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# Kinetoplastid diseases: Insights into the mechanisms of drug action and resistance for novel drug discovery

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ABSTRACT

*Trypanosoma* and *Leishmania* species are kinetoplastid protozoan parasites that cause diseases which result in significant disability-adjusted life years (DALYs) and agricultural losses in the developing world. Despite the progress recorded in understanding biology and chemotherapy of these pathogens of neglected diseases, treatment failure, due to drug resistance or toxicity-driven non-compliance remain major challenges. Advances in molecular parasitology have led to the identification of specific transport mechanisms, druggable targets and persister-like cells, which play distinct roles in the overall success of therapies. Transporters and other cellular transport mechanisms affect the internalisation of drugs and their intracellular availability which determine drug activity. Thus, we reviewed kinetoplastid drug transport mechanisms, molecular drug targets and persisters to highlight mechanisms of action and development of resistance for antikinetoplastid drugs, with the aim of providing novel insights for drug discovery programmes.

## 1. Introduction

Kinetoplastids are protozoan parasites characterized by a dense network of concatenated DNA within the mitochondrion called kinetoplast (De Rycker et al., 2023). Click or tap here to enter text. They are unicellular pathogens that parasitise some plants and most animals, severely impacting global health and economy (Filardy et al., 2018). Two genera, *Trypanosoma* and *Leishmania* bring tryapnosomatids to prominence due to their role as human pathogens. The human pathogens are *Trypanosoma brucei gambiense* and *T. b. rhodensiense* (causing human African trypanosomiasis or sleeping sickness), *Trypanosoma cruzi* (causing Chagas disease) and *Leishmania* spp. (causing leishmaniasis). While *T. congolense*, *T vivax* and *T. brucei* infect animals causing severe chronic debilitating disease (nagana) with high mortality if left untreated. Other pathogenic veterinary trypanosomes include *T. simiae* (which infects pigs), *T. evansi* (which causes surra), *T. equiperdum* (causative agent of dourine in equids). Human kinetoplastid diseases have been designated as part of the neglected tropical diseases (NTDs) by the World Health Organization, which affect more than a billion people across 149 countries and generate a cumulative economic burden amounting to billions of dollars every year (Brigger, 1944). *Trypanosoma* and *Leishmania* are obligatorily dixenous parasites that exhibit zoonotic or anthroponotic life cycles, and are transmitted by hematophagous insects and other vectors (Kaufer et al., 2017).

Drugs used in the treatment of kinetoplastid diseases are far from ideal due to increasing rates of drug resistance, toxicity, high cost, and limited access. Therefore, a thorough understanding of the parasite biology and their interaction with drugs, at the cellular and molecular levels is necessary for the discovery of new therapeutic targets and agents. Recent advances in parasitology, chemistry, genomics and bioinformatics have generated vast information, which opens new opportunities for novel drug concepts and compound screening technologies. The presence of unique ultrastructural and metabolic features in these protozoan parasites presents druggable targets that can be exploited through the available screening tools (Menzies et al., 2018). The mitochondrion of kinetoplatid is a fascinating organelle that is essential to

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their survival in the mammalian hosts and contains several therapeutic targets. Due to the variety of information uncovered about the structure and function of mitochondria in numerous cell types, including protozoa and cancerous mammalian cells, the past ten years have rekindled interest in mitochondrial research for drug development (Ebiloma et al., 2019). A greater knowledge of mitochondrial function (Pedra-Rezende et al., 2022) and drug mechanism of action (Pedra-Rezende et al., 2022) has been made possible by several scholarly reviews in this area.

Target-based screening is one of the most important strategies for drug discovery against kinetoplastid parasites. It involves screening for inhibitors against a purified protein (such as an enzyme) and the efficacy of compounds shown to modulate the protein are evaluated in a cellular model (Balogun et al., 2019a). The first step in target-based drug discovery involves the identification of specific putative molecular targets that play a crucial role in the pathophysiology of a particular disease (Herrera-Acevedo et al., 2022), which is followed by the design of small molecules that will disrupt the function of those specific targets. The drug target molecules should be either absent in the mammalian host or structurally dissimilar to those found in the host (Chatelain and Ioset, 2018). Important druggable targets in kinetoplastid parasites have been identified and elucidated (Field et al., 2017b; Kourbeli et al., 2021a), which offers prospects for the discovery of novel and more effective drugs against these NTDs. Hence, this review present important druggable kinetoplastid proteins with the aim of highlighting their biological functions at the molecular level, to further understand the mechanisms of drug resistance and stimulate novel drug discovery.

## 2. Chemotherapy of kinetoplastid diseases

### 2.1. Chemotherapy of Chagas disease

To date, only two drugs have been approved for the treatment of Chagas disease, the nitroheterocyclic compounds benznidazole and nifurtimox (Fig. 1) de Melo et al. (2025). Click or tap here to enter text. Such treatments are currently indicated for acute cases, congenital infections, reactivations and patients in the chronic phase without symptoms or with mild cardiac or digestive involvement (Alonso-Vega et al., 2021). These drugs are effective in the acute phase, whereas in adults with chronic infection, clinical trials have shown limitations, and both drugs cause several toxic effects leading to their restricted use (Coura, 2014). Benznidazole is better tolerated in adults, although 15–30% of patients are unable to finish the standard 60-days course, mainly because of skin and nervous system complications. In children, nifurtimox is better tolerated than benznidazole (Rao et al., 2019). The mechanism that results in the activation of nitroheterocyclic drugs in trypanosomes is achieved through the involvement of the nitroreductase enzyme and thus, reduced activity of this enzyme leads to resistance to benznidazole and nifurtimox (Murta et al., 2024)Click or tap here to enter text. Benznidazole prevents the multiplication of the parasites through covalent modifications of biomolecules. Nifurtimox generates nitro radical anions and becomes reactive in the presence of oxygen (Jacobs et al., 2011). Nifurtimox is no longer used as a therapeutic agent of Chagas disease in Brazil because it causes neurological disorders or psychiatric episodes as side effects (Spaulding et al., 2019). Progress has been made in the research of new promising drugs against the disease. E1224, a pro-drug of ravuconazole, has shown some efficacy in treating the disease in infected patients. Furthermore, its combination with benznidazole is more effective as compared to monotherapy-based regimens (Chatelain and Ioset, 2018).

### 2.2. Chemotherapy of African trypanosomosis

The five drugs approved for the treatment of human African trypanosomosis include pentamidine, melarsoprol, eflornithine, suramin, and

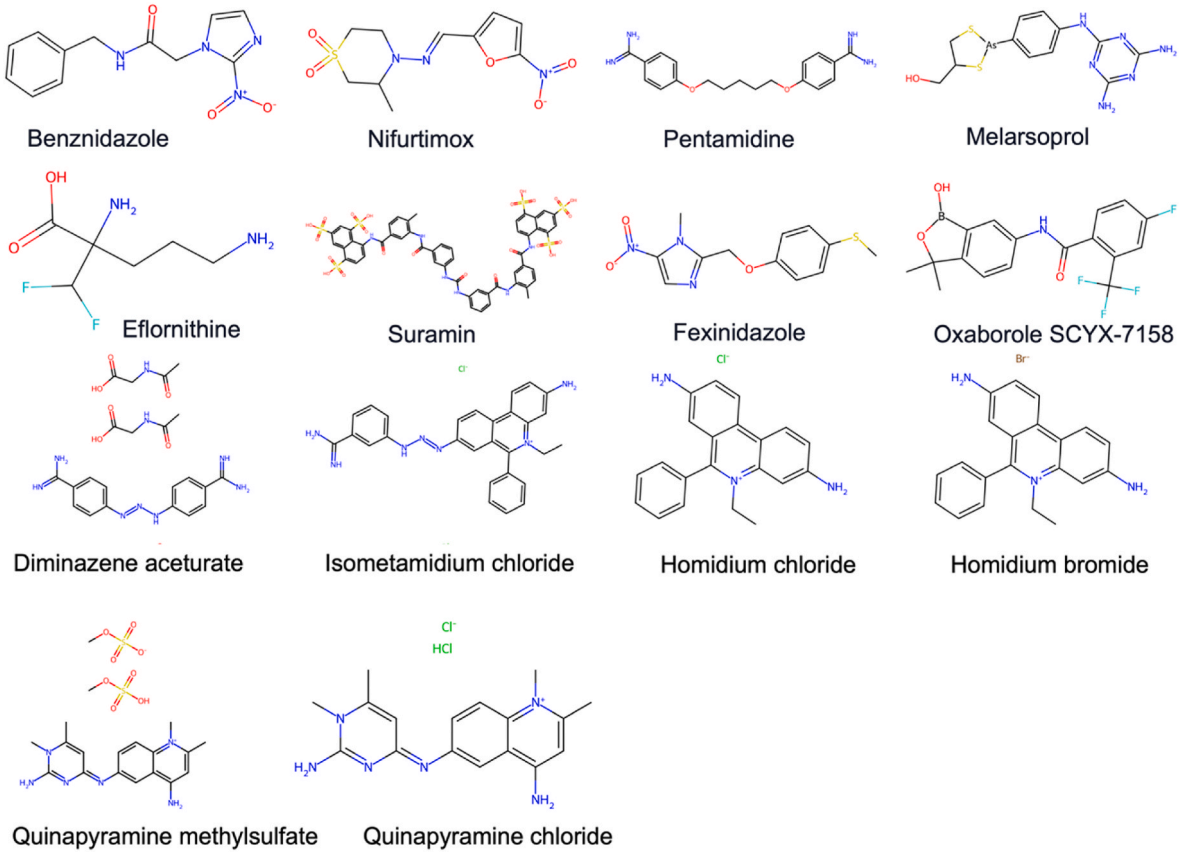


**Fig. 1.** Chemical structures of chemotherapeutic agents against trypanosome parasites.

nifurtimox (Fig. 1) (Cullen and Mocerino, 2017; Kourbeli et al., 2021a). Pentamidine and suramin are used in monotherapies in the early stage of *T. brucei gambiense* and *T. brucei rhodesiense* infections, whereas melarsoprol is used for the second stage of the disease (Babokhov et al., 2013). Nifurtimox has been used since 2009 in combination with eflornithine, mostly in the second stage of *T. brucei gambiense* infection. The combination therapy protocols for these two drugs have been improved, although there are still practical restrictions to their potential use in large-scale applications (Filardy et al., 2018). Pentamidine presents relative toxicity and patients need to be monitored during therapy. This drug acts by preventing the synthesis of trypanosomal proteins, nucleic acids, phospholipids, and folate. It also causes the inhibition of enzymes of the polyamine synthesis and the RNA polymerase activity (Simarro et al., 2012).

Suramin is used for the treatment of first-stage human African trypanosomosis caused by *T. brucei rhondesiense*. This compound was first introduced in 1917, and is considered a broad-spectrum drug, with promising results against other trypanosomes. However, its mechanism of action has not been fully elucidated (Wiedemar et al., 2020).

