



HAL
open science

Identification of a kappa-opioid agonist as a potent and selective lead for drug development against human African trypanosomiasis

Deuan C. Jones, Irene Hallyburton, Laste Stojanovski, Kevin D. Read, Julie A. Frearson, Alan H. Fairlamb

► To cite this version:

Deuan C. Jones, Irene Hallyburton, Laste Stojanovski, Kevin D. Read, Julie A. Frearson, et al.. Identification of a kappa-opioid agonist as a potent and selective lead for drug development against human African trypanosomiasis. *Biochemical Pharmacology*, 2010, 80 (10), pp.1478. 10.1016/j.bcp.2010.07.038 . hal-00626235

HAL Id: hal-00626235

<https://hal.science/hal-00626235>

Submitted on 24 Sep 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Identification of a kappa-opioid agonist as a potent and selective lead for drug development against human African trypanosomiasis

Authors: Deuan C. Jones, Irene Hallyburton, Laste Stojanovski, Kevin D. Read, Julie A. Frearson, Alan H. Fairlamb

PII: S0006-2952(10)00576-9
DOI: doi:10.1016/j.bcp.2010.07.038
Reference: BCP 10671

To appear in: *BCP*

Received date: 24-5-2010
Revised date: 21-7-2010
Accepted date: 27-7-2010

Please cite this article as: Jones DC, Hallyburton I, Stojanovski L, Read KD, Frearson JA, Fairlamb AH, Identification of a kappa-opioid agonist as a potent and selective lead for drug development against human African trypanosomiasis, *Biochemical Pharmacology* (2010), doi:10.1016/j.bcp.2010.07.038

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



1
2 **Identification of a kappa-opioid agonist as a potent and selective lead for drug**
3 **development against human African trypanosomiasis**
4
5
6
7
8
9

10 Deuan C. Jones^a, Irene Hallyburton^a, Laste Stojanovski^a, Kevin D. Read^a, Julie A. Frearson^a
11 and Alan H. Fairlamb^{a*}
12
13
14
15
16

17 ^aDivision of Biological Chemistry & Drug Discovery, College of Life Sciences, University of
18 Dundee, Dundee, UK.
19
20
21
22
23
24

25 **Running title:** Phenotypic screening of *Trypanosoma brucei*
26
27
28
29
30
31
32
33
34
35
36
37
38
39

40 *Corresponding author.
41

42 Division of Biological Chemistry & Drug Discovery, College of Life Sciences, University of
43 Dundee, Dundee DD1 5EH, UK
44
45

46 Tel.: +44 1382 385155, Fax: +44 1382 385542, e-mail: a.h.fairlamb@dundee.ac.uk
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Abstract

1
2
3 A resazurin-based cell viability assay was developed for phenotypic screening of the LOPAC
4
5 1280 'library of pharmacologically active compounds' against bloodstream forms of *T. brucei*
6
7 *in vitro* identifying 33 compounds with EC₅₀ values < 1 μM. Counter-screening versus
8
9 normal diploid human fibroblasts (MRC5 cells) was used to rank these hits for selectivity,
10
11 with the most potent (< 70 nM) and selective (> 700-fold) compounds being suramin and
12
13 pentamidine. These are well known antitrypanosomal drugs which demonstrate the robustness
14
15 of the resazurin cell viability assay. The most selective novel inhibitor was (+)-trans-(1*R*,2*R*)-
16
17 U50,488 having an EC₅₀ value of 60 nM against *T. brucei* and 270-fold selectivity over
18
19 human fibroblasts. Interestingly, (-)-U50,488, a known CNS-active κ-opioid receptor agonist
20
21 and other structurally related compounds were > 70-fold less active or inactive, as were
22
23 several μ- and κ-opioid antagonists. Although (+)-U50,488 was well tolerated by the oral
24
25 route and displayed good pharmaceutical properties, including high brain penetration, the
26
27 compound was not curative in the mouse model of infection. Nonetheless, the divergence of
28
29 antinociceptive and antitrypanosomal activity represents a promising start point for further
30
31 exploratory chemistry. Bioinformatic studies did not reveal any obvious candidate opioid
32
33 receptors and the target of this cytostatic compound is unknown. Among the other potent, but
34
35 less selective screening hits were compound classes with activity against protein kinases,
36
37 topoisomerases, tubulin, as well as DNA and energy metabolism.
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52

53 **Keywords:** Phenotypic screening; African trypanosomiasis; Target identification; Target
54
55 validation; U50,488
56
57
58
59
60
61
62
63
64
65

1 Introduction

1
2
3 Human African trypanosomiasis (HAT) is a disease endemic to the sub-Saharan region of
4
5 Africa and is caused by two subspecies of the protozoan parasite *Trypanosoma brucei*. *T. b.*
6
7 *gambiense* is responsible for the chronic form of the disease found in western and central
8
9 Africa, accounting for over 90 % of reported cases of the disease, whereas *T. b. rhodesiense* is
10
11 responsible for the more acute form of the disease located in eastern Africa [1]. Only 10-15%
12
13 of the 60 million people at risk of the disease are under surveillance [1] and the death rate is
14
15 currently estimated at 30,000 per annum [2]. *T. b. brucei* and other *Trypanosoma spp* are
16
17 responsible for related veterinary diseases of economic importance, such as nagana in cattle.
18
19
20
21

22
23 Of the four drugs that are currently registered for use against HAT, pentamidine and
24
25 suramin are used against the early stage of the disease; and melarsoprol and eflornithine
26
27 (difluoromethylornithine, DFMO) are used against the late stage of the disease, when the
28
29 infection has spread to the central nervous system (CNS). These treatments are beset with
30
31 problems such as difficulties in administration (none are given orally), cost, duration of
32
33 treatment, toxicity and resistance [3]. Melarsoprol treatment is highly toxic and responsible
34
35 for iatrogenic deaths in 5% of patients. Eflornithine therapy is only effective against
36
37 *T.b.gambiense* infections and presents severe economic and logistical problems in resource
38
39 poor settings due to the need for 4 daily intravenous infusions over 14 days [4]. A recent
40
41 clinical trial suggests the duration and frequency of treatment can be reduced by combination
42
43 of eflornithine with nifurtimox [5], which may act as an interim solution until better and safer
44
45 drugs are developed. The recent failure of the first orally active first stage drug, pafuramidine
46
47 (DB289) [6], and rumours of increasing failures with eflornithine therapy underlines the
48
49 urgent need for novel therapeutics.
50
51
52
53
54
55

56
57 Whilst many dominant paradigms of drug discovery focus on screening against
58
59 molecular targets [7], there has been a resurgence of interest in phenotypic screening against
60
61
62
63
64
65

1 whole parasites [8]. Phenotypic screening, particularly when in combination with a
2 mammalian counter-screen, has the distinct advantage of addressing key druggability and
3 toxicity issues early in drug discovery, thereby reducing attrition at later stages in
4 development. In some cases phenotypic screening may identify novel molecular targets
5 thereby accelerating drug development. However, understanding the mode of action of
6 phenotypic screening hits can prove challenging since many drugs act by modulation of
7 multiple intracellular targets (“network pharmacology”) [9]. Hit identification for novel
8 targets is best approached with a screening library of diverse chemical space, but whole cell
9 assays typically have a much lower throughput than molecular-target-based screens. Hence,
10 we chose to screen a library of known pharmacologically-active compounds against *T. brucei*
11 cultured *in vitro* and to counterscreen actives against a human fibroblast cell line (MRC5
12 cells) to eliminate non-selective inhibitors. Potent and selective hits from such an approach
13 can present exploitable shortcuts, particularly if they have already been used in humans with
14 known dosing and toxicity information. Developing these hits could represent a low-risk, low-
15 cost strategy for tackling orphan diseases of the poor [7].

16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37 The Library of Pharmacologically Active Compounds from Sigma-Aldrich (LOPAC
38 1280; international version) contains 1268 compounds that are ligands for many enzymes,
39 receptors and ion channels in other organisms. Many are drug-like molecules and some are
40 CNS active – an important consideration when seeking a replacement therapy for late-stage
41 trypanosomiasis. Here we report over 30 compounds with EC₅₀ values against *T. brucei* less
42 than 1 μM, a concentration that should be readily achieved in plasma. Of these, one third have
43 >20-fold selectivity with the κ-opioid receptor agonist U50,488 showing the greatest potency
44 and selectivity. Some preliminary structure-activity relationships of CNS-active μ- and κ-
45 opioid receptor agonists and antagonists are reported.

2 Materials and Methods

2.1 Chemicals and materials

The LOPAC 1280 library (international version) was purchased from Sigma-Aldrich (Gillingham, UK). Pentamidine isethionate was obtained from Research Biochemicals International, eflornithine was a gift from Merrell Dow Research Institute (Ohio, USA) and melarsoprol a gift from Rhone-Poulenc (France). U69593, U5449A and naloxone were obtained from Alexxis Biochemicals (Nottingham, UK). Norbinaltorphimine, (-)-U50,488, (+)-U50,488, naltrexone, DIPPA (2-(3,4-dichlorophenyl)-*N*-methyl-*N*-[(1*S*)-1-(3-isothiocyanatophenyl)-2-(1-pyrrolidinyl)ethyl]acetamide hydrochloride) and 4-P-PDOT (cis-4-phenyl-2-propionamidotetralin) were obtained from Tocris Bioscience (Avonmouth, UK). DMSO was purchased from VWR international and HPLC-grade methanol and acetonitrile from Fluka. Resazurin, thioglycerol and PEG400 were obtained from Sigma-Aldrich (Gillingham, UK). Sterile 96-well plates were obtained from Greiner Bio-one (Stonehouse, UK).

