHCO_3$, twice with water, once with brine), dried (Na_2SO_4), and evaporated in vacuo to a brown oily crude product, **8** (780 mg). This material was purified via reverse-phase HPLC to afford a pale yellow, solid trifluoroacetate salt, which was partitioned between CH_2Cl_2 and saturated aqueous $NaHCO_3$. The organic phase was washed (once with water; once with brine), dried (Na_2SO_4), and evaporated in vacuo to pure (HPLC), tan solid, **8** (310 mg). This compound in methanol was treated with ethereal HCl, to form the acid-addition salt, and concentrated to a solid, which was redissolved in 0.1 N HCl and lyophilized to give a pale yellow solid, **8**•HCl (380 mg, 35%). ^1H NMR (CD_3OD) δ 8.29 (s, 1H), 8.09 (d, 1H, J = 7.9 Hz), 7.58–7.55 (m, 1H), 7.39–7.33 (m, 1H), 7.27–7.21 (m, 1H), 4.30–4.15 (m, 4H), 3.56 (d, 2H, J = 7.1 Hz), 3.40–3.33 (m, 4H), 2.45 (s, 3H), 1.40–1.25 (m, 1H), 0.69–0.62 (m, 2H), 0.47–0.42 (m, 2H); ESI-MS m/z 433 (MH^+). Anal. Calcd for $C_{21}H_{26}N_8S$•2.0 HCl•3.0 H_2O: C, 45.90; H, 6.23; N, 20.39; Cl, 12.90; KF, 9.83. Found: C, 45.83; H, 5.97; N, 20.10; Cl, 13.35; KF, 8.67.

Compound ^1H NMR Data. Spectral data are for trifluoroacetate salts in CD_3OD (unless otherwise indicated).

Compound **2**: δ 8.28 (s, 1H), 7.68 (d, 1H, J = 6.7 Hz), 7.32–7.15 (m, 3H), 4.09–4.08 (m, 4 H), 3.32 (s, 3H), 3.26-3.22 (m, 4+H), 2.33 (s, 3H).

Compound **3** (HCl salt): δ 8.31 (s, 1H), 7.64–7.60 (m, 1H), 7.33–7.17 (m, 3H), 4.20–4.05 (m, 4 H), 3.56 (d, 2H, J = 7.0 Hz), 3.27–3.24 (m, 4H), 2.33 (s, 3H), 1.40–1.25 (m, 1H), 0.67–0.60 (m, 2H), 0.46–0.41 (m, 2H).

Compound **4**: δ 8.27 (s, 1H), 7.78 (d, 2H, J = 7.5 Hz), 7.44–7.38 (m, 2H), 7.19–7.14 (m, 1H), 4.21–4.18 (m, 4 H), 3.49 (d, 2H, J = 6.8 Hz), 3.34-3.31 (m, 4+H), 1.32–1.20 (m, 1H), 0.62–0.55 (m, 2H), 0.40–0.35 (m, 2H).

Compound **5**: δ 8.27 (s, 1H), 7.64–7.57 (m, 2H), 7.31–7.27 (m, 1H), 7.00 (d, 1H, J = 7.6 Hz), 4.21–4.19 (m, 4 H), 3.49 (d, 2H, J = 7.1 Hz), 3.35-3.30 (m, 4H), 2.90 (br s, 1H), 2.38 (s, 3H), 1.28–1.22 (m, 1H), 0.61–0.56 (m, 2H), 0.40–0.36 (m, 2H).

Compound **6** (DMSO-d_6): δ 8.40-8.38 (m, 1H), 8.37 (s, 1H), 7.17–7.05 (m, 3H), 4.30–4.10 (m, 4 H), 3.96 (s, 3H), 3.49 (d, 2H, J = 6.8 Hz), 3.30-3.20 (m, 4H), 1.30–1.22 (m, 1H), 0.60–0.54 (m, 2H), 0.39–0.35 (m, 2H).

Compound **7**: δ 8.44 (d, 1H, J = 7.6 Hz), 8.25 (s, 1H), 7.11–7.00 (m, 3H), 4.30–4.10 (m, 6 H), 3.49 (d, 2H, J = 6.8 Hz), 3.35-3.30 (m, 4H), 1.49 (t, 3H), 1.30–1.20 (m, 1H), 0.63–0.57 (m, 2H), 0.41–0.36 (m, 2H).

Compound **8** (HCl salt): δ 8.29 (s, 1H), 8.09 (d, 1H, J = 7.9 Hz), 7.58–7.55 (m, 1H), 7.39–7.33 (m, 1H), 7.27–7.21 (m, 1H), 4.30–4.15 (m, 4 H), 3.56 (d, 2H, J = 7.1 Hz), 3.40–3.33 (m, 4H), 2.45 (s, 3H), 1.40–1.25 (m, 1H), 0.69–0.62 (m, 2H), 0.47–0.42 (m, 2H).

Compound **9**: δ 8.29 (s, 1H), 7.84–7.82 (m, 1H), 7.57–7.54 (m, 1H), 7.35 (dd, 1H, J = 7.9, 8.0 Hz), 7.13–7.06 (m, 1H), 4.30–4.20 (m, 4 H), 3.50 (d, 2H, J = 7.0 Hz), 3.40–3.33 (m, 4H), 2.75 (br s, 1H), 2.54 (s, 3H), 1.35–1.20 (m, 1H), 0.65–0.57 (m, 2H), 0.45–0.37 (m, 2H).
Compound **10**: δ 8.29 (s, 1H), 7.77–7.74 (m, 2H), 7.37–7.34 (m, 2H), 4.22–4.13 (m, 4 H), 3.50 (d, 2H, J = 7.0 Hz), 3.37–3.33 (m, 4H), 2.74 (br s, 1H), 2.51 (s, 3H), 2.17 (s, MeCN), 1.30–1.20 (m, 1H), 0.63–0.57 (m, 2H), 0.42–0.37 (m, 2H).