Melarsoprol administration presents high toxicity that leads to reactive encephalopathy (Seixas et al., 2020) and death. This led to its replacement with eflornithine that has been in the market since 2001. However, eflornithine requires large doses of administration (56 infusions during 14 days in 14 L of sterile saline) and consequently, daily visits of patients to health facilities and hospitals. This becomes impractical since rural habitants who are mostly affected by the disease do not have easy access to hospitals; thus, the toxic but easily administered melarsoprol is still being used for the treatment of human African trypanosomosis. Moreover, eflornithine presents a high risk of resistance to patients, resulting in the possibility of untreated second-stage disease. The concept of combining therapies started to gain ground, as a way of optimizing drug efficacy and lowering the resistance to therapy Barbosa et al. (2024). Click or tap here to enter text. Various clinical studies have been performed using a combination of eflornithine, melarsoprol, and nifurtimox. All the studies on the administration of melarsoprol reported high toxicity of the drug, while promising results were reported for nifurtimox-eflornithine combination therapy (NECT therapy) for the treatment of second-stage disease caused by *T. brucei gambiense*. To eliminate the difficulty in the administration of eflornithine, the World Health Organization (WHO) designed a medical kit, consisting of the drug in an accessible form, and trained health workers to implement the therapy (Lindner et al., 2025). Click or tap here to enter text. The NECT therapy is not effective against *T. brucei rhodesiense,* thus melarsoprol remains the only efficient drug for infection caused by this parasite.

Currently, new drugs designed to improve patient care are being considered to meet current elimination targets. Most of these have been optimized for clinical trials (Cullen and Mocerino, 2017). Fexinidazole and oxaborole SCYX-7158 (Fig. 1), are already being studied in clinical evaluation as oral therapies for *T. brucei* infections (Jacobs et al., 2011).

### 2.3. Chemotherapy of veterinary trypanosomatids

Chemotherapy and chemoprophylaxis are the mainstay of veterinary trypanosomoses control measures and will probably remain so for a long time, despite of the problems of drug resistance and toxicity (Baldissera et al., 2016), as no vaccine has been developed against the disease. The drugs currently in use in animal trypanosomosis include: diminazene aceturate (Berenil®), isometamidium chloride (Samorin®), homidium chloride (Novidium®), homidium bromide (Ethidium®), quinapyramine methylsulfate (Antrycide®), and quinapyramine methylsulfate/chloride (Antrycide prosalt®) (Fig. 1). These trypanocides have been in use for over 50 years. They have small therapeutic indices and can also cause local irritation at the injection site. Most importantly, extensive utilization in the past has led to the appearance of resistant parasites in the field, and the fact that many of these trypanocides are chemically related has exacerbated the situation with the onset of cross-resistance (Eghianruwa and Oridupa, 2018). Diminazene aceturate is used exclusively as a therapeutic agent, while isometamidium has both prophylactic and curative properties. Homidium has limited prophylactic properties, and its use has dwindled due to carcinogenic concerns. The decision to use therapeutic or prophylactic drug depends on several factors, including the risk of infection, drug availability and distribution logistics (GIORDANI et al., 2016).

### 2.4. Chemotherapy of leishmaniasis

There are only a few drugs available for the treatment of leishmaniasis and most of them have been in use for many years. These drugs are used as monotherapy or in combination. Since there is no vaccine available for the prevention of leishmaniases, the control of the disease depends mainly on chemotherapy. The therapeutic scheme depends on the type of the disease (cutaneous or visceral leishmaniasis), the parasite species, the geographic location, and probable underlying diseases of the patient. The significant toxicity, low efficacy, and increasing drug resistance are some of the most important problems of the current drugs. The high cost and poor availability of drugs, along with the long duration of therapy and the need for hospitalization are other concerns, especially in countries where the disease is endemic (Mansuri et al., 2020; Rao et al., 2019).

Pentavalent antimonials are the oldest category of antileishmanial drugs. The use of antimony for the treatment of leishmaniasis has been known for century and the first pentavalent antimony compound was synthesized and proved effective against visceral leishmaniasis in 1925. Today, there are two pentavalent antimonials (sodium stibogluconate and meglumine antimonite, Fig. 2) that are used as first-line monotherapy for visceral leishmaniasis, and they still are in canine leishmaniasis (Olías-Molero et al., 2021). These drugs inhibit enzymes of the glycolytic pathway and fatty acid oxidation in *Leishmania* parasites (Mansuri et al., 2020).

Amphotericin B is a polyene antibiotic that has been used as antileishmanial drug for several decades (Fig. 2). Amphotericin B increases the membrane permeability of *Leishmania* spp. by binding to ergosterol of the parasite membrane (Olías-Molero et al., 2021). This drug is used as a second line drug for visceral leishmaniasis, and even though it exhibits major side effects such as nephrotoxicity (Mansuri et al., 2020), it is recommended by the WHO as a first-line therapy in South Asia, where the cure rate of antimonial drugs is low. AmBisome®, a liposomal form of amphotericin B, is very effective and devoid of the side effects of the conventional amphotericin B, however, its high price makes it un-affordable for many patients and countries, especially for some endemic countries (Uliana et al., 2018).

Paromomycin is an aminoglycoside antibiotic, which has been used as antileishmanial agent since the 1980s and is currently administered as a second-line drug, but it is not used as a single agent, because of the risk of developing drug resistance (Fig. 2) (Mansuri et al., 2020; Uliana et al., 2018). Its mechanism of action is apparently related to the inhibition of protein synthesis by specifically binding to 16S ribosomal RNA (Olías-Molero et al., 2021).

Miltefosine is an alkylphospholipid derivative (Fig. 2), approved for the treatment of leishmaniasis in 2002. It was first developed as an antineoplastic agent because of its ability to induce selective apoptosis in tumor cells but was also found to have activity against *Leishmania* spp (Bora et al., 2024)Click or tap here to enter text. Miltefosine modulates parasite membrane receptors and changes the structure of the cell membrane by inhibiting the metabolism of phospholipids. It also affects various intracellular pathways leading to regulated cell death (Basmaciyan et al., 2018). The use of miltefosine is being restricted because of its significant toxicity and teratogenic properties (Mansuri et al., 2020). Combination therapy with at least two drugs is proposed to increase the therapeutic efficacy and avoid drug resistance. The use of a combined dosage form also aims to shorten the duration of therapy and reduce the drug doses as well as to eliminate the toxicity and increase

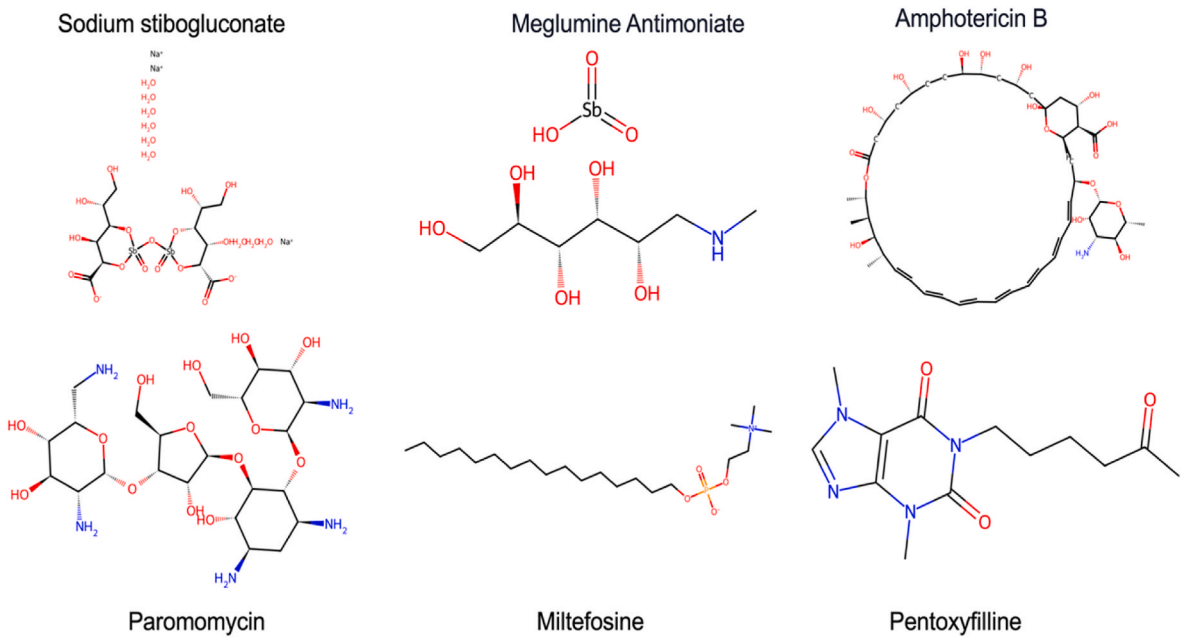


**Fig. 2.** Chemical structures of chemotherapeutic agents against leishmania parasites.

the compliance of patients. More so, this therapeutic approach is cost effective. There are several forms that are used, such as sodium stibogluconate with paromycin and amphotericin B or paromycin in combination with miltefosine. There are also some repurposed drugs, e.g., pentoxyfilline (Fig. 2), allopurinol, and various azoles that are used in combination dosage forms, along with antileishmanial drugs (Uliana et al., 2018).

Although the chemotherapeutic agents currently used in the treatment of leishmaniasis infections have been in circulation for decades and are far from perfect, commercial interests in developing new therapeutics is limited by their affordability and accessibility to the affected poor population (de Menezes et al., 2015). In addition, the advances in understanding the biology of these parasites have translated into the discovery of only a few new chemotherapeutic compounds in the last decades. Yet, the need for the development of completely new classes of therapeutic agents, with reduced host toxicity and improved administration profile remains high (Field et al., 2017b). Many drug discovery projects are now focusing on transporter proteins because of their roles in drug action and resistance.