2.2 Trypanosome culture

Bloodstream form *T. brucei brucei* cells (strain 427, 'single marker') were grown at 37°C and 5% CO₂ in a modified HMI9 medium [10] (HMI9-T where 0.2 mM 2-mercaptoethanol was replaced with 0.056 mM thioglycerol). Stock cultures were maintained in T75 vented cap culture flasks (Greiner, Stonehouse, UK) and sub-cultured every 2 to 3 days by 500-fold dilution into fresh medium. For microtitre plate assays, cells were counted using a Casy cell counter TT (Sharfe systems) and diluted appropriately.

2.3 Mammalian cell culture

1 Normal human MRC5 cells (diploid foetal lung fibroblasts) were used as a counter-screen for
2 non-selective inhibitors. Stabilates were obtained from the European Collection of Cell
3 Cultures (ECACC) were grown at 37°C and 5% CO₂ in a humidified incubator in Eagle's
4 Minimal Essential Medium (Sigma-Aldrich, Gillingham, UK) supplemented with 10% foetal
5 bovine serum (Invitrogen, Paisley, UK). Stock cultures were maintained in T75 vented cap
6 culture flasks with half the medium changed each day. Cells were split once confluent as
7 follows: medium was removed and the monolayer washed with PBS (137 mM NaCl, 2.7 mM
8 KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4). Cells were detached by incubation for 2 min
9 with 1 ml of trypsin EDTA solution (0.5 mg ml⁻¹ trypsin, 0.2 mg ml⁻¹ EDTA·4Na in Hanks
10 balanced salt solution supplied by Invitrogen, Paisley, UK). Detachment was confirmed by
11 microscopy. Fresh media was added to the cells to subculture into new flasks at twofold to
12 fourfold the original volume as required.

2.4 Linearity of assay

13 *T. brucei* bloodstream-form cells and human fibroblasts were pipetted into 96-well plates to
14 give a range of densities in a total volume of 200 µl culture medium. Resazurin (20 µl of a
15 500 µM stock; 45.5 µM final concentration) was added immediately to *T. brucei* cells or the
16 next day for human fibroblasts, following incubation overnight to allow attachment to the
17 plastic plates. Fluorescence was then measured at intervals up to 5 h (excitation of 528 nm
18 and emission of 590 nm) on a Biotec Instruments FLX 800 fluorescent plate reader.

2.5 DMSO-tolerance of the assay

19 DMSO was serially diluted across a 96-well plate in 200 µl of the appropriate media. For *T.*
20 *brucei*, 100 µl aliquots were then transferred to a plate containing 100 µl cells at 2 x 10³ ml⁻¹.

Human fibroblasts were plated at $2 \times 10^4 \text{ ml}^{-1}$ in 100 μl of medium and incubated overnight to allow cells to adhere before the addition of medium containing DMSO (100 μl) over a range of concentrations. Parasites and mammalian cells were then incubated for 3 days, after which 20 μl 500- μM resazurin was added to each well. Plates were incubated for a further 4 h before measuring fluorescence as above.

2.6 EC_{50} determinations against *T. brucei*

Test compounds were dissolved in DMSO at 20 or 10 mM except for eflornithine which was dissolved in water and sterilised by filtration. The control drug (pentamidine) was dissolved in DMSO at 100 μM . HMI9-T media (148.5 μl) was added to column 2 (B2-G2) of a sterile 96-well culture plate. HMI9-T +1 % DMSO (100 μl) was added to all remaining wells. Test compound solutions (1.5 μl) were added to column 2 (B2-G2). Pentamidine was placed on row G of all plates as a control. Three-fold serial dilutions were carried out by transferring 50 μl from column 2 to the adjacent column (100 μl). The process was repeated up to column 10. HMI9-T containing 2×10^3 trypanosomes ml^{-1} (100 μl) was added to all wells except column 1. HMI9-T (100 μl) was added to column 1. Column 1 and column 11 served as controls without cells and without test-compound respectively. Cells were incubated for 3 days, after which 20 μl 0.5 mM resazurin was added to each well, before measuring fluorescence after 4 h incubation. Data were processed using GRAFIT (version 5.0.4; Erithacus software) and fitted to a 3-parameter equation, where the data are corrected for background fluorescence, to obtain the effective concentration inhibiting growth by 50% (EC_{50}):

$$y = \frac{y_{\max}}{1 + \left(\frac{i}{\text{EC}_{50}}\right)^S} \quad (1)$$

where y_{\max} is the uninhibited fluorescence value, i the inhibitor concentration and s is the Hill slope of the curve.

Measurements for each compound were carried out on 3 separate occasions and the mean weighted to the standard error calculated using the following formulas, where 'a' is the standard error of EC₅₀ determination 'A' etc.

$$\text{weighted mean} = \frac{(A/a^2) + (B/b^2) + (C/c^2)}{(1/a^2) + (1/b^2) + (1/c^2)} \quad \text{error} = \frac{(1/a) + (1/b) + (1/c)}{(1/a^2) + (1/b^2) + (1/c^2)} \quad (2)$$

2.7 Production of LOPAC daughter plates.

Latch-racks containing the LOPAC library at 10 mM dissolved in DMSO were stored frozen at -20°C. Racks were thawed at room temperature for 3 h and centrifuged in a Beckman CS-15R centrifuge at 1,100g for 2 min to return any condensation on the lids to the solution. Racks were then mixed vigorously for 5 min on a platform shaker. Aliquots (50 µl) of each rack were transferred to v-bottomed polypropylene 96-well plates (Matrix) using a Janus liquid handler (Perkin-Elmer) to create a primary daughter-set. DMSO (98 µl) was plated out to columns 2-11 of fresh v-bottom polypropylene 96-well plates using a Precision 200 liquid handler (Biotek). An aliquot (2 µl) of the primary daughter set was transferred to the DMSO-containing plates using a Platemate 2X2 liquid handler (Matrix) to create secondary daughter sets at 200 µM. At this stage, 50 µl of 200 µM pentamidine was added to wells E1-H1, 50 µl DMSO was added to the remaining wells of column 1 and 12 for use as control wells. Duplicate daughter sets (set A and set B) created in this way were used for two independent screening runs to control for errors in liquid handling. Daughter sets were sealed using a nitrogen-flushing heat sealer (K Biosystems) and stored at -20°C.

2.8 Screening of the LOPAC library against *T. brucei*

Daughter sets were thawed and mixed thoroughly, before 1 μl was transferred to an empty 96-well tissue-culture plate (Greiner) using a Platemate 2X2 liquid handler (Matrix). Plates were transferred to a tissue culture hood and 180 μl media added to each well using a WellMate dispenser (Matrix). HMI9-T (19 μl) was added to wells A1 – D1 and 19 μl media containing *T. brucei* ($1.05 \times 10^4 \text{ ml}^{-1}$) was added to all other wells using a Iact2 pipettor (Matrix). Wells A1-D1 served as background (no cell) controls; wells E1-H1 served as pentamidine controls (1 μM); and column 12 served as a full signal control (cells, no compound). Plates were incubated for 3 days after which 20 μl 0.5 mM resazurin was added to each well. Plates were incubated for a further 4 h before measuring fluorescence as above. Fluorescence signal for each well was background subtracted and expressed as percentage of the full signal controls. Z-prime (Z') values were calculated for all plates using the following equation [11]:

$$Z' = 1 - \frac{3\sigma(\text{full signal}) + 3\sigma(\text{background signal})}{\mu(\text{full signal}) - \mu(\text{background signal})} \quad (3)$$

where μ and σ represent the means and standard deviations, respectively

2.9 EC₅₀ determinations against mammalian cells.

Compounds with submicromolar EC₅₀ values against *T. brucei* were tested against human fibroblasts as follows. Cells were detached, counted as described above and diluted to $2 \times 10^4 \text{ ml}^{-1}$. Cells (100 μl) were added to columns 1 to 11 and 100 μl media was added to column 12 of a 96 well plate. The plate was then incubated for 24 h to allow attachment. Test compounds and Doxorubicin as a control, were serially diluted in DMSO in a 96-well poly-propylene V-bottomed plate using a Janus liquid handler (Perkin-Elmer). Samples from the test compound dilution series and QC plates (2 μl) were transferred to duplicate tissue culture 96-well plates using a PlateMate 2X2 liquid handler (Matrix). These plates were transferred to a tissue

1 culture hood and 200 μ l media added to all wells. From these plates, 100 μ l was transferred to
2 the cell-containing plates set up the previous day. Plates were incubated for 3 days after which
3
4 20 μ l 0.5 mM resazurin was added to each well. Plates were incubated for a further 4 h before
5
6 measuring fluorescence as above. Fluorescence signal for each well was background
7
8 subtracted and expressed as percentage inhibition of the full signal control wells. EC₅₀ curve
9
10 fitting employed a 4 Parameter Logistic dose response curve using IDBS XLFit 4.2 Model
11
12
13
14 205:

$$17 \quad y = A + \frac{B - A}{1 + \left(\frac{C}{x}\right)^D} \quad (4)$$

22 Where A is the minimum y-value; B is the maximum y-value; C is the EC₅₀; and D is the Hill
23
24 slope. All test compound curves had floating maximum and minimum and pre-fit was used
25
26 for all 4 parameters.
27
28

32 2.10 Homology searching and protein alignments

35 Sequences for κ -opioid receptor protein were retrieved from the National Centre for
36
37 Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) for sheep (accession AAY66995),
38
39 zebrafish (accession NP_878306), rough skinned newt (accession AAU15126), mouse
40
41 (accession NP_035141 and AAA56759) and guinea pig (accession AAA67171). Global
42
43 alignments were carried out using ClustalW and *T. brucei* GeneDB was searched using the
44
45 TBLASTN algorithm [12].
46
47
48
49
50
51
52
53

54 2.11 Effect of (+)-U50,488 on cell growth.

