Supporting Information

5-Fluoro-imidazo[4,5-b]pyridine is a privileged fragment that conveys bioavailability to potent trypanosomal methionyl-tRNA synthetase inhibitors

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**Compound synthesis.** Unless otherwise stated, all chemicals were purchased from commercial suppliers and used without further purification. Microwave irradiation was performed in a CEM Discover System. The final purity of all compounds was determined by analytical LCMS with a Phenomenex Onyx Monolithic C18 column (4.6 mm x 100 mm). The products were detected by UV at 220 nm. All compounds were determined to be >95% pure by this method. The purification by preparative HPLC was performed on a Waters Xterra Prep RP18 OBD 5µM (19 mm x 50 mm) with CH$_3$CN/H$_2$O and 0.1% TFA as eluent. The mass spectra were recorded with the Agilent Ion Trap Mass Spectrometer. NMR spectra were recorded with a Bruker 500 MHz spectrometer at ambient temperature. For all new compounds, syntheses and characterization data are presented below.

**General procedure for compounds 3-7:**

![Chemical structure](image)

2-bromo-5-fluoro-1H-benzo[d]imidazole was synthesized following the method described in the literature.\(^1\) The mixture of 2-bromo-5-fluoro-1H-benzo[d]imidazole (20 mg, 0.093 mmol) and tert-butyl 3-aminopropylcarbamate (48.5 mg, 0.28 mmol, 3 equiv.) in 0.5 ml of pyridine was microwave irradiated at 100 °C for 30 min. After solvent was removed, the residue was dissolved in ethyl acetate, washed with water, brine and dried over Na$_2$SO$_4$. The organic extract was purified via flash chromatography on silica gel to give tert-butyl 3-(6-fluoro-1H-benzo[d]imidazol-2-ylamino)propylcarbamate in 72% yield. LC/MS: 309.5 [M+H]$^+$. Tert-butyl 3-(6-fluoro-1H-benzo[d]imidazol-2-ylamino)propylcarbamate (15 mg, 0.049 mmol) was treated with DCM (2ml) and TFA (1ml) at room temperature for 30 min. The solvents were completely removed. To the residue in 2ml of methanol was added DIPEA (20 µl, 11.5 mmol), 0.1 ml of acetic acid, and 3,5-dichlorobenzaldehyde (10.4 mg, 0.059 mmol). The mixture was stirred at room temperature for 10 min, then NaBH$_3$CN (6.3 mg, 0.1 mmol) was added. The reaction mixture was stirred at room temperature for 4 hours. After solvent was removed, the residue was purified via silica gel chromatography eluted with MeOH/DCM and further purified by using preparative HPLC. Conversion of TFA salt to HCl salt was performed via adding HCl in methanol and removing solvents to give compound 3 in 78% yield with purity >97%. \(^1\)H NMR (500 MHz, MeOD) δ 7.61 (m, 3H), 7.42 (m, 2H), 7.22 (m, 2H), 4.30 (s, 2H), 3.62 (t, \(J = 6.9\) Hz, 2H), 3.27 (m, 2H), 2.19 (m, 2H). LC/MS: (ESI) 368.3 [M+H]$^+$. Compound 4 was synthesized using 2-bromo-5,6-difluoro-1H-benzo[d]imidazole in 48% yield in two steps. Purity >95%. \(^1\)H NMR (500 MHz, MeOD) δ 7.61 (m, 3H), 7.40 (t, \(J = 8.2\) Hz, 2H), 4.30 (s, 2H), 3.61 (t, \(J = 6.8\) Hz, 2H), 3.27 (m, 2H), 2.19 (m, 2H). LC/MS: (ESI) 386.6 [M+H]$^+$. Compound 5 was synthesized using 2-bromo-1H-imidazo[4,5-b]pyridine in 52% yield in two steps. Purity >97%. \(^1\)H NMR (500 MHz, MeOD) δ 7.98 (d, \(J = 6.2\) Hz, 1H), 7.88 (d, \(J = 7.7\) Hz,
Compound 6 was synthesized using 2-bromo-5-fluoro-1H-imidazo[4,5-b]pyridine in 55% yield in two steps. Purity >97%. $^1$H NMR (500 MHz, MeOD) $\delta$ 7.87 (m, 1H), 7.59 (s, 2H), 7.56 (s, 1H), 6.97 (s, 1H), 4.27 (s, 2H), 3.59 (t, J = 6.9 Hz, 2H), 3.24 (m, 2H), 2.16 (m, 2H). LC/MS: (ESI) 369.5 [M+H]$^+$. 