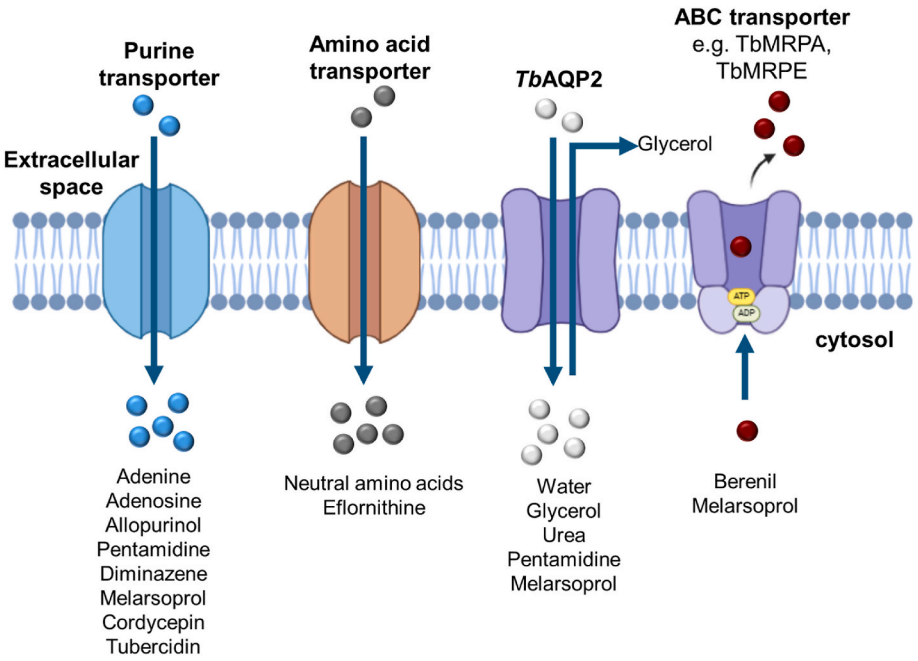


**Fig. 3.** Drug transporters involved in drug action and resistance in *T. brucei.* Purine transporters mediate the uptake of purine nucleosides and serve as high-affinity transporters for adenosine analogs, pentamidine, and diminazene. The amino acid transporter facilitates the uptake of neutral amino acids across the parasite membrane and mediates eflornithine transport. *Tb*AQP2, uniquely expressed in *T. brucei*, enables the movement of water, glycerol, and urea across the plasma membrane while functioning as a high-affinity transporter for pentamidine and melarsoprol. ABC transporters are efflux pumps that expel toxic drugs out of the parasite and contribute to drug resistance.

## 3. Drug transporters in kinetoplastids

The intracellular concentrations of metabolites are regulated by membranes and transporter proteins (permeases). Transporters are broadly categorized into channels and carriers. They are integral membrane proteins that allow the passage of nutrients, ion molecules, and macromolecules, such as proteins, from one intracellular compartment to another (Landfear, 2008; Valera-Vera et al., 2020). Membrane transporters that are essential to parasite survival are often targeted for drug discovery, either for delivery of antiparasitic agents into parasites or as drug targets (Fig. 3). Some currently used antiprotozoal drugs enter into the parasite through membrane transporters, hence, decreased expression or mutations causing loss of function in these transporters are largely responsible for drug resistance. Similarly, drug resistance can occur due to overexpression or an increase in the activity of transporters or efflux pumps involved in the efflux of toxic drugs out of the parasite cells (Landfear, 2008; Possart et al., 2021a). The delivery of antiprotozoal agents via membrane transporters exploits the differences in the host and parasite transporters. The parasites' membrane transporters that are important for chemotherapeutics drug development are usually absent in or substantially different from that of their hosts, thus, they can selectively take up therapeutic antimetabolites. Transporters are important in understanding the mechanisms of drug action and resistance.

### 3.1. Purine transporters

Unlike their vertebrate hosts, kinetoplastids are auxotrophic for purines and are unable to synthesize purine *de novo*; therefore, they depend on the purine salvage pathway which involve enzymes with distinct substrate specificities. This distinction between the parasite and its host is exploited in drug development for the selective delivery of antiprotozoal drugs into the parasite's intracellular space. Kinetoplastids depend on their purine transporters for scavenging purines from their hosts' environment. This phenomenon is the basis for the development of purine analogs as broad-spectrum antiprotozoals. An example of this is allopurinol which is used in the treatment of leishmaniasis (Campagnaro, 2022). The purine salvage pathway is an attractive target for chemotherapeutic interventions. Transporters with physiological functions in nutrient uptake can mediate the import of drugs that are analogs of their natural ligands or that bear minimal structural similarity to them (Landfear, 2008).

The *T brucei* AT1 (TbAT1), also known as P2 is an adenine/adenosine permease that acts as a carrier for diminazene, pentamidine and arsenical drugs such as melarsoprol (Geiser et al., 2005). In contrast to the human purine transporters, P2 has strict substrate specificity as they transport adenine and adenosine, indicating that the presence of an amidine motif is required for transport to occur (Possart et al., 2021). P2 transporters interact with diamidines (pentamidine and diminazene) and arsenicals via a common structural motif. Therefore, the loss of P2 transporter activity in *T. brucei* confers resistance to pentamidine and melarsoprol (Mä;ser et al., 1999). P2 is also a high-affinity transporter for the adenosine analogs cordycepin and tubercidin. High Affinity Pentamidine Transporter (HAPT1) and Low Affinity Pentamidine Transporter (LAPT1) are two other pentamidine transporters that have been identified in trypanosomes. NT11.1, also called AT-A naturally transports adenine, xanthine, and hypoxanthine, is exploited as transporter of pentamidine in *T. brucei*. NT12.1 (AT-E) transports adenine and pentamidine (Possart et al., 2021).

LmaNT3, a purine nucleobase transporter in Leishmania major is responsible for the uptake of allopurinol, a purine analog in *L. major*. Pyrazolopyrimidines, hypoxanthine analogs, and formycin B, an inosine analog are toxic to *Leishmania* parasites because they can be used as substrates by the parasite's purine salvage enzymes but not by the host's (Mansour, 2002). In L. *donovani*, Formycin B is taken up by LdNT2 which is an inosine/guanosine transporter and tubercidin, a cytotoxic adenosine analog is transported by LdNT1 adenosine/pyrimidine nucleoside permeases (Vasudevan et al., 1998).

### 3.2. Amino acid transporters

Amino Acid Transporter 6 (AAT6) is a permease for neutral amino acids usedfor the delivery of trypanocidal amino acid derivates such as eflornithine (Vincent et al., 2010). Bloodstream and procyclic forms of trypanosomes that have AAT6 downregulated are less sensitive to eflornithine (Mathieu et al., 2014; Vincent et al., 2010). AAT6 is a single-copy gene and is not essential for the survival of *T. brucei* when tested *in vitro* condition. This means that there are likely other transporters with overlapping substrate selectivity with AAT6 (Possart et al., 2021). Proline is the primary source of energy for *T. brucei* in tsetse fly but downregulation of TbAAT6 in the presence of proline and glucose showed that TbAAT6 is not essential for the growth and survival of *T. brucei* (Mathieu et al., 2014).

Polyamines are essential in *Trypanosoma cruzi* and are involved in the regulation of cell growth and differentiation. *T. cruzi* parasites are auxotrophic for polyamines and are unable to synthesize putrescine because they lack arginine and ornithine decarboxylase, therefore, they exclusively depend on their polyamine transporters to obtain polyamines from their hosts. So far, *T. cruzi* polyamine transporter 12 (*Tc*PAT12) is the only polyamine transporter that has been identified in *T. cruzi*, and probably the only polyamine transporter found in the parasite (Martin et al., 2014). This makes TcPAT12 an attractive target for drug development against Chagas disease.

### 3.3. Aquaglyceroporin channels

Aquaglyceroporin channels allow the flux of water, glycerol, and urea across the plasma membrane of kinetoplastids (Possart et al., 2021). The aquaglyceroporin channels in *L. major*, LmAQP1 are sensitive to Sb (III), the active agent of antimonial drugs like Pentostam and Glucantime, and AS(III) (Landfear, 2008). Deletion of one allele of *LmAQP1* gene has been shown to confer increased resistance to Sb (III) and As (III) (Landfear, 2008).

TbAQP2, an aquaglyceroporin exclusively expressed in *T. brucei* is responsible for the influx and efflux of glycerol and works as a high-affinity carrier for pentamidine and melarsoprol in the bloodstream forms of *T. brucei*. Overexpression of aquaglyceroporin channels increases sensitivity of *T. brucei* to drugs. Melarsoprol resistance is caused by the loss of TbAQP2 or the appearance of TbAQP2/3 chimeras (Campagnaro, 2022). Genetic disruption of Tb*AQP*2 gene has been shown to cause resistance to pentamidine and mela rsoprol. Field isolates of *T. b. gambiense* with mutations to the AQP gene are resistant to melarsoprol, and expression of wild type *AQP2* gene in these isolates restored the sensitivity of the parasite to melarsoprol (Baker et al., 2012).

### 3.4. ATP-binding cassette (ABC) transporters

The ABC transporters are noted for their ability to efflux cytotoxic compounds out of the cells thereby conferring drug resistance to parasites. The genome of *T. brucei* and *L. major* are predicted to encode 18 and 36 ABC transporters respectively (Landfear, 2008). Two groups of ABC transporters, the PGPA and PGPE proteins present in several *Leishmania* spp mediate resistance against As(III) and Sb(III) by promoting efflux of metal thiol conjugates (Klokouzas, 2003). The MDR1 homologues which confer resistance to organic compounds such as vinblastine have been identified in several *Leishmania* species as well (Hendrickson et al., 1993). Several intracellular organelles that are components of the secretory apparatus of *L. entiettii* have been shown to contain MDR1 which mediates the export of cytotoxic substances out of the parasite (Dodge et al., 2004). Resistance to melarsoprol and Berenil® in *T. brucei* is mediated by TbMRPA and TbMRPE, respectively

(Landfear, 2008). Pgp and ABC transporters play critical roles in the decreased uptake or increased efflux of drugs leading to drug resistance in parasites (Garcia-Salcedo et al., 2016).

### 3.5. Strategies to overcome transport-related drug resistance

Transporter-related drug resistance can be overcome through strategies that deliver drug molecules into the parasite's intracellular space via non-transporter routes. Nanobodies and nanoparticles can easily traverse parasite membranes and have been shown to facilitate drug delivery while bypassing transporters (Garcia-Salcedo et al., 2016). In these approaches, drugs are either coated with nanoparticles or conjugated to nanobodies to be delivered into the parasites without the help of transporters. The parasites readily internalize these conjugates, increasing their sensitivity to the drugs, demonstrating the potential of nanobodies and nanoparticles in overcoming transporter-related drug resistance (Arias et al., 2015; Garcia-Salcedo et al., 2016).

Another strategy involves inhibiting efflux pumps, which expel drugs from the parasite and prevent their accumulation to therapeutic levels. Inhibitors of ABC transporters, such as calcium channel blockers, calmodulin antagonists, and certain antibiotics, have been reported to enhance the sensitivity of *Leishmania* and *Trypanosoma cruzi* to drugs (Garcia-Salcedo et al., 2016).

Since drug resistance is primarily due to transporter issues rather than the drugs themselves, modifying and optimizing drug structures to utilize alternative transporter systems within the parasite – while preserving their therapeutic efficacy – offers another viable approach. Identifying novel drug transporters in kinetoplastids is crucial for mitigating transporter-related drug resistance in kinetoplastid diseases.

## 4. Kinetoplastid drug targets

In general, drug discovery projects target the metabolic, physiological or morphological differences between pathogens and their hosts. Many of such differences exist between kinetoplastids and their mammalian host. Here, we discuss key metabolic pathways and enzymes in kinetoplastid parasites with remarkable differences to their hosts, highlighting how some current drug discovery approaches are exploiting these pathways (Fig. 4).

