Pharmacokinetics and pharmacodynamics utilizing unbound target tissue exposure as part of a disposition-based rationale for lead optimization of benzoxaboroles in the treatment of Stage 2 Human African Trypanosomiasis

**INTRODUCTION**

Prediction of the therapeutic dose and success of an anti-parasitic agent requires an understanding of the complex interaction between the inherent potency of the agent, the clinically relevant exposure required within the host’s target tissue and hence the organism, and the duration that exposure has to be sustained in that tissue to effect a cure. Potential disconnects between exposure in plasma and the target tissue must be considered early to ensure drug exposure is assessed in the pharmacologically relevant compartment (Louie et al. 2005). For example, in malaria, leishmaniasis, human African trypanosomiasis and African animal trypanosomiasis the relevant compartments would include blood erythrocytes, macrophages, brain and testes, respectively.

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**SUMMARY**

This review presents a progression strategy for the discovery of new anti-parasitic drugs that uses *in vitro* susceptibility, time-kill and reversibility measures to define the therapeutically relevant exposure required in target tissues of animal infection models. The strategy is exemplified by the discovery of SCYX-7158 as a potential oral treatment for stage 2 (CNS) Human African Trypanosomiasis (HAT). A critique of current treatments for stage 2 HAT is included to provide context for the challenges of achieving target tissue disposition and the need for establishing pharmacokinetic–pharmacodynamic (PK–PD) measures early in the discovery paradigm. The strategy comprises 3 stages. Initially, compounds demonstrating promising *in vitro* activity and selectivity for the target organism over mammalian cells are advanced to *in vitro* metabolic stability, barrier permeability and tissue binding assays to establish that they will likely achieve and maintain therapeutic concentrations during in-life efficacy studies. Secondly, *in vitro* time-kill and reversibility kinetics are employed to correlate exposure (based on unbound concentrations) with *in vitro* activity, and to identify pharmacodynamic measures that would best predict efficacy. Lastly, this information is used to design dosing regimens for pivotal pharmacokinetic–pharmacodynamic studies in animal infection models.

Key words: *In vitro* susceptibility, time-kill, pharmacokinetics–pharmacodynamics, tissue disposition, benzoxaboroles, HAT.
In this review, we describe a data-driven strategy (Fig. 1) developed during lead optimization studies that led to the discovery and development of SCYX-7158 as a potential oral treatment for Human African Trypanosomiasis (HAT). The strategy employed target tissue disposition, complemented with in vitro susceptibility, time-kill and reversibility data to identify the pharmacokinetic–pharmacodynamic measures that were associated with efficacy in a murine model of stage 2 (CNS) HAT. Data are presented for milestone compounds evaluated during lead optimization (Fig. 2).

Human African Trypanosomiasis is caused by infection with the eukaryotic protozoan parasite Trypanosoma brucei, which is transmitted with the saliva through the bite of an infected tsetse fly. HAT currently affects thousands of individuals in sub-Saharan Africa (WHO, 2006). Complete clearance of the parasite from the body presents a key tissue distribution challenge for drug discovery efforts insomuch as it initially lives in the bloodstream (Stage 1 disease) of the infected individual, then later migrates across the blood–brain barrier (BBB) to establish the fatal, if untreated, central nervous system (CNS) infection (Stage 2). For complete cures it is important to maintain therapeutic drug levels in the haemolymphatic and CNS compartments to address both stages of the disease. The time between initial infection and CNS infiltration is highly variable, and dependent upon the strain of parasite (e.g. Trypanosoma brucei gambiense vs Trypanosoma brucei rhodesiense) (Masocha et al. 2007). Because the symptoms of HAT prior to CNS infiltration by the parasite are relatively mild and non-specific (e.g. transient fever, general
malaise), and good diagnostic tests for the infection are not available, victims commonly progress to the CNS stage of the disease prior to seeking treatment.

Drugs to treat HAT, while available, are undesirable due to toxicity, difficulty of administration and ineffectiveness, particularly with respect to Stage 2 disease. The shortcomings of these treatments are discussed herein with emphasis on CNS disposition challenges and how they were addressed during the discovery of SCYX-7158. Data are compared for milestone compounds in the optimization campaign: AN2920, an early anti-trypansomal compound in the benzoxaborole series; AN3520, the first compound with oral pharmacokinetics commensurate with efficacy in the acute (Stage 1) murine HAT model, and SCYX-6759, the first compound to achieve 100% cures in the CNS (Stage 2) murine model following oral administration.

The optimization strategy employed in vivo time-kill and reversibility studies complemented with in vivo exposure data based on the total and unbound concentrations in plasma and target tissues to predict whether discovery-phase compounds had sufficient merit to progress to in vivo efficacy models, and if so, to design dosing regimens based on either single or multiple daily administrations that were most likely to provide sterile cures in rodent models. Taken together, we believe that the strategy, methods and data presented in this review provide a valuable starting point for other parasitic disease targets.

MATERIALS AND METHODS

Materials

Investigational compounds and the deuterium-labelled internal standard SCYX-5232 were synthesized by SCYNEXIS, Inc. (Research Triangle Park, NC) and were characterized by 1H-nmr and LC-MS before use. All compounds were purified to >95%. HPLC grade solvents were acquired from Fisher Scientific (Pittsburgh, PA). Formic acid (Fluka Analytical, ≥98% purity) was purchased from Sigma-Aldrich (St. Louis, MO) and ammonium formate (Alfa Aesar, 99% purity) was purchased from VWR International, LLC (West Chester, PA). Phosphate buffered saline (PBS) was from Gibco (Invitrogen Corporation, Carlsbad, CA).

