Chemical Validation of Methionyl-tRNA Synthetase (MetRS) as a Druggable Target in *Leishmania donovani*

Leah S. Torrie, Stephen Brand, David A. Robinson, Eun Jung Ko, Laste Stojanovski, Frederick R.C. Simeons, Susan Wyllie, John Thomas, Lucy Ellis, Maria Osuna-Cabello, Ola Epemolu, Andrea Nühs, Jennifer Riley, Lorna MacLean, Sujatha Manthri, Kevin D. Read, Ian H. Gilbert*, Alan H. Fairlamb, Manu De Rycker*.

Drug Discovery Unit, Division of Biological Chemistry and Drug Discovery, School of Life Sciences, Sir James Black Centre, University of Dundee, Dundee DD1 5EH, U.K.

* co-corresponding authors

**SUPPLEMENTARY FIGURE 1: Determination of the Oligomeric State of *LdMetRS***

SEC–MALS elution profiles with the elution peak shown in red and the molar mass over elution peaks shown in black. Analysis of the *LdMetRS* SEC–MALS data suggests it forms a stable monomer under physiological conditions, with the calculated average molar mass of 69 ± 0.5 kDa in agreement with the monomeric size of the protein.
SUPPLEMENTARY FIGURE 2: DDD806905 \( K_{\text{app}} \) Determination

(A) The active enzyme concentration of \( Ld\text{MetRS} \) was determined by titrating this enzyme in the presence of a fixed, high concentration of 1 \( \mu \text{M} \) DDD806905. The active enzyme concentration can be determined from the velocity versus apparent enzyme concentration plot, with the x-axis
intersect defining the point where the active enzyme concentration equals the inhibitor concentration. From this analysis, the point of intersection was found to be 780 nM, indicating that 78% of this LdMetRS protein contains active enzyme, therefore in the LdMetRS enzyme reaction containing 50 nM total protein, the active enzyme concentration is 39 nM. (B) Using this defined active enzyme concentration of 39 nM as a constant, DDD806905 is found to return a $K_i^{app}$ of 41 nM following fitting to the Morrison equation. All data shown as mean ± SD (n≥3 technical replicates).
**SUPPLEMENTARY FIGURE 3: Sequence Alignment of TbMetRS and LdMetRS**

Sequence alignment of *T. brucei* MetRS (Tb927.10.1500) and *L. donovani* MetRS (LdBPK_210890.1) showing a high level of sequence conservation. Residues that line the ligand binding site are highlighted with upward arrows. Residues that line the expanded methionine pocket are highlighted with blue arrows and those that line the auxiliary pocket are highlighted with red arrows. The CP domain (*Tb*MetRS residues 354 to 406), which moves significantly to allow the binding of DDD806905, is highlighted in magenta.
Co-operativity between the *Ld*MetRS methionine and ATP binding sites is highlighted with the methionine $K_m$ varying depending on the ATP concentration used in the assay. (A) When ATP is used at a fixed, saturating concentration of 400 μM ($10 \times K_m$), the methionine $K_m$ is shown to be 170 μM. (B) However, if the fixed ATP concentration is reduced to 80 μM ($2 \times K_m$), the methionine $K_m$ increases to 1500 μM. All data shown as mean ± SD (n=3 technical replicates).

**SUPPLEMENTARY FIGURE 4: Co-operativity Between ATP and Methionine Binding Pockets**
SUPPLEMENTARY FIGURE 5: DDD806905 IC\textsubscript{50} Under Different Substrate Conditions

(A) The DDD806905 IC\textsubscript{50} shifted from 110 nM to 433 nM when the methionine concentration was increased from a sub-\(K_m\) concentration of 50 µM (closed circles) to 500 µM (open circles) (ATP concentration fixed at 500 µM). (B) The DDD806905 IC\textsubscript{50} shifted from 98 nM to 380 nM when the ATP concentration was increased from 50 µM (closed circles) to 500 µM (open circles) (methionine concentration fixed at 500 µM). (C) The DDD806905 IC\textsubscript{50} shifted from 37 nM to 433 nM when both the methionine and ATP concentrations were increased from 50 µM (closed circles) to 500 µM (open circles). All data are shown as mean ± SD (n=3 technical replicates).
SUPPLEMENTARY FIGURE 6: DDD806905 Inhibits Mitochondrial Protein Synthesis

(A) The effect of DDD806905 and (B) linezolid (known inhibitor of mitochondrial protein synthesis) on mitochondrial protein synthesis was tested by comparing levels of a cytoplasmic translated protein (SDH-A) and a mitochondrial translated protein (COX-1). The graphs plot the ratio of normalised COX-1 levels over normalised SDH-A levels against compound concentration. Any value below 1 reflects selective inhibition of mitochondrial protein synthesis. Plots are representative of
three independent experiments. Average data from the three experiments: DDD806905 IC$_{50}$ 1.68 µM (95% CI 1.00 – 2.84 µM), Linezolid IC$_{50}$ 15.1 µM (95% CI 7.8 – 29.2 µM).
SUPPLEMENTARY FIGURE 7: DDD806905 Leishmania Intracellular Amastigote EC₅₀ Determination

DDD806905 inhibits growth of Leishmania intracellular amastigotes with an EC₅₀ of 2.9 µM (95% CI 2.2 – 3.9 µM) (n = 3 biological replicates). The representative potency determination shown returns a DDD806905 EC₅₀ of 2.8 µM and a Hill slope of 45 (data shown as mean ± SD, n=8 technical replicates).
SUPPLEMENTARY FIGURE 8: DDD806905 Integrated Pharmacokinetics for Mouse Visceral Leishmaniasis Efficacy Study

(A) The blood concentration time profile in infected mice following first dose on day 1. (B) The blood concentration time profile in infected mice following first dose on day 10. In (A) and (B) mean whole blood total concentrations are shown in closed squares (mean ± SD, n=3), with mean blood free concentrations shown in closed circles. Dashed line shows MIC from intracellular *Leishmania* assay. The maximum concentration in blood (C\textsubscript{max}) was 2,600 ng/ml and 3,200 ng/ml for day 1 and day 10 respectively, T\textsubscript{max} was at 2h, AUC\textsubscript{(0-8h)} was 950,000 ng-min/ml on day 1 and 1,400,000 ng-min/ml on day 10.
SUPPLEMENTARY FIGURE 9: DDD806905 EC\textsubscript{50} in \textit{Leishmania} Promastigotes Using Varying Serum Concentrations

With increasing serum concentration, the EC\textsubscript{50} of DDD806905 in \textit{Leishmania} promastigotes increases linearly (solid line). Extrapolation of this linear plot (dashed line) reveals a predicted EC\textsubscript{50} of 1.75 µM in 100% serum and 0.12 µM in no serum.
SUPPLEMENTARY FIGURE 10: pH Effect of Ionisation and Lipophilicity of DDD806905