Compound **11**: δ 9.13 (d, 1H, J = 8.2 Hz), 8.47 (s, 1H), 8.00–7.97 (m, 1H), 7.78–7.73 (m, 1H), 7.33–7.28 (m, 1H), 4.26–4.22 (m, 4 H), 3.45 (d, 2H, J = 7.0 Hz), 3.37–3.32 (m, 4H), 3.16 (s, 3H), 2.76 (br s, 1H), 1.30–1.15 (m, 1H), 0.60–0.54 (m, 2H), 0.37–0.32 (m, 2H).

Compound **12** ($CDCl_3$): δ 10.28 (s, 1H), 9.94 (br s, 1H), 8.91 (d, 1H, J = 7.6 Hz), 8.52 (s, 1H), 7.61–7.58 (m, 1H), 7.47–7.42 (m, 1H), 7.10–7.06 (m, 1H), 4.35–4.25 (m, 4 H), 3.44–3.31 (m, 6H), 2.81 (q, 2H), 2.02 (br s, 6+H), 1.22 (t, 3H), 1.17–1.14 (m, 1H), 0.64-0.59 (m, 2H), 0.34–0.31 (m, 2H).

Compound **13**: δ 8.20-8.18 (m, 1H), 8.09 (s, 1H), 7.63 (d, 1H, J = 7.3 Hz), 7.51–7.47 (m, 1H), 7.14–7.10 (m, 1H), 3.99–3.97 (m, 4 H), 3.30 (d, 2H, J = 6.9 Hz), 3.14–3.12 (m, 4H), 3.11 (CH_3OD), 1.09–1.05 (m, 1H), 0.44–0.39 (m, 2H), 0.22–0.18 (m, 2H).

Compound **14**: δ 8.27 (s, 1H), 7.83–7.81 (m, 1H), 7.34–7.18 (m, 3H), 4.10–4.00 (m, 4 H), 3.48-3.47 (m, 2H), 3.30-3.20 (m, 4H), 2.77-2.71 (m, 2H), 2.03 (MeCN), 1.35–1.20 (m, 4H), 0.60–0.57 (m, 2H), 0.39–0.36 (m, 2H).

Compound **15**: δ 8.28 (s, 1H), 7.53–7.51 (m, 1H), 7.41–7.39 (m, 1H), 7.27-7.23 (m, 2H), 4.08–3.98 (m, 4 H), 3.51 (d, 2H, J = 7.1 Hz), 3.20-3.16 (m, 5H), 1.27–1.23 (m, 1H), 1.21 (d, 6H, J = 7.1 Hz), 0.60–0.58 (m, 2H), 0.40–0.37 (m, 2H).

Compound **16**: δ 8.16 (s, 1H), 8.05 (d, 1H, J = 7.9 Hz), 7.20-7.01 (m, 3H), 4.08–4.05 (m, 4 H), 3.38 (d, 2H, J = 7.2 Hz), 3.28-3.25 (m, 4H), 3.21 (CH_3OD), 1.88-1.83 (m, 1H), 1.18-1.13 (m, 1H), 1.01-0.94 (m, 2H), 0.62-0.58 (m, 2H), 0.52–0.46 (m, 2H), 0.30–0.27 (m, 2H).

Compound **17**: δ 8.27 (s, 1H), 8.20-8.17 (m, 1H), 7.27–7.19 (m, 3H), 4.18–4.14 (m, 4 H), 3.48 (d, 2H, J = 7.1 Hz), 3.37-3.33 (m, 4+H), 1.30-1.22 (m, 1H), 0.60-0.54 (m, 2H), 0.39-0.34 (m, 2H).

Compound **18**: δ 8.42-8.40 (m, 1H), 8.27 (s, 1H), 7.54-7.52 (m, 1H), 7.43-7.39 (m, 1H), 7.18-7.15 (m, 1H), 4.25–4.10 (m, 4 H), 3.47 (d, 2H, J = 6.8 Hz), 3.35-3.30 (m, 4H), 2.82 (br s, 1H), 2.03 (MeCN), 1.27–1.23 (m, 1H), 0.60–0.55 (m, 2H), 0.39–0.35 (m, 2H).

Compound **20**: δ 8.07 (s, 1H), 4.07–4.03 (m, 4H), 3.33 (d, 2H, J = 7.0 Hz), 3.20–3.12 (m, 4+H), 3.11 (CH_3OD), 2.90–2.80 (m, 1H), 2.55 (br s, 1H), 1.20–1.00 (m, 1H), 0.80–0.70 (m, 2H), 0.50–0.45 (m, 2H), 0.41–0.36 (m, 2H), 0.21–0.16 (m, 2H).

Compound **21**: δ 8.32 (s, 1H), 4.23–4.20 (m, 4H), 4.11–4.07 (m, 1H), 3.55 (d, 2H, J = 7.0 Hz), 3.46–3.33 (m, 4H), 2.10–1.70 (m, 5H), 1.60–1.20 (m, 6H), 0.64–0.58 (m, 2H), 0.43–0.38 (m, 2H).

