Supporting information

Repurposing of a Nucleoside Scaffold from Adenosine Receptor Agonists
to Opioid Receptor Antagonists

Dilip K. Tosh,\textsuperscript{a}† Antonella Ciancetta,\textsuperscript{a}‡ Philip Mannes,\textsuperscript{a} Eugene Warnick,\textsuperscript{a}
Aaron Janowsky,\textsuperscript{b} Amy J. Eshleman,\textsuperscript{b} Elizabeth Gizewski,\textsuperscript{c} Tarsis F. Brust,\textsuperscript{d} Laura M. Bohn,\textsuperscript{d}
John A. Auchampach,\textsuperscript{c} Zhan-Guo Gao,\textsuperscript{a} and Kenneth A. Jacobson\textsuperscript{a}* 

\textsuperscript{a}Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892 USA.
\textsuperscript{b}VA Portland Health Care System, Research Service (R&D-22), and Departments of Psychiatry and Behavioral Neuroscience, Oregon Health and Science University, 3710 S.W. U.S. Veterans Hospital Blvd., Portland, Oregon 97239 USA.
\textsuperscript{c}Department of Pharmacology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, Wisconsin 53226 USA.
\textsuperscript{d}Departments of Molecular Medicine and Neuroscience, 130 Scripps Way, The Scripps Research Institute, Jupiter, Florida 33458 USA.

† Contributed equally.

‡ present address: Queen’s University Belfast, School of Pharmacy, 96 Lisburn Rd, Belfast BT9 7BL, UK.
| Contents                                                                 | Page     |
|------------------------------------------------------------------------|----------|
| Chemical synthesis (with Schemes S1-S4)                                | S3-S10   |
| Reagents for diverse and off-target binding activities                 | S11      |
| Binding inhibition curves (KOR and TSPO, Figures S1-S4)               | S12-S15  |
| Binding inhibition curves (other off-targets, Figure S5)              | S16-S22  |
| Parameters for interaction with DAT and NET (Table S1)                | S23      |
| Lack of correlation of binding at KOR and TSPO (Fig. S6)              | S24      |
| Molecular modeling procedures and results (Table S2 and Figure S7)    | S25-S30  |
| Pharmacokinetic methods and ADME-toxicity data for 28 (Tables S3-S7    | S31-S40  |
| and Figures 8-9)                                                      |          |
Chemical synthesis

Scheme S1. Synthesis of C2-H analogues. Reagents and conditions: (i) 7-deaza-6-chloro-purine, Ph$_3$P, DIAD, THF, rt (ii) 40% MeNH$_2$, MeOH; (iii) 10% TFA, MeOH, 70 °C.

Ethyl (3aR,3bS,4aS,5R,5aS)-5-(4-chloro-7H-pyrrolo[2,3-d]pyrimidin-7-yl)-2,2-dimethyltetrahydrocyclopropa[3,4]cyclopenta[1,2-d][1,3]dioxole-3b(3aH)-carboxylate (60)

DIAD (0.22 mL, 1.13 mmol) was added to a solution of triphenylphosphine (0.296 g, 1.13 mmol) and 7-deaza-6-chloro-purine (0.173 g, 1.13 mmol) in dry THF (4 mL) at 0 °C and after addition it was stirred at room temperature for 10 min. A solution of compound 59 (0.137 g, 0.566 mmol) in THF (2 mL) was added to the reaction mixture, and the mixture stirred overnight at room temperature. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (hexane:ethyl acetate = 5:1) to give compound 60 (0.134 g, 63%) as a colorless foamy solid. $^1$H NMR (CD$_3$OD, 400 MHz) δ 8.52 (s, 1H), 7.55 (d, $J$ = 4.0 Hz, 1H), 6.68 (d, $J$ = 4.0 Hz, 1H), 5.85 (d, $J$ = 7.2 Hz, 1H), 4.86 (s, 1H), 4.79 (d, $J$ = 7.2 Hz, 1H), 4.27-4.20 (m, 2H), 2.28-2.23 (m, 1H), 1.54-1.50 (m, 3H), 1.31-1.26 (m, 6H). HRMS calculated for C$_{18}$H$_{21}$N$_3$O$_4$SCl (M + H)$^+$: 378.1221; found 378.1226.

(3aR,3bS,4aS,5R,5aS)-N,2,2-Trimethyl-5-(4-(methylamino)-7H-pyrrolo[2,3-d]pyrimidin-7-yl)tetrahydrocyclopropa[3,4]cyclopenta[1,2-d][1,3]dioxole-3b(3aH)-carboxamide (62)

40% methylamine solution (3.0 mL) was added to a solution of compound 60 (134 mg, 0.35 mmol) in methanol (3 mL) and the mixture stirred at room temperature overnight. Solvent
was evaporated under vacuum and the residue was purified on flash silica gel column chromatography (CH$_2$Cl$_2$:MeOH=45:1) to give compound 62 (92 mg, 72%) as a colorless syrup. $^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 8.16 (s, 1H), 7.07 (d, $J$ = 3.6 Hz, 1H), 6.59 (d, $J$ = 3.6 Hz, 1H), 5.67 (d, $J$ = 6.8 Hz, 1H), 5.10 (s, 1H), 4.67 (d, $J$ = 6.8 Hz, 1H), 3.06 (s, 3H), 2.82 (s, 3H), 2.13-2.09 (m, 1H), 1.58-1.53 (m, 4H), 1.46 (d, $J$ = 5.2 Hz, 1H), 1.27 (s, 3H). HRMS calculated for C$_{18}$H$_{24}$N$_5$O$_3$ (M + H)$^+$: 358.1879; found 358.1874.

$^{(15,2R,3S,4R,5S)}$-2,3-Dihydroxy-N-methyl-4-(4-(methylamino)-7H-pyrrolo[2,3-d]pyrimidin-7-yl)bicyclo[3.1.0]hexane-1-carboxamide (17)

10% TFA (2 mL) was added to a solution of compound 62 (60 mg, 0.168 mmol) in MeOH (2 mL) and heated at 70 $^\circ$C for 3 h. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (CH$_2$Cl$_2$:MeOH = 10:1) to give compound 17 (45 mg, 85%) as a colorless syrup. $^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 8.25 (s, 1H), 7.37 (d, $J$ = 3.6 Hz, 1H), 6.86 (br s, 1H), 5.13 (s, 1H), 4.96 (d, $J$ = 6.4 Hz, 1H), 3.93 (d, $J$ = 6.4 Hz, 1H), 3.19 (br s, 3H), 2.02-1.98 (m, 1H), 1.87 (d, $J$ = 4.8 Hz, 1H), 1.41-1.38 (m, 1H). HRMS calculated for C$_{15}$H$_{20}$N$_5$O$_3$ (M + H)$^+$: 318.1566; found 318.1566.

**Scheme S2.** Synthesis of $^\delta$-substituted analogues. Reagents and conditions: (i) $R^3$NH$_2$, Et$_3$N, MeOH, rt; (ii) 40% MeNH$_2$, MeOH; (iii) 2-chloro-5-ethynylthiophene, Pd(Ph$_3$P)$_2$Cl$_2$, CuI, Et$_3$N, DMF, rt; (iv) 10% TFA, MeOH, 70 $^\circ$C.

$^{(15,2R,3S,4R,5S)}$-4-(2-((5-Chlorothiophen-2-yl)ethynyl)-6-((3,3,3-trifluoropropyl)-amino)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide (19)

10% TFA (2 mL) was added to a solution of compound 95a (38 mg, 0.065 mmol) in MeOH (2 mL) and heated at 70 $^\circ$C for 3 h. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (CH$_2$Cl$_2$:MeOH = 35:1) to give compound 19 (29 mg, 82%) as a brown syrup. $^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 8.13 (s, 1H), 7.31 (d, $J$ = 4.0 Hz, 1H), 7.02 (d, $J$ = 4.0 Hz, 1H), 5.04 (d, $J$ = 5.2 Hz, 1H), 4.01 (d, $J$ = 6.0 Hz, 1H), 3.89 (br s,
was purified on flash column chromatography (hexane:ethyl acetate = 2:1) to give the desired product at room temperature overnight. The reaction mixture was evaporated under vacuum and the residue was purified on flash silica gel column chromatography (hexane:ethyl acetate = 2:1) to give compound 93a (40 mg, 79%) as a colorless syrup.

**Ethyl (3aR,3bS,4aS,5R,5aS)-5-(2-iodo-6-((3,3,3-trifluoropropyl)amino)-9H-purin-9-yl)-2,2-dimethyltetrahydrocyclopropa[3,4]cyclopenta[1,2-d][1,3]dioxole-3b(3aH)-carboxylate (93a)**

3,3,3-trifluoropropan-1-amine (49 mg, 0.43 mmol) and triethylamine (0.12 mL, 0.87 mmol) was added to a solution of compound 92 (44 mg, 0.87 mmol) in methanol (2 mL) and the mixture stirred at room temperature overnight. The reaction mixture was evaporated under vacuum and the residue was purified on flash column chromatography (hexane:ethyl acetate = 2:1) to give the desired product 93a (40 mg, 79%) as a colorless syrup. 

**Ethyl (3aR,3bS,4aS,5R,5aS)-5-(2-iodo-6-((3,3,3-trifluoropropyl)amino)-9H-purin-9-yl)-2,2-dimethyltetrahydrocyclopropa[3,4]cyclopenta[1,2-d][1,3]dioxole-3b(3aH)-carboxamide (93b)**

40% methyamine solution (2.0 mL) was added to a solution of compound 93a (38 mg, 0.065 mmol) in methanol (2 mL) and the mixture stirred at room temperature overnight. Solvent was evaporated under vacuum and the residue was purified on flash silica gel column chromatography (CH3Cl2:MeOH=35:1) to give compound 94a (24 mg, 67%) as a colorless syrup. 

**Ethyl (3aR,3bS,4aS,5R,5aS)-5-(2-iodo-6-((3,3,3-trifluoropropyl)amino)-9H-purin-9-yl)-2,2-trimethyltetrahydrocyclopropa[3,4]cyclopenta[1,2-d][1,3]dioxole-3b(3aH)-carboxamide (94a)**

Compound 94b (70%) was prepared from compound 93b following the same method as for compound 94a. 