56 Flasks of *T. brucei* were seeded at 1×10^4 cells ml⁻¹ and incubated in the presence of the
57
58 known inhibitors pentamidine and eflornithine, and the LOPAC hit (+)-U50,488 for 3 days.
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Flasks were set up in triplicate for each inhibitor at multiples of EC_{50} along with 3 control flasks with no inhibitor added. Pentamidine and (+)-U50,488 were added in DMSO in a volume of 0.1% of the culture. Eflornithine was dissolved directly in HMI9-T media and sterilised by 0.22 μ M filtration before diluting into the cell culture. Cell densities were determined at intervals using a haemocytometer and generation times calculated using GraFit (version 5.0.13; Erithacus software) using the following equation:

$$N = N_0 2^{t/g} \quad (5)$$

where N_0 and N are the number of cells at time zero and time t , respectively, and g is the time per generation. For low cell densities, samples were concentrated 150-fold by centrifugation and resuspension in an appropriate volume of medium.

2.12 DMPK and efficacy studies

All animal experiments were carried out following local ethical review and under UK regulatory licensing in accordance with the European Communities Council Directive (86/609/EEC). NMRI outbred mice were purchased from Harlan laboratories, UK.

In brief, a 96-well equilibrium dialysis apparatus (HT Dialysis LLC, Gales Ferry, CT) was used to determine the free fraction in plasma for (+)-U50,488. Membranes (12-14 kDa cut-off) were conditioned in deionised water for 60 min, followed by conditioning in 80:20 deionised water:ethanol for 20 min, and then rinsed in isotonic buffer before use. Frozen female mouse plasma was thawed, centrifuged (2100g, 10 min), spiked with (+)-U50,488 (10 μ g g^{-1}), and 150- μ l aliquots (n=6 replicate determinations) loaded into the 96-well equilibrium dialysis plate. Dialysis against isotonic buffer (150 μ l) was carried out for 5 h at 37°C using an orbital microplate shaker at 125 revolutions min^{-1} . At the end of the incubation period, aliquots of plasma or buffer were transferred to Micronic tubes (Micronic B.V., the

1 Netherlands) and the composition in each tube balanced with control fluid, such that the
2 volume of buffer to plasma is the same. Sample extraction was performed by the addition of
3
4 400 μl of acetonitrile containing a structural analogue of (+)-U50,488 as internal standard.
5
6 Samples were allowed to mix for 1 min and then centrifuged at 2100g in 96-well blocks for
7
8 10 min. All samples were analysed by UPLC/MS/MS on a Quattro Premier XE Mass
9
10 Spectrometer (Waters Corporation, USA) and the unbound fraction determined as the ratio of
11
12 the peak area in buffer to that in plasma.
13
14
15

16
17 To determine brain penetration, (+)-U50,488 was administered intravenously to
18
19 female NMRI mice (n=3) at a dose level of 2mg kg^{-1} (freshly prepared in 5% DMSO v/v in
20
21 sterile saline). A blood sample (50 μl) was collected by cardiac puncture from each animal
22
23 into Micronic tubes containing deionised water (100 μl) at 5 min post-dose and the brain of
24
25 each animal removed into tared Covaris glass tubes. Blood and brains were stored at -80°C
26
27 until analysis.
28
29
30

31
32 To determine exposure, (+)-U50,488 was administered by oral gavage using a 20-
33
34 gauge feeding tube fitted with a 1-ml syringe to female NMRI mice (n=3) at a dose level of
35
36 150 mg kg^{-1} (freshly prepared in DMSO, PEG400, milliQ water (1:8:11 v/v respectively)).
37
38 Blood samples (10 μl) were collected from the tail vein of each animal into Micronic tubes
39
40 containing deionised water (20 μl) at 0.25, 0.5, 1, 2, 4, 6 and 8 h post-dose and stored at -
41
42 80°C until analysis.
43
44
45

46
47 For analysis, brain tissue was homogenised in 50% v/v methanol in deionised water
48
49 (1:2 w/v) using a Covaris S2 (K Biosciences, Hoddesdon, UK). Extraction of blood and brain
50
51 samples was then performed by a method based on protein precipitation using acetonitrile
52
53 containing a structural analogue of (+)-U50,488 as an internal standard. Levels of (+)-
54
55 U50,488 were determined in these extracted samples by UPLC-MS/MS on a Quattro Premier
56
57 XE mass spectrometer (Waters corporation, USA). A calibration curve was constructed in
58
59
60
61
62
63
64
65

1 blood and brain homogenate to cover at least 3 orders of magnitude for (+)-U50,488 (range 2
2 – 2000ng ml⁻¹).
3
4

5 For the efficacy study, groups of three female NMRI mice were infected with the
6 bloodstream form of *T. brucei brucei* (strain 427) by a single intraperitoneal injection of 10⁴
7 parasites in 0.2 ml HM19-T medium. Three days following infection, mice were either dosed
8 by oral gavage with (+)-U50,488 (150 mg kg⁻¹, freshly prepared in DMSO, PEG400, milliQ
9 water (1:8:11 v/v respectively)) or left untreated. Dosing of (+)-U50,488 treated mice was
10 then repeated in an identical manner every 4 h for a further 32 h. Thereafter, animals were
11 inspected daily for clinical signs of infection and wet smears of tail blood were examined
12 microscopically as appropriate. Parasitaemia was determined using a Neubauer
13 haemocytometer and mice were monitored for 30 days. Mice that exceeded a parasitaemia
14 >10⁸ ml⁻¹ were humanely killed since prior experience indicated that animals would succumb
15 to an overwhelming infection by the following day.
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

3 Results

3.1 Linearity of the resazurin cell viability assays

Alamar Blue has been used previously as an *in vitro* viability assay for *T. brucei* [13]. This proprietary reagent contains resazurin [14], a dye which is metabolically reduced in cells to the highly fluorescent product resorufin. Using the less expensive and widely available reagent resazurin, the linearity and dynamic range of the assay was determined for parasite and human fibroblasts. To determine the dynamic range of the assay, bloodstream forms of *T. brucei* were incubated at 37 °C in culture medium at a range of cell densities containing 440 µM resazurin and fluorescence measured at intervals. For each time point fluorescence was proportional to cell density with linear correlation coefficients (r^2) >0.997 (Fig. 1A). Likewise, fluorescence increased linearly with time at any cell density (Fig. 1C, r^2 >0.996). Thus fluorescence is proportional to cell density up to 2×10^5 cells per well within a 2 to 5 h incubation period. Similar results were obtained for human fibroblasts (Fig. 1B and 1D) up to 3×10^4 cells per well within a 2 to 6 h incubation. In further assays, final cell densities and resazurin-incubation times did not exceed these ranges.

3.2 DMSO-tolerance of the resazurin cell viability assays

Test-compounds were supplied as 10 mM stocks dissolved in DMSO and so it was necessary to determine the tolerance of the assays for this solvent. Both cell lines showed a similar sensitivity to DMSO with IC_{50} values of 0.80 ± 0.06 and 1.04 ± 0.08 % (v/v) for *T. brucei* and human fibroblasts, respectively (data not shown). DMSO (0.5%) was selected as an acceptable compromise allowing compound addition up to 50 µM with an acceptable attenuation of fluorescence of 20% for the *T. brucei* assay and 15% for the MRC5 assay. All subsequent assays contained 0.5% DMSO.

3.3 Sensitivity to standard drugs

Five drugs currently used in treatment of HAT were tested for growth inhibition (Fig. 2A). The most potent of these were pentamidine, suramin and melarsoprol, with EC₅₀ values in the low nanomolar range, similar to those published for *T. brucei rhodesiense* and *T. brucei gambiense* by Raz *et al* [13] and for various *T. brucei gambiense* stocks by Likeufack *et al.* [15]. Eflornithine was the least effective drug (EC₅₀ 22.4 ± 1.1 µM). Since eflornithine induces physiological activation of mitochondrial metabolism [16], it could potentially yield erroneous results due to increased reduction of resazurin in drug-treated cells. However, this does not appear to be the case as direct counting of cell growth returned a similar EC₅₀ of 17.1 ± 0.1 µM. Nifurtimox was intermediate in potency (EC₅₀ 1.8 µM) in good agreement with previous reports [17;18]. These results provide further validation of resazurin as a suitable substitute for Alamar blue reagent. Pentamidine isethionate was chosen for use as a ‘per-plate’ control for all future assays. Similarly, the anticancer compound doxorubicin [19] was used as a control for MRC5 assays (Fig. 2B).