Compound 7 was synthesized using 2-bromo-1H-imidazo[4,5-b]pyridine and tert-butyl 3-amino-2,2-difluoropropylcarbamate in 39% yield in two steps. Purity >95%. $^1$H NMR (500 MHz, MeOD) $\delta$ 7.96 (d, J = 4.0 Hz, 1H), 7.49 (d, J = 7.6 Hz, 1H), 7.28 (s, 2H), 7.25 (s, 1H), 6.99 (m, 1H), 3.93 (t, J = 13.6 Hz, 2H), 3.81 (s, 2H), 2.95 (t, J = 13.9 Hz, 2H). LC/MS: (ESI) 386.9 [M+H]$^+$. 

General procedure for compounds 8-9:

A mixture of 3,5-dichlorobenzylamine (264 mg, 1.5 mmol) and (R)-(−)-3-bromo-2-methyl-1-propanol (76.5 mg, 0.5 mmol), DIPEA (87 µl, 0.5 mmol) and trace amount of KI in ethanol (1.0 ml) was microwave irradiated at 90 °C for 30 min. To the solution was added (Boc)$_2$O (273 mg, 1.25 mmol) and DIPEA (260 µl, 1.5 mmol) in 2 ml of acetonitrile and the solution was stirred at room temperature overnight. After solvents were removed, the residue was dissolved in ethyl acetate, washed with water, brine and dried over Na$_2$SO$_4$. The organic extract was purified via flash chromatography on silica gel to obtain compound 15 in 50% yield. MS: 371.0 [M+Na]$^+$. 

To compound 15 (87 mg, 0.25 mmol) in 20 ml of dry DCM at -10 °C was added DIPEA (78 µl, 0.45 mmol) and methanesulfonyl chloride (30 µl, 0.39 mmol). The mixture was stirred at -10 °C for 30 min. After solvent was removed, the residue was dissolved in 5 ml of DMF and NaN$_3$ (33 mg, 0.51 mmol) was added. The mixture was stirred at 40-45 °C overnight. Solvent was removed and the residue was extracted with ethyl acetate. The organic extract was washed with water, brine and dried over Na$_2$SO$_4$. The residue was purified via flash chromatography on silica gel to obtain compound 16 in 84% yield. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.28 (m, 1H), 7.10 (s, 2H), 4.37 (s, 2H), 3.24 (m, 4H), 2.04 (br, 1H), 1.48 (m, 9H), 0.97 (d, J = 6.7 Hz, 3H). MS: 395.8 [M+Na]$^+$. 

A mixture of compound 16 (31 mg, 0.083 mmol), PPh$_3$ (66 mg, 0.25 mmol) in 1 ml of THF and 15 µl of water was stirred at room temperature overnight. Purification was performed via flash chromatography on silica gel to obtain compound 17 in 90% yield. MS: 347.8 [M+H]$^+$. 

The mixture of 2-bromo-1H-imidazo[4,5-b]pyridine (10 mg, 0.05 mmol) and compound 17 (26 mg, 0.075 mmol) in 0.5 ml of pyridine was microwave irradiated at 100 °C for 30 min. After solvent was removed, the residue was dissolved in ethyl acetate, washed with water, brine and dried over Na$_2$SO$_4$. The organic extract was purified via flash chromatography on silica gel and...
further treated with DCM (2 ml) and TFA (1 ml) at room temperature for 30 min. After the solvents were completely removed, the residue was purified by using preparative HPLC. Conversion of TFA salt to HCl salt was performed via adding HCl in methanol and removing solvents to give compound 8 with 62% yield and >97% purity. ¹H NMR (500 MHz, MeOD) δ 8.03 (m, 1H), 7.56 (s, 2H), 7.46 (s, 1H), 7.32 (m, 1H), 7.00 (m, 1H), 4.59 (m, 2H), 3.63 (m, 2H), 3.40 (m, 2H), 2.20 (m, 1H), 1.28 (m, 3H). LC/MS: (ESI) 365.5 [M+H]+.

