Chapter 1

Review of Literature on Leishmaniasis: Introduction, Current Scenario, and Future Prospects

Abstract

Despite its classification as a neglected tropical illness, leishmaniasis presents a significant socioeconomic challenge globally, characterized by its high rates of mortality and morbidity. With no available vaccines and the growing resistance of current chemotherapeutic treatments, options for managing this disease remain limited. This underscores the critical need to identify parasitic pathways that can be targeted to address this neglected tropical disease. Understanding the life cycle of leishmania, particularly the adaptive mechanisms of the amastigote stage is imperative. Studies indicate that lesion amastigotes enter a semi-quiescent state to evade host cell microbiocidal processes and exhibit a stringent response. Their reliance on the TCA cycle to produce essential molecules such as glutamate, glutamine, and aspartate for nucleotide, thiol, and amino sugar synthesis is crucial for survival. Without these, the parasite cannot persist. This study focuses on citrate synthase, the first enzyme of the TCA cycle, which plays a pivotal role in sustaining the cycle, generating ATP through oxidative phosphorylation, and facilitating fatty acid and cholesterol synthesis in the cytosol. Beyond its functional significance, citrate synthase exhibits significant sequential and structural differences from its human counterpart. This project aims to develop an alternative chemotherapy for leishmaniasis by circumventing current limitations and targeting citrate synthase as a pharmacological intervention point.

Keywords: Leishmaniasis, Leishmania, TCA cycle, Citrate synthase, and Chemotherapeutics.

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1.1 Introduction

Infectious diseases rank as the third leading cause of death globally, with many falling under the category of neglected diseases. Leishmaniasis, a neglected tropical disease, holds a significant position following malaria, according to the World Health Organization (Sangshetti et al., 2015). It is transmitted by various species of protozoan parasites from the order Kinetoplastidae, family Trypanosomatidae, and genus Leishmania through the bite of infected phlebotomine sand flies of the genera Phlebotomus and Lutzomyia (Irshad, 2022). These parasites undergo a digenetic life cycle, beginning as motile, flagellated promastigote forms in the gut of sand fly vectors. Upon infecting mammalian hosts, they transform into non-motile, non-flagellated amastigote forms, surviving and multiplying within the phagolysosomal compartments of macrophages (Sasidharan and Saudagar, 2021). Leishmaniasis manifests in three main clinical forms: Visceral (VL), Cutaneous (CL), and mucocutaneous leishmaniasis (MCL), each varying in immune-pathologies, morbidity, and mortality rates. This disease is widespread across tropical and subtropical regions, spanning 98 countries in Europe, Africa, Asia, and the Americas. An estimated 12 million individuals worldwide are infected with various Leishmania species, with approximately 350 million individuals residing in endemic areas at risk of infection, posing a significant global concern. The surge in leishmaniasis cases is attributed to socio-economic factors, demographic shifts, migration, urbanization, deforestation, therapy resistance, inadequate healthcare coverage, and vector control strategies (Arenas et al., 2017). There are no effective vaccines available against these diseases. One of the significant reasons is inadequate knowledge of the precise immune mechanism to control parasite growth. The complete control of disease is based on chemotherapy, but it also has several drawbacks, such as increased drug resistance, cost, adverse side effects, and efficacy [Singh et al., 2012]. Thus, searching for alternative drugs against the parasite is required. To advance the development of new drugs, we must focus on a parasitic component essential for

survival and characterize the point where rational drug design leads to targeting the inactivation of these components and their activities. It is necessary to focus on the Leishmania life cycle, especially the amastigote stage and its adaptive mechanisms [Ranjan and Dubey, 2023a]. The studies indicate that lesion amastigote enters a semi-quiescent state and shows a stringent response to evade the activation of host cell microbiocidal processes. The 13C tracer studies show that they preferentially utilize sugar for growth and development. Most of the endolysosomal system compartments of macrophages, except the phagolysosome compartment, contain a low luminal concentration of sugars [Saunders et al., 2014]. Amastigotes rely on the Tricarboxylic acid (TCA) cycle to produce glutamate, glutamine, and aspartate, which are needed for nucleotide, thiol, and amino sugar production. In addition to amino sugars, amastigotes use fatty acids as a substantial carbon source. The complete inhibition of amastigote growth and survival upon inhibition of the TCA cycle enzymes responsible for synthesizing these amino acids implies that it is necessary for the amastigote stage of the Leishmania life cycle [McConville et al., 2015]. Here, we have focused on Citrate synthase, the first TCA cycle enzyme involved in the TCA cycle continuation and ATP production in mitochondria and fatty acid & cholesterol synthesis in the cytosol. It also functions as an inflammatory signal and plays a crucial role in regulating immune cell metabolism. This enzyme has substantial sequential and structural differences with human citrate synthase. In this study, we want to explore this enzyme as a possible drug target against leishmaniasis and find a few FDA-approved inhibitors through a drug repurposing approach that could become key players in the fight against this illness.

1.2 Leishmania: the discovery

In ancient human history, few reports exist on the occurrence of leishmaniasis by different names like Oriental sore and Nile pimple [Frías et al., 2013]. However, from the 16th century onwards, several skin infections that seemed to be Oriental sores were reported from different