### 4.1. Carbohydrate metabolism

Carbohydrate metabolism is an important metabolic pathway in *Kinetoplastida*, as in other life forms. In the catabolism of hexose sugars, the enzyme hexokinase (HK) catalyzes the first committed step leading to the phosphorylation and activation of glucose to glucose-6-phosphate (G-6-P). The G-6-P that results from the action of HK either completely metabolizes via the glycolytic pathway or enters alternative pathways

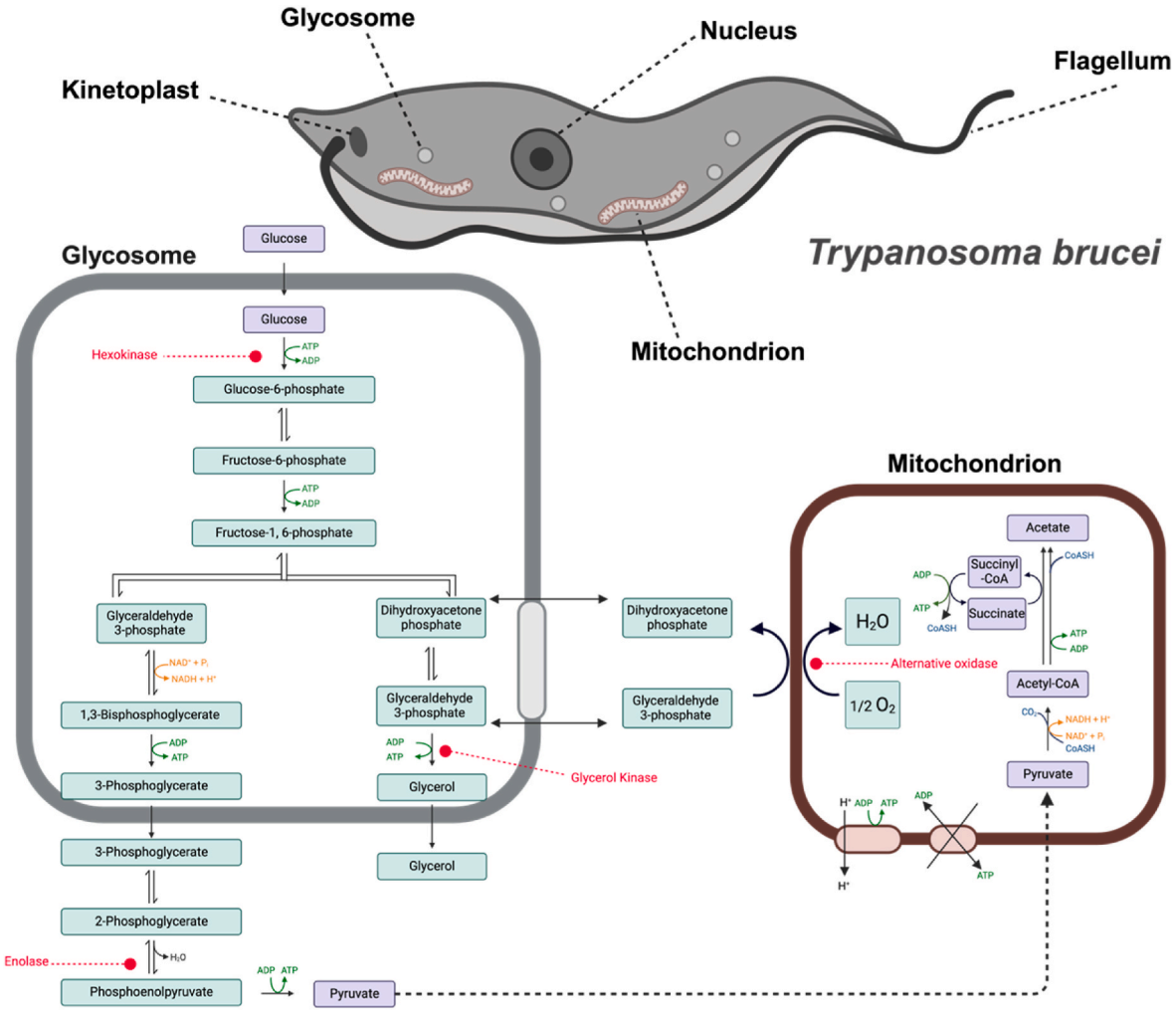


**Fig. 4.** Compartmentalized carbohydrate metabolism in trypanosomes, highlighting areas of possible therapeutic intervention with red dots. **ATP** Adeneosine triphosphate**, ADP** Adeneosine diphosphate, **NADH** reduced nicotinamide adenine dinucleotide, **NAD** $^{+}$ oxidized nicotinamide adenine dinucleotide, **CoASH** reduced Coenzyme A. *Created in BioRender. Danazumi, A. (2025)* https://BioRender.com/k19i282.(For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

including the pentose phosphate pathway that produces a reduced nicotinamide adenine dinucleotide phosphate (NADPH) and five–carbon sugar intermediates (Littleflower et al., 2024). Click or tap here to enter text. Therefore, HK is a critical enzyme in the metabolism of carbohydrates and is present in cells of all living organisms. The production of energy, ATP, by trypanosomatid parasites was reported to be largely and/or exclusively dependent on the glycolytic pathway (Fig. 4) (Michels et al., 2021). Thus, trypanosomatids HKs could serve as drug targets for leishmaniasis, trypanosomiasis and Chagas disease (Fig. 3). Two similar hexokinase genes, (*HK-1 and HK-2*) have been shown to encode HK in *T. brucei* and *Leishmania* spp, and a different one encodes HK in *T. cruzi* (Morris et al., 2006). In fact (Chambers et al., 2008), have shown that HK-1 and -2 from *T. brucei* possess unique carboxyl terminal domains that give rise to a unique oligomer. Moreover, they revealed the relevance of HK to the viability of trypanosomatid parasites through the knock down of *T. brucei* HK by RNA interference. It was recently shown that *T. b. brucei* HK1 shares 48% and 68% homology with HK from *T. cruzi* and *T. b. gambiense*, respectively, but only 26% with the human hexokinase IV (Ojo et al., 2019).

Similarly, the bloodstream form *T. brucei* relies absolutely on glycolysis for ATP production (Steketee et al., 2021a), making its HK a potential target for chemotherapeutic intervention. Studies have shown effective inhibition of trypanosomes HK by mixed-type inhibitors of *T. brucei* HK, Ebselen and SID-17387000 (Sharlow et al., 2010), the benzamidobenzoic acid derivatives with dual inhibitory activity against both *T. brucei* and *L. major* HKs (Flaherty et al., 2017) and the 25 novel inhibitors of *T. cruzi* HK with excellent *in vitro* trypanocidal activities and low toxicities against NIH-3T3 cells (Mercaldi et al., 2019). As a contribution to the continued search for candidate drugs and mechanisms of action against trypanosomatid diseases (Larit et al., 2021), used molecular docking techniques to show that quercetin and myricetin could also potentially function as inhibitors of *T. brucei* HK.

The pentose phosphate pathway (PPP) is distinguished by two separate carbohydrate metabolic phases: oxidative and non-oxidative. After two steps of enzymatic processes catalyzed by glucose-6-phosphate dehydrogenase and phospho-pentose isomerase, the oxidative phase processes D-glucose-6-phosphate to produce D-ribose-5-phosphate and NADPH as products. Most organisms can synthesize nucleic acids and attain redox stability with the resultant products (Maslowska et al., 2019). In the non-oxidative phase, the reversible switching of D-ribose-5-phosphate and D-ribulose-5-phosphate is catalyzed by the coordinated activities of enzymes such as ribose-5-phosphate isomerase, ribulose-5-phosphate epimerase, transaldolase, and transketolase.

In trypanosomastids, specifically *Trypanosome brucei*, other enzymes of the PPP non-oxidative phase except ribose-5-phosphate isomerase seem to be developmentally regulated. The activities of ribose-5-phosphate epimerase and transketolase were absent in the bloodstream form, but present in procyclic form of the parasite (Chen et al., 2020). This implies that in the mammalian host, the bloodstream form of *Trypanosoma brucei* restricts sugar metabolism to the production of ribose-5-phosphate and NADPH through the oxidative phase. In the context of an *in vivo* infection, this is most likely to satisfy the parasites' extraordinarily rapid multiplication rate and/or to protect themselves against a range of reactive oxygen and nitrogen species (Comini et al., 2013). To convert ribulose-5-phosphate (Ru-5-P) to ribose 5-phosphate, ribose-5-phosphate isomerase (R-5-$P_i$) must be present (R–5P). Trypanosomatids possess an R-5-$P_i$ Type B, which is structurally different from R-5-$P_i$ Type A found in humans (Kaur et al., 2012), and this, combined with the unfavorable phenotype seen in *rpiA-/rpiB* knockout *Escherichia coli* (*E. coli*) (Nakahigashi et al., 2009), could suggest R-5-$P_i$B as an appealing drug target candidate that begs thorough delineation. Despite the essentiality of R-5-$P_i$B in trypanosomatids, not much progress has been made in the targeting of this enzyme for drug discovery. Nevertheless, some putative inhibitors of *L. infantum* and *T. cruzi* R-5-$P_i$B have been reported in studies using *in silico* methods involving molecular docking and dynamic simulations (Dickie et al., 2021). Natalia et al. (2021) reported a compound from a series of *T. cruzi* R-5-$P_i$-B substrate analogs that inhibit the enzyme in a mechanism that involves a Cys69 residue in the active site. The work of (Soares et al., 2019) further confirms the requirement of Cys69 for the enzyme's catalysis, suggesting that the residue is a suitable point of targeting for future investigations.

Enolase is yet another crucial metalloenzyme in glycolytic and gluconeogenic pathways that catalyzes the reversible dehydration reaction involving 2-phosphoglycerate (2-PG) and phosphoenolpyruvate (PEP). Just as with other glycolytic enzymes, enolase reaction are traceable to virtually all living organisms including trypanosomatids. The relevance of the glycolytic pathway in energy metabolism, generation of high-energy phosphate intermediates, and its organismal ubiquity make it an indispensable target for the design of drugs against parasitic infections. While the sequence of enolase is evolutionarily conserved across various species, the enzyme could still serve as a target against human parasitic diseases. This is especially possible considering the report of (Kang et al., 2008) which reveals that the human enolase possesses a unique surface structure in comparison to other organisms. Like R-5-$P_i$-B, most of the progress in this direction is mostly predictive (Vidhya et al., 2020). Notwithstanding, these predictions could guide the selection of protein motifs to target. Indeed, Navarro et al. (2007) implicated lysine-specific to *T. brucei* enolase to be closer to the catalytic site than crystal models predict and also address the question of whether cysteine-specific to parasite enzymes are accessible to modification of chemical reagents. Therefore, attention should be given to experimental validations of the reported simulations to aid the discovery of inhibitors of kinetoplastids enolase. Additionally (Wachsmuth et al., 2017), demonstrated how interfacial protein-protein interactions could be an advantageous lead for the development of peptide inhibitors against critical oligomeric enzymes of kinetoplastid organisms. They did this by using a computational alanine scanning approach. It was discovered that *T. brucei* UDP-glucose 4-epimerase (UGE) contains two hotspots, V-119 and I-123 amino acids, which are adjacently located on the same helical surface and contain the 111-P-L-K-Y-Y-D-N-N-V-V-G-I-L-R-L-L helix.