(A) Ionisation profile of DDD806905 over the pH range 0 to 14. Percentage of unionised species (black line), ionised species one (green line), ionised species two (red line) and ionised species three (blue line) of DDD806905 is shown. The figure shows that as the pH decreases, the percentage of the unionised species (black) also decreases and the percentage of ionised species increases. The points at which the lines cross represent the pKₐs (3.4, 7.5 and 11). (B) Lipophilicity profile of DDD806905 over pH range 1 – 12. As the pH decreases, the logD of DDD806905 decreases (red line represents extrapolated data).
SUPPLEMENTARY TABLE 1: Enzymatic and Phenotypic Potencies of *Ld*MetRS Inhibitors

| Compound ID | Structure | *Ld*MetRS IC₅₀ (µM) | Leishmania Promastigote EC₅₀ (µM) | Leishmania Axenic Amastigote EC₅₀ (µM) |
|-------------|-----------|---------------------|----------------------------------|--------------------------------------|
| 1           | ![Structure 1](image1.png) | 3.9                 | 1.5                              | >50                                  |
| 2           | ![Structure 2](image2.png) | 1.7                 | 2.2                              | >50                                  |
| 3           | ![Structure 3](image3.png) | 69                  | 7.1                              | >50                                  |
| 4           | ![Structure 4](image4.png) | 2.8                 | 1.3                              | 38                                   |
| 5           | ![Structure 5](image5.png) | 46                  | 6.2                              | 29                                   |
| 6           | ![Structure 6](image6.png) | 14                  | 6.2                              | >50                                  |
| 7  | ![Molecule 1] | 79 | 19 | >50 |
|----|--------------|----|----|-----|
| 8  | ![Molecule 2] | 47 | 33 | >50 |
| 9  | ![Molecule 3] | >100 | 7.8 | >50 |
| 10 | ![Molecule 4] | 4.8 | 6.5 | >50 |
| 11 | ![Molecule 5] | 0.17 | 0.54 | 6.2 |
| 12 | ![Molecule 6] | 5.6 | 9.5 | >50 |
| 13 | ![Molecule 7] | 37 | 19 | >50 |
|   |   |   |   |
|---|---|---|---|
| 14|   | 12| 7.6| >50 |
| 15|   | 5.4| 4.1| >50 |
| 16|   | 5.9| 4.9| >50 |
| 17|   | 0.38| 0.87| 19 |
| 18|   | 10| >50| >50 |
| 19|   | 44| 21| >50 |
| 20|   | 2.1| 43| >50 |
|    |    |    |    |    |
|----|----|----|----|----|
| 21 | ![Image](image1.png) | >100 | >50 | >50 |
| 22 | ![Image](image2.png) | 55 | 22 | >50 |
| 23 | ![Image](image3.png) | 2.0 | 0.87 | 45 |
| 24 | ![Image](image4.png) | 8.9 | 2.8 | >50 |
| DDD806905 | ![Image](image5.png) | 0.09 | 0.44 | 14 |
**SUPPLEMENTARY TABLE 2: Data Measurement and Refinement Statistics**

| Data Measurement | TbMetRS:DDD00806905 |
|------------------|----------------------|
| PDB code         | 5NFH                 |

| **Data Measurement** | |
|----------------------|---------------------|
| **Source**           | DLS I02             |
| **Space Group**      | P2_{1}2_{1}2_{1}    |
| **Unit Cell Dimensions (Å)** | a=87.09, b=105.99, c=207.07 |
| **Resolution (Å)**   | 50.0-2.80 (2.90-2.80) |
| **Observations**     | 218279              |
| **Unique Observations** | 47875              |
| **Rmerge (%)**       | 13.6 (70.0)         |
| **I/σI**             | 9.0 (2.0)           |
| **CC(1/2)**          | 0.99 (0.68)         |
| **Completeness (%)** | 99.8 (99.9)         |
| **Redundancy**       | 4.6 (4.5)           |

| **Refinement Statistics** | |
|---------------------------|-----------------|
| **Resolution Range (Å)**  | 50.0 – 2.80     |
| **R-factor (R_{work}/R_{free})** | 19.5 /23.0     |
| **Number of atoms**       | 8367/28/51/263  |
| **Mean B-factor (Å²)**    | 46/53/57/37     |
| **RMS bond length deviation (Å)** | 0.012          |
| **RMS bond angle deviation (°)** | 1.32           |

\(^{a}\) Values in parentheses are for reflections in the highest resolution shell

\(^{b}\) R_{merge} = \sum |I - <I>| / \sum <I>

\(^{c}\) A cutoff criterion for resolution limits was applied on the basis of the mean intensity correlation coefficient of half-subsets of the data set (CC_{1/2} > 0.5)

\(^{d}\) R-factor = \sum |F_{obs} - F_{calc}| / \sum |F_{obs}|
° Number of atoms of protein/ligand/heteroatoms/water

 Mean B-factor for protein/ligand/heteroatoms/water
MATERIALS AND METHODS

LdMetRS Protein Expression and Purification

An *E.coli* codon optimised (Genscript) version of the *Ld*MetRS gene (LdBPK_210890) was cloned into pET15b TEV vector using *Nde*1 and *Xho*1 restriction sites. The vector had been previously modified to include a HIS tag and TEV cleavage site at the N-terminus.

The plasmid was transformed into BL21 DE3 *E.coli* cells. One litre cultures were grown in autoinduction + AMP media at 37 °C at 200 rpm until the OD$_{600}$ reached 0.8. The temperature was then reduced to 22 °C at 200 rpm overnight. Cells were harvested by centrifugation at 3,000 g for 20 min and stored at -20 °C.

The pellets were resuspended in 150 ml 20 mM HEPES, 500 mM NaCl, 5% Glycerol, 1 mM TCEP, 10 μM ZnCl$_2$ and 20 mM Imidazole pH 7.0 (Buffer A). DNase I (Sigma) and protease inhibitors (Pierce) were added. The cells were lysed using the Continuous Flow Cell Disrupter (Constant Systems) at 30 KPSI. The lysate was centrifuged at 40,000 g for 20 min and the supernatant was then filtered (0.2 μm).

The sample was loaded onto a 5 ml HiTrap His column (GE Healthcare) equilibrated with buffer A on an AKTA Purifier (GE). The column was then washed with 10 column volumes of buffer A. A step gradient of 5% Buffer B (A + 500 mM imidazole) was used to remove Histidine-rich protein contaminants. A gradient of 5 to 50% B over 20 column volumes was used to elute the *Ld*MetRS. The protein was then concentrated to 11 ml ready for gel filtration. Gel filtration was carried out on a Superdex 200 26/60 prep grade column (GE) in 20 mM HEPES, 500 mM NaCl, 5% Glycerol, 1 mM TCEP and 10 μM ZnCl$_2$ pH7.0. 10% glycerol was added for storage at -80 °C.

TbMetRS Protein Expression and Purification

A codon optimized gene encoding for *Tb*MetRS (Uniprot ID Q38C91) was synthesized by GenScript (USA) for expression in *E.coli*. The gene encoded a truncated form of *Tb*MetRS (residues 273-773) with three point mutations: K452A, K453R and E454A.

The gene was synthesized with an N-terminal His tag followed by a PreScission Protease cleavage site and cloned into a pET15b vector (Novagen) using *Nco*I and *Xho*I restriction sites.
For expression, the plasmid was transformed into BL21 (DE3) cells by a standard heat shock method and plated onto LB agar containing 50 mg/ml carbenicillin. A single colony was used to inoculate a 100 ml starter culture that was grown overnight at 37 °C 200 rpm, prior to inoculating 6 l of autoinduction media. Cells were incubated for 4 h at 37 °C 200 rpm before the temperature was dropped to 22 °C and growth continued for a further 20 h. Cells were subsequently pelleted at 3,500 g 4 °C for 30 min and frozen until required.

The cell pellet was re-suspended in 3-4 pellet volumes of 25 mM HEPES, 500 mM NaCl, 10% Glycerol, 20 mM imidazole, 2 mM DTT pH7.0, Complete Protease Inhibitor Tablet (Roche) and DNase (0.1 mg/ml) before lysis on a Continuous Flow Cell Disrupter (Constant Systems).

Solid debris was removed by centrifugation at 40,000g for 20 min then filtered to 0.22 µm. The sample was then loaded onto a 5 ml HiTrap HIS HP (GE) column pre equilibrated with 25 mM HEPES, 500 mM NaCl, 10% Glycerol, 20 mM imidazole, 2 mM DTT pH7.0 (Buffer A) using an AKTA Purifier system (GE) at 5 ml/min. The column was then washed for 10 column volumes with Buffer A. A gradient of 0-50% B (A+500 mM imidazole) was used for the elution. At 5% B a His rich peak was eluted. At 6.8% B the gradient was held to elute the protein.

The protein was dialyzed overnight at 4 °C in Buffer A in the presence of PreScission Protease (PP) (5mg) before the sample was ran over 1 ml of Glutathione Sepharose 4B beads (GE) to remove the protease.