parts of the Middle East, and these infections were named based on places from where they first appeared [Steverding, 2017]. The Scottish physician and naturalist Alexender Russell (1715–1768) provided comprehensive information regarding the dry and wet types of Oriental sores in 1756 and mentioned that without treatment, these conditions could be cured in eight months to a year. Additionally, he mentioned that the most effective plaster against this sickness was mercurial. The locals referred to these forms as the disease's male and female forms. *L. major* induced the wet zoonotic CL, while *L. tropica* generated the dry anthroponotic CL. The first documented report of MCL was written by the Spanish chronicler Pedro Pizarro (1515-1602) in 1571, who described how coca producers on the eastern slopes of the Peruvian Andes suffered from lip and nose deformities [Lainson, 2010]. There have been no credible reports concerning VL until the 19th century. In 1832, military surgeon William Twining (1790–1835) published a book that gave more detailed information regarding the signs and symptoms of the kala-azar. However, in 1824-1825, the first kala-azar outbreak was reported in the village of Mahomedpore, thirty miles east of Jessore, in Lower Bengal, India [Gibson, 1983]. From there, the disease spread westward till it arrived in Burdwan, West Bengal, in 1860. After that, it became an epidemic and moved north of Bengal and Assam in the ensuing years. The mortality of kala-azar patients in the impacted areas was approximately thirty percent [Gibson, 1983]. This illness persisted to be endemic in numerous places for the ensuing decades. The word kala-azar was first coined in the late 19th century, and it literally means ‘black disease.’ The term "kala-azar" alludes to the grayish discoloration that occurs on the skin of individuals with light complexion when the disease progresses. At the end of the 19th century, efforts were made to identify the causative agent that causes the various types of leishmaniasis. The Scottish pathologist William Boog Leishman (1865–1926), who was serving in the British army in India, found the ovoid corpses from the smears of the dead soldier with splenomegaly and emaciation in November 1900 in Dum-Dum, a town close to Calcutta.

In addition, he discovered comparable types of bodies from the experimentally infected white rat. He published his results in 1903 and proposed that the ovoid bodies were degenerated trypanosomes, and trypanosomiasis was the cause of the notorious "Dum-dum fever" (Leishman, 1903). A few weeks later, the Irish physician Charles Donovan (1863-1951), a physiology professor at the Madras Medical College, also found identical ovoid bodies as Leishman from the splenic sample infected with remittent fever and enlarged spleens (Donovan, 1903). Afterward, In November 1903, the British physician Ronald Ross (1857-1932), who was authorized to investigate the kala-azar in India, published a paper and concluded that the ovoid bodies found by Leishman and Donovan were a unique protozoan organism rather than a degenerated trypanosome and suggested the scientific name *Leishmania donovani* [Leishman and Ross, 1903]. Following that, several other species of Leishmania, such as *Leishmania tropica,* were discovered in 1903 by American pathologist James Homer Wright (1869–1928); *Leishmania infantum* in 1908 by the French bacteriologist Charles Jules Henery Nicolle (1866–1936) and *L.tropica* and *L.major* by Bray in 1973 based on their difference in morphologies [Bray, 1973] were reported. Some of other New World Leishmania species that have been identified as causing agents of CL and MCL are *L. mexicana* (1953), *L. guyanensis* (1954), *L. amazonesis,* and *L. panamensis* (1972), *L. venezuelensis* (1980), *L. lainsoni* (1987), *L. naffi* & *L. shawi* (1989), *L. lindenbergi* (2002), and *L. waltoni* (2015) (Lainson, 2010), (Shaw, 2015). Currently, 53 species have been identified, while several are still up for debate. However, 20 species among them can infect people and cause leishmaniasis [Sasidharan and Saudagar, 2021].

The British-Israeli parasitologist Saul Adler (1895–1966) provided conclusive evidence that sand flies were the kala-azar vector in 1941 [Adler and Ber, 1941]. According to Maroli (2013), leishmaniasis has been linked to 42 Phlebotomus species and 56 Lutzomyia species in the Old and New Worlds, respectively, up to this point [Maroli et al., 2013].

1.3 Life-cycle of Leishmania

Leishmania has a digenetic life cycle that cycles between two hosts, Insect and Mammals. Sandflies from the Phlebotomus genus in Old World Leishmania and the Lutzomyia genus in New World Leishmania species are the insect vectors for leishmaniasis. The leishmania parasite undergoes morphological differentiation, forming promastigote and amastigote each time, which alternate between the host and the vector. According to Figure 1.1, the leishmanial amastigote is first picked up by sandflies during their consumption of blood meals from the diseased host. The amastigotes inside the sandfly developed into procyclic promastigotes in the midgut of the animal, which after that transformed into non-dividing nectomonad promastigotes. Additionally, the leptomonad promastigotes differentiated into metacyclic promastigotes and proceeded to the sandfly proboscis, after that, they prepared themselves to be transmitted to the mammalian host in the event of a subsequent bite. This life cycle stage, known as metacyclogenesis, occurs over seven to ten days. During the subsequent blood meal, the infected sandflies inject the infective metacyclic promastigote and their saliva into the host. The promastigotes attach to the plasma membrane, initiate the phagocytosis processes, enter the macrophages, and infect the parasitophorous vacuole. Within this vacuole, they differentiate into the amastigote form and begin multiplying by binary fission, increasing in number until the macrophages burst and all the mature amastigotes are released, reinfecting the other phagocytic cells and repeating the cycle. This phase typically lasts a month [Sasidharan and Saudagar, 2021]. In addition to mammals, the parasite also infects various hosts, including dogs, cats, foxes, jackals, and rodents. These mammals act as a "reservoir" host as they cannot directly infect people. They also needed sandflies as a transmission intermediary.

1.3.1 Promastigote stage: The promastigotes are flagellated and motile forms of the parasite with a narrow body shape between 15 to 20 μm long and 1.5 to 3.5 μm wide. The flagellar Length ranges from 15 to 28 μm, as shown in Figure 1.2, which aids the parasite in attaching