Compound **22**: δ 8.27 (s, 1H), 7.77 (d, 1H, J = 6.8 Hz), 7.31–7.13 (m, 3H), 4.10–4.07 (m, 4 H), 3.60-3.57 (m, 2H), 3.27-3.24 (m, 4+H), 2.34 (s, 3H), 1.82-1.72 (m, 2H), 1.05-1.00 (m, 3H).

Compound **23**: δ 8.27 (s, 1H), 7.78 (d, 1H, J = 7.1 Hz), 7.31–7.15 (m, 3H), 4.10–4.07 (m, 4 H), 3.62-3.57 (m, 2H), 3.27-3.24 (m, 4+H), 2.34 (s, 3H), 1.80-1.70 (m, 2H), 1.46-1.35 (m, 6H), 0.95-0.90 (m, 3H).

Compound **24**: δ 8.26 (s, 1H), 7.81 (d, 1H, J = 8.3 Hz), 7.31–7.12 (m, 3H), 4.10–4.00 (m, 5 H), 3.27-3.24 (m, 4+H), 2.35 (s, 3H), 2.03-1.72 (m, 5H), 1.53-1.20 (m, 5H).

Compound **25**: δ 8.28 (s, 1H), 7.96 (d, 1H, J = 7.1 Hz), 7.27–7.26 (m, 2H), 7.13-7.11 (m, 1H), 4.20–3.96 (m, 8 H), 3.28-3.26 (m, 4+H), 2.37 (s, 3H), 1.36-1.31 (m, 6H).

Compound **27**: δ 8.29 (s, 1H), 7.82 (d, 1H, J = 7.9 Hz), 7.40–7.24 (m, 7H), 7.17–7.12 (m, 1H), 4.90 (s, 2H), 4.09–4.06 (m, 4H), 3.26–3.22 (m, 4+H), 2.35 (s, 3H).

Compound **28**: δ 8.35 (s, 1H), 7.83 (d, 1H, J = 7.9 Hz), 7.29–7.23 (m, 3H), 7.16–7.09 (m, 2H), 6.97-6.94 (m, 1H), 4.99 (s, 2H), 4.09–4.05 (m, 4H), 3.26–3.22 (m, 4+H), 2.35 (s, 3H).

Compound **29**: δ 8.32 (s, 1H), 7.89 (d, 1H, J = 8.0 Hz), 7.74 (d, 1H, J = 3.3 Hz), 7.50 (d, 1H, J = 3.3 Hz), 7.30–7.24 (m, 2H), 7.16–7.10 (m, 1H), 5.11 (s, 2H), 4.12–4.08 (m, 4H), 3.28–3.24 (m, 4H), 2.73 (br s, 1H), 2.36 (s, 3H).

Compound **30**: δ 8.29 (s, 1H), 7.72 (d, 1H, J = 7.6 Hz), 7.32–7.15 (m, 3H), 4.2–3.8 (br m), 3.54 (d, 2H, J = 7.2 Hz), 3.3-3.20 (br m), 2.34 (s, 3H), 2.2–2.0 (br m, 2+H), 1.35-1.25 (m, 1H), 0.64–0.58 (m, 2H), 0.43–0.40 (m, 2H).

Compound **31** (DMSO-d_6): δ 9.64 (br s, 1H), 9.12 (s, 1H), 8.06 (s, 1H), 8.00–7.94 (m, 1H), 7.54 (d, 1H, J = 7.5 Hz), 7.07–7.00 (m, 2H), 6.92–6.87 (m, 1H), 5.20 (br s, 3+H), 4.60-4.30 (br m, 2H), 3.23–3.14 (m, 4 H), 3.00-2.70 (m, 4H), 2.58 (s, 3H), 2.26 (DMSO), 2.05 (s, 3H), 0.97–0.93 (m, 1H), 0.24–0.18 (m, 2H), 0.10–0.05 (m, 2H).

Compound **32** (DMSO-d_6): δ 9.24 (s, 1H), 8.27 (s, 1H), 8.20-8.10 (m, 1H), 7.90-7.80 (m, 1H), 7.35–7.25 (m, 2H), 7.15–7.05 (m, 1H), 3.75–3.60 (m, 8 H), 3.39-3.37 (m, 2H), 2.29 (s, 3H), 1.25–1.15 (m, 1H), 0.46–0.40 (m, 2H), 0.35–0.30 (m, 2H).

Compound **33**: δ 8.23 (s, 1H), 7.77 (d, 1H, J = 8.0 Hz), 7.31–7.13 (m, 3H), 4.8–4.75 (m, 2+H), 3.54–3.50 (m, 2 H), 3.0–2.80 (m, 4H), 2.34 (s, 3H), 2.0–1.80 (m, 3H), 1.35–1.10 (m, 3H), 0.64–0.60 (m, 2H), 0.42–0.37 (m, 2H).

Compound **34**: δ 8.14 (s, 1H), 7.73 (d, 1H, J = 8.2 Hz), 7.21–7.05 (m, 3H), 4.92–4.84 (m, 2+H), 3.40–3.38 (m, 4+ H), 3.20 (CH_3OD), 2.98-2.84 (m, 1H), 2.26 (s, 3H), 2.0-1.90 (m, 2H), 1.46-1.30 (m, 2H), 1.20–1.10 (m, 1H), 0.51–0.48 (m, 2H), 0.31–0.26 (m, 2H).