A second 5 ml HiTrap HIS HP (GE) column was performed to remove uncleaved protein before the sample was concentrated for gel filtration. The sample was run on a Superdex 200 26/60 column pre-equilibrated with 25 mM HEPES, 500 mM NaCl, 10% Glycerol, 2 mM DTT, 10 mM L-methionine pH7.0. Purified *TbMetRS* was concentrated to 10 mg/ml prior to crystallisation.

**Size Exclusion Chromatography and Multi Angle Light Scattering (SEC–MALS)**

SEC–MALS experiments were performed on a Dionex Ultimate 3000 HPLC system with an inline Wyatt miniDAWN TREOS MALS detector and Optilab T-rEX refractive index detector. Superdex 200 10/300 GL SEC column was used to perform SEC experiment at a flow rate of 0.3 ml/min of buffer solution. The buffer conditions were 20 mM HEPES-KOH pH 7.5 with 150 mM sodium chloride concentrations. Around 40 µl of 4 mg/ml protein solution was loaded into SEC column. Molar masses spanning elution peaks were calculated using ASTRA v6.0.0.108 (Wyatt).
**LdMetRS Assay Development and Kinetic Parameter Determinations**

Activity of the LdMetRS enzyme was determined by monitoring levels of pyrophosphate released during the first step of the enzymatic reaction. The pyrophosphate formed was converted to two inorganic phosphate molecules using a pyrophosphatase enzyme and levels of the resulting phosphate molecules measured using the BIOMOL® Green reagent (Enzo Life Sciences)².

Following optimization of the assay buffer and determination of the enzyme linearity, 384-well plate based assays were carried out at room temperature in a 50 μl reaction volume containing 30 mM Tris, pH 8.0, 140 mM NaCl, 30 mM KCl, 40 mM MgCl₂, 0.01% (v/v) Brij-35, 1 mM DTT, 1 U/ml pyrophosphatase, 50 nM recombinant LdMetRS, and varying concentrations of substrates. Michaelis constants for the ATP and methionine substrates were determined in an end point assay using the above buffer and enzyme conditions. Data were fitted to the modified Michaelis-Menten equations 1 and 2 respectively.

Equation 1 (Hill equation):

\[ v = \frac{V_{\text{max}} [S]^n}{K^n + [S]^n} \]

Equation 2 (High substrate inhibition Michaelis-Menten equation):

\[ v = \frac{V_{\text{max}}}{1 + \frac{K_{\text{m}}}{S} + \frac{S}{K_i}} \]

**LdMetRS Inhibitor Studies**

All test compounds were solubilized in 100% DMSO to a concentration of 10 mM. To generate IC₅₀ data for the compounds, assay ready plates containing 10-point inhibitor curves (half-log serial dilutions) were prepared using an Echo® 550 (Labcyte).

Following preparation of the inhibitor curves, assays were carried out at room temperature in clear, flat bottom, polystyrene, 384-well plates (Greiner). Each assay was performed in a 50 μl reaction volume containing 30 mM Tris; pH 8.0, 140 mM NaCl, 30 mM KCl, 40 mM MgCl₂, 0.01% (v/v) Brij-35, 1 mM DTT, 1 U/ml pyrophosphatase, 50 nM recombinant LdMetRS, 50 μM methionine and 100 μM ATP.
Assays were performed by adding 25 μl buffer with enzyme to assay plates (with buffer only added to ‘no enzyme’ control wells) before the reaction was initiated with the addition of a 25 μl mix containing methionine, ATP and pyrophosphatase to all wells. Following a 120 min reaction at room temperature the assay was stopped with the addition of 50 μl BIOMOL® Green. The BIOMOL® Green signal was allowed to develop for 30 min before the absorbance of each well was read at 650 nm using an EnVision multilabel plate reader (PerkinElmer Life Sciences). All liquid dispensing steps were carried out using a Thermo Scientific WellMate dispenser (Matrix).

ActivityBase from IDBS (version 8.0.5.4) was used for data processing and analysis. All IC\textsubscript{50} curve fitting was undertaken using ActivityBase XE (version 7.7.1) from IDBS. A four-parameter logistic dose-response curve was utilized with prefit used for all four parameters.

For DDD806905 \( K_i^{app} \) determinations, data fitting to the Morrison equation (equation 3) was carried out using GraFit v6.0 (Erithacus Software). It should be noted that data for fitting to the Morrison equation was normalised to a ‘100 μM DDD806905’ control rather than a ‘no enzyme’ control as described above.

Equation 3 (Morrison equation):

\[
\frac{v_i}{v_0} = 1 - \frac{\left( [E]_t + [I]_t + K_i^{app} \right) - \sqrt{\left( [E]_t + [I]_t + K_i^{app} \right)^2 - 4 [E]_t [I]_t}}{2 [E]_t}
\]

\text{LdMetRS Mode of Inhibition Studies}

To establish the mode of inhibition of DDD806905, data sets (generated using the BIOMOL® Green assay platform previously described) were collected by varying both the inhibitor and substrate concentrations. Using GraFit v6.0 (Erithacus Software) each data set was individually fitted to the Michaelis-Menten equation or the modified high substrate inhibition Michaelis-Menten equation described above (equation 2), and the resulting Lineweaver-Burk plots were examined for diagnostic patterns of competitive, mixed, or uncompetitive inhibition.

Data sets were then globally fitted to the appropriate model (with equations 4 and 5 used for competitive and mixed inhibition respectively). If more than one model appeared possible, then data were fitted to both and examined for significance using the F-test function in GraFit.
Equation 4 (Competitive inhibition equation):

\[
v = \frac{V_{\text{max}} [S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S]}
\]

Equation 5 (Mixed inhibition equation):

\[
v = \frac{V_{\text{max}} [S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + \left(1 + \frac{[I]}{K_i}\right)[S]}
\]

**TbMetRS Crystallisation**

Crystals of TbMetRS:Met were obtained by incubating TbMetRS with 10 mM methionine and 1 mM TCEP before crystallisation by sitting drop vapour diffusion against a reservoir solution containing 2.0-2.3 M Ammonium Sulphate, 0.2 M NaCl, 0.1 M Na Cacodylate pH 6.0-6.6. Single crystals grew after 3-4 days incubation at 18 °C. To obtain ternary TbMetRS:Met:Ligand complexes, TbMetRS:Met crystals were soaked for 10-30 seconds in a 10 μl drop consisting of 1 μl of ligand (20 mM in 20% DMSO), 4 μl reservoir solution and 5 μl glycerol. Crystals were flash frozen immediately in liquid nitrogen in preparation for data collection.

Diffraction data were measured at Diamond Light Source beamline I02. Data were integrated using XDS 3 and scaled using Aimless 4. Structure solution was carried out using MOLREP 5 with TbMetRS (4EGA) used as a search model. The structure was refined with REFMAC5 6 from the CCP4 suite 7, ligand topologies generated using PRODRG 8 and manual model alteration carried out using COOT 9. Data measurement and model refinement statistics are presented in Supplementary Table 2. Coordinate files and associated experimental data have been deposited in the Protein Data Bank (PDB) with accession code 5NFH.

