SUPPLEMENTAL MATERIALS

Standard Operation Procedure (SOP) for *Trypanosoma brucei* Trypanothione Synthetase High-Throughput Screening

Description

The assay is designed in a 384 well plate to screen 4,210 compounds from *LOPAC® (SIGMA), Selleck Chemicals, NIH Clinical* and *TOCRIS* libraries (pilot screening) and then 47,414 small molecules from *ENAMINE* library, against the enzyme trypanothione synthetase from *Trypanosoma brucei* (*Tb*TryS). *Tb*TryS uses glutathione, spermidine and ATP as substrates. ATP is hydrolyzed to produce inorganic phosphate and this is quantified using a commercial version of malachite green called BIOMOL GREEN^{TM} reagent.

All the steps were performed by the ADS (Assay Development and Screening group), expect for those specifically pointed, which were performed by the LRL (Leishmania Research Laboratory) or the ALM (Automation and Logistics Management group).

Purpose

Find inhibitors of *Tb*TryS.

Equipment and consumables required

- **Equipment**
  - Personal Pipettor liquid handler (Apricot Designs)
  - VCode (Agilent Technologies. Room - R5.29)
  - Matrix Wellmate Microplate Dispenser (Thermo Fisher Scientific)
  - Multidrop™ Combi Reagent Dispenser (Thermo Fisher Scientific)
  - 8ch. Small-bore Disposable cartridge (tube) for Wellmate (Thermo Fisher Scientific, 201-30002)
  - Robotic arm Plate Handler II (Perkin Elmer)
  - Envision multilabel reader (Perkin Elmer)
  - Centrifuge (Eppendorf)
  - Pipette 100p, 200p, and 1000p (Gilson)
  - Pipette aid
- **Consumables**
  - 50 mL conical tubes (Falcon 352070)

- 384 well plate (Greiner 781101)
- Finntip 50 (Thermo Fisher Scientific 9400360)
- 5 mL serological pipette (Falcon 357543)
- 10 mL serological pipette (Falcon 357551)
- 25 mL serological pipette (Falcon 357525)
- 250 mL storage bottle (Corning CC-430281)
- 500 mL storage bottle (Corning CC-430282)
- 1 L storage bottle (Corning CC-430518)
- Reservoir (Corning CT-4870)
- Bioharzard autoclave bag

Chemicals and Solutions

- Spermidine (Sigma 85558)
- ATP (Sigma A2383)
- Glutathione (Sigma G4251)
- DTT (APPLICHEM A1101)
- BIOMOL GREEN^{TM} reagent (Enzo Life Science BML-AK111)
- DMSO (Sigma 472301)
- EDTA (Sigma E5134)
- NaCl (Sigma 71376)
- HEPES (Sigma H3375)
- $MgSO_4$ (Sigma 230391)
- Reaction buffer: 100 mM HEPES, 0.5 mM EDTA, 10 mM $MgSO_4$, pH 7.4
- Enzyme stock: *Tb*TryS enzyme in reaction buffer 1 mM DTT and 150 mM NaCl

Preparation of Materials

1. Stock reagent solution (LRL)

Reaction buffer and enzyme (dilution prepared from enzyme stock in reaction buffer)

2. Plate barcoding (ALM)

The Barcode of plates are labelled by the VCode

3. Controls stock solution

Prepare 20% DMSO in 3’ distilled water (2 mL of DMSO + 8 mL of 3’ distilled water)

Assay process

1. Wellmate preparation

- Connect the Wellmate dispense cartridge into Wellmate machine
- Turn on the Wellmate (Switch is backside of the machine)
- Control the speed of the Wellmate dispense (speed 2)
- Wash the tubing with 70% EtOH
- Wash the tubing with distilled water

2. Enzyme preparation (LRL)

3. Master mix (MM) preparation (LRL)

4. Compound plate preparation with the Personal Pipettor liquid handler (ALM)

- 96 well Mother Plate (MPL): 100 μL compounds at 10 mM (DMSO 100%)
- 384 well Intermediate Plate (IPL): dilution 1:5 (20 μL + 80 μL DMSO) are performed to obtain a 2 mM solution (DMSO 100%)
- 384 Daughter Plate (DPL): dilution 1:5 (20 μL + 80 μL 3' distilled water) in 384 Daughter Plate (DPL) to obtain a 0.4 mM (20% DMSO-80% distilled water)

Note: Column 1 and 12 in the 96 well plate and 1, 2, 23 and 24 in 384 well plate are used for controls