**Ethyl (3aR,3bS,4aS,5R,5aS)-5-(2-iodo-6-((3,3,3-trifluoropropyl)amino)-9H-purin-9-yl)-N,2,2-trimethyltetrahydrocyclopropa[3,4]cyclopenta[1,2-d][1,3]dioxole-3b(3aH)-carboxamide (94b)**

Compound 94b (70%) was prepared from compound 93b following the same method as for compound 94a.
\((3aR,3bS,4aS,5R,5aS)-5-(2-((5-Chlorothiophen-2-yl)ethynyl)-6-((3,3,3-trifluoropropyl)amino)-9H-purin-9-yl)\)-N,2,2-trimethyltetrahydro-cyclopropa[3,4]cyclopenta[1,2-d][1,3]dioxole-3b(3aH)-carboxamide (95a)

PdCl\(_2\)(PPh\(_3\))\(_2\) (8.1 mg, 0.01 mmol), CuI (1.1 mg, 0.05 mmol), 5-chlorothienyl (49 µL, 0.34 mmol) and triethylamine (80 µL, 0.7 mmol) was added to a solution of compound 94a (33 mg, 0.05 mmol) in anhydrous DMF (1.2 mL) and the mixture stirred at room temperature overnight. Solvent was evaporated under vacuum and the residue was purified on flash silica gel column chromatography (hexane:ethyl acetate = 1:1) to give compound 95a (26 mg, 78%) as a brownish syrup.

\[^{1}H\text{NMR (CD}_3\text{OD, 400 MHz) \delta 8.16 (s, 1H), 7.39 (d, J = 4.0 Hz, 1H), 7.04 (d, J = 4.0 Hz, 1H), 5.79 (d, J = 6.4 Hz, 1H), 5.02 (s, 1H), 3.89 (br s, 2H), 2.82 (s, 3H), 2.68-2.54 (m, 2H), 2.17-2.13 (m, 1H), 1.56-1.53 (m, 4H), 1.43 (t, J = 5.2 Hz, 1H), 1.31 (s, 3H).\] HRMS calculated for C\(_{25}\)H\(_{25}\)N\(_6\)O\(_3\)F\(_3\)SCl (M + H\(^+\)) : 581.1349; found 581.01349.

\((3aR,3bS,4aS,5R,5aS)-5-(2-((5-Chlorothiophen-2-yl)ethynyl)-6-((3-hydroxypropyl)amino)-9H-purin-9-yl)\)-N,2,2-trimethyltetrahydro-cyclopropa[3,4]cyclopenta[1,2-d][1,3]dioxole-3b(3aH)-carboxamide (95b)

Compound 95b (70%) was prepared from compound 94b following the same method as for compound 95a. \[^{1}H\text{NMR (CD}_3\text{OD, 400 MHz) \delta 8.16 (s, 1H), 7.38 (d, J = 4.0 Hz, 1H), 7.04 (d, J = 4.0 Hz, 1H), 5.80 (d, J = 6.4 Hz, 1H), 5.02 (s, 1H), 3.70-3.67 (m, 4H), 2.82 (s, 3H), 2.17-2.13 (m, 1H), 1.94-1.88 (m, 2H), 1.57-1.53 (m, 4H), 1.42 (t, J = 5.2 Hz, 1H), 1.31 (s, 3H).\] HRMS calculated for C\(_{25}\)H\(_{28}\)N\(_6\)O\(_4\)SCl (M + H\(^+\)) : 543.1581; found 543.1572.

Scheme S3. Synthesis of 1-deaza analogues. Reagents and conditions: (i) 2-chloro-5-ethynylthiophene, Pd(Ph\(_3\))\(_2\)Cl\(_2\), CuI, Et\(_3\)N, DMF, rt; (ii) 10%TFA, MeOH, 70 °C.

Ethyl (1S,2R,3S,4R,5S)-4-(5-((5-Chlorothiophen-2-yl)ethynyl)-7-(methylamino)-3H-imidazo[4,5-b]pyridin-3-yl)-2,3-dihydroxybicyclo[3.1.0]hexane-1-carboxylate (26)

PdCl\(_2\)(PPh\(_3\))\(_2\) (8.7 mg, 0.012 mmol), CuI (1.2 mg, 0.006 mmol), 5-chlorothienyl (53 mg, 0.37 mmol) and triethylamine (86 µL, 0.6 mmol) was added to a solution of compound 96 (31 mg, 0.06 mmol) in anhydrous DMF (1.0 mL) and heated at 65 °C overnight. Solvent was evaporated under vacuum and the residue was roughly purified on flash silica gel column chromatography (hexane:ethyl acetate = 1:1) and the resulting compound was dissolved in MeOH (2 mL) and 10% TFA (2 mL) and heated at 70 °C for 2 h. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (CH\(_2\)Cl\(_2\), MeOH = 30:1) to give compound 26 (18 mg, 63%) as a brown syrup. \[^{1}H\text{NMR (CD}_3\text{OD, 400 MHz) \delta 8.01 (s, 1H), 7.25 (d, J = 4.0 Hz, 1H), 7.01 (d, J = 4.0 Hz, 1H), 6.67 (s,}\]
1H), 5.16 (d, J = 6.8 Hz, 1H), 4.29-2.22 (m, 2H), 4.18 (d, J = 6.4 Hz, 1H), 3.04 (s, 3H), 2.25-2.21 (m, 1H), 1.98 (t, J = 5.2 Hz, 1H), 1.68-1.64 (m, 1H), 1.30 (t, J = 7.2 Hz, 3H). HRMS calculated for C_{22}H_{22}ClN_{4}O_{4}SCl (M + H)^{+}: 473.1050; found 473.1052.

Scheme S4. Synthesis of 7-deaza 5′-keto analogues. Reagents and conditions: (i) 2,2,6,6-tetramethylpiperidine, n-BuLi, Bu_3SnCl, THF, -78 °C; (ii) I_2, THF, rt; (iii) MeNH_2·HCl, Et_3N, MeOH, rt; (iv) 2-Chloro-5-ethynylthiophene, Pd(Ph_3P)_2Cl_2, CuI, Et_3N, DMF, rt; (v) 10%TFA, MeOH, 70 °C.

1-((3aR,3bS,4aS,5R,5aS)-5-(4-Chloro-2-(tributylstannyl)-7H-pyrrolo[2,3-d]pyrimidin-7-yl)-2,2-dimethyltetrahydrocyclopropa[3,4]cyclopenta[1,2-d][1,3]dioxol-3b(3aH)-yl)pentan-1-one (63)

n-BuLi (1.32 mL, 1.6M solution in hexane) was added dropwise to a solution of 2,2,6,6-tetramethyl pyridine (0.36 mL, 2.12 mmol) in THF (3 mL) at -78 °C. After 5 min, a solution of compound 36 (160 mg, 0.42 mmol) in THF (1 mL) was added and finally Bu_3SnCl (0.57 mL, 2.12 mmol) to the reaction mixture and the mixture stirred for 1.5 h under the same conditions. The reaction mixture was quenched with saturated NH_4Cl solution. Aqueous layer was extracted with ethyl acetate (3 times) and the combined organic layer was washed with brine, dried over Na_2SO_4, filtered and evaporated. The residue was roughly purified on flash silica gel column chromatography (hexane:ethyl acetate = 10:1) to give compound 63 (121 mg, 42%) as a syrup.

1-((3aR,3bS,4aS,5R,5aS)-5-(4-Chloro-2-iodo-7H-pyrrolo[2,3-d]pyrimidin-7-yl)-2,2-dimethyltetrahydrocyclopropa[3,4]cyclopenta[1,2-d][1,3]dioxol-3b(3aH)-yl)pentan-1-one (64)

Iodine (69 mg, 0.272 mmol) was added to a solution of compound 63 (121 mg, 0.181 mmol) in THF (3 mL) and the mixture stirred for 4 h at room temperature. Na_2S_2O_3 solution was
added to the reaction mixture and aqueous layer was extracted with ethyl acetate (3 times). The combined organic layer was washed with brine, dried (Na$_2$SO$_4$), filtered and evaporated. The residue was purified on flash silica gel column chromatography (hexane:ethyl acetate = 8:1) to give compound 64 (85 mg, 93%) as a colorless syrup. $^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 8.43 (d, $J$ = 7.2 Hz, 1H), 7.06 (d, $J$ = 7.2 Hz, 1H), 6.02 (d, $J$ = 6.4 Hz, 1H), 4.98 (s, 1H), 4.96 (d, $J$ = 6.4 Hz, 1H), 2.69-2.57 (m, 2H), 2.28-2.25 (m, 1H), 1.75-1.74 (m, 1H), 1.58-1.54 (m, 5H), 1.36-1.26 (m, 6H), 0.91 (t, $J$ = 7.2 Hz, 3H). HRMS calculated for C$_{20}$H$_{24}$N$_3$O$_3$I (M + H)$^+$: 516.0551; found 516.0557.

$1$-((3a$^R$,3b$^S$,4a$^S$,5$^R$,5a$^S$)-5-(2-Iodo-4-(methylamino)-7$^H$-pyrrolo[2,3-d]pyrimidin-7-yl)-2,2-dimethyltetrahydrocyclopropa[3,4]cyclopenta[1,2-d][1,3]dioxol-3b(3a$^H$)-yl)pentan-1-one (65)

MeNH$_2$HCl (55 mg, 0.82 mmol) and triethyl amine (0.23 mL, 1.65 mmol) was added to a solution of compound 64 (85 mg, 0.16 mmol) in methanol (3 mL) and the mixture stirred at room temperature overnight. The reaction mixture was evaporated under vacuum and the residue was purified on flash silica gel column chromatography (hexane:ethylacetate = 1:1) to give compound 65 (60 mg, 72%) as a syrup. $^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 7.99 (s, 1H), 6.03 (d, $J$ = 7.2 Hz, 1H), 4.93 (d, $J$ = 6.8 Hz, 1H), 4.88 (s, 1H), 3.03 (s, 3H), 2.76-2.63 (m, 2H), 2.16-2.12 (m, 1H), 1.75-1.70 (m, 1H), 1.60-1.50 (m, 6H), 1.42-1.21 (m, 5H), 0.94 (t, $J$ = 7.2 Hz, 3H). HRMS calculated for C$_{21}$H$_{28}$N$_4$O$_3$I (M + H)$^+$: 511.1206; found 511.1202.