Pharmacokinetic studies in mice

The CNS disposition and pharmacokinetics of the benzoxaboroles were evaluated in infected and uninfected mice following oral administration. In vivo phases of single-dose rodent PK studies were performed at Vivisource (Waltham, MA) following Institutional Animal Care and Use Committee (IACUC) review in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved facility. Studies in Trypanosoma brucei (TREU 667 strain) infected mice, or uninfected satellite animals, were performed after IACUC review at Pace University (New York, NY).

Male CD-1 or female Swiss Webster mice (approximately 25 g) were administered test compounds by oral gavage either once (QD) or twice per day (Q12 h) with doses ranging between 6 and 100 mg kg\(^{-1}\) as described below. All compounds were formulated as an in situ sodium salt in 2% (v/v) ethanol, 5% (m/v) dextrose, to yield typically clear colourless solutions that were administered at 4 mL kg\(^{-1}\). Blood samples for pharmacokinetic studies were collected into polypropylene tubes containing K\(_2\)EDTA anticoagulant and stored on wet ice until centrifuged and processed to plasma. Plasma was stored at −80°C. Blood samples from mice were collected via cardiac puncture under terminal anaesthesia. Rodents were euthanized in a CO\(_2\) chamber before collection of terminal blood or tissue samples. Brain tissues were blotted dry and stored at approximately −80°C.

Analysis of test compounds in biological samples

Tissue homogenization. On the day of analysis either fresh tissue (for binding studies) or freshly thawed tissue samples from PK studies were blotted dry, weighed and homogenized in 1 volume of PBS. The density of brain tissue used for the determination of volume was 1·043 g mL\(^{-1}\) (Allen et al. 1959).

Whole tissue samples were homogenized in parallel by means of a Precellys® 24 bead mill homogenizer (Bertin Technologies, France) using 1·5 mL Precellys tubes containing 1·4 mm diameter ceramic beads, (CK14), and a single 23 sec cycle of 6500 pulses per minute. Parallel bead homogenization increased throughput and avoided potential cross-contamination of samples as each was wholly contained and homogenized within its own sealed vial with no direct contact between the homogenizer instrument or other samples.

After homogenization, an additional volume of PBS was added to reduce viscosity. All tissue homogenates were treated in a bath sonicator at 22°C for approximately 5 min to remove air bubbles.

Preparation of biological samples for LC-MSMS. Plasma (50 μL) was treated with 4 volumes of ice-cold methanol (containing 25 ng mL\(^{-1}\) of either deuterium (d\(_6\))-labelled SCYX-7158 internal standard (SCYX-5232) for SCYX-7158 analyses or a related benzoxaborole for other compounds) to precipitate proteins. Treated samples were gently mixed on a 96-well plate shaker for approximately 10 min then centrifuged at approximately 3000 g and 15°C for
High-performance liquid chromatography with tandem mass spectrometry (LC-MSMS). All biological samples were assayed by a verified LC-MSMS method employing two dimensional chromatographic separation to overcome ion suppression (Annesley, 2003) caused by co-elution of endogenous components with benzoxaboroles. In summary, treated samples were loaded onto Phenomenex (Torrance, CA) Synergi Polar RP, 4 μ, 50 × 2 mm extraction columns and subsequently back-eluted onto a Phenomenex Luna C8(2) 3 μ, 50 × 2 mm analytical columns protected by a Phenomenex C8 guard cartridge. Mobile phases for extraction and analytical columns consisted of 5 mM ammonium formate and 0.1% (v/v) formic acid in water (A), and 5 mM ammonium formate and 0.1% (v/v) formic acid in methanol (B). Samples were loaded onto the extraction column in 40% B and back-eluted onto the analytical column using a linear gradient to 50% B between 0.55 to 1.55 min after loading the sample. A linear gradient from 5 to 95% B over 4.5 min was employed for analytical chromatography. Test compounds eluted at approximately 3 min, and the total run time was 6 min.

The instrumentation for two dimensional LC-MSMS comprised two Agilent 1100 series pumps (Agilent Technologies Inc., Santa Clara, CA), interfaced to either a API-3000 or API-4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) via a turbo-ion electrospray source operated in positive ionization mode. Eluent flow between extraction and analytical columns and the mass spectrometers was directed using two Valco 10-port valves (Valco Instruments Company Inc., Houston, TX). Samples were introduced to the system via CTC Pal Autosampler (Leap Technologies, Carboro, NC) set to inject 20 μL aliquots of sample extract through a 10 μL sample loop. The liquid chromatography system and all peripherals were controlled from within Analyst V.1.4.2 software.

Instrumental conditions for the mass spectrometer and precursor to product ion transitions were selected and optimized for sensitivity and selectivity. The MS/MS transitions for AN2920, AN3520, SCYX-6759 and SCYX-7158 were 292-2/133-2, 322-0/89-2, 340-1/191-1 and 368-0/191-0, respectively.