**Leishmania tarentolae In Vitro Translation**

Protein translation in a Leishmania cell extract was studied using a commercially available cell-free protein expression kit (Jena Bioscience). Reactions were carried out in black 384-well, small-volume plates (Greiner) in 10 μl reaction volumes containing 7 μl L. tarentolae cell extract, 1 μl eGFP DNA template and 2 μl nuclease free water (‘no DNA’ wells were included as negative controls). eGFP expression was monitored over time using a BMG PheraStar plate reader (Excitation 485 nm; Emission 520 nm).
When test compounds were to be screened, the compound of interest (solubilised in 100% DMSO) was dispensed into assay plates using an Echo® 550 (Labcyte) before *L. tarentolae* extract and eGFP template DNA were added to assay wells as described above. The reaction was run at room temperature for 120 min before eGFP fluorescence intensity was measured. IC\textsubscript{50} curve fitting was undertaken in GraFit version 6.0 (Erithacus Software) using a four-parameter logistic dose-response curve (equation 6), with $s$ the slope.

\begin{equation}
\begin{align*}
y' &= \frac{\text{Range}}{1 + \left(\frac{x}{\text{IC}_{50}}\right)^s} + \text{Background}
\end{align*}
\end{equation}

**HeLa In Vitro Translation**

Protein translation in a HeLa cell extract was studied using a commercially available in vitro translation kit (Thermo Scientific). Reactions were carried out in black 384-well, small-volume plates (Greiner) in 10 µl reaction volumes containing 5 µl HeLa cell extract, 1 µl accessory proteins, 2 µl reaction mix, 0.8 µl GFP DNA template and 1.2 µl nuclease free water (‘no DNA’ wells were included as negative controls). GFP expression was monitored over time using a BMG PheraStar plate reader (Excitation 485 nm; Emission 520 nm).

When testing DDD806905 and the positive control compound cycloheximide, compounds (solubilised in 100% DMSO) were dispensed into assay plates using an Echo® 550 (Labcyte) before HeLa extract, accessory proteins, reaction mix and GFP template DNA were added to assay wells and GFP expression monitored as described above.

**Mitochondrial protein synthesis assay**

The effect of DDD806905 on mitochondrial protein synthesis was determined by BioDuro using the MitoBiogenesisTM In-Cell ELISA Kit (Abcam). The kit reports the ratio of a nuclear (SDH-A) and a mitochondrial (COX-1) translated protein which is used to determine if there is a mitochondrial specific protein synthesis effect. In the assay the levels of SDH-A and COX-1 are measured in H9C2 cells. These are normalised to DMSO treated cells (vehicle) and then the ratio of normalised SDH-A over COX-1 is calculated. Dose response curves are calculated with this data and an IC\textsubscript{50} is reported.
**Leishmania** Cell Lines and Animal Strains

THP-1 (human monocytic leukemia) cells were obtained from the Health Protection Agency Culture Collections. The cells were screened for mycoplasma and maintained in RPMI plus 10% (vol/vol) heat-inactivated foetal bovine serum (FBS). *L. donovani* (MHOM/SD/62/1S-CL2D, LdBOB) axenic amastigotes and promastigotes were maintained as described previously[^10]. Every 5 weeks, the parasites were cycled between developmental stages. For amastigote differentiation, 20 μl of metacyclic promastigote culture was inoculated in amastigote media. For the reverse process, 20 μl of dense amastigote culture was inoculated in promastigote media.

For **in vivo** efficacy studies the sodium stibogluconate sensitive *Leishmania donovani* (WHO designation: MHOM/ET/67/HU3, also known as LV9 or L82) is used. Female BALB/c mice (~20 g), specific pathogen free and Syrian hamsters – *Mesocricetus auratus* – are supplied by accredited suppliers and maintained under containment level 3 conditions.

Animal Ethics

All regulated procedures on living animals were carried out under the authority of a project license approved by the University’s Ethical Review Committee (ERC) and issued by the Home Office under the Animals (Scientific Procedures) Act 1986, as amended in 2012 (and in compliance with EU Directive EU/2010/63). The ERC has a general remit to develop and oversee policy on all aspects of the use of animals on University premises and is a subcommittee of the University Court, its highest governing body.

Mouse Pharmacokinetics (efficacy study)

Following the first dose on day 1 and on day 10 blood samples were taken from 3 infected mice at 30 min, 1, 2, 4, 8 hours post-dose and mixed with two volumes of distilled water. Each diluted sample was frozen and thawed 3 times within the Containment Level 3 suite before being transferred for bioanalysis. After suitable sample preparation, the analyte concentration in blood was determined by UPLC-MS/MS using a Waters Xevo™ TQs [TQ³, n=3]. The pharmacokinetic parameters were calculated by noncompartmental analysis of the corrected blood concentration versus time curves of DDD806905 using PK solutions software v 2.0 (Summit Research Services, USA).

Mouse Pharmacokinetics (naive)

Test compound was dosed as a bolus solution intravenously at 3 mg free base/kg (dose volume: 5 mL/kg; dose vehicle: 10% DMSO; 40% PEG400; 50% MilliQ H₂O) to female Balb/c mice (n=3). Blood
samples were taken from each mouse at 1, 3, 5, 15 and 30 minutes, 1, 2, 4, 6, 8 and 24 hours post-dose and mixed with two volumes of distilled water. After suitable sample preparation, the concentration of the test compound in blood was determined by UPLC-MS/MS using a Waters Xevo™ TQs [TQn, n=3]. Pharmacokinetic parameters were derived from the mean blood concentration time curve using PKsolutions software v 2.0 (Summit Research Services, USA).

**In vivo Leishmaniasis Animal Model**

*L. donovani* amastigotes were isolated from the spleen of a heavily infected donor hamster and an inoculum containing $\sim 1 \times 10^8$ amastigotes/ml in DMEM (Dulbecco’s Modified Eagle’s Medium) was prepared. Groups of five randomly assorted mice were infected intravenously (tail vein) with a 0.2 ml bolus (equivalent to $\sim 2 \times 10^7$ amastigotes/ml) on day 0. From day 7 post-infection, groups of mice were treated with either vehicle only (orally), with miltefosine (30 mg/kg orally) or with DDD806905 (50 mg/kg orally). Miltefosine was dosed once daily for 10 days, with vehicle and DDD806905 administered twice daily over the same interval. Drug dosing solutions were prepared fresh daily and the vehicle was 10% DMSO; 40% PEG400; 50% MilliQ water.

On day 19 post-infection, all animals were euthanized humanely and parasite burdens were determined microscopically on Rapi-Diff II liver smears. Parasite load is expressed in Leishman-Donovan Units (LDU) (mean number of amastigotes per 500 liver cell x mg weight of liver).

**Knock-out and overexpressor generation**

Unsuccessful experiments to generate *Leishmania* MetRS knock-out or overexpressor cells were carried out following standard methods as described in.

**Leishmania Promastigote Assay**

Compounds to be tested were pre-dispensed into white, clear bottom 384-well plates (Greiner). For potency determinations, ten-point one in three dilution curves were generated. The top concentration was 50 μM and on each plate a control curve of Amphotericin B was included. LdBOB promastigotes were added to all wells containing compound (5000 cells per well, 50μl) using a Thermo Scientific WellMate dispenser (Matrix). Media only was dispensed into control columns. After a 68 h incubation at 37°C under 5% CO$_2$ in a humidified incubator, resazurin was added to each well at a final concentration of 0.5 mM and the plates were incubated for a further 4 h. Plates were then sealed with clear film and resorufin fluorescence was detected using a Victor 3 plate reader (Perkin Elmer) with excitation at 528 nm and emission at 590 nm. Serum shift experiments were
carried out as described above using 5%, 10% and 20% FBS in the media. To examine the effect of the methionine concentration on the potency of DDD806905 parasites were grown in the presence of drug and in the presence (or absence) of 20-fold excess methionine (0.3 g/l). IC\textsubscript{50} curve fitting was undertaken in GraFit version 6.0 (Erithacus Software) using a four-parameter logistic dose-response curve (equation 6), with s the slope.

