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

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ABSTRACT

A resazurin-based cell viability assay was developed for phenotypic screening of the LOPAC 1280 'library of pharmacologically active compounds' against bloodstream forms of *Trypanosoma brucei in vitro* identifying 33 compounds with EC_{50} values <1 μM. Counter-screening vs. normal diploid human fibroblasts (MRC5 cells) was used to rank these hits for selectivity, with the most potent (<70 nM) and selective (>700-fold) compounds being suramin and pentamidine. These are well-known antitrypanosomal drugs which demonstrate the robustness of the resazurin cell viability assay. The most selective novel inhibitor was (+)-*trans*-(1*R*,2*R*)-U50,488 having an EC_{50} value of 60 nM against *T. brucei* and 270-fold selectivity over human fibroblasts. Interestingly, (−)-U50,488, a known CNS-active κ-opioid receptor agonist and other structurally related compounds were >70-fold less active or inactive, as were several μ- and κ-opioid antagonists. Although (+)-U50,488 was well tolerated by the oral route and displayed good pharmaceutical properties, including high brain penetration, the compound was not curative in the mouse model of infection. Nonetheless, the divergence of antinociceptive and antitrypanosomal activity represents a promising start point for further exploratory chemistry. Bioinformatic studies did not reveal any obvious candidate opioid receptors and the target of this cytostatic compound is unknown. Among the other potent, but less selective screening hits were compound classes with activity against protein kinases, topoisomerases, tubulin, as well as DNA and energy metabolism.

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1. Introduction

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

Of the four drugs that are currently registered for use against HAT, pentamidine and suramin are used against the early stage of the disease; and melarsoprol and eflornithine (difluoromethylornithine, DFMO) are used against the late stage of the disease, when the infection has spread to the central nervous system (CNS). These treatments are beset with problems such as difficulties in administration (none are given orally), cost, duration of treatment, toxicity and resistance [3]. Melarsoprol treatment is highly toxic and responsible for iatrogenic deaths in 5% of patients. Eflornithine therapy is only effective against *T. b. gambiense* infections and presents severe economic and logistical problems in resource poor settings due to the need for 4 daily intravenous infusions over 14 days [4]. A recent clinical trial suggests the duration and frequency of treatment can be reduced by combination of eflornithine with nifurtimox [5], which may act as an interim solution until better and safer drugs are developed. The recent failure of the first orally active first stage drug, pafuramidine (DB289) [6], and rumours of increasing failures with eflornithine therapy underlines the urgent need for novel therapeutics.

Whilst many dominant paradigms of drug discovery focus on screening against molecular targets [7], there has been a resurgence of interest in phenotypic screening against whole parasites [8]. Phenotypic screening, particularly when in combination with a mammalian counter-screen, has the distinct advantage of addressing key druggability and toxicity issues early in drug discovery, thereby reducing attrition at later stages in development. In some cases phenotypic screening may identify

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novel molecular targets thereby accelerating drug development. However, understanding the mode of action of phenotypic screening hits can prove challenging since many drugs act by modulation of multiple intracellular targets (''network pharmacology'') [9]. Hit identification for novel targets is best approached with a screening library of diverse chemical space, but whole cell assays typically have a much lower throughput than molecular-target-based screens. Hence, we chose to screen a library of known pharmacologically active compounds against *T. brucei* cultured *in vitro* and to counter-screen actives against a human fibroblast cell line (MRC5 cells) to eliminate non-selective inhibitors. Potent and selective hits from such an approach can present exploitable shortcuts, particularly if they have already been used in humans with known dosing and toxicity information. Developing these hits could represent a low-risk, low-cost strategy for tackling orphan diseases of the poor [7].

The Library of Pharmacologically Active Compounds from Sigma–Aldrich (LOPAC 1280; international version) contains 1268 compounds that are ligands for many enzymes, receptors and ion channels in other organisms. Many are drug-like molecules and some are CNS active—an important consideration when seeking a replacement therapy for late-stage trypanosomiasis. Here we report over 30 compounds with EC_{50} values against *T. brucei* less than 1 μM, a concentration that should be readily achieved in plasma. Of these, one third have >20-fold selectivity with the κ-opioid receptor agonist U50,488 showing the greatest potency and selectivity. Some preliminary structure–activity relationships of CNS-active μ- and κ-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. b. brucei* cells (strain 427, 'single marker') were grown at 37 °C and 5% CO_2 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 2–3 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

Normal human MRC5 cells (diploid foetal lung fibroblasts) were used as a counter-screen for non-selective inhibitors. Stabilates were obtained from the European Collection of Cell Cultures (ECACC) were grown at 37 °C and 5% CO_2 in a humidified incubator in Eagle's Minimal Essential Medium (Sigma–Aldrich, Gillingham, UK) supplemented with 10% foetal bovine serum (Invitrogen, Paisley, UK). Stock cultures were maintained in T75 vented cap culture flasks with half the medium changed each day. Cells were split once confluent as follows: medium was removed and the monolayer washed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). Cells were detached by incubation for 2 min with 1 ml of trypsin EDTA solution (0.5 mg ml^{-1} trypsin, 0.2 mg ml^{-1} EDTA·4Na in Hanks balanced salt solution supplied by Invitrogen, Paisley, UK). Detachment was confirmed by microscopy. Fresh medium was added to the cells to subculture into new flasks at 2- to 4-fold the original volume as required.

2.4. Linearity of assay

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

2.5. DMSO tolerance of the assay

DMSO was serially diluted across a 96-well plate in 200 μl of the appropriate medium. For *T. brucei*, 100 μl aliquots were then transferred to a plate containing 100 μl cells at 2×10^3 ml^{-1}. Human fibroblasts were plated at 2×10^4 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 medium (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. Threefold 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. Columns 1 and 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}):

$$y = \frac{y_{max}}{1 + (i/EC_{50})^s} \quad (1)$$

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

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