Oral eflornithine treatment of late-stage human African trypanosomiasis

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ABSTRACT

Human African trypanosomiasis is a fatal disease unless treated. It is a parasitic vector borne disease endemic in sub-Saharan African countries. Eflornithine is a recommended treatment for *gambiense* human African trypanosomiasis (g-HAT) in the later disease stage when the parasites have infected the central nervous system. Eflornithine is currently dosed as a racemic mixture of D- and L-eflornithine via repeated intravenous infusions, which comes with several disadvantages. The work in this thesis aimed to assess the feasibility of an oral eflornithine treatment. A chiral liquid chromatography method was developed for separation and preparation of the D- and L-eflornithine enantiomers from the racemic mixture. The acquired enantiopure material was used to determine that L-eflornithine had higher antiparasitic *in vitro* potency compared to D-eflornithine. The *in vitro* findings were used with a mathematical modeling approach to predict survival in late-stage g-HAT patients treated with L-eflornithine using pharmacodynamic time-to-event modeling. The *in vivo* pharmacokinetics in the rat after oral or intravenous doses of enantiopure L-eflornithine was characterized using nonlinear mixed effects modeling and compared to the racemic mixture. Moreover, the distribution of D- and L-eflornithine to the third brain ventricle from the systemic circulation was examined using *in vivo* microdialysis. Clinical pharmacokinetics in plasma and cerebrospinal fluid for L-eflornithine was modeled using literature data. The pharmacokinetic model was used to predict drug exposure and estimate the probability of target attainment for oral L-eflornithine-based treatments against late-stage g-HAT. L-eflornithine administered as monotherapy dosed at 750 mg/kg/day four or twelve times daily could serve as efficacious regimens. In combination with nifurtimox, dose regimens of L-eflornithine at 375 mg/kg/day dosed two, four or twelve times daily could be efficacious. These results are based on *in vitro* and preclinical *in vivo* data as well as clinical data using a translational modeling and simulation approach. Future clinical pharmacokinetic studies are warranted to assess the feasibility of an oral L-eflornithine-based treatment and to establish optimal treatment strategies against late-stage g-HAT.

Keywords: Sleeping Sickness; Neglected Tropical Diseases; Enantiomers; Nonlinear Mixed Effects Modeling; Pharmacokinetics; Pharmacodynamics
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SAMMANFATTNING PÅ SVENSKA

Behandling av afrikansk sömnsjuka med eflornitin

I 36 länder i Afrika söder om Saharaöknen finns en parasitsjukdom som är dödlig om den inte behandlas med rätt läkemedel. Denna sjukdom heter afrikansk trypanosomiasis. Den är även känd vid namnet afrikansk sömnsjuka efter dess typiska symtom där sömncykeln påverkas hos drabbade patienter. Sjukdomen sprids av tsetse-flugan som lever på blod från människor och djur. När en fluga med parasiter biter en människa förs parasiterna över till blodet. I blodet förökar sig parasiterna och efter en tid, om inte sjukdomen behandlas, tar de sig in i det centrala nervsystemet, det vill säga hjärnan. Läkemedlet eflornitin är en av de rekommenderade behandlingarna mot sömnsjuka då parasiterna har tagit sig till centrala nervsystemet. Eflornitin är ett läkemedel med speciella egenskaper som består av två molekyler som är varandras spegelbilder, så kallade enantiomerer. Detta innebär att de har olika tredimensionella strukturer, vilket i eflornitins fall påverkar hur läkemedlet tas upp i kroppen och potentiellt hur bra det fungerar som behandling mot parasitinfektionen.

Målet med projekten som är inkluderade i denna avhandling är att ta reda på hur dagens behandling med eflornitin kan förbättras. Idag doseras eflornitin i en 50/50-blandning av båda enantiomererna som ett intravenöst dropp. Denna behandling kräver nålar, sterilt vatten, sjukhusutrustning samt utbildad personal som kan ge behandlingen. Med en möjlig framtida behandling där substansen doseras oralt, det vill säga via munnen i form av exempelvis en lösning, suspension, kapsel eller tablett skulle det vara mycket lättare att behandla denna dödliga sjukdom i de drabbade områdena i Afrika. Hypotesen var att en oral dosering av den ena enantiomeren har bättre effekt mot parasitinfektionen än den andra och därmed kan vara ett framtida behandlingsalternativ mot afrikansk sömnsjuka. För att undersöka detta har de två enantiomererna av läkemedlet, kallade L-eflornitin och D-eflornitin, separerats med kemiska metoder för att kunna studera dem var för sig. Tester i laboratorium visade att L-eflornitin var 9-faldigt mer potent än D-eflornitin i sin hämning av parasiters tillväxt.

Med hjälp av matematiska modeller har dessa fynd sedan använts för att prediktera hur effektiv behandlingen med L-eflornitin skulle vara mot afrikansk sömnsjuka. Dessa prediktioner visade att L-eflornitin är bättre än behandling med 50/50-blandningen. Dock bör en oral L-eflornitin-behandling troligtvis kombineras med andra tillgängliga läkemedel mot afrikansk sömnsjuka för att ge tillräcklig effekt. Utöver detta har L-eflornitins

absorption, fördelning och elimination från kroppen studerats med hjälp av studier i råttor. Dessa experiment har genomförts för att öka kunskapen kring hur väl en potentiell framtida oral behandling med L-eflornitin skulle fungera hos patienter med afrikansk sömnsjuka. Denna studie visade att upptaget från mag- och tarmkanalen till blodet av L-eflornitin ej förbättrades när det doserades som rent L-eflornitin jämfört med 50/50-blandningen. Fördelningen i kroppen samt eliminationen var också oförändrade för rent L-eflornitin i jämförelse med 50/50-blandningen. Resultaten visade även att båda enantiomererna av eflornitin kunde nå cerebrospinalvätskan i råttornas hjärna, det organ där parasiterna finns under det senare skedet av afrikansk sömnsjuka.

Slutligen har klinisk data samt de nya experimentella fynden från de tidigare delprojekten kunnat användas för att ta fram en matematisk modell. Denna modell användes för att undersöka hur mycket L-eflornitin som skulle kunna ta sig från mag- och tarmkanalen via blodet till centrala nervsystemet hos människor drabbade av afrikansk sömnsjuka. Analysen visade att en oral formulering med L-eflornitin borde doseras 750 mg/kg/dag för att ha tillräcklig effekt och att kombinationen med L-eflornitin och ett annat läkemedel som heter nifurtimox hade möjliggjort en dossänkning av L-eflornitin till 375 mg/kg/dag. I framtiden kan den matematiska modellen användas för att guida beslut kring designen av kliniska studier där L-eflornitin kan testas med slutmålet att förbättra dagens behandling mot den dödliga parasitsjukdomen afrikansk sömnsjuka.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals:

I. **Boberg M**, Jonson AC, Leek H, Jansson-Löfmark R, Ashton M. Chiral chromatographic isolation on milligram scale of the human African trypanosomiasis treatment D- and L-eflornithine. *ACS Omega*, 2020; 5(37): 23885-91

II. **Boberg M**, Cal M, Kaiser M, Jansson-Löfmark R, Mäser P, Ashton M. Enantiospecific antitrypanosomal *in vitro* activity of eflornithine. *PLoS Neglected Tropical Diseases*, 2021; 15(7): e0009583

III. Amilon C*, **Boberg M***, Tärning J, Äbelö A, Ashton M, Jansson-Löfmark R. Population pharmacodynamic modeling of eflornithine-based treatments against late-stage *gambiense* human African trypanosomiasis and efficacy predictions of L-eflornithine-based therapy. *AAPS J*. 2022; 24(3): 48

* Authors contributed equally

IV. **Boberg M**, Akhondipour Salehabad Y, Oladetoun-Ageh E, Vallöf D, Jansson-Löfmark R, Ashton M. Enantiospecific pharmacokinetics after enantiopure and racemic dosing of eflornithine in the rat. *In manuscript*

V. **Boberg M**, Jansson-Löfmark R, Na-Bangchang K, Ashton M. Pharmacokinetics of racemic eflornithine in human plasma and cerebrospinal fluid: Clinical perspectives for L-eflornithine against human African trypanosomiasis. *In manuscript*

CONTENT

ABBREVIATIONS

CYP	Cytochrome P450
g-HAT	*Gambiense* human African trypanosomiasis
HAT	Human African trypanosomiasis
HPLC	High-performance liquid chromatography
IC_{50}	50% inhibitory concentration
ID_{50}	Dose resulting in 50% inhibition of baseline hazard
I_{max}	Maximum inhibition of the drug
M&S	Modeling and simulation
SFC	Supercritical fluid chromatography
UV-Vis	Ultraviolet visible
γ	Hill slope characterizing the concentration-effect relationship steepness

DEFINITIONS IN SHORT

Enantiomer	One of two molecules that are non-superimposable mirror images of each other, i.e., have different three-dimensional structures. This means that the molecule is chiral (hand in Ancient Greek: *kheír*), similar to the left and right hand.
In vitro studies	Latin for "within the glass" studies that are conducted without whole living organisms using, for instance, cells that are cultured in a test tube and studied in a laboratory.
In vivo studies	Latin for "within a living organism" studies that are conducted with whole living organisms such as an animal.
Neglected tropical diseases	Infectious diseases common in tropical areas in Africa, Asia and Latin America affecting the world's poorest populations where the high needs of treatments have been neglected.
Pharmacodynamics	What the drug does to the body.
Pharmacokinetics	What the body does to the drug.
Racemic mixture	A 50/50 mixture of two enantiomers, also known as racemate.

1 INTRODUCTION

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a parasitic disease that is fatal unless treated [1]. The parasite strains *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* cause the disease and are spread via bites from tsetse flies (Figure 1) in endemic areas in sub-Saharan Africa [2]. HAT is listed as one of the prioritized neglected tropical diseases by the World Health Organization as the research resources have been low despite high disease burden in one of the most poor areas in the world [3]. It is estimate that 51 million people live in areas with risk of infection and, of these, five million people live in areas with moderate or higher infection risk [4]. The *Trypanosoma brucei gambiense* subspecies is responsible for over 95% of the HAT cases [4]. Eflornithine (Figure 2) is a recommended treatment against *gambiense* HAT (g-HAT) in the later disease stage when the parasites have invaded the central nervous system [1, 5]. Eflornithine is currently dosed as a racemate of D- and L-eflornithine via repeated intravenous infusions. This route of administration for eflornithine is associated with logistical difficulties and high demand on the healthcare systems in endemic areas. This thesis focuses on strategies how to improve the eflornithine-based treatment against late-stage g-HAT and investigate the feasibility of an oral treatment using non-clinical methods.

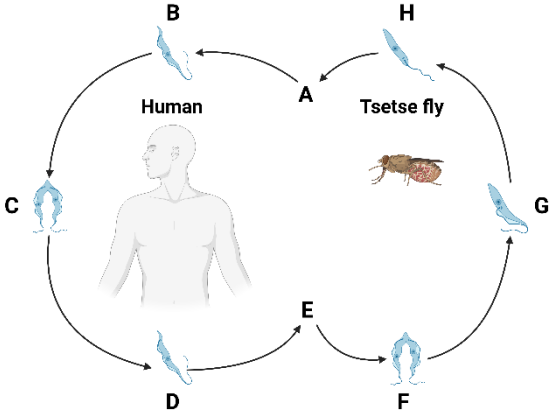

Figure 1. *Trypanosoma brucei gambiense and rhodesiense parasite life cycle in humans and tsetse flies. A) Tsetse fly with Trypanosoma parasites bites human, B) parasites transform into bloodstream trypomastigotes, C) parasites multiply in the human body, D) parasites are detectable in blood samples for diagnosis, E) tsetse fly bites a human with trypanosomes in the blood stream, F) bloodstream trypomastigotes turn into procyclic trypomastigotes in the midgut of the tsetse fly, G) parasites exit the midgut and transform to epimastogotes and H) parasites multiply in salivary gland and become metacyclic trypomastigotes. Figure created with BioRender.com.*

The hypothesis is that one of the eflornithine enantiomers has favourable pharmacokinetics and/or is more active against the parasitic infection and would be a feasible treatment against late-stage g-HAT. This thesis is based on five subprojects in which Paper I presents how the D- and L-eflornithine enantiomers were isolated from the racemic mixture using semi-preparative chiral chromatography. In Paper II, a higher antiparasitic *in vitro* potency was discovered for L-eflornithine compared to D-eflornithine or racemic eflornithine. The new knowledge about the superior L-eflornithine potency gained from Paper II was integrated in a pharmacodynamic time-to-event model in Paper III. In this time-to-event analysis, literature data from three clinical trials with racemic eflornithine were used to design the pharmacodynamic model and predict survival for L-eflornithine against late-stage g-HAT. In Paper IV, the *in vivo* pharmacokinetics of L-eflornithine in the rat was investigated to characterize the oral absorption and drug disposition of enantiopure L-eflornithine in comparison to racemic eflornithine. In Paper V, a clinical pharmacokinetic model for L-eflornithine is presented to translate the findings from the previous *in vitro* and *in vivo* studies to perform dose predictions in late-stage g-HAT patients.