**Leishmania Axenic Amastigote Assay**

The *Leishmania* axenic amastigote assay was performed using the method published in Nühs *et al*.\textsuperscript{15}

**Leishmania Intracellular Amastigote Assay**

The intramacrophage *Leishmania* assay was performed using a modified version of the method described in De Rycker *et al*.\textsuperscript{16} For potency determinations, ten-point, one in three dilution curves were created with the highest concentration being 50 µM and on each plate a control curve of amphotericin B was included. Controls were as follows: columns 11 and 12: DMSO, columns 23 and 24: amphotericin B (final concentration 2 µM). 350 nl of compound was pre-dispensed into 384-well sterile intermediary plates. To the intermediary plates, 35 µl of THP-1 media was added and plates were shaken for >5 min to ensure complete mixing. THP-1 cells (8,000 per well, 50 µl) were plated into black clear-bottom 384-well plates (Corning) in presence of 20 nM phorbol 12-myristate 13-acetate (PMA). After 20 min at room temperature, the plates were incubated at 37°C under 5% CO\textsubscript{2} in a humidified incubator for 75 h. The cells were then washed with 450 µl sterile phosphate buffered saline (PBS) supplemented with 1 mM CaCl\textsubscript{2}, 0.5 mM MgCl\textsubscript{2}, 0.1% (w/v) bovine serum albumin (PBS-A) and amastigotes were added to all wells at a multiplicity of infection of 5 (40,000 amastigotes per well). After 40 min at room temperature, plates were returned to the incubator. Amastigotes were incubated in the presence of THP-1 macrophages for 16 h. Any remaining extracellular amastigotes were subsequently removed by washing with PBS-A followed by addition of 25 µl of the compound pre-dilutions using an Ipette-pro pipetting station (FluidX). The final dilution of each compound was 200-fold. Plates were incubated for 96 h and then washed (250 µl PBS-A) and fixed (4 % (v/v) formaldehyde-PBS, 30 min, room temperature). After fixation, the wells were washed with 250 µl PBS, stained (10 µg/ml DAPI, 0.4 µg/mL HCS Cellmask Deep Red in PBS + 0.1% (v/v) Triton X-100, 30 min, room temperature) and washed with 250 µl PBS. Finally, PBS + 0.05% (v/v) thimerosal was added to the wells, the plates were sealed and imaged on a high-content microscope (GE IN Cell 2000) using a 10x objective. Image analysis was carried out with GE IN Cell Analyzer 1000 Workstation using the “Multi Target Analysis” module. For each well, i) THP-1 cell count (cytotoxicity readout) and ii) average number of amastigotes per cell (potency readout) were
calculated, both in terms of pEC\textsubscript{50} values. MIC (minimum inhibitory concentration) values are defined as the lowest concentration tested at which maximal inhibition is reached.

**Lysosomotropism Assay**

THP-1 cells were differentiated in 96-well plates with PMA as described above. Following differentiation the cells were treated for 30 min with dose-response curves of test compounds in presence of 100 nM LysoTracker Red DND-99 (Thermo Fisher) and 5 µg/ml Hoechst 33342. Next cells were washed 3 times with Hanks Buffered Saline Solution (Gibco) and imaged on a Perkin Elmer Operetta system. An image analysis algorithm to measure mean Lysotracker Red DND-99 intensity per cell was developed in Columbus. Mean Intensity was normalised using a no dye control (100% effect) and DMSO treated wells stained with Lysotracker Red DND-99 (0% effect control). Dose-response curves were fitted for the test compounds using a four-parameter equation (equation 5 above) and constrained to 0 and 100% where necessary.

**pK\textsubscript{a} measurements**

To measure the pK\textsubscript{a} of DDD806905, a fast UV-metric method was performed using a Sirius T3 instrument (Sirius Analytical, U.K.). The calculation of the pK\textsubscript{a} was determined using the Sirius T3 refine software (Sirius Analytical, U.K.). In brief, the UV-metric pK\textsubscript{a} method is based on measuring pH during an acid-base titration and monitoring UV absorbance shift. The ionisation curve (Bjerrum curve) was then used to determine the pH where exactly 50% of neutral and 50% of ionised species was present. DDD806905 must be fully dissolved throughout the titration and so the titration is set to start at the pH where DDD806905 is in its fully ionised state. The appearance of precipitate was monitored throughout the experiment by a turbidity sensing device. DDD806905 was predicted to be poorly soluble (ACD labs) and so the titration was performed in the presence of methanol. The pK\textsubscript{a}s of DDD806905 was determined at the pH where there was maximal change in UV absorbance with respect to pH. The pH range of the titration was between 2 and 12. The pK\textsubscript{a} values were then extrapolated to an aqueous pK\textsubscript{a} using a graphical method based on the work by Yasuda and Shedlovsky\textsuperscript{17,18} using the Sirius T3 refine software\textsuperscript{19}.

**LogD\textsubscript{7.4} measurements**

LogD\textsubscript{7.4} values were quantified using a Sirius T3 instrument (Sirius Analytical, U.K.). The lipophilicity assay was a standard potentiometric titration performed in the presence of three varying octanol-water ratios. The instrument software calculated the pH of each data point using mass and charge balance equations and then fitted this theoretical data to the experimental data. The logP value
which gave the best fit to the data was then recorded as the logP. Using the logP and an experimentally determined pKₘ (see above), the software generated a lipophilicity plot showing changes in the logD with increasing pH and from this plot a logD₇.₃ and logD₅.₆ values were recorded.

**Plasma Protein Binding Determination**

A 96-well equilibrium dialysis apparatus was used to determine the free fraction in plasma for DDD806905 (HT Dialysis LLC, Gales Ferry, CT). Membranes (12-14 kDA cut-off) were conditioned in deionised water for 60 minutes, followed by conditioning in 80:20 deionised water:ethanol for 20 minutes, and then rinsed in isotonic buffer before use. Female CD1 mouse plasma or human plasma was removed from the freezer and allowed to thaw on the day of experiment. Thawed plasma was then centrifuged (Allegra X12-R, Beckman Coulter, USA), spiked with test compound (final concentration 10 µg/ml), and 150 µL aliquots (n=6 replicate determinations) loaded into the 96-well equilibrium dialysis plate. Dialysis vs isotonic buffer (150 µl) was carried out for 5 hours in a temperature controlled incubator at ca. 37°C (Barworld scientific Ltd, UK) using an orbital microplate shaker at 100 revolutions/minute (Barworld scientific Ltd, UK). At the end of the incubation period, 50 µl aliquots of plasma or buffer were transferred to micronic tubes (Micronic B.V., the Netherlands) and the composition in each tube balanced with control fluid (50 µl), such that the volume of buffer to plasma is the same. Sample extraction was performed by the addition of 200 µl of acetonitrile containing an appropriate internal standard. Samples were allowed to mix for 1 minute and then centrifuged at 3000 rpm in 96-well blocks for 15 minutes (Allegra X12-R, Beckman Coulter, USA) after which 150 µl of supernatant was removed to 50 µl of water. All samples were analysed by UPLC-MS/MS on a Quattro Premier XE Mass Spectrometer (Waters Corporation, USA). The unbound fraction (fu) was determined as the ratio of the peak area in buffer to that in plasma.

**Medicinal Chemistry**

All compounds in Supplementary Table 1 provided by GSK.

**Preparation of compounds in Table 1.**

**Preparation of 2-((((3-(((4,6-dichloro-1H-indol-2-yl)methyl)amino)propyl)amino)quinazolin-4(1H)-one dihydrochloride (DDD806905.2HCl):**
Preparation of intermediate 2-(3-aminopropylamino)-3H-quinazolin-4-one:

A mixture of 2-methylsulfanyl-3H-quinazolin-4-one, prepared according to Moreno et al. 20, (1.0 g, 5.20 mmol) and propane-1,3-diamine (8.0 g, 107.9 mmol) was heated at 130°C in a microwave tube for 1 h. The reaction was then concentrated in vacuo and the residual powder triturated with DCM, stirred vigorously for 30 min and collected by filtration. The greyish powder was then washed with cold diethyl ether and the powder dried in vacuo to give 2-(3-aminopropylamino)-3H-quinazolin-4-one (1.06 g, 4.63 mmol, 89%) yield as a fluffy white powder.