3.4 Screening of the LOPAC collection

Initially, the LOPAC collection of pharmacologically active compounds was screened against *T. brucei* at a fixed concentration of 1 µM on two independent occasions. This identified 52 compounds producing a mean signal of less than 50% of the control on at least one occasion: an initial hit rate of 4.3%. Hits were analysed by LC-MS confirming a purity of at least 95% for all compounds (data not shown).

Triplicate independent EC₅₀ determinations were carried out for each of these hits against *T. brucei*. The resulting EC₅₀ values for each hit were in good agreement with

1 coefficients of variation typically less than 20%. Means weighted to standard error for each
2 compound were determined and at this point 19 hits with EC₅₀ values of greater than 1 μM
3 were rejected (Table 1), leaving 33 confirmed sub-micromolar EC₅₀ hits against *T. brucei*
4 (Table 2).
5
6
7
8
9

10 Selective activity against the parasite is an essential feature of drug leads [20]. We
11 therefore determined EC₅₀ values of our hits against MRC5 cells, a human embryonic lung
12 cell line used in similar previous studies [21]. Coefficients of variation between the resulting
13 4 determinations were typically less than 40%. Means weighted to standard error were
14 determined and used to calculate the fold-specificity for *T. brucei* over human fibroblasts
15 (Table 2). Two known trypanocidal drugs, pentamidine and suramin, which happen to be
16 present in the LOPAC library returned specificities of 3,500-fold and >700-fold, respectively.
17 The highest specificity of the remaining hits (270 fold) was demonstrated by (+)-U50,488,
18 with an EC₅₀ of 59 nM. The mixture of enantiomers, (±)-U50,488, showed a similar
19 specificity, but only half the potency of the 1*R*,2*R* *trans* enantiomer, suggesting that the
20 growth inhibition is largely due to (+)-U50,488. Only two other hits returned specificities
21 greater than 100-fold: dequalinium dichloride, an broad-range antimicrobial known to act
22 against plasmodium in mice [22] and oligomycin A, a macrolide antibiotic that inhibits
23 membrane associated mitochondrial ATPase [23]. Only one compound (vincristine sulphate,
24 an inhibitor of microtubule assembly [24]) demonstrated greater specificity for human
25 fibroblasts compared to *T. brucei*. Among the other potent, but less selective screening hits
26 were compound classes with activity against protein kinases, topoisomerases, tubulin, as well
27 as DNA and energy metabolism. The average Z' scores throughout the testing was 0.66 for *T.*
28 *brucei* assays and 0.57 for MRC5 assays, values characteristic of high quality cell-based
29 screening assays.
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

3.5 Confirmation of inhibition by (+)-U50,488 and other opioid receptor ligands

1
2
3 A sample of (+)-U50,488 and eight other compounds known to act on μ - or κ -opioid receptors
4
5 were purchased for EC₅₀ determination. The mean weighted to standard error was 58.9 nM for
6
7 the authentic (+)-U50,488 sample compared to the 60 nM value found during the library
8
9 screening. Of the other opioid receptor ligands with structural similarity to (+)-U50,488, three
10
11 displayed EC₅₀ values ~5 μ M (Fig. 3) and one (U69593) was inactive. Of the compounds
12
13 showing structural similarity to morphine, only buprenorphine showed weak activity. Thus,
14
15 the original (+)-U50,488 hit had the highest potency of this group of compounds and was 70-
16
17 times more potent than its trans enantiomer, (-)-U50,488.
18
19
20
21
22
23
24
25

3.6 κ -Opioid receptor homology searching

26
27
28
29 Global alignments of the amino acid sequences of the κ -opioid receptor from sheep, zebrafish,
30
31 rough skinned newt, mouse and guinea pig return similarity scores of 60-99%. TBLASTN
32
33 searching of the *T. brucei* GeneDB with these query sequences revealed several predicted
34
35 genes with only short stretches of alignment. Global alignments of these predicted genes with
36
37 genes with only short stretches of alignment. Global alignments of these predicted genes with
38
39 the known receptor sequences returned similarity scores of only 9 to 19%.
40
41
42
43
44

3.7 Effect of (+)-U50,488 on *T. brucei* in vivo

45
46
47 Pharmacokinetic studies on (+)-U50,488 in uninfected mice were performed. The compound
48
49 is not tightly bound to mouse plasma proteins (fraction unbound is 0.69) and is well tolerated
50
51 when administered at 150 mg kg⁻¹ via the oral route. The brain to blood ratio was 8.2, an
52
53 excellent property for a stage 2 candidate HAT drug. The peak free blood concentration of
54
55 (+)-U50,488 was 800 ng ml⁻¹ 1 h after dosing with a half-life of 2.6 \pm 0.04 h (Fig. 4). Based
56
57 on these findings, 3 mice were infected with *T. brucei* and dosed orally at 150 mg kg⁻¹ every 4
58
59
60
61
62
63
64
65

1 h for 32 h to ensure that the free blood level remained above the EC₉₉. However, this regimen
2 was not curative, with only one mouse showing a transient reduction in parasitaemia followed
3
4 by relapse, in sharp contrast to our recent studies on inhibitors of *N*-myristoyltransferase [25]
5
6 and nitroaromatic drugs [18].
7
8
9

10 11 12 **3.8 Effect of (+)-U50,488 and HAT drugs on *T. brucei* culture growth**

13
14
15 To examine the cause of the poor trypanocidal activity in vivo, (+)-U50,488 was compared
16
17 with the known HAT drugs pentamidine and eflornithine for their ability to kill or slow the
18
19 growth of *T. brucei* cells in culture (Fig. 5). Exposure to pentamidine (10 x EC₅₀) caused the
20
21 cell density to drop below the limit of detection (1 x 10³ cells ml⁻¹) by 40 h with no viable
22
23 cells visible by microscopy, indicating a cytotoxic effect. Eflornithine arrested cell growth
24
25 after 30 h and cell density did not change thereafter, indicating a cytostatic effect. In contrast,
26
27 exposure to (+)-U50,488 at 10 x EC₅₀ only had a minor effect on growth, reducing the
28
29 doubling time from 6.8 h to 9.7 h. Further inspection of the EC₅₀ curves suggested that this
30
31 unexpected result might be attributed to a low Hill slope for this compound. Based on the EC₅₀
32
33 values and Hill slopes for pentamidine and eflornithine (8 nM, *s* = 3.1 and 22.4 μM, *s* = 4.0 ,
34
35 respectively) EC₉₉ values of 37 nM and 77 μM can be calculated, values which are less than
36
37 10-times EC₅₀. In contrast, (+)-U50,488 (EC₅₀ 60 nM, *s* = 1.8) has a predicted EC₉₉ value of
38
39 740 nM, which is greater than 10-times the EC₅₀. Thus, the observed partial inhibition of
40
41 growth is consistent with exposure to this concentration of drug. In a series of additional
42
43 experiments the effect on growth of 50-, 100- and 500-times the EC₅₀ for (+)-U50,488 was
44
45 examined. These concentrations showed a dose dependent reduction in growth rate, but only
46
47 at the highest drug concentration (30 μM, 500-times EC₅₀) was it possible to achieve a
48
49 cytotoxic effect (Fig. 5, closed squares).
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

4 Discussion

1
2
3 Our experiments establish that resazurin can be used as a cheaper substitute for the Alamar
4
5 blue formulation for growth inhibition assays for bloodstream-form *T. brucei* or human
6
7 fibroblasts. However, this assay method does not necessarily indicate cell death. Inhibitors
8
9 could be cytocidal, cytostatic, or interfering with the reduction of resazurin without effect on
10
11 cell growth. Lead compounds must therefore be assessed for cytotoxicity by alternative means
12
13 such as direct cell counting of viable parasites (Fig. 5) or a ‘live-dead assay’ [26]. The HMI9-
14
15 T media used in this assay has been shown to mask inhibition of certain metabolic pathways
16
17 such as folate metabolism and thymidylate biosynthesis [27] and consequently these
18
19 inhibitors are unlikely to be identified with these culture conditions. Given this proviso, our
20
21 results indicate that this semi-automated medium-throughput screening method against *T.*
22
23 *brucei* is reproducible and robust. With further automation and miniaturisation it could be
24
25 used to screen much larger compound collections and other special focussed sets available in
26
27 our Drug Discovery Unit [28].
28
29
30
31
32
33
34

35 The drugs currently used to treat HAT show a wide range of potencies in this assay,
36
37 ranging from 8 nM for pentamidine and melarsoprol to 1.8 μ M for nifurtimox and 22 μ M for
38
39 eflornithine. Despite many current drug-discovery paradigms being driven by potency - at
40
41 least at the early stages of lead identification - the example of eflornithine demonstrates that
42
43 lower potency compounds may still emerge as effective treatments. Any lead offering
44
45 improvement on either the mode of administration or cost of eflornithine regimen would be an
46
47 advance in the treatment of HAT.
48
49
50
51