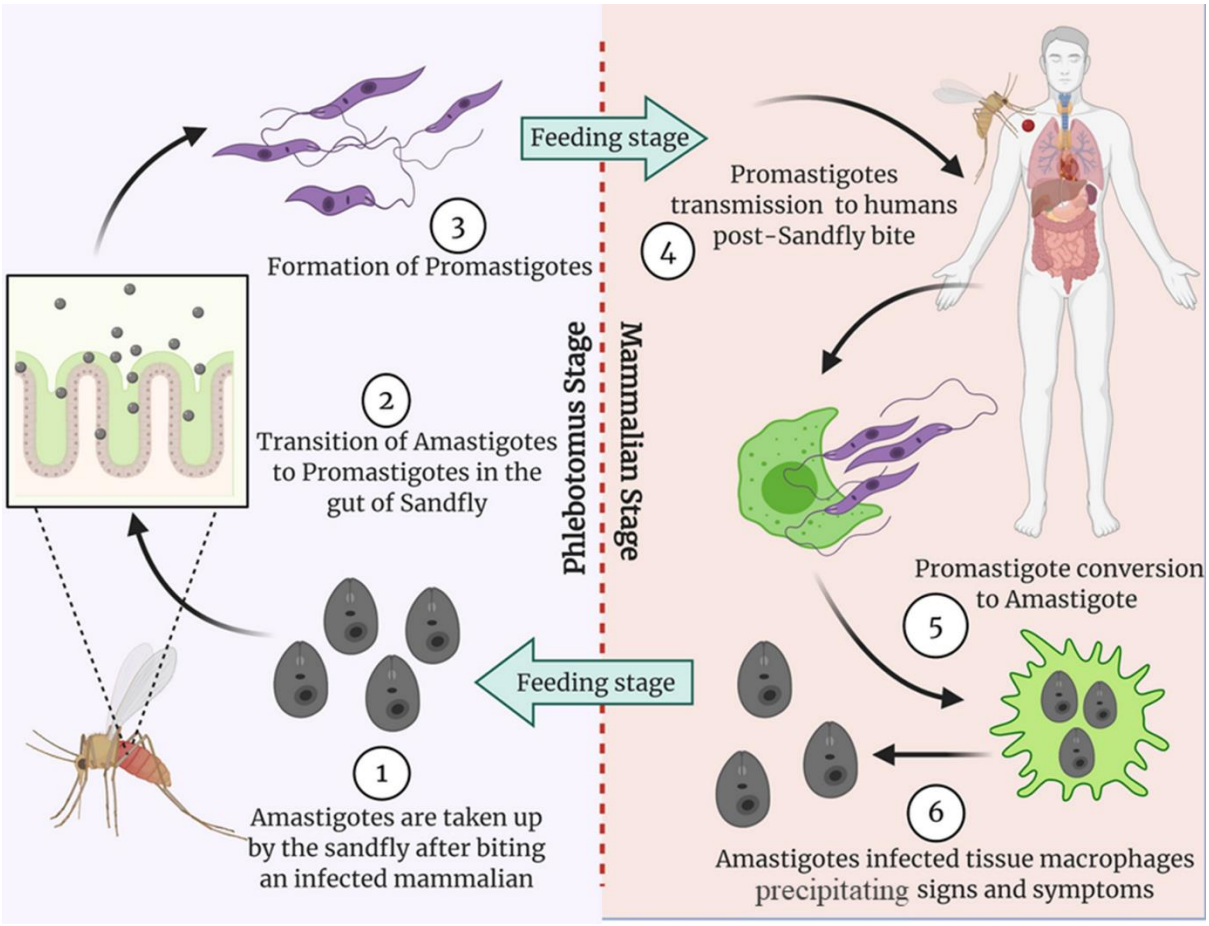


Figure 1.1: Life Cycle of Leishmania and its stages; (1) Sandfly acquire amastigotes from infected mammalian host; **(2)** Amastigotes conversion into the promastigotes in sandfly gut; **(3, 4)** Transmission of promastigotes from sandfly to the healthy mammalian host by sandfly bite; **(5, 6)** Promastigotes conversion to amastigotes which infect the macrophages and induce a symptomatic condition. (**Adopted from Jamshaid et al., J Nanobiotechnol, 2021, 19, 106)**

itself to the gut of the sandfly.

1.3.2 Amastigote stage: The amastigotes are non-flagellated and non-motile forms of the parasite with an oval body shape measuring 3 to 6 μm in length and 1 to 3 μm in width. It lacks external flagella, as shown in Figure 1.2, and is present in the mononuclear phagocytes and the circulatory systems of humans.

1.4 Types of Leishmaniasis

Leishmaniasis, according to clinical manifestations, depends on the parasite's characteristics and on the genetic aspects of the host that determine the effectiveness of the immune response,

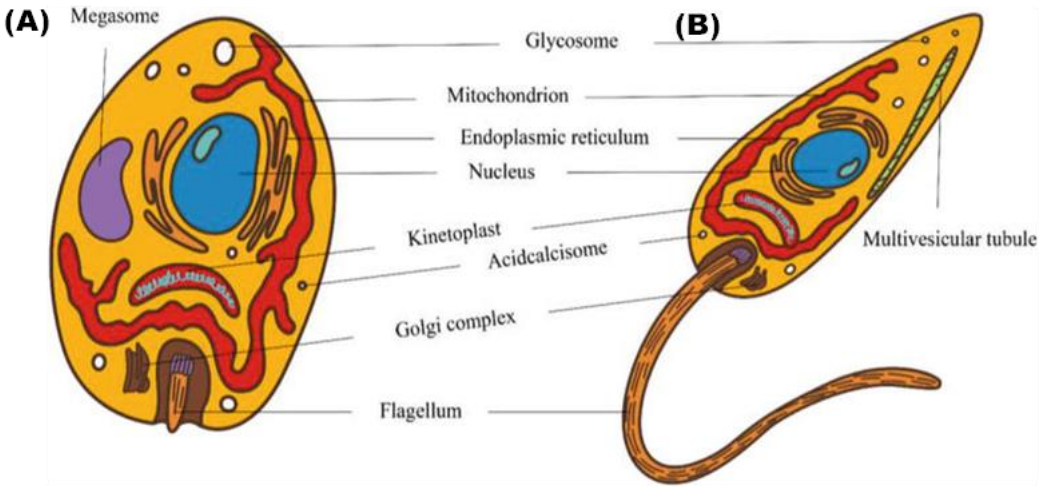


Figure 1.2: Life stages of Leishmania (A) Ultrastructure of Amastigote stage of Leishmania species reside within the human macrophages. (B) The Ultrastructure of the Promastigote stage of Leishmania species resides inside the midgut of the sandfly. **(Adopted from Gabrille et al., Leishmaniasis- General Aspects of a Stigmatized Disease, Intech open, 2021).**

which is divided into three types. 1) Cutaneous (Localized and disseminated), 2) Mucocutaneous, and 3) Visceral or Kala-azar

1) Cutaneous Leishmaniasis

This form of leishmaniasis is characterized by skin ulcers on the body's exposed parts, such as the face, arms, and legs. It is of two types.

