

Protozoan persister-like cells and drug treatment failure

Michael P. Barrett^{1*}, Dennis E. Kyle², L. David Sibley³, Joshua B. Radke³ and Rick L. Tarleton²

Abstract | Antimicrobial treatment failure threatens our ability to control infections. In addition to antimicrobial resistance, treatment failures are increasingly understood to derive from cells that survive drug treatment without selection of genetically heritable mutations. Parasitic protozoa, such as *Plasmodium* species that cause malaria, *Toxoplasma gondii* and kinetoplastid protozoa, including *Trypanosoma cruzi* and *Leishmania* spp., cause millions of deaths globally. These organisms can evolve drug resistance and they also exhibit phenotypic diversity, including the formation of quiescent or dormant forms that contribute to the establishment of long-term infections that are refractory to drug treatment, which we refer to as ‘persister-like cells’. In this Review, we discuss protozoan persister-like cells that have been linked to persistent infections and discuss their impact on therapeutic outcomes following drug treatment.

Persisters

A subpopulation of slow-growing or growth-arrested cells that have a decreased susceptibility to killing by normally effective cytotoxic agents. Persisters survive treatment with drugs because of their altered metabolism during a temporary state of quiescence and are genetically identical to other, drug-susceptible cells in the population. Persisters may arise stochastically or in response to environmental cues such as nutrient starvation.

Treatment failure of antimicrobial agents is a serious global health threat¹. Drug resistance emerges from mutations in the microbial genome that enable microorganisms to survive in the presence of drugs² and that are propagated in a genetically stable manner. Microorganisms can also be refractory to drugs without the selection of genetic mutations. In 1944, Irish physician Joseph Bigger³ noted that in a population of *Staphylococcus aureus* a small proportion of cells were refractory to penicillin; cells within this subpopulation are termed ‘persisters’. The formation of non-replicating but viable cells is common among bacteria^{4–7} and a fundamental and ancient evolutionary adaptive mechanism⁸, whereby a small subpopulation of cells spontaneously enter dormancy or quiescence, terms we use interchangeably in this Review, given that different authors have used the terms arbitrarily in their studies describing cells that are non-replicating or replicating very slowly with associated metabolic alterations such as diminished DNA synthesis, and widespread down-regulation of protein translation when compared with replicative cells. These cells are less vulnerable to a range of transient environmental stresses, including immunological attack and drug treatment⁸. Once those conditions pass, this population can return to a proliferative state and recolonize the environment. Although the dormant state enables bacteria to survive stress, it is not necessarily triggered by stress; instead dormancy occurs spontaneously^{4–7}, and recent preliminary data point to new approaches to identify and isolate low numbers of spontaneously arising *Mycobacterium smegmatis*

persisters⁹ (that is, a subpopulation of cells is prepared for the sudden onset of stress).

Eukaryotic cells also enter quiescent/dormant states that affect drug susceptibility, and drug-tolerant persister-like cell types have been described for both fungi¹⁰ and cancer cells¹¹. Protozoan parasites are eukaryotes that cause millions of deaths each year. Treatment failure of antiparasitic drugs is of concern, and drug resistance mechanisms in parasitic protozoa are increasingly understood and have recently been reviewed elsewhere¹².

Protozoa use various mechanisms to establish persistent infections, including mechanisms to manipulate and avoid host immunity^{13,14}. In this Review, we focus on parasite stages that are capable of entering a quiescent/dormant state and relate this phenomenon to drug treatment failure. In bacteria, metabolic divergence from proliferative cells is a hallmark of quiescence¹⁵, and a number of pathways implicated in metabolic adaptation have been identified; for example, toxin–antitoxin systems¹⁶ and stress response pathways^{4–7}.

Several studies have shown that parasitic protozoa can generate various forms during their life cycle that have been linked to persistence. In particular, the hypnozoite stages of *Plasmodium vivax* and related *Plasmodium* species have been shown to cause persistent infections and they have been linked to drug treatment failure. Moreover, it has been shown that *Plasmodium falciparum* parasites can enter a transient growth-arrested state as ring forms in erythrocytes, during which time they are refractory to artemisinin-based drugs.

¹Wellcome Centre for Integrative Parasitology, Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, UK.

²Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, GA, USA.

³Department of Molecular Microbiology, Washington University School of Medicine, St Louis, MO, USA.

*e-mail: michael.barrett@glasgow.ac.uk

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Hypnozoite

A small dormant form of *Plasmodium vivax* and *Plasmodium ovale* (and *Plasmodium cynomolgi* in monkeys) that persists in host hepatocytes following infection of sporozoites.

Artemisinin

An antimalarial drug derived from the sweet wormwood plant, *Artemisia annua*. The structure comprises a sesquiterpene lactone containing an endoperoxide responsible for activity.

Tachyzoite

A rapidly growing intracellular form of *Toxoplasma* that divides asexually and undergoes successive rounds of lytic growth in a wide range of nucleated cells before being controlled by an efficient immune response. Cause of acute infection and disease manifestation in humans.

In *Toxoplasma gondii*, the switch between the rapidly proliferative tachyzoite form and the slow-replicating tissue cyst-bound bradyzoite form underpins chronic infections that are refractory to treatment. In addition, it was recently shown that *Trypanosoma cruzi* can form dormant, transiently non-proliferating amastigote forms¹⁷. Finally, evidence indicates *Leishmania* spp. form semiquiescent amastigotes¹⁸. These protozoan parasites exhibit a loss of sensitivity to drugs that kill more rapidly proliferating cells because of phenotypic differences related to slow or arrested growth.

In this Review, we describe these forms as ‘persister-like cells’. We describe the current knowledge of the observed forms and their possible role in persistent infections and treatment failure and we outline possible treatment options. Key features of bacterial persister cells and their protozoan persister-like counterparts described in this Review are compared in TABLE 1.

Plasmodium species

More than 200 million people are infected with malaria-causing parasites of the genus *Plasmodium*, which cause the death of approximately half a million individuals annually¹⁹. The parasites have a complex life cycle involving both an *Anopheles* mosquito vector and a

vertebrate host. Infection begins when sporozoite forms of human infectious *Plasmodium* species are injected by female *Anopheles* mosquitoes during a blood meal. Sporozoites rapidly invade liver cells, where the parasites undergo a proliferative stage, including substantial nuclear division without cell division to produce a schizont. Subsequent cytokinesis results in the formation of multiple merozoites that are released into the bloodstream and invade erythrocytes (developing into the erythrocytic stages). During infection with *P. vivax* in humans and *Plasmodium cynomolgi* in monkeys, a small number of liver-stage parasites enter a different developmental stage, termed the ‘hypnozoite’, which can remain dormant for many months to years before entering the proliferative stage through schizogony. Within the erythrocyte, differentiation to a ring form then a feeding trophozoite that consumes haemoglobin precedes a further round of schizogony and the release of multiple merozoites. Depending on the species, this asexual erythrocyte cycle takes either 2 days or 3 days. Some schizonts, instead of producing typical merozoites that proceed through further rounds of asexual reproduction, produce cells that on reinfection of the next erythrocyte will form either male or female gametocytes. These non-proliferating gametocytes recombine

Table 1 | Characteristics of bacterial persisters and protozoan persister-like cells

Organism	Cell growth	Drug sensitivity	Genetic background	Metabolism	Reactivation	Persistent infection and treatment outcome
Bacterial persister ⁶	Subpopulation of growth-arrested or slow-growing cells	Transient decreased sensitivity to drugs	Genetically identical to parent population	Metabolically distinct from parent population	Recrudescence leads to the generation of a replicating population that can in turn generate low numbers of persisters	Contributes to persistent infections and treatment failure without resistance
<i>Trypanosoma cruzi</i>	Subpopulation of growth-arrested cells	Transient decreased sensitivity to drugs	Presumed genetically identical to parent population	Presumed metabolically distinct from parent population	Recrudescence leads to the generation of a replicating population that can in turn generate low numbers of persister-like cells	Contributes to persistent infections and treatment failure without resistance
<i>Leishmania</i> spp.	Subpopulation of growth-arrested cells	Suspected transient sensitivity to drugs	Presumed genetically identical to parent population	Presumed metabolically distinct from parent population	Recrudescence presumed to lead to the generation of a replicating population	Contribute to persistent infections and treatment failure without resistance (for example, post-kala azar dermal leishmaniasis and leishmaniasis recidivans)
<i>Plasmodium falciparum</i> artemisinin-refractory cells	Subpopulation of growth-arrested cells	Transient decreased sensitivity to artemisinin	Mutations (for example, <i>P. falciparum</i> kelch protein 13) confer increased survival possibly by increasing incidence of growth-arrested cells	Presumed metabolically distinct from parent population	Recrudescence leads to the generation of a replicating population	Contribute to persistent infections and recrudescence after artemisinin treatment
<i>Plasmodium vivax</i> hypnozoites	Subpopulation of growth-arrested cells	Insensitive to most erythrocytic stage plasmodicidal drugs	Genetically identical to parent population	Metabolically distinct from parent population	A full life cycle needs to be completed before more hypnozoites can form	Contribute to persistent infections and treatment failure without resistance
<i>Toxoplasma gondii</i> bradyzoites in tissue cysts	Subpopulation of semidormant cells	Current therapies that are effective against tachyzoites are not effective against bradyzoites	Genetically identical to tachyzoite population	Metabolically distinct from tachyzoite population	Reactivation of chronic infection occurs when bradyzoites differentiate back to the actively replicating tachyzoite stage	Contribute to persistent infection treatment failure without resistance.