52 The EC₅₀ values determined by Likeufack *et al* against *T. brucei gambiense* vary by a
53
54 maximum of 4.5-fold between stocks [15], and the values we report against *T. brucei brucei*
55
56 fall within these ranges. The same is true for the values reported by Raz *et al* [13] for
57
58 *T.b.rhodesiense*. However, although *T. brucei brucei*, which is non-pathogenic to humans, is
59
60
61
62
63
64
65

1 a suitable model for HAT, it is important to test advanced lead compounds against these
2 clinically relevant sub-species.
3

4
5 The 33 nanomolar inhibitors of *T. brucei* identified from the LOPAC collection
6 included pentamidine and suramin which acted as internal controls. The minor differences in
7 EC₅₀ values between the library screening and our testing of standard compounds could be
8 due to higher purity in the original samples purchased and evaporation in the smaller volumes
9 of the library samples. Original hits that were rejected at the 1 μ M cut-off include several
10 compounds that have known pharmacological activity in animal models. Testing these against
11 rodent models of HAT at the already-established maximum tolerated doses could identify
12 additional promising leads.
13
14
15
16
17
18
19
20
21
22
23

24
25 Six out of the 33 hits with EC₅₀ values less than 1 μ M showed more than 100-fold
26 selectivity for *T. brucei*. Pentamidine and suramin were the most selective compounds
27 identified, illustrating the target selectivity (>1000 fold) that should be aimed for at the final
28 stages of lead development. The most selective compounds previously unknown to inhibit *T.*
29 *brucei* were from isomers of U50,488, a known agonist of the κ -opioid receptor [29]. This
30 compound shows good brain penetration and therefore potentially able to access a late-stage
31 HAT infection, a key feature specified by the current target product profile from WHO.
32
33
34
35
36
37
38
39
40
41
42

43 From the limited number of commercially available opioid-receptor ligands, (+)-
44 U50,488 was the most potent (Fig. 4). Other compounds showed either weak or no activity.
45 The complete lack of activity of norbinaltorphimine against *T. brucei* suggests that possible
46 CNS side effects of a treatment based on (+)-U50,488 could be reduced by co-administration
47 of a κ -opioid antagonist. Whilst the (-)-U50,488 isomer is a stronger agonist of the κ -opioid
48 receptor than the (+)-U50,488 isomer, the latter is almost 70 times more effective against *T.*
49 *brucei*. κ -Opioid receptors typically share high similarity at the amino acid level across
50 species as indicated by our homology searching. However, consistent with the poor activity of
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 other opioid-receptor ligands, we found no convincing homologue of the receptor in the *T.*
2 *brucei* genome. An opioid receptor agonist (endomorphin-1; YPWF-NH₂) has been shown to
3
4 inhibit a drug-metabolite transporter from the malaria parasite *Plasmodium falciparum* [30].
5
6 Mutations in this transporter confer resistance to chloroquine, but unfortunately the
7
8 physiological substrates are not known [31]. Moreover, no convincing homologues could be
9
10 identified in the *T. brucei* genome database. Thus the target for U50,488 remains to be
11
12 identified in *T. brucei*.
13
14
15

16 The in vivo and in vitro experiments indicate that further optimization of (+)-U50,488
17
18 is required to achieve efficacy. Although the compound has good pharmacological properties,
19
20 including high CNS penetration, it was not curative using a therapeutic schedule which was
21
22 predicted to maintain free blood exposure above the predicted EC₉₉. The reason for this is not
23
24 entirely clear. The in vitro studies demonstrated that the compound is cytostatic at 100-times
25
26 the EC₅₀, like eflornithine, rather than cytotoxic, like pentamidine. Thus, continuous exposure
27
28 above the minimum inhibitory concentration may be required to allow sufficient time for the
29
30 immune system to eliminate non-dividing parasites, as is the case with eflornithine.
31
32
33

34 Alternatively, differences in the physiological environment in vivo and in vitro may be
35
36 responsible for the lack of efficacy. Further studies on the mode of action of (+)-U50,488
37
38 would be informative. Although a cytotoxic effect was observed at high concentrations (30
39
40 μM, 500-times EC₅₀), this could be due to additional off-target effects and, in any case, is
41
42 above the maximum free blood concentration achievable under the dosing schedule used here.
43
44
45
46
47
48

49 Future work will involve further medicinal chemistry to explore structure-activity-
50
51 relationships, improve potency and the Hill slope. It remains to be seen whether the cytostatic
52
53 effect of U50,488 is a function of the target(s) or the inhibitor itself. Identifying the
54
55 intracellular target of U50,488 could provide a new avenue for drug discovery efforts against
56
57
58
59 *T. brucei*.
60
61
62
63
64
65

Acknowledgements

This work was supported by grants from the Wellcome Trust (WT079838, WT077705, WT083481) and the Drugs for Neglected Diseases initiative. We thank Bhavya Rao for technical assistance in the testing of compounds against human fibroblasts, Robert Kime and Suzanne Norval for DMPK studies, Frederick Simeons for assistance with animal efficacy testing and Magnus Alphey for critical reading of the manuscript.

Accepted Manuscript

1 **Figure Legends**

2 **FIG. 1. Linearity of the Resazurin-based assay.**

3 *T. brucei* bloodstream-form cells (panels A and C) or human fibroblasts (panels B and D)
 4 were diluted across a range of densities on 96-well plates. Resazurin was added immediately
 5 in the case of *T. brucei* or the following day for human fibroblasts. Fluorescence signal from
 6 *T. brucei* plates was proportional to cell density at various time points (panel A, open circles 2
 7 h; closed circles 3 h; open squares 4 h; closed squares 5 h) and linear with respect to time at
 8 various cell densities (panel C, open circles 1×10^6 cells per well; closed circles 5×10^5 cells
 9 per well; open squares 2.5×10^5 cells per well; closed squares 1.25×10^5 cells per well).
 10 Fluorescence signal from MRC5 plates was proportional to cell density at various time points
 11 (panel B, open circles 1 h; closed circles 2 h; open squares 3 h; closed squares 4 h; open
 12 triangles 5h; closed triangles 6 h) and linear with respect to time at various cell densities
 13 (panel D, open circles 1×10^4 cells per well; closed circles 1.5×10^4 cells per well; open squares
 14 2×10^4 cells per well; closed squares 2.5×10^4 cells per well; open triangles 3×10^4 cells per
 15 well).

18 **FIG. 2. Response of *T. brucei* and human fibroblasts to standard drugs**

19 *T. brucei*, 200 cells per well (panel A) or MRC5, 2000 cells per well (panel B) were incubated
 20 for 3 days with a range of inhibitor concentrations. Resazurin was added approximately 4
 21 hours before determining per centage inhibition of the fluorescent signal compared to controls
 22 minus inhibitor. The EC_{50} values determined were as follows: pentamidine (open circles) 8.10
 23 ± 0.54 nM; melarsoprol (closed circles) 7.52 ± 0.28 nM; suramin (open squares) 20.1 ± 1.1

1 nM; nifurtimox (closed squares) $1.81 \pm 0.13 \mu\text{M}$; eflornithine (open triangles) $22.4 \pm 1.1 \mu\text{M}$.

2 Doxorubicin yielded an EC_{50} of $85.9 \pm 38.2 \text{ nM}$.

3
4
5
6
7
8 **FIG. 3. Structure activity relationships of opioid receptor ligands against *T. brucei***

9
10
11 These compounds were screened for potency against *T. brucei*. EC_{50} values are recorded as
12 the weighted mean of 3 determinations. Norbinaltorphimine was tested at a maximum
13 concentration of $50 \mu\text{M}$ due to solubility issues.
14
15
16
17
18
19
20
21

22 **FIG. 4. Pharmacokinetic properties of (+)-U50,488 in mice**

23
24
25 10 Data show the mean and standard error for the free blood concentration in 3 mice following a
26 single oral dose at 150 mg kg^{-1} . The calculated half life is $2.60 \pm 0.04 \text{ h}$. The dashed lines
27 indicate the EC_{50} obtained in vitro against *T. brucei* and the predicted EC_{90} and EC_{99} values for
28 growth inhibition.
29
30
31
32
33
34
35
36
37
38
39
40

41 **FIG. 5. Effect of inhibitors on *T. brucei* growth in vitro**

42 16 *T. brucei* were grown in 10-ml cultures in the presence of the inhibitors at 10 times (open
43 symbols) and 500 times (closed symbols) their EC_{50} values. Cells were counted using a
44 haemocytometer over a three day period. Values are the mean and standard deviation of three
45 flasks and are representative of three independent experiments. No drug, circles; (+)-U50,488,
46 squares; eflornithine triangles; pentamidine, inverted triangle. Cell counts below the limits of
47 detection are indicated by a skull and crossbones symbol.
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