1) Localized cutaneous leishmaniasis

This type of leishmaniasis is characterized by a local increase in temperature and swelling, which initially starts as a papule at the bite site and further rises in size as crusts and then ulcerates, as shown in Figure 1.3 (A). In 90% of the cases, it may take 3 to 18 months to heal. In old-world cutaneous leishmaniasis, the incubation period lasts from 2 weeks to several Months, and in some cases, it may take up to 3 years, whereas, in the case of new-world cutaneous leishmaniasis, the incubation period usually takes 2 to 8 weeks [Piscopo and Azzopardi, 2007]. It occurs in the exposed part of the body, such as ears, especially the helix, and the anti-helix areas, nose, upper lip, cheeks, legs, hands, forearms, and ankles. It is caused

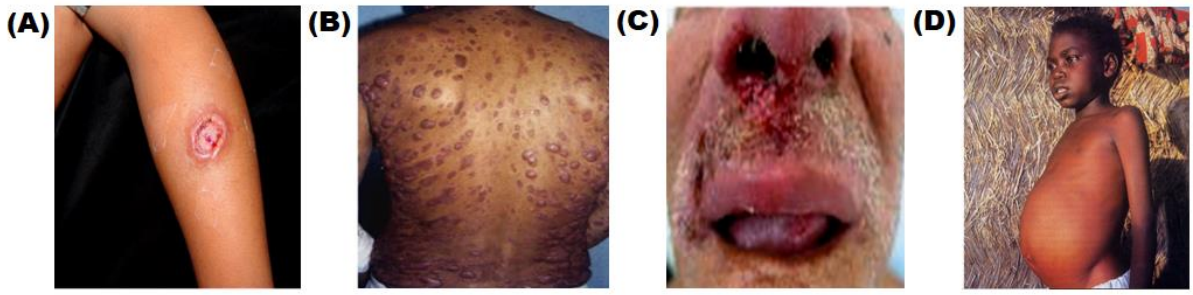


Figure 1.3: Different types of Leishmaniasis. (A) Localized cutaneous Leishmaniasis **(B)** Diffused cutaneous Leishmaniasis **(C)** Mucocutaneous Leishmaniasis(**Adopted from Torres-Guerrero et al., F1000 Research, 2017, 6 (F1000 Faculty Rev),750) (D)** Visceral Leishmaniasis (**Adopted from Opperdoes, 2004**)

by *L.major, L.braziliensis, L.tropica, and L.panamensis*.

II) Diffuse cutaneous leishmaniasis

This type of leishmaniasis is caused by poor cell-mediated immune responses allowing dissemination through tissue, lymph, and blood pathways, developing lesions in most skin except the scalp, as shown in Figure 1.3 (B). It is observed in Central America, Amazonian Brazil, Venezuela, Ethiopia, and Kenya and is caused by several leishmania species such as *L.mexicana, L.amazonensis, and L.braziliensis* [Arenas et al., 2017].

2) Mucocutaneous Leishmaniasis

This form of leishmaniasis is characterized by invasion and destruction of the nasopharyngeal mucosa region, as shown in Figure 1.3 (C). These cases occur in South America, Bolivia, Brazil, and Peru caused by leishmania species such as *L.braziliensis, L.guyanensis,* and *L.panamensis* [Davies et al., 2000].

3) Visceral Leishmaniasis

This form of leishmaniasis is characterized by lymphadenopathy, hepatomegaly, splenomegaly, pallor, anemia, leukopenia, thrombocytopenia, fever, night sweats, weakness, anorexia, asthenia, cutaneous pigmentation, and weight loss, as shown in Figure 1.3 (D). This illness, also known as kala-azar (black fever), spread over a large part of South and East Asia, Africa, the Mediterranean, and South America. It is caused by *L.donovani* (India and Eastern Africa),

L.infantum (Mediterranean area), *L.chagasi, L.amazonesis,* and *L.tropica* in South America. The incubation period of this disease is 3 to 8 months; if left untreated, it can cause death. The people who are at risk of this disease are immunocompromised and undernourished individuals [Arenas et al., 2017].

4) Post-Kala-azar dermal leishmaniasis

It is a skin manifestation that is the sequel of visceral leishmaniasis and appears as a macular, papular, or nodular rash, usually on the face, upper arms, and trunk. It occurs in East Africa and the Indian subcontinent, where 5-10% of kala-azar-infected persons reported this condition. It usually appears 6 months to 1 year after infection, which was previously cured.

1.5 Distribution of leishmaniasis (worldwide & India)

Although Leishmaniasis is classified under the umbrella of neglected tropical disease according to the World Health Organization (WHO) report, it continues to be a significant health problem in four eco-epidemiological regions of the world: the Americas, East Africa, North Africa and West and South-East Asia. According to the WHO 2022 report, out of the 200 countries and territories, 90 countries were considered to be endemic for cutaneous leishmaniasis (CL), and 80 were considered to be endemic for visceral leishmaniasis (VL). According to data, 71 out of 200 countries were endemic to CL and VL. The regions that are reported to be endemic to both cutaneous and visceral leishmaniasis are American regions (AMR), African regions (AFR), Eastern Mediterranean region (EMR), and South-East Asia region (SEAR). Very few cases are found in the European region, and there are no cases in the Western Pacific region. According to the report, in 2022, 205986 new cases of CL and 12842 new cases of VL were found. 94% of the new CL cases were reported from EMR and AMR regions. The EMR and Algeria constituted an eco-epidemiological “hotspot” region, reporting 79% of all CL cases. The eight countries, such as Afghanistan, Algeria, Brazil, Colombia, Iran, Iraq, Peru, and the