In vitro prediction of BBB permeability and Pgp-mediated efflux transport

The propensity of test compounds to cross the BBB was examined using an in vitro MDCKII-hMDR1 monolayer assay (Polli et al. 2001). In summary, MDCK-II-hMDR1 cells (Netherlands Cancer Institute, Amsterdam, the Netherlands) were seeded at a density of 3 × 10^5 cells per well onto microporous polycarbonate membranes in 12 well Costar Transwell plates (Corning Inc., Lowell, MA). The cells were incubated for 3 days during which time they formed confluent monolayers. Trans-epithelial resistance (TEER) was measured for each insert to ensure the integrity of the monolayer (acceptable TEER >160 Ω cm^2). The permeability and susceptibility for Pgp-mediated efflux was evaluated by adding each compound at a concentration of 3 μM, in the presence or absence of 2 μM GF120918 (a potent Pgp inhibitor), to the apical compartment. Competency of the Pgp efflux transporter was confirmed by assay of propranolol (non-substrate) and amprenavir (substrate). Cell monolayers were incubated in triplicate with shaking (160 rpm) at 37 °C in a 5% CO_2-enriched humidified atmosphere for 1 h. Samples were removed from the apical and basolateral compartments after incubation and assayed for test compound concentrations by LC-MSMS. Values for mass balance, P_appA–B, P_appA–B+GF918, and absorption quotient (AQ) were calculated for each compound (Troutman and Thakker, 2003; Thiel-Demby et al. 2004). Acceptance criterion for mass balance was 70–120%.

Plasma protein and tissue binding

Binding to plasma proteins, brain homogenates or proteins in serum-fortified HMI-9 media was determined by rapid equilibrium dialysis (RED) (Pierce, Rockford, IL) using a 48-well plate-based format according to the manufacturer’s instructions. Briefly, test compound was added to fresh plasma (Bioreclamation, Liverpool, NY) at the required concentrations, freshly prepared brain homogenate or media. Duplicate aliquots of each sample were transferred into the sample chambers of the RED devices, and dialysis buffer (BupH PBS) was added to the buffer chambers. The plates were sealed and incubated at 37 °C for 4 h. After dialysis, samples collected from the buffer and tissue chambers were treated with ice-cold methanol (3 volumes for plasma or HMI-9 media, 4 volumes for brain) to precipitate proteins. The treated samples were centrifuged for 10 min at approximately 3000 g at 15 °C. The supernatants were assayed for test compound by LC-MSMS. Calibration standards and quality control samples were prepared in matched matrix and assayed with samples. Values for unbound and bound fractions and mass balance were calculated.
Concordance of binding for each batch of plasma was confirmed by assay of warfarin, imipramine and carbamazepine. Acceptance criterion for mass balance was 70–120%.

In vitro time-kill assays and reversibility of trypanocidal effects

The time and concentration dependency for oxaborole-mediated killing of T. b. brucei, under constant (time-kill) and transient (reversibility) drug-pressure, was determined as described previously (Nare et al. 2010).

In summary, time-kill kinetics were determined using the CellTiter Glo kit (Promega Inc., Madison, WI) to measure trypanosome ATP content as a real-time indicator of viability. Test compounds were evaluated over the concentration range 0.01–10.0 μg mL$^{-1}$ to provide concentrations from sub-therapeutic levels to >5 fold the predicted minimum inhibitory concentrations (MIC) or maximal effects. Test solutions were prepared in HMI-9 media and dispensed into white wall-clear bottom 96-well plates (Corning Inc., Lowell, MA). Trypanosomes (1 × 10$^4$) were added to each well, incubated at 37 °C/5% CO$_2$ and data are presented as composite mean values.

To assess the potential effects of protein binding on trypanocidal activity, parasite viability assays were performed in the presence of increasing concentrations (2.5–50%) of foetal calf serum (Invitrogen, Carlsbad, CA) in HMI-9 media. T. b. brucei parasites were conditioned to the test serum concentrations for at least one round of passage prior to assay. Test compounds were added, incubated for 72 h, signal measured and IC$_{50}$ determinations were conducted as described above using the resazurin endpoint. The fold change in IC$_{50}$ from minimal serum concentration to maximal serum concentration was calculated and used to compare compounds and their potential for being restrictively protein bound.

Efficacy in the murine model for chronic (Stage2, CNS) HAT

The efficacy of benzoxaboroles was evaluated in a murine model of Stage 2 (CNS) HAT (Nare et al. 2010; Jacobs et al. 2011). In summary, mice (n = 10 per group) were infected by bolus i.p. injection of 1 × 10$^7$ T. b. brucei (TREU 667) parasites. A single bolus i.p. dose (10 mg kg$^{-1}$) of berenil (Sigma-Aldrich, St. Louis, MO) was administered on day 4 as a positive treatment control for haemolympathic-stage infection. Infection of the remaining animals proceeded for 21 days to establish a CNS infection before treatment with either a single dose of berenil (as above) or test compounds by the oral route either once or twice daily over 7 days at the doses indicated below. Animals were checked at least weekly for parasitaemia and were immediately removed from cages and euthanized upon recrudescence. Animals were considered cured if they were aparasitaemic for at least 180 days after treatment.