References

1. Garcia A, Courtin D, Solan P, Koffi M, Jamonnea V. Human African trypanosomiasis: connecting parasite and host genetics. *Trends Parasitol* 2006;22:405-409.
2. Stuart KD, Brun R, Croft SL, Fairlamb AH, Gurtler RE, McKerrow JH, Reed S, Tarleton RL. Kinetoplastids: related protozoan pathogens, different diseases. *J Clin Invest* 2008;118:1301-1310.
3. Renslo AR, McKerrow JH. Drug discovery and development for neglected parasitic diseases. *Nat Chem Biol* 2006;2:701-710.
4. Pépin J, Milord F. The treatment of human African trypanosomiasis. *Adv Parasitol* 1994;33:1-47.
5. Priotto G, Kasparian S, Mutombo W, Ngouama D, Ghorashian S, Arnold U, Ghabri S, Baudin E, Buard V, Kazadi-Kyanza S, Ilunga M, Mutangala W, Pohlig G, Schmid C, Karunakara U, Torreele E, Kande V. Nifurtimox-eflornithine combination therapy for second-stage African *Trypanosoma brucei gambiense* trypanosomiasis: a multicentre, randomised, phase III, non-inferiority trial. *Lancet* 2009;374:56-64.
6. Thuita JK, Karanja SM, Wenzler T, Mdachi RE, Ngotho JM, Kagira JM, Tidwell R, Brun R. Efficacy of the diamidine DB75 and its prodrug DB289, against murine models of human African trypanosomiasis. *Acta Trop* 2008;108:6-10.
7. Frearson JA, Wyatt PA, Gilbert IH, Fairlamb AH. Target assessment for antiparasitic drug discovery. *Trends Parasitol* 2007;23:589-595.
8. Mackey ZB, Baca AM, Mallari JP, Apsel B, Shelat A, Hansell EJ, Chiang PK, Wolff B, Guy KR, Williams J, McKerrow JH. Discovery of trypanocidal compounds by whole cell HTS of *Trypanosoma brucei*. *Chem Biol Drug Des* 2006;67:355-363.
9. Hopkins AL. Network pharmacology: the next paradigm in drug discovery. *Nat Chem Biol* 2008;4:682-690.
10. Hirumi H, Hirumi K. Continuous cultivation of *Trypanosoma brucei* blood stream forms in a medium containing a low concentration of serum protein without feeder cell layers. *J Parasitol* 1989;75:985-989.
11. Zhang JH, Chung TDY, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* 1999;4:67-73.
12. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic Local Alignment Search Tool. *J Mol Biol* 1990;215:403-410.
13. Raz B, Iten M, Grether-Buhler Y, Kaminsky R, Brun R. The Alamar Blue® assay to determine drug sensitivity of African trypanosomes (*T.b.rhodesiense* and *T.b.gambiense*) in vitro. *Acta Trop* 1997;68:139-147.

14. O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem* 2000;267:5421-5426.
15. Likeufack ACL, Brun R, Fomena A, Truc P. Comparison of the in vitro drug sensitivity of *Trypanosoma brucei gambiense* strains from West and Central Africa isolated in the periods 1960-1995 and 1999-2004. *Acta Trop* 2006;100:11-16.
16. Giffin BF, McCann PP. Physiological activation of the mitochondrion and the transformation capacity of DFMO induced intermediate and short stumpy bloodstream form trypanosomes. *Am J Trop Med Hyg* 1989;40:487-493.
17. Wilkinson SR, Taylor MC, Horn D, Kelly JM, Cheeseman I. A mechanism for cross-resistance to nifurtimox and benznidazole in trypanosomes. *Proc Natl Acad Sci USA* 2008;105:5022-5027.
18. Sokolova AY, Wyllie S, Patterson S, Oza SL, Read KD, Fairlamb AH. Cross-resistance to nitro drugs and implications for treatment of human African trypanosomiasis. *Antimicrob Agents Chemother* 2010;54:2893-2900.
19. Arcamone F, Cassinel G, Fantini G, Grein A, Orezzi P, Pol C, Spalla C. Adriamycin, 14-hydroxydaunomycin, a new antitumor antibiotic from *S.Peucetius* var caesius. *Biotechnology and Bioengineering* 1969;11:1101-1110.
20. Caffrey CR, Steverding D, Swenerton RK, Kelly B, Walshe D, Debnath A, Zhou YM, Doyle PS, Fafarman AT, Zorn JA, Land KM, Beauchene J, Schreiber K, Moll H, Ponte-Sucre A, Schirmeister T, Saravanamuthu A, Fairlamb AH, Cohen FE, McKerrow JH, Weisman JL, May BC. Bis-acridines as lead anti-parasitic agents: structure activity analysis of a discrete compound library *in vitro*. *Antimicrob Agents Chemother* 2007;51:2164-2172.
21. Girault S, Grellier P, Berecibar A, Maes L, Mouray E, Lemiere P, Debreu MA, Davioud-Charvet E, Sergheraert C. Antimalarial, antitrypanosomal, and antileishmanial activities and cytotoxicity of bis(9-amino-6-chloro-2-methoxyacridines): Influence of the linker. *J Med Chem* 2000;43:2646-2654.
22. Rodrigues JR, de Dominguez NG. *Plasmodium berghei*: *in vitro* and *in vivo* activity of dequalinium. *Exp Parasitol* 2007;115:19-24.
23. Lardy HA, Johnson D, McMurray WC. Antibiotics as tools for metabolic studies. I. A survey of toxic antibiotics in respiratory, phosphorylative and glycolytic systems. *Arch Biochem Biophys* 1958;78:587-597.
24. Jordan MA, Himes RH, Wilson L. Comparison of the effects of vinblastine, vincristine, vindesine, and vinepidine on microtubule dynamics and cell proliferation in vitro. *Cancer Res* 1985;45:2741-2747.
25. Frearson JA, Brand S, McElroy SP, Cleghorn LAT, Smid O, Stojanovski L, Price HP, Guther MLS, Torrie LS, Robinson DA, Hallyburton I, Mpamhanga CP, Brannigan JA, Wilkinson AJ, Hodgkinson M, Hui R, Qiu W, Raimi OG, van Aalten DMF, Brenk R, Gilbert IH, Read KD, Fairlamb AH, Ferguson MAJ, Smith DF, Wyatt PG. *N-*

myristoyltransferase inhibitors as new leads to treat sleeping sickness. *Nature* 2010;464:728-732.

26. Turner CMR, McLellan S, Lindergard LAG, Bisoni L, Tait A, MacLeod A. Human infectivity trait in *Trypanosoma brucei*: stability, heritability and relationship to *sra* expression. *Parasitol* 2004;129:445-454.
27. Sienkiewicz N, Jaroslowski S, Wyllie S, Fairlamb AH. Chemical and genetic validation of dihydrofolate reductase-thymidylate synthase as a drug target in African trypanosomes. *Mol Microbiol* 2008;69:520-533.
28. Brenk R, Schipani A, James D, Krasowski A, Gilbert IH, Frearson J, Wyatt PG. Lessons learnt from assembling screening libraries for drug discovery for neglected diseases. *ChemMedChem* 2008;3:435-444.
29. Vonvoigtlander PF, Lahti RA, Ludens JH. U-50,488 - A selective and structurally novel non- μ -(κ) opioid agonist. *J Pharmacol Exp Ther* 1983;224:7-12.
30. Martin RE, Marchetti RV, Cowan AI, Howitt SM, Broer S, Kirk K. Chloroquine transport via the malaria parasite's chloroquine resistance transporter. *Science* 2009;325:1680-1682.
31. Sanchez CP, Stein WD, Lanzer M. Is PfCRT a channel or a carrier? Two competing models explaining chloroquine resistance in *Plasmodium falciparum*. *Trends Parasitol* 2007;23:332-339.

TABLE 1. Initial submicromolar hits from the LOPAC library returning EC₅₀ versus *T. brucei* greater than 1 μ M.

Compound	EC ₅₀ vs. <i>T. brucei</i> (μ M)
Parthenolide	1.06 \pm 0.08
CB 1954 ^a	1.08 \pm 0.09
Cyclosporin A	1.14 \pm 0.17
Bromoacetyl alprenolol menthane	1.26 \pm 0.14
Indirubin-3'-oxime	1.28 \pm 0.09
Dipropyldopamine hydrobromide	1.29 \pm 0.06
SB 224289 hydrochloride ^b	1.35 \pm 0.28
R(-)-Propylnorapomorphine hydrochloride	1.52 \pm 0.21
1,3-PBIT dihydrobromide ^c	1.58 \pm 0.08
Caffeic acid phenethyl ester	1.64 \pm 0.13
Kenpaullone	1.86 \pm 0.27
CGS-15943 ^d	1.97 \pm 0.21
A-77636 hydrochloride ^e	2.21 \pm 0.15
Retinoic acid p-hydroxyanilide	2.23 \pm 0.27
6-Hydroxymelatonin	3.31 \pm 0.23
S-(4-Nitrobenzyl)-6-thioinosine	3.78 \pm 0.55
S-(p-Azidophenacyl)glutathione	>10
Dephostatin	>10
2',3'-Dideoxycytidine	>10

^a 5-(1-Aziridinyl)-2,4-dinitrobenzamide

^b 1'-Methyl-5-([2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl] carbonyl)-2,3,6,7-tetrahydro-spiro(furo[2,3-f]indole-3,4'-piperidine)

^c Phenylene-1,3-bis(ethane-2-isothiourea) dihydrobromide

^d 9-Chloro-2-(2-furyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine

^e (-)-(1R,3S)-3-Adamantyl-1-(aminomethyl)-3,4-dihydro-5,6-dihydroxy-1H-2-benzopyran hydrochloride hydrate