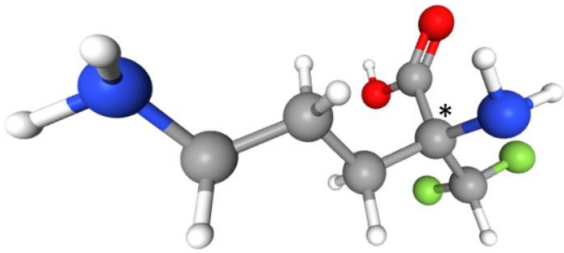

Figure 2. *Chemical structure of eflornithine with asterisk showing the chiral center. White – Hydrogen, Blue – Nitrogen, Gray – Carbon, Red – Oxygen, Green – Fluorine. Figure created with PubChem (CID 3009), URL: pubchem.ncbi.nlm.nih.gov/compound/3009#section=3D-Conformer.*

1.1 HUMAN AFRICAN TRYPANOSOMIASIS

1.1.1 EPIDEMIOLOGY

HAT is endemic in 36 countries in sub-Saharan Africa [6]. The number of HAT cases have decreased over the last decades and, in 2020, fewer than 700 new cases were reported [7]. In contrast, approximately 27,000 new HAT cases were reported in 2001 [8]. HAT has earlier been defined as the third most important parasitic disease defined by its global disease burden, after malaria and schistosomiasis when adjusting for lost quality adjusted life years [9]. The country with most cases reported is the Democratic Republic of the Congo with 395 reported cases in 2020. The ambition by the World Health Organization

was to eliminate HAT as a public health problem by 2020, which has been achieved in some countries, and to eliminate disease transmission of g-HAT by 2030 [10].

1.1.2 SYMPTOMS AND DIAGNOSIS

There are two disease stages of HAT: early-stage or late-stage. The symptoms of early-stage and late-stage HAT may overlap with the main difference that symptoms associated with late-stage HAT involve the central nervous system. In early-stage HAT, also known as the haemolymphatic stage, the *Trypanosoma brucei* parasites are present in the blood stream and lymph nodes. This leads to symptoms including fever, headache and nausea during the first weeks of infection [11]. Another characteristic symptom for HAT is the Winterbottom's sign, a swelling of the lymph nodes along the neck as well as a chancre at the site of the tsetse fly bite [11]. Parasites can be found in various organs throughout the body with liver, spleen and heart as examples [9]. If untreated, the parasitic infection will progress into a late-stage infection. This meningo-encephalitic stage is characterized by symptoms related to when parasites have infected the central nervous system such as confusion, tremor, agitation, numbness and affected sleep cycle [9, 12]. The changed sleep pattern has given the common name, sleeping sickness, for HAT. Fatigue and sleepiness during the day and insomnia during the night are common symptoms for late-stage HAT patients. The sleep cycle itself is also affected by the parasitic infection. A regular sleep cycle for a healthy adult has a periodicity of around 90 minutes with initial non-rapid eye movement sleep, which is followed by rapid eye movement sleep [13]. In late-stage HAT patients, this normal sleep cycle is reversed and the patients enter the sleep cycle with the rapid eye movement stage [14]. This symptom is suggested to be useful to diagnose HAT patients and is reversible with adequate treatment [15]. If not treated, the late-stage HAT patient will have seizures, fall into coma and die [1, 16, 17].

HAT can be diagnosed by microscopic examination of various body fluids, e.g., blood or cerebrospinal fluid. The parasites can also be stained prior to examination on glass slides to increase the visibility in a microscope. Another common diagnosis method, especially when screening populations for undiagnosed g-HAT patients, is the Card Agglutination Trypanosomiasis Test that was developed in the 1970's [18]. This is a serological test where an antigen for the bloodstream form of *Trypanosoma brucei gambiense* is used. Another parasite detection method for blood samples is mini anion exchange centrifugation technique, which is a much more sensitive diagnostic method but is less resource-effective [18]. This is a potential issue out in the field in rural areas. An alternative, however less sensitive, technique is the capillary

tube centrifugation method where a blood sample from a finger prick is examined. Another way to detect the presence of *Trypanosoma* parasites in a sample is the loop-mediated isothermal amplification method. This method amplifies the parasite DNA and has an advantage over methods using polymerase chain reactions as it can be monitored via spectrophotometers or even visually [19]. To determine if a HAT patient with a positive blood test has early-stage or late-stage HAT, a lumbar puncture is carried out to examine if trypanosomes are present in cerebrospinal fluid. For late-stage HAT, detection of trypanosomes and/or a count of white blood cells in cerebrospinal fluid above the cut-off value of five white blood cells per μL are widely used diagnostic criteria [20, 21]. This cut-off value for late-stage HAT has been debated and a count of up to 20 white blood cells per μL has been suggested as an alternative threshold [22].

1.1.3 *TRYPANOSOMA BRUCEI* PARASITES

The *Trypanosoma brucei* parasites (Figure 3) are kinetoplastids, meaning that the parasites have a large mass DNA called kinetoplast in the base of their flagella. Humans and animals can be infected by various *Trypanosoma brucei* subspecies and *Trypanosoma brucei brucei* is an example of subspecies that can infect cattle and gives rise to the disease called animal African trypanosomiasis [23]. There are two subspecies that cause disease in humans. In western and central parts on the African continent, the *Trypanosoma brucei gambiense* type is found while *Trypanosoma brucei rhodesiense* parasites are spread in the eastern parts. The disease progression for the two subspecies is different with *Trypanosoma brucei rhodesiense* leading to a more acute infection in contrast to *Trypanosoma brucei gambiense* where the patient can be infected for months without developing major symptoms [11].

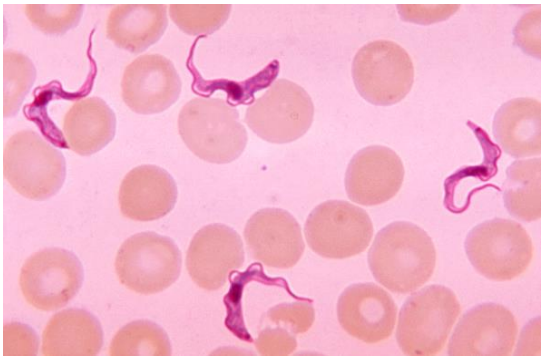

Figure 3. *Trypanosoma parasites (pink) in blood smear from a human African trypanosomiasis patient. Image is free of copyright restrictions and provided by Dr. Myron G. Schultz and Centers for Disease Control and Prevention, Public Health Image Library (ID 613).*

1.2 RECOMMENDED PHARMACOLOGICAL TREATMENTS

1.2.1 EFLORNITHINE

Eflornithine was registered as a late-stage g-HAT treatment with intravenous dosing in the 1990's [5]. Eflornithine is administered as a racemic mixture of D-eflornithine and L-eflornithine and can be used as monotherapy or in combination with the oral drug nifurtimox [24]. In the monotherapy regimen, eflornithine is dosed at 400 mg/kg/day every six hours for 14 days. In the nifurtimox-eflornithine combination therapy regimen, eflornithine is dosed less frequently, at 400 mg/kg/day every 12 hours for seven days [24].

The mechanism of action for eflornithine is exhibited by irreversible inhibition of the target enzyme ornithine decarboxylase [25]. This enzyme is responsible for biosynthesis of polyamines such as putrescine, spermine and spermidine that are critical for cell growth and division [26]. Eflornithine is a trypanostatic drug meaning that the decreased polyamine levels prevent parasite growth [27]. The turnover rate of ornithine decarboxylase is higher in mammalian cells compared to *Trypanosoma brucei* parasites making the parasites more susceptible to eflornithine treatment [28, 29]. Eflornithine is limited to treat infections by *Trypanosoma brucei gambiense* and not *Trypanosoma brucei rhodesiense* [11]. The main reason is the approximately four-fold higher ornithine decarboxylase turnover rate in *Trypanosoma brucei rhodesiense* parasites making these parasites less susceptible [30]. Examples of adverse events associated with eflornithine treatment are nausea, abdominal pain, diarrhoea and vomiting [29]. More severe adverse events including anaemia, convulsions and hearing loss may also occur when treated with eflornithine. The adverse events are generally reversible when the treatment is ended [29].

Eflornithine is a zwitterionic compound with a molecular weight of 182.2 g/mol. It is a hydrophilic amino acid derivate and has three pKa values at 10.4, 6.4 and 0.08 [31]. After intravenous dosing, eflornithine has a half-life of approximately three hours and is eliminated via renal excretion [29, 32]. The renal clearance of eflornithine is 2 mL/min/kg and the volume of distribution is 0.35 L/kg [29]. Eflornithine has no known metabolites [33]. In plasma, eflornithine has a high unbound fraction as it is not bound to plasma proteins [29]. Eflornithine can reach the cerebrospinal fluid from the systemic circulation [34, 35]. However, preclinical studies indicate that the blood-brain barrier permeability is poor [36]. Pharmacokinetic data from late-stage g-HAT patients showed that the cerebrospinal fluid to plasma concentrations ratio is variable [35]. For late-stage g-HAT, the pharmacological effect is dependent on adequate drug exposure in the central nervous system to elicit its irreversible

inhibition of the ornithine decarboxylase enzyme [25]. A clinical cut-off value for total D- and L-eflornithine concentrations of 50 μM in cerebrospinal fluid has been suggested to avoid treatment failure for racemic eflornithine [35]. Moreover, eflornithine has enantioselective oral absorption with higher absorption for D-eflornithine compared to L-eflornithine [37]. In terms of drug disposition, no enantioselectivity has been observed in clinical [37] or preclinical studies [38, 39].

1.2.2 NIFURTIMOX-EFLORNITHINE COMBINATION THERAPY

Nifurtimox was originally discovered as a treatment against Chagas disease [40, 41], also known as American trypanosomiasis that is caused by *Trypanosoma cruzi* parasites. Additionally, it was shown that nifurtimox was active against *Trypanosoma brucei gambiense* parasites [42]. Nifurtimox is a nitrofuran compound currently dosed orally at 5 mg/kg three times daily for ten days in combination with intravenous eflornithine for seven days against late-stage g-HAT [24, 43]. This combination is the first-line treatment for more severe late-stage g-HAT once the white blood cell count in cerebrospinal fluid is over 100 per μL and for children younger than six years or with a body weight below 20 kg and white blood cell count in cerebrospinal fluid over five per μL [24].

The mechanism of action for nifurtimox is not fully understood, however, the nitro-heterocyclic structure is suggested to produce trypanocidal free radicals rendering its effect [44]. The adverse events for nifurtimox-eflornithine combination therapy are generally similar to those observed for eflornithine monotherapy. However, the combination therapy was associated with a reduced frequency of adverse events such as fever, neutropenia, hypertension, diarrhoea or infections [45-47].

The nifurtimox-eflornithine combination therapy is the only drug combination therapy available against late-stage g-HAT. By combining two or more drugs with different targets it is possible to decrease the risk of drug resistance [48]. Additionally, the combination may lead to the possibility to reduce doses, shorter treatment durations and/or fewer adverse events [49]. For late-stage g-HAT, other drug combinations have been studied, such as nifurtimox and melarsoprol in combination as well as eflornithine with melarsoprol, however, the nifurtimox-eflornithine combination therapy was more effective and safer combination treatment [17].

1.2.3 FEXINIDAZOLE

Fexinidazole was approved against g-HAT in 2018, making it the first new treatment in decades [50]. It has been suggested that fexinidazole is

biologically activated via parasite nitroreductase enzymes that generate its antitrypanosomal mechanism of action [51]. The activation results in reactive amines that damage DNA and proteins in the *Trypanosoma* parasites [24]. Fexinidazole is a 5-nitroimidazole that can be dosed orally [51] and absorption is higher if dosed orally under fed conditions [52]. The oral route of administration makes it more convenient to dose than other parenteral HAT treatments. Oral administration increases adherence, requires no intravenous infusions and potentially reduce hospitalizations [53]. Moreover, fexinidazole is efficacious against both early-stage and late-stage g-HAT. This makes lumbar puncture for determination of disease stage unnecessary for patients older than six years and with a bodyweight over 20 kg [54].