Compound **35**: δ 8.24 (br s, 1H), 7.70–7.60 (m, 1H), 7.30–7.10 (m, 3H), 3.50–3.25 (m, 6 H), 3.21 (CH_3OD), 3.10–2.90 (m, 2H), 2.25 (s, 3H), 2.17–2.11 (m, 1H), 2.06 (s, MeCN), 1.75–1.60 (m, 2H), 1.20–1.05 (m, 1H), 0.54–0.45 (m, 2H), 0.30–0.25 (m, 2H).

Compound **36**: δ 8.33-8.31 (m, 1H), 8.26 (s, 1H), 7.59–7.56 (m, 1H), 7.40–7.35 (m, 1H), 7.18–7.14 (m, 1H), 4.25–4.10 (m, 4 H), 3.63 (d, 2H, J = 7.1 Hz), 3.33-3.30 (m, 4H), 2.85-2.70 (m, 1H), 2.44 (s, 3H), 2.20–1.75 (m, 6H).

Compound **37**: δ 8.10 (s, 1H), 8.07–8.06 (m, 1H), 7.41–7.38 (m, 1H), 7.21–7.15 (m, 1H), 7.04–6.99 (m, 1H), 4.01–3.98 (m, 4 H), 3.55–3.51 (m, 2H), 3.17–3.14 (m, 4H), 3.13 (CH_3OD), 2.28 (s, 3H), 1.99 (s, MeCN), 1.52–1.45 (m, 2H), 0.71–0.58 (m, 1H), 0.37–0.31 (m, 2H), 0.07–0.00 (m, 2H).

Compound **38**: δ 8.27 (s, 1H), 8.22-8.20 (m, 1H), 7.57–7.55 (m, 1H), 7.37–7.33 (m, 1H), 7.21–7.17 (m, 1H), 4.18–4.15 (m, 4 H), 3.84-3.82 (m, 2H), 3.70-3.68 (m, 2H), 3.39 (s, 3H), 3.33-3.30 (m, 4+H), 2.44 (s, 3H).

Compound **39**: δ 8.34 (s, 1H), 8.33-8.32 (m, 1H), 7.56 (d, 1H, J = 7.9 Hz), 7.37–7.27 (m, 2H), 7.18–7.09 (m, 2H), 6.97–6.94 (m, 1H), 4.97 (s, 2 H), 4.16-4.13 (m, 4H), 3.30-3.28 (m, 4H), 2.44 (s, 3H).

Compound **40**: δ 8.35-8.33 (m, 1H), 8.32 (s, 1H), 7.75 (d, 1H, J = 3.3 Hz), 7.53-7.50 (m, 2H), 7.37–7.34 (m, 1H), 7.16–7.11 (m, 1H), 5.11 (s, 2H), 4.30–4.10 (m, 4H), 3.40–3.20 (m, 4H), 2.43 (s, 3H).

Compound **41**: δ 8.74 (d, 1H, J = 0.67 Hz), 8.44–8.36 (m, 2H), 8.29 (s, 1H), 7.96 (d, 1H, J = 8.1 Hz), 7.87–7.82 (m, 1H), 7.61–7.58 (m, 1H), 7.42–7.36 (m, 1H), 7.22–7.16 (m, 1H), 5.11 (s, 2H), 4.23–4.19 (m, 4H), 3.36–3.33 (m, 4H), 2.45 (s, 3H).

Compound **42**: δ 8.22 (s, 1H), 8.19-8.17 (m, 1H), 7.58–7.55 (m, 1H), 7.39-7.35 (m, 1H), 7.24–7.20 (m, 1H), 4.30–4.10 (br s, 4 H), 3.34-3.30 (m, 4+H), 2.44 (s, 3H).

Compound **43**: δ 8.38 (d, 1H, J = 8.3 Hz), 8.24 (s, 1H), 7.59–7.56 (m, 1H), 7.41–7.35 (m, 1H), 7.20–7.14 (m, 1H), 4.50-4.44 (m, 1H), 4.30–4.20 (m, 1H), 3.75–3.65 (m, 2 H), 3.49 (d, 2H, J = 6.8 Hz), 3.42–3.37 (m, 1H), 2.44 (s, 3H), 2.20–2.10 (m, 1H), 1.84-1.68 (m, 3H), 1.28–1.21 (m, 1H), 0.63–0.59 (m, 2H), 0.41–0.38 (m, 2H).

Compound **44**: δ 8.34 (dd, 1H, J = 1.2, 8.3 Hz), 8.24 (s, 1H), 7.58–7.56 (m, 1H), 7.40–7.36 (m, 1H), 7.20–7.16 (m, 1H), 4.51-4.47 (m, 1H), 4.30–4.20 (m, 1H), 3.74–3.63 (m, 2 H), 3.52-3.48 (m, 2H), 3.40–3.37 (m, 1H), 2.44 (s, 3H), 2.20–2.10 (m, 1H), 1.93-1.71 (m, 3H), 1.31–1.23 (m), 0.63–0.60 (m, 2H), 0.42–0.39 (m, 2H).

Compound **45**: δ 8.40–8.35 (m, 1H), 8.22 (s, 1H), 7.60–7.55 (m, 1H), 7.40–7.30 (m, 1H), 7.20–7.10 (m, 1H), 5.10–4.90 (m, 2H), 3.55–3.45 (m, 2 H), 3.10–2.85 (m, 4H), 2.47 (s, 3H), 2.10–1.80 (m, 3H), 1.40–1.20 (m, 3H), 0.65–0.55 (m, 2H), 0.45–0.35 (m, 2H).