### 4.2. Cysteine proteases

Cysteine proteases (CP) occupy a central position in the pathogenesis and survival of many parasitic protozoans. They participate in cell penetration, immune evasion, autophagy, proteolysis of host proteins, apoptosis, maintenance of flagellum structural integrity, as well as invasion of the blood-brain barrier (Siqueira-Neto et al., 2018), making them potential targets for the development of new antileishmanial and trypanocidal drugs. Kinetoplastid parasites express a wide range of CPs, but by far, the CA papain-like family is the most studied and abundant CPs in these organisms (Judice et al., 2021). CPs from this family are considered virulent factors since their knockdown is associated with a severe reduction in infectivity of *L. Mexicana* and L. *chagasi*, for example (Mundodi et al., 2005). The most well-characterized CPs in Leishmania are CPA, CPB, and CPC. These CPs are expressed at higher levels in the amastigote stage of the parasites, which is the stage that infects mammals. CPA and CPB are involved in the interaction between Leishmania and its host, with CPA playing a role in infectivity and CPB modulating host immune responses. CPC is involved in cell death and secretome remodeling in Leishmania (Siqueira-Neto et al., 2018).

The best-characterized CPs in *T. brucei* are cathepsin L (TbCatL) and cathepsin B (TbCatB). TbCatL promotes the crossing of the blood-brain barrier by activating host G-protein-coupled receptors, protects the parasite from lysis by host serum, and degrades internalized variant surface-glycoprotein-bound antibodies (Uzureau et al., 2013). TbCatB is involved in the degradation of host transferrin in the parasite endosomal/lysosomal compartment and potentially in cytokinesis (O'Brien et al., 2008). Accordingly, both TbCatL and TbCatB have been validated as drug targets via RNA interference. The inhibitor K11777 targets TbCatL and showed synergy when combined with eflornithine and may

be considered for development as a new therapy for African trypanosomiasis (Steverding, 2015). Inhibitors from a variety of chemical classes have been tested against Leishmania CPs, including peptidomimetics, dipeptides, ketones, benzophenones, quinolines, organic tellurium, and aziridines (Judice et al., 2021). Natural and semi-synthetic bi-flavonoids from *Garcinia brasilienses* have also shown potent activity against promastigote and amastigote forms of Leishmania (de Almeida et al., 2015). However, many of these inhibitors have also been associated with cytotoxicity in mammalian cells. Thus, there is ongoing research to develop more effective and safer inhibitors of CPs for the treatment of parasitic infections (Judice et al., 2021).

### 4.3. Energy metabolism

The mitochondrial electron transport chain plays a key role in the metabolism of prokaryotes and eukaryotes. It is comprised of protein complexes that are involved in maintaining the balance of NADH and NAD + through electron transfer along the respiratory chain (Wu et al., 2020). Blocking the electron transport chain can result in the generation and accumulation of harmful reactive oxygen species (ROS) within eukaryotic cells, which can lead to the pathogen's death (Boniface et al., 2020). In *Leishmania* parasites, type 2 NADH dehydrogenase, which catalyzes the transfer of electrons from NADH to ubiquinone in the respiratory chain, is a potential target for inhibiting the parasite's growth as it has been established to be essential (Duarte et al., 2021). A 6-methoxyquinalidine derivative was shown to be effective at inhibiting the NADH dehydrogenase enzyme in *L. infantum* and exhibited anti-leishmanial activity at very low concentrations (Stevanović et al., 2018). On the contrary, *T. brucei* type 2 NADH dehydrogenase was reported to be important dispensable (Surve et al., 2017).

An atypical cytochrome-independent alternative oxidase (AOX) is found in the respiratory chain of some trypanosomatids and is absent in mammals (Menzies et al., 2018). AOX is mainly found in the long slender bloodstream form of *T. brucei*, which relies on glucose as a major energy source. It acts as a terminal oxidase enzyme in the aerobic respiratory pathway, allowing the parasite to re-oxidize NADH formed during glycolysis using oxygen (Balogun et al., 2019a, 2019b, 2019c; Ebiloma et al., 2019). While the activity of cytochrome oxidase can be replaced by AOX in *T. brucei*, both molecules coexist in another life cycle stage of the parasite called the procyclic form. It is unclear if AOX is present in other pathogenic trypanosomatids, but other parasites in the same family have been observed to consume oxygen in a cytochrome oxidase-independent manner (Zíková et al., 2017). This raises the question of whether an inhibitor of AOX could be effective in treating Chagas disease and leishmaniasis, as these parasites have a similar active mitochondrial metabolism to the procyclic form of *T. brucei* (Pedra-Rezende et al., 2022).

So far, the most effective inhibitor of trypanosome alternative oxidase (TAO) discovered is ascofuranone (AF), which has an $IC_{50}$ value in the sub-nanomolar range (0.13 nM). However, it is most potent against *T. brucei* bloodstream form when glycerol kinase (GK) is also inhibited (Shiba et al., 2013). Trypanosomal GK is a glycosomal enzyme that can catalyze both the thermodynamically favorable reaction of converting glycerol using ATP into G3P & ADP, and the thermodynamically unfavorable reverse reaction of converting ADP & G3P into ATP & glycerol (Balogun et al., 2013). Since the reverse reaction of GK prevents the bloodstream form *T. brucei* from AF toxicity, both TAO and TGK must be concomitantly inhibited to eventually kill the parasites. Efforts in this direction have yielded interesting *in-vitro* preliminary results (Balogun et al., 2019c; Tauheed et al., 2022).

### 4.4. Folate metabolism

The interest in targeting trypanosomatids folate metabolism was born out of the auxotrophic nature of the parasites for folate (Possart et al., 2021). Because trypanosomatids cannot ability to synthesize folate *de novo*, they salvage the metabolite from their host, where it undergoes two reduction steps to form tetrahydrofolate, which is channeled into pyrimidine biosynthesis as a methyl group donor. This reduction step is mainly catalyzed by dihydrofolate reductase (DHFR) in a reaction that requires NADPH as a reducing equivalent (Gibson et al., 2016). Consequently, the inhibition of DHFR offers a potential therapeutic point against Leishmania and trypanosomes. However, when DHFR is inhibited, pteridine reductase 1 (PTR1) expression is upregulated, and the enzyme compensates for the missing role of DHFR. In normal physiological settings, PTR1 catalyzes the reduction of pterin to tetrahydrobiopterin and only accounts for 10% of folate reduction to THF (Possart et al., 2021). Notwithstanding, it is established that simultaneous inhibition of the enzymes is necessary to block folate metabolism and effectively kill the parasites. Hence, current research projects on targeting trypanosomatids folate metabolism focus on the design of compounds with dual inhibitory activity against both DHFR and PTR1 (Possart et al., 2021).

### 4.5. Polyamine biosynthesis

Polyamines such as Putrescine, spermidine, and spermine are small metabolites characterized by possession of several positively charged amine groups under physiological environment. They are ubiquitous across life domains and occupy critical positions in many biological processes. Because of their crucial role in rapidly dividing cells, their metabolism has been the subject of research of cancer and protozoan pathogens (Phillips, 2018). Due to the difference in pathophysiology of Leishmania and African trypanosomes, especially with regards to their localization in the host system, the enzymes of therapeutic interest in polyamine biosynthetic pathway slightly differ in the two parasites. Ornithine decarboxylase (ODC) and Adenosylmethionine decarboxylase (AdoMetDC) are the best described and validated targets in *T. brucei*, while ODC and spermidine synthase (SpdSyn) represent well characterized targets in Leishmania parasites (Phillips, 2018). The anti-trypanosomal drug DL-α-Difluoromethylornithine (DFMO) targets the polyamine pathway of *T. brucei* by inhibiting ODC, a strategy that has yielded remarkable results in the clinical management of African trypanosomiasis (LoGiudice et al., 2018). The success of this drug against African trypanosomes stimulated efforts towards repurposing of DFMO against Leishmania ODC, given that the enzyme is equally essential in the infective stage of the parasite (Salem et al., 2024.)Click or tap here to enter text. However, DFMO efficacy varies among Leishmania species. *L. donovani* and *L. infantum* are highly susceptible to the drug, whereas *L. mexicana* and *L. major* are insensitive (Salem et al., 2024). Click or tap here to enter text.

A potent *T. brucei* AdoMetDC inhibitor MDL 73811, and its derivatives have shown remarkable efficacy against the parasite both *in-vitro* and *in-vivo*. However, these compounds suffer from poor brain penetration, rendering them ineffective against the late CNS stage of the disease (Bacchi et al., 2009). Attempts towards designing MDL 73811 analogs with improved brain penetration have been made but complications associated with synthetic routes impeded progress with this series (Brockway et al., 2017). Despite the essentiality of SpdSyn in Leishmania, not much progress has been recorded in targeting the enzyme. Nevertheless, v. M et al. (2018) reported the *in-silico* identification of some putrescine analogs as potential inhibitors of *L. donovani* SpdSyn.

At the intersection of polyamine biosynthesis and redox metabolism lies an important enzyme called Trypanothione reductase (TR). TR maintains trypanothione in the reduced state, a metabolite formed from the conjugation of two glutathione molecules with spermidine in a process occurring exclusively in trypanosomatids (Battista et al., 2020). Trypanothione is the primary antioxidant defense and the regulator of the parasites' redox balance (Ilari et al., 2016). Therefore, inhibiting TR is associated with oxidative stress that kills the parasites. Indeed, TR is a well-characterized and validated drug target against both Leishmania

and Trypanosomes. In fact, antimonials, which are currently used to treat leishmaniasis, disrupt trypanothione metabolism by inhibiting TR (Baiocco et al., 2009). In addition, TRs from different parasites share high sequence homology, making them an excellent target for the design of a single broad-spectrum anti-Trypanosomatids drug (Ilari et al., 2018). The biggest drawback of TR as a drug target is its high efficiency/turnover, which necessitates the use of potent inhibitors with IC50 in the low micromolar range to exhibit significant potency on the parasite redox system and survival (Battista et al., 2020).