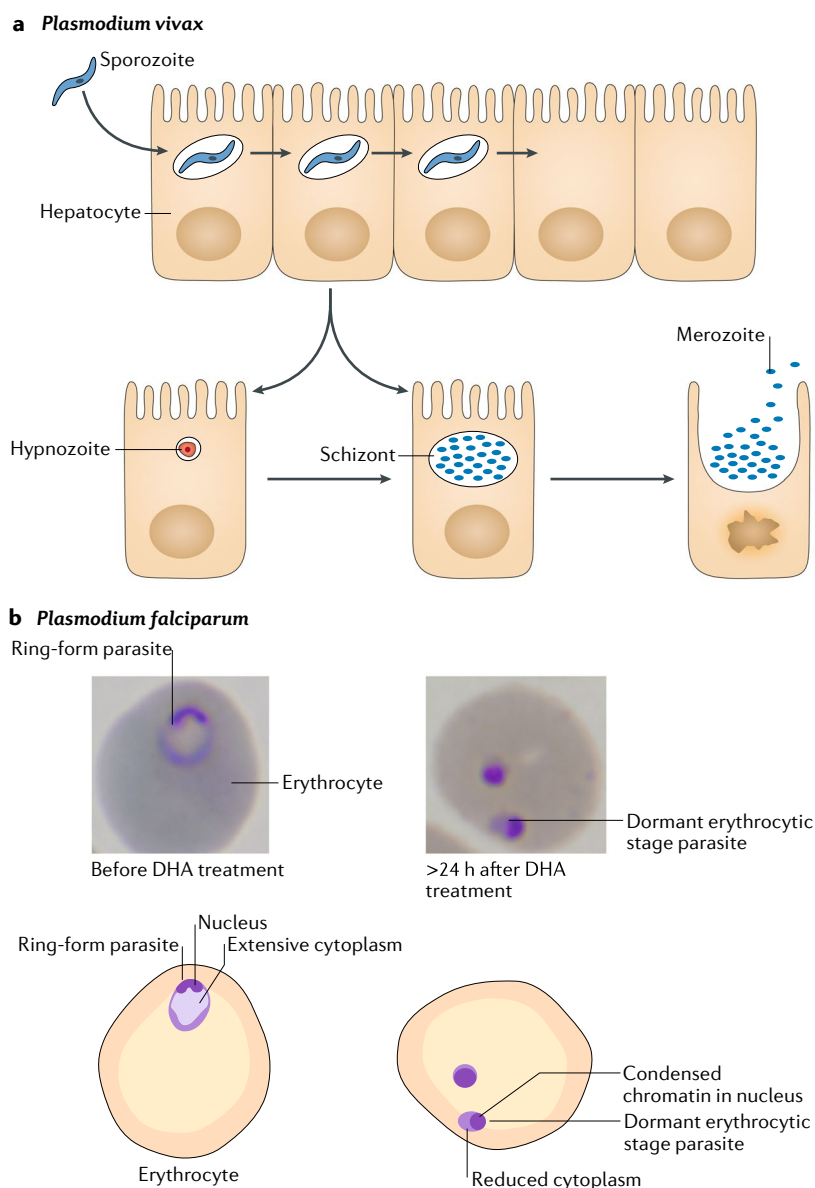


Fig. 1 | Persister-like cell types of *Plasmodium* spp. **a** | The *Plasmodium vivax* hypnozoite. *P. vivax* sporozoites injected into the host by infected *Anopheles* mosquitoes rapidly infect hepatocytes. In the liver cells, the parasites undergo a proliferative stage, including substantial nuclear division without cell division to produce a schizont (schizogony). The schizonts differentiate into merozoite forms, which burst from hepatocytes and enter the bloodstream, where they invade erythrocytes. Alternatively, some sporozoites will form hypnozoites, which can persist in a growth-arrested state for weeks to many months before entering schizogony and completing the developmental cycle in the liver. **b** | Artemisinin-refractory dormant ring stages in *Plasmodium falciparum*. Exposure of synchronous ring-stage *P. falciparum* parasites to dihydroartemisinin (DHA) induces cell-cycle arrest. These dormant ring-stage forms are characterized morphologically by condensed chromatin and reduced cytoplasm. By contrast, pre-exposed ring-stage forms have a relatively diffuse nucleus and large cytoplasmic area. Dormant ring-stage parasites persist for several days before resuming normal growth. DHA-induced ring-stage dormancy is observed in artemisinin-susceptible and artemisinin-resistant *P. falciparum*^{45,47}. The top panel shows Giemsa-stained parasites (purple) inside erythrocytes. The lower panel provides a representation of the upper panel in which key structures visible in light microscopy are visible. The diameter of the erythrocyte is 6 μ m. Part **a** adapted with permission from REF.¹⁵⁷, Elsevier. Part **b** courtesy of Dennis E. Kyle, University of Georgia, Athens, USA.

when they enter a mosquito during a blood meal, creating a zygote that further matures into a motile ookinete, which can migrate out of the mosquito midgut, where it matures into an oocyst. Within the oocyst, sporozoite forms develop before bursting from the oocyst, and these move to the salivary gland of the mosquito, from where the sporozoites are injected into the human host during the next blood meal of the mosquito¹⁹. Sporozoites are a non-replicative form of the parasites. Persistent forms, outlined below, that affect therapeutic outcomes are known for some *Plasmodium* species that dwell in the liver and also during the erythrocytic stages. Other quiescent stages, for example the gametocytes, have also been considered as an important target for novel chemotherapy as they are refractory to most drugs used against replicative bloodstream forms²⁰.

The hypnozoite. Hypnozoites have been indicated in persistent infections with *P. vivax*, and they have been linked to drug treatment failure for some forms of malaria, particularly those caused by *P. vivax* and *Plasmodium ovale* in humans and the monkey malaria parasite *P. cynomolgi*.

Hypnozoites can persist for weeks, months or even years before activation by unknown cues promotes schizogony^{21,22}. These growth-arrested parasites arise spontaneously from a small number of the invading sporozoites, while most commence schizogony almost immediately (FIG. 1a). Relapse arising from differentiation of hypnozoites into proliferative liver-stage schizonts that in turn create merozoites may be more rapid in tropical strains, whereas in more temperate climates, hypnozoite activation can occur 1 year or more after infection, to coincide with warm weather and high mosquito prevalence²³. Morphologically, hypnozoites are uninucleate, non-growing stages first described in liver biopsy samples from *P. vivax*-infected chimpanzees and *P. cynomolgi*-infected rhesus monkeys^{24,25}. Hypnozoite-driven relapse is a major impediment to malaria elimination globally.

The biology of hypnozoites is poorly understood, given the difficulties of studying a very rare parasite inside host liver cells and the lack of appropriate rodent models. However, several studies probing *P. vivax* or *P. cynomolgi* biology following sporozoite invasion of either immortalized hepatoma cells or primary hepatocytes have revealed key insights into hypnozoite biology. For example, histone acetyltransferase inhibitors can activate hypnozoites²⁶ and the assessment of liver-stage active drugs has been possible^{27,28}. Transcriptome analysis of *P. cynomolgi*²⁹ and *P. vivax*²⁷ has revealed stage-specific gene expression, and comparison of *P. cynomolgi* liver stages at 9–10 days after infection³⁰ with those at 6–7 days²⁹ showed that schizonts express many genes and that most metabolic pathways are active. By contrast, hypnozoites exhibit a marked decrease in the expression of genes for most biochemical pathways between day 6 and day 9, which indicates progressive entry into a quiescent state. However, similarly to other known persister cell types, quiescence does not involve the complete downregulation of metabolic pathways. Several pathways remain active, including

Bradyzoite

A persistent form of *Toxoplasma* found in tissue cysts, which are intracellular and commonly found in skeletal muscle and neurons in the brain. Cysts are not cleared by active immunity or drug therapy. Reactivation can lead to serious disease, especially in those with compromised immune function.