Compound **46**: δ 8.64–8.62 (m, 1H), 8.26 (s, 1H), 7.60–7.57 (m, 1H), 7.35–7.30 (m, 1H), 7.17–7.11 (m, 1H), 4.45–4.35 (m, 2H), 4.10–4.0 (m, 2 H), 3.45 (d, 2H, J = 6.8 Hz), 3.15-3.05 (m, 1H), 2.44 (s, 3H), 1.25–1.20 (m, 1H), 0.62–0.56 (m, 2H), 0.39–0.34 (m, 2H).

Compound **47**: δ 8.56 (d, 1H, J = 8.3 Hz), 8.26 (s, 1H), 7.59 (d, 1H, J = 7.6 Hz), 7.37-7.31 (m, 1H), 7.19–7.14 (m, 1H), 4.41 (s, 4 H), 4.33 (s, 4H), 3.46 (d, 2H, J = 7.2 Hz), 2.44 (s, 3H), 1.29–1.21 (m, 1H), 0.64–0.58 (m, 2H), 0.41–0.36 (m, 2H).

Compound **48**: δ 8.40–8.30 (m, 1H), 8.25 (s, 1H), 7.58–7.55 (m, 1H), 7.39–7.31 (m, 1H), 7.23–7.18 (m, 1H), 4.10–4.0 (m, 2H), 3.53 (d, 2 H, J = 7.2 Hz), 3.30–3.25 (m/s, 5+H), 2.65 (s, 3H), 2.43 (s, 3H), 1.30–1.21 (m, 1H), 0.64–0.58 (m, 2H), 0.43–0.38 (m, 2H).

Compound **49**: δ 8.09–8.06 (m, 1H), 8.02 (s, 1H), 7.39–7.36 (m, 1H), 7.20–7.11 (m, 1H), 7.02–6.96 (m, 1H), 4.79–4.75 (m, 2H), 3.31 (d, 2 H, J = 7.0 Hz), 3.11 (CH_3OD), 2.88–2.80 (m, 4H), 2.73 (s, 6H), 2.25 (s, 3H), 2.10–1.90 (m, 1H), 1.75–1.60 (m, 2H), 1.30–1.20 (m, 3H), 0.47–0.35 (m, 2H), 0.29–0.19 (m, 2H).

Compound **50**: δ 8.31-8.29 (m, 1H), 8.28 (s, 1H), 7.59-7.56 (m, 1H), 7.39–7.33 (m, 1H), 7.21-7.16 (m, 1H), 5.10-4.95 (br m, 2+H), 3.70-3.45 (m, 4+H), 3.40-3.35 (m, 4+H), 3.20–3.10 (m, 2+H), 2.96 (s, 3H), 2.72 (br s, 1H), 2.45 (s, 3H), 1.35–1.25 (m, 1H), 0.61–0.55 (m, 2H), 0.39–0.34 (m, 2H).

Cloning, Expression, and Purification of KHK-C.[26c] Human ketohexokinase isoform C (KHK-C), with the amino acid sequence MGSSHHHHHHSSGLVPRGSQILCVGLVVLDVISLV-DKYPKEDSEIRCLSQRWQRGGNASNSCTVLSLLGAPCAFMGSMAPGHVADFLVADFRRRGVDVS QVAWQSKGDTPSSCCIINNSNGNRTIVLHDTSLPDVSATDFEKVDLTQFKWIHIEGRNASEQVKM LQRIDAHNTRQPPEQKIRVSVEVEKPREELFQLFGYGDVVFVSKDVAKHLGFQSAEEALRGLYGR VRKGAVLVCAWAEEGADALGPDGKLLHSDAFPPPRVVDTLGAGDTFNASVIFSLSQGRSVQEALR FGCQVAGKKCGLQGFDGIV, was cloned into a pET28a vector modified with a BamHI restriction site at Gly/Ser of the thrombin cleavage site. The plasmid was transformed into an *E. coli* BL21 (DE3) cellular background from a single colony. The strain was grown overnight at 37°C in Luria Broth (LB) supplemented with kanamycin at 50 μg/mL. This subculture was supplemented with 0.5% glucose + kanamycin (50 μg/mL) until the optical density (OD; A600) reached 0.8. The culture was chilled to 15°C on ice, induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and grown for an additional 16 h at 15°C. Cell pellets were collected by centrifugation at 4000 x *g* for 15 min at 4°C. The cells were lysed on ice. The lysates were centrifuged at 20,000 x *g* for 30 min. The soluble protein fraction was subjected to Ni^{2+}-NTA (nitrilotriacetic acid) affinity chromatography (His-Trap™) and KHK samples were eluted with an imidazole gradient (0-500 mM). Buffer exchange was completed by gel-filtration chromatography in 25 mM Tris-HCl (pH 8) and 250 mM NaCl. The resulting recombinant KHK-C protein was greater than 95% homogeneous and was flash frozen at a concentration of 31 μM using liquid nitrogen. It was stored at –80°C for later use.

Fluorescence Polarization (FP) Assay. In vitro activity was assessed with fluorescence polarization using the ADP[2] assay platform developed by BellBrook Labs (Transcreener® Assay). The homogeneous assay, which was performed in 384-well plates, is based on the immunodetection of ADP produced/inhibited during the kinase reaction as a function of test compound. The KHK kinase reaction was run for 12-15 min as a function of test compounds under the following conditions: 100 nM enzyme concentration, 100 μM ATP, 200 μM D-fructose substrate in an assay buffer of 50 mM HEPES, pH 7.5, 4 mM $MgCl_2$, 0.01% Brij-35. Under these conditions, less than 10% substrate turnover was observed, such that the reaction can be assumed to be under initial rate conditions; 60 μg/mL of antibody was used for detection. The IC_{50} values reported for most compounds were determined with $n = 1$. The IC_{50} values for the following compounds were determined with $n > 1$ [compound, IC_{50} ± standard deviation (nM); n is given in parentheses]: **3**, 210 ± 88 (6); **6**, 100 ± 35 (3); **8**, 12 ± 6 (22); **13**, 3000 ± 1400 (2); **16**, 380 ± 28 (2); **41**, 9.8 ± 4.5 (3); **42**, 7.1 ± 4.4 (2); **47**, 8.0 ± 7.1 (2), **50**, 110 ± 75 (3).