Fexinidazole is dosed once daily for 10 days. During the first four days, loading doses at 1800 mg are administered and followed by six days of maintenance dosing at 1200 mg [24]. Adverse events associated with fexinidazole treatment are vomiting and nausea. Less common, but more severe, adverse events are QT interval prolongations and neutropenia [24]. In a clinical study, it was determined that fexinidazole was non-inferior to nifurtimox-eflornithine combination therapy [55]. However, as discussed in a Cochrane review by Lutje *et al.*, death due to late-stage g-HAT was higher in the cohort treated with fexinidazole (9 deaths/264 treated [3.4%]) at 24 months in comparison to the nifurtimox-eflornithine combination therapy cohort (2 deaths/130 treated [1.5%]) [53]. The efficacy in late-stage g-HAT patients with white blood cell counts over 100 per μL in cerebrospinal fluid was inferior for fexinidazole in comparison to the nifurtimox-eflornithine combination therapy [56]. Therefore, fexinidazole is currently used against less severe late-stage g-HAT cases. The development of fexinidazole is, despite this, an important step forward and this recently approved drug has the potential to simplify treatment and improve the clinical practice for g-HAT [57]. Especially for pediatric patients, as the fexinidazole efficacy was high in this patient population [58].

1.2.4 MELARSOPROL

Melarsoprol was introduced as a treatment option against HAT in 1949 [59]. This drug is an arsenical derivate with a therapeutic history that originated from findings made by the physician and missionary David Livingstone in 1847 [60]. Melarsoprol is able to permeate the blood-brain barrier and can be used to treat late-stage HAT [61]. Melarsoprol is dosed intravenously at 2.2 mg/kg once daily for ten days [62] and is currently a rescue treatment if other recommended HAT treatments fail to clear the parasitic infection [24]. Melarsoprol is associated with a life-threating adverse reaction known as post-treatment reactive encephalopathy [63, 64]. This serious adverse reaction is manifested by fever, seizure and coma and occurs in about five to ten out of

100 HAT patients treated with melarsoprol [65]. Out of these, 50% die from this adverse reaction, which equals to five out of 100 melarsoprol treated HAT patients. To reduce the risk of this possibly fatal adverse event, prednisolone may be used in a 12-day concomitant treatment to protect the patient. Other adverse events associated with melarsoprol treatment are skin rash, malaise and cardiac failure [24]. Despite over hundred years of usage, the mechanism of action for arsenical compounds like melarsoprol is still to be elucidated [66].

1.2.5 SURAMIN

Suramin is a recommended treatment for early-stage HAT caused by *Trypanosoma brucei rhodesiense* [67]. Suramin is a polysulfonated naphthyl urea that is soluble in water. Suramin treatment is initiated with a low test dose to detect early signs of a rare hypersensitivity reaction to the drug. If this initial dose is tolerated, the treatment continues with five intravenous doses every seven days of 20 mg/kg, not exceeding a total dose of 1 g [68]. Suramin was discovered in 1916 [45] and is based on previous work from trypanocidal dye molecules with Nagana Red, Trypan Red [69] and Trypan Blue [70, 71].

The exact mechanism of action for suramin against HAT is unknown but it has been suggested to inhibit glycolytic enzymes in *Trypanosoma brucei* parasites [72]. However, alternative antitrypanosomal mechanisms of action for suramin have also been proposed [73]. The molecular structure of suramin violates Lipinski's rule of five with a molecular weight of 1297 g/mol and over 10 hydrogen-bond donors and 20 hydrogen-bond acceptors [74]. Thus, oral absorption of this molecule is highly unlikely. Adverse events associated with suramin treatment are, e.g., nephrotoxicity, dermatitis, neuropathy and the mentioned hypersensitivity reaction after dosing [75].

1.2.6 PENTAMIDINE

Pentamidine is a synthetic aromatic diamidine that was registered in 1940 as an early-stage g-HAT therapy [21, 76]. It is administered once daily for seven days via intramuscular injections at 4 mg/kg [24]. In a study with early-stage g-HAT patients, the half-life varied between 10 to 46 hours for the first dose and 22 to 62 hours after the 10^{th} dose [77]. Pentamidine is suggested to be extensively distributed in peripheral tissue with a high volume of distribution at steady state [78]. Although pentamidine is considered as a well-tolerated therapy, about 10% of the patients experience hypotension as an acute adverse reaction. In addition, nephrotoxicity is a more severe adverse reaction associated with pentamidine treatment [21]. The mechanism of action for pentamidine is not fully understood, however, with its cure rate at 94% it is considered as an effective treatment against early-stage g-HAT [79].

Pentamidine is not effective against late-stage g-HAT as it is effluxed by drug transporters from the central nervous system to the systemic circulation [45].

1.2.7 SUBSTANCES IN RESEARCH AND DEVELOPMENT

There is still a high need for new treatment options for HAT patients. New substances are currently in various research and development stages. This includes the compound acoziborole, a benzoxaborole that is currently in clinical phase 2/3 studies [80]. It is investigated as a treatment of both stages of g-HAT with oral single dosing and is a promising future g-HAT treatment [53, 81]. Single dosing is possible since the half-life for acoziborole is approximately 400 hours [82]. Adverse events associated with acoziborole treatment include headache, nausea and diarrhoea. Acoziborole treatment was not associated with any serious adverse events in a phase I study [83]. Other candidate drugs that have previously been studied are the furamidine compound DB-75 and its orally dosed prodrug DB-289 that failed to reach the clinic due to intolerable toxicity and inferior efficacy to pentamidine [84, 85]. Compounds still in development are the diamidine compound DB-829 as well as its oral prodrug DB-868 [86, 87]. Earlier in the drug development pipeline are the oxaborole compounds SCYX-1330682 or SCYX-1608210 [82].

1.3 ENANTIOMERS IN PHARMACOLOGY

The three-dimensional structures of a drug molecule and its target, for example a protein, are important to take into consideration. The chemical interaction(s) between the target protein and a chiral drug molecule can be different for its enantiomers, consequently leading to stereoselective pharmacodynamics. In other cases, undesired adverse effects can be caused by one of the enantiomers or one enantiomer can have different pharmacological responses [88]. Chiral drugs exhibiting stereoselective pharmacodynamics are the calcium channel blocker, verapamil [89], β_1 receptor blocker metoprolol [90], antiplatelet clopidogrel [91] and antidepressant citalopram [92]. Moreover, the pharmacokinetics of the enantiomers can be different with the proton pump inhibitor omeprazole as an example [93, 94]. The (S)-omeprazole enantiomer was found to be metabolized to a lower degree by the polymorphic cytochrome P450 (CYP) 2C19 enzyme compared to (R)-omeprazole [95]. This leads to higher drug exposure and effect of (S)-omeprazole. Another drug with stereospecific pharmacokinetics is salbutamol, an asthma and chronic obstructive pulmonary disease treatment. Salbutamol exhibits stereoselective elimination with a higher clearance of one of the enantiomers [96]. The antidepressant mirtazapine, which enantiomers are

suggested to be metabolized by different CYP enzymes is another example [97].

Apart from the pharmacokinetic and pharmacodynamic characteristics, knowledge concerning the toxicology of the individual enantiomers is critical. An example is the chiral drug thalidomide [98] used for treatment of anxiety and insomnia that caused teratogenic effects derived from its (S)-enantiomer [99]. For thalidomide, purification of enantiopure (R)-enantiomer would not have been feasible to avoid these horrible adverse events since the drug is converted back to a 50/50 ratio of the two enantiomers in the body [100]. Based on the examples and the increasing knowledge of risks and benefits for enantiomers, regulatory agencies such as European Medicines Agency and US Food and Drug Administration require data on the pharmacokinetics, pharmacodynamics and toxicology for the racemate and the individual enantiomers for new chiral drugs [101, 102].

1.4 ORAL DRUG ABSORPTION

Oral dosing is non-invasive, allows better adherence and decreases the need of trained medical staff for drug administration compared to, e.g., intravenous infusions [53]. There are several factors that limit uptake of drugs in the gastrointestinal tract after oral administration. Some factors are part of normal physiology to protect the body from hazardous substances or pathogens and enable absorption of nutrients and water [103, 104]. The factors limiting drug absorption are commonly categorized in physiological factors or drug-dependent physicochemical properties. Physicochemical properties that may affect drug absorption are for instance water solubility, ionization, polar surface area and molecular weight [105]. Lipophilic drugs are more likely to permeate through the epithelial barrier in the gastrointestinal tract via a passive transcellular mechanism. However, drug molecules that are poorly soluble in gastrointestinal fluids, i.e., too lipophilic, are absorbed to a lower extent compared to compounds that are able to dissolve in gastrointestinal fluids. Lower absorption also occurs for drug molecules that are ionized at intestinal pH, compared to drug molecules that are unionized at these conditions. A molecular weight over 500 g/mol is associated with lower absorption than smaller drug molecules as exemplified by the Lipinski's rule of five [74]. In addition, the drug formulation with its excipients may also affect the rate and/or extent of drug absorption, with extended-release formulation as an example where the drug release from the oral solid dosage form is modified.

Bioavailability is a pharmacokinetic parameter that characterizes the oral absorption of a drug. Oral bioavailability is defined as the rate and extent to which a drug molecule was delivered from its oral formulation and reached the systemic circulation in unchanged, not metabolized, form after oral dosing [106]. The bioavailability parameter describes multiple processes, as it contains three parts: fraction absorbed, first pass extraction in the liver and drug metabolism in gastrointestinal epithelial cells (Figure 4). Hepatic and gastrointestinal metabolism are dependent on expression and activity of drug-metabolizing enzymes. For instance, the drug-metabolizing enzyme family carboxylesterases are expressed in both liver and the gastrointestinal tract. However, the enzyme carboxylesterase 1 is expressed to a higher degree in the liver than carboxylesterase 2, which is expressed more in the gastrointestinal tract [107]. Another example of a drug-metabolizing enzyme expressed in the intestine is CYP3A4 that metabolizes drugs and may decrease the bioavailability of drugs that are CYP3A4 substrates [108]. The drug-metabolizing enzyme activity may also vary between individuals, as seen for the β_1 receptor blocker metoprolol where the drug clearance varied between individuals due to metabolism by the polymorphic enzyme CYP2D6 [109].

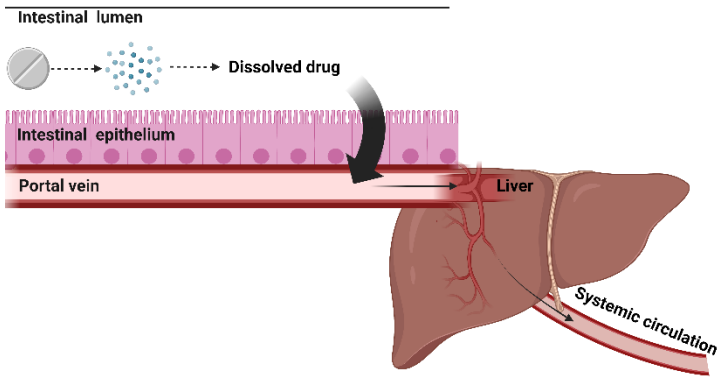

Figure 4. *Processes that determine the bioavailability of a drug after oral administration. After disintegration of the formulation and dissolution, the drug molecules are absorbed across the intestinal epithelium to the portal vein. This blood vessel supplies the liver with blood and in case of no first-pass extraction by hepatic enzymes, the drug molecules will reach the systemic circulation in unchanged form. Figure created with BioRender.com.*

The drug absorption after oral dosing includes different mechanisms that are passive or active, i.e., requires energy (Figure 5). The physicochemical properties of the drug molecule determine by which mechanism a drug is

absorbed or not at all [110]. The passive uptake mechanisms encompass transcellular or paracellular uptake mechanisms [111].

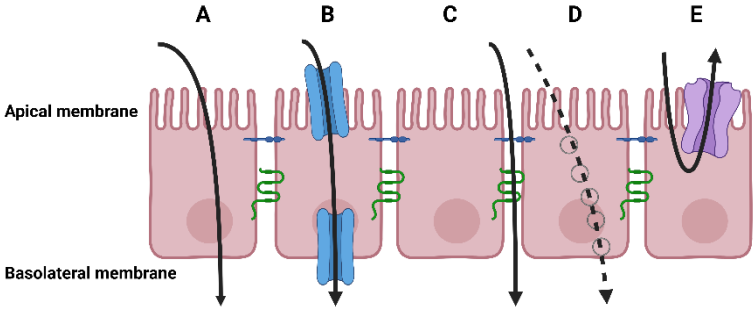

Figure 5. *Examples of drug absorption (A – D) or efflux (E) mechanisms in the intestinal epithelium. A) Passive transcellular, B) active transport, C) passive paracellular, D) transcytosis and E) efflux. Figure created with BioRender.com.*

There are many gastrointestinal transporters involved in active drug transport from the gastrointestinal tract to the systemic circulation with organic anion transporting polypeptides as examples [112, 113]. The subtype organic anion transporting polypeptide 2B1 is suggested to be involved in the uptake of rosuvastatin [114] as well as furosemide [115]. Due to the three-dimensional structure of the drug transporter, the interaction between the transporter protein and the drug molecule is specific and can be enantioselective [116]. An example of an enantioselective transporter is the proton-coupled folate transporter that is responsible for uptake of the immunosuppressant drug methotrexate [117]. Other drug uptake mechanisms include endocytosis and transcytosis that are receptor-dependent and commonly involved in absorption of large macromolecules via internalization in cellular vesicles [118, 119]. There are also transporters that efflux the drug back to the intestinal lumen with P-glycoprotein as a clinically relevant example [120].