RESULTS AND DISCUSSION

Until recently, progress towards new treatments for HAT had been slow, owing to a lack of commercial interest and, most notably, a poor understanding of CNS disposition and pharmacokinetic–pharmacodynamic (PK–PD) relationships for Stage 2 drugs in particular. This shortcoming is currently being addressed by academic and non-profit drug research and development (R&D) organizations including the Drugs for Neglected Diseases initiative (DNDi), whose success has been demonstrated by delivery of two HAT compounds, Fexinidazole (Torreele et al. 2010) and SCYX-7158 (Jacobs et al. 2011), into clinical development. The target product profile (TPP) for these new agents included the requirement for oral delivery and the ability to treat both stages of the disease using a single dosing regimen. The PK–PD strategy described herein reflects the approach developed for the
optimization and selection of SCYX-7158 as a pre-clinical and clinical candidate for the treatment of Stage 1 and Stage 2 HAT.

Pharmacokinetic–pharmacodynamic measures
Pharmacokinetic–pharmacodynamic (PK–PD) relationships establish whether a drug’s efficacy increases with dose and, if so, help determine which PK parameter can best be used with MIC to predict outcome. Typical measures used to predict efficacy are summarized in Fig. 3 and include: (a) the time above MIC ($t >$MIC), (b) the ratio of peak concentration and MIC ($C_{\text{max}}$/MIC), (c) the ratio of the area under the concentration vs time profile (AUC) while concentration is greater than MIC, or (d) the ratio of the area under the concentration vs time profile (AUC) measured over the dosing interval or 24 h to MIC (AUC$_{0-24h}$/MIC) (Mueller et al. 2004). The most predictive measure guides whether an optimized dosing regimen employs either a fewer number of larger doses where efficacy is driven by $C_{\text{max}}$, or more frequent but lower doses to maintain an AUC/MIC (average concentration/MIC) multiple. In either regimen it is generally desirable to maintain tissue concentrations above the MIC throughout the treatment period to lower the risk of the target organism generating resistance.

Target tissue disposition for HAT
CNS disposition challenges for currently approved Stage 2 HAT drugs. One current first line treatment for Stage 2 HAT, melarsoprol, has been shown to be highly toxic, causing drug-induced death in 2–10% of patients (Schmid et al. 2004; Doua et al. 2005; Kuepfer et al. 2012). Nonetheless, it is active in treating both T. b. gambiense and T. b. rhodesiense. Reports of treatment failures with melarsoprol (Matovu et al. 2001) are suggestive of development of drug resistance (Delespaux and de Koning, 2007) although evidence of significant change in the sensitivity of T. b. gambiense strains to melarsoprol appears elusive (Likefack et al. 2006). Another possibility is that treatment failures may arise from inconsistent or inadequate CNS exposure of this drug. Indeed, failure to achieve sustained cerebrospinal fluid (CSF) exposure (a surrogate for the unbound fraction in brain tissue (Shen et al. 2004; Summerfield and Jeffer, 2006; Lin, 2008)) is thought to contribute to the high (3–30%) failure rate with melarsoprol treatment (Legros et al. 1999). Pre-clinical and clinical studies support this claim. Following intravenous administration (doses of 2.2–3.6 mg kg$^{-1}$) maximum concentrations of melarsoprol in the CSF of uninfected vervet monkeys were very low (<50 ng mL$^{-1}$) despite serum levels of the drug being substantially higher (1.7–3.8 μg mL$^{-1}$) (Burri et al. 1994). Equivalent concentrations of melarsoprol have been reported in both the serum (0.87–2.2 μg mL$^{-1}$) and CSF (39–79 ng mL$^{-1}$) of human patients 24 h after the last injection of a multi-day treatment regimen (Burri and Keiser, 2001). The in vitro MIC reported for melarsoprol is 2–100 ng mL$^{-1}$ (Burri et al. 1994) suggesting therapeutic levels may neither be achieved nor sustained. Increasing dose is contra-indicated because of a higher risk of melarsoprol-induced encephalopathy.
An alternative treatment for Stage 2 HAT, eflorenceine, while better tolerated than melarsoprol, is potentially less attractive because it is active only against T. b. gambiense and must be administered by large intravenous infusion doses (400 mg kg\(^{-1}\) day\(^{-1}\)) four times per day over fourteen days (Priotto et al. 2008). This treatment paradigm is very difficult to achieve in disease-endemic regions. Recently, clinical studies have shown that treatment duration maybe shortened to 7 days using a combination therapy of nifurtimox and twice daily eflorenceine (Priotto et al. 2007); however, the complexity of parenteral administration remains.

Like melarsoprol, CNS exposure of eflorenceine is limited. In mice, maximum concentrations of eflorenceine only reach 10–40 nM, representing <0.1% of the administered eflorenceine dose, and far below the reported in vitro MIC for the drug (ca. 50 μM) (Sanderson et al. 2008). In this study, the authors included uninfected mice as controls to allow comparison of CNS exposure in healthy animals to those infected with T. b. brucei GVR35, a strain of the parasite commonly used in murine models of Stage 2 HAT (Jennings and Gray, 1983) as it readily establishes a CNS infection. The permeability of eflorenceine across the BBB was similar between infected and uninfected animals for 21 days following infection, although greater permeability was observed for late-stage infected mice at days 28 and 35 post-infection. Sanderson et al. (2009) have also reported that the integrity of the BBB is maintained until late stage CNS infection. These observations, coupled with reports of interactions of T. brucei with the BBB in both in vitro (Grab et al. 2004) and in vivo (Masocha et al. 2007) models suggest a dynamic translocation of parasites between the periphery and the brain, with only modest modification of the BBB. Consequently, stage 2 drugs must be able to circumvent the physical and biological barriers present in the fully intact BBB to the extent that they achieve and maintain therapeutic levels within brain parenchyma and CSF. Reports for the pharmacokinetics of eflorenceine in human patients are sparse but are consistent with those derived from animal models, suggesting that CSF levels of eflorenceine at steady state are generally low (5–150 nM) relative to concentrations required to be efficacious in vitro (Milford et al. 1993).