Cellular Assay. A HepG2 cell line was purchased from American Type Culture Collection (ATCC; Cat # CRL-11997) and maintained in growth media consisting of Dulbecco's Modification of Eagle's Medium (DMEM) with 4.5 g/L glucose (Cellgro #10-013-CV), supplemented with 10% fetal bovine serum (Cellgro #35-011-CV) and 100 U/mL each penicillin and streptomycin (Cellgro #30-002-CI). The cells were maintained in a humidified 5% CO_2 atmosphere at 37°C. The cells for testing were passed and seeded on 96-well plates (Falcon #3072), at ~40% confluence in 100 μL of standard media. After overnight incubation of the cells, ~80% confluent, were washed with sterile PBS buffer. The media was replaced with conditioned media (DMEM), supplemented with 10% fetal bovine serum, penicillin, streptomycin) with and without inhibitor test compound. The compounds were prepared as 10 mM DMSO stock solutions, so the final concentration was 0.1%. After an incubation period of 30 min at 37°C, fructose was added to a concentration of 15 mM. After an additional incubation period of 3 h, the cells were washed several times with cold PBS buffer. Cold hypotonic lysis buffer, 30 μL/well of 10 mM ammonium acetate pH 7.4, was added to the plates and they were allowed to rest on ice for 1-2 min. The solution was pipetted up/down (for mechanical lysis) and then frozen at –80°C. The samples were scraped and the lysates were centrifuged at 15,000 x *g* at 4°C for 20 min. Supernantant samples were collected and analyzed for levels of fructose-1-phosphate (F1P). Cell lysate samples were analyzed directly by using LC-MS. Each test sample or control (10 μL) was injected onto an Agilent 1100/Applied Biosystems 4000 Q-Trap LC-MS system for analysis. The mass spectrometer was operated in electrospray-ionization mode with selected reaction monitoring (SRM). F1P was quantified by monitoring SRM chromatographic peak areas against a standard calibration curve from 0.1 to 50 μM, prepared in incubation buffer. The IC_{50} values for the compounds in Table 4 were determined with $n > 1$, except for **38** [compound, IC_{50} ± standard deviation (nM); n is given in parentheses]: **3**, 2400 ± 920 (36); **8**, 400 ± 110 (56); **40**, 270 ± 77 (3); **41**, 270 ± 86 (9); **42**, 78 ± 49 (2); **46**, 590 ± 230 (3); **47**, 8.0 ± 7.1 (2). A negative control, the 3-methoxy isomer of **6** (KHK IC_{50} >9,000 nM), gave the following result: IC_{50} = 26,000 ± 18,000 nM ($n = 6$).

X-ray Crystallography. The KHK protein used in the crystallography studies was cloned, expressed and purified following a published protocol.[26c] KHK was crystallized by using published conditions[26c] (also see PDB ID: 2HLZ). The KHK crystals were soaked overnight in a 5 mM solution of the compound of interest. The next day, the crystals were transferred to a cryoprotectant solution and quickly frozen by immersion in liquid nitrogen. X-ray diffraction data for cocrystals containing **3**, **8**, and **47** were collected on the IMCA-CAT beamline BM-17 at the Argonne National Laboratory. Diffraction data were indexed, integrated, and scaled using d*trek (Pflugrath, J. W. The finer things in X-ray diffraction data collection. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1999**, *D55*, 1718–1725). The crystals belong to the $P2_12_12_1$ space group, with two KHK molecules in the asymmetric unit. The KHK structure was determined with the PHENIX suite (Adams, P. D.; Afonine, P. V.; Bunkóczi, G.; Chen, V. B.; Davis, I. W.; Echols, N.; Headd, J. J.; Hung, L.; Kapral, G. J.; Grosse-Kunstleve, R. W.; McCoy, A. J.; Moriarty, N. W.; Oeffner, R.; Read, R. J.; Richardson, D. C.; Richardson, J. S.; Terwilliger, T. C.; Zwart, P. H. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2010**, *D66*, 213–221) by using pdb coordinates 3NBV as the search model.[26c] The atomic coordinates and structure factors for the KHK complexes with **3**, **8**, and **47** have been deposited in the Protein Data Bank under accession codes 3QA2, 3Q92, and 3QAI, respectively [Rutgers University, New Brunswick, NJ, Protein Data Bank, Research Collaboratory for Structural Bioinformatics (http://www.rcsb.org)].