5. Compound/ DMSO transfer by Personal Pipettor liquid handler (Apricot Designs) (ALM)

Dispense 1 μL of compound or DMSO from the DPL to the assay plate

6. Dispense reaction buffer ("inhibition control")

- Prepare Wellmate equipment and reaction buffer as above
- Prime the cassette tubing with reaction buffer
- Dispense 10 μL (final volume 11 μL) of enzyme buffer (not the enzyme) into columns **23 and 24** by Wellmate

7. Dispense enzyme

- Prepare Wellmate equipment an enzyme as above
- Prime the cassette tubing with enzyme
- Dispense 10 μL of the prepared enzyme dilution to columns **1 to 22** in the plates by Wellmate
- Speed down 200*g*, 1 s

8. Incubation for 1 h at room temperature (RT)

9. Dispense MM

- Prepare Wellmate equipment and MM as above
- Prime the cassette tubing with MM

- Dispense 5 µL (final volume 16 µL) of the prepared MM solution in the whole plate by Wellmate
- Speed down 200*g*, 1 s

10. Incubation for 1 h at RT

11. Dispense EDTA 210 mM

- Prepare Wellmate equipment and EDTA as above
- Prime the cassette tubing with EDTA
- Dispense 5 µL (final volume 21 µL) of the EDTA 210 mM to stop the reaction by Wellmate
- Speed down 200*g*, 1 s

12. Transfer plate to the tower in Cell explore (room - R5.11)

- Put the plates to stacker (hotel, check the position, number and turn)

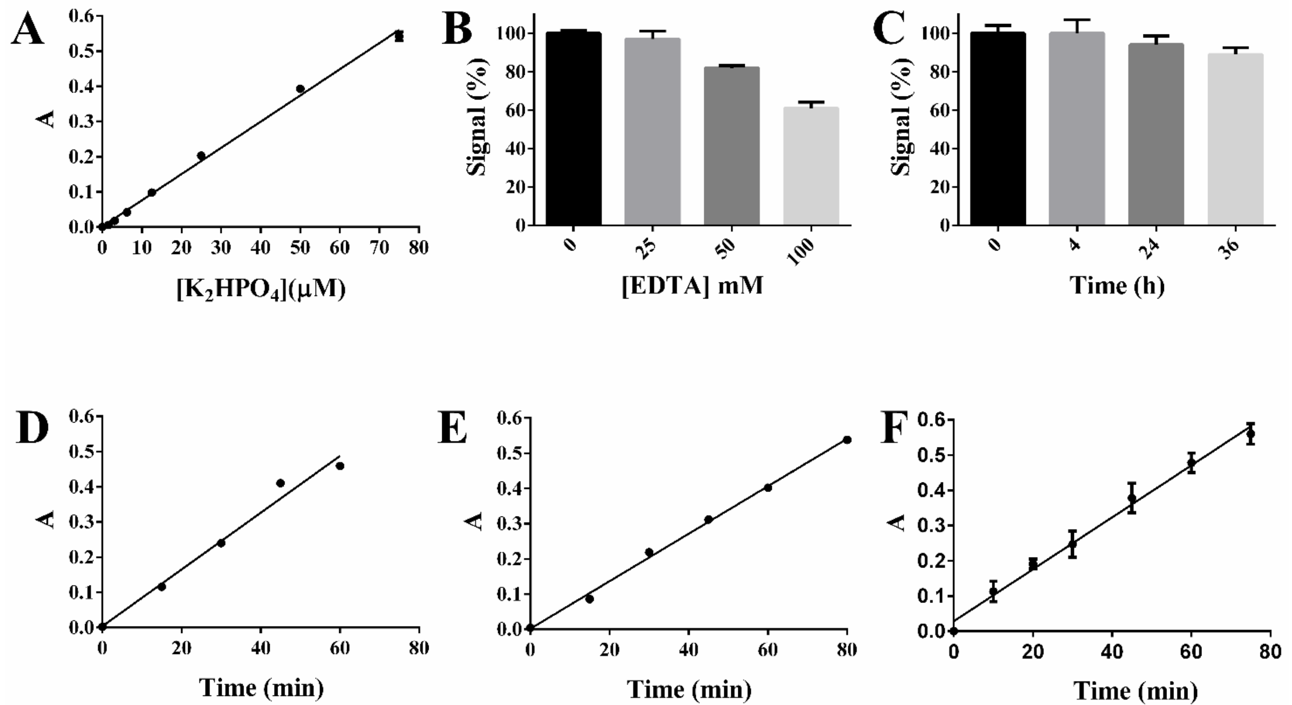
13. Preparation Cell explore (by ALM)