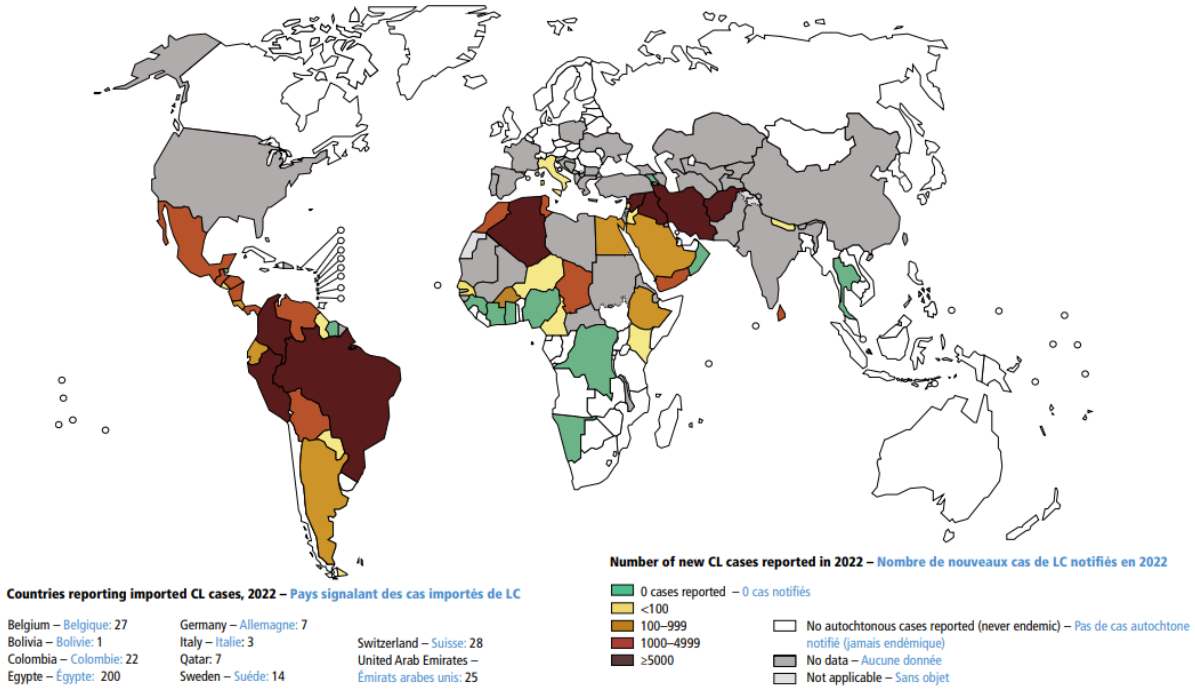
Syrian Arab Republic, reported more than 5000 CL cases, as shown in Figure 1.4 (A) [Ruiz-postigo et al., 2023].

44% of the new VL cases were reported from EMR, followed by 33% cases of AFR and 14% and 8% cases. The eco-epidemiological hotspots region considered for VL is East Africa (Eritrea, Ethiopia, Kenya, Somalia, South Sudan, Sudan, and Uganda) with 72% of the total cases, followed by Brazil with 13% and the Indian subcontinent (Bangladesh, India and Nepal) with 8% of total cases. There are four countries such as Brazil, Kenya, South Sudan, and Sudan, which report more than 1000 VL cases, and there are 11 countries such as Eritrea, Ethiopia, India, Nepal, Somalia, Uganda, and Yemen which report 95% of all cases worldwide as shown in Figure 1.4 (B) [Ruiz-postigo et al., 2023].

In India, an estimated 165.4 million population are at risk of visceral leishmaniasis, and there are four states, i.e., Bihar (33 districts, 458 blocks), Jharkhand (4 districts, 33 blocks), West Bengal (11 districts, 120 blocks), and Uttar Pradesh (6 districts, 22 blocks) which are endemic to visceral leishmaniasis. India has reported 810 cases and 39 deaths of visceral leishmaniasis in the year 2022. The majority of visceral leishmaniasis cases and deaths were found in Bihar, although it is decreasing in a time-dependent manner [Kumar Mahto et al., 2023].

India has stepped up multiple efforts in the eradication of visceral leishmaniasis. It has strengthened a widespread network of comprehensive primary healthcare facilities, prioritizes health education, and has established a national health strategy to attain universal health coverage [Kumar Mahto et al., 2023].

(A)



(B)