Compound 9 was synthesized using 2-bromo-5-fluoro-1H-imidazo[4,5-b]pyridine and compound 17 in 70% yield and >97% purity. ¹H NMR (500 MHz, MeOD) δ 7.88 (m, 1H), 7.64 (s, 2H), 7.54 (s, 1H), 6.97 (d, J = 8.4 Hz, 1H), 4.30 (m, 2H), 3.58-3.38 (m, 2H), 3.10-2.96 (m, 2H), 2.42 (m, 1H), 1.20 (d, J = 6.7 Hz, 3H). LC/MS: (ESI) 383.5 [M+H]+.

General procedure for compounds 10-13:

(R)-6,8-dichlorochroman-4-amine and 3-(1H-imidazo[4,5-b]pyridin-2-ylamino)propanal were synthesized following the method described in the literature. To a solution of (R)-6,8-dichlorochroman-4-amine (21.8 mg, 0.1 mmol) in 5 ml of methanol was added 0.15 ml of HOAc and 3-(1H-imidazo[4,5-b]pyridin-2-ylamino)propanal (22.8 mg, 0.12 mmol). The mixture was stirred at room temperature for 30 min, and NaBH₃CN (12.6 mg, 0.2 mmol) was added. The reaction mixture was stirred at 50 °C for 24 hours. After solvent was removed, the residue was first purified via silica gel chromatography eluted with MeOH/DCM and further purified by using preparative HPLC. The combined collections were neutralized with NaHCO₃ and most solvent was removed, the residue was extracted with ethyl acetate and washed with brine and dried over Na₂SO₄ to give compound 10 in 72% yield and >97% purity. ¹H NMR (500 MHz, MeOD) δ 7.94 (d, J = 5.0 Hz, 1H), 7.48 (dd, J = 7.7, 1.3 Hz, 1H), 7.33 (d, J = 2.5 Hz, 1H), 7.27 (d, J = 2.5 Hz, 1H), 6.98 (dd, J = 7.7, 5.2 Hz, 1H), 4.42 (m, 1H), 4.32 (m, 1H), 3.87 (t, J = 4.9 Hz, 1H), 3.54 (t, J = 7.3 Hz, 2H), 2.84 (m, 2H), 2.08 (m, 2H), 1.93 (m, 2H). LC/MS: (ESI) 393.6 [M+H]+.

Compound 11 was synthesized using of 3-(5-fluoro-1H-imidazo[4,5-b]pyridin-2-ylamino)propanal in 78% yield and >97% purity. ¹H NMR (500 MHz, MeOD) δ 7.91 (m, 1H), 7.62 (d, J = 1.9 Hz, 1H), 7.54 (d, J = 1.8 Hz, 1H), 7.00 (d, J = 8.3 Hz, 1H), 4.68 (m, 1H), 4.51 (m, 2H), 3.66 (m, 2H), 3.45 (m, 2H), 2.54 (m, 1H), 2.51 (m, 1H), 2.25 (m, 2H). LC/MS: (ESI) 411.3 [M+H]+.

Compound 12 was synthesized using 3-(1H-imidazo[4,5-b]pyridin-2-ylamino)propanal and (R)-6,8-dichloro-1,2,3,4-tetrahydroquinolin-4-amine in 78% yield and >97% purity. ¹H NMR (500 MHz, MeOD) δ 7.92 (m, 1H), 7.45 (m, 1H), 7.08 (m, 2H), 6.96 (m, 1H), 3.75 (m, 1H), 3.47 (m, 4H), 2.80 (m, 2H), 2.05 (m, 1H), 1.85 (m, 3H). LC/MS: (ESI) 392.6 [M+H]+.