- Prepare Combi equipment and BIOMOL GREEN™ reagent as above
- Prime the cassette tubing with BIOMOL GREEN™ reagent
- Open the Cell explore control program and load protocol
- Start program by ALM
- Dispense 60 µL of BIOMOL GREEN™ reagent (final volume 81 µl) to all the wells in the whole plate by Combi by automatic
- Incubation 20 min in RT by automatic
- Reading plate with ENVISION by automatic
- After reading plate put the stacker (hotel) by automatic

14. Collect plate and seal plate

- When the reading is done, remove the plates carefully
- Put the plate back in the carrier, and transport to out of room - R5.11

Figure S1.



TryS assay performance. A) $A_{620\ nm}$ *vs.* K_2HPO_4 concentration µM. Under reaction conditions resembling the screening assay for *Leishmania infantum* trypanothione synthetase (*Li*TryS) and using KH_2PO_4 as inorganic phosphate source, the detection limit, slope and linearity range of the colorimetric reaction with BIOMOL GREEN^{TM} was estimated to be ~ 0.9 µM, 0.0074 ± 0.0002 UA/µM and 1.6-75 µM, respectively. **B) Signal (%) *vs.* EDTA concentration mM.** EDTA was added at 0, 25, 50 and 100 mM to a solution containing at 50 µM KH_2PO_4. Although it decreases ~18% assay sensitivity, 50 mM EDTA was selected to stop the TryS reaction via Mg^{++} chelation. **C) Signal (%) *vs.* Time (h).** Signal stability (50 µM KH_2PO_4 + BIOMOL GREEN^{TM} + 50 mM EDTA) was evaluated over time. After 36 h, signal drop down only ~ 11%. For the three experiments outlined above, KH_2PO_4 was dissolved in *Li*TryS master mix. Although the GSH concentration using in the master mix varies for each enzyme, in the range used, this reagent does not affect signal development (Benítez, 2016). **$A_{620\ nm}$ *vs.* Time (h) for D)** *Trypanosoma cruzi* TryS, **E)** *Li*TryS and **F)** *T. brucei* TryS. The development of signal (A_{620nm}) was determined at different time points for each TryS. All assays displayed a linear relation for signal *vs.* time at least up to 60 min.