For eflornithine, various models have been used to investigate the oral drug absorption characteristics. *In vitro* methods with Caco-2 cells [39] or the Ussing chamber have been tested. However, they were not able to mimic the observed enantioselective absorption of D- and L-eflornithine in humans [37]. In contrast to *in vitro* methods*, in vivo* models using laboratory rats were more useful to study the enantioselectivity in oral drug absorption [38, 39].

1.5 THE BLOOD-BRAIN BARRIER

The blood-brain barrier is an anatomical separation of brain blood vessels and extracellular fluid in the brain tissue. The small blood vessels, known as capillaries, in the brain have a thin vessel wall. The vessel wall consists of

endothelial cells, connective tissue and nervous tissue. The cells in the vessel wall are interconnected by tight junctions and form a barrier with the physiological function to maintain brain homeostasis, regulate uptake and efflux of endogenous and exogenous molecules [121, 122]. The blood-brain barrier is far from a passive permeable membrane and should be considered as an active organ that protects the brain from harmful substances meanwhile maintaining vital organ homeostasis [123]. Around the capillaries, the basal lamina surrounds the vessel alongside pericytes and astrocytic endfeet that affect the exposure of substances in the central nervous system (Figure 6). In this endothelium, transporters take up important polar molecules such as glucose or amino acids to the brain [122]. These transporters can also take up drug molecules that may elicit their pharmacological effect inside the central nervous system such as the opioid oxycodone [124]. In the luminal membrane in the blood-brain barrier, P-glycoprotein is an important efflux transporter alongside the breast cancer resistance protein that may efflux drugs from the central nervous system [125, 126].

It is of high relevance to study the drug concentrations inside the blood-brain barrier to establish the relationship between pharmacokinetics and pharmacodynamics in the target tissue for a drug that elicit its effect in the central nervous system. Experiments involving microdialysis methods have been essential and allowed for detailed examination of drug transport across the blood-brain barrier [127]. Microdialysis has been used extensively in non-clinical studies to gain more understanding of free drug concentrations in the central nervous system with the analgesic opioid morphine as an example [128].

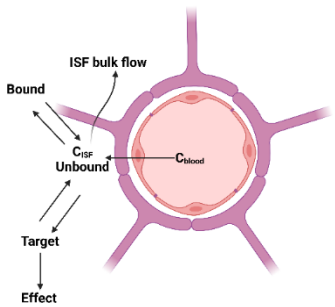

Figure 6. *The drug concentration in the blood (C_{blood}) drives the potential passage across the blood-brain barrier with endothelium, pericytes and astrocytic endfeet to the interstitial space in the brain. The drug either can be unbound in the interstitial fluid (C_{ISF} unbound) or bound to brain tissue components. When unbound, the drug can interact with the target and elicit its pharmacological effect. The drug can be eliminated from the brain via the interstitial fluid (ISF) bulk flow. Figure created with BioRender.com.*

The cerebrospinal fluid surrounds the brain and the spinal cord and can be sampled from humans by lumbar puncture. Therefore, this fluid is of particular interest in research for quantification of drug concentrations in the central nervous system. For HAT, cerebrospinal fluid samples can be used for diagnosis and stage determination of disease. Choroid plexus is the brain tissue that produces the cerebrospinal fluid. The choroid plexus is found in the brain ventricles and makes up a barrier itself, the blood-cerebrospinal fluid barrier. This is a leaky barrier with larger gaps in the endothelial cells in comparison to the blood-brain barrier. Another group of structures in the central nervous system that is more permeable than the blood-brain barrier is the circumventricular organs. These are located around the third and fourth ventricles in the brain. The circumventricular organs are important with regards to *Trypanosoma brucei* parasite infection as it has been suggested that the parasites may enter through these more permeable structures into the central nervous system from the blood stream [129]. This infiltration triggers inflammatory responses from white blood cells that promote the *Trypanosoma parasite* migration inside the central nervous system. In a rodent model, the cytokine interferon-gamma and CXC motif chemokine ligand 10 were needed for *Trypanosoma* parasites to infect the central nervous system [130]. The parasite entry mechanism in HAT patients is potentially similar and may occur via an inflammatory-dependent disruption of the blood-brain barrier and/or the blood-cerebrospinal fluid barrier [131]. The dysfunction of the protective mechanisms in the central nervous system is not unique for *Trypanosoma brucei* parasites as inflammation generally may induce changes in expression and/or activity of drug transporters [132]. It has also been shown in a preclinical study that inflammation in the central nervous system can alter the expression of the efflux transporter P-glycoprotein and affect drug disposition of digoxin [133].

1.6 CHIRAL CHROMATOGRAPHY

Enantiomers of a chiral compound have to be isolated to enable studies of the individual enantiomers. Chiral chromatography is a resource-efficient approach in the early drug discovery and development stages to isolate enantiomers with high purity from a racemic mixture [134, 135]. In these early stages, many drug molecules with varying physicochemical properties are evaluated in various *in vitro* and preclinical *in vivo* screenings. Moreover, only low quantities of each enantiomer are needed initially and higher amounts are required in the later drug development stages [136]. For larger studies where grams to kilograms of the drug substances are needed, high-performance liquid chromatography (HPLC) has been commonly used [137]. Now, supercritical

fluid chromatography (SFC) can be deemed as a gold standard method for preparative enantiomer isolation in early drug development [138, 139]. An alternative approach to obtain pure enantiomers would be asymmetric drug molecule synthesis [135]. This is a more time-consuming approach, where only one of the enantiomers is obtained for each synthesis cycle [137].

1.7 *IN VITRO* ASSAYS

Laboratory models of diseases are useful in drug development when performing screening studies to assess the efficacy of various compounds. In contrast to *in vivo* models, *in vitro* models are a relatively simple and less complex option that offer an early indication on what substance to bring forward in the drug development and guide decision-making. For HAT, *in vitro* assays have provided valuable information on compound efficacy against *Trypanosoma brucei* parasites. Moreover, the use of *in vitro* assays have enabled the development of new treatments and investigations of potential combination treatments against HAT [80, 140]. The assays are useful tools to, e.g., investigate treatment resistant parasites and develop strategies to circumvent emerging resistance in endemic areas in sub-Saharan Africa [141, 142]. For *Trypanosoma brucei* parasites, many types of *in vitro* assays exist that range from relatively quick parasite counts to more sophisticated methods with radioactive nucleotides or fluorescent measurements [143-146]. Generally, results obtained from *in vitro* assays are derived from the drug concentration-effect relationship and are used to determine relevant pharmacodynamic parameters like maximum inhibition (I_{max}), 50% inhibitory concentration (IC_{50}) and/or Hill slope (γ) using the *in vitro* data.

1.8 PHARMACOKINETIC AND PHARMACODYNAMIC MODELING

1.8.1 NONLINEAR MIXED EFFECTS MODELING

In pharmacokinetic and pharmacodynamic studies, nonlinear mixed effects modeling is commonly used. Modeling is performed to fit models to the data to describe both population parameters and variability parameters, known as fixed effects and random effects, respectively [147]. In its early applications, it was shown how the method could be useful by finding the right dose for patients treated with digoxin [148]. The approach allows for estimations of parameters using sparse pharmacokinetic and/or pharmacodynamic data, i.e., few samples from each individual in the study. Over the years, the research

field has expanded and nonlinear mixed effects modeling is commonly used in drug research and development.

In nonlinear mixed effects modeling, the fixed effects describe the structure of the model with pharmacokinetic parameters such as clearance, volume of distribution or first-order absorption rate constant for the typical individual. The random effects in a model describe the observed variability in the data. Variability can come from various sources and may be divided into three subcategories: interindividual variability, interoccasion variability or residual variability. Mathematically, the relationship between an individual estimate (θ_{ind}) and the typical individual (θ_{pop}) can be described by the interindividual variability (η_{ind}) in, for example, an exponential relationship as shown in Equation 1:

$$\theta_{ind} = \theta_{pop} * e^{\eta_{ind}} \quad \text{Eq. 1}$$

In this mathematical relationship, normal distribution of η_{ind} with a mean equal to zero and variance of ω^2 is assumed and that θ_{ind} is log-normally distributed. Moreover, the pharmacokinetics may in some cases change over time for a drug that is administered repeatedly. Examples of mechanisms for this interoccasion variability are autoinduction of hepatic drug-metabolizing enzyme expression(s) or food intake affecting the drug absorption. This variability could also be characterized in the model. Moreover, pharmacokinetics may vary between individuals due to a known factor, i.e., a covariate. Gender or ethnicity are examples of categorical covariates and body weight or kidney function are continuous covariates that are commonly assessed in nonlinear mixed effects modeling.

The variability that remains unexplained by the estimated interindividual and/or interoccasion variability as well as covariates for an individual is known as the residual variability. Residual variability may stem from, e.g., measurement error in the bioanalysis, errors in sample storage and/or inaccurate dosing or sampling records. In nonlinear mixed effects modeling, the residual variability is commonly estimated by additive, proportional or combined residual error models. In the equations below, $y_{i,j}$ describe the j^{th} observation for the i^{th} individual of the individual prediction (IPRED). The difference between the individual observation and the prediction can be described by ε in the equations (Equation 2 – 4). The mean for ε is assumed to be equal to zero with a variance of σ^2.

Additive: $y_{i,j} = IPRED_{i,j} + \varepsilon_{i,j}$ Eq. 2

Proportional: $y_{i,j} = IPRED_{i,j} + IPRED * \varepsilon_{i,j}$ Eq. 3

Combined: $y_{i,j} = IPRED_{i,j} + IPRED * \varepsilon_{prop} + \varepsilon_{add}$ Eq. 4

1.8.2 MODEL INFORMED DRUG DEVELOPMENT

Mathematical models and statistics can be used to describe the pharmacokinetics and/or pharmacodynamics of a drug on a quantitative level. A solid knowledge concerning the links between dose, concentration and effect is key in pharmacokinetic and pharmacodynamic studies. Mathematical models and statistics may be applied to interpret the data and can be used to predict various scenarios using simulations [149]. Modeling and simulation (M&S) in drug development can be useful, since it increases knowledge of risks versus benefits, potentially reduces the number of patients needed in a clinical trial, brings down costs for the clinical studies, reduces the risk of failure in the clinical trial and leads to possibilities of optimized and/or personalized treatment [150, 151]. Moreover, pharmacokinetic and pharmacodynamic M&S in preclinical research provides a solid scientific foundation around, for example, dose selection or optimization when planning clinical programs for compounds in early drug research and development [152, 153]. M&S can also be valuable in early research and development in the phase of identifying the right compound to bring forward in the pipeline and enable effective communication around uncertainty with predicted human dose levels or other findings to make the right data-driven decisions [154].

1.8.3 TIME-TO-EVENT ANALYSIS

Modeling can also be useful to determine the time from start of drug treatment to a defined event. An event can for example be treatment failure, relapse or death. By combining the knowledge about pharmacokinetics, pharmacodynamics and survival in a specific study population, the time-to-event can be determined [155]. In time-to-event modeling, the probability of survival is determined by using the baseline hazard for event in a survival function. The survival function contains the parameters baseline hazard and might also have a shape factor parameter of, e.g., a Weibull function that describes the distribution of the hazard.

Generally, the aim with time-to-event analysis is to determine what clinical efficacy a treatment has at different doses or in combination with other treatments. With a time-to-event model, various treatment scenarios can be simulated using the model to predict survival at certain time points. This hypothesis generating approach with M&S enables data-driven decisions to be made when investigating new treatments or exploring new dose regimens. Examples of this are models describing the antiemetic effect of ondansetron or efficacy of Parkinson's disease treatments [156, 157]. Other examples of time-to-event models in the field of parasitic diseases display how pharmacokinetics and survival for malaria treatments can be simultaneously modeled [158-160].

2 AIM

The overall aim of this thesis was to assess the feasibility of an oral treatment with eflornithine against late-stage g-HAT using non-clinical methods and explore strategies to improve efficacy. The hypothesis was that an enantiopure eflornithine-based treatment could be a feasible oral treatment against late-stage g-HAT.