**Progression pathway and identification of pharmacokinetic–pharmacodynamic measures based on unbound target tissue exposure**

A data-driven strategy (Fig. 1) was developed during lead optimization studies for SCYX-7158 that has broad applicability in drug discovery for parasitic disease targets. The strategy employs ‘Tier 1’ in vitro metabolism and disposition assays, with in vitro parasite susceptibility assays, to prevent progression of compounds that would likely fail in animal infection models. Tier 1 assays may also prioritize compounds for ‘Tier 2’ in vitro target tissue disposition, and in vitro time-kill and reversibility assays used to identify the pharmacokinetic–pharmacodynamic measures that may be associated with efficacy in animal infection models such as the murine model of Stage 2 (CNS) HAT. In vitro and in vivo PK–PD measures may then be correlated to provide a rationale for clinical dosing regimens.

**Tier 1: goals for potency and drug-like properties during lead optimization**

In vitro susceptibility of T. brucei. Demonstration of in vitro potency and selectivity against a target organism vs host is typically the first hurdle in advancing a compound through the progression pathway to pre-clinical candidate selection. The milestone oxaborole carboxamides (Fig. 2, Table 1) each exhibited potent activity against T. b. brucei, T. b. rhodesiense and T. b. gambiense in vitro, and achieved >40 fold selective inhibition of parasite compared to the L929 mammalian cell line employed as an in vitro predictor of cytotoxicity and therefore selectivity (Nare et al. 2010). The IC\(_{50}\) is defined as the concentration of compound that is required to decrease parasite viability by 50%, and the minimum inhibitory concentration (MIC) is the lowest concentration that completely inhibits parasite growth (Jacobs et al. 2011).

In vitro DMPK assays: establishing drug-like properties. Compounds with in vitro anti-parasitic activity must also demonstrate adequate ‘drug-like’ properties; namely, solubility, in vitro metabolic stability in the presence of liver sub-cellular fractions (typically microsomes or S9 fraction) (Mandagere et al. 2002), non-restrictive binding to plasma proteins (Mackichan, 2005; Summerfeld et al. 2006) and, for oral compounds, permeability in either MDCKI-hMDR1 or Caco-2 monolayer assays (Mandagere et al. 2002). The latter can also provide insight on the compound’s ability to cross blood-tissue barriers such as the blood–brain, blood–testicular or blood-retinal barriers, and interact with drug efflux or uptake transporters that can either attenuate or enhance disposition within a target tissue.

The ability of a drug to cross the BBB and gain entry into the CNS compartment reflects interplay between membrane permeability, protein binding and drug transporters, notably efflux transporters such as P-glycoprotein (Pgp) or Breast Cancer Related Protein (BCRP). It is also worth considering that these parameters may impact the ability of a compound to penetrate the target parasite.
In a study comparing the properties of CNS and non-CNS antihistamines, compounds with high brain-to-plasma concentration ratios typically achieved high passive permeability (>150 nm s$^{-1}$) in MDCK-MDR1 transport assays, moderate protein binding (<84%), and were not Pgp substrates (Mahar Doan et al. 2004). In contrast, compounds with low brain-to-plasma ratios typically displayed low passive permeability (<100 nm s$^{-1}$), were substrates for Pgp and had higher binding to plasma proteins. Exceptions, such as terfenidine, were noted which, although being Pgp substrates, had high passive permeability that was able to overcome the transporter-mediated efflux (Polli et al. 2001). *In vitro* permeability, although a helpful predictor of CNS penetration, reflects the potential rate and is a surrogate for the actual extent of brain drug delivery. The latter is the more helpful parameter for developing models and data on unbound tissue exposure are preferred for developing PK–PD models.

*In vitro* protein binding and permeability data for the milestone benzoxaboroles during the discovery of SCYX-7158 are presented in Table 1. Oxaboroles as a class generally met the criteria for CNS penetration with milestone compounds demonstrating very high permeability values (PappA$^{\text{B}}$, >350 nm s$^{-1}$) in the MDCK-MDR1 transport assay, and were not substrates for the Pgp efflux transporter (AQ<0·3 (Thiel-Demby et al. 2004)). Less desirably, protein binding was modest to high 93·2–98·7% and likely hampered CNS penetration such that in single-dose studies in mice the brain to plasma ratio was typically less than unity (Fig. 4). Protein binding was also a concern because it may also attenuate *in vivo* potency.