### 4.6. Purine salvage pathway

A critical biochemical distinction between most parasites and their hosts lies in the metabolism of purines. Parasites, including the kinetoplastid protozoans, lack the system for the *de novo* synthesis of purine nucleoside and therefore rely on imported DNA to obtain purines through the purine salvage pathway to fulfill their physiological demands. Enzymes and transporters regulating this pathway are, in some cases, dissimilar enough from the hosts' enzymes and have attracted great interest over the past decades for the design of broad-spectrum antiprotozoal drugs. Nucleoside hydrolase (NH) is an enzyme of the purine salvage pathway that facilitates the hydrolysis of the *N*-glycosidic bond of β-ribonucleosides. Although this protein is found across almost all domains of life, neither its gene nor its activity has been reported in mammals. For this reason, inhibiting the NH of Leishmania and Trypanosomes, as with other protozoans, has been validated as a viable therapeutic strategy against these parasites. Immucillins and N-aryl methyl-substituted iminoribitol are nucleoside analogs showing the most potent inhibitory activity against *L. infantum*, *T. vivax* and *T. b. brucei* nucleoside hydrolases (Kourbeli et al., 2021a).

Nevertheless, it has been shown that inhibiting NH alone is insufficient to compromise the purine salvage pathway in Kinetoplastid parasites (Berg et al., 2010). Therefore, the therapeutic potential of other essential proteins in this pathway has been explored. Purine phosphoribosyl transferases (PPRTs) play a role in converting phosphoribosyl pyrophosphate to monophosphate nucleotides. Both sequence and structural differences between Human and kinetoplastid PPRTs have been reported and could allow for specific targeting of the former enzymes. Therefore, PPRTs of Leishmania and trypanosomes have been identified as promising targets for chemotherapy (Glockzin et al., 2022). The search for inhibitors of PPRTs mainly focuses on nucleoside analogs such as acyclic nucleoside phosphonates (Terán et al., 2019). The role of nucleoside diphosphate kinases (NDPK) in converting free purine and pyrimidines into nucleosides and nucleotides brings them under the spotlight of many drug-discovery projects against kinetoplastid parasites. The enzyme's involvement in many cellular processes and the glycosomal localization of one of its isoforms suggest that the NDPK may be involved in unusual processes crucial to the parasite, further strengthening its potential as a drug target (Martin et al., 2014). Although no potent and specific inhibitor of trypanosomatids NDPK has been reported to date, preliminary studies have shown the effectiveness of BTB13319 and a pyrrole-indolinone against Leishmanial NDPK (Mishra et al., 2017).

Other essential elements in the purine salvage pathway of the trypanosomatids are the purine nucleotide transporters. The focus on designing antiprotozoal drugs that are substrates of nucleoside transporters was fueled by the findings that pentamidine and melarsoprol are transported by *T. brucei* adenosine transporter (*Tb*AT1) and that these drugs are selectively recognized through their amidine structural recognition motif, which is not recognized by their mammalian counterparts (Campagnaro, 2022). Although research interest on this subject matter was higher in the 1990s and early 2000s, the recent discovery of tubercidin and its derivatives (P1-like transporters substrates) as highly effective trypanocides and antileishmanial compounds seems to have renewed interest in the search for nucleoside transporters substrates (Campagnaro, 2022; Hulpia et al., 2019a).

### 4.7. Sterol biosynthesis

Isoprenoid compounds, including sterols, are found in both prokaryotic and eukaryotic cells. In eukaryotes, sterols are typically the most abundant isoprenoid group and play a structural role in cellular membranes. Trypanosomatids rely on a metabolic pathway that produces a specific type of sterol called ergosterol and other 24-methyl sterols for their growth and survival (Souza and Rodrigues, 2009). Because these sterols are not found in mammalian cells, essential and distinctive steps or enzymes in their biosynthetic pathways become attractive drug targets. 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGR), being the rate-limiting enzyme in ergosterol biosynthesis, is the first enzyme of interest in this pathway. Statins, such as Atorvastatin and mevastatin, and non-statins, including resveratrol and glycyrrhizic acid, were reported to impede the growth of both promastigotes and intracellular amastigotes of *L. donovani* without causing harm to the host. (Singh et al., 2018).

Squalene synthase is another enzyme involved in the production of sterols in kinetoplastid parasites. Its primary function is to catalyze the dimerization of two farnesyl pyrophosphate molecules to produce squalene, which is then converted into ergosterol in trypanosomatids, or cholesterol in humans (Chawla and Madhubala, 2010). It was found that inhibiting or reducing the levels of squalene synthase by RNA interference resulted in the impairment of the growth of *T. brucei, T. cruzi,* and *L. mexicana* (Pérez-Moreno et al., 2012). Two quinuclidine derivatives, ER119884 and E5700, have demonstrated effective inhibition of squalene synthase of *L. amazonensis* and *T. cruzi* at sub-nanomolar concentrations (Rodrigues et al., 2008).

As its name implies, farnesyl pyrophosphate synthase (FPPS) is another important enzyme that synthesizes farnesyl pyrophosphate (FPP), an important intermediate located at the intersection of various metabolic pathways, including sterol metabolism. The FPPS reaction occurs in two steps; The first step involves the reaction of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate to form a 10-carbon compound called geranyl pyrophosphate (GPP). In the second step, GPP is combined with another molecule of IPP to create FPP (Mukherjee et al., 2019). The essentiality of FPPS in *T. brucei* was investigated using gene knockdown. The result was slowed growth of *T. brucei,* eventually leading to cell death (Montalvetti et al., 2003). Mukherjee et al. (2019) established that FPPS is required for the survival of leishmania parasites in both vector and mammalian stages and that the deletion of FPPS reduced the ability of the parasites to cause disease in animal models. Despite not having desirable drug-like properties, bisphosphonates are still the most effective inhibitors of the enzyme farnesyl pyrophosphate synthase. The potential of bisphosphonates to act as antiparasitic agents is due to their accumulation in organelles called acidocalcisomes, which have a similar composition to bone minerals and therefore facilitate their antiparasitic activity (Park et al., 2021).

Still on sterol metabolism, 14-α-demethylase (CYP51), a cytochrome P450 monooxygenase, facilitates the removal of the 14-α-methyl group from the sterol core during ergosterol biosynthesis through a three-step oxidative process (Lepesheva and Waterman, 2011). The essentiality of the enzyme was highlighted by the antiparasitic effects of CYP51 inhibitors against *T. brucei*, suggesting that it could be a viable target against African sleeping sickness (Lepesheva et al., 2007). While CYP51 was confirmed to be essential in *Leishmania donovani* (McCall et al., 2015), Xu et al. (2014) proved that CYP51-mutants *Leishmania major* only showed remarkable defects but were viable and replicating, suggesting that the enzyme is dispensable in some species. In support of this thesis, inhibition or genetic inactivation of CYP51 in *Leishmania major* leads to abnormalities in the plasma membrane and mitochondria, as well as increased sensitivity to heat and reduced virulence but not death (Mukherjee et al., 2020). Notwithstanding, the advantage of targeting sterol 14-alpha demethylase as an antiprotozoal drug is that imidazole and triazole derivatives, which inhibit this enzyme, are already effectively used as antifungal agents in both clinical and agricultural settings

(Zonios and Bennett, 2008).

## 5. Drug resistance in kinetoplastid diseases

Drug resistance has remained a major problem in the treatment of kinetoplastid infections. When the treatment of a parasite disease with a standard antiparasitic at the indicated dosage and administration route showed either reduced or no activity, drug resistance is suspected (Assefa and Shibeshi, 2018). Such suspected cases of antikinetoplastid resistance can be confirmed through a variety of drug sensitivity assessments on parasite populations recovered from patient blood or lesions carried out *in vivo and in vitro* (Hadighi et al., 2006). However, treatment failure does not always arise because of drug resistance, as treatment success depends on several factors in the host-pathogen-drug interaction (Sereno et al., 2019). New infection is possible after treatment upon another exposure such as vector bite especially in highly endemic areas (Rowlands et al., 2001). For example, DNA markers indicated that *T. congolense* sample collected from diminazene-treated cattle in Ethiopia contains populations from both new infection (40%) and actual relapse (37·5%) (Moti et al., 2015). Another crucial factor is the innate resistance of some species to a particular drug even in the absence of previous exposure to the drug (Chitanga et al., 2011). Recent *in vitro* studies indicated that differences in sensitivity to multiple drugs between AAT pathogens, *T. congolense* and *T. brucei* result from disparities in membrane transporters (Ungogo et al., 2022a) and divergent metabolism (Steketee et al., 2021b).

Drug resistance has remained an important challenge to the control of kinetoplastid diseases, given the critical role chemotherapy plays in the absence of vaccines for these diseases until recently. In African trypanosomes, trypanocide resistance has been reported in 21 out of the 37 countries in the tsetse belt, 10 countries of which have also documented cases of multiple drug resistance (Assefa and Shibeshi, 2018). A recent meta-analysis reported a prevalence rate of 30% for drug resistance in African animal trypanosomes (Okello et al., 2022). The prolonged treatment regimen for Chagas disease and drug toxicity affect compliance to the two antichagasic drugs resulting in drug resistance (ALSFORD et al., 2013). Widespread resistance has been reported against antileishmanial drugs particularly the pentavalent antimonials sodium stibogluconate (SSG) and meglumine antimoniate (MA), with prevalence as high as 60% in countries such as India (Sundar et al., 2019). Antikinetoplastid drug resistance is probably underreported, especially in places where treatment outcome is not followed up or where studies were not carried out or published (Melaku and Birasa, 2013), since most of the available information on drug resistance arises from local case reports (Mamoudou et al., 2008).

Acquired antikinetoplastid resistance usually develops in parasites following repeated drug underdosing arising from several circumstances (Leach and Roberts, 1981). In a laboratory setting, resistant parasites are generated through adaptation to gradually increasing concentration of a drug over several passages *in vivo* (Tihon et al., 2017), or *in vitro* (Carruthers et al., 2021). Such resistant strains exhibit decreased sensitivity to other drugs with shared mechanisms of uptake or action (Fig. 5), a term described as cross-resistance (Assefa and Shibeshi, 2018; P and De Koning, 2020), as observed between antichagasic drugs nifurtimox and benznidazole (Wilkinson et al., 2008a), and between diamidines and melaminophenyl arsenicals used in the treatment of African trypanosomiasis (Teka et al., 2011).