Amastigote

In *Trypanosoma cruzi*, an intracellular, replicative cell within the mammalian host cell cytoplasm (*T. cruzi* amastigotes reside in the cytoplasm of various host cell types but show preferential persistence in muscle and adipose tissues). In *Leishmania*, an intracellular form of *Leishmania* with a truncated flagellum that replicate in mammalian cells, including macrophages and dendritic cells, within the acidic phagolysosome compartment of these cells.

Sporozoite

In *Plasmodium* parasites, a form forming in mosquitoes, where it migrates to salivary glands and is injected during a blood meal. It migrates to the liver and invades hepatocytes. In *Toxoplasma*, an infectious form found in oocysts, the product of the sexual phase, which is shed into the environment and contaminates food and water, leading to transmission. The sporozoite persists in a semidormant state within the oocysts, surviving for many months in the environment.

Schizont

A multinucleated form of the *Plasmodium* parasite that forms through multiple nuclear divisions preceding cellular division to release merozoite forms. Can occur either in erythrocytes or before the erythrocytic cycle in hepatocytes.

Merozoites

Forms of *Plasmodium* parasites that emerge from liver cells after differentiation and invade red cells, where the asexual life cycle progression ultimately creates many more merozoites, which burst from infected red cells and invade new ones.

those involved in protection against oxidative and other stresses, protein export, ATP maintenance and protein post-translational modifications, including those involved in genome structure (for example, histone methylation and acetylation)³⁰, which suggests that epigenetic changes may contribute to the regulation of gene expression and heterochromatin structure in hypnozoites.

Fluorescent reporters have allowed the enrichment of *P. cynomolgi* liver stages³¹. A miniaturized culture system of *P. vivax*-infected primary human hepatocytes has offered new models to study liver-stage infection^{28,32} and can be used for high-content imaging-based assays to identify new hypnozoitocidal drugs. Immunodeficient mice, engrafted with human hepatocytes, have allowed the study of *P. vivax* liver stages³³, including the elucidation of the hypnozoite maturation process, which allowed the testing of putative hypnozoitocidal drugs and the identification of upregulated in infectious sporozoites protein 4 (UIS4) as a liver-specific marker for hypnozoites^{33,34}. However, transcripts that are found only in hypnozoites and not other liver stages were not identified³¹.

Therapeutic options for hypnozoites are very limited. Most erythrocytic stage active drugs are inactive against hypnozoites. This may be due to the absence of pathways such as haem degradation, which is targeted by chloroquine, and the absence of pathways that are targeted by artemisinins. However, primaquine and its newly approved analogue tafenoquine are 8-aminoquinolines with proven efficacy against hypnozoites (and also liver-stage schizonts). These drugs are most effective when given in combination with chloroquine or quinine, which have some synergistic effect with primaquine^{35,36}. Primaquine is metabolized in the liver by the host cytochrome P450 2D6 into short-lived reactive metabolites. Different cytochrome P450 2D6 variants have been associated with reduced primaquine metabolism in the host and diminished primaquine efficacy^{37,38}. However, reduced cytochrome P450 2D6 activity is not associated with decreased tafenoquine efficacy^{39,40} even though tafenoquine metabolites have been shown to eliminate liver-stage parasites in rodents⁴¹. This suggests that unmetabolized tafenoquine or metabolites created by different metabolic routes may have activity against liver stages for this drug. Unfortunately, 8-aminoquinoline metabolites can also cause serious side effects in glucose 6-phosphate dehydrogenase (G6PD)-deficient patients, in whom haemolytic anaemia may occur. An estimated 35% of people at risk of malaria caused by *P. vivax* are therefore not eligible for primaquine treatment either due to G6PD deficiency or due to impaired cytochrome P450 2D6 activity⁴². G6PD diagnostic tests will be used in conjunction with tafenoquine following its approval in the United States and Australia for human use to exclude enzyme-deficient patients from treatment. New hypnozoitocidal drugs without G6PD liabilities are urgently needed, and the recent breakthroughs in hypnozoite models described above might expedite the discovery of novel avenues to target hypnozoites and thus increase treatment success.

Recently discovered phosphatidylinositol 4-kinase (PI4K) inhibitors⁴³ generated some excitement given their potency against liver stages of *P. cynomolgi* in vitro, yet treatment with such inhibitors failed to prevent relapses in rhesus monkeys challenged with sporozoites of *P. cynomolgi*⁴⁴. Despite this result, important insights into hypnozoite biology emerged from these studies; crucially, it was shown that hypnozoites mature by day 4–5 after infection of hepatocytes into PI4K inhibitor-refractory forms, whereas exposure to these inhibitors during early infection eliminates liver-stage infection.

Artemisinin-induced dormant ring stages. In addition to hypnozoites, recent evidence has pointed to another type of transiently growth-arrested form of *P. falciparum* that profoundly affects malaria therapy. Exposure of ring stages of *P. falciparum* to artemisinin derivatives (for example, dihydroartemisinin (DHA)) can induce a cell-cycle arrest that is time and dose dependent. These non-replicating ring-stage parasites persist in erythrocytes for days to weeks before they resume normal asexual growth^{45,46}. This artemisinin-induced dormancy is characterized phenotypically by small parasites showing condensed nuclei (FIG. 1b). Whether such forms can also arise spontaneously is not known.

Artemisinin-induced dormancy has been confirmed in *P. falciparum* from lines that are refractory to the drug and also lines that are susceptible to the drug^{45,47}, which suggests the involvement of an adaptive stress response in the formation of this dormant ring form⁴⁸. Dormant ring stages are physiologically distinct from normal ring-stage forms as shown by upregulation of fatty acid and pyruvate metabolism⁴⁹ and maintenance of mitochondrial membrane potential⁵⁰. Although artemisinin-based drugs induce dormancy during ring-stage development, other current antimalarials, including quinolines, antifolates and atovaquone, do not seem to affect ring forms. However, the antifolate pyrimethamine can induce ring-stage arrest and dormant ring forms in the asexual cycle but not in the cycle where the drug is applied but rather in the following cycle. Hence, parasites initially exposed to pyrimethamine develop into mature schizonts, some of which produce merozoites that invade new erythrocytes and only then arrest development as dormant ring stages⁴⁸. These observations explain the results of studies with pyrimethamine^{51,52} that found recrudescence parasites following drug exposure. This suggests the possibility of adaptive fitness advantages of dormancy (starting at different parts of the life cycle) for parasite survival against a range of stresses rather than a specific response to artemisinins. Additional drug or nutritional stressors can arrest development of intraerythrocytic stages of *P. falciparum*. These include isoleucine starvation⁵³, atovaquone exposure⁵⁴, and difluoromethylornithine-induced depletion of polyamines⁵⁵. However, these conditions block trophozoite development and thus differ from artemisinin-induced cell-cycle arrest in ring-stage parasites. Artemisinins (which are activated by cleavage of their endoperoxide bridge) alkylate many proteins in the parasite^{56,57}, which leads to protein misfolding and ubiquitinylation and subsequent induction of the unfolded protein response involving protein

Trophozoite

A form in the *Plasmodium* parasite life cycle following the ring stage that consumes host haemoglobin before entering schizogony.

Haemolytic anaemia

Anaemia ensuing from lysis of red blood cells, for example due to oxidative stresses induced by primaquine in individuals that are deficient in glucose 6-phosphate dehydrogenase.

degradation by the proteasome. However, treatment with artemisinins also diminishes proteasome activity⁵⁸, and parasite death seems to be linked to the accumulation of damaged protein⁵⁸. Inhibition of translation by the chemotherapeutic agent cyclophosphamide was shown to antagonize the antimalarial activity of DHA⁵⁸. Growth-arrested ring stages induced by artemisinin are likely to have diminished protein synthesis, which would explain why the drug loses activity against such forms.

Parasites isolated from patients in whom artemisinin treatment failed in South-East Asia were characterized by low clearance rates from blood⁵⁹. However, these parasites did not show alterations in artemisinin sensitivity in classic in vitro potency tests conducted over 72 h (REF.⁶⁰). The reasons for this became clear in subsequent studies into understanding the nature of artemisinin treatment failure. In vitro selection of resistance in increasing doses of artemisinin⁶¹ revealed that these parasites also failed to yield discriminatory susceptibility to the drug in classic assays but did show an enhanced rate of dormant ring form formation. Mutated alleles of the gene *Pfk13*, encoding kelch protein 13 (Pfk13), were associated with parasites from artemisinin treatment failures in field studies as well as the in vitro selected line⁶². In each case, classic in vitro sensitivity assays could not detect the loss of response to artemisinins. However, a ring-stage survival assay⁶³, where early rings (forming within the first 3 h of erythrocyte invasion) from artemisinin-refractory lines were exposed to 700 nM artemisinin for 6 h, showed they had enhanced ability to survive compared with sensitive lines. The same phenotype is found when various mutant alleles of *Pfk13* are engineered into wild-type parasites⁶⁴.