Table S1: Inhibition of Tri-Tryp trypanothione synthetase (TryS).					
			TryS inhibition ± SD at 25 µM (%) ***or IC₅₀ (± SD) (µM)***		
#	**Compounds**	**Core**	***TcTryS***	***LiTryS***	***TbTryS***
1	Aurintricarboxylic acid	Singleton	5.5 ± 2.8	***4.6 ± 0.7***	***8.2 ± 0.6***
2	4-Chloromercuribenzoic acid	Singleton	32.0 ± 9.7	1.6 ± 14.1	***2.2 ± 0.05***
3	Morin (Flavonol)	Singleton	NA	NA	NA
4	6-Hydroxy-DL-DOPA	Singleton	***16.8 ± 2.8***	***4.0 ± 1.4***	***13.0 ± 3.5***
5	NH125	Singleton	***15.5 ± 2.9***	***25.7 ± 2.6***	***12 ± 2.5***
6	Ebselen	Singleton	***13.8 ± 1.5***	***2.6 ± 0.2***	***5.3 ± 0.2***
7	Sanguinarine chloride	Singleton	***40.0 ± 1.2***	***11.6 ± 0.4***	***3.3 ± 0.4***
8	Calmidazolium chloride	Singleton	***2.7 ± 0.5***	***1.6 ± 0.8***	***9.5 ± 2.5***
9	Piceatannol	Singleton	NA	NA	NA
10	SCH 202676 hydrobromide	Singleton	43.4 ± 5.7	***3.0 ± 0.8***	***3.7 ± 0.5***
11	SAM002548975 (Raloxifene)	Singleton	24.5 ± 7.0	41.3 ± 4.9	NA
12	PD 404,182	Singleton	10.9 ± 5.6	8.9 ± 5.9	***15.3 ± 1.1***
13	Demethylasterriquinone B1	Singleton	27.7 ± 7.6	69.3 ± 3.5	31.0 ± 7.0
14	CGP 71683 hydrochloride	Singleton	34.7 ± 7.6	***13.9 ± 1.8***	14.4 ± 8.2
15	Carvedilol	Singleton	NA	15.2 ± 12.7	23.7 ± 6.4
16	Tin protoporphyrin IX dichloride	Singleton	18.7 ± 10.4	5.1 ± 7.4	13.8 ± 11.2
17	PQ 401	Singleton	6.2 ± 5.6	NA	***1.5 ± 0.4***
18	Tetrindole mesylate	Singleton	28.9 ± 7.5	NA	26.0 ± 4.6
19	WIN 64338 hydrochloride	Singleton	25.9 ± 6.5	70.9 ± 6.8	9.2 ± 7.4
20	Nutlin-3	Singleton	NA	NA	39.8 ± 5.2
21	Z102601424	CPA	NA	12.6 ± 15.1	NA
22	Z1081732798	CP	6.2 ± 0.9	10.4 ± 8.9	38.1 ± 7.2
23	Z109494586	CPz	7.1 ± 4.0	16.8 ± 17.9	***8.0 ± 1.8***
24	Z113169536	CPz	NA	NA	NA
25	Z1188133056	Singleton	NA	NA	***~ 25***
26	Z1223157898	Singleton	NA	NA	40.0 ± 5.8
27	Z1291366164	Singleton	NA	NA	24.3 ± 2.4
28	Z13598484	Singleton	NA	NA	NA
29	Z13601063	Singleton	NA	NA	***28.0 ± 4.5***
30	Z145119580	AMPh	NA	NA	15.5 ± 9.4
31	Z145386962	AMTPh	NA	NA	NA
32	Z147103388	Singleton	NA	NA	***1.2 ± 0.2***
33	Z1547375509	Singleton	NA	28.5 ± 41.1	***15.2 ± 3.7***
34	Z165123846	AMPh	NA	27.7 ± 28.1	NA
35	Z167605396	APh	NA	NA	NA
36	Z19222119	Singleton	NA	NA	NA
37	Z199816662	Singleton	NA	NA	NA
38	Z212978220	Singleton	NA	NA	40.4 ± 5.8
39	Z21459859	Singleton	13.3 ± 3.3	NA	***36.2 ± 2.6***
40	Z220425080	Singleton	NA	NA	NA
41	Z223988664	APh	NA	NA	10.3 ± 6.6
42	Z224029074	APh	NA	NA	NA
43	Z225233562	AMTPh	NA	NA	***20.3 ± 4.6***
44	Z225677598	CPA	5.5 ± 0.8	NA	37.1 ± 4.7
45	Z227072034	AMTPh	NA	NA	***8.4 ± 0.9***
46	Z227978766	Singleton	12.9 ± 6.1	74.4 ± 18.1	NA
47	Z24194344	CPh	NA	NA	NA
48	Z244772346	AMPh	NA	NA	***28.2 ± 1.3***
49	Z27611228	CPz	NA	NA	7.2 ± 3.0
50	Z280049026	Singleton	NA	NA	NA
51	Z29053217	A2T	NA	NA	NA
52	Z29458018	A2T	NA	13.3 ± 10.5	NA
53	Z300598652	Singleton	NA	NA	NA
54	Z318180488	AMPh	NA	NA	***13.1 ± 0.7***
55	Z32477432	CP/APh	NA	NA	NA
56	Z325706758	CPh	NA	NA	34.1 ± 6.0
57	Z33583129	APh	NA	NA	NA
58	Z339431458	CPz	NA	NA	27.0 ± 28.4
59	Z339866610	CPA	NA	NA	***26.4 ± 0.9***
60	Z339869040	AMPh	NA	NA	27.5 ± 9.5
61	Z356216222	Singleton	NA	NA	NA
62	Z357307658	Singleton	NA	NA	18.6 ± 5.6
63	Z363062290	CPA	8.8 ± 7.3	NA	***18.4 ± 2.5***

64	Z367586420	CPA	NA	NA	12.7 ± 9.8
65	Z423190460	Singleton	NA	NA	NA
66	Z423365454	CP	NA	NA	NA
67	Z431613218	CP	NA	NA	31.8 ± 2.1
68	Z436803656	AMPh	NA	NA	29.0 ± 5.1
69	Z44454993	Singleton	NA	NA	NA
70	Z46336887	AMPh	NA	NA	NA
71	Z51971326	AMTPh	NA	NA	***19.9 ± 2.7***
72	Z55659431	Singleton	NA	NA	NA
73	Z572699208	CPA	NA	NA	***~ 25***
74	Z64547920	CPh	NA	NA	35.8 ± 3.9
75	Z650921460	AMPh	NA	NA	NA
76	Z666948054	Singleton	NA	NA	37.1 ± 13.9
77	Z804115708	Singleton	NA	NA	NA
78	Z89695286	Singleton	NA	19.7 ± 7.4	11.6 ± 4.6
79	Z95751789	AMPh	NA	NA	NA
80	Z95976280	CPA	NA	NA	***12.0 ± 2.6***
81	Z98408834	CP	NA	NA	33.7 ± 6.1
82	Z2241128018	Singleton	NA	43.1 ± 4.2	NA