Compound 13 was synthesized using 3-(5-fluoro-1H-imidazo[4,5-b]pyridin-2-ylamino)propanal and (R)-6,8-dichloro-1,2,3,4-tetrahydroquinolin-4-amine in 75% yield and >97% purity. ¹H NMR (500 MHz, MeOD) δ 7.43 (m, 1H), 7.15 (m, 2H), 6.48 (d, J = 8.2 Hz, 1H), 3.92 (m, 1H), 3.47 (m, 4H), 2.85 (m, 2H), 2.20 (d, J = 17.5 Hz, 1H), 1.97 (m, 3H). LC/MS: (ESI) 410.4 [M+H]+.
Protein expression and purification
Protein expression, purification, crystallization and structure determination were previously
reported in detail.\textsuperscript{3-4} Full-length and truncated (237-773) \textit{Tb}MetRS was cloned into the
AVA0421 vector through ligation-independent cloning for expression in \textit{Escherichia coli}. Purification of recombinant protein was carried out by a Ni-NTA affinity column, removal of the
N-terminal hexa-histidine by 3C protease at 4°C, and finally size-exclusion chromatography on a
Superdex 75 column (Amersham Pharmacia Biotech). Final protein buffer contains 25 mM
HEPES, 500 mM NaCl, 2 mM DTT, 5% glycerol and 0.025% NaN\textsubscript{3} at pH 7.0. Full-length 
\textit{Tb}MetRS was used in thermal shift assays. Site-directed mutagenesis of surface residues
452KKE\textsubscript{454} to ARA on the truncated \textit{Tb}MetRS was required to obtain well-diffracting crystals,
and needed the presence of methionine. Therefore, 10 mM L-methionine was added to protein
preparations used for crystallization.

X-ray crystallography
\textit{Tb}MetRS surface mutant at 10 mg/ml was crystallized through vapor diffusion equilibrated
against a reservoir containing 2.0 to 2.3 M (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 0.2 M NaCl and 0.1 M sodium cacodylate
pH 6.2 to 6.8, in the presence of 10 mM methionine.\textsuperscript{3-4} Crystals were subsequently soaked in 2
mM inhibitor solution containing 30 % glycerol as cryo-protectant. For \textit{Tb}MetRS•Met crystals
soaked in compounds 5, 6 or 13, data were collected in-house using a MicroMax-007 HF
rotating anode (Rigaku) equipped with VariMax HF (Osmic) optics and a Saturn 994 (Rigaku)
CCD detector at a wavelength of 1.54 Å. For \textit{Tb}MetRS•Met crystals soaked in compounds 7, 8
or 11, data were collected at Stanford Synchrotron Radiation Lightsource synchrotron beamlines
9-2 and 12-2 at wavelength of 1 Å. Data integration was performed using either HKL2000\textsuperscript{5} or
XDS\textsuperscript{6} while data scaling was done with Aimless.\textsuperscript{7} Phase determination was carried out with
Phase\textsuperscript{8} using previously reported structures of \textit{Tb}MetRS with similar inhibitors.\textsuperscript{3-4} Model
building/rebuilding and refinement were performed using Coot\textsuperscript{9} and REFMAC5\textsuperscript{10}, respectively.
The refinement restraints for all ligands were generated by the Grade web server.\textsuperscript{11} The final
crystallographic data collection and refinement statistics are given in Table S1.

\textit{Tb}MetRS•Met crystallized in space group \textit{P} 2\textsubscript{1}2\textsubscript{1}2\textsubscript{1} with two \textit{Tb}MetRS subunits in one
asymmetric unit. Soaking of \textit{Tb}MetRS•Met in inhibitor solution resulting in the replacement of
methionine by the inhibitor through conformational selection in one of the subunits,
accompanied by extensive changes in the conformation of the same subunit. Detailed
descriptions of the structures in complex with compounds 1 and 2 were reported earlier.\textsuperscript{3-4}
Table S1. Crystallographic data collection and refinement statistics.

| Compound | 5  | 6  | 7  | 8  | 11 | 13 |
|----------|----|----|----|----|----|----|
| PDB ID   | 4ZT2 | 4ZT3 | 4ZT4 | 4ZT5 | 4ZT6 | 4ZT7 |