The function of Pfk13 is not known, although roles in protein ubiquitinylation have been predicted⁶⁵. This includes ubiquitinylation of *P. falciparum* phosphatidylinositol 3-kinase (PfPI3K), and increased phosphatidylinositol 3-phosphate (PtdIns3P) levels have also been linked to diminished sensitivity to artemisinins⁶⁶. It was recently shown that parasites with *Pfk13* mutant alleles have different kinetics of haemoglobin digestion⁶⁷, which could also decrease the activation of the drug. Recently, a coronin-encoding gene, which like *Pfk13* encodes a member of the WD40-propeller domain protein family, was identified in another *P. falciparum* line selected in vitro over a prolonged period for resistance to artemisinin⁶⁸. Phenotypically, parasites with *P. falciparum* coronin gene mutant alleles mirror those of parasites with *Pfk13* mutant alleles in that the ring-stage survival assay is required to discriminate sensitive and artemisinin-refractory lines. The *Pfk13* and *P. falciparum* coronin gene mutations are, therefore, somewhat analogous to the so-called high persister (Hip) mutations that are known to enhance the rate at which persisters form in bacteria and yeasts¹⁶. Many of the known Hip genes are part of toxin–antitoxin protein pairs, and given the predicted capacity of Pfk13 to combine in protein-complex formations⁶⁹, it will be of interest to determine whether analogous functions can be found for Pfk13, *P. falciparum* coronin and any other proteins that confer enhanced dormancy and artemisinin refractoriness as they are identified.

Recent studies evaluated the ability of antimalarial drugs to prevent the recrudescence of artemisinin-induced dormant ring stages. Mefloquine exposure reduced the rate of recrudescence but did not completely abrogate activation and growth⁷⁰. However, PI4K inhibitors and imidazolopiperazines did block recrudescence of dormant ring forms of artemisinin-susceptible and artemisinin-resistant *P. falciparum*^{71,72}. A tetroxane-based compound was shown to eliminate parasites carrying *Pfk13* mutant alleles⁷³ probably because of its long elimination half-life ensuring it remains at parasitocidal levels for more than 4 days and thus can kill drug-induced dormant ring forms as they reactivate⁷³. New data suggest that phytohormones⁷⁴, including gibberellin and abscisic acid, activate DHA-induced dormant rings 24–48 h earlier than controls treated with DHA alone and left to recover. This work offers a potential new route to drug discovery through reactivation of dormant forms, thereby resensitizing them to current drugs. Reactivation is a more viable strategy for tropical infectious diseases than seeking molecules that sustain the growth-arrested state, as has been proposed for cancer cell persisters¹¹, given economic considerations in long-term provision of therapy for diseases that most commonly occur in underprivileged areas. An additional study demonstrated the potential role of eukaryotic initiation factor 2 α (eIF2 α) phosphorylation as a mechanism promoting dormancy in ring stages in *Plasmodium berghei*⁷⁵, which suggests that inhibition of eIF2 α phosphorylation might prevent parasites entering dormancy²⁰ and thus retaining sensitivity to artemisinins. The role of eIF2 α phosphorylation in controlling dormancy was initially described in sporozoites⁷⁶, and the phosphorylation status of eIF2 α has since been proposed as a key regulator of different dormancy in apicomplexans²⁰.

Toxoplasma gondii

T. gondii, the causative agent of toxoplasmosis, is a tissue cyst-forming coccidian and a member of the phylum Apicomplexa^{77,78}. The ability to generate dormant (or semidormant forms that grow slowly as opposed to being growth arrested) yet highly infective forms as part of the developmental life cycle is critical to the persistent nature of these parasites. *T. gondii* is one of the most widespread protozoan parasites infecting a diverse range of warm-blooded vertebrate hosts, including humans⁷⁹. Its complex, multihost developmental life cycle is defined by sexual and asexual stages, each with distinct yet related end forms that enable the parasite to persist in nature. The natural life cycle for *T. gondii* involves small rodents that serve as intermediate hosts and cats that serve as the definitive host⁷⁹. Within enterocytes of the cat gut, the parasite undergoes sexual development, culminating in shedding of oocysts in the faeces⁷⁹. After shedding, oocysts undergo meiosis to form eight haploid sporozoites, which remain dormant and do not divide, despite surviving in the environment for long periods⁷⁹. Following oral infection, oocysts hatch, which enables sporozoites to invade the intestine and differentiate into replicating tachyzoites, which disseminate throughout the host, thus causing acute disease⁷⁹. In response to immune pressure and environmental stress, the parasite

differentiates into a life-cycle stage called the 'bradyzoite', which forms tissue cysts within host cells and gives rise to persistent infection⁷⁹. Bradyzoites are often referred to as dormant, quiescent or latent forms; however, these terms are not strictly accurate as bradyzoites remain metabolically active, and do replicate, albeit slowly and asynchronously⁸⁰. However, within a given tissue cyst, some bradyzoites seem to be non-replicative and may represent a truly dormant reservoir. Infections in the intermediate host are likely to persist for life in the host and are characterized by slow expansion of bradyzoite numbers within tissue cysts and periodic rupture to release bradyzoites that can infect host cells and produce new tissue cysts or alternatively reconvert to actively replicating tachyzoites. This balance is controlled by immune surveillance that can suppress active parasite growth but not eliminate the tissue cyst reservoir⁸¹.

Dormant forms of Toxoplasma: the sporozoite and bradyzoite.

Outside of cats, truly dormant oocyst forms of *Toxoplasma* can remain viable in the environment for many years before being ingested by an intermediate host. Hatching of the oocyst in the gut releases highly invasive sporozoites that invade epithelial cells and then differentiate into proliferative tachyzoites, which disseminate widely before differentiating into tissue cysts containing bradyzoites. The bradyzoite is the source of transmission between intermediate hosts and to the definitive cat host. Tissue cysts are also the source of lifelong infections, having the potential to reactivate in a chronically infected host⁸². Humans serve as intermediate hosts and therefore the life cycle is similar to that described earlier for rodents. During the bradyzoite stage, infections are largely asymptomatic in immunocompetent individuals, with disease arising when these infections reactivate in immunocompromised patients. Individuals undergoing chemotherapy or organ transplantation or patients with untreated HIV are at risk of reactivation when bradyzoites emerge from tissue cysts and reconvert to rapidly growing tachyzoites, which resume lytic growth and destroy their host cells. The process of reactivation is particularly severe when tissue cysts harboured within the central nervous system undergo reactivation^{83,84}. In the absence of appropriate antiparasitic treatment, the resulting syndrome of toxoplasmic encephalitis⁸⁵ can become life-threatening.

The tachyzoite-to-bradyzoite transformation controls the transition between acute to chronic infection, while the reverse switch from semidormant bradyzoites to actively replicating tachyzoites is the key to disease reactivation. Gene expression analyses indicate that only a few hundred genes differ between tachyzoite and bradyzoite stages, whereas other stages (sporozoites and merozoites) show much greater differences⁸⁶. However, the molecular basis of these life cycle transitions is not well defined. Slowing of the tachyzoite replication cycle⁸⁷ coincides with the expression of a bradyzoite-specific transcriptional cascade^{88–90}, which includes stage-specific roles for apicomplexan AP-2 transcription factors^{91–95} that have also been implicated in transcriptional regulation of development in *Plasmodium* spp. (that is, ookinete, sporozoite and gametocyte stages)^{96–98}.

These tightly regulated developmental responses enable parasites to balance dissemination within the host and chronic infection that allows host-to-host transmission⁹⁹. Studies have shown that stressors such as nutrient limitation¹⁰⁰, high pH¹⁰¹ and nitric oxide exposure¹⁰² can induce bradyzoite differentiation. Infection of long-lived, differentiated cells such as skeletal muscle¹⁰³ and neurons¹⁰⁴ also induces bradyzoite formation and persistent infections^{105,106}. Therefore, host immune pressure, environmental stressors and parasite-specific genetic programmes can result in the formation of bradyzoite-containing tissue cysts. Triggering differentiation back to actively growing tachyzoites is also not fully understood, but might arise from abundance of nutrients or absence of immune pressure. Either model might explain why bradyzoites used to infect naive mice quickly revert to tachyzoite growth before later developing into new bradyzoites and forming tissue cysts⁸². This switching ability is not understood but it probably underlies the permissive nature of transmission between intermediate hosts, which include nearly all warm-blooded animals, which are usually infected by ingestion of semidormant tissue cysts¹⁰⁷. This oral transmissibility of the asexual, tissue stages sets *T. gondii* apart from its close relatives that have more restricted modes of transmission.