$1$-((3a$^R$,3b$^S$,4a$^S$,5$^R$,5a$^S$)-5-(2-((5-Chlorothiophen-2-yl)ethynyl)-4-(methylamino)-7$^H$-pyrrolo[2,3-d]pyrimidin-7-yl)-2,2-dimethyltetrahydrocyclopropa[3,4]cyclopenta[1,2-d][1,3]dioxol-3b(3a$^H$)-yl)pentan-1-one (66)

PdCl$_2$(PPh$_3$)$_2$ (4.67 mg, 0.006 mmol), CuI (1.0 mg, 0.005 mmol), 2-chloro-5-ethynylthiophene (28 mg, 0.166 mmol) and triethylamine (0.16 mL, 0.33 mmol) was added to a solution of compound 65 (17 mg, 0.033 mmol) in anhydrous DMF (1.2 mL) and was heated at 65 °C under for 2 h. Solvent was evaporated under vacuum and the residue was purified on flash silica gel column chromatography (hexane:ethyl acetate = 1:1) to give compound 66 (13 mg, 74%) as a yellow syrup. $^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 8.14 (s, 1H), 7.25 (d, $J$ = 4.0 Hz, 1H), 7.03 (d, $J$ = 4.0 Hz, 1H), 6.95 (s, 1H), 5.94 (d, $J$ = 7.2 Hz, 1H), 5.12 (s, 1H), 4.95 (d, $J$ = 6.4 Hz, 1H), 3.07 (s, 3H), 2.62-2.56 (m, 2H), 2.28-2.25 (m, 1H), 1.73-1.69 (m, 1H), 1.55-1.49 (m, 5H), 1.34-1.26 (m, 6H), 0.93 (t, $J$ = 7.2 Hz, 3H). HRMS calculated for C$_{27}$H$_{30}$N$_4$O$_3$SCl (M + H)$^+$: 525.1727; found 525.1730.

$1$-((1S,2$^R$,3S,4$^R$,5$^S$)-4-(5-Chlorothiophen-2-yl)ethynyl)-4-(methylamino)-7$^H$-pyrrolo[2,3-d][pyrimidin-7-yl]-2,3-dihydroxybicyclo[3.1.0]hexan-1-yl)pentan-1-one (44)

10% TFA (2.5 mL) was added to a solution of compound 66 (13 mg, 0.024 mmol) in MeOH (2.5 mL) and heated at 70 °C for 3 h. Solvent was evaporated and the residue was purified on flash silica gel column chromatography (CH$_2$Cl$_2$:MeOH = 35:1) to give compound 44 (10 mg, 87%) as a yellowish syrup. $^1$H NMR (CD$_3$OD, 400 MHz) $\delta$ 8.12 (s, 1H), 7.22 (d, $J$ = 4.0
Hz, 1H), 7.00 (d, J = 4.0 Hz, 1H), 6.9 (s, 1H), 5.49 (d, J = 6.8 Hz, 1H), 4.98 (s, 1H), 4.42-4.39 (m, 1H), 3.06 (s, 3H), 2.70-2.65 (m, 2H), 2.17-2.13 (m, 1H), 1.73 (t, J = 5.2 Hz, 1H), 1.67-1.64 (m, 1H), 1.54-1.47 (m, 2H), 1.37-1.27 (m, 2H), 0.92 (t, J = 7.2 Hz, 1H). HRMS calculated for C_{24}H_{26}N_{4}O_{3}SCl (M + H)^+: 485.1414; found 485.1407.

4-Chloro-7-(triisopropylsilyl)-7H-pyrrolo[2,3-d]pyrimidine (68)

NaH (61 mg, 2.73 mmol) was added portion wise to a solution of compound 67 (262 mg, 1.71 mmol) in anhydrous THF (5 mL) at 0 °C and the mixture stirred for 20 min. Then, TIPSCl (0.58 mL, 2.73 mmol) was added dropwise and the mixture stirred for 3 h under same condition. The reaction was quenched by addition of saturated NH_4Cl solution and the aqueous layer was extracted with ethyl acetate (3 times). The combined organic layer was dried (Na_2SO_4), filtered, evaporated and the residue was purified on flash silica gel column chromatography (hexane:ethyl acetate = 20:1) to give compound 68 (488 mg, 92%) as a colorless syrup.

{\textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400 MHz) \(\delta\) 8.55 (s, 1H), 7.60 (d, \(J\) = 3.2 Hz, 1H), 7.00 (d, \(J\) = 3.2 Hz, 1H), 1.97-1.89 (m, 3H), 1.15 (d, \(J\) = 7.2 Hz, 18H). HRMS calculated for C_{14}H_{23}N_{4}SiCl (M + H)^+: 310.1381; found 310.1383.

4-Chloro-2-(tributylstannyl)-7-(triisopropylsilyl)-7H-pyrrolo[2,3-d]pyrimidine (69)

n-BuLi (5.6 mL, 1.6 M solution in hexane) was added dropwise to a solution of 2,2,6,6-tetramethyl pyridine (1.5 mL, 9.0 mmol) in THF (10 mL) at -78 °C. After 5 min, a solution of compound 68 (559 mg, 9.0 mmol) in THF (5 mL) was added, followed by Bu\textsubscript{3}SnCl (2.43 mL, 9.0 mmol) to the reaction mixture and the mixture stirred for 2 h under the same conditions. The reaction mixture was quenched with saturated NH_4Cl solution. Aqueous layer was extracted with ethyl acetate (3 times) and the combined organic layer was washed with brine, dried over Na_2SO_4, filtered and evaporated. The residue was purified on flash silica gel column chromatography (hexane:ethyl acetate = 60:1) to give compound 69 (832 mg, 77%) as a colorless syrup.

{\textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400 MHz) \(\delta\) 7.24 (d, \(J\) = 3.6 Hz, 1H), 7.00 (d, \(J\) = 3.2 Hz, 1H), 1.97-1.89 (m, 6H), 1.38-1.12 (m, 12H), 1.13 (d, \(J\) = 7.6 Hz, 18H), 0.94 (t, \(J\) = 7.2 Hz, 9H). HRMS calculated for C_{26}H_{49}N_{4}SiClSn (M + H)^+: 596.2432; found 596.2440.

4-Chloro-2-iodo-7-(triisopropylsilyl)-7H-pyrrolo[2,3-d]pyrimidine (70)

Iodine (528 mg, 2.08 mmol) was added to a solution of compound 69 (832 mg, 1.38 mmol) in THF (10 mL) and the mixture stirred for 2 h at room temperature. Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3} solution was added to the reaction mixture and aqueous layer was extracted with ethyl acetate (3 times). The combined organic layer was washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was purified on flash silica gel column chromatography (hexane:ethyl acetate = 60:1) to give compound 70 (413 mg, 68%) as a colorless syrup.

{\textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400 MHz) \(\delta\) 7.21 (d, \(J\) = 3.6 Hz, 1H), 7.00 (d, \(J\) = 3.2 Hz, 1H), 1.70-1.62 (m, 3H), 1.14 (d, \(J\) = 7.6 Hz, 18H). HRMS calculated for C_{14}H_{22}N_{4}SiClI (M + H)^+: 436.0347; found 436.0340.

4-Chloro-2-iodo-7H-pyrrolo[2,3-d]pyrimidine (71)

TBAF (1.1 mL, 1M solution in THF) was added to a solution of compound 70 (413 mg, 0.94 mmol) in dry THF at 0 °C and the mixture stirred for 1 h. Solvent was evaporated and the
residue was purified on flash silica gel column chromatography (hexane:ethyl acetate=2:1) to give compound 71 (212 mg, 80%) colorless powder. \(^1\)H NMR (CD\(_3\)OD, 400 MHz) \(\delta\) 7.44 (d, \(J = 3.6\) Hz, 1H), 6.61 (d, \(J = 3.6\) Hz, 1H). HRMS calculated for C\(_6\)H\(_4\)N\(_3\)ClI (M + H) \(^+\): 436.0347; found 436.0340.
Reagents for diverse and off-target binding activities (measured by PDSP, except for A3AR).

\( K_i \) determinations and binding profiles in a broad screen of receptors and channels were generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, Contract # HHSN-271-2008-00025-C (NIMH PDSP). The NIMH PDSP is directed by Bryan L. Roth MD, PhD at the University of North Carolina at Chapel Hill and Project Officer Jamie Driscol at NIMH, Bethesda MD, USA. For experimental details please refer to the PDSP web site http://pdsp.med.unc.edu/ and click on "Binding Assay" or "Functional Assay" on the menu bar. The interactions assayed were: Affinity at histamine receptors will be reported separately.

Radioligands:
- \([^3]H\)Tyr-D-Ala-Gly-Phe-D-Leu (\([^3]H\)DADLE, 97) for DOR.
- \([^3]H\)N\(^-\)methyl-2-phenyl-N\-[\(5R\),7\(S\),8\(S\)-7-(pyrrolidin-1-yl)-1-oxaspiro[4.5]dec-8-yl]acetamide (\([^3]H\)U69593, 98) for KOR.
- \([^3]H\)Ala\(^2\)-MePhe\(^4\)-Glyol\(^5\)-Enkephalin (\([^3]H\)DAMGO, 99) for MOR.
- \([^3]H\)nociceptin 100 for NOP.
- \([^3]H\)Ala\(^2\)-MePhe\(^4\)-Glyol\(^5\)-Enkephalin (\([^3]H\)DAMGO, 99) for MOR.
- \([^3]H\)PK11195, 101 for rat TSPO (1 nM).
- \([^{125}]I\)\(^6\)-(4-amino-3-iodobenzyl)adenosine-5'-N-methyl-uronamide (\([^{125}]I\)AB-MECA, 102) for hA3AR.
- \([^3]H\)methyl (1\(R\),2\(S\),3\(S\))-3-(4-fluorophenyl)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate (\([^3]H\)WIN35428, 103) for hDAT.
- \([^3]H\)\(R\),\(S\)-3-(2-methoxyphenoxy)-N-methyl-3-phenylpropan-1-amine (nisoxetine, 104) for hNET.
- \([^3]H\)\(\pm\)-1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile (citalopram, 105) for hSERT.
- \([^3]H\)3-(3-methyl-3-butenyl)-1,2,3,4,5,6-hexahydro-6,11-dimethyl-8-hydroxy-2,6-metheno-3-benzazocine (pentazocine, 106) for guinea pig \(\sigma_1\) receptor.
- \([^3]H\)1,3-di-(2-tolyl)guanidine (DTG, 107) for rat \(\sigma_2\) receptor.