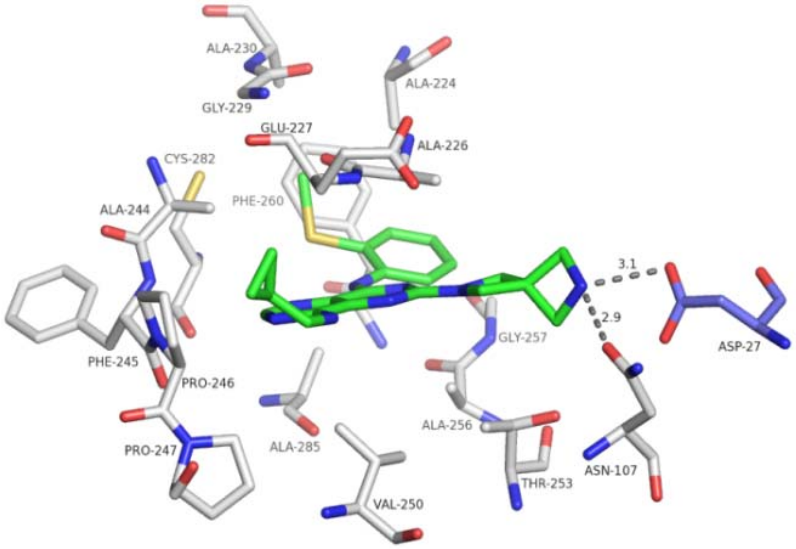

Figure S1. View of **47**·KHK as stick models (C, green; N, blue; S, yellow), showing the ligand and neighboring KHK residues (labeled) in subunit *a* (C, white; N, blue; O, red; S, yellow), with Asp-27 in subunit *b* (C, light blue; N, blue; O, red). The heavy atom for the conserved water was not adequately represented in the electron density map in this case. The H-bonds from the azetidine nitrogen to Asp-27B (3.1 Å) and to Asn-107 (2.9 Å) are denoted by dashed lines.

Pharmacokinetics (PK). This study was conducted by WuXi PharmaTec (Shanghai, P.R. China). Eight male Sprague–Dawley rats (~250 g) were used. The rats were segregated into two groups, four animals per study, for oral and intravenous administration experiments. Compound **8** as a free base was dissolved in 20% hydroxypropyl-β-cyclodextrin in water (w/v) and the solution was adjusted to pH 4.0 with 1 N HCl. Oral dose (10 mg/kg) administration was by gavage. Blood samples were collected at 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h after administration. Intravenous dose administration (2 mg/kg) was in the jugular vein, and blood samples were collected at 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h after administration. Complete plasma profiles were taken from four different animals, with plasma samples being assayed by using LC-MS/MS. Noncompartmental pharmacokinetic analysis was performed with a commercial software package (WinNonlin Professional version 5.2, Pharsight, Mountain View, CA). Analysis was carried out on the plasma concentration time profiles obtained from each animal (n = 4). Oral PK parameters observed were maximum plasma concentration (C_{max}), time to reach the maximum plasma concentration (t_{max}), plasma half-life ($t_{1/2}$), bioavailability (F), and exposure of the compound calculated by the area under the curve (AUC_{last}; AUC_{inf}). PK parameters after iv administration were volume of distribution (Vd_{ss}), total plasma clearance (CL), and area under the curve (AUC_{last}; AUC_{inf}).

Panel of 31 Kinases. The panel of kinases listed in the table below, which is distributed among the range of kinase families (see Figure S2), was assayed by Invitrogen.

kinase	full name
ABL1	Abelson murine leukemia viral oncogene homolog 1
ACVR1B (ALK4)	activin receptor-like kinase 4
AKT1 (PKB alpha)	protein kinase B alpha
AMPK A1/B1/G1	AMP-activated protein kinase A1/B1/G1
AURKA (Aurora A)	aurora A
CAMK1D (CaMKI delta)	calmodulin-dependent kinase 1 delta
CAMK2A (CaMKII alpha)	calmodulin-dependent kinase 2 alpha
CDK1/cyclin B	cyclin-dependent kinase 1
CHEK1 (CHK1)	checkpoint kinase 1
CHEK2 (CHK2)	checkpoint kinase 2
CSNK1D (CK1 delta)	casein kinase 1 delta
DAPK3 (ZIPK)	death-associated kinase 3
EGFR (ErbB1)	epidermal growth factor receptor
EPHB1	ephrin B1
GSK3B (GSK3 beta)	glycogen synthase kinase 3 beta
INSR	insulin receptor
IRAK4	interleukin-1 receptor-associated kinase 4
JAK2	Janus kinase 2
MAPK13 (p38 delta)	mitogen-activate
MST4	mammalian homolog Ste20-like kinase
NEK2	NIMA (never in mitosis in *Aspergillus nidulans*)-related kinase 2
NTRK1 (TRKA)	neurotrophic tyrosine kinase receptor type 1
PAK3	p21-activated protein kinase 3
PDGFRB (PDGFR beta)	platelet-derived growth factor beta chain
PIM2	provirus integration site for Moloney murine leukemia virus 2
PLK3	polo-like kinase 3
PRKACA (PKA)	protein kinase A
PRKCQ (PKC theta)	protein kinase C theta
ROCK1	Rho-dependent protein kinase
RPS6KA3 (RSK2)	p90 ribosomal S6 kinase
SRC	sarcoma kinase