Human infections arise either from ingestion of oocysts shed into the environment from the feline definitive host¹⁰⁸ or from consumption of undercooked meat contaminated with cyst-bound bradyzoites¹⁰⁹. Mothers first infected with *T. gondii* during pregnancy can transmit infection via tachyzoites crossing the placental barrier and infecting the fetus¹¹⁰. Although most infections are subclinical or result only in mild disease in healthy adults, hypervirulent strains of *T. gondii* in South America cause an increased incidence of ocular toxoplasmosis in immunocompetent patients¹¹¹.

Current therapies for toxoplasmosis based on pyrimethamine–sulfadiazine or trimethoprim–sulfamethoxazole target DNA synthesis by inhibiting the folate pathway and are effective at blocking replication and killing tachyzoites⁸⁵. Adverse reactions are common and include low tolerance due to bone marrow toxicity and allergy to sulfa components, while potential teratogenicity prevents use during the first trimester of pregnancy⁸⁵. Clinical resistance to treatment (defined by the emergence of mutations rendering parasites stably insensitive to a drug) is rare as human infections are zoonotic (and food animals are not routinely treated for toxoplasmosis) and human-to-human transmission is uncommon.

Although current therapies can control acute infection, owing to their mode of action, chronic infections are unaffected. Replicating tachyzoites are vulnerable, but slowly or asynchronously replicating bradyzoites are not⁸⁵ (FIG. 2). Typically, bradyzoite numbers expand as they replicate, so tissue cysts contain 100–500 individual parasites over the course of 1–2 months (suggesting an average doubling time of several days), whereas tachyzoites double in number every 6–8 h (REF.⁸⁰). Although pyrimethamine eliminates tachyzoites in culture overnight, a 4-h exposure followed by drug wash out does not inhibit parasite replication, and

Trypomastigotes

In *Trypanosoma cruzi*, non-replicative, flagellated, extracellular cells that can invade host cells or be transmitted to reduviid vectors during a blood meal.

hence continuous exposure of replicating tachyzoites over prolonged periods is required for effective treatment¹¹². Neither the blood–brain barrier^{85,113} nor the heavily glycosylated tissue cyst wall^{114,115} is a physical barrier to pyrimethamine, which suggests that the semi-dormant state of the bradyzoite is responsible for treatment failure. Alternative treatments such as clindamycin (which inhibits protein synthesis in the vital apicoplast organelle) also target actively replicating forms⁸⁵.

New drugs to treat toxoplasmosis are actively being investigated. The Malaria Box and Pathogen Box compounds selected in high-throughput screens against *P. falciparum* and other pathogens were screened to find compounds that hit biological targets shared among apicomplexans¹¹⁶. A number of other chemical libraries have been tested against *T. gondii* to possibly identify new therapeutic compounds^{117–120}, including all FDA-approved drugs, seeking compounds for repurposing. In addition, screening of compounds that are active against malaria identified relatively few candidates that were effective against *T. gondii*¹²¹. Atovaquone¹²² and ELQ-300 (REF.¹²³) are effective against tachyzoites and both compounds reduced tissue cyst numbers in chronically infected mice but complete cure was not achieved. Intriguingly, primaquine, used to treat patients with *P. vivax* hypnozoites, has modest activity against *T. gondii* tachyzoites, but its therapeutic use against the bradyzoite has not been evaluated¹²⁴. In separate studies, inhibitors of calcium-dependent protein kinase 1 (CDPK1), an essential enzyme in *T. gondii*, have shown promise for

eradicating infection in immunocompromised mice¹¹². Learning more about essential processes in bradyzoites may lead to therapies that control or eliminate this stage.

Trypanosoma cruzi

T. cruzi, which causes Chagas disease, afflicts millions of people, mostly in Latin America¹²⁵, and is vectored by blood-feeding insects of the Triatominae subfamily of reduviid bugs. Infections in humans and in many other mammals are often low level but lifelong and generally immune controlled. The preferential persistence of *T. cruzi* in muscle, and the continued immune response to parasites in heart and other muscle tissues, probably accounts for the cardiac and gut pathology associated with Chagas disease. Infection with *T. cruzi* generally begins with invasion by flagellated (non-replicative) trypomastigotes transmitted in bug faeces as they feed on their hosts. Trypomastigotes enter host cells and differentiate into amastigotes, which divide, notably in the host cell cytoplasm and not in a vacuole, by binary fission. Amastigotes reconvert to trypomastigotes, which are released from the lysed host cell and invade new cells or are available in the blood for transmission. The intracellular replication cycle generally lasts for 4–5 days, although it can be slower for some isolates¹²⁶.

T. cruzi amastigotes are the primary targets of both immune control and drug treatment. Benznidazole and nifurtimox have rapid trypanocidal activity against *T. cruzi* and have been used therapeutically for decades. However, long treatment courses (30–60 days) are required and outcomes are unpredictable. These and other observations prompted the suggestion of drug-insensitive and potentially dormant subpopulations of *T. cruzi* in hosts¹²⁷.

Dormant amastigote forms of *T. cruzi*. Recently, non-replicating intracellular *T. cruzi* amastigotes were demonstrated in both mice and cultured host cells¹⁷. These dormant parasites occur spontaneously in the absence of drug pressure but resist killing by multiple trypanotoxic compounds that are highly active against replicating parasites. Entering the quiescent state seems not to be a response to a lack of nutrients or to the presence of other obvious stressors, as non-replicating amastigotes are frequently present in host cells alongside actively dividing amastigotes. Furthermore, these non-replicating amastigotes sense the signals that trigger amastigote-to-trypomastigote differentiation, as they differentiate synchronously with the actively dividing forms in the same host cell (FIG. 3). Trypomastigotes derived from non-replicating amastigotes can invade new host cells and re-establish both actively dividing and non-dividing progeny, a hallmark of phenotypic heterogeneity in bacterial persisters. Formerly dormant amastigotes emerging after a 30-day continuous drug exposure *in vitro* are equally sensitive to the drug as the parent (untreated) population¹⁷, and hence drug exposure has not selected for genetically resistant parasites, instead suggesting that dormancy is the cause of drug refractoriness. Transient dormancy (that is, dormant cells that will reactivate) among a small subpopulation of intracellular *T. cruzi* amastigotes therefore underlies

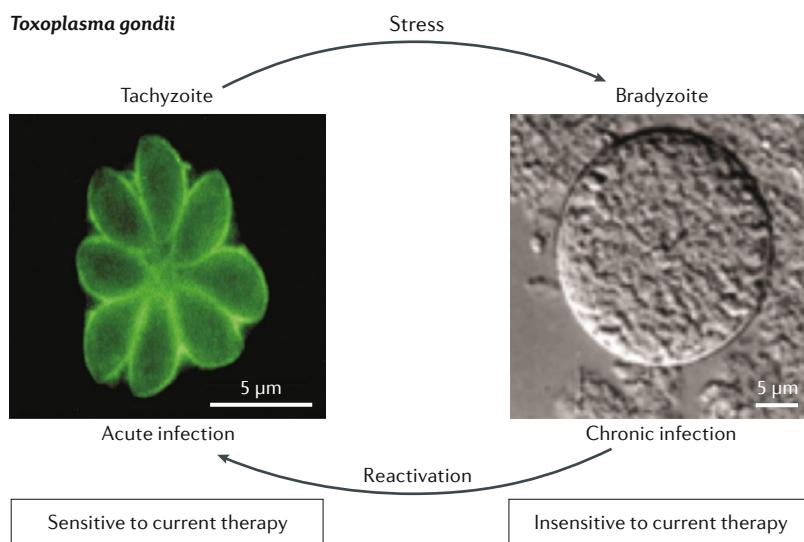


Fig. 2 | *Toxoplasma gondii* tachyzoites and bradyzoites. *T. gondii*, the causative agent of toxoplasmosis, undergoes asexual replication in nucleated host cells of intermediate hosts (such as rodents and humans). During initial infection, tachyzoites disseminate throughout the host and cause acute disease. In response to immune pressure and environmental stress, the parasite differentiates into bradyzoites, which form tissue cysts within host cells and give rise to persistent infection. Shown are microscopy images of the tachyzoite (visualized using polyclonal rabbit anti-surface antigen 1 antibody with Alexa Fluor 488) and a tissue cyst containing bradyzoites (brightfield image). Current therapies are successful only against the acute phase and fail to eliminate parasites during the chronic stage. See REF.⁸⁰ for further details on bradyzoite development. Left image courtesy of L. David Sibley, Washington University School of Medicine, USA. Right image adapted with permission from REF.⁸¹, Springer Nature Limited.