Alternate DAT radioligands used in the laboratory of A. Janowsky:
- \([^{125}]I\)methyl (1\(R\),2\(S\),3\(S\))-3-(4-iodophenyl)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate (RTI-55, 118)
- \([^3]H\)\(\pm\)-5-(4-chlorophenyl)-3,5-dihydro-2\(H\)-imidazo[2,1-a]isoindol-5-ol (mazindol, 119)

Reference compounds for nonspecific binding
- hDOR: natrindole, 108 (Ki = 0.81±0.08 nM)
- hKOR: Salvinorin A, 109 (Ki = 1.93±0.45 nM)
- hMOR: morphine, 110 (Ki = 3.29±0.50 nM)
- hNOP: 7'-[\(4-(2,6\)-dichlorophenyl)-1-piperidinyl]methyl]-6,7,8,9-tetrahydro-1-methyl-5\(H\)-benzocyclohepten-5-ol (SB612111), 111 (Ki = 6.58±1.42 nM)
- rTSPO: 4'-chlorodiazepam (Ro5-4864), 112 (Ki = 27.6±2.3 nM)
- mA3AR: adenosine-5'-N ethyluronamide (NECA), 113 (Ki = 0.45±0.13 nM)
- hA3AR: adenosine-5'-N ethyluronamide (NECA), 113 (Ki = 35±12 nM)
- hDAT: 1-[\(2-[bis-(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine (GBR12909), 114 (Ki = 3.04±0.21 nM)
- hNET: desipramine, 115 (Ki = 3.16±0.28 nM)
- hSERT: amitriptyline, 116 (Ki = 4.46±0.55 nM)
- gp-\(\sigma_1\) receptor: haloperidol, 117 (Ki = 3.29±0.50 nM)
- r-\(\sigma_2\) receptor: haloperidol, 117 (Ki = 24.7±1.8 nM)
Figure S1. A-D. Inhibition of radioligand binding at hDOR by (N)-methanocarba nucleosides: 5′-ethyl esters 28, 29 and 43 and 4′-truncated 56 (○), in comparison to pharmacological standard 17-cyclopropylmethyl-6,7-dehydro-4,5-epoxy-3,14-dihydroxy-6,7,2′,3′-indolomorphinan (●, natrindole, 108).
Figure S2. A-F. Inhibition of radioligand binding at hKOR by (N)-methanocarba nucleosides: 5′-ethyl esters 28, 30, 39, 43 and 82 and 4′-truncated 54 (σ), in comparison to pharmacological standard Salvinorin A (●, methyl (2S,4aR,6aR,7R,9S,10aS,10bR)-9-(acetyloxy)-2-(furan-3-yl)-6a,10b-dimethyl-4,10-dioxodecahydro-2H-benzo[f]isochromene-7-carboxylate, 109).
Figure S3. A-D. Inhibition of radioligand binding at hMOR by (N)-methanocarba nucleosides: 5′-ethyl esters 28, 29 and 42 and 4′-truncated 56 (σ), in comparison to pharmacological standard morphine (●, 110).
Figure S4. A-F. Inhibition of radioligand binding at rTSPO, by (N)-methanocarba nucleosides: 5′-ethyl esters 28, 29 and 42 and 4′-truncated 52, 55 and 56 (σ), in comparison to a pharmacological standard, Ro5-4864 (●, 4′-chlorodiazepam, 112).
Diverse and off-target binding activities (measured by PDSP, representative binding curves):

**Figure S5.** Unless noted in the text, no significant interactions (<50% inhibition at 10 µM) for any of the nucleosides were found at the following sites (human unless noted): 5HT₁A, 5HT₁B, 5HT₁D, 5HT₁E, 5HT₂A, 5HT₂B, 5HT₂C, 5HT₃, 5HT₅A, 5HT₆, 5HT₇, α₁A, α₁B, α₁D, α₂A, α₂B, α₂C, β₁, β₂, β₃, BZP rat brain site, D₁, D₂, D₃, D₄, D₅, delta opioid receptor (DOR), GABAₐ, M₁, M₂, M₅, µ opioid receptor (MOR), σ₁, σ₂, DAT, NET, SERT. Binding at histamine receptors will be reported elsewhere.

Compd. 4 (PDSP 26565, MRS5698) at KOR

Compd. 9 (PDSP 44289, MRS5703) at TSPO

Compd. 27 (PDSP 44066, MRS7315) at TSPO

Compd. 4 (PDSP 26565, MRS5698) at TSPO

Compd. 27 (PDSP 44066, MRS7315) at 5HT₂A
Compd. 28 (PDSP 42677, MRS7299) at TSPO

Compd. 28 (PDSP 42677, MRS7299) at NOP

Compd. 29 (PDSP 42678, MRS7300) at TSPO

Compd. 29 (PDSP 42678, MRS7300) at NOP

Compd. 29 (PDSP 42678, MRS7300) at MOR
Compd. 31 (PDSP 40444, MRS7252) at KOR

Compd. 32 (PDSP 43918, MRS7304) at TSPO

Compd. 33 (PDSP 43919, MRS7305) at TSPO

Compd. 34 (PDSP 44068, MRS7317) at KOR

Compd. 35 (PDSP 44069, MRS7318) at KOR

Compd. 36 (PDSP 44070, MRS7319) at KOR
Compd. 39 (PDSP 44229, MRS7331) at DOR

Compd. 39 (PDSP 44229, MRS7331) at KOR

Compd. 40 (PDSP 44295, MRS7335) at DAT

Compd. 43 (PDSP 44344, MRS7343) at DOR

Compd. 43 (PDSP 44344, MRS7343) at KOR

Compd. 43 (PDSP 44344, MRS7343) at TSPO
Compd. 48 (PDSP 44287, MRS5708) at TSPO

Compd. 52 (PDSP 44288, MRS5775) at TSPO

Compd. 54 (PDSP 45174, MRS7358) at MOR

Compd. 55 (PDSP 45175, MRS7359) at 5HT2B

Compd. 55 (PDSP 45175, MRS7359) at NET
Compd. 56 (PDSP 45298, MRS7393) at MOR

Compd. 58 (PDSP 45299, MRS7394) at 5HT2B

Compd. 73 (PDSP 42476, MRS7420) at MOR

Compd. 73 (PDSP 42476, MRS7420) at TSPO

Compd. 77 (PDSP 45746, MRS7414) at DOR
Compd. 77 (PDSP 45746, MRS7414) at 5HT2C

Compd. 77 (PDSP 45746, MRS7414) at KOR

Compd. 82 (PDSP 48475, MRS7419) at KOR

Compd. 82 (PDSP 48475, MRS7419) at DOR

**Other off-target screening**

Screening of compounds 14 and 18 at 240 GPCRs and 486 kinases was performed by DiscoverX Corp. (Fremont, CA). Assays all at 10 µM (percent of control shown):

gpcrMAX and OrphanMAX (report NIH_PANLC04648D)

14: A3AR agonist 168%; A3AR antagonist -111%; A2bAR antagonist -9%. No other GPCRs registered >30% in either agonist or antagonist mode.

18: A3AR agonist 158%; A3AR antagonist -95%; A2bAR antagonist 36%. No other GPCRs registered >30% in agonist or >50% in antagonist mode.

KINOMEScan (report NIH045-01-p-00001)

14: MEK6 (MAP2K6) 17% activity remaining. No other kinases registered >50% inhibition.
18: VRK2 31% activity remaining; LATS2 26% activity remaining. No other kinases registered >50% inhibition.
Table S1. Binding and inhibition parameters at DAT and NET (determined in the laboratory of A. Janowsky).

| Compound, (MRS number)a | hDAT | hNET |
|-------------------------|------|------|
|                         | [¹²⁵I]118 binding EC₅₀ or Ki (nM) | [³H]119 binding EC₅₀ or Ki (nM) | [³H]DA uptake IC₅₀ (nM) | [¹²⁵I]118 binding EC₅₀ or Ki (nM) | [³H]119 binding EC₅₀ or Ki (nM) | [³H]NE uptake IC₅₀ (nM) |
| 11 (5676)               | 70 ± 26# 690 ± 180% | 128 ± 18# 438 ± 41% | 92 ± 16 | 1760 ± 640# 371 ± 67% | >10 µM NE | >10 µM |
| 14 (5980)               | 35.1 ± 8.4# 550 ± 110% | 131 ± 31# 403 ± 33% | 253 ± 92 | 1180 ± 360# 386 ± 73% | >10 µM NE | >10 µM |
| 15 (7036)               | 9.1 ± 1.7# 217 ± 24% | 870 ± 130# 369 ± 31% | 229 ± 30 | 670 ± 200# 285 ± 22% | >10 µM NE | 6110 ± 570 |
| 18 (7154)               | 1120 ± 220# 268 ± 32% | ND | >8700 | 900 ± 320# 159 ± 1% | ND | ND |
| 24 (7232)               | 34 ± 13# 434 ± 64% | 267 ± 80# 455 ± 39% | 107 ± 14 | 490 ± 120# 365 ± 11% | >8,400 NE | 3980 ± 330 |
| 28 (7299)               | 1410 ± 340# 320 ± 23% | 2160 ± 550# 267 ± 17% | >9800 | >10 µM NE | >10 µM NE | >10 µM |
| 30 (7251)               | 294 ± 82# 461 ± 67% | 535 ± 29# 347 ± 60% | 1145 ± 52 | 980 ± 270# 302 ± 24% | >10 µM NE | 7900 ± 1400 |

a – data for 11, 14, 15, 18, 24 and 30 are from Tosh et al.33 Values for all compounds at hSERT (binding of [¹²⁵I]118 or [¹²⁵I]119 and serotonin uptake inhibition) are >10 µM or inactive.

ND, not determined.