Preparation of intermediate 4,6-dichloro-1H-indole-2-carbaldehyde:

A solution of ethyl 4,6-dichloro-1H-indole-2-carboxylate (4.0 g, 15.5 mmol) in THF (100 mL) at 0°C was treated dropwise with LiAlH₄ (2 M in THF) (16.0 mL, 32.0 mmol). The reaction was then slowly allowed to warm to room temperature and stirred for 18 h before being carefully quenched by the careful dropwise addition of 3 mL of 2 M sodium hydroxide solution, followed by 100 mL of potassium sodium tartrate solution. The reaction mixture was stirred for 30 min prior to dilution with diethylether (100 mL) and stirring for 1 h before removal of the organic phase and extraction of the aqueous phase with diethylether (3 x 50 mL). The combined organic extracts were dried (MgSO₄), filtered and concentrated in vacuo. Chromatography (SiO₂, 50 to 100% EtOAc : hexanes) gave (4,6-dichloro-1H-indol-2-yl)methanol (2.50 g, 11.57 mmol, 65%). This was immediately dissolved in DCM (250 mL) and treated portionwise with manganese dioxide (5.03 g, 57.9 mmol) at room temperature and the reaction stirred overnight at 40°C. The reaction was allowed to cool prior to filtration through a short pad of celite. The celite was washed with ether (200 mL) and the filtrate concentrated in vacuo to the title compound (2.39 g, 11.0 mmol, 95%) as a fluffy off-white powder.
Preparation of 2-((3-(((4,6-dichloro-1H-indol-2-yl)methyl)amino)propyl)amino)quinazolin-4(1H)-one dihydrochloride (DDD806905.2HCl):

A slurry of 2-(3-aminopropylamino)-3H-quinazolin-4-one (2.39 g, 11.0 mmol) and 4,6-dichloro-1H-indole-2-carbaldehyde (2.39 g, 11.2 mmol) in THF (250 mL) at room temperature needed to be warmed to 55°C in order to obtain a solution. After stirring at 55°C for 60 mins sodium triacetoxyborohydride (4.75 g, 22.4 mmol) was added portionwise and the resulting solution was stirred for 72 h. The reaction was then cooled to room temperature then quenched by careful addition of 25 mL NaOH solution. The reaction was then diluted with brine (100 mL) and diethylether (100 mL) and stirred for 2 h, the organic phase separated, combined with organic extracts of the aqueous layer (3 x 50 mL EtOAc), the combined organics dried (MgSO₄) and concentrated in vacuo. The residual gum was subjected to chromatography (SiO₂, 100% EtOAc moving to 20% 7 N methanolic ammonia in EtOAc) to give 1.85 g of the title compounds as the free base. This solid was then slurried in dry diethylether and treated dropwise with 1 M HCl in diethylether and the resulting precipitate collected by filtration and dried to give the title compound (2.55 g, 5.16 mmol, 46%) as a white flowing powder. ¹H NMR (500 MHz, DMSO-d₆): δ11.48 (s, 1H), 8.38 (s, 1H), 7.89 (d, J = 7.9 Hz, 1H), 7.53 (t, J = 7.9 Hz, 1H), 7.36 (s, 1H), 7.21 (d, J = 8.2 Hz, 1H), 7.13 - 7.06 (m, 2H), 6.36 (s, 2H), 3.85 (s, 2H), 3.41 (s, 2H), 2.61 (t, J = 6.7 Hz, 2H), 1.76 - 1.67 (t, J = 6.8 Hz, 2H). HRMS (m/z): [M+H]+ calcd for C₂₀H₂₀Cl₂N₅O, 416.1045; found 416.1059.

Compound 25 was provided by GSK

Preparation of 2-((3-(3,5-dichlorobenzyl)amino)propyl)amino)quinazolin-4(3H)-one hydrochloride (compound 26):

Preparation of intermediate 2-(3,3-diethoxypropylamino)-3H-quinazolin-4-one:
A mixture of 2-methylsulfanyl-3H-quinazolin-4-one, prepared according to Moreno et al.\textsuperscript{20} (10.0 g, 50.1 mmol) and 3,3-diethoxypropan-1-amine (38.3 g, 260.1 mmol) were heated at 110°C for 24 h. The reaction was then concentrated in vacuo to give a black residual oil which was azeotroped with EtOAc three times until a crude solid was formed. The solid was triturated from EtOAc and collected by filtration to give a grey/white powder which was recrystallised from methanol to give the title compound (13.0 g, 42.3 mmol, 81%) as a white powder.

\textbf{Preparation of intermediate 3-[(4-oxo-3H-quinazolin-2-yl)amino]propanal:}

\[
\begin{align*}
\text{A solution of 2-(3,3-diethoxypropylamino)-3H-quinazolin-4-one (5.0 g, 15.4 mmol) in dioxane (100 mL) was treated with 2 M hydrochloric acid (10 mL) and the reaction heated to 70°C for 4 h. The reaction was then concentrated in vacuo, diluted with EtOAc and azeotroped with EtOAc several times until a solid was obtained, which was triturated from diethylether, collected by filtration and dried to give the title compound (3.0 g, 12.5 mmol, 81%) as a grey/white powder which was used crude in subsequent reductive aminations.}
\end{align*}
\]

\textbf{Preparation of 2-[[3-[(3,5-dichlorobenzyl)amino]propyl]amino]quinazolin-4(3H)-one hydrochloride (compound 26):}

\[
\begin{align*}
\text{A solution of (3,5-dichlorophenyl)methanamine (202.6 mg, 1.15 mmol) and 3-[(4-oxo-3H-quinazolin-2-yl)amino]propanal (250.0 mg, 1.15 mmol) in DMF (4.0 mL) and acetic acid (1 mL) at room temperature was stirred for 30 min prior to addition of sodium cyanoborohydride (40.5 mg, 0.65 mmol). The reaction was heated in a sealed tube in a microwave at 80°C for 2 h. The reaction was then concentrated in vacuo to give a gum. This was then diluted with DCM (20 mL), washed with aqueous ammonia solution (20 mL), dried (MgSO}_4) and concentrated in vacuo to give a white powder which was purified by chromatography (SiO}_2, 100% EtOAc moving to 10% MeOH : EtOAc with 1% ammonia) to give the title compound (45.0 mg, 0.098 mmol, 9%) as a white powder. ^1H NMR (500 MHz, DMSO-d6): 8\textsuperscript{9.60} (m, 2H), 9.13 (s, 1H), 8.01 (d, J = 7.5 Hz, 1H), 7.79 (d, J = 7.5 Hz, 2H), 7.73 - 7.38 (m, 4H), 4.20 (s, 2H), 3.73 (s, 2H), 3.11 (s, 2H), 2.03 (s, 2H). HRMS (m/z): [M+H]^+ calcd for C\textsubscript{18}H\textsubscript{19}Cl\textsubscript{2}N\textsubscript{4}O, 377.0943; found 377.0930.}
\end{align*}
\]
Preparation of \( N-(3,5\text{-dichlorobenzyl})-3-\{4\text{-oxo-1,4\text{-dihydroquinazolin-2-yl}}\text{amino}\}\text{propanamide (compound 27)}:\)

\[
\begin{array}{c}
\text{O} & \text{N} & \text{H} & \text{O} \\
\downarrow & & & \\
\text{N} & \text{H} & \text{N} & \text{O} \\
\end{array}
\quad \quad
\begin{array}{c}
\text{O} & \text{N} & \text{H} & \text{O} \\
\downarrow & & & \\
\text{N} & \text{H} & \text{N} & \text{O} \\
\end{array}
\quad \quad
\begin{array}{c}
\text{O} & \text{N} & \text{H} & \text{N} & \text{Cl} \\
\downarrow & & & & \\
\text{N} & \text{H} & \text{N} & \text{O} & \text{Cl} \\
\end{array}
\]

Preparation of intermediate \( 3-\{4\text{-oxo-1H-quinoxaline-2-yl} \text{amino}\}\text{propanoic acid:} \)

To a solution of \( 3-\{4\text{-oxo-1H-quinoxaline-2-yl} \text{amino}\}\text{propanal} \) (500 mg, 2.30 mmol) in Acetone (5 mL)/water (2 mL), was added KMnO\(_4\) (728 mg, 4.60 mmol) in one portion. The reaction mixture was stirred at room temperature for 45 min and then the reaction mixture was treated with solid NaHSO\(_3\) to give a thick brown sludge. This was filtered through a pad of celite eluting with acetone and water to give a pale yellow solution. The solvent was removed \textit{in vacuo} to give an off-white solid. LC/MS analysis of the solid indicated presence of some of the aldehyde. The solid was triturated with a 1:1 mixture of EtOAc:MeOH to remove the unreacted aldehyde to give \( 3-\{4\text{-oxo-1H-quinoxaline-2-yl} \text{amino}\}\text{propanoic acid} \) (505 mg, 1.73 mmol, 75 \%) as an off-white solid.