TABLE 2 Submicromolar hits against *T. brucei* ranked by selectivity versus MRC5 cells

Compound	EC ₅₀ vs. <i>T. brucei</i> (nM)	EC ₅₀ vs. MRC5 (nM)	Selectivity
Pentamidine isethionate	3.7 ± 0.10	13 000 ± 6 800	3400
Suramin hexasodium	68 ± 3.5	>50 000	>710
(+)-trans-(1R,2R)-U-50488	59 ± 2.2	16 000 ± 4 900	270
(±) trans-U-50488 methanesulfonate	113 ± 7.6	26 000 ± 8 700	230
Dequalinium dichloride	43 ± 8.8	6 100 ± 1 500	140
Oligomycin A	150 ± 3.1	18 000 ± 2 600	120
Mitoxantrone	2.4 ± 0.20	110 ± 37	46
8-(4-Chlorophenylthio)-cAMP sodium	400 ± 54	18 000 ± 12 000	44
Chelerythrine chloride	59 ± 5.7	2 500 ± 480	42
Pimozide	340 ± 19	14 000 ± 3 300	41
8-Cyclopentyl-1,3-dipropylxanthine	530 ± 53	17 000 ± 7 900	31
4-Chloromercuribenzoic acid	690 ± 85	19 000 ± 3 000	27
Dihydroergocristine methanesulfonate	890 ± 57	20 000 ± 770	23
Dequalinium analog, C-14 linker	70.0 ± 4.6	1 000 ± 290	14
Calmidazolium chloride	510 ± 66	6 600 ± 750	13
Purvalanol A	820 ± 55	10 000 ± 3 200	12
Beta-Lapachone	850 ± 130	8 600 ± 2 900	10
Vinblastine sulphate salt	94 ± 6.0	780 ± 910	8.3
CGP-74514A ^a	210 ± 8.3	1 500 ± 620	7.1
Sanguinarine chloride	240 ± 17	1 400 ± 350	5.8
Diphenyleneiodonium chloride	120 ± 7.6	700 ± 870	5.7
Sphingosine	820 ± 78	4 600 ± 2 500	5.7
Quinacrine dihydrochloride	380 ± 13	1 900 ± 440	5.0
SKF 89145 hydrobromide ^b	610 ± 44	2 900 ± 200	4.7
Calcimycin	180 ± 13	750 ± 140	4.2
Idarubicin	20 ± 1.5	78 ± 29	3.9
Emetine dihydrochloride hydrate	7.9 ± 0.53	30 ± 3.1	3.8
Ellipticine	460 ± 45	1 700 ± 620	3.7
Mevastatin	940 ± 50	2 000 ± 360	2.2
Taxol	3.3 ± 0.071	6.6 ± 9.8	2
(S)-(+)-Camptothecin	530 ± 13	800 ± 99	1.5
Alsterpaullone	400 ± 40	510 ± 170	1.3
Vincristine sulphate	30 ± 1.0	0.60 ± 1.0	0.02

^a *N*²-(cis-2-Aminocyclohexyl)-*N*⁶-(3-chlorophenyl)-9-ethyl-9H-purine-2,6-diamine HCl

^b 4-(3,4-dihydroxyphenyl)-6-methyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine HBr

Figure

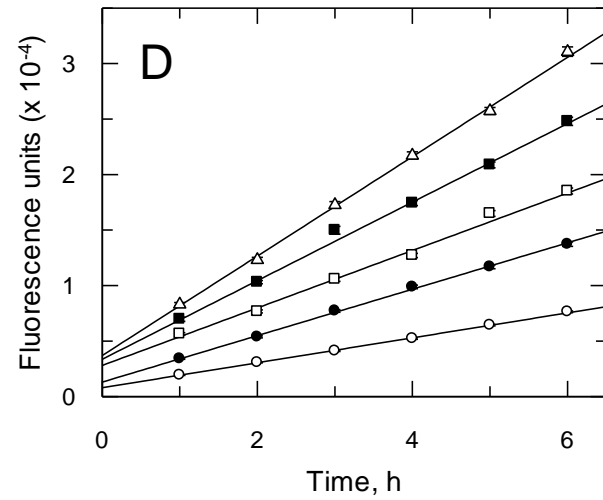
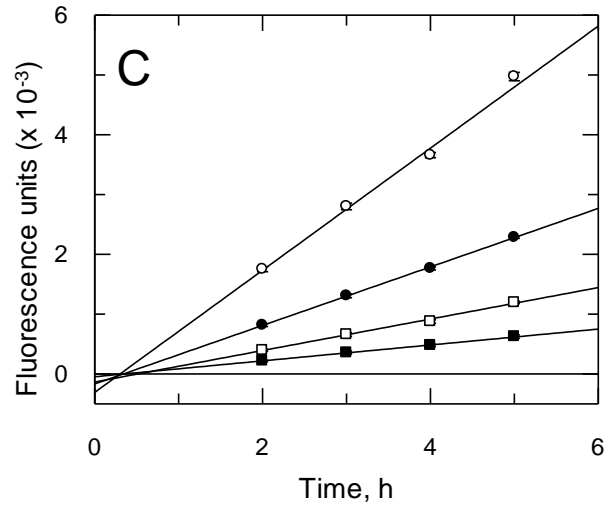
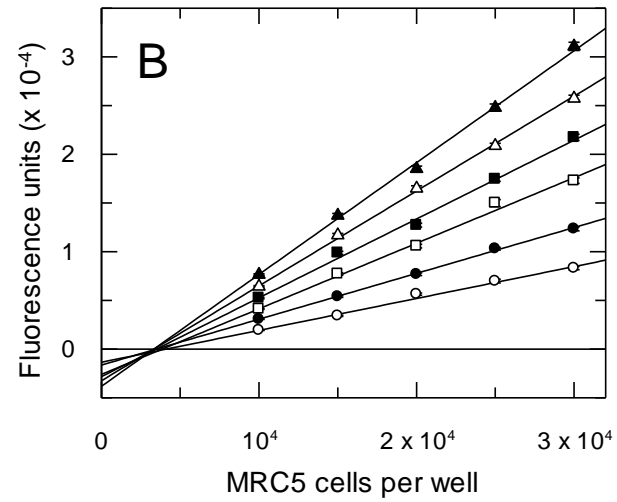
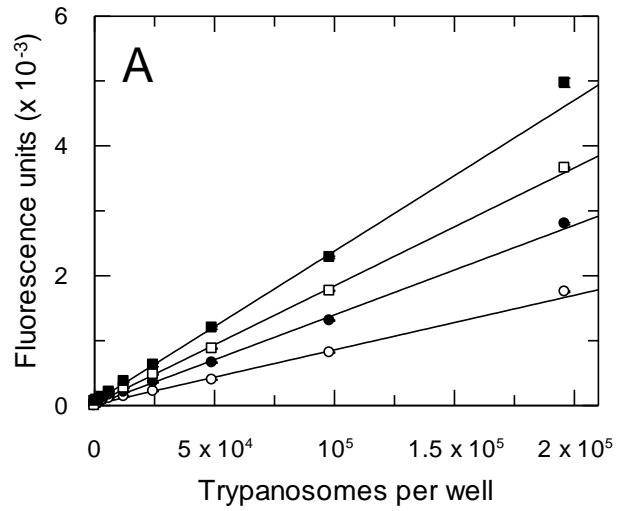


Figure 1 Jones et al

Figure

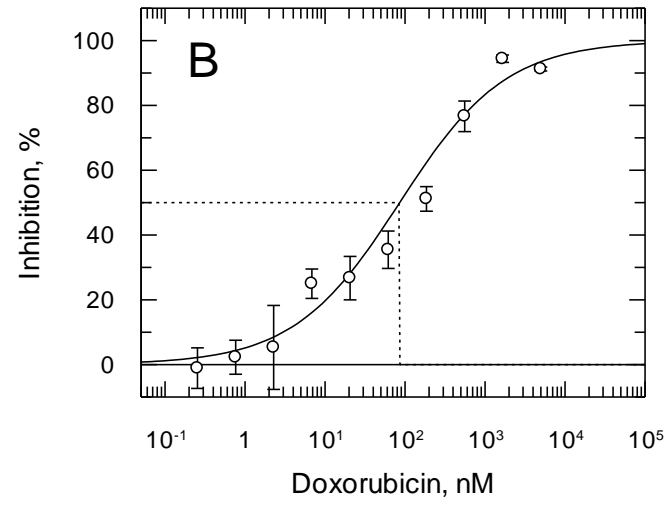
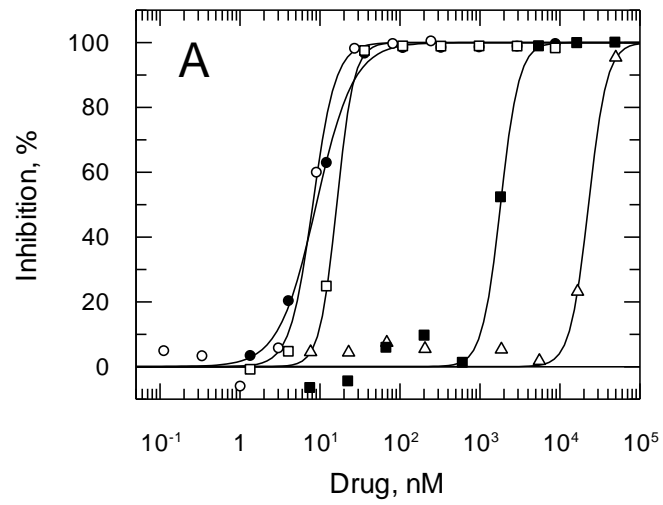
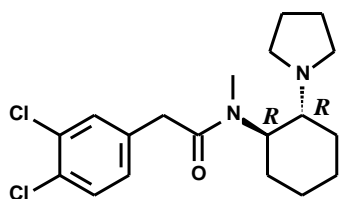
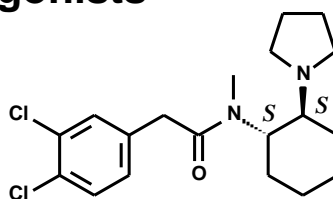