[¹²⁵I]methyl (1R,2S,3S)-3-(4-iodophenyl)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate (RTI-55, 118)

[³H](±)-5-(4-chlorophenyl)-3,5-dihydro-2H-imidazo[2,1-a]isoindol-5-ol (mazindol, 119)
Figure S6. Lack of correlation of binding at KOR and TSPO (primary binding data from PDSP).
Molecular Modeling Methods

**Computational facilities.** Ligand geometry optimization, homology modeling and molecular docking simulations were carried out using a 6 Intel® Xeon® E5-1650 v3 CPU workstation. Membrane MD simulations were run on one NVIDIA® 980Ti GTX and one NVIDIA® 1080 GTX.

**Ligand Preparation.** Selected compounds were built using Maestro¹ and subjected to gas phase geometry optimization with the Jaguar 9.1 quantum chemistry package² using density functional theory (DFT) with the B3LYP hybrid functional and the 6-31G** basis set.

**Homology modeling.** The hKOR homology model was built upon the X-ray structure of the JDTic-hKOR complex.³ The template structure and the amino acid sequence were retrieved from the RCSB PDB database⁴ (http://www.rcsb.org, PDB ID: 4DJH) and the Universal Protein Resource⁵ (UniProt IDs: P41445), respectively. The X-ray structure was pre-processed as follows: after manual removal of the lysozyme (T4L) fusion protein and solvent atoms, ionization states of protein sidechains and hydrogen positions were assigned with the Protein Preparation Wizard⁶ tool by retaining the co-crystallized ligand and water molecules. Prime 4.5⁷ was used to build the homology model (ClustalW alignment method and knowledge-based building method) and to reconstruct and refine missing loop domains. N-terminal and C-terminal portions were not modeled if their lengths exceeded those of the template.

**Protonation state of His291 (6.52).** After building the hKOR homology model, we carefully assessed the protonation state of His291 (6.52) by examining the H-bond pattern with surrounding residues and water molecules and by comparing the KOR structure with the X-ray structures of other ORs (namely MOR, PDB ID: 4DKL and DOR, PDB ID: 4N6H). In the X-ray structures, His(6.52) establishes a H-bond with the aromatic hydroxyl moiety of the co-crystallized morphinan antagonists through the interplay of two highly conserved water molecules. This H-bond pattern, therefore, requires His291 (6.52) protonated on the Nε atom (hereby denoted as HSE). However, as a preliminary docking analysis of 28 did not predict the presence of a H-bond donor moiety in the proximity of His291 (6.52), we also considered the residue protonated on the Nδ (hereby denoted as HSD). We therefore and carried out MD simulations by considering both HSE and HSD protonation states for His291 (6.52) and compared the two sets of results.

**Induced Fit Docking (IFD).** Compound 28 was docked to the homology model by means of the IFD⁸ procedure based on Glide search algorithm using the Standard Protocol (SP) and OPLS3 force field.⁹ The centroid of the co-crystallized JDTic ligand³ was selected as center of the Glide grid (inner box side = 10 Å; outer box side = auto). The ligand was initially docked rigidly into the receptor by applying a scaling factor of 0.5 to both ligand and protein van der Waals (vdW) radii. Up to 20 poses were collected, and the sidechains of residues within 5 Å of the ligand were refined with Prime.⁷ The ligand was re-docked into the newly generated receptor conformations with Glide⁸ by generating up to 10 poses using the SP scoring function and reverting the vdW radii scaling factors to their default values.

**Molecular dynamics (MD).** The best obtained docking complex of 28 was subjected to 30 ns of membrane MD simulations run in triplicate by considering His291 (6.52) both as HSE and HSD. The ligand-protein systems were embedded in a 1-palmitoyl-2-oleoyl-sn-glycero-3-
phosphocholine (POPC) lipid bilayer (80x80 Å wide, generated through the VMD Membrane Plugin tool) according to the orientation suggested by the “Orientations of Proteins in Membranes (OPM)” server. Overlapping lipids (within 0.6 Å) were removed upon protein insertion and the systems were solvated with TIP3P water and neutralized by Na+/Cl− counterions (final concentration 0.154 M). MD simulations with periodic boundaries conditions were carried out with ACEMD using CHARMM36 force field for lipid and protein atoms, and CGenFF(3.0.1) force field for ligand atoms. Ligand parameters were obtained by analogy through the ParamChem service (https://cgenff.paramchem.org, accessed 08/2017, version 1.0.0) with no further optimization. The force constants of the υ0 and υ1 dihedral angles of the (N)-methanocarba ring (coinciding with υ1 and υ2, respectively) was increased to 4.0 kcal/mol to avoid unfeasible south ring puckering. The systems were equilibrated through a stepwise procedure: in the first phase, after 2500 cycles of conjugate-gradient minimization aimed at reducing steric clashes arising from the manual setup of the system, 10 ns of MD simulation were performed in the NPT ensemble, restraining protein (and ligand) atoms by a force constant of 1 kcal/mol·Å. In the second phase, once water molecules diffused inside the protein cavity and the lipid bilayer reached equilibrium, the constraints on the ligand atoms were maintained whereas those on protein atoms were removed except for alpha carbon atoms (force constant = 0.5 kcal/mol·Å) for other 10 ns. During the equilibration procedure, the temperature was maintained at 310 K using a Langevin thermostat with a low damping constant of 1 ps, and the pressure was maintained at 1 atm using a Berendensen barostat. Bond lengths involving hydrogen atoms were constrained using the M-SHAKE algorithm with an integration timestep of 2 fs. The equilibrated systems were subjected to 30 ns of unrestrained MD simulations (NVT ensemble, damping constant of 0.1 ps) run in triplicates. Long-range Coulomb interactions were handled using the particle mesh Ewald summation method (PME) with grid size rounded to the approximate integer value of cell wall dimensions. A non-bonded cutoff distance of 9 Å with a switching distance of 7.5 Å was used.

**MD Trajectory Analysis.** MD trajectory analysis was performed with an in-house script exploiting the NAMD 2.10 mdenergy function and the RMSD trajectory tool (RSMDTT) implemented in VMD. Selection of a representative trajectory was based upon the total ligand–protein interaction energy (IEtot) expressed as the sum of van der Waals (IEvdW) and electrostatic (IEele) contribution as previously described. For both the HSD and HSE systems the trajectory characterized by low average ligand RMSD value and achieving the ligand-protein complex with the lowest IE value was selected for a more detailed analysis and visualization (see Table S2).

**Docking of compounds 22, 24, 30-37.** The ligand-protein complexes characterized by the lowest IE values extracted from the MD trajectories for both the HSE and HSD systems were minimized and prepared for docking calculations by retaining water molecules directly interacting with the ligand or connecting surrounding protein residues with the conserved Asp138(3.32). Selected compounds were docked to the 28-hKOR MD so-refined structures with Glide by generating up to 5 poses using the SP scoring function. The centroid of 28 was selected as center of the Glide grid (inner box side = 10 Å; outer box side = 30 Å).
Molecular Modeling References

1. Schrödinger Release 2016-3: Maestro, Schrödinger, LLC: New York, NY, 2016.
2. Schrödinger Release 2016-3: Jaguar, Schrödinger, LLC, New York, NY, 2016.
3. Wu, H.; Wacker, D.; Mileni, M.; Katritch, V.; Han, G. W.; Vardy, E.; Liu, W.; Thompson, A. A.; Huang, X. P.; Carroll, F. I.; Mascarella, S. W.; Westkaemper, R. B.; Mosier, P. D.; Roth, B. L.; Cherezov, V.; Stevens, R. C. Structure of the human κ-opioid receptor in complex with JDTic. *Nature* **2012**, *485*, 327-332.
4. Bernstein, F. C.; Koetzle, T. F.; Williams, G. J.; Meyer Jr., E. E.; Brice, M. D.; Rodgers, J. R.; Kennard, O.; Shimanouchi, T.; Tasumi, M. The Protein Data Bank: A computer-based archival file for macromolecular structures. *J. Mol. Biol.* **1977**, *112*, 535–542.
5. The UniProt Consortium. UniProt: a hub for protein information. *Nucleic Acids Res.* **2015**, *43*, D204–D212.
6. Sastry, G. M.; Adzhigirey, M.; Day, T.; Annabhimoju, R.; Sherman, W. Protein and ligand preparation: parameters, protocols, and influence on virtual screening enrichments *J. Comput. Aided Mol. Des.* **2013**, *27*, 221–234.
7. Schrödinger Release 2016-3: Prime, Schrödinger, LLC: New York, NY, 2016.
8. Schrödinger Release 2016-3: Schrödinger Suite 2016-3 Induced Fit Docking protocol; Glide, Schrödinger, LLC, New York, NY, 2016; Prime, Schrödinger, LLC, New York, NY, 2016.
9. Harder, E.; Damm, W.; Maple, J.; Wu, C.; Reboul, M.; Xiang, J. Y.; Wang, L.; Lupyan, D.; Dahlgren, M. K.; Knight, J. L.; Kaus, J. W.; Cerutti, D. S.; Krilov, G.; Jorgensen, W. L.; Abel, R.; Friesner, R. A. OPLS3: A force field providing broad coverage of drug-like small molecules and proteins. *J. Chem. Theory Comput.* **2016**, *12*, 281–296.
10. Humphrey, W.; Dalke, A.; Schulten, K. VMD - Visual molecular dynamics. *J. Molec. Graphics*, **1996**, *14*, 33–38.
11. Lomize, M. A.; Pogozheva, I. D.; Joo, H.; Mosberg, H. I.; Lomize, A. L. OPM database and PPM web server: resources for positioning of proteins in membranes. *Nucleic Acids Res.* **2012**, *40*, D370–376.
12. Jorgensen W. L.; Chandrasekhar, J.; Madura, J. D.; Impey, R. W.; Klein, M. L. Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* **1983**, *79*, 926–935.
13. Harvey, M.; Giupponi, G.; De Fabritiiis, G. ACEMD: Accelerated molecular dynamics simulations in the microseconds timescale. *J. Chem. Theory and Comput.* **2009**, *5*, 1632–1639.
14. Best, R. B.; Zhu, X.; Shim, J.; Lopes, P. E. M.; Mittal, J.; Feig, M.; MacKerell Jr., A. D. Optimization of the additive CHARMM all-atom protein force field targeting improved sampling of the backbone phi, psi and side-chain chi1 and chi2 dihedral angles. *J. Chem. Theory Comput.* **2012**, *8*, 3257–3273.
15. Klauda, J. B.; Venable, R. M.; Freites, J. A.; O’Connor, J. W.; Tobias, D. J.; Mondon-Ramirez, C.; Vorobyov, I.; MacKerell Jr., A. D.; Pastor, R. W. Update of the CHARMM all-atom additive force field for lipids: validation on six lipid types. *J. Phys. Chem. B*, **2010**, *114*, 7830–7843.
16. Vanommeslaeghe, K.; MacKerell, A. D., Jr., Automation of the CHARMM General Force Field (CGenFF) I: bond perception and atom typing. *J. Chem. Inf. Model.* **2012**, *52*, 3144-3154.
17. Vanommeslaeghe, K.; Raman, E. P.; MacKerell, A. D., Jr., Automation of the CHARMM General Force Field (CGenFF) II: assignment of bonded parameters and partial atomic charges. *J. Chem. Inf. Model.* **2012**, *52*, 3155-3168.
18. Kräutler, V.; Van Gunsteren, W. F.; Hünenberger, P. H. A Fast SHAKE algorithm to solve distance constraint equations for small molecules in molecular dynamics simulations. *J. Comput. Chem.* **2001**, *22*, 501–508.