The first 20 compounds belong to the pilot screening and the rest to the large (*ENAMINE* library) screening. Compounds inhibiting TryS by 45-55% at 25 µM an estimated value of ~25 µM is provided. When enzyme inhibition is ≤ 0.0 ± 5.0 % the activity is expressed as not active (NA). #, Number; IC_{50}, Half-maximum inhibitory concentration; SD, Standard deviation; *Tc*, *Trypanosoma cruzi*; *Li*, *Leishmania infantum*; *Tb*, *Trypanosoma brucei*. Compounds core structure abbreviations: CPA; Carboxy piperidine amide, AMTPh; Amide methylene thiazole phenyl, AMPh; Amide methylene phenyl, APh; Amide phenyl, CP; Carboxy piperidine, CPz; Carboxy piperazine and CPh; Carboxy phenyl.
Structural information of molecules belonging to the *ENAMINE* library can be retrieved from the company website (https://www.enaminestore.com/search) using the corresponding compound code.

Equations for substrate inhibition
(Torrie, 2009)

Competitive high substrate inhibition

$$1/v = (K_M/X \times V_{max})\ (1 + i/K_i) + (X/K_i^x \times V_{max})\ (1 + i/K_i) + (1/\ V_{max})$$

Uncompetitive high substrate inhibition

$$1/v = (K_M/X \times V_{max}) + (X/K_i^x \times V_{max}) + (i/K_i \times V_{max}) + (1/\ V_{max})$$

Allosteric high substrate inhibition

$$1/v = (K_M/X \times V_{max}) + (X/K_i^x \times V_{max})\ (1 + i/K_i) + (1/\ V_{max})$$

All the equations obey the following general equation:
A/X (exponential term) + X/B (lineal term) + C

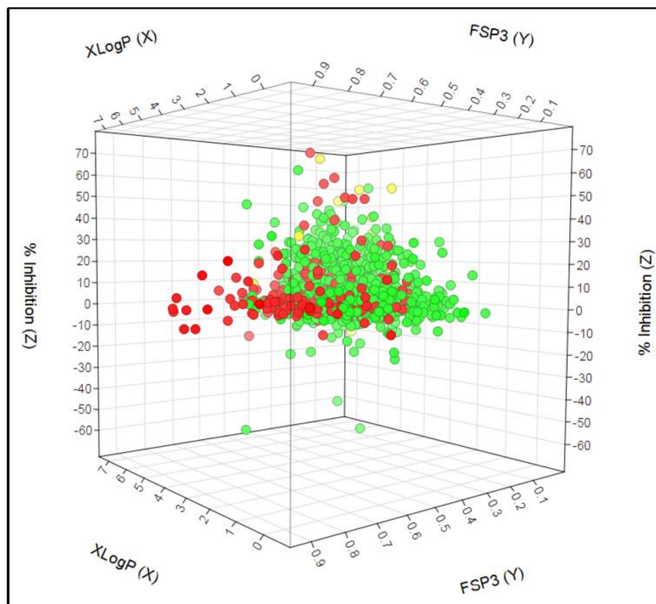
Because of the high substrate inhibition exhibited by *Tb*TryS with GSH and because it was not possible to decrease the concentration of this substrate below 75 μM, the equations used to analyze data from varying GSH concentrations were simplified to the exponential term.

i, inhibitor concentration.

X, substrate concentration.

K_M, K_i, V_{max}, are constants.

Figure S2. Drug-likeness analysis.



Drug-likeness analysis of scaffold type *vs.* *Tb*TryS. *Tb*TryS inhibition (%) (Z axis) *vs.* drug-likeness metrics, FSP3 (fraction of sp3 hybrized or saturated carbons) (Y axis) and XLogP (lipophilicity, based on atoms contributions) (X axis) values. Scaffolds are showed as dots: CPA in red, AMTPh scaffold in yellow and AMPh scaffold in green. The CPA compounds may have better drug-likeness properties than the other scaffolds. FSP3 score of these compounds was greater than 0.3 (suggesting more flexible molecules) and XLogP values were less than 5. Compounds core structure abbreviations: CPA; Carboxy piperidine amide, AMTPh; Amide methylene thiazole phenyl and AMPh; Amide methylene phenyl.