**Data collection**

| Space group | \( P \overline{2}_1 \overline{2}_1 \overline{2}_1 \) | \( P \overline{2}_1 \overline{2}_1 \overline{2}_1 \) | \( P \overline{2}_1 \overline{2}_1 \overline{2}_1 \) | \( P \overline{2}_1 \overline{2}_1 \overline{2}_1 \) | \( P \overline{2}_1 \overline{2}_1 \overline{2}_1 \) | \( P \overline{2}_1 \overline{2}_1 \overline{2}_1 \) |
| Cell dimensions \( a, b, c (\text{Å}) \) | 87.1, 106.1, 207.7 | 86.7, 106.1, 207.5 | 87.5, 106.1, 207.1 | 86.8, 106.0, 207.7 | 87.1, 106.2, 206.6 | 86.6, 105.9, 207.3 |
| Resolution (Å) | 35.4 – 2.70 | 38.6 – 2.80 | 38.6 – 2.30 | 39.4 – 2.35 | 38.5 – 2.25 | 37.8 – 2.40 |
| \( R_{merge} \) | 0.164 (0.941) | 0.195 (0.938) | 0.098 (0.995) | 0.174 (1.88) | 0.116 (1.01) | 0.136 (0.818) |
| \( \text{Observed reflections} \) | 351017 (25864) | 253141 (23139) | 583582 (31075) | 1208827 (68815) | 611580 (29847) | 525460 (18813) |
| \( \text{Unique reflections} \) | 53554 (4475) | 47919 (4624) | 86315 (4500) | 80610 (4543) | 91234 (4448) | 75309 (4363) |
| \( \text{Mean} \, I / \sigma I \) | 10.5 (1.9) | 9.8 (1.8) | 17.0 (2.2) | 17.5 (1.8) | 12.6 (1.9) | 15.3 (1.8) |
| \( \text{Completeness} (%) \) | 99.7 (97.6) | 99.9 (100) | 99.9 (100) | 100 (100) | 99.7 (99.7) | 99.9 (99.3) |

**Refinement**

| Resolution (Å) | 35.4 – 2.70 | 38.6 – 2.80 | 38.6 – 2.30 | 38.7 – 2.35 | 37.9 – 2.25 | 37.8 – 2.40 |
| Reflections used | 50766 | 45441 | 81926 | 76494 | 86564 | 71386 |
| \( R_{work} / R_{free} \) | 0.194/0.228 | 0.209 (0.249) | 0.200 (0.225) | 0.200 (0.229) | 0.208 (0.233) | 0.207 (0.236) |
| Number of atoms | | | | | | |
| Protein | 8337 | 8293 | 8238 | 8259 | 8329 | 8339 |
| Met | 9 | 9 | 9 | 9 | 9 | 9 |
| Other solvent ligands | 48 | 51 | 23 | 47 | 16 | 22 |
| Water | 325 | 328 | 458 | 444 | 616 | 529 |
| Average B-factors (Å²) | | | | | | |
| Protein | 50.2 | 49.1 | 45.8 | 52.4 | 42.6 | 38.8 |
| Met | 38.4 | 26.9 | 31.5 | 37.4 | 29.4 | 24.2 |
| Other solvent ligands | 69.1 | 65.3 | 73.0 | 82.9 | 79.5 | 58.7 |
| Water | 37.6 | 28.3 | 44.3 | 48.8 | 44.4 | 32.6 |
| R.m.s. deviations | | | | | | |
| Bond lengths (Å) | 0.007 | 0.007 | 0.007 | 0.007 | 0.007 | 0.007 |
| Bond angles (°) | 1.12 | 1.09 | 1.11 | 1.09 | 1.12 | 1.12 |
| Ramachandran plot\(^a\) | | | | | | |
| Favored (%) | 99 | 98 | 98 | 98 | 98 | 98 |
| Outlier (%) | 0 | 0 | 0 | 0 | 0 | 0 |
| Compound | | | | | | |
| Number of atoms | 23 | 24 | 25 | 24 | 27 | 27 |
| Average B-factors (Å²) | 39.7 | 60.2 | 60.0 | 50.4 | 37.0 | 32.3 |
| LLDF\(^b\) | -0.93 | 0.05 | 0.85 | -0.91 | -0.65 | -0.85 |
| RSR\(^c\) | 0.13 | 0.19 | 0.17 | 0.11 | 0.10 | 0.10 |