Genetic studies of drug-resistant parasites and high throughput genome-wide studies have unravelled the molecular mechanisms of resistance to many drugs, especially in the important human kinetoplastid pathogens (Fig. 5) (ALSFORD et al., 2013). The genome of drug-resistant parasites shows mutations in genes encoding for surface proteins involved in drug internalisation, importantly drug transporters (Munday et al., 2015), and cellular targets (de Koning, 2017a). In *T. brucei*, the aminopurine (P2/TbAT1) transporter and the aquaglyceroporin-2 (AQP2) were found to be the transporters of diamidine and melaminophenyl arsenicals, which also determine resistance to such drugs (Baker et al., 2012; Matovu et al., 2003; Munday et al., 2015). In addition, the eflornithine-resistant *T. brucei* strains were shown to bear deletions in the amino acid transporter *TbAAT6* gene, which was validated to be the transporter for eflornithine (Vincent et al., 2010). The roles of TbAT1, AQP2 and TbAAT6 in drug resistance have been independently validated through genome-wide RNAi screens (Alsford et al., 2012; Baker et al., 2011; Schumann Burkard et al., 2011). Other African trypanosomes outside the brucei group, *T. congolense* and *T. vivax* lack the syntenic and functional equivalent of *TbAT1* and *TbAQP2* (Alghamdi et al., 2020; Munday et al., 2013), and this has proved to be an important factor in their reduced capacity to internalize multiple drugs or to be susceptible to them (Fig. 4) (Ungogo et al., 2022b). Thanks to elucidated structure-activity relationship (SAR) and minding models (Alghamdi et al., 2020; Munday et al., 2015), the modification of diamidine binding motifs has generated promising nucleoside drugs and diamidine derivatives that by-pass TbAT1 (Hulpia et al., 2019a) and TbAQP2 (Robertson et al., 2021) resulting in activity against resistant parasites and veterinary trypanosomes lacking both transporters (Robertson et al., 2021; Ungogo et al., 2023). Among intracellular transporters, a lysosomal membrane transporter of a major facilitator superfamily of transporters (MFST, *Tb927.9.6360-80*) has been linked to suramin resistance (Alsford et al., 2012) and resistance to the antileishmanial, paromomycin and other aminoglycosides (Collett et al., 2019) in *T. brucei,* likely through export of endocytosed drugs from the lysosome into the cytosol (Zoltner et al., 2016). Interestingly, recent studies have reported a variant surface glycoprotein (VSGsur) that is switched in suramin-resistant *T. brucei* (Wiedemar et al., 2018, 2019).

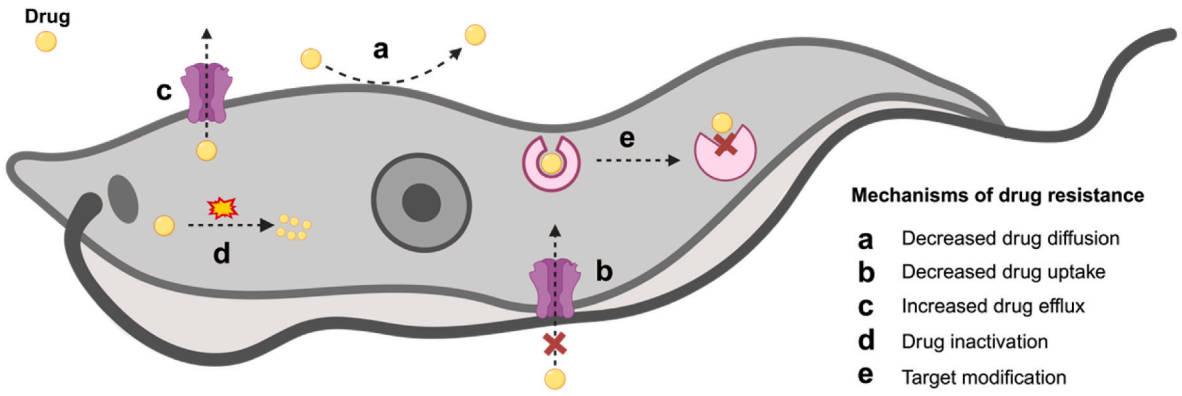


**Fig. 5.** Mechanisms of drug resistance in kinetoplastid parasistes. Drug resistance could arise from decreased drug diffusion through the cell membrane (a); decreased drug uptake by transporters (b); increased drug efflux by transporters (c); drug inactivation in the cell; or modification of drug target (e). *Created in BioRender. Danazumi, A. (2025)* https://BioRender.com/m14f596.

Further studies showed that, unlike other VSGs, the VSGsur binds tightly to suramin, preventing the internalisation of the drug (Zeelen et al., 2021). In *Leishmania*, the *L. donovani* miltefosine transporter (LdMT) determines miltefosine resistance, with even a single point mutation resulting in a significant reduction in uptake and sensitivity to the drug (Seifert et al., 2007; Srivastava et al., 2017). Other transporters where copy number variation is involved in kinetoplastid drug resistance include *Leishmania* AQP1 linked to antimonial resistance (Potvin et al., 2021) and a drug metabolite transporter (DMT) involved in isometamidium resistance in *T. congolense* (Tihon et al., 2017). Beyond the transporters involved in drug uptake, mutations or differential expressions in drug efflux pumps such as the ATP-binding cassette (ABC) transporters limiting the intracellular concentrations of drugs and were shown to be responsible for isometamidium-resistance in *Trypanosoma* (Tihon et al., 2017) and antimonial-resistance in *Leishmania* (Légaré et al., 2001).

As the nitrofuran-based drugs nifurtimox and benznidazole need to be activated by the NADH-dependent nitroreductase (NTR), mutations in *T. cruzi* NTR were observed in drug-resistant *T. cruzi* clinical isolates and laboratory-generated strains (Wilkinson et al., 2008a). Cellular target mutations associated with drug resistance include the highly conserved CPSF3 related to acozibarole in African trypanosomes (Wall et al., 2018). Many trypanocides such as diamidines, phenanthridines and quinapyramine target kinetoplast, mitochondria or both, and it appears that kinetoplast loss or reduced mitochondrial membrane potential present key steps in the adaptation of trypanosomes to these drugs (Carruthers et al., 2021; Dean et al., 2013; Eze et al., 2016). As a consequence of such organelle dysfunction, mutation or deletion of genes, drug-resistant parasites often show fitness cost such as reduced growth rate as observed in benznidazole-resistant *T. cruzi* (Campos et al., 2014). As multiple cellular organelles and molecules may be involved in the internalisation and action of drugs, drug resistance is not always a function of a single point mutation, and disruption in any factor involved directly or indirectly in drug action can possibly affect drug sensitivity.

Despite the development of a few genotypic screens for drug resistance in some kinetoplastid diseases, translation into affordable diagnostic tools has been challenging. As the development of new antikinetoplastid drugs remains comparatively slow due to low profit projections, especially for veterinary diseases, optimizing current drugs becomes imperative (Giordani et al., 2016; Richards et al., 2021). Strategies would include control of counterfeit drugs (Kingsley, 2015), rational drug use (Richards et al., 2021), optimisation of drug dosage and dosage regimen based on the parasite strains in a particular endemic area (Quebrada Palacio et al., 2018) to improve both compliance and cure rate (Sundar et al., 2019). However, a comprehensive approach involving multisector collaboration and involvement of all key stakeholders including clinicians, veterinarians, farmers, policy makers and law enforcement agencies is required.

## 6. Persister-like cells and drug resistance in kinetoplastids

Drug resistance in kinetoplastids can result from persister cells, which develop resistance to medications without undergoing mutation. They arise either randomly or in response to environmental pressure. Persisters are characterized by altered metabolism, reduced protein synthesis, slow or arrested growth rate and increased resistance to drug treatments and immune responses. The term was first used by an Irish physician, Joseph Bigger, when he observed a subpopulation of *Staphylococcus aureus* was refractory to penicillin (Brigger, 1944).

Bacteria are known for the formation of non-replicating but viable cells (persisters) that spontaneously enter dormancy or quiescence state when the environment is not favorable (Barrett et al., 2019; Van den Bergh et al., 2017). These cells have the potential to return to the proliferative state and recolonize the environment when it becomes favorable. Eukaryotes also exhibit this mechanism that affects drug susceptibility. Of the kinetoplastid parasites, the phenomenon of persistence or dormancy is extensively reported in Chagas disease and leishmaniasis.

Although mutation in drug transport mechanisms or drug targets is known as the molecular mechanism underlining the development of resistance, how cells become persisters is not well elucidated. Persistance is thought to result from either varying degrees of metabolic quiescence, which makes cells or parasites less susceptible to medications that target processes found in actively dividing and growing cells, or by other physiological changes that lessen drug effect (Balaban et al., 2019). Drugs that target DNA replication and cell division may not be effective if the cell is not dividing or is dividing very slowly. It has also been proposed that changes in the control of transporters may lower drug uptake or efflux, hence affecting drug levels in the pathogen (De Rycker et al., 2023).

### 6.1. Persistence or dormancy in Chagas disease

*In vitro* and *in vivo* spontaneous development of persisters has been reported in *T. cruzi* and like other infections, the development of persister phenotype is linked to quiescence (MacLean et al., 2018; Ward et al., 2020). However, the coexistence of non-replicating amastigotes in host cells with actively dividing amastigotes suggests that entering the quiescent state is not a reaction to nutritional deficiency or the presence of other stressors. These non-replicating amastigotes also differentiate concurrently with the forms that are actively dividing in the same host cell, which allows them to sense the cues that initiate amastigote-to-trypomastigote differentiation. An important feature of phenotypic variability in bacterial persisters is the ability of trypomastigotes, which are descended from non-replicating amastigotes, to enter new host cells and re-establish both actively dividing and non-dividing progeny. Following a 30-day continuous treatment regimen, previously dormant amastigotes emerge. Drug exposure has not selected for genetically resistant parasites, showing that dormancy is the cause of drug refractoriness. *T. cruzi* dormant amastigotes were shown to be resistant to an extended drug treatment of 30-day in the *in vitro* and *in vivo* studies. (Sánchez-Valdéz et al., 2018). Treatment failure of anti-*T. cruzi* drugs that kill actively dividing amastigotes is therefore caused by transient dormancy, or dormant cells that will reactivate, in a tiny subpopulation of intracellular *T. cruzi* amastigotes. Dormant amastigotes show lower levels of reporter protein expression than replicating amastigotes, indicating decreased protein synthesis capability and altered metabolism (Sánchez-Valdéz et al., 2018). However, the metabolic status of non-replicating amastigotes has not yet been investigated.

Although dormancy in *T. cruzi* is still being debated, dormant amastigotes are not actively transmitted. Rather, trypomastigotes enter the host's bloodstream and they are ingested by the vector during blood meal. Instead of being actively transferred, *T. cruzi* immune detection and control are typically quite effective at maintaining low parasite loads. Only dormant amastigotes in host cells are likely to escape detection by $CD8^+$ T cells or other immune effectors, and as a result, they could serve as reservoirs for the slow but steady development of trypomastigotes. Reseeding tissue sites with fresh persister-like parasites could increase the length of time over which transmission is possible and promote parasite persistence. The variable nature of *T. cruzi* infection may also be explained by the sporadic reactivation of these dormant parasites, that is, the emergence of novel parasite replication sites that arise and vanish in various anatomical regions (Garg, 2025). Click or tap here to enter text. Dormant amastigotes may serve as a stable "stem-like" cell if the gene resorting results in impaired progeny in replicating forms since *T. cruzi* rarely performs sexual recombination. Rather, it recombines and resorts existing genes to create genetic and antigenic diversity (Desale et al., 2023). Click or tap here to enter text.