The specific aims were:

1. To isolate the eflornithine enantiomers from racemic mixture (Paper I)

2. To determine the antitrypanosomal *in vitro* activities for the eflornithine enantiomers and racemic eflornithine (Paper II)

3. To *i)* assess the clinical efficacy of three racemic eflornithine treatments and *ii)* predict efficacy of an L-eflornithine-based treatment by integration of clinical and *in vitro* data with a pharmacodynamic time-to-event modeling and simulation approach (Paper III)

4. To characterize the *(i)* enantioselective *in vivo* plasma pharmacokinetics of enantiopure L-eflornithine or racemic eflornithine and *(ii) in vivo* pharmacokinetics in brain dialysate of D- and L-eflornithine administered to the rat (Paper IV)

5. To integrate *in vitro*, preclinical *in vivo* and clinical data to enable dose predictions for L-eflornithine-based treatment regimens against late-stage g-HAT (Paper V)

3 MATERIALS AND METHODS

3.1 CHIRAL CHROMATOGRAPHY

3.1.1 CHROMATOGRAPHIC SYSTEMS AND CONDITIONS

The Acquity UPC2 system was used for the SFC runs. This instrument had photodiode array and single quadrupole detectors. The system was used with 3.5 mL/min flow rate and 120 bar backpressure at 40°C. The software Empower (Version 3, Waters, Milford, MA, USA) was used. Supersep™ 150 with ultraviolet-visible (UV-Vis) spectroscopy detection and Proficy HMI/SCADA iFIX (Version 5.1, Novasep, Pompey, France) software was used for the preparative SFC work. For the analytical HPLC runs, Alliance 2695 with Waters Micromass ZQ detector (Waters, Milford, USA) system and MassLynx (Version 4.1) software were used. The runs were conducted at a flow rate of 1 mL/min at 25°C. For HPLC in semi-preparative scale, Interchim puriFlash® 4250 using puriFlash AS-I sampler, UV-Vis, evaporative light scattering and mass spectrometry detectors and Interchim soft (Version 5.1c.09, Montluçon, Cedex, France) software were utilized. All acquired material from the preparative work were evaporated with Rotavapor® R II rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) to concentrate the material before it was precipitated using a Biotage® V-10 Evaporator system (Biotage AB, Uppsala, Sweden).

3.1.2 CHIRAL STATIONARY PHASES AND MODIFIERS

For SFC, the following chiral stationary phases were evaluated: Lux Cellulose 3, Lux Cellulose 4 and Lux Amylose 1 from Phenomenex (Torrance, CA, USA); tert-butylcarbamoylquinine (tBuCQN), Chiralcel OJ and Chiralpak (AS, AD, IA, IB, IB N-3, IC, ID, IE, IG) from Chiral Technologies (Illkirch, France); Kromasil CelluCoat from Eka Chemicals (Bohus, Sweden); Chirobiotic T and Chirobiotic V2 from Astec (Whippany, NJ, USA); (S,S)Whelk-O1 from Regis Technologies (Morton Grove, IL, USA); and Chiralart Cellulose SJ from YMC (Kyoto, Japan). The chiral column dimension for SFC was 150 mm x 4.6 mm with 3 µm particle size. Chiral stationary phases tested in HPLC mode were: tBuCQN; Chirobiotic (R, T, T2, TAG, V, V2); (S,S)Whelk-O1; Chiralpak (AD, IA, IB, IC, ID); and Reprosil Chiral-NR from Dr Maisch (Ammerbuch, Germany). Chiral stationary phase dimension in HPLC mode was 250 mm x 4.6 mm and particle size 5 µm.

Methanol, ethanol or isopropyl alcohol with and without additives were evaluated in the SFC mode. The modifiers were tested with a gradient from 5

– 40%. Additionally, acetonitrile with and without additives was included in the HPLC mode alongside modifiers tested in SFC mode. Isocratic methods were used in the HPLC mode.

3.2 ANTITRYPANOSOMAL *IN VITRO* ACTIVITY

3.2.1 PARASITE CULTURES AND DRUG EFFICACY ASSAY

The antitrypanosomal *in vitro* activity was investigated using three strains of *Trypanosoma brucei gambiense* parasites. The strains STIB930, K03048 and 130R were previously isolated from patient samples [161-163]. The parasites were incubated in HMI-9 medium at 37°C [164]. The drug sensitivity of the *Trypanosoma brucei gambiense* parasites was tested using the Alamar Blue assay where the parasite growth inhibition at different drug concentrations was determined [143]. L-eflornithine, D-eflornithine and the racemic mixture of the two eflornithine enantiomers were tested. For more detailed description of the *in vitro* assay, see Paper II.

3.2.2 *IN VITRO* DATA ANALYSIS

An I_{max} model (Equation 5) was fitted to the *in vitro* activity data using naïve pooled data analysis in the software Phoenix (Version 8.2, Certara, Princeton, NJ, USA) with the purpose to model the antitrypanosomal *in vitro* activity for L-eflornithine, D-eflornithine and racemic mixture. This allowed for estimation of the parameters I_0 representing baseline effect without drug exposure, IC_{50}, γ and I_{max} for L-eflornithine, D-eflornithine and racemic mixture.

$$\text{Inhibition} = I_0 - \frac{I_{max} \times \text{Concentration}^{\gamma}}{IC_{50}^{\gamma} + \text{Concentration}^{\gamma}} \qquad \text{Eq. 5}$$

Parameter precision, change in -2log likelihood (Δ-2LL) and goodness-of-fit plots were used for discrimination between models with $\gamma = 1$ or estimated γ. The likelihood-ratio test was used to discriminate between nested models where a decrease in -2LL >3.84 was considered as a statistically significant improvement of the model ($P<0.05$, one degree of freedom), assuming a χ^2 distribution for Δ-2LL. For more details regarding the modeling of the *in vitro* data, see Paper II. Bootstrap was used (n = 1,000) to obtain 95% confidence intervals (95% CI) of the parameter estimates and 5th and 95th percentiles for the model predictions in the model validation step.

3.3 PHARMACODYNAMIC TIME-TO-EVENT MODELING

3.3.1 CLINICAL STUDY DATA

Data on late-stage g-HAT treatment outcome, i.e., death or cure, from three clinical studies with racemic eflornithine monotherapy administered intravenously or in combination with oral nifurtimox [165, 166] or oral racemic eflornithine monotherapy [34] were collected from the literature. In total, data from 1,248 late-stage g-HAT patients were analyzed.

3.3.2 TIME-TO-EVENT DATA ANALYSIS

The time-to-event pharmacodynamic modeling was performed using the Laplacian estimation method in NONMEM (Version 7.4, ICON Development Solutions, Ellicott City, MD, USA) [167]. For model assessments and/or diagnostics, Piraña (Version 2.9.8), Rstudio (Version 1.3.1093), the R software (Version 4.1.1, The R foundation for Statistical Computing), Perl-speaks-NONMEM (PsN) (Version 4.8.1, Department of Pharmaceutical Biosciences, Uppsala University, Uppsala, Sweden) [168] and Xpose (Version 4.7.1) [169, 170] were used. Models were assessed using visual predictive checks (n = 1,000) of the respective Kaplan-Meier curves and bootstrap (n = 1,000) to obtain confidence intervals for the model parameters.

The total racemic eflornithine dose for each treatment in the respective study was implemented as a time constant effect in the pharmacodynamic modeling. The effect eflornithine treatment has on the baseline hazard (BASE) was modeled using an I_{max} model (Equation 6). In this equation, the model parameters were BASE, the dose associated with 50% reduction in baseline hazard (ID_{50}) and total eflornithine dose administered in each study (D) to estimate the time-to-event (I_{max} and slope (n) were set as equal to 1 with assumed possibility of full BASE inhibition).

$$Time\ to\ event = BASE \times \left(1 - \frac{Imax \times D^n}{ID_{50}{}^n + D^n}\right) \qquad \text{Eq. 6}$$

To predict outcome for L-eflornithine, the estimated *in vitro* potency difference for racemic mixture versus L-eflornithine treatment was determined by a mathematical modeling approach in the software Phoenix (Version 8.3, Certara, Princeton, NJ, USA). Potency for the 1:2 L-eflornithine:D-eflornithine plasma exposure ratio in late-stage g-HAT patients after oral dosing of racemate [37] was estimated by a mathematical model for competitive target-ligand interaction of D-eflornithine and L-eflornithine [171, 172]. The estimated difference in potency was implemented in the

pharmacodynamic model by adjusting the ID_{50} parameter according to the derived *in vitro* potency ratio for the predicted IC_{50} for the 1:2 L-eflornithine:D-eflornithine plasma exposure ratio for oral racemic eflornithine and IC_{50} for L-eflornithine.

3.4 PHARMACOKINETICS OF EFLORNITHINE IN THE RAT

For the *in vivo* study, male Sprague-Dawley rats (Charles River, Italy) were used. They weighed 250 – 270 g at arrival to the certified animal facility Experimental Biomedicine at University of Gothenburg. The rats were fed *ad libitum*, maintained in groups at a 12/12 hour light/dark cycle at 19 – 21°C with 40 – 70% humidity for at least seven days before the experiments started. The study was approved by the Ethics Committee for Animal Research, Gothenburg, Sweden (2127/19). The detailed surgery protocols to catheterize the rats as well as installation of microdialysis probes in the third brain ventricle are provided in Paper IV.

3.4.1 STUDY DESIGN

The study included five study arms. For the oral absorption study, L-eflornithine or racemic eflornithine formulations in saline were administered via oral gavage or intravenous bolus dose to determine the *in vivo* pharmacokinetics. In the microdialysis study, oral racemic eflornithine formulation was administered via oral gavage.

3.4.2 BIOANALYSIS

To quantify plasma and brain dialysate concentrations of D-eflornithine and L-eflornithine, a validated HPLC method was used [173]. In brief, precipitation of plasma was performed with 300 µL cold methanol. After centrifugation, the supernatant was transferred to new vials and evaporated before addition of 75 µL deionized water. The samples were placed in injections vials and analyzed by HPLC. The brain dialysate samples (60 µL) were transferred to injections vials and analyzed. To allow enantioselective quantification, derivatization of D-eflornithine and L-eflornithine with *ortho*-phthalaldehyde and N-acetyl-L-cysteine was done by the Endurance Prospect 2 (SparkHolland, Emmen, Holland) autoinjector before the sample was injected on two interconnected C18-columns (Chromolith Performance, RP-18e, Merck KGaA, Darmstadt, Germany). The HPLC system had two Shimadzu LC-10AD pumps (Shimadzu Corporation, Kyoto, Japan), FP-920 fluorescence detector (Jasco, Tokyo, Japan) and Shimadzu SPD-10A UV-Vis detector.

3.4.3 NONLINEAR MIXED EFFECTS MODELING

The software Phoenix was used for pharmacokinetic modeling. Rstudio and the R software were used for model assessments, diagnostics and data visualisation. The purpose of the pharmacokinetic modeling was to characterize the pharmacokinetics of enantiopure L-eflornithine and determine whether enantioselective pharmacokinetics occurred. The first-order conditional estimation method was used to model the *in vivo* pharmacokinetic data from plasma to assess the fit of one- or two-compartment drug disposition models. To describe the absorption data of D-eflornithine and L-eflornithine, different oral absorption models were fitted to the data. Tested models had a first-order absorption rate constant with or without lag time or were parameterized with a fixed or estimated number of transit compartments with or without a first-order absorption rate constant [174, 175]. The brain dialysate data were modeled by a sequential modeling approach with mid-time of each 30-minutes sampling window used as time input. The plasma pharmacokinetics was fixed before in and out flows from the brain compartment were estimated.

Model selection was based on physiological plausibility of parameters, parameter precision, change in -2LL and goodness-of-fit plots. Likelihood-ratio test was performed for discrimination between two nested models. A drop in -2LL greater than 3.84 was considered as a statistically significant improvement of the pharmacokinetic model ($P<0.05$) assuming a χ^2 distribution for Δ-2LL. Model evaluation were done by visual predictive checks and bootstrap (n = 1,000) to resample data sets and obtain confidence intervals of the pharmacokinetic parameter estimates. For more details around the nonlinear mixed effects modeling, see Paper IV.

3.5 CLINICAL PHARMACOKINETICS OF EFLORNITHINE

The pharmacokinetic modeling was performed with the purpose to simultaneously model plasma and cerebrospinal fluid concentration-time data from late-stage g-HAT patients. Pharmacokinetic parameters on influx and efflux rates between the plasma and cerebrospinal fluid compartments were estimated. Predictions by the pharmacokinetic model enabled the probability of target attainment analysis for different L-eflornithine-based dose regimens.