Preparation of \( N-(3,5\text{-dichlorobenzyl})-3-\{4\text{-oxo-1,4\text{-dihydroquinazolin-2-yl}}\text{amino}\}\text{propanamide (compound 27)}:\)

To a solution of \( 3-\{4\text{-oxo-1H-quinoxaline-2-yl} \text{amino}\}\text{propanoic acid} \) (250 mg, 0.86 mmol) and diisopropylethylamine (222 mg, 1.72 mmol) in DMF (5 mL), were added (3,5-dichlorophenyl)methanamine (181 mg, 1.03 mmol) and TBTU (413 mg, 1.29 mmol) and the reaction stirred at room temperature overnight. LC/MS analysis showed the mass of the desired product. The reaction mixture was diluted with EtOAc and water and partitioned. The aqueous layer was further extracted with EtOAc and the combined organics were washed with brine, dried over MgSO\(_4\) and concentrated. The crude residue was dry-loaded onto silica and purified by column chromatography (SiO\(_2\), 100\% EtOAc moving to 10\% MeOH : EtOAc). The fractions containing product were combined, however impurities were still present. The residue was triturated with DCM and the resulting yellowish solid was collected by filtration to give the title compound (26 mg, 0.063 mmol, 7 \%) as a pale yellow solid.
\[^1^\text{H}-\text{NMR}\ (500\ \text{MHz},\ \text{DMSO-d6}):\ \delta\ 10.85\ (s,\ 1\text{H}),\ 8.52\ (d,\ J = 5.6,\ 5.6\ \text{Hz},\ 1\text{H}),\ 7.88\ (d,\ J = 7.9\ \text{Hz},\ 1\text{H}),\ 7.57\ (d,\ J = 7.6,\ 7.6\ \text{Hz},\ 1\text{H}),\ 7.47\ (s,\ 1\text{H}),\ 7.30,\ (s,\ 2\text{H}),\ 7.26\ (d,\ J = 7.9\ \text{Hz},\ 1\text{H}),\ 7.10\ (d,\ J = 7.6,\ 7.6\ \text{Hz},\ 1\text{H}),\ 6.38\ (d,\ J = 5.6,\ 5.6\ \text{Hz},\ 1\text{H}),\ 4.31\ (d,\ J = 6.0\ \text{Hz},\ 2\text{H}),\ 3.59\ (q,\ J = 6.0\ \text{Hz},\ 2\text{H}).\ HRMS\ (m/z):\ [M+H]^+\ \text{calcd for}\ \text{C}_{18}\text{H}_{16}\text{Cl}_2\text{N}_4\text{O}_2,\ 391.0723;\ \text{found}\ 391.0736.\]

\[\]

**Preparation of 3,5-dichloro-N-(3-((4-oxo-1,4-dihydroquinazolin-2-yl)amino)propyl)benzenesulfonamide (compound 28):**

\[\]

To a suspension of 2-(3-aminopropylamino)-1H-quinazolin-4-one (100 mg, 0.46 mmol) and DIPEA (118 mg, 0.92 mmol) in DMF (1 mL) a solution of the 3,5-dichlorobenzenesulfonyl chloride (112 mg, 0.46 mmol) in DMF (1 mL) was added and the reaction was stirred at room temperature for 1 h. LC/MS indicated complete consumption of SM. The reaction mixture was diluted with DCM and quenched with 1 N NaOH solution. The reaction mixture was then neutralised with 2 N HCl, on neutralising precipitate formation was noted. The precipitate was collected by filtration and dried in vacuo to give the title compound (43 mg, 0.096 mmol, 21 %) as an off-white solid.

\[^1^\text{H}-\text{NMR}\ (400\ \text{MHz},\ \text{DMSO-d6})\ 8.05-8.02\ (m,\ 1\text{H}),\ 7.96-7.94\ (m,\ 2\text{H}),\ 7.78\ (s,\ 2\text{H})\ 7.73-7.66\ (m,\ 1\text{H}),\ 7.49-7.37\ (m,\ 1\text{H}),\ 7.31-7.21\ (m,\ 1\text{H}),\ 3.41\ (br\ s,\ 2\text{H}),\ 2.94-2.89\ (m,\ 2\text{H}),\ 1.74-1.71\ (m,\ 2\text{H}).\ HRMS\ (m/z):\ [M+H]^+\ \text{calcd for}\ \text{C}_{17}\text{H}_{16}\text{Cl}_2\text{N}_4\text{O}_3\text{S},\ 427.0393;\ \text{found}\ 427.0396.\]

\[\]

**Preparation of 2-((5-(3,5-dichlorophenyl)pentyl)amino)quinazolin-4(1H)-one (compound 29):**

\[\]

**Preparation of intermediate 5-(3,5-dichlorophenyl)pentan-1-amine:**

A solution of tert-butyl N-pent-4-enylcarbamate (1.0 g, 5.4 mmol) in THF (10 mL) at room temperature was treated dropwise with 9-BBN (0.5 M in THF) (10.9 mL, 5.94 mmol). The solution
was then sealed in a microwave tube and heated to 90°C for 1 h. The solution was then concentrated to one half its original volume then transferred into a solution containing 1-bromo-3,5-dichloro-benzene (1.22 g, 5.4 mmol), Pd(PPh3)4 (156 mg, 0.14 mmol) and potassium phosphate (1.72 g, 8.1 mmol) in DMF (10 mL) and the resulting mixture was heated with stirring in a MW to 100°C for 1 h. The reaction was then concentrated in vacuo, the resulting gum diluted with DCM (50 mL), washed with brine (10 mL), the organic phase separated then dried (MgSO4), treated at room temperature with 1 mL of TFA and stirred overnight. The solution was then concentrated in vacuo, redissolved in DCM (20 mL), treated dropwise with 7 N ammonia in methanol (5 mL) and concentrated. The residual gum was chromatographed (SiO2, 100% EtOAc moving to 5% MeOH:EtOAc) to give the title compound (760.0 mg, 3.24 mmol, 60%) as a white powder.

Preparation of 2-((5-(3,5-dichlorophenyl)pentyl)amino)quinazolin-4(1H)-one (compound 29):

A solution of 2-methylsulfanyl-3H-quinazolin-4-one (176.0 mg, 0.92 mmol) and 5-(3,5-dichlorophenyl)pentan-1-amine (212.5 mg, 0.92 mmol) in Ethanol (4.0 mL) was heated in the microwave at 150°C for 5 h. The reaction was allowed to cool to room temperature then diluted with methanol (15 mL) and the resulting precipitate removed by filtration, washed with ice-cold MeOH then dried in vacuo to give the title compound (176.0 mg, 0.46 mmol, 50%) as an off-white powder. 1H NMR (500 MHz, DMSO-d6): δ10.77 (s, 1H), 7.87 (d, J = 7.8 Hz, 1H), 7.56 (t, J = 7.0 Hz, 1H), 7.39 (s, 1H), 7.29 (s, 2H), 7.23 (d, J = 8.1 Hz, 1H), 7.09 (t, J = 7.4 Hz, 1H), 6.20 (s, 1H), 3.51 (m, 2H), 2.61 (t, J = 7.5 Hz, 2H), 1.65 - 1.55 (m, 4H), 1.37 - 1.32 (m, 2H). HRMS (m/z): [M+H]+ calcd for C19H20Cl2N3O, 376.0983; found 376.0975.