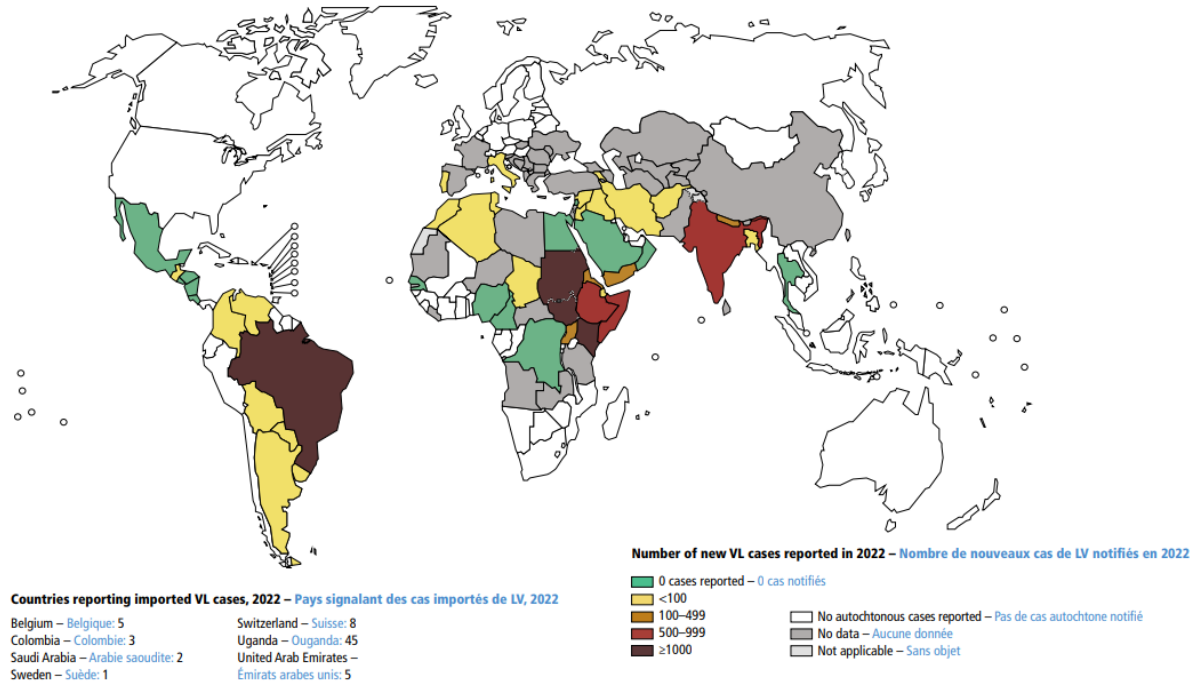


Figure 1.4: Worldwide distribution of Cutaneous & Visceral Leishmaniasis (A) Imported & new cases of Cutaneous leishmaniasis & **(B)** Imported & new cases of Visceral leishmaniasis. **Data source: World Health Organization**

1.6 Risk factors associated with Leishmaniasis

There are multiple risk factors associated with leishmaniasis. The major one is shown below, with other risk factors in Table 1.1.

1. Socio-economic condition

Leishmaniasis, especially the visceral form, basically affects economically weak people and marginalized societies who are forced to stay close to water resources, in humid houses, and in the vicinity of accumulated rubbish, sewerage, and farms of livestock [Boelaert et al., 2009]. These places are risk factors contributing to the growth and multiplication of sandflies. The risk of disease in those individuals is very high who are living in close contact with asymptomatically infected individuals and also living in crowded and poor communities [Picado et al., 2014].

2. Malnutrition

People having diets lacking protein, iron, vitamin A, and zinc are more prone to risk to infection [Oryan and Akbari, 2016].

3. Population Mobility

The increased number of travels from tropical and subtropical regions and the immigration of dogs have significantly increased the incidence of this disease in non-endemic countries. These people have more infection risk than natural people [Mettler, 2005].

4. Environmental and Climatic change

The prevalence and progression of leishmaniasis are intricately linked to environmental factors and natural phenomena. Forest fragmentation, climatic variations, vegetation coverage, and land surface temperatures are significant determinants that influence the proliferation and abundance of sandflies, consequently contributing to the outbreak of leishmaniasis. The conversion of forests into alternative forms of land use enhances the habitat for reservoir hosts such as dogs, foxes, and rodents, thereby amplifying the prevalence of leishmaniasis and the

Table 1. 1: Risk factors of emergence and re-emergence of leishmaniasis and their effects. (Adopted from Oryan et al., Asian Pac. J. Trop. Med, 2016, 9, 10, 925-932)

Risk factors	Effect
Organ transplant	Prescribed immunosuppressive drugs prevent T-cell activation and indirectly defense mechanisms against intracellular parasites.
Travel to endemic areas	Increasing travel to tropical and subtropical countries and dog importation to non-endemic regions increases the risk of leishmaniasis.
Canine leishmaniasis	Transportation of dogs from canine leishmaniasis endemicity region to non-endemic region causes venereal and transplacental transmission in non-endemic areas dogs.
Leishmania/HIV co-infections	The prevalence of HIV and existing leishmania in discarded syringes of addicted persons increases the incidence of visceral leishmaniasis in adults in addition to children.
Climate change	Temperature and environmental variation cause the abundance of sandflies.
Household-level	The design and type of construction material of the house and the presence of domestic animals increase the abundance of sandflies and the incidence of leishmaniasis.
Social condition	The worse socio-economic status of people in marginalized societies and proximity to water bodies, rubbish, sewerage, and farms increase the exposure of sandflies.
Environmental factors	Deforestation, climatic, and temperature changes increase the growth of sandflies.

populations of sandfly vectors (Patz et al., 2000; Gebre-Michael et al., 2004).

1.7 Current therapeutic approach and their drawbacks

Currently, there are a few existing drugs, such as pentavalent antimonials, pentamidine, various formulations of amphotericin B, paromomycin, and miltefosine, which are in use for the treatment of different forms of leishmaniasis with several drawbacks. Detailed information related to pre-existing drugs is provided in Table 1.2.

1. Antimonials

Pentavalent antimonials (SbV) have been used as first-line chemotherapy in the form of stibogluconate since 1945. Earlier, it was used as trivalent antimony (Sb^{+3}) against the treatment of VL, which was later replaced by pentavalent antimony, also called urea stibamine by Brahmachari as it shows less toxicity compared to Sb^{+3} and effective against VL in India [Haldar et al., 2011]. Basically, the pentavalent antimony functions as a prodrug that transforms into its trivalent form during the action against the parasite. Although the drug's exact mode of action is unknown, DNA topoisomerase I may be inhibited. The drug preferentially accumulates intracellularly in the parasites through modification of the aqua glyceroporin AQP1 gene transporter, which results in excess production of thiols and overexpression of ABC transporters [Marquis et al., 2005];[Légaré et al., 2001]. Glucantime® and Pentostam® are two distinct formulations of pentavalent antimony with about 90% efficacy each [Wortmann et al., 2002]. The reason for the restricted use of this drug includes severe adverse effects such as cardiotoxicity, pancreatitis, hepatotoxicity, and nephrotoxicity, as well as the establishment of resistance and treatment failures in subcontinent countries [Singh et al., 2023].