3.5.1 LITERATURE DATA

Clinical literature data from late-stage g-HAT patients from a study in Côte d'Ivoire were analyzed and patient characteristics are available in full

elsewhere [34]. In brief, 12 late-stage g-HAT patients were dosed 100 mg/kg and 13 late-stage g-HAT patients were dosed 125 mg/kg four times daily for two weeks, in the clinical study. Plasma samples were previously analyzed using an enantioselective analytical method [173] and modeled. The plasma pharmacokinetic modeling work is presented in full elsewhere [37]. Cerebrospinal fluid samples were also analyzed at the same time but were not included in the pharmacokinetic modeling. To acquire the cerebrospinal fluid samples, lumbar punctures were performed on day 10 before the first drug administration and before the last drug administration on the final study day. Authors of the previous publication [37] provided the data set on the D- and L-eflornithine concentrations in plasma and cerebrospinal fluid. The data set also included adverse events frequency data for malaise and diarrhoea that were used to determine potential enantioselectivity for these adverse events.

3.5.2 NONLINEAR MIXED EFFECTS MODELING

Pharmacokinetic modeling was performed with Phoenix software. Rstudio (Version 2021.09.0 build 351), the R software and the R package mrgsolve (Version 0.11.1) were used for model assessments, diagnostics, data visualisation and/or simulations. The model selection and discrimination was similar to the method described previously in Section 3.4.3 for Paper IV. In brief, assessment of goodness-of-fit plots, physiological plausibility and parameter estimation precisions were made and Δ-2LL >3.84 ($P<0.05$) was considered as a statistically significant improvement for nested models. The first-order conditional estimation method was used in the pharmacokinetic modeling of D- or L-eflornithine in plasma and cerebrospinal fluid. Drug distribution between plasma and cerebrospinal fluid was modeled using an effect compartment model or a peripheral compartment model with fixed volume. The total human cerebrospinal fluid volume ranges between 90 – 200 mL [176]. The peripheral compartment model with a fixed volume had an assumed cerebrospinal fluid volume of 150 ml based on literature data [177]. The plasma pharmacokinetics was fixed to prior parameter estimates from the previous publication with its model validation presented in full elsewhere [37].

3.5.3 EXTERNAL MODEL VALIDATION

Cerebrospinal fluid concentration data [35] and plasma concentrations data after oral or intravenous dosing [32] were used in the external model validation. The data on total eflornithine concentrations were digitized from plots using WebPlotDigitizer (Version 4.5, Pacifica, California, USA). The data were transformed to D- and L-eflornithine concentrations based on assumptions that intravenous data have a 50:50 L-eflornithine:D-eflornithine exposure ratio and oral data a 1:2 L-eflornithine:D-eflornithine plasma

exposure ratio for racemic eflornithine as previously seen in late-stage g-HAT patients [37].

3.5.4 PROBABILITY OF TARGET ATTAINMENT

Simulated cerebrospinal fluid concentrations of L-eflornithine by the final pharmacokinetic model were used in the probability of target attainment analysis. For racemic eflornithine, total concentrations of D- and L-eflornithine in cerebrospinal fluid over 50 μM have been associated with cure [35]. The *in vitro* IC_{50} ratio for L-eflornithine (5.5 μM) and racemic eflornithine (9.1 μM) is 1.65 [178]. Derived from this *in vitro* IC_{50} ratio, cerebrospinal fluid concentrations of L-eflornithine over the threshold concentration of 30.3 μM, i.e., 50 μM/1.65, were defined as attaining target. Similarly, the threshold concentration for L-eflornithine in combination with oral nifurtimox was based on findings from our previous pharmacodynamic modeling study [179]. In this analysis, nifurtimox exhibited a 2.52-fold apparent potentiation on the treatment with racemic eflornithine that lowers the threshold concentration in the present study to 12 μm, i.e., 30.3 μM/2.52. The probability of target attainment was defined as the fraction of predicted L-eflornithine concentrations in cerebrospinal fluid over 12 μM for L-eflornithine in combination with nifurtimox. A dose regimen was determined as adequate with probability of target attainment ≥ 90%.

4 RESULTS AND DISCUSSION

4.1 CHIRAL CHROMATOGRAPHIC ISOLATION OF D- AND L-EFLORNITHINE

The isolation of the eflornithine enantiomers from the racemic mixture is presented in Paper I. The eflornithine enantiomers were isolated on milligram scale using HPLC with the chiral stationary phase Chirobiotic R. In the optimized method, a mobile phase with 90/10 methanol/water (v/v%) and the additives 175 mM acetic acid and 35 mM triethylamine was used (Figure 7). The recovery was 49% and 48% for D-eflornithine and L-eflornithine, respectively. The enantiomeric purity was 99.1% for D-eflornithine and 95.7% for L-eflornithine. A previously developed HPLC method, with the chiral stationary phase Chirobiotic TAG on analytical scale [180] was used to determine the order that D- and L-eflornithine eluted from the chiral stationary phase Chirobiotic R.

A SFC method would allow for a more efficient isolation and is commonly used in early drug discovery for racemic compounds [135]. Unfortunately, none of the chiral stationary phase and modifier combinations tested in the present study could separate the eflornithine enantiomers without derivatization. The eflornithine enantiomers could be separated in the SFC mode when derivatized with ortho-phthalaldehyde. This was done firstly on analytical scale, using the chiral stationary phase Chiralpak IG and 35% ethanol with ammonia in CO_2. In semi-preparative scale, the ortho-phthalaldehyde derivates degraded in the process and no enantiopure material could be collected by this SFC method. Poor stability of the eflornithine-ortho-phthalaldehyde derivate has previously been shown [181]. Other derivatization agents could have been tested to potentially separate the eflornithine enantiomers. However, derivate stability and removal of the derivatization agent from the eflornithine enantiomers after separation could still be challenging. The developed SFC method for ortho-phthalaldehyde derivates of eflornithine could still be useful on analytical scale in, for instance, future bioanalytical applications. Asymmetric, i.e., stereoselective, drug synthesis would be an alternative approach to the semi-preparative chiral chromatography approach used in the present study [135]. However, only one of the two enantiomers would be obtained each synthesis cycle using this technique [137].

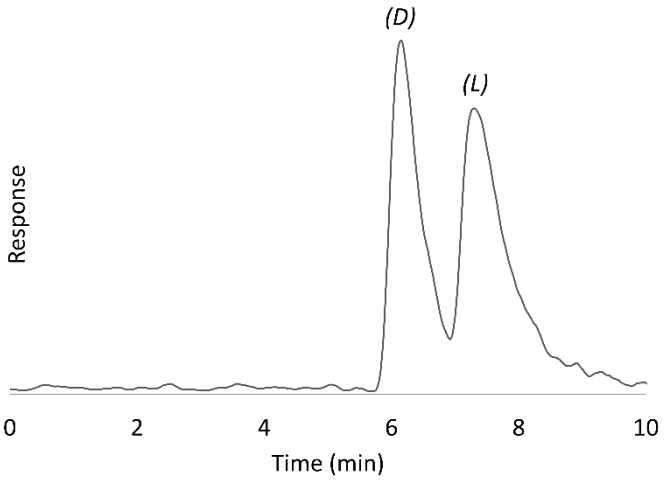

Figure 7. *Analytical scale separation of D- and L-eflornithine using Chirobiotic R and the mobile phase 90/10 methanol/water (v/v% [175 mM acetic acid and 35 mM triethylamine]) at 40 °C, flow rate of 1 mL/min. Reproduced with permission from ACS Omega: [182].*

4.2 ENANTIOSPECIFIC ANTITRYPANOSOMAL *IN VITRO* ACTIVITY OF EFLORNITHINE

The *in vitro* antitrypanosomal activities for L-eflornithine, D-eflornithine and the racemic mixture are presented in Paper II. The *in vitro* parasite growth of the *Trypanosoma brucei gambiense* strains was inhibited with the highest potency by L-eflornithine. The IC_{50} estimates for L-eflornithine were 4.1 μM (95% CI: 3.1 to 5.0), 8.9 μM (7.0 to 11) and 7.7 μM (6.8 to 8.5) for the STIB930, K03048 and 130R parasite strains, respectively. D-eflornithine exhibited the lowest potency with its IC_{50} estimates 39 μM (29 to 49) for STIB930, 73 μM (62 to 85) for K03048 and 76 μM (66 to 86) for 130R. The IC_{50} estimates for the racemic mixture of eflornithine were 6.4 μM (5.2 to 7.7), 17 μM (15 to 18) and 14 μM (12 to 17) for the STIB930, K03048 and 130R strains, respectively.

When estimating an overall *in vitro* potency for all three strains simultaneously, L-eflornithine exhibited the lowest IC_{50} estimate of 5.5 μM (4.5 to 6.6). D-eflornithine was less potent, with an approximately 9-fold increase in IC_{50} of 50 μM (42 to 57) (Figure 8). IC_{50} for the racemic mixture was 9.1 μM (8.1 to 10) which was 1.7-fold less potent than L-eflornithine. The lower potency for the racemate is suggested to be a consequence of the dilution

at a 1:1 ratio of L-eflornithine with less potent D-eflornithine. Moreover, a time-dependence in the antitrypanosomal activity was observed for L-eflornithine, D-eflornithine and the racemic mixture with steeper concentration-inhibition relationship, with higher estimates of γ after 72 h than after 24 h or 48 h. Moreover, lower IC_{50} estimates were also observed after 72 h compared to after 24 h or 48 h. These findings suggest that the *Trypanosoma brucei gambiense* parasites required a certain time of eflornithine exposure to enable proper growth inhibition. This time-dependent *in vitro* growth inhibition of *Trypanosoma brucei gambiense* parasites has also been observed for other g-HAT treatments [140].

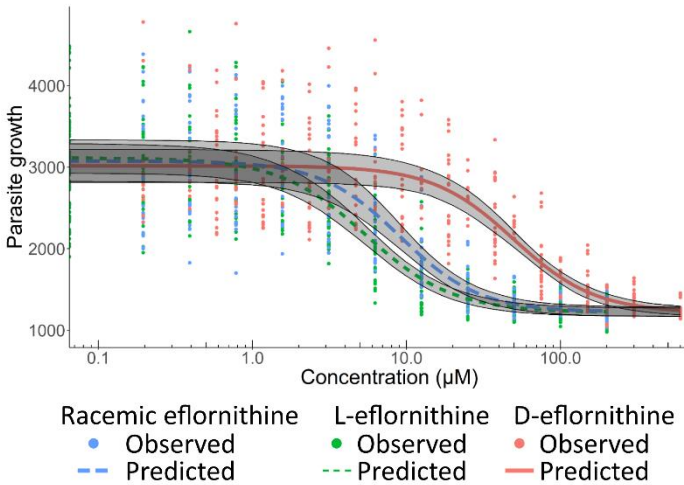

Figure 8. *Antitrypanosomal in vitro activity against Trypanosoma brucei gambiense parasites for racemic eflornithine (blue), L-eflornithine (green) and D-eflornithine (red). Dots represent observed parasite growth data, lines the model predictions and gray areas the 5th to 95th percentiles of predictions. Reproduced with permission from PLoS NTD: [178].*

The total concentrations of racemic eflornithine in cerebrospinal fluid are recommended to exceed 50 μM to avoid treatment failure [35]. This recommended concentration is 5.5-fold higher than the estimated overall *in vitro* potency for racemic eflornithine at 9.1 μM. With the lower overall *in vitro* IC_{50} estimate for L-eflornithine, this therapeutic threshold concentration could potentially be lowered for an enantiopure formulation with L-eflornithine. In a clinical study with a number of 25 late-stage g-HAT patients, cerebrospinal fluid concentrations for L-eflornithine over 23 μM were associated (not statistically significant) with higher probability of treatment success when treated with racemic eflornithine orally [37]. This concentration of L-eflornithine was over the *in vitro* 90% inhibitory concentration value of 17 μM

and approximately 4-fold higher compared to the estimated overall *in vitro* IC_{50} for L-eflornithine.

Three strains of *Trypanosoma brucei gambiense* parasites were tested in the current study, which is a strength. However, if more strains would have been studied, the results would be more generalizable and give higher confidence in future extrapolations using the presented *in vitro* findings. Moreover, using an *in vivo* infection model would also increase the confidence in the findings, as immune system components are not present in the *in vitro* assay. As the transporter TbAAT6 has been associated with resistance to eflornithine treatment [183, 184], it would also be relevant to study the potential enantioselectivity of this and/or other transporters. With radiolabelled eflornithine, it could be possible to determine if a potential enantioselective transport into the *Trypanosoma brucei gambiense* occurs. Overall, the *in vitro* findings with higher potency for L-eflornithine may be useful when predicting efficacious doses using pharmacokinetic and/or pharmacodynamic models.