**Table 1. In vitro activity of milestone benzoxaboroles against *T. brucei* subspecies**

<table>
<thead>
<tr>
<th>Compound</th>
<th>T. b. brucei</th>
<th>T. b. rhodesiense</th>
<th>T. b. gambiense</th>
</tr>
</thead>
<tbody>
<tr>
<td>STIB929</td>
<td>4·27</td>
<td>1·13</td>
<td>0·29</td>
</tr>
<tr>
<td>STIB930</td>
<td>4·90</td>
<td>1·13</td>
<td>0·29</td>
</tr>
<tr>
<td>AN2920</td>
<td>0·17</td>
<td>0·04</td>
<td>0·29</td>
</tr>
<tr>
<td>AN3520</td>
<td>0·04</td>
<td>NA</td>
<td>0·29</td>
</tr>
<tr>
<td>SCYX-6759</td>
<td>0·07</td>
<td>0·06</td>
<td>0·29</td>
</tr>
<tr>
<td>SCYX-7158</td>
<td>0·07</td>
<td>0·06</td>
<td>0·29</td>
</tr>
</tbody>
</table>

Serum shift assay and the impact of drug binding to plasma proteins or tissues on potency. Drug distribution in plasma and tissue reflects equilibrium between bound and unbound (free) fractions, where the unbound fraction is deemed to be pharmacologically active and also the target for drug clearance mechanisms. As unbound drug is cleared from a compartment either by elimination mechanisms or, for example, uptake into a target cell or parasite, bound drug in plasma or tissue interstitial spaces dissociates to re-establish the equilibrium. Restrictive or non-restrictive binding has been used qualitatively to describe the dissociation rate ($K_d$) that, rather than the binding extent, influences whether binding impacts potency (Fig. 5) (MacKichan, 2005). Despite its importance, $K_d$ is rarely measured during lead optimization of ‘small molecule’ drugs owing to the complexity of the measurement. More frequently, the *in vitro* ‘serum-shift’ assay is performed as a surrogate that assesses the impact of increasing serum concentration on potency (IC$_{50}$). In this assay, the trypanocidal activity of benzoxaboroles appeared only modestly attenuated by
In a comparison, the fold changes in IC₅₀ between 2·5 and 50% calf serum for SCYX-7158 and suramin were 3·6 and 26, respectively, suggesting SCYX-7158 is less restrictively bound (Fig. 6).

A marked loss in potency in a serum-shift assay provides a warning that in vitro potency in a low serum media may not translate to in vivo efficacy where the candidate drug maybe bound thereby achieving sub-therapeutic concentrations. As a general strategy it is more reliable to build PK–PD models based on unbound concentration in the target compartment rather than total drug levels.

### Table 2. Comparison of in vitro protein binding and MDCKII-hMDR1 permeability

<table>
<thead>
<tr>
<th>In vitro study</th>
<th>Compound</th>
<th>MN2920</th>
<th>AN3520</th>
<th>SCYX-6759</th>
<th>SCYX-7158</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding to plasma protein</td>
<td>Human plasma (1 μg mL⁻¹, n=3)</td>
<td>93·2±0·836</td>
<td>95·3±0·425</td>
<td>97·7±0·053</td>
<td>98·7±0·018</td>
</tr>
<tr>
<td>Unbound fraction (%)</td>
<td>6·8</td>
<td>4·7</td>
<td>2·3</td>
<td>1·3</td>
<td></td>
</tr>
<tr>
<td>Mass balance (%)</td>
<td>106</td>
<td>110</td>
<td>97</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>MDCK-MDR1 Permeability</td>
<td>Incubation concentration (μM)</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Papp, A→B direction (nm s⁻¹)</td>
<td>438</td>
<td>424</td>
<td>379</td>
<td>414·8</td>
<td></td>
</tr>
<tr>
<td>Papp, A→B direction+GF918 (nm s⁻¹)</td>
<td>465</td>
<td>434</td>
<td>386</td>
<td>426·9</td>
<td></td>
</tr>
<tr>
<td>Mass balance (A→B, A→B+GF918, %)</td>
<td>94%, 99%</td>
<td>92%, 90%</td>
<td>89%, 95%</td>
<td>79%, 75%</td>
<td></td>
</tr>
<tr>
<td>Absorption quotient</td>
<td>0·06</td>
<td>0·02</td>
<td>0·02</td>
<td>0·03</td>
<td></td>
</tr>
<tr>
<td>P-gp classification</td>
<td>Non-substrate</td>
<td>Non-substrate</td>
<td>Non-substrate</td>
<td>Non-substrate</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4. Total concentration vs time curves for AN2920, AN3520, SCYX-6759 and SCYX-7158 in male CD-1 mice following a single oral dose. Data are normalized to a 50 mg kg⁻¹ dose to allow direct comparison of exposure between compounds. The MIC line is defined as the lowest concentration of each compound that completely inhibits visible parasite growth. Data points for plasma (solid lines) and brain (dotted lines) represent a single mouse at each time point.
Unbound fraction and concentrations in target tissues. CSF has been employed successfully as a surrogate for the unbound fraction in brain tissue. However, systemic drugs can enter the CSF by 2 main routes; either indirectly via passage across the BBB followed by diffusion from the brain interstitial fluid into the CSF, or directly via passage across the choroid plexus. In the latter instance, drug in CSF would thus not represent unbound concentration in brain interstitial fluid. A further complication of using CSF is the practical challenge of collecting an adequate volume from mice that is also free of contamination by blood. Consequently, in vitro ultrafiltration or equilibrium dialysis techniques, with either fortified samples of tissue homogenate or ex vivo tissue samples (Fridén et al. 2007; Watson et al. 2009), are most practicable for assessing unbound concentrations in tissues.