Preparation of 2-[3-[(4,6-dichloro-1H-indol-2-yl)methyl-methyl-amino]propylamino]-1H-quinazolin-4-one (compound 30):

Preparation of intermediate 1-(4,6-dichloro-1H-indol-2-yl)-N-methyl-methanamine:
To a solution of 4,6-dichloro-1H-indole-2-carbaldehyde (200 mg, 0.54 mmol) in Ethanol (1 mL) methanamine (101 mg, 1.07 mmol) was added and the reaction was heated to 60 °C overnight. The solvent was removed under reduced pressure and 1H NMR of the intermediate confirmed the formation of the imine. The intermediate was redissolved in Ethanol (1 mL) and sodium borohydride (40 mg, 1.07 mmol) was added in one portion and the reaction was monitored by TLC and LC/MS. After stirring at RT for 3 h, complete consumption of starting material was noted and the reaction mixture was partitioned between 2N NaOH (10 mL) and EtOAc (20 mL), a precipitate formed at this point which was filtered off. LC/MS and NMR analysis of the precipitate showed it not to be product. The aqueous layer was further extracted with EtOAc (2 x 20 mL) and the combined organics were washed with brine, dried over MgSO4 and concentrated. 1H NMR of the crude reaction mixture showed it to be composed mostly of desired product. The residue was dry-loaded onto silica and purified by column chromatography (SiO2, 100% EtOAc moving to 10% 7 N methanolic ammonia in EtOAc) to give the title compound as a yellow oil.

Preparation of 2-[3-[(4,6-dichloro-1H-indol-2-yl)methyl-methyl-amino]propylamino]-1H-quinazolin-4-one (compound 30):

To 3-[(4-oxo-1H-quinazolin-2-yl)amino]propanal (142 mg, 0.65 mmol) a solution of 1-(4,6-dichloro-1H-indol-2-yl)-N-methyl-methanamine (100 mg, 0.39 mmol) in DMF (1 mL) / acetic acid (1 mL) was added and the reaction mixture was allowed to stir at 60 °C for 2 hours. The reaction mixture was allowed to cool to RT and sodium triacetoxyborohydride (231 mg, 1.09 mmol) was added in one portion and then heated again at 60 °C overnight. The main peak in the LC/MS of the crude reaction mixture corresponded to the mass of the desired product. The reaction mixture was allowed to cool to RT and it was partitioned between 2N NaOH (10 mL) and EtOAc (20 mL). The aqueous layer was further extracted with EtOAc (2 x 20 mL) and the combined organics were washed with brine (20 mL) and dried over MgSO4 and concentrated to give a brown solid. The residue was dry-loaded onto silica and purified by column chromatography (SiO2, 100% EtOAc moving 10% MeOH in EtOAc). The fractions containing the desired product were combined, however impurities were still present. The orange oil was triturated with a 1:1 mixture of heptane:Et2O and the resulting solid was collected by filtration to give the title compound (82mg, 0.18 mmol, 41%) as a pale orange solid. 1H NMR (500 MHz, DMSO-d6): 11.49 (s, 1H), 10.92 (s, 1H), 7.88 (dd, J = 7.9 Hz, 1.5 Hz, 1H), 7.57 - 7.53 (m, 1H), 7.35 (s, 1H), 7.22 (d, J = 8.2 Hz, 1H), 7.11 (d, J = 1.5 Hz, 1H), 6.36 (s, 1H), 6.30 (s, 1H), 3.67 (s, 2H), 3.40-
3.36 (m, 2H), 2.45 (dd, J = 6.9 Hz, 6.9 Hz, 2H), 2.20 (s, 3H), 1.80 - 1.73 (m, 2H), 1.28 - 1.23 (m, 2H); HRMS (m/z): [M+H]^+ calcd for C_{21}H_{22}Cl_2N_5O, 430.1196; found 430.1199.

**Preparation of 2-(3-(((4,6-dichloro-1H-indol-2-yl)methyl)amino)propoxy)quinazolin-4(1H)-one (compound 31):**

![Chemical Structure of Compound 31]

**Preparation of intermediate tert-butyl N-[3-[[4-oxo-1H-quinazolin-2-yl]oxy]propyl]carbamate:**

A mixture of 2-hydroxy-3H-quinazolin-4-one (408 mg, 2.52 mmol), tert-butyl N-(3-bromopropyl)carbamate (500 mg, 2.10 mmol) and sodium carbonate (445 mg, 4.20 mmol) was suspended in DMF (5 mL). The reaction mixture was heated 60 °C overnight. TLC showed a new spot which ran higher than the starting material, indicating formation of product. The reaction mixture was cooled to room temperature and partitioned between water (20 mL) and EtOAc (40 mL). The aqueous layer was further extracted with EtOAc (2 × 30 mL) and the combined organics were washed with brine (40 mL), dried over MgSO₄ and concentrated **in vacuo**. The colourless residue was purified by column chromatography (SiO₂, 0-100% gradient of EtOAc in heptane). Fractions containing the higher running spot were combined and the solvent removed to give a colourless foam which consisted mainly of the desired product, however impurities were still present. The colourless foam was triturated with Et₂O to give tert-butyl N-[3-[[4-oxo-1H-quinazolin-2-yl]oxy]propyl]carbamate (170 mg, 0.51 mmol, 24%) as a colourless solid.

**Preparation of intermediate 2-(3-aminoproxy)-1H-quinazolin-4-one hydrochloride:**

![Chemical Structure of Compound 31 Hydrochloride]

To a suspension of tert-butyl N-[3-[[4-oxo-1H-quinazolin-2-yl]oxy]propyl]carbamate (110 mg, 0.34 mmol) in 1,4-dioxane (1 mL), was added hydrochloric acid (4 M in 1,4-dioxane) (0.69 mmol) dropwise and the reaction mixture was stirred at room temperature overnight. LC/MS seemed to indicate complete deprotection. The solvent was removed under reduced pressure to give 2-(3-aminoproxy)-1H-quinazolin-4-one hydrochloride (89 mg, 0.33 mmol, 91%) a colourless foam.
Preparation of 2-(3-(((4,6-dichloro-1H-indol-2-yl)methyl)amino)propoxy)quinazolin-4(1H)-one (compound 31):

To a suspension of 2-(3-aminopropoxy)-1H-quinazolin-4-one hydrochloride (85 mg, 0.33 mmol) in DMF (0.5 mL)/acetic acid (0.5 mL) was added sodium triacetoxyborohydride (140 mg, 0.66 mmol) and the reaction was stirred at room temperature overnight. LC/MS showed formation of some product, however reaction very messy. The reaction mixture was partitioned between 2N NaOH (10 mL) and EtOAc (20 mL). The aqueous layer was further extracted with EtOAc (2 × 20 mL) and the combined organics were washed with brine (20 mL), dried over MgSO₄ and concentrated in vacuo.

The crude residue was dry-loaded onto silica and purified by column chromatography (SiO₂ 0-100% gradient of 10% MeOH/EtOAc in heptane) to give fractions containing the desired product, however impurities still present. All the fractions containing product were combined and the solvent removed in vacuo. The resulting orange residue was triturated with DCM to give the title compound (12 mg, 0.026 mmol, 8%) as an orange solid. ¹H-NMR (500 MHz, DMSO-d6) 11.43 (br s, 1H), 7.93 (dd, J = 8.0, 1.3 Hz, 1H), 7.67-7.64 (m, 1H), 7.36 (s, 1H), 7.22-7.17 (m, 2H), 7.09 (d, J = 1.65 Hz, 1H), 6.32 (s, 1H), 3.97 (t, J = 7.2 Hz, 2H), 3.84 (s, 2H), 2.56 (t, J = 7.0 Hz, 2H), 1.80-1.76 (m, 2H). HRMS (m/z): [M+H]+ calcd for C₂₀H₁₈Cl₂N₄O₂, 417.0880; found 417.0886. 
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