4.3 PHARMACODYNAMIC TIME-TO-EVENT MODELING OF EFLORNITHINE

The time-to-event modeling for three eflornithine-based treatments and pharmacodynamic model predictions for L-eflornithine treatment against late-stage g-HAT are presented in Paper III. The pharmacodynamic model described the time-to-event for late-stage g-HAT patients adequately with baseline hazards estimates at 0.035, 0.026 or 0.077 $month^{-1}$ for intravenous eflornithine as monotherapy, intravenous eflornithine + oral nifurtimox or oral eflornithine monotherapy, respectively. Estimations for the doses associated with 50% reduction in baseline hazard were 159 g, 60 g or 291 g for intravenous eflornithine as monotherapy, intravenous eflornithine + oral nifurtimox or oral eflornithine monotherapy, respectively. The pharmacodynamic modeling-based analysis showed that the combination of intravenous eflornithine and oral nifurtimox was slightly superior compared to intravenous eflornithine monotherapy in terms of survival after 12 months follow-up. This finding concurs with a previous study where nifurtimox-eflornithine combination therapy was concluded to be a suitable treatment [47]. Moreover, the analysis in the present study showed that oral eflornithine monotherapy was inferior to both the nifurtimox-eflornithine combination therapy as well as the intravenous eflornithine monotherapy.

To predict survival for an L-eflornithine-based treatment, the ID_{50} parameter in the pharmacodynamic model was adjusted. This adjustment was based on the *in vitro* IC_{50} ratio for L-eflornithine and predicted IC_{50} for the 1:2 L-

eflornithine:D-eflornithine plasma exposure ratio in late-stage g-HAT patients after oral dosing of racemate [37]. Predicted IC_{50} for the 1:2 L-eflornithine:D-eflornithine ratio using the mathematical modeling approach was 13.4 µM (Figure 9).

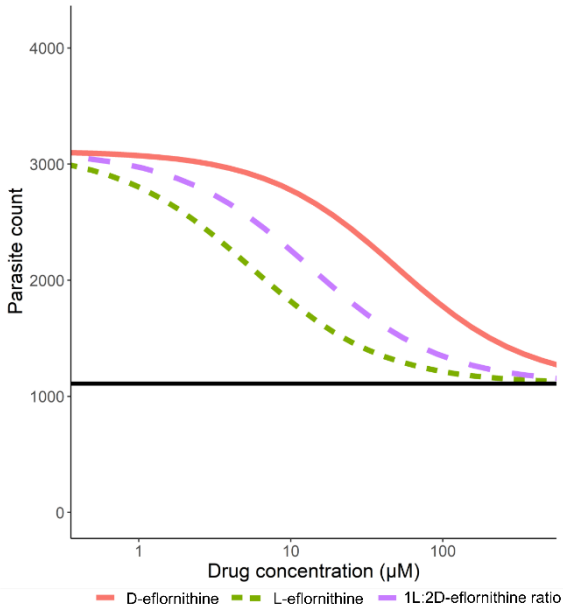

Figure 9. *In vitro efficacy predictions for the 1:2 L-eflornithine:D-eflornithine plasma exposure ratio (purple dashed line). The predictions were performed to obtain a potency estimate for oral racemic eflornithine using a mathematical modeling approach with experimental in vitro data [178] for L-eflornithine (green dashed green) and D-eflornithine (red solid line). Swedish National Data Service (SND-ID: 2021-45) database provided the data set [185]. Figure reproduced with permission from The AAPS Journal: [179].*

Thus, the ID_{50} parameter for the oral eflornithine monotherapy was adjusted by 2.46-fold (13.4 µM/5.5 µM) from 291 g to 118 g to predict survival for an oral L-eflornithine monotherapy using the pharmacodynamic time-to-event model. An assumption in the prediction was that the oral bioavailability for L-eflornithine would be unchanged when administering an enantiopure formulation with L-eflornithine compared to racemate. Oral racemic eflornithine monotherapy had a median survival of 68% (95% CI: 48 to 84) at 12 months. L-eflornithine monotherapy at the same total dose of 333 g, which is equal to 100 to 125 mg/kg dosed four times daily, had a higher predicted median survival of 80% (62 to 92) at 12 months follow-up (Figure 10).

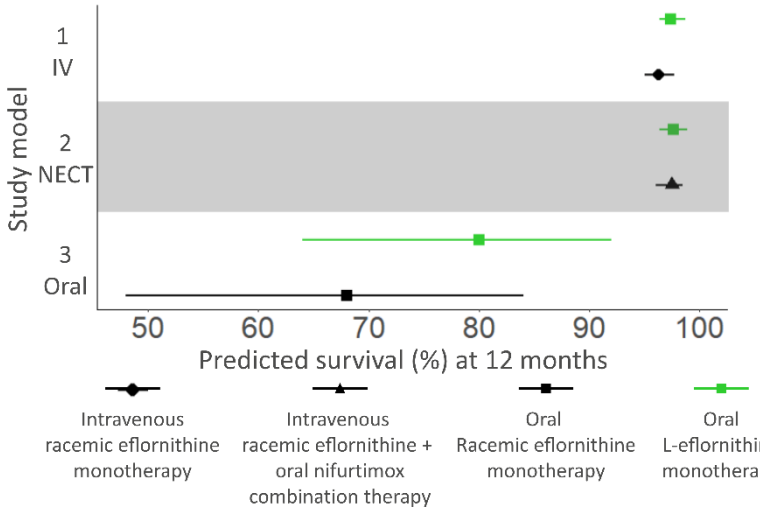

Figure 10. *Predicted survival after 12 months for intravenous racemic eflornithine monotherapy (black diamond represents median), intravenous racemic eflornithine in combination with oral nifurtimox (black triangle represents median) and oral racemic eflornithine monotherapy (black square represents median) against late-stage gambiense human African trypanosomiasis. Predicted survival after 12 months for oral L-eflornithine monotherapy at 333 g total dose (green squares represent median). Error bars show 95% confidence intervals for the predictions (n = 1,000). The gray area covers predictions based on the study with nifurtimox-eflornithine combination therapy. Figure reproduced with permission from The AAPS Journal: [179].*

A limitation in the pharmacodynamic modeling work is that the baseline hazard was not estimated with data from a placebo cohort. All patients in the included studies were treated with eflornithine or the nifurtimox-eflornithine combination therapy and placebo data were not found in the literature. Simultaneous estimation of an overall baseline hazard for all three treatments was tested. This overall estimation failed, as the parameter precision using this model was too poor. This led to the individual estimations of baseline hazards for each study. A second limitation is that a dose-response model was presented in the current study. A pharmacokinetic and pharmacodynamic concentration-response model would be preferable. A concentration-response model would provide information about exposures that lead to a certain outcome, i.e., cure or treatment failure. It was not possible to design such model since individual pharmacokinetic data were not available in all studies.

The racemic eflornithine treatment against late-stage g-HAT is associated with adverse events like diarrhoea, vomiting and/or nausea [186, 187]. To what

degree and with what mechanism the individual enantiomers potentially are contributing to the adverse event profile, or whether the total dose of eflornithine is the better predictor, remain to be determined. High levels of eflornithine in cerebrospinal fluid may lead to convulsions [35]. The adverse events related to eflornithine were generally reversible by lowering the doses or stopping the treatment [188]. Similar total doses of racemic eflornithine and L-eflornithine could potentially be dosed in future clinical studies if the eflornithine enantiomers contribute equally to the adverse event profile and potentially at the same time lead to higher survival. For the reported gastrointestinal adverse events [186], it is likely that these were caused by osmotic, i.e., not enantioselective, effects by the high amount of eflornithine administered orally [188]. In comparison to the racemic eflornithine monotherapy, the combination of eflornithine and nifurtimox was associated with less adverse events [45, 47]. It would therefore be relevant from a drug safety perspective to treat patients with the combination of L-eflornithine and nifurtimox, and/or potentially fexinidazole [50]. Moreover, appropriate next steps would be to determine the maximum tolerated oral dose for L-eflornithine alongside with the clinical minimum effective concentration in cerebrospinal fluid and/or plasma for late-stage g-HAT patients with and without other available treatments.

4.4 ENANTIOSPECIFIC *IN VIVO* PHARMACOKINETICS OF EFLORNITHINE

The *in vivo* pharmacokinetics of L-eflornithine after oral or intravenous administration of an enantiopure formulation or as racemic mixture are presented in Paper IV. A one-compartment drug disposition model with constant clearance adequately described the dispositions of L-eflornithine and D-eflornithine in the rat. The oral absorption was described by the transit compartment model (n = 1) with first-order absorption rate constant. For L-eflornithine, when dosed as an enantiopure formulation, the estimated clearance was 4.2 mL/min (95% CI: 2.9 to 6.3) and volume of distribution was 202 mL (141 to 273). For L-eflornithine dosed as racemic eflornithine, the clearance estimate was 3.3 mL/min (2.7 to 4.2) and volume of distribution was estimated to 173 mL (140 to 224). The estimated oral bioavailability for L-eflornithine as enantiopure formulation was 9.2% (3.8 to 17) in comparison to 17% (12 to 24) for L-eflornithine dosed in the racemic mixture. The overlap in the confidence interval for the bioavailability parameter suggested that a formulation with enantiopure L-eflornithine would not increase nor decrease the bioavailability. However, the parameter was estimated with poor precision. Since the oral absorption of eflornithine in rats and humans are suggested to be similar [37, 39], this finding indicated that the bioavailability in humans

may be comparable for an enantiopure L-eflornithine formulation as well. The bioavailability in humans could be determined in a future clinical study with oral administration of an enantiopure L-eflornithine formulation to late-stage g-HAT patients or healthy volunteers.

For D-eflornithine dosed as racemic eflornithine, clearance was estimated to 3.1 mL/min (2.5 to 3.7) and volume of distribution to 181 mL (150 to 226). The estimated oral bioavailability for D-eflornithine was 43% (32 to 55) when dosed as racemic mixture. This was approximately 2.5-fold higher than the bioavailability of L-eflornithine in the racemic eflornithine formulation. This enantioselectivity, with higher bioavailability for the less potent D-eflornithine [178], was in line with observations from previous preclinical studies with oral dosing of racemic eflornithine [38, 39]. More knowledge concerning the mechanism(s) of this disfavoured absorption of L-eflornithine would be valuable. There are different suggested hypotheses with, e.g., enantioselective paracellular absorption, formation of a chemical complex with a 1:2 ratio between D:L-eflorntihine or transport via active mechanism(s) [39]. With data from the present *in vivo* study, the hypothesis that chiral complex formation between one D-eflornithine and two L-eflornithine molecules in the gastrointestinal tract would hamper the absorption is not likely since oral absorption of enantiopure L-eflornithine was investigated in the current study.

The pharmacokinetics of D- and L-eflornithine in brain dialysate from the third brain ventricle in the rat after oral administration of racemic eflornithine were described by an effect-compartment model. The *in vitro* relative recovery values were used as correction factors to obtain concentrations in the brain dialysate. The *in vitro* relative recovery was 12.5% (standard error of the mean [SEM]: 0.9) and 12.2% (SEM: 0.9) for L-eflornithine and D-eflornithine, respectively. The estimated brain influx and efflux rate ratios were similar for L-eflornithine and D-eflornithine at 6.3% (4.0 to 8.7) and 5.5% (3.7 to 7.6), respectively. The similar ratios indicated that the uptake mechanism(s) to the third brain ventricle in the rat was not enantioselective.

The present study has its limitation. Uninfected rats were used to determine the *in vivo* pharmacokinetics of D- and L-eflornithine and parasitic infection has previously been associated with higher maximum plasma concentration in mice [189]. Potentially, the cytokines in the central nervous system could affect the endothelial barrier, which may lead to higher blood-brain barrier permeability [190-192]. It would be relevant to carry out future work with a *Trypanosoma brucei* infected *in vivo* model. Investigations with this type of *in vivo* model would also enable efficacy studies for various drug combinations with L-eflornithine and other available g-HAT treatments.

4.5 CLINICAL PHARMACOKINETICS OF EFLORNITHINE IN CEREBROSPINAL FLUID

The clinical pharmacokinetics of D- and L-eflornithine in plasma and cerebrospinal fluid after oral administration of racemic mixture are presented in Paper V. Pharmacokinetics of D- and L-eflornithine in cerebrospinal fluid after oral dosing were adequately described by the final pharmacokinetic model. The cerebrospinal fluid to plasma exposure ratio was similar for D- and L-eflornithine, suggesting that the extent of D- or L-eflornithine transport to and from cerebrospinal fluid was not enantioselective. External validation of the pharmacokinetic model with data from Milord *et al*. [35] and Haegele *et al*. [32] showed that the model was able to describe cerebrospinal fluid data but overpredicted plasma concentrations in the elimination phase. The cerebrospinal fluid to plasma ratio shows high interindividual variability in late-stage g-HAT patients, but eflornithine can reach the cerebrospinal fluid from plasma [34, 35]. A limitation in the present study is that only two cerebrospinal fluid samples from each patient were available for the pharmacokinetic modeling. A second limitation is that the M&S work was based on data from only 25 late-stage g-HAT patients, which comes with the assumption that the pharmacokinetic M&S work was generalizable and represented the pharmacokinetics in the late-stage HAT patient population in an adequate way.