At steady state, permeable compounds unaffected by active uptake or efflux mechanisms should achieve generally similar unbound concentrations in tissue and plasma although the unbound fractions may differ. The binding of SCYX-7158 to mouse and human plasma was concentration dependent, with the unbound fraction increasing from 0.3 to 4.6% and from 0.3 to 3.9% in mouse and human plasma, respectively (Fig. 7). The most rapid increase in the unbound fraction occurred between 0.1 and 10 μg mL$^{-1}$ SCYX-7158. Interestingly, the binding to mouse brain tissue was generally similar across the concentration-range studied (Fig. 7). The relationship between unbound fraction in each matrix and total concentration was calculated to allow conversion from total to free concentrations in PK studies. The SCYX-7158 concentration dependent binding to proteins in the cell culture media used for in vitro trypanosomal assays was also determined to allow analysis of time-kill and reversibility data, based on free concentrations and to identify the IC$_{50u}$ and MIC$_{u}$ based on free concentration.

To progress to Tier 2 assays, a compound should be non-cytotoxic to mammalian cells and suitable for administration by a route and dose likely to achieve and ideally maintain $\geq$ ED$_{90}$ unbound concentration in plasma or tissue.

Tier 2: goals for tissue disposition and efficacy in infection models

In vitro time-kill and reversibility of exposure to benzoxaboroles. Time kill and reversibility curves based on total drug exposure for the milestone benzoxaboroles, including SCYX-7158, have been reported previously (Nare et al. 2010; Jacobs et al. 2011). In summary, time-kill studies demonstrated that the onset of trypanocidal behaviour was rapid (<24 h) and concentration dependent, reaching maximal effect at approximately 4 fold the MIC.
Transient exposure to benzoxaboroles in reversibility studies further demonstrated that a persistent effect on trypanosome survival was dependent on both exposure time and concentration.

To elucidate whether either drug concentration, AUC/MIC or exposure time have the greater impact on trypanosome survival, and to allow correlation between in vitro and in vivo efficacy endpoints, exposure time and concentration in the reversibility assay were considered in the context of exposure expressed as AUC (area under the concentration vs time curve) for unbound concentrations (Fig. 8).

The lowest AUC affording a 100% trypanocidal effect ($5.81 \mu g \cdot h \cdot mL^{-1}$) was achieved following incubation with an unbound concentration of $0.242 \mu g \cdot mL^{-1}$ ($C_t$, $0.625 \mu g \cdot mL^{-1}$) SCYX-7158 for 24 h. Interestingly, at the next highest concentration studied ($C_m$, $1.11 \mu g \cdot mL^{-1}$ equivalent to a $C_t$, $1.25 \mu g \cdot mL$) this target AUC was achieved by 6 h;
However, parasites were able to tolerate the higher concentration, for 8 h without impact on survival thereby suggesting treatment time rather than absolute concentration may be the more important factor in determining efficacy. This observation will be important when designing a dosing regimen for in vivo efficacy studies because it implies sustaining exposure above an MIC level (AUC/MIC) is more important than achieving a maximum concentration. Further, tolerability in clinical treatment regimens is often limited by the maximum concentration achieved shortly after dosing so the opportunity for lower doses and hence more modest maximal concentrations may improve safety margins and patient compliance.

In vivo pharmacokinetics and CNS penetration of oxaboroles. Total brain concentration is a helpful first step in assessing CNS penetration relative to MIC to prevent progression of compounds that do not achieve sufficient exposure and would likely fail in Stage 2 efficacy models. A standard in vivo protocol would involve dosing the target pre-clinical efficacy species, usually rodents, by intended administration route and then collecting paired blood and tissue samples at selected times post-dose to allow calculation of the tissue-to-plasma (or whole blood) ratio for each sample pair and, more reliably, the ratio based on overall exposure (AUC, area under the concentration vs time profile). Total brain exposure is first corrected for the amount of drug in the residual blood using the following parameters: plasma volume, ~15 μl g⁻¹ brain tissue (Vérant et al. 2007; Friden et al. 2009), murine microvascular haematocrit, 30% (Vérant et al. 2007); density of brain tissue, 1·043 g mL⁻¹ (Allen et al. 1959), and density of blood 1·046 g mL⁻¹ at 30% haematocrit (Friedman, 1959).

AN2920, the original lead compound, achieved moderate plasma exposure maintaining concentrations above its MIC to approximately 7 h post dose, consistent with Stage 1 efficacy (Fig. 4); but CNS exposure above MIC was only achieved for 1 h. AN3520 was selected based on improved plasma exposure and potency and modestly extended CNS exposure; but concentrations in CNS were not consistent with likely efficacy in the CNS model of the disease even if dosed twice daily. In contrast, SCYX-6759 and SCYX-7158 maintained total CNS concentrations above their MIC for approximately 10 and 20 h, respectively, potentially consistent with twice or once daily dosing in a Stage 2 model. Both compounds were progressed to dose range-finding studies in acute and chronic murine models of HAT (Nare et al. 2010; Jacobs et al. 2011). PK–PD models based on the unbound brain concentrations of SCYX-7158 vs cure rate in the chronic HAT model are described below.