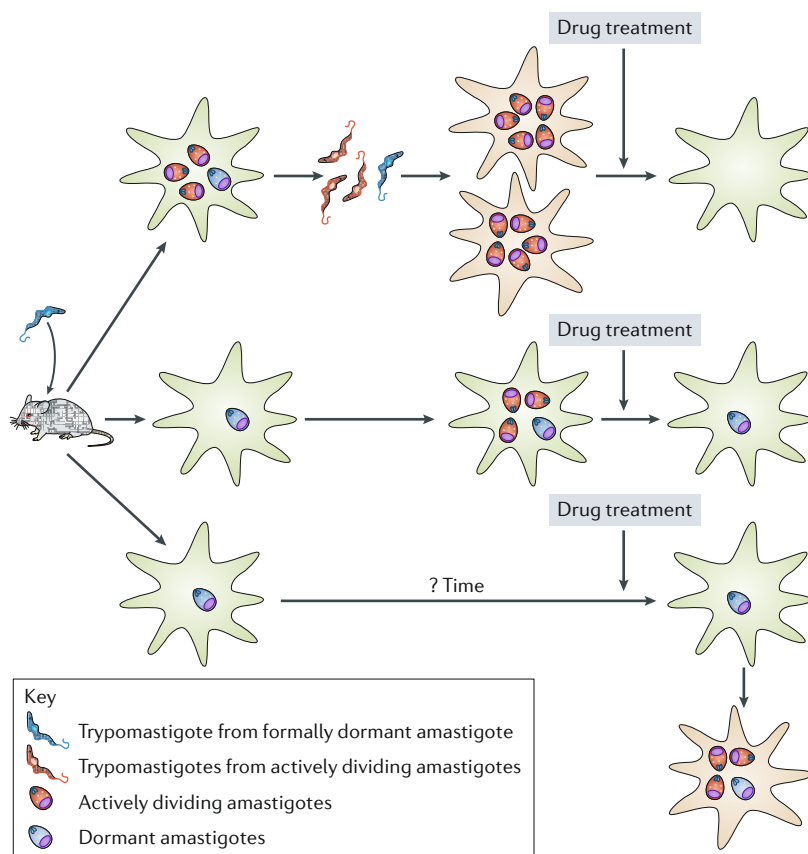


Fig. 3 | Dormant amastigote forms of *Trypanosoma cruzi*. Following invasion of host cells, *T. cruzi* trypomastigotes (shown in blue; indicating no or minimal proliferation) differentiate into non-replicating amastigotes. The presence or absence of dormant parasites determines treatment outcomes. Amastigotes may not replicate (bottom part, blue amastigotes, dormant parasites), undergo minimal replication (top and middle parts, red amastigotes) before becoming dormant or actively replicate (top part, second round of invasion, red amastigotes). Replicating parasites are eliminated by drug treatment and under appropriate conditions (for example, long treatment period), possibly resulting in sterile cure (top part). However, parasites that are dormant during the time of drug exposure (middle and bottom parts, blue amastigotes) resist drug clearance and can revive the infection by resuming replication after drug treatment (bottom part).

treatment failure of anti-*T. cruzi* compounds that kill actively dividing amastigotes. Although the metabolic state of non-replicating amastigotes has yet to be probed, dormant amastigotes have lower levels of expression of reporter proteins, which suggests decreased protein synthetic capacity and altered metabolism¹⁷.

Many questions remain concerning dormancy in *T. cruzi*. Dormant amastigotes are not actively transmitted; instead trypomastigotes must reach the host bloodstream to be taken up by blood-feeding vectors. So what is the selective advantage of dormancy? Immune detection and control of *T. cruzi* is generally highly effective in keeping parasite load low. Host cells containing only dormant amastigotes probably evade surveillance by CD8⁺ T cells or other immune effectors and thus could function as reservoirs for the low but continuous production of trypomastigotes. This could extend the timeframe over which transmission is possible and contribute to parasite persistence by reseeding tissue sites with new persister-like parasites. The occasional reactivation of these dormant parasites might also account for

the dynamic nature of *T. cruzi* infection (for example, the appearance of new sites of parasite replication, appearing and disappearing in different anatomical locations)¹²⁸.

Hosts with shorter *T. cruzi* infections (for example, children) may be more responsive to drug treatment than those with longer infections (decades in humans); of note, this conclusion is still debated and is complicated by how cure is determined^{129–131}. If true, longer infections could result in increased seeding of tissues with dormant parasites, some of which may have exceptional longevity in the dormant state and thus avoid clearance by current, time-limited, treatment protocols.

As *T. cruzi* rarely undergoes sexual recombination, instead recombining and resorting existing genes to generate genetic and antigenic diversity¹³², dormant amastigotes might function as a stable ‘stem-like’ cell should the gene resorting yield compromised progeny in replicating forms.

It seems that the length of time that amastigotes stay dormant, or at least for the ability of an infection to persist under drug pressure, is finite. Sterile cure with drugs can be achieved, in some cases, with treatment courses as short as 10–20 days^{133,134} and some, albeit not all, in vitro cultures clear parasites following a 30-day drug exposure¹⁷. For some *T. cruzi* strains, 16 days of in vitro exposure to benznidazole is sufficient to sterilize the culture¹³⁵. However, 30-day and even 60-day treatment regimens in humans and other animals often fail, and dormancy in combination with other features of the infection (for example, the establishment of infection in a wide range of tissues which differ in drug penetration) is likely to be responsible. On the basis of this, currently available drugs might be more efficacious if used over longer periods (for example, several months), possibly at lower frequency of dosing to keep the overall dose down. Indeed pulse-treating with benznidazole once every 5 days¹³⁶ or even once every 7 days (J. Bustamante, unpublished observations) over several months can be as or even more efficacious than shorter, daily treatment protocols.

New trypanocidal compounds that can target dormant forms are clearly desirable. Nifurtimox and benznidazole both require metabolic activation by trypanosomal enzymes¹³⁷, the activity of which is likely to be reduced in dormant forms. Therefore, establishing screens specific for these dormant forms may identify a new generation of drugs. The observation that non-dividing amastigotes can respond to the cues that trigger stage conversion to trypomastigotes also offers the possibility of finding molecules that might activate dormant parasites and sensitize parasites to existing drugs.

Leishmania species

The leishmaniasis represent a series of conditions caused by more than 20 different species of the genus *Leishmania*¹³⁸ that belong to the same phylogenetic order, the Kinetoplastida, as African and American trypanosomes. Leishmaniasis span self-healing cutaneous disease to a frequently fatal visceral form. Around one million clinical cases are reported each year, with ~10-fold that number of people having subclinical infections in nearly 100 countries in the tropics and

Promastigote

A form of *Leishmania*, with an anterior flagellum, that replicates within the midgut of the sandfly vector, which transmits these parasites. Several distinctive other forms exist in the sandfly vector too.

subtropics¹³⁸. The parasites are transmitted by sandflies (FIG. 4). In the sandfly, a series of different flagellated promastigote forms complete the life cycle, culminating in the formation of growth-arrested metacyclic promastigotes in the mouth parts ready for transmission to the vertebrate host. There they enter phagocytes,

where they transform into amastigote forms within the phagolysosome to establish infection.

Successful treatment of leishmaniasis is notoriously difficult. Pentavalent antimony formulations were the mainstay of treatment for many years¹³⁹ but toxicity and parasite drug resistance increasingly limit use.