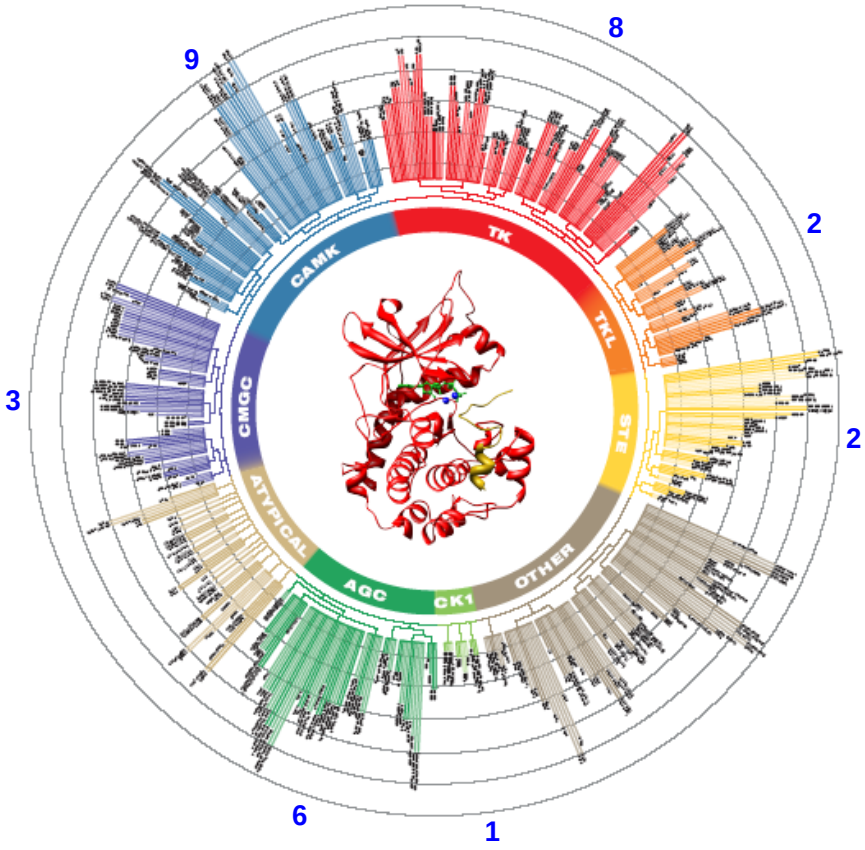

Figure S2. Representation of the 518 kinases according to families, with the distribution of tested kinases denoted by the blue numbers. The graphic is attributed to Invitrogen.

Off-target Action on Receptors and Ion Channels. Binding affinity to diverse receptors and ion channels was conducted for **8** at 10 μM by CEREP. The results are presented below.

Binding Assay	% of Inhibition
Adenosine A1 (h)	<10
Adenosine A2A (h) (agonist radioligand)	92
Adenosine A3 (h) (agonist radioligand)	96
alpha 1 adrenergic (non-selective)	<10
alpha 2 adrenergic (non-selective)	<10
beta 1 adrenergic (h) (agonist radioligand)	<10
Angiotensin AT1 (h)	<10
Benzodiazepine BZD (central) (agonist radioligand)	16
Bradykinin B2 (h) (agonist radioligand)	<10
Cholecystokinin CCK1 (CCKA) (h) (agonist radioligand)	11
Dopamine D1 (h)	45
Dopamine D2S (h)	28
Endothelin ETA (h) (agonist radioligand)	<10
GABA (non-selective) (agonist radioligand)	<10
Galanin GAL2 (h) (agonist radioligand)	17
CXC chemokine CXCR2 (IL-8B) (h) (agonist radioligand)	<10
C-C chemokine CCR1 (h) (agonist radioligand)	<10
Histamine H1 (h)	13
Histamine H2 (h)	14
Melanocortin MC4 (h) (agonist radioligand)	14
MT1 (ML1A) (h) (agonist radioligand)	39

Muscarinic M1 (h)	46
Muscarinic M2 (h)	60
Muscarinic M3 (h)	23
Neurokinin NK2 (h) (agonist radioligand)	54
Neurokinin NK3 (h)	<10
Neuropeptide Y Y1 (h) (agonist radioligand)	<10
Neuropeptide Y Y2 (h) (agonist radioligand)	24
Neurotensin NTS1 (NT1) (h) (agonist radioligand)	<10
delta 2 opioid (DOP) (h) (agonist radioligand)	79
kappa opioid (KOP) (agonist radioligand)	24
mu opioid (MOP) (h) (agonist radioligand)	59
Nociceptin/orphanin FQ (N/OFQ) peptide NOP (ORL1) (h) (agonist radioligand)	<10
Serotonin 5-HT1A (h) (agonist radioligand)	<10
Serotonin 5-HT1B	11
Serotonin 5-HT2A (h)	39
Serotonin 5-HT2B (h) (agonist radioligand)	95
Serotonin 5-HT3 (h)	22
Serotonin 5-HT5A (h) (agonist radioligand)	33
Serotonin 5-HT6 (h) (agonist radioligand)	23
Serotonin 5-HT7 (h) (agonist radioligand)	12
Somatostatin sst (non-selective) (agonist radioligand)	<10
VPAC1 (VIP1) (h) (agonist radioligand)	<10
Vasopressin V1a (h) (agonist radioligand)	<10
Ca2+ channel (L, verapamil site) (phenylalkylamine)	11
KV channel	<10
SKCa channel	<10
Na+ channel (site 2)	17
Cl- channel (GABA-gated)	<10
norepinephrine transporter (h)	81
dopamine transporter (h)	46

Binding or Cellular Functional Data Follow-up (EC_{50} or IC_{50} in μM):

- adenosine A2A functional: inactive
- adenosine A3 functional: EC_{50} = 15 μM (no adverse effects)
- M2 muscarinic functional: IC_{50} = 16 μM
- delta 2 opioid binding: IC_{50} = 5 μM
- mu opioid functional: inactive
- NK2 functional: inactive
- norepinephrine transporter binding: IC_{50} = 0.83 μM
- serotonin 5-HT2B functional: EC_{50} = 2.5 μM (Note: compound interference with functional assay)