Values in parentheses are for highest-resolution shell

\(^a\) Ramachandran Plot statistics as reported by the wwPDB validation report

\(^b\) Local ligand density fit as reported by the wwPDB validation report

\(^c\) Real space R value as reported by the wwPDB validation report
Figure S1. Electron densities of inhibitors bound to *Tb*MetRS.
σA-weighted *F*<sub>obs</sub> − *F*<sub>calc</sub> electron densities calculated by omitting bound inhibitors are shown in green (positive) and red (negative) contoured at the 3.5 σ level, and σA-weighted 2*F*<sub>obs</sub> − *F*<sub>calc</sub> electron densities in light blue at the 1.5 σ level. Inhibitors are depicted in ball and stick models while subunit B of *Tb*MetRS is shown in ribbon representation.
Figure S2. Comparison of structures of TbMetRS in complex with compound 5 or compound 6. The fluorinated compound 6 (carbon atoms in pink) binds to TbMetRS as designed with its fluorine atom almost on top of dummy atom position 1 (0.1 Å shift) based on compound 5 (carbons in green).

Thermal shift assay
Thermal shift assays were performed in white 96-well plates (Bio-Rad) using the fluorescent dye SYPRO Orange (Invitrogen). 100 µl of 9.6 mg/ml protein was diluted in 1.2 ml assay buffer (100 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM ATP-Mg) containing 2 µl of SYPRO Orange DMSO solution sold as “5000x”. 1 µL of 20 mM stock solution of test compounds in DMSO (100 µM final concentration) was pipetted into 9 µl of assay buffer and mixed with 10 µl of the above protein solution in each well. The 96-well plate was sealed with a transparent film (Bio-Rad) and centrifuged for 3 min at 800 rpm to ensure that samples were at the bottom of their wells and any bubble was removed. Temperature-dependent fluorescence increase reporting protein denaturation was measured in triplicate using a DNA Engine Opticon 2 instrument (Bio-Rad) from 25 to 90 °C in steps of 0.2 °C (excitation 470-505 nm; emission 540-700 nm). A Tm for each well was determined with the First Derivative method, which used Opticon Monitor software (Bio-Rad) to smooth the raw fluorescence data (converting each data point into a moving average of itself and adjacent points) and then finding the temperature at which the upward slope of the fluorescence versus temperature curve was the steepest—that is, the temperature at which the first derivative of this curve was maximal.

Enzyme Inhibition Assay and Data
Aminoacylation assays were performed using TbMetRS (at 10 nM) or human mitochondrial MetRS (at 13 nM) as described previously.13-14 Briefly, reactions were performed in 96-well filter plates with Durapore membranes (Millipore) in volumes of 75 µL. The reaction was performed after pre-incubating the enzymes and the compounds; and the TbMetRS condition had a final concentrations of 0.1 mM ATP, 240 nM [3H]L-methionine (83 Ci/mmol), and 400 µg/mL bulk E. coli tRNA (Sigma-Aldrich or the equivalent from Roche). The human mitochondrial MetRS reaction had a final concentration of 2.5 mM ATP, 240 nM [3H]L-methionine (83 Ci/mmol), and 200 µg/mL bulk E. coli tRNA (Sigma-Aldrich or the equivalent from Roche). After incubating the reactions at room temperature for 120 min (TbMetRS) or 60 min (human
mitochondrial MetRS), the reactions were stopped by the addition of 100 µL/well cold 10% trichloroacetic acid. After filtration, washing, and drying, 25µL/well scintillation fluid was added, and the counts of the aminoacylated tRNA on the plates were determined using a scintillation plate counter.