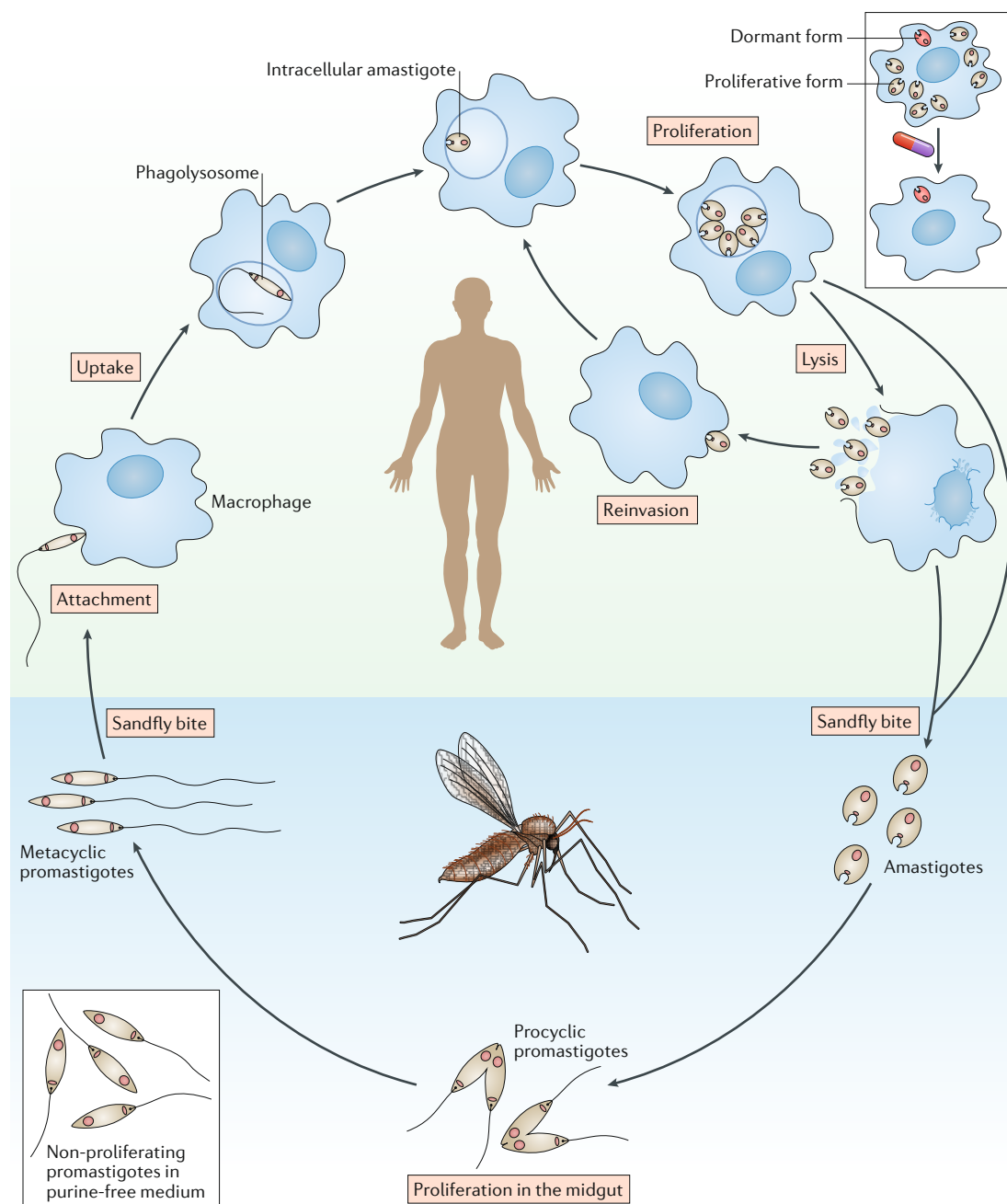


Fig. 4 | *Leishmania* life cycle showing impact of persisters. *Leishmania* spp. alternate between motile promastigotes in the sandfly vector and intracellular amastigotes in the mammalian host. Following transmission of infective, non-dividing metacyclic promastigotes via the bite of a sandfly, promastigotes enter host phagocytes and differentiate within phagolysosomes into amastigote forms (with a truncated flagellum) and undergo replication. Subsequently, host cells burst and the released amastigotes enter further host cells. Alternatively, amastigotes may be taken up during a blood meal by a sandfly, in which they differentiate into proliferating promastigotes (procyclic promastigotes) in the midgut. Studies have identified discrete replicating and non-dividing intracellular amastigote populations, and it is likely that growth-arrested leishmanial amastigote forms contribute to drug treatment failure and persistent infections (top-right panel). Promastigotes have been shown to enter a non-proliferative but viable state in purine-depleted medium (bottom-left panel). Amended from <https://doi.org/10.1128/CMR.14.2.229-243.2001>, *Clin. Microbiol. Rev.* **14**, 229–243 (2001)¹⁵⁸, with permission from the American Society for Microbiology.

Ergosterol

Major sterol of the *Leishmania* plasma membrane, also found in fungi. Binds to amphotericin B.

Miltefosine and paromomycin are used in some settings¹³⁹. Amphotericin B, which binds to ergosterol found in both fungal and leishmanial membranes but not mammals, has become the drug of choice in many countries (its liposomal formulation, AmBisome, being safer than the non-encapsulated drug). Visceral leishmaniasis is caused by *Leishmania donovani* or *Leishmania infantum* in the Old World and *Leishmania chagasi* in the New World. The cutaneous form is generally caused by *Leishmania major* or *Leishmania tropica* in the Old World and a variety of different species in the New World. Mucocutaneous disease is caused by parasites of the *Viannia* group: *Leishmania (Viannia) braziliensis* or *Leishmania (Viannia) guyanensis*. Post-kala azar dermal leishmaniasis (PKDL) occurs in patients with visceral leishmaniasis, emerging as a tegumental condition several months to years after treatment¹⁴⁰. For cutaneous leishmaniasis, a post-treatment

relapsing form is known as leishmaniasis recidivans¹⁴¹. Both conditions are likely to arise from persister-type parasites; that is, parasites that have survived drug treatment due to transient drug refractoriness during dormancy. They reawaken into an immunologically altered environment as the immune system has responded to the primary infection, and in currently ill-understood ways, they re-establish a pathological condition in the skin. Drug resistance mechanisms have been discovered¹⁴², and it is clear that drug treatment failure due to stably transmitted genetic mutation is an increasing problem. For example, treatment failure with antimony formulations in the Indian subcontinent has been traced to mutated aquaporin 1 (which is encoded by the *AQP1* gene), which transports antimony into the cells¹⁴³. A genetic locus associated with miltefosine resistance in field isolates has been identified¹⁴⁴, and changes in genes involved in sterol metabolism have been implicated in amphotericin B resistance^{145,146}. However, treatment failures without classic genetic-based heritable resistance are also widespread (for example, PKDL and leishmaniasis recidivans), which indicates that *Leishmania* spp., like *T. cruzi*, produce persister-like cells.

Box 1 | Methods to study protozoan persister-like cells

Fluorescent probes

Fluorescent probes have become essential to visualize dormant protozoan cells. Labelling parasites using classic fluorescent reporters such as GFP, DsRed or Tmtomato are used to identify the parasites themselves. Labels that enter cells on a pulsed exposure and are then either diluted out during cell division or remain at their initial pulse level in non-replicating cells allow differentiation between replicating and non-replicating cells. CellTrace Violet, carboxyfluorescein succinimidyl ester and CellTracker Red are examples of dyes used for this purpose. Fluorescence-activated cell sorters can be used to sort differentially labelled populations for further analysis.

DNA replication probes

Non-replicating cells do not incorporate new bases into their DNA, while replicating cells do. The thymidine analogues 5-ethynyl-2'-deoxyuridine and 5-bromo-2'-deoxyuridine have both been shown to be effectively incorporated into replicating DNA of *Leishmania* spp. and *Trypanosoma cruzi*, whereas non-replicating cells did not incorporate the activated derivatives of these analogues. To visualize their incorporation it is necessary to fix cells and use specific antibodies to detect them, precluding their use in sorting live cells. *Toxoplasma gondii*¹⁵⁵ and *Plasmodium* spp.¹⁵⁶ do not salvage thymidine and hence can be labelled only with 5-bromo-2'-deoxyuridine and similar probes after stable transfection of thymidine kinase.

Sorting quiescent and replicating cells

Cells that have been labelled with fluorescent dyes (see above) can be separated using fluorescence-activated cell sorters. This approach can also be used to sort cells in which fluorescent reporters (for example, GFP) are regulated by stage-specific promoters, for example to separate tachyzoites from bradyzoites of *T. gondii*. Another simple method for enrichment of persister-like cells is to treat a mixed population with varying doses of antimicrobials that kill replicating cells but not quiescent cells followed by the analysis of the viable cells remaining after treatment.

Metabolomics

Untargeted metabolomics analysis allows the comprehensive profiling of metabolite levels in different cell types. As persister-like cells have altered metabolism when compared with proliferative cells, but genome sequences are unchanged, metabolomics offers a key route to profile the differences in biochemical physiology that distinguish cell types.

Transcriptomics and proteomics

Also relating to the fact that the genomes of persister-like cells are unaltered, profiling the transcriptome and proteome of different cell types can report on adaptations to the quiescent cell phenotype and point to metabolic pathways that might be targeted in these cells.

Single cell transcriptomics

Profiling transcription in single cells, rather than population averages, will allow a more detailed understanding of the changes at the transcriptional level that could be linked to the formation of persister-like cells.