19. Essmann, U.; Perera, L.; Berkowitz, M. L.; Darden, T.; Lee, H.; Pedersen L. G. A. Smooth particle mesh Ewald method. *J. Chem. Phys.* **1995**, *103*, 8577–8593.

20. Phillips, J. C.; Braun, R.; Wang, W.; Gumbart, J.; Tajkhorshid, E.; Villa, E.; Chipot, C.; Skeel, R. D.; Kale, L.; Schulten, K. Scalable molecular dynamics with NAMD. *J. Comput. Chem.* **2005**, *26*, 1781–1802.

21. Tosh, D. K.; Ciancetta, A.; Warnik, E.; Crane, S.; Gao, Z.-G.; Jacobson K. A. Structure-Based Scaffold Repurposing for GPCRs: Transformation of Adenosine Derivatives into Selective 5HT$_{2B}$/5HT$_{2C}$ Serotonin Receptor Antagonists. *J. Med. Chem.* **2016**, *59*, 11006-11026.

22. Schrödinger Release 2017-1: Glide, Schrödinger, LLC: New York, NY, 2017.
Molecular Modeling Results

Table S2. Parameters considered for the selection of a representative trajectory among three replicas (30 ns each) of the 28-hKOR_HSD and 28-hKOR_HSE complexes: protein alpha carbon atoms (Cα) average RMSD, ligand average RMSD, and minimum of the ligand-protein Interaction Energy (IEmin). RMSD values are in Å and IE values are in kcal/mol. Selected run (shown in Video S1) are marked in bold.

| Replica | IEmín [kcal/mol] | RMSDlig (ave) [Å] | RMSDca (ave) [Å] | His291 (6.52) |
|---------|------------------|--------------------|-----------------|--------------|
| Run1    | **-110.210**     | 1.977              | 1.660           | HSD          |
| Run2    | -89.511          | 2.557              | 1.671           | HSD          |
| Run3    | -89.543          | 2.169              | 1.818           | HSD          |
| Run1    | **-90.800**      | 1.191              | 1.874           | HSE          |
| Run2    | -90.524          | 1.120              | 1.667           | HSE          |
| Run3    | -88.318          | 1.674              | 1.769           | HSE          |
Figure S7. Superimposition of the WaterMap calculated by Goldfeld et al. (Goldfeld, D.; Murphy, R.; Kim, B.; Wang, L.; Beuming, T.; Abel, R.; Friesner, R. A. Docking and free energy perturbation studies of ligand binding in the kappa opioid receptor. *J. Phys. Chem. B* **2015**, *119*, 824-835.) on the KOR X-ray structure (grey ribbons) and the hypothetical binding mode of 28 (cyan carbon atoms, balls and stick representation) at the hKOR HSE model (cyan ribbons) suggested by our MD simulations. Transparent green and red spheres correspond to “happy” and “unhappy” water molecules, respectively. Highlighted water molecules are represented with a higher sphere radius. Green and red circle highlight the proximity or superimposition between hydrophobic substituents and “unhappy” water molecules and hydrophilic substituents with “happy” water molecules, respectively. Water molecules in the proximity of the ligand (radius = 5Å) detected during our MD simulations are represented as cyan spheres.
| TABLE S3. Study Title: PHARMACOKINETICS OF 28 (MRS7299) INTRAVENOUS AND ORAL ADMINISTRATION IN MALE SPRAGUE DAWLEY RATS, by GVK, Hyderabad, India |
| Study Details |
| Study Number | 0462-18-DMPK |
| Test Article Name | MRS7299 |
| Formulation IV solution | IV: DMSO:20%HPBCD (10:90) |
| Formulation PO solution | PO: DMSO: 20%HPBCD (10:90) |
| Species | Male SD Rats |
| Feed Condition | IV: Fed, PO: Fasting Overnight, Feed 4 hr postdose |
| Study Design | Single compound dosing for IV and PO |
| Matrix and Anticoagulant | Plasma and Li-heparin |
| LLOQ (ng/mL) | 1.00 |
| ULOQ (ng/mL) | 1000.00 |
| A. Plasma concentration (ng/mL) of MRS7299 after IV (0.5 mg/kg) dose administration to male Sprague Dawley rats |
| Time (h) | Rat-1 | Rat-2 | Rat-3 | Mean | Std Dev | % CV |
| 0.08 | 26.94 | 12.45 | 23.29 | 20.89 | 7.54 | 36.07 |
| 0.25 | 17.15 | 12.30 | 7.81 | 12.42 | 4.67 | 37.61 |
| 0.50 | 18.27 | 8.94 | 8.14 | 11.78 | 5.63 | 47.79 |
| 1.00 | 15.24 | 8.27 | 5.57 | 9.69 | 4.99 | 51.48 |
| 2.00 | 8.21 | 5.95 | 3.11 | 5.76 | 2.56 | 44.39 |
| 4.00 | 3.03 | 1.58 | 1.03 | 1.88 | 1.03 | 54.96 |
| 8.00 | BLQ | BLQ | BLQ | BLQ | NA | NA |
| 12.00 | BLQ | BLQ | BLQ | BLQ | NA | NA |
| 24.00 | BLQ | BLQ | BLQ | BLQ | NA | NA |
| Dose (mg/kg) | 0.50 | 0.50 | 0.50 | 0.50 | 0.00 | 0.00 |
| C0 (ng/mL) | 33.72 | 12.53 | 40.09 | 28.78 | 14.43 | 50.14 |
| T1/2 (h) | 1.33 | 1.36 | 1.24 | 1.31 | 0.06 | 4.81 |
| AUC0-last (ng·h/mL) | 40.67 | 23.67 | 18.30 | 27.55 | 11.68 | 42.38 |
| AUC0-infi (ng·h/mL) | 46.46 | 26.77 | 20.13 | 31.12 | 13.69 | 44.00 |
| AUCExtra (%) | 12.47 | 11.55 | 9.12 | 11.04 | 1.73 | 15.69 |
| Cl (ml/min/kg) | 179.37 | 311.35 | 413.87 | 301.53 | 117.56 | 38.99 |
| Vd (L/kg) | 20.58 | 36.55 | 44.25 | 33.79 | 12.08 | 35.74 |
| MRT0-last (h) | 1.34 | 1.41 | 1.12 | 1.29 | 0.15 | 11.80 |
| Rsq | 0.9973 | 0.9735 | 0.9998 | 0.9902 | 0.01 | 1.46 |
| B. Plasma concentration (ng/mL) of MRS7299 after PO (1.00mg/kg) dose administration to |
male Sprague Dawley rats

| Time (h) | Rat-1 | Rat-2 | Rat-3 | Mean | Std Dev | % CV |
|----------|-------|-------|-------|------|---------|------|
| 0.25     | 4.05  | 10.35 | 2.85  | 5.75 | 4.03    | 70.06|
| 0.50     | 3.52  | 19.55 | 3.56  | 8.88 | 9.24    | 104.13|
| 1.00     | 4.43  | 18.30 | 5.93  | 9.55 | 7.61    | 79.68|
| 2.00     | 2.05  | 12.90 | 2.82  | 5.92 | 6.05    | 102.21|
| 4.00     | 1.49  | 8.53  | 1.69  | 3.90 | 4.01    | 102.68|
| 8.00     | BLQ   | 3.03  | BLQ   | NA   | NA      | NA   |
| 12.00    | BLQ   | 1.05  | BLQ   | NA   | NA      | NA   |
| 24.00    | BLQ   | BLQ   | BLQ   | NA   | NA      | NA   |
| Dose (mg/kg) | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 | 0.00 |
| Cmax (ng/mL) | 4.43 | 19.55 | 5.93 | 9.97 | 8.33 | 83.55 |
| T<sub>max</sub> (h) | 1.00 | 0.50 | 1.00 | 0.83 | 0.29 | 34.64 |
| AUC<sub>0-last</sub> (ng·h/mL) | 10.04 | 79.79 | 12.13 | 33.99 | 39.68 | 116.76 |
| AUC<sub>0-inf</sub> (ng·h/mL) | 14.50 | 83.80 | 16.40 | 38.23 | 39.47 | 103.24 |
| AUC<sub>Extra</sub>% | 30.76 | 4.79 | 26.07 | 20.54 | 13.84 | 67.40 |
| MRT<sub>0-last</sub> (h) | 1.67 | 3.59 | 1.75 | 2.34 | 1.08 | 46.34 |
| F (%) | 18.22 | 134.64 | 22.01 | 58.29 | 66.15 | 113.48 |
| Rsq | 0.8300 | 1.0000 | 0.9149 | 0.9149 | 0.08 | 9.29 |
| T1/2 (h) | 2.07 | 2.65 | 1.75 | 2.16 | 0.45 | 20.97 |