Data for some compounds (1, 2, 5, 6, 10, 11, and 13) were reported earlier with apparent IC\textsubscript{50} values against \textit{Tb}MetRS and human mitochondrial MetRS, many of which were below enzyme concentrations used.\textsuperscript{14} Table S2 contains additional assay results (additional runs with different batches of enzymes) of all the compounds in this manuscript.

|                | \textit{T. brucei} MetRS IC\textsubscript{50} (nM) | Human mitochondrial MetRS IC\textsubscript{50} (nM) | Selectivity Index (IC\textsubscript{50} human/IC\textsubscript{50} \textit{T. brucei}) |
|----------------|-----------------------------------------------|-------------------------------------------------|---------------------------------------------------------------------------------|
| 1              | <10                                          | <13                                             | N/A                                                                             |
| 2              | <10                                          | 157±15                                          | >15.7                                                                           |
| 3              | <10                                          | 635±93                                          | >63.5                                                                           |
| 4              | <10                                          | 7037±964                                        | >704                                                                            |
| 5              | <10                                          | <13                                             | N/A                                                                             |
| 6              | <10                                          | 19.5±8.5                                        | >2                                                                              |
| 7              | <10                                          | 269±103                                         | >26.9                                                                           |
| 8              | <10                                          | 20±8                                            | >2                                                                              |
| 9              | <10                                          | 19.5±0.5                                        | >2                                                                              |
| 10             | <10                                          | 17.5±3.5                                        | >1.8                                                                            |
| 11             | <10                                          | 20.5±7.5                                        | >2                                                                              |
| 12             | <10                                          | <13                                             | N/A                                                                             |
| 13             | <10                                          | <13                                             | N/A                                                                             |

**\textit{T. brucei} growth inhibition assay**

The assay is performed in the same procedure as described in a previous publication.\textsuperscript{15} \textit{T. brucei} (bloodstream form strain 427 from Dr. K. Stuart, Seattle Biomedical Research Institute, Seattle, WA) was cultured in HMI-9 medium containing 10% fetal bovine serum, penicillin, and streptomycin at 37°C with 5% CO\textsubscript{2}. Drug sensitivity of the \textit{T. brucei} strain was determined in 96
well microtiter plates in triplicate with an initial inoculum of $1 \times 10^4$ trypomastigotes per well. Compound stock solutions were prepared in DMSO at 20 mM and added in serial dilutions for a final volume of 200 µl/well. Parasite growth was quantified at 48 H by the addition of Alamar Blue (ThermoFisher Scientific, Waltham, MA). Pentamidine isethionate (Aventis, Dagenham, UK) was included in each assay as a positive control.

**PK studies in mice**
The assay is performed in the same procedure as described in previous publications. Test compound was administered to mice by oral gavage followed by blood sampling at intervals of 30, 60, 120, 240, 360, and 480 min. Compound was dosed orally at 50 mg/kg in 0.2 mL of dosing solution (7% Tween 80, 3% ethanol, 5% DMSO, 0.9% saline). Experiments were performed with groups of three mice per compound as published. Blood samples were analyzed by extracting plasma or dried blood spots (compounds 4, 7, and 12, with correction for plasma/blood partition) in acetonitrile for measurements of compound concentrations by liquid chromatography/tandem mass spectrometry.

**Distribution of compounds between mouse plasma and brain**
This experiment is adapted from a previous reported procedure. Mice (in groups of 3) were injected with test compounds (5 mg/kg IP) and sacrificed at 60 min for collection of plasma and brains. Compound was dissolved in 400 µl of dosing solution (7% Tween80, 3% EtOH, 5% DMSO, 0.9% saline) for IP injections. The brains were weighed and immediately frozen, then later homogenized in acetonitrile using a Dounce homogenizer. Compound recovery was determined by liquid chromatography/tandem mass spectrometry analysis relative to a standard compound amount. Blood was taken from the same mice to determine the compound concentration in plasma. The concentration of compound in the brain was obtained by dividing the moles of compound in the brain by the brain volume (obtained from the brain weight assuming 1 g is 1 mL) and correcting for the brain vasculature volume of 3% by weight.

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