Tier 3: Pharmacokinetics–pharmacodynamics

Pharmacokinetics–pharmacodynamics of SCYX-7158 in the Stage 2 (CNS) murine HAT model. In vitro reversibility assays for SCYX-7158 indicated that exposure time and AUC/MIC were important in predicting efficacy. The minimum AUC based on unbound concentration and 24 h exposure for complete and irreversible inhibition of parasite growth was 5·81 μg h mL⁻¹. This corresponds to a target average unbound concentration at steady-state (C_uSS(AVE)) in brain of 0·242 μg mL⁻¹ which, by definition, corresponds to the unbound MIC. Tantalizingly, partial trypanocidal activity, 24% reduction in survival relative to untreated control parasites (Fig. 8), was also observed in the reversibility assay at a lower concentration (C_u, 0·123 μg mL⁻¹; C_o, 0·313 μg mL⁻¹) after 24 h incubation, thereby suggesting that prolonging exposure may allow for a lower dose.

Pharmacokinetic studies were performed in CD-1 mice to establish AUC₀−₂₄ following single and 7 × once daily oral doses of 6·25, 12·5, 25, 50 and 100 mg kg⁻¹ SCYX-7158 (Jacobs et al. 2011). Steady-state data were generated in uninfected mice dosed concurrently with those being treated following infection with T. b. brucei (TREU 667) for the Stage 2 HAT model. Values for unbound AUC₀−₂₄ in brain were calculated using the single and steady-state exposure data (non-parametric super-positioning indicated steady-state was mostly attained on day 2 so day 7 data were employed for days 2–7 rather than modelled data for days 2–6) and compared to the target unbound AUC from the in vitro reversibility studies (Fig. 9).

All animals treated with the lowest oral dose (6·25 mg kg⁻¹ day⁻¹) for 7 days were cleared of haemolymphatic T. b. brucei but demonstrated recrudescence of parasitaemia around day 35 after infection. Treatment with 12·5 mg kg⁻¹ SCYX-7158 once daily for 7 days achieved an 80% cure rate, and higher doses (≥25 mg kg⁻¹ day⁻¹) afforded complete cures. Comparison of unbound AUC₀−₂₄ in brain tissue (Fig. 9) on study days 1–7 demonstrated that animals receiving 25 mg kg⁻¹ approached the reversibility assay MIC_u AUC₀−₂₄ (5·81 μg h mL⁻¹) on day 1 (4·98 μg h mL⁻¹) and exceeded it for treatment days 2–7 (7·15 μg h mL⁻¹) suggesting that the reversibility data were predictive of efficacy. Interestingly, the unbound AUC₀−₂₄ for the animals receiving 12·5 mg kg⁻¹ SCYX-7158 was approximately 50% of the target MIC_u AUC (day 1, 2·41 μg h mL⁻¹ and day 2–7, 2·98 μg h mL⁻¹) yet yielded 80% cures, thus suggesting prolonged exposure at lower AUC can achieve cures.

Examination of the unbound concentration vs time curves (not shown) for the animals receiving 12·5 mg kg⁻¹ SCYX-7158 indicated that unbound plasma concentrations in brain tissue exceeded the...
MIC$_u$ (0·242 μg mL$^{-1}$) and IC$_{50u}$ (0·082 μg mL$^{-1}$) for only 4 and 10 h, respectively on each day. In vitro reversibility data indicated that both exposure time and AUC are important for efficacy and thus the administration of the 12·5 mg kg$^{-1}$ as a divided dose (2 × 6·25 mg kg$^{-1}$) on a Q12 h regimen may improve efficacy beyond 80% by maintaining exposure for longer, albeit with lower values for Cmax. This hypothesis could be also evaluated in vitro by extending exposure times in the reversibility assay.

The need for continued exposure was further indicated in a separate murine Stage 2 HAT study where infected mice were treated with 50 mg kg$^{-1}$ SCYX-7158 for either 1, 3, 5 or 7 days. As expected, a single oral 50 mg kg$^{-1}$ dose achieved an unbound AUC$_{0−24}$ (18·2 μg h mL$^{-1}$) that markedly exceeded the in vitro MIC$_u$ AUC (5·8 μg h mL$^{-1}$) allowing animals to achieve a potentially therapeutic CNS exposure after a single treatment; however, 100% cure rates were only achieved after ≥3 days of dosing. No animals were cured after a single dose, thereby suggesting in vitro exposure beyond 24 h is required.

CONCLUSIONS

In conclusion, we have successfully utilized a data-driven strategy based on target tissue disposition, complemented with in vitro susceptibility, time-kill and reversibility assays to deliver SCYX-7158 as a potential oral treatment for stage 2 (CNS) HAT. The in vitro reversibility assay was employed to correlate exposure based on unbound concentrations with activity, and to identify that exposure time with AUC/MIC were the PK–PD measures that would best predict efficacy in a murine model of chronic (CNS) HAT. Finally, pharmacokinetic studies in uninfected mice dosed concurrently with those infected with T. b. brucei (TREU 667) demonstrated that 100% cure rates are achieved when the unbound AUC$_{0−24}$ in brain tissue meets or exceeds the MIC$_u$ AUC determined in the in vitro reversibility assay. Both the in vitro reversibility assay and the murine model suggest that cures maybe achieved at a lower exposure providing exposure is maintained for extended periods. Additional studies to explore this possibility further are planned and will be reported in due course.

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