2. Amphotericin

Amphotericin B (AmB) is a polyene antifungal compound produced by the fermentation of *Streptomyces nodosusparenteris* and is used to treat leishmaniasis in antimonials-resistant endemic regions. It is presently the 1st line drug for VL treatment. This medication affects the

ergosterol in plasma membranes, changing their permeability and resulting in intracellular component leakage, ion balance changes, and, ultimately, cell death [Ramos et al., 1996]. The success rate of this medication is more than 90%, and it is advised for use by pregnant women and HIV; nevertheless, it is also quite toxic and expensive [Singh et al., 2023]. Compared to non-liposomal amphotericin B, lipid formulations of the drug, such as AmBisome ®, Amphocil ®, and Abelcet ®, are less toxic and more effective. The main limitation of this formulation includes its expensive price, mode of administration, and inability to withstand high temperatures [Singh et al., 2012].

3. Miltefosine

Miltefosine, an alkyl phosphocholine derivative, was initially identified as an antineoplastic medication for treating cutaneous tumors. However, it is currently regarded as a reliable chemotherapeutic drug against leishmaniasis [Dorlo et al., 2012]. This medication works by interfering with the architecture of the cell membrane by impeding the metabolism of phospholipids by inhibiting the formation of phosphatidylcholine and phosphatidylethanolamine by lowering choline levels [Rakotomanga et al., 2007]. It is the only medication taken orally to treat leishmaniasis with an efficiency index of 95% in a clinical trial in India. The major drawback of this medication is the lengthy half-life in the organism (>120 h) and its tetragenicity. The efficacy of miltefosine in murine models varies depending on the leishmania species [Singh et al., 2023].

4. Pentamidine

Pentamidine is an aromatic diamidine used as a second-line drug in the antimonial-resistant region, but its use was forfeited due to increasing resistance and toxicity. The mechanism of action of this drug is inhibition of polyamine biosynthesis and the disruption of membrane potential. The significant side effects of Pentamidine are hypotension, diabetes mellitus, and renal impairment [Le Pape, 2008].

5. Paromomycin

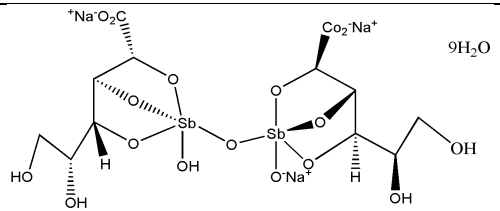
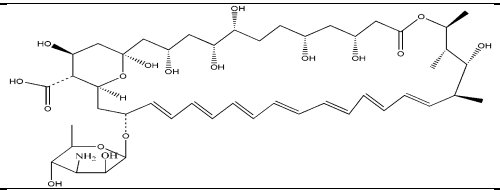
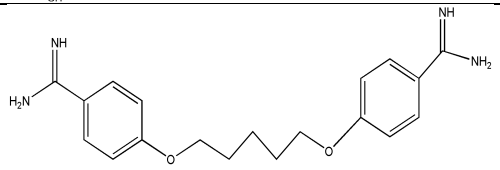
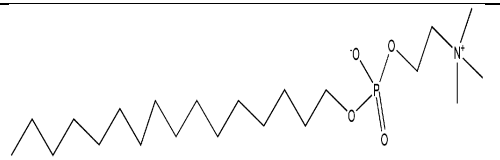
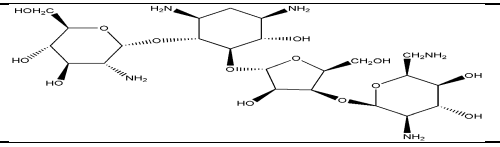
Paromomycin, a broad-spectrum aminoglycoside antibiotic produced from culture filtrates of *Streptomyces krestomyceticus,* has been used to treat Leishmaniasis (VL and CL). It is more effective in the treatment of CL. Paromomycin is available as an intramuscular injection to treat systemic infection (i.e., VL) and as an ointment formulation to treat local skin infections (i.e., CL). It impairs the mitochondrial membrane potential, inhibits protein synthesis, and leads to respiratory dysfunction. It also alters membrane fluidity and lipid metabolism [Freitas-Junior et al., 2012]. A dose of 16 mg/kg body weight is required daily for 21 days. The side effect of this drug is elevated hepatic transaminases, pain at the injection site, nausea, abdominal cramps, and diarrhea [Sangshetti et al., 2015].

1.8 The current problem associated with Treatment failure

1. Emergence of drug-resistant Leishmania species

The emergence of drug-susceptible leishmania species, which may be caused by both inherent and acquired drug resistance, is one of the particular causes of treatment relapse. When a parasite lacks an initial pharmacological reaction, it is said to have inherent resistance, and when it has acquired resistance after being exposed to the same medicine numerous times, it is said to have acquired resistance [Hendrickx et al., 2018]. To overcome these problems, no validated standard operating procedure is available to define species-specific breakpoint concentrations of drugs. Over the past years, multiple antileishmanial drug-resistant cases have been reported, increasing with time. The most familiar example is antimony (SbV) resistant cases in India, which have risen even in Nepal and Bangladesh. It enforced the use of another antileishmanial drug, such as Miltefosine, and a liposomal formulation of Amphotericin-B. However, Miltefosine and Amphotericin B-resistant cases have surfaced in a few regions.