Figure 2 Jones et al

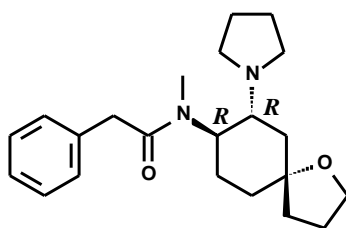
κ-Opioid agonists



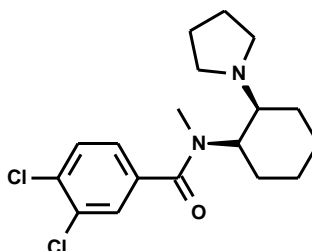
(+)-U50,488
 $0.06 \pm 0.01 \mu\text{M}$



(-)-U50,488
 $4.11 \pm 0.25 \mu\text{M}$

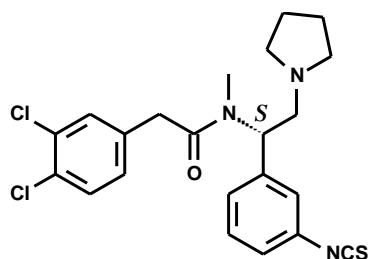


U69,593
 $> 100 \mu\text{M}$

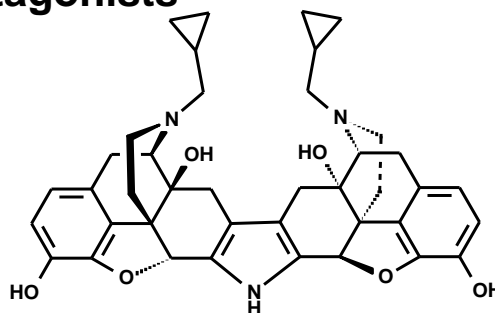


(+)-U54,494A
 $5.72 \pm 0.42 \mu\text{M}$

κ-Opioid antagonists

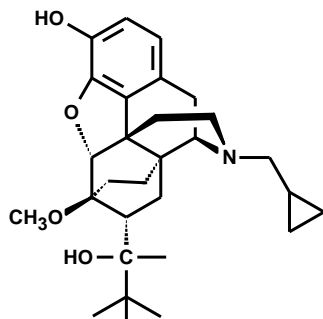


DIPPA
 $4.58 \pm 0.34 \mu\text{M}$



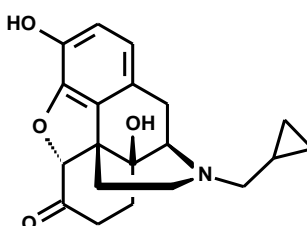
Norbinaltorphimine
 $> 50 \mu\text{M}$

μ-Opioid partial agonist

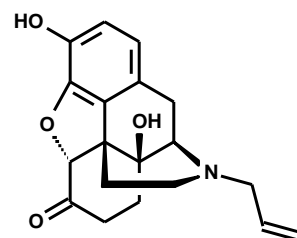


Buprenorphine
 $13.7 \pm 1.6 \mu\text{M}$

Broad spectrum opioid antagonists



Naltrexone
 $> 100 \mu\text{M}$



Naloxone
 $> 100 \mu\text{M}$

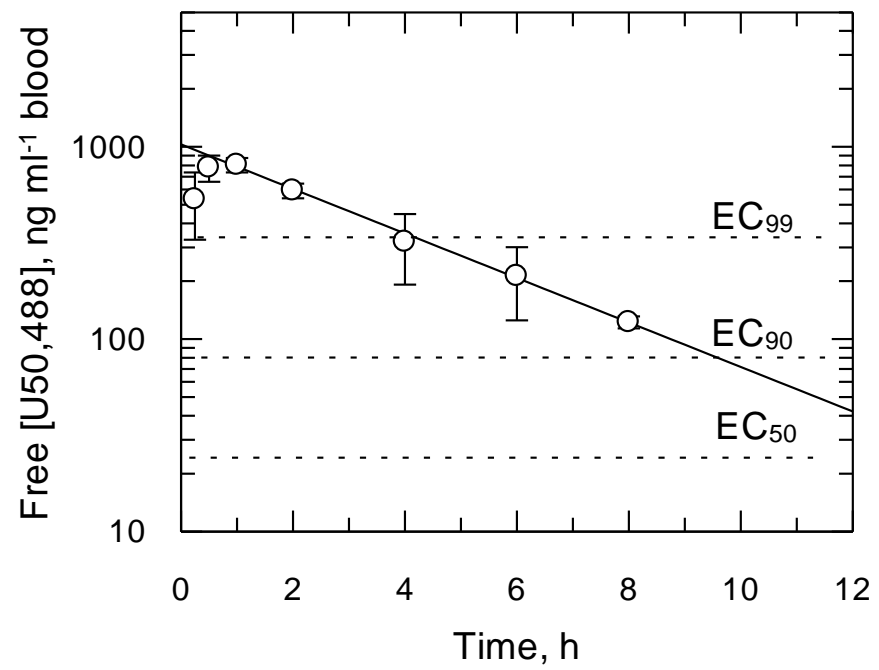


Figure 4 Jones et al

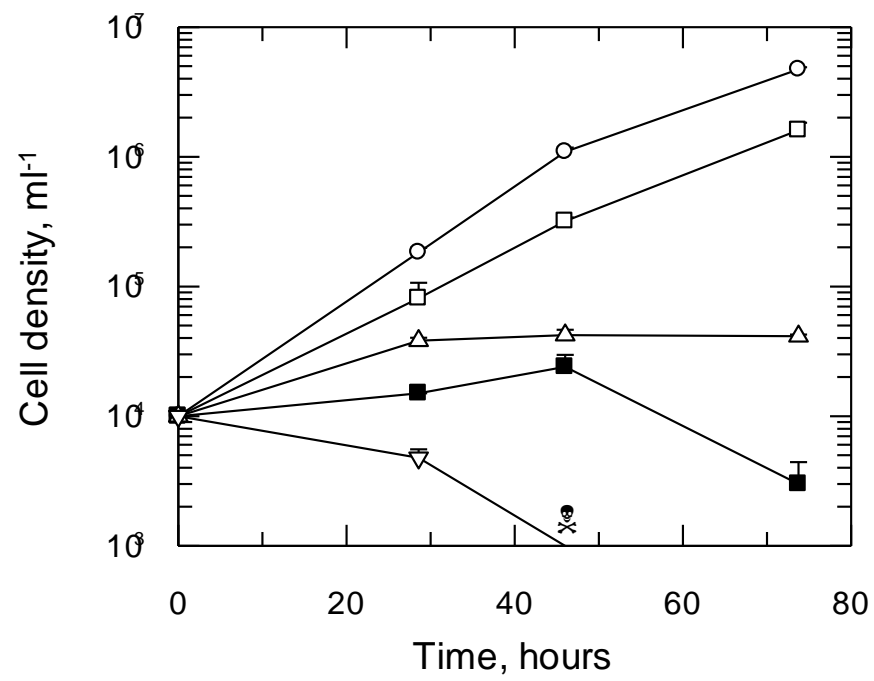
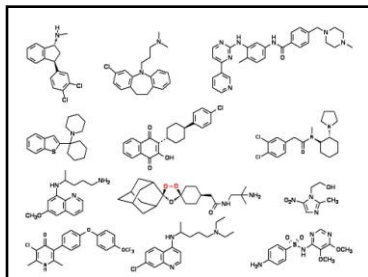
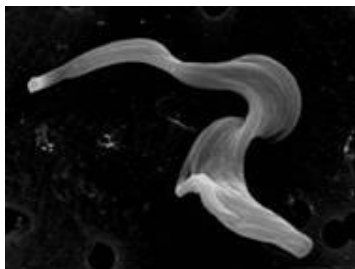


Figure 5 Jones et al

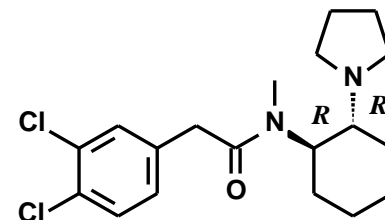
LOPAC Library



Phenotypic screen



(+)-U50,488



Phenotypic screening of the LOPAC library identified several potent and selective inhibitors of African trypanosomes. The κ -opioid agonist (+)-U50,488 represents a novel lead for drug discovery against sleeping sickness.

Accepted Manuscript