There is a need for new trypanocidal substances that can target dormant forms. Both nifurtimox and benznidazole need trypanosomal

enzymes (Wilkinson et al., 2008a) to be metabolically activat. It is possible that the activities of these enzymes could be diminished in dormant forms. As a result, developing screening procedures tailored toward these dormant forms may reveal a new class of medications. The discovery that non-diverging amastigotes can react to the cues that cause stage conversion to trypomastigotes opens up the possibility of discovering chemicals that could reawaken latent parasites and make parasites more susceptible to already available medications (Barrett et al., 2019).

### 6.2. Persistence or dormancy in leishmaniasis

The relapse illnesses occasionally observed in patients treated against Viannia group of leishmaniasis (post-Kala azar dermal leishmaniasis) and leisshmaniasis recidivans are probably caused by persister-type parasites. *Leishmania* spp. are primarily found in dendritic cells and macrophages in mammals. It was shown that *L. mexicana* doubled in size every 12 days in non-resolving infections in Balb/c mice (Kloehn et al., 2015), and it was inferred that amastigotes are semi-quiescent in mice. Additionally, it was discovered that amastigotes (as a whole population) in mice have a strict metabolism, utilizing metabolites more efficiently than the cultured promastigote form (Saunders et al., 2014) and consuming them in relatively small amounts. This study did not include the potential of dividing and non-dividing subpopulations. However, in a C57BL/6J mouse model of *L. major* cutaneous illness, distinct populations of non-diverging and reproducing intracellular amastigotes were identified (Mandell and Beverley, 2017). Based on their capacity to integrate the nucleotide analogue 5-bromo-2′-deoxyuridine into DNA, two separate populations could be distinguished 4 months after infection; an actively dividing population that doubled roughly every 60 h, and a non-dividing, dormant population. Amastigotes divide at a similar pace in the early stages of infection, although with a fewer dormant forms (Barrett et al., 2019). The phenomenon of persister is being speculated in non-replicating *L. donovani* following the production of THP-1 macrophages in the *in vitro* experiment (Tegazzini et al., 2016). These growth-arrested *Leishmania* forms may hold the key to understanding why medication frequently fail in the absence of genetically chosen resistant parasites (Wasunna et al., 2016). However, *in vitro* comparison of amastigotes and promastigotes of *L. (Viannia) braziliensis* reveals no development in macrophages and lower quantities of protein, RNA, mitochondrial kDNA, and ATP (Jara et al., 2017).

Because persister cells are dispersed throughout several host organs, difficult to reach, and difficult to study *in vivo*. Other life cycle phases of *Leishmania* spp. would be expected to develop quiescent forms as an evolutionarily old trait, rather than as an adaptation to mammalian parasitism, to permit population-level survival amid environmental challenges. Non-replicative forms play crucial functions in the life cycle as well (Barrett et al., 2019). For instance, the transmitted metacyclic form is comparable to the sporozoite of non-replicating Plasmodium species. In purine-depleted culture conditions, *Leishmania donovani* promastigotes assume a non-proliferative but viable quiescent state (Carter et al., 2010). In another study, purine transporters and purine salvage enzymes were increased within 24 h of purine deprivation. However, after 48 h, there had been a significant remodeling of the proteome: oxidative stress response pathways had increased while protein synthesis and DNA replication and repair enzymes had decreased (Martin et al., 2014). These changes are all characteristics of persister-like cells. The leishmanial promastigote model may provide a feasible way to investigate the mechanisms underlying the formation of persister-like cells and direct research in less commonly studied amastigotes because nutrient starvation responses have significant mechanistic overlap with the formation of persister cells in bacteria (Michiels et al., 2016) and fungi (Delarze and Sanglard, 2015) as well.

### 6.3. Persistence or dormancy in African trypanosomiasis

The concept of persisters in African trypanosomiasis is not yet fully elucidated. This may not be unconnected to the significant progress made on the containment of the infection on the African continent. However, the existence of *T. brucei* in short-stumpy and long-slender forms, wherein, the former is dormant, non-dividing cells while the latter is a proliferative form of the cells informs the possibility of the existence of persister cells in this species. The colonization of adipose tissue, skin and nervous system of the mammalian host during infection (Luzak et al., 2021) could be responsible for the heterogenicity of stumpy and slender forms seen in the *T. brucei*. Adipose tissue is the largest reservoir of the *T. brucei* (Trindade et al., 2016). Interestingly, the adipose tissue forms (ATFs) of *T. brucei* fcan re-infect the blood circulation and have metabolism that appears to be suited to catabolize fatty acids, demonstrating that they are not terminally specialized to exist only in adipose tissue. A major proportion of ATFs population grows heterogeneously, synthesizes proteins at a slower rate, and is resistant to pharmacological treatment, which contributes to disease chronicity (Trindade et al., 2016, 2022). Compared to blood-stream forms (BSFs), the ATF population was more diverse, with around 40% of the population exhibiting a slower rate of proliferation. Because of this population heterogeneity, the method of adaptation to living in adipose tissue may be more intricate than cell plasticity, which allows cells to temporarily adjust to their environment by uniformly changing their growth rate (Trindade et al., 2022). Paradoxically, the authors conclude that both the blood-stream forms and ATFs are neither dormant nor quiescent.

#### 6.3.1. Treatment strategies against persisters and prospects for new therapy

Since the observation of the existence of persisters in trypamastigotes and their link with the increased incidences of drug resistance, efforts are being made to develop new treatment protocols and strategies to overcome them. Exploring longer treatment times is one strategy. This strategy is based on persister parasites' inherent dynamics, which all persisters eventually develop a resistance to pharmacological therapy. This strategy has difficulties due to the lack of knowledge about the dynamics of spontaneous persister and the possibility of drug-induced persistence. These difficulties may be overcome by long-term intermittent therapy; proof of concept for this strategy was shown in mice treated with benznidazole (Álvarez et al., 2020). Álvarez et al. (2020) reported that a greater dose of benznidazole (2.5–5 times the usual dose) resulted in a higher and longer reduction of parasite populations. The persister condition was cleared more quickly with the larger dose than with the standard dose, and intermittent (weekly) treatment for 30 weeks resulted in a parasitological cure. They hypothesize that this is caused by continuing therapy after the parasites have gone dormant or are no longer present as persisters. However, intermittent low-dose treatment for 30 weeks did not clear the persister. Therefore, longer treatment periods and larger doses are both necessary to eradicate persisters (Fig. 6). However, there is no clinical information to show if this high-dose intermittent treatment could be used to treat people. A pilot clinical investigation found that intermittent benznidazole therapy was no more successful than regular daily therapy (Álvarez et al., 2020). Nonetheless, this strategy might lessen side effects, and longer intermittent dosing schedules might have a higher efficacy. The primary disadvantage from the standpoint of public health is that shorter treatments are favoured over lengthier ones. Finding substances that kill persistent parasites is a more direct strategy that might help reduce the length of the treatment. Due to their slower metabolism, persister parasites may resist therapy, but they are still likely to maintain the pathways necessary for their existence. The effectiveness of this strategy depends on improved knowledge of persister biology and extensive phenotypic screening to find chemicals that target persisters (De Rycker et al., 2023). Combination therapy, which is an extension of this strategy, might be able to address the problems the persister group poses.

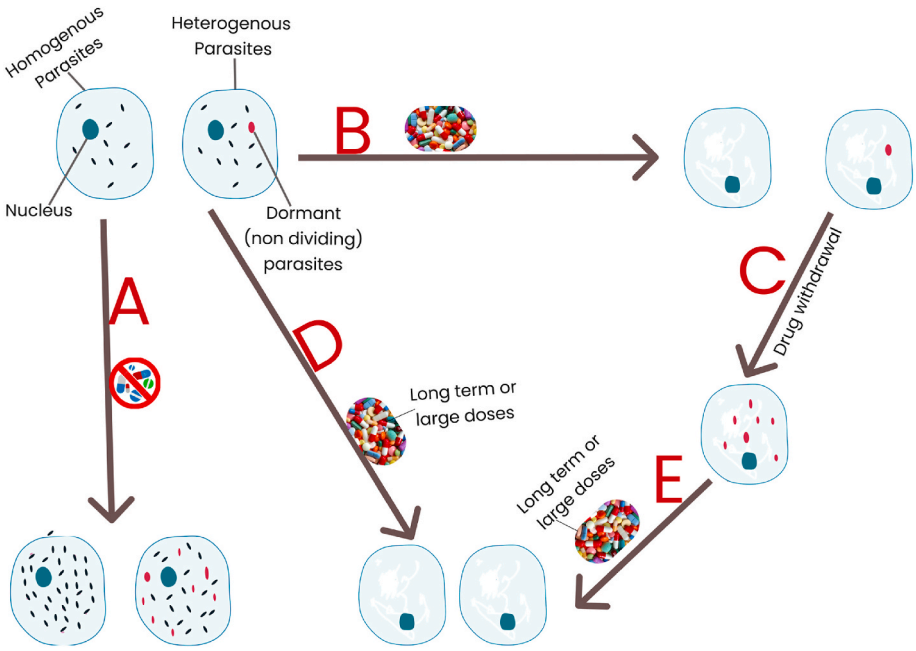


**Fig. 6.** Persistence (dormancy) and drug resistance. Cell with homogenous parasites represents infected cells with classical pathogens; while cell with heterogeneous parasites represents infected cells with classical parasites and a dormant parasite. In the absence of drug (A), the parasites in the two cells proliferate to establish infection. Administration of drug therapy at a usual recommended dosage regimen eliminate the classical pathogens completely and keep dormant (persiter) cells at bay (B). Drug withdrawal from the completion of conventional dosage regimen results in the proliferation of persister cell that was initially kept at bay by the drug (C). However, longer term treatments or administration of larger doses completely cure the cells of both classical and dormant pathogens (D) and prevent the occurrence of pathway C. Similarly, longer term therapies or larger doses have the potential to completely cure cells with proliferated dormant pathogens following initial drug withdrawal (E).

## 7. Concluding remarks

Advances in drug discovery and the understanding of drug action and resistance have highlighted the roles of transporters, efflux pumps, drug targets, and persister cells in resistance mechanisms of kinetoplastids. These insights are crucial for developing novel therapeutics to address current treatment challenges in kinetoplastid diseases. The identification of new drug targets, alternative transport pathways, and AI/ML-driven drug discovery can accelerate solutions to drug resistance. Sustained research in these areas is essential for translating discoveries into effective, clinically viable treatments.

## CRediT authorship contribution statement

**Abdullah M. Tauheed:** Writing – review & editing, Writing – original draft, Resources, Methodology, Data curation, Conceptualization. **Ammar U. Danazumi:** Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Data curation. **Oluwafemi A. Adepoju:** Writing – review & editing, Writing – original draft, Resources, Data curation. **Patricia I. Kobo:** Writing – review & editing, Writing – original draft, Resources, Data curation. **Auwal Adamu:** Writing – review & editing, Writing – original draft, Resources, Data curation. **Emmanuel O. Balogun:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Data curation, Conceptualization.

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## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Reports a relationship with that includes: Has patent pending to. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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