Table 1. 2: Currently available antileishmanial drug and their related information. (Adopted from Ranjan et al., Life Sciences, 2023, 322, 121314)

Drug	Structure	Route of Administration with dosage regimen	Mechanism of action	Toxicity/ Side effects	References
Pentavalent antimonials		Intramuscularly or intravenously (20 mg/kg daily for 20-30 days)	Conversion of Pentavalent form into trivalent form (the lethal and active form of the drug). Inhibition of glycolysis, fatty acid β-oxidation, phosphorylation of ADP, Trypanothione reductase, and TCA cycle inside amastigote.	Cardiac arrhythmias, ventricular tachycardia, ventricular fibrillation, pain at the site of injection, gastrointestinal problems, and stiff joints	[Sangshetti et al., 2015]; [Freitas-Junior et al., 2012]; [Jardim et al., 2018]
Amphotericin B and Liposomal amphotericin B		Intravenously by infusion (1 mg/kg alternatively for 30 days)	Bind to the ergosterol component of the parasite cell membrane; this leads to changes in membrane permeability, leakage of intracellular components, alteration of ion balance, and, finally, cell death.	High fever with rigor and chills, Nephrotoxicity, thrombophlebitis, severe hypokalaemia, and even death	[Singh et al., 2012]; [Kumar et al., 2018]
Pentamidine		Intravenously or intramuscularly (2-4 mg/kg alternatively for 30 to 40 days.	Inhibition of polyamine biosynthesis and topoisomerase II of mitochondrial *kinetoplast*- DNA, membrane potential disruption.	myalgia, pain at the injection site, nausea, headache, numbness, hypotension, diabetes mellitus and renal impairment	[Singh et al., 2019]
Miltefosine		Oral (50-100mg/day for 28 days)	Inhibition of phosphatidylcholine biosynthesis, alteration of phospholipid & sterol composition, and calcium homeostasis.	Gastrointestinal disturbance, teratogenicity hepatotoxicity, and renal toxicity	[Sangshetti et al., 2015]; [Saudagar et al., 2014]
Paromomycin		Intramuscularly and topically (11 mg/kg daily for 21 days)	Inhibition of protein synthesis by binding to 30S subunit of ribosome. Respiratory dysfunction.	pain at the injection site, abdominal cramps, nausea, ototoxicity, and diarrhea	[Singh et al., 2016]; [Kumar et al., 2018]

The ABC transporters and conjugated thiol system influence the development of a resistant leishmanial strain. Antimony resistance is linked to the ABCC3 transporter, whereas pentamidine resistance is linked to the Pentamidine resistant protein-1 transporter. The P-glycoprotein & ATP-mediated efflux pump and the shorter medication residence time inside the parasite may also contribute to treatment failure [Croft et al., 2006].

2. Drug Toxicity

In addition to resistance emergence, the anti-leishmanial drug leads to toxicity, such as SbV, and causes renal failure, pancreatitis, and cardiomyopathy. Even the patient undergoing SSG treatment encounters pain at the injection site and feels nausea & vomiting [Nieto et al., 2003]. Amphotericin-B, despite its effectiveness, causes infusion-related problems and nephrotoxicity [Sundar et al., 2007]. Pentamidine even encounters a similar situation. Miltefosine is accompanied by gastrointestinal distress, hemolysis, and nephrotoxicity. Paromomycin (PR) is also associated with nephrotoxicity and vestibular and cochlear side effects [Davidson et al., 2009], [Aruleba et al., 2020].

3. Anti-Leishmanial drugs with meager GIT absorption

Except for Miltefosine, all of the anti-leishmanial medications now on the market can be injected. These are administered intravenously due to their all-around low gastrointestinal absorption and susceptibility to harm from the stomach's acidic pH [Jamshaid et al., 2021].

4. The other drug-related factor

I. Quality of drug

For a treatment to be successful, the quality of the drugs is crucial. Many fake and subpar medications, some of which are mislabelled, have subtherapeutic quantities of the active component or even have no active medication, are sold in endemic nations. The overall responsiveness of treatment is either diminished or eliminated as a result of these malpractices, which also raises the possibility of future drug resistance [Hendrickx et al., 2018].

II. Drug formulation and stability

The retention of drug efficacy and stability has become essential as the liposomal formulation of Amphotericin B, a highly effective antileishmanial drug, requires less than 25°C temperature to maintain its effectiveness and stability.

III. Pharmacokinetic and Pharmacodynamic Properties of anti-leishmanial drug

Knowing how the medicine functions at the target location and behaves in animal models and human patients can help define the drug's therapeutic efficacy. Otherwise, predicting its plasma concentration and the actual intracellular drug concentration to which the parasite is exposed will be challenging [Hendrickx et al., 2018]. Due to the parasite's deep cutaneous layer residence and the limited cutaneous absorption qualities of the antileishmanial medications currently on the market, treating CL can be challenging [Jamshaid et al., 2021].

5. Host-related factor

Immunosuppression is one of the risk factors for VL infection; due to this, HIV-positive people, children, and the elderly are more prone to infection than immunocompetent patients. The VL-HIV co-infections have been reported since the early 2000s in Brazil. The data from 2017 indicates that 8% of VL-HIV infections are from Latin America, and 95.3% of these cases are from Brazil. Currently, the endemicity of VL-HIV is most predominant in Eastern Africa [Wamai et al., 2020]. The factor behind these problems is a limited understanding of the precise immune mechanism to control parasite growth.

The emergence of drug resistance and the stated problem jeopardizes the chemotherapy-based Leishmaniasis control program. Thus, there is a need for pharmacovigilance as well as continued drug development. To advance the development of new drugs, we must focus on a parasitic component essential for survival and characterize the point where rational drug design leads to targeting the inactivation of these components and

their activities, which necessitated the need to focus on the Leishmania life cycle and their metabolism.

1.9 Outline of promastigote and amastigote metabolism

Leishmania promastigote form utilizes both glucose and amino acids as energy sources. The catabolism of these substrates involves both glycolysis and the TCA cycle linked with the electron transport chain. As this stage stays in the midgut of sandflies, there is evidence that it utilizes other sugar besides glucose. The glyoxylate cycle was absent in this stage, which means it does not serve fatty acid as the sole substrate for gluconeogenesis. In addition to carbon dioxide, its metabolism includes other end-products such as succinate, acetate, pyruvate, D-lactate, alanine, ammonia, and urea [Opperdoes and Michels, 2008]. Amastigote resides in the phagolysosomes, which have a low pH (~ 5.4) and hydrolases and NADH oxidase enzymes that produce an anti-microbial oxidative burst.