C. Plasma concentration (ng/mL) of MRS7299 after PO (3.00mg/kg) dose administration to male Sprague Dawley rats

| Time (h) | Rat-1 | Rat-2 | Rat-3 | Mean | Std Dev | % CV |
|----------|-------|-------|-------|------|---------|------|
| 0.25     | 22.36 | 11.52 | 51.58 | 28.49 | 20.72 | 72.74 |
| 0.50     | 62.63 | 20.02 | 62.51 | 48.39 | 24.57 | 50.77 |
| 1.00     | 59.14 | 64.33 | 74.34 | 65.94 | 7.73 | 11.72 |
| 2.00     | 40.62 | 24.70 | 45.60 | 36.97 | 10.92 | 29.53 |
| 4.00     | 17.08 | 8.36 | 13.45 | 12.96 | 4.38 | 33.79 |
| 8.00     | 1.39  | 1.32  | 8.33  | 3.68  | 4.03  | 109.43 |
| 12.00    | BLQ   | BLQ   | 1.05  | BLQ   | NA   | NA   |
| 24.00    | BLQ   | BLQ   | BLQ   | BLQ   | NA   | NA   |
| Dose (mg/kg) | 3.00 | 3.00 | 3.00 | 3.00 | 0.00 | 0.00 |
| Cmax (ng/mL) | 62.63 | 64.33 | 74.34 | 67.10 | 6.33 | 9.43 |
| T<sub>max</sub> (h) | 0.50 | 1.00 | 1.00 | 0.83 | 0.29 | 34.64 |
| AUC<sub>0-last</sub> (ng·h/mL) | 172.52 | 113.29 | 223.20 | 169.67 | 55.01 | 32.42 |
| AUC<sub>0-inf</sub> (ng·h/mL) | 174.95 | 116.02 | 226.26 | 172.41 | 55.16 | 32.00 |
| AUC<sub>Extra</sub>% | 1.39 | 2.35 | 1.35 | 1.70 | 0.56 | 33.28 |
| MRT<sub>0-last</sub> (h) | 2.24 | 2.18 | 2.90 | 2.44 | 0.40 | 16.39 |
| F (%) | 93.70 | 62.14 | 121.18 | 92.34 | 29.54 | 32.00 |
| Rsq | 0.9930 | 0.9983 | 0.9374 | 0.9762 | 0.03 | 3.46 |
| T1/2 (h) | 1.21 | 1.43 | 2.02 | 1.55 | 0.42 | 26.89 |

D. Plasma concentration (ng/mL) of MRS7299 after PO (10.00mg/kg) dose administration
TABLE S4. Bioanalytical method for compound 28

| Study Number   | 462-18-DMPK          |
|----------------|----------------------|
| Study Type     | IV-PO-PK study       |
| Instrument ID  | API 4000             |

**COMPOUND DETAILS:**

| Name of Compound | Molecular weight Free Form | Molecular weight Salt Form | Purity/potency / assay | Diluents-Master stock preparation |
|------------------|-----------------------------|----------------------------|------------------------|----------------------------------|
| Analyte          | MRS7299                     | 472.90                     | 100%                   | DMSO                             |
| Internal Standard| Telmisartan                 | 514.60                     | 98%                    | DMSO                             |

**CHROMATOGRAPHY:**

| LC Gradient:  |
|---------------|
| Time (min)    | %A   | %B   |
| 0.01          | 95   | 5    |
| 0.80          | 5    | 95   |
| 2.50          | 5    | 95   |
| 2.60          | 95   | 5    |
| 3.50          | 95   | 5    |
| Mobile Phase (A)| 0.1% Formic acid in Milli Q water |
| Mobile Phase (B)| 100% Methanol                   |
| Column                  | PHENOMENEX C18, 50°4.6 mm, 5 µ |
|------------------------|--------------------------------|
| Injection Volume (µL)  | 20                             |
| Flow Rate (mL/min)     | 1                              |
| Run Time(min)          | 4                              |
| Sample Cooler Temperature (°C) | 15      |
| Column Oven Temperature (°C) | 40        |
| Rinsing Solution       | Acetonitrile:Methanol:water::20:60:20,V/V |

**SAMPLE PREPARATION:**

**Extraction Technique**

Protein Precipitation

**Extraction Solvent**

Acetonitrile Containing IS

**Calibration Curve & QC preparation:** 2.0 µL of calibration curve standards added to 48 µL of blank matrix and added 25µL of milli-Q-water precipitated with 200 µL of Acetonitrile containing internal standard at 200 ng/mL conc. then Vortexed for 5 min at 850 rpm, centrifuged at 4000 rpm for 5 min at 4 °C, from this 110 µL of supernatant was separated and diluted with 130 µL of water.

**Sample Preparation:** 50 µL of sample was taken and precipitated with 200 µL of Acetonitrile containing internal standard at 200 ng/mL conc. then Vortexed for 5 min at 850 rpm, centrifuged at 4000 rpm for 5 min at 4 °C, from this 110 µL of supernatant was separated and diluted with 130 µL of water.

**MASS SPECTROMETRIC CONDITION:**

| Ionization Mode-Polarity | ESI-positive |
|--------------------------|--------------|
| Name of Compound         | Retention Time (Min) | MRM Transitions | Declustering Potential (DP) | Entrance Potential (EP) | Collision Energy (CE) |
| Analyte                  | MRS-7299      | 1.56            | 473.50 288.90               | 151 10 44               |
| Internal Standard        | Telmisartan   | 1.36            | 515.30 276.10               | 65 10 60               |

**Collision Cell Exit Potential (CXP)**: 12

**Collision Gas (CAD)**: 8

**Curtain Gas (CUR)**: 25

**Nebulizer Gas (GS1)**: 50

**Heater Gas (GS2)**: 50

**Ion spray voltage (V)**: 5500

**Temperature (TEM)**: 500

**Interface Heater (ihe)**: ON
TABLE S5. Formulation for in vivo administration of 28.

| FORMULATION DETAILS: | Intravenous (0.5 mg/kg) | FORMULATION DETAILS: | Per-Oral (1 mg/kg) |
|----------------------|--------------------------|----------------------|-------------------|
| **Study No.**        | 462-18-DMPK              | **Study No.**        | 462-18-DMPK       |
| **Compound ID**      | MRS7299                  | **Compound ID**      | MRS7299           |
| **Molecular weight** | 472.9                    | **Molecular weight** | 472.9             |
| **Dose (mg/kg)**     | 0.5                      | **Dose (mg/kg)**     | 1                 |
| **Dose Volume (mL/kg)** | 5                        | **Dose Volume (mL/kg)** | 5               |
| **Dose Concentration (mg/mL)** | 0.1                      | **Dose Concentration (mg/mL)** | 0.2            |
| **Administration Route** | IV (G1-1)              | **Administration Route** | Per Oral (G2-2) |
| **Species/Strain/Sex** | Rat / SD / Male          | **Species/Strain/Sex** | Rat / SD / Male   |
| **Test Item Weighed:** | 21.10 mg                 | **Total Volume of Vehicle Required:** | 5.0 mL          |
| **Total Volume of Vehicle Required:** | 5.0 mL for IV Formulation | **Volume of Vehicle added:** | 5.0 mL          |
| **Formulation/Vehicle Used:** | DMSO, 20% HPβCD         | **Formulation/Vehicle Used:** | DMSO, 20% HPβCD |
| **Preparation Procedure and Documentation:** | Volume of Vehicle added: | | | |
| Stock Preparation: 21.10 mg of compound was dissolved in 1.055 mL of DMSO to make 20 mg/mL concentration. Required volume 0.025 mL of DMSO stock solution was taken in a vial to this 0.475 mL of DMSO was added vortexed then 4.5 mL of 20% HPβCD was added vortexed, sonicated to make a formulation of 0.1 mg/mL concentration. | The final dosing formulation was clear and transparent in appearance. | Required volume 0.050 mL of DMSO stock solution was taken in a vial to this 0.450 mL of DMSO was added vortexed then 4.5 mL of 20% HPβCD was added vortexed, sonicated to make a clear formulation. | The final dosing formulation was clear, transparent solution. |

TABLE S6. Cytochrome P450 inhibition of compound 28.

| Compound    | IC₅₀ (µM) | 1A2 | 2C9 | 2C19 | 2D6 | 3A4 |
|-------------|-----------|-----|-----|------|-----|-----|
| MRS7299     | 26.0      | 14.5| 13.9| 14.1 | 10.7|     |

| Compound    | CACO-2 cell permeability of compound 28: Average Values |
|-------------|--------------------------------------------------------|
|             | P<sub>app</sub> (10⁻⁶ cm/sec) | Efflux Ratio | A to B % Recovery | B to A % Recovery | Classification |
|             | Apical to Basal | Basal to Apical |                 |                   |               |
| MRS7299     | 1.42          | 22.21            | 15.63            | 18.00             | 44.59          | Low       |
| Propranolol | 21.24         | 9.99             | 0.47             | 66.42             | 84.71          | High      |
| Atenolol    | 0.32          | 0.78             | 2.40             | 86.58             | 103.64         | Low       |

Procedures for determining CYP Inhibition:
Incubation in human microsomes, pH 7.4; Six concentrations in duplicate to determine IC₅₀; CYP panels (1A2, 2C9, 2C19, 2D6, 3A4) 5 in 1 cocktail substrates: CYP3A4 (Midazolam), CYP2D6 (Dextromethorphan), CYP1A2 (Tacrine),CYP2C9 (Diclofenac), CYP2C19 (Mephenytoin); Analysis of substrate by LC-MS/MS.