For eflornithine, the plasma and cerebrospinal fluid concentrations of eflornithine drive antitrypanosomal effect in late-stage g-HAT patients. For racemic eflornithine, concentrations >50 μM, which is 5.5 times higher than the *in vitro* IC_{50} of 9.1 μM for racemic eflornithine [178], were associated with successful treatment [35]. The *in vitro* findings in Paper II showed that L-eflornithine had higher *in vitro* potency compared to D-eflornithine [178]. Different oral dose regimens for L-eflornithine were therefore simulated using the pharmacokinetic model to predict L-eflornithine concentrations in cerebrospinal fluid. These predicted concentration-time profiles were used in the probability of target attainment analysis. The clinically relevant cut-off value of 30.3 μM, corresponding to 5.5 times the *in vitro* IC_{50} of 5.5 μM for L-eflornithine [178], was used to define target attainment. To achieve over 90% probability of target attainment, L-eflornithine doses over 750 mg/kg/day administered four or twelve times daily were needed. This total daily dose is higher than the highest racemic eflornithine dose of 500 mg/kg/day administered in the clinical study [37]. It is critical to be cautious and monitor the occurrence of potential adverse events if this high dose level is used in a

future clinical study. The L-eflornithine nifurtimox (15 mg/kg/day nifurtimox doses) combination therapy exhibited a probability of target attainment over 90% for L-eflornithine doses of 375 mg/kg/day administered two, four or twelve times daily. This total daily dose is lower than the highest 500 mg/kg/day dose in the clinical study with racemic eflornithine [37]. The probability of target attainment analysis and the threshold concentrations were based on *in vitro* findings. It is therefore important to assess the generalizability of these translations carefully if using the findings to design future studies.

It would be valuable to establish the maximum tolerated oral L-eflornithine dose and minimum effective concentration for L-eflornithine in a prospective clinical study. Furthermore, investigating potential combination treatments with L-eflornithine and nifurtimox and/or fexinidazole would be of high relevance. In Paper III, data suggested that tolerated oral doses of L-eflornithine should be administered concomitantly with the other treatment options to avoid treatment failure [179]. Adverse events associated with eflornithine treatment are malaise and diarrhoea [29]. The probability of target attainment analysis was performed with higher doses of L-eflornithine than the tolerated oral doses [34]. If adverse events such as malaise and diarrhoea might be dependent on doses and/or exposures of D- or L-eflornithine in the clinical setting is yet to be determined. However, the limited data in the present study, in terms of both dose range and number of patients in the clinical study [34], suggested that none of the enantiomers were individually driving the frequencies of malaise or diarrhoea adverse events.

To summarize, the pharmacokinetic model showed that D- and L-eflornithine exposures after oral dosing of racemate in cerebrospinal fluid were not enantioselective. Model predictions showed that high doses of 750 mg/kg/day of L-eflornithine as monotherapy would be required to achieve adequate probability of target attainment. The combination of L-eflornithine and nifurtimox required lower L-eflornithine doses of 375 mg/kg/day administered two, four or twelve times daily to render adequate probability of target attainment. The pharmacokinetic M&S work presented in the current study could serve as a starting point for dose selection and designing future clinical studies with L-eflornithine-based treatments.

5 CONCLUSION

The pharmacokinetics and pharmacodynamics of L-eflornithine have been studied in this thesis with a translational M&S approach to assess its feasibility as a late-stage g-HAT treatment. To enable preclinical studies, enantiopure material of D-eflornithine and L-eflornithine was isolated by a chiral chromatography method. The *in vitro* and preclinical *in vivo* data were analyzed using nonlinear mixed effects modeling. The findings were used in a translational M&S approach to predict drug exposure and probability of target attainment of the more potent L-eflornithine enantiomer in late-stage g-HAT patients by the final pharmacokinetic model. The main conclusions were:

D-eflornithine and L-eflornithine enantiomers were isolated by a milligram scale high-performance liquid chromatography method with the chiral stationary phase Chirobiotic R.

Antitrypanosomal *in vitro* potency was higher for L-eflornithine than D-eflornithine for all three strains of T*rypanosoma brucei gambiense* parasites studied. The overall *in vitro* potency was 9-fold higher for L-eflornithine compared to D-eflornithine.

Using a pharmacodynamic time-to-event model, a median survival of 80% for oral L-eflornithine as monotherapy at doses anticipated to be tolerated in late-stage g-HAT patients could be expected based on the model predictions.

Bioavailability of enantiopure L-eflornithine *in vivo* was similar to that of L-eflornithine administered as racemic mixture, however, estimated with poor precision. After intravenous dosing, the plasma pharmacokinetics was similar for enantiopure L-eflornithine and L-eflornithine in the racemic mixture suggesting that enantiopure L-eflornithine will not provide higher absolute bioavailability. Moreover, uptake to the third brain ventricle in the rat of D-eflornithine and L-eflornithine was not enantioselective.

Oral monotherapy of L-eflornithine dosed at 750 mg/kg/day four or twelve times daily could be efficacious regimens based on predictions by the final pharmacokinetic model and probability of target attainment analysis. In combination with nifurtimox, L-eflornithine doses at 375 mg/kg/day administered two, four or twelve times daily could be efficacious against late-stage g-HAT.

6 FUTURE PERSPECTIVES

6.1.1 A FUTURE WITH AN ORAL EFLORNITHINE THERAPY?

For the most severe late-stage g-HAT patients, the current treatment is repeated intravenous infusions of racemic eflornithine as monotherapy or in combination with oral nifurtimox [24]. An oral treatment with eflornithine would make logistics easier and reduce burden on healthcare systems in the endemic areas in sub-Saharan Africa [34]. With the discovered higher antitrypanosomal *in vitro* potency for L-eflornithine, a treatment with enantiopure L-eflornithine could potentially be a way forward to enable an oral eflornithine-based therapy. The absorption, distribution, metabolism and excretion profile for L-eflornithine in the racemic mixture is well known since it has been used to treat late-stage g-HAT for decades [5]. However, the main hurdle for an oral eflornithine therapy has been the lower bioavailability with lower oral absorption for L-eflornithine compared to D-eflornithine [37].

The latest developed drug against g-HAT, fexinidazole [55, 57], fulfilled several of the target product profile criteria as it can be used treat both early-stage and late-stage g-HAT, is dosed orally for 10 days and is a tolerated treatment by g-HAT patients. With that said, survival after two years was lower for the patient cohort treated with fexinidazole (3.4%) compared to the nifurtimox-eflornithine combination therapy cohort (1.5%) [53]. The observed difference in treatment failures indicates that there is room for improvement where a potential future oral L-eflornithine-based treatment may be a feasible option to treat late-stage g-HAT. Another future approach would be to determine the clinical efficacy of combination therapies including the promising candidate drug acoziborole [83]. By combining treatments that differ in their mechanisms of action, the drug resistance development risk is suggested to be reduced [48]. In the latest treatment recommendations against late-stage g-HAT, the nifurtimox-eflornithine combination therapy is the only drug combination against g-HAT [12, 47, 193]. Resistance against nitro drugs such as nifurtimox and fexinidazole has been demonstrated in laboratory studies [194, 195]. If drug resistance for nitro drugs develops in endemic areas, a future L-eflornithine-based treatment could potentially be a solution as an oral therapy.

The combination of available g-HAT treatments would also be interesting to study in a potential continuation of the preclinical evaluation work of L-eflornithine. Knowledge gained about oral absorption and *in vivo* pharmacokinetics of L-eflornithine when administered concomitantly with nifurtimox, fexinidazole and/or acoziborole would be clinically relevant.

Moreover, it may be useful to evaluate the blood-brain barrier permeability of drugs in infected *in vivo* models as infection may affect the pharmacokinetics [36]. The data would potentially elucidate how inflammation may affect late-stage g-HAT treatment exposures in the central nervous system as cytokines have been shown to affect blood-brain barrier permeability [190, 192, 196]. With regards to bioavailability, other pharmaceutical strategies to enhance the bioavailability of L-eflornithine would be relevant to study in the future. Various salt forms of L-eflornithine and/or permeation enhancers used in clinical and preclinical studies [197] to increase the permeability of drugs could be tested. Apart from the perspectives for L-eflornithine as g-HAT treatment, there could also be a future for an enantiopure eflornithine-based cancer treatment against, for example, malignant gliomas [198] as eflornithine was initially developed as an anticancer drug [199].

6.1.2 FUTURE MODEL INFORMED DRUG DEVELOPMENT

The pharmacokinetic and pharmacodynamic M&S work in this thesis shows what potential L-eflornithine-based treatments may hold. In the future, pharmacokinetic and pharmacodynamic M&S may enable the design of studies to gain more knowledge about the risk-benefit ratio for treatments, improve chances of successful clinical trials, optimize current treatments and enable personalized dosing. For neglected tropical diseases in general, few of the published pharmacokinetic studies use the findings for simulations or extrapolations [200]. A neglected tropical disease like g-HAT would benefit from M&S where scarce data can be used to characterize key pharmacokinetic and pharmacodynamic relationships [200]. In the future, machine learning methods may be used to describe these relationships for neglected tropical disease treatments and predict drug exposure as has been shown for the immunosuppressive drug tacrolimus [201] as well as the opioid remifentanil [202]. The work presented in this thesis with the integration of preclinical data and literature data on clinical pharmacokinetics of L-eflornithine could serve as a preliminary indication for future clinical studies. An approach with pharmacokinetic and pharmacodynamic M&S using clinical data from g-HAT patients could decrease the risk of treatment failure meanwhile maintaining drug-related adverse events at a minimum by a more personalized treatment approach. Moreover, it is also central to keep the interplay between the patient, parasite and drug in mind [203]. Pharmacokinetic and pharmacodynamic M&S would be able to describe this complex interplay. Prospective clinical studies with samples from late-stage g-HAT patients could provide such important information too. New clinical data may also confirm if the findings regarding enantioselective pharmacodynamics and pharmacokinetics are adequately translated from the used non-clinical models to humans.

6.1.3 A FUTURE WITHOUT HUMAN AFRICAN TRYPANOSOMIASIS?

The epidemiological data with decreasing numbers of HAT cases are promising. With over 25,000 reported HAT cases annually in 2000 and in 2001 [8, 10], the goal was set out by the World Health Organization to eliminate HAT as a public health problem by 2020. This has been achieved in some sub-Saharan countries, with Togo and Côte d'Ivore as examples of nations that have eliminated HAT [7]. The active screening campaigns as well as the passive surveillance in endemic countries have been effective. However, the g-HAT situation can vary between nations and countries in the region may have health care system challenges that affect both disease elimination progress and data collection [7]. Climate change may also affect the location where tsetse flies are able to live in the future which may lead to new disease areas, as exemplified for Zimbabwe [204]. Misdiagnosis or delayed diagnosis could also be challenging with the currently lower HAT incidence and healthcare personnel need to be alert and keep testing patients with suspected *Trypanosoma brucei* infections [205].

In the future with more than one recommended treatment, also with a potential acoziborole therapy, it must be established what g-HAT patient that would benefit the most from what treatment. To conduct these future studies, challenges with logistics in the most rural areas of sub-Saharan Africa need to be addressed including sample collection, cold chain transportation and laboratory facilities. Furthermore, available funding is limited to develop new treatments for HAT and other neglected tropical diseases [206]. Research and development for new treatments and optimization of currently available treatments are crucial to treat and eliminate HAT. Currently, novel antitrypanosomal targets are evaluated in the drug discovery phase including *Trypanosoma brucei* phosphofructokinase, *Trypanosoma brucei* phosphodiesterase or the Cdc-like kinase CLK1 [207]. Studies with adequate pharmacokinetic and pharmacodynamic analyses have, unfortunately, been absent for many neglected tropical diseases in general [200]. For late-stage g-HAT, the findings based on pharmacokinetic and pharmacodynamic M&S presented in this thesis could be a starting point for designing studies with an L-eflornithine-based treatment. Hopefully, the clinical situation with arsenic derivate treatments like melarsoprol will be history for HAT patients as better treatments are or become available. A forthcoming future world where HAT is eliminated would be second to none. Whether the clinical use of an L-eflornithine-based treatment is part of that future or not is still to be explored.

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