Dormant forms of *Leishmania* species. In mammals, *Leishmania* spp. dwell primarily within macrophages and dendritic cells. The growth rate of *Leishmania mexicana* in non-resolving infections in Balb/c mice¹⁴⁷ was determined to be slow but constant, doubling every 12 days, and it was concluded that amastigotes are semi-quiescent in mice. Moreover, in mice, amastigotes (as an overall population) were shown to have a stringent metabolism, consuming relatively sparing quantities of metabolites as a source for carbon and energy and using them relatively efficiently compared with the cultured promastigote form¹⁴⁸. The possibility of dividing and non-dividing subpopulations was not taken into account in that study. However, discrete replicating and non-dividing intracellular amastigote populations were discriminated in a C57BL/6J mouse model of *L. major* cutaneous disease¹⁸. Four months after infection, two distinct populations could be distinguished on the basis of their ability to incorporate the nucleotide analogue 5-bromo-2'-deoxyuridine into DNA: one actively dividing population that doubled around every 60 h and a non-dividing, dormant population. In the early days following infection, amastigotes divide at a similar rate (around every 60 h) but fewer dormant forms are found. Learning more about the comparative phenotypes of dormant and replicating *Leishmania* spp. amastigotes will shed more light on these persister-like cells.

Other *Leishmania* species also exhibit potential persister-like cellular states. Comparison of *L. (Viannia) braziliensis* promastigotes and amastigotes¹⁴⁹ showed that amastigotes have diminished levels of protein, RNA, mitochondrial kinetoplast DNA and ATP and exhibit no growth in macrophages in vitro. Untargeted metabolomics showed that axenic amastigotes also had diminished levels of multiple amino acids and polyamine pathway metabolites compared with promastigotes. Metabolomics offer a key route to understanding the persister phenotype (BOX 1).

Non-replicating *L. donovani* was also found in in vitro cultivated THP-1 macrophages¹⁵⁰, prompting speculation that these too represent persister-like cells. Although in their infancy, studies of these growth-arrested leishmania forms will probably provide answers to the frequent drug treatment failures in the absence of genetically selected resistant parasites¹⁵¹.

Studying persister cells in vivo is challenging owing to low abundance and inaccessibility, their being distributed across multiple host organs (BOX 1). As an evolutionarily ancient trait⁸, rather than an adaptation to mammalian parasitism, other life cycle stages of *Leishmania* spp. might be expected to form quiescent forms to allow population-level survival during environmental stresses. Non-replicative forms also have important roles in the life cycle; for example, the transmitted metacyclic form is analogous to the non-proliferative *Plasmodium* spp. sporozoite. *L. donovani* promastigotes enter a non-proliferative but viable quiescent state in purine-depleted culture conditions¹⁵². Proteomics analysis¹⁵³ revealed that purine transporters and purine salvage enzymes were upregulated within 24 h of purine deprivation (presumably in an attempt to restore purine levels). However, after 48 h, a profound remodelling of the proteome had occurred: the levels of enzymes involved in DNA replication and repair and in protein synthesis were diminished, while proteins associated with some oxidative stress response pathways were increased, all hallmarks of persister-like cells. In bacteria⁷ and fungi¹⁰ too, nutrient starvation responses

have considerable overlap mechanistically with the formation of persister cells, and hence the leishmanial promastigote model may offer a tractable means of probing mechanisms of persister-like cell formation and guide studies in less readily studied amastigotes (BOX 1).

Very recently insertion of a GFP gene into the ribosomal gene locus of both *L. mexicana* and *L. braziliensis* was reported in a preliminary study¹⁵⁴. Transformation of promastigotes to amastigotes in both species led to pronounced reduction in expression of the reporter gene, consistent with decreased translation in amastigotes. 5-Bromo-2'-deoxyuridine labelling showed that parasites with low GFP expression levels were also non-replicative. Axenic amastigotes were shown to form two populations (bright and dull with respect to GFP expression, and were presumed to represent proliferative and non-replicating cells, respectively). All macrophage-resident amastigotes had dull expression. With use of CellTracker Deep Red staining, in vivo, parasites retaining the dye (and thus not replicating) could be found up to 120 days after infection. GFP fluorescent parasites at this time lacked the dye, indicating that this dual staining approach can distinguish non-proliferating (CellTracker positive) and proliferating (GFP positive, CellTracker negative) parasites. The toolkit necessary to probe *Leishmania* persisters more deeply is thus emerging.

To date, testing the susceptibility to drugs of dormant *Leishmania* spp. amastigotes has not been performed. However, by analogy to other systems — and given the widespread nature of drug treatment failure (including PKDL and leishmaniasis recidivans) — it is probable that these parasite forms are refractory to treatment with antileishmanial drugs, and by learning more about their biology, we will be able to improve therapeutic interventions.

Concluding comments

Persister populations are increasingly appreciated as a problem leading to drug treatment failure in bacteria, fungi and cancer cells. Many parasitic protozoa also display the dormant/quiescent properties characteristic of persister phenotypes, ranging from discrete life cycle stages (for example, non-dividing *P. vivax* hypnozoites and slow-growing *T. gondii* bradyzoites) to the stochastically arising, non-dividing amastigotes of *T. cruzi* to the slow-growing and non-replicating amastigotes of *Leishmania* spp. As in bacteria, these protozoan persister-like cells exhibit drug tolerance and are likely to be responsible for frequent drug treatment failure. Besides some advances, many challenges remain in studying protozoan persister-like cells: these cells are generally of low abundance in mammalian hosts and are either non-replicating or only slowly growing; their tissue distribution is not well defined in mammalian hosts and they can be difficult to find; and in vitro methods for their cultivation are not well developed.

Understanding the mechanisms of dormancy, which involves reversible metabolic changes (FIG. 5), might lead to new treatment strategies. For example, screens that specifically target persister cells may lead to the discovery of compounds that facilitate sterile cure, either alone or together with current compounds that eliminate actively

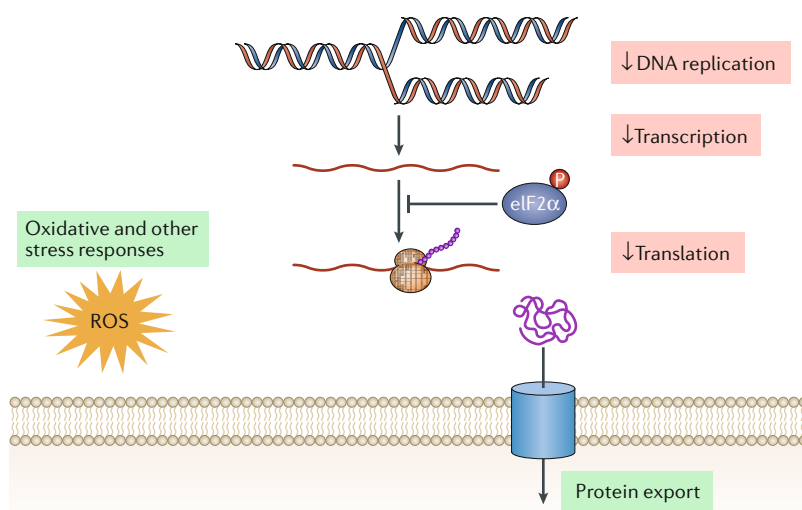


Fig. 5 | Metabolic changes in protozoan persisters. Currently, our knowledge is limited regarding metabolic changes across protozoan persister-like cells. Studies have shown that in *Plasmodium* spp. and *Toxoplasma gondii* DNA replication, general transcription and protein synthesis are decreased in persister-like cells. However, some biochemical pathways are sustained, such as protein export in *Plasmodium cynomolgi* hypnozoite forms and also oxidative and other stress responses in *Plasmodium* and *T. gondii* bradyzoites. In addition, in purine-depleted culture conditions, *Leishmania donovani* non-proliferative promastigotes exhibit decreased DNA replication and repair and protein synthesis, whereas proteins associated with some oxidative stress response pathways are increased. Finally, in apicomplexan protozoa, phosphorylation of parasite eukaryotic initiation factor 2 α (eIF2 α) has been implicated in the establishment of dormancy (possibly by inhibiting global protein synthesis), although the role of eIF2 α phosphorylation in the formation of persister-like cells in kinetoplastid protozoa has yet to be investigated. ROS, reactive oxygen species.

replicating cells. It may also be possible to use current drugs in different ways; for example, pulsing therapy at intervals that mean reactivated persisters that emerge are confronted with a drug to which they are sensitive. Alternatively, small molecules that trigger reactivation could resensitize the parasites to currently used drugs.

Finally, the identification of small molecules that modulate dormancy will also provide new tools for understanding the molecular basis of persister phenotypes.

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Author contributions

M.P.B. researched data for article and provided substantial contribution to discussion of the content. M.P.B., D.E.K., L.D.S., J.B.R. and R.L.T. wrote the article and reviewed/edited the manuscript before submission.

Competing interests

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