Assay procedure:
To 158µL of HLM working stock solution to reaction plate (0.2 mg/mL) 2 µL of inhibitor/test compound/vehicle control is spiked. 20 µL of substrate pool is added to the reaction. The plate is pre-incubated at 37 °C for 5 min. The reaction is initiated by adding NADPH. The plate is incubated 37 °C for 10 min. The reaction is terminated adding cold acetonitrile (100 µL/well). The samples are centrifuged at 4,000 rpm for 20min to precipitate protein. After precipitation, 200 µL of supernatant is analysed by LC/MS/MS.
Table S7. CACO-2 Cell Assay of 28.

| Section | Description |
|---------|-------------|
| 4.1     | **Cell seeding density** | 12000 cells/well (96 well plate). |
| 4.2     | **Reagent Preparation** | |
| 4.2.1   | **Preparation of (Dulbecco’s Modified Eagles Medium) DMEM medium pH 7.4:** | 5 mL of 100 mM Sodium pyruvate, 5 mL of 100X non essential amino acids, 5 mL of Penstrep was added to 100 mL of heat inactivated fetal bovine serum to 385 mL of DMEM aseptically and mixed thoroughly. |
| 4.2.2   | **Preparation of Hank’s Balanced salt solution (HBSS) pH 7.4:** | One vial of Hank’s balanced salt (Sigma-H1387) was dissolved in 900 mL of milli Q water; adjusted the pH to 7.4 and made up the volume to 1000 mL with the same. The solution was filter sterilized and store at 4°C. |
| 4.2.3   | **Preparation & Dilution of Test Compound:** | 10 mM stock solution of test compound was prepared in DMSO. 10 mM stock was diluted with HBSS Buffer to a final concentration of 10 µM. |
| 4.3     | **Assay:** | |
|         | Revival of Caco-2 cells: As per SOP-BIO-IA-TCL-013-00 | |
|         | Sub culturing of Caco-2 cells: As per SOP-BIO-TCL-013-00 | |
| 4.4     | **Seeding of the cells:** | 250 µL of DMEM was added to the basal compartment of 96 well multi-screen Caco-2 plate and seeded 12000 cells/well (0.16 x 10⁶ cells/ml) in all the apical wells required and one well with only media as blank without cells. Placed the Caco-2 plate in CO₂ incubator at 37°C for proliferation of cells. |
| 4.5     | **Pre-Assay Preparation:** | On the day of assay, medium was removed and washed twice with HBSS Buffer and incubated with HBSS buffer for 30 min in an incubator and wells with TEER values greater than 230 ohm.cm² were selected for the incubation. |
| 4.6     | **Apical to Basal permeability with 2% BSA:** | 75 µL of test compound was added to apical wells and 250 µL of HBSS buffer with 2% BSA was added to basal wells. 25 µL of basal samples was collected at 120 min and processed as stated below. |
| 4.7     | **Basal to Apical permeability with 2% BSA:** | 250 µL of test compound was added to basal wells and 75 µL of HBSS buffer with 2% BSA was added to apical wells. 25 µL of apical samples was collected at 120 min and processed as stated below. |
| 4.8     | **Sample processing:** | Single point calibration curve in HBSS buffer with 2% BSA was used. Donor samples were diluted 1:1 with HBSS containing 2% BSA and receiver samples were diluted with 1.1 HBSS buffer and precipitated with 200 µL of acetonitrile containing internal standard and vortexed for 5 min @ 1000 rpm, centrifuged at 4000 rpm for 10 min. 100 µL of supernatant was diluted with 200 µL of water and submitted for LC-MS/MS analysis. |
| 4.9     | **Calculations:** | Papp = $\frac{dQ}{dT} \times \frac{1}{Co} \times \frac{1}{A}$, $dQ$ is amount permeated to the receiver compartment of the 96 well filter plate; $dT$ is Time of incubation of drug on the cell monolayer; $Co$ is initial concentration of drug in the apical compartment of the well; $A$ is surface area of the filter. |
Recovery: \( \frac{(dQ \text{ of Apical} + dQ \text{ of Basal})}{\text{Standard } dQ} \times 100 \)

| Generic Gradient method for LC-MS/MS: |  |
|--------------------------------------|---|
| Column                               | KINETEX EVO C18 100Å° 50*4.6mm 5µ |
| Mobile Phase:                        |  |
| Aqueous Reservoir (A) A               | 10 mm Ammonium acetate+0.1% Formic Acid in water |
| Organic Reservoir (B)                | 100 % Methanol |
| Flow Rate                            | 1 mL/min |
| Injection volume                     | 10µL |

**Figure S8. Lack of HEP-G2 cell toxicity of 28.**

180 µl of HEPG2 cells were seeded at a density of 5,000 cells/well in a white opaque plate and incubated for 24 h at 37°C, 5% CO₂ incubator. Cells were incubated with MRS7299 or standard (puromycin, CC₅₀ 0.7 µM) for 72 h at 37°C in a 5% CO₂ incubator. Cell Titer-Glo Luminescent Cell Viability Assay Reagent was added, and the incubation was continued for 30 min. Luminescence signal was captured using Envision Multi-label 2104 plate reader.

| Results: |  |
|----------|---|
| Compound ID | CC₅₀, µM | pCC₅₀ | CC₅₀_Lower 95% Confidence interval | CC₅₀_Upper 95% Confidence interval | Max Response | Max response concentration, µM | HillSlope | No. of points used to derive DRC |
|----------|---|---|---|---|---|---|---|---|
| MRS-7299 | >30 | <4.5 | - | - | 24 | 30 | - | 11 |
| "Puromycin" (reference) | 0.7 | 6.1 | 0.5 | 0.97 | 95 | 10 | 1.3 | 11 |
Procedure for determining human plasma stability of 28:
1 mM stock of test compound was prepared in acetonitrile: water by diluting from 10 mM stock (i.e. 10 µL of 10 mM stock solution was added to 90 µL of acetonitrile: water (50:50)). 25 µM stock of test compound was prepared in acetonitrile: water by diluting from 1mM stock (i.e. 2.5 µL of 1mM stock solution was added to 97.5 µL of acetonitrile: water (50:50)). The frozen plasma was thawed at room temperature and centrifuged at 1400x RCF 4ºC, for 15 minutes. Approximately 90% of the clear supernatant fraction was transferred to a separate tube and was used for the assay.

For 0 min samples, plasma was heat inactivated at 56ºC for 45 min. To 72 µL of heat inactivated plasma, 3 µL of 25 µM test compound was added. A 25 µL aliquot of the mixture was taken and crashed with 200 µL of acetonitrile containing internal standard and further processed along with other time points.

For other time point samples, final working stock of 1 µM was prepared by diluting in plasma (i.e. 8 µL of 25 µM acetonitrile: water stock was added to 192 µL of plasma). 200 µL of plasma containing the test compound was incubated for 120 min at 37 ºC in shaker water bath with gentle shaking. 25 µL aliquot of sample at 0 and 120 min was precipitated immediately with 200 µL of acetonitrile containing internal standard and centrifuged at 4000x RCF, 4ºC for 20 minutes. 150 µL of supernatant was diluted with 150 µL of water and analyzed on LC-MS/MS.

Calculation: % remaining of the test substance = (Peak Area ratio at time (min)) *100 / (Peak Area Ratio at 0 min)

Bioanalysis:
Generic Gradient method for LC-MS/MS:
Instrumentation ABSCIEX 4500QTRAP, exion LC
Column KINETEX 5µ EVO C18 100A° 50*4.6mm
Mobile Phase
Aqueous Reservoir (A) 0.1% FORMIC ACID IN WATER
Organic Reservoir (B) METHANOL
Flow rate 1.0 mL/min
Injection volume 15 µL

Procedure for determining stability in simulated body fluids:
Preparation of phosphate buffer solution for intestinal fluid assay:
Sodium hydroxide (pellets, 0.42 g), monobasic potassium phosphate (3.954 g), and sodium chloride (6.186 g) were dissolved in 500 mL of purified water in a 1 L of volumetric container. The pH of the buffer was adjusted to exactly 6.5 using either 1 N sodium hydroxide or 1 N hydrochloride and the volume made up to 1 L with water.

Preparation and Dilution of Test Compound:
10 mM stock solution of test compound was prepared in DMSO and diluted with water: acetonitrile (1:1) to a concentration of 1 mM. Working concentration of 100 µM was prepared by further dilution with water: acetonitrile (1: 1).

Preparation of FaSSGF buffer, pH 1.60:
1.999 g of NaCl was dissolved in 900 mL of milli Q water. pH was adjusted to 1.6 with HCl and final volume was made up to 1000 mL with milli q water
0.060 g of SIF powder was added to 1000 mL of NaCl/HCl solution
Preparation of FaSSIF buffer, pH 6.50:
0.420 g of NaOH, 3.438 g of NaH$_2$PO$_4$, 6.186 g of NaCl was added to 900 mL of milli q water. pH was adjusted to 6.5 with 1N NaOH/1N HCl.
2.240 g of SIF powder was added to 1000 mL of buffer and allowed to stand at room temperature for 2 h, to opalescence.

Assay Procedure:
a. Incubation mixture: 5 µL Test Cpd. (100 µM stock) + 495 µL of buffer system (FaSSGF, FaSSIF), Incubated on shaking water bath at 37°C
b. Sample preparation: 50 µL incubation mixture + 200 µL of acetonitrile containing internal standard + Vortex 5 min @ 1200 rpm + Centrifuge 10 min @ 4000 rpm. Supernatant diluted 2 fold with water and injected on LC-MS/MS.
**Figure S9.** hERG inhibition assay of 28 (fluorescent method):

![Graph showing hERG inhibition assay results](image)

| Cpd ID  | Cpd Type | IC50, nM | pIC50 | pIC50_Lower 95CL | pIC50_Upper 95CL | % Max Inh | % Max Conc, μM | Hillslope | No. of points used for curve |
|---------|----------|---------|-------|------------------|-----------------|----------|----------------|-----------|-----------------------------|
| MRS-7299| TEST     | >30000  | <4.52 | -                | -               | 45       | 30             | -         | 11                          |
| E-4031  | TEST     | 18.1    | 7.74  | 8.03             | 7.46            | 104      | 30             | 0.7       | 11                          |

**Protocol:**

1. **Predictor™ hERG membrane, assay buffer, control compound and tracer were thawed to room temperature**

2. Serial half log dilutions of control and test compounds from 3mM stocks were made in DMSO and a 4x stock of respective concentration was made in assay buffer

3. 10μl/well of 2x hERG membrane was dispensed into a 384 black low volume plate except for the tracer control wells

4. 5μl of compounds and control were added from the 4x stocks to the respective assay wells

5. 5μl Tracer was added into respective assay wells

6. **Incubation at 25°C for 2 hrs in dark**

7. The plate was read on Perkin Elmer’s ENVISION multimode reader using Tamra FP filters

8. **Data analyzed in Microsoft Excel and GraphPad Prism**

^ Half log dilutions, starting from 30μM through 3nM for all the test compound i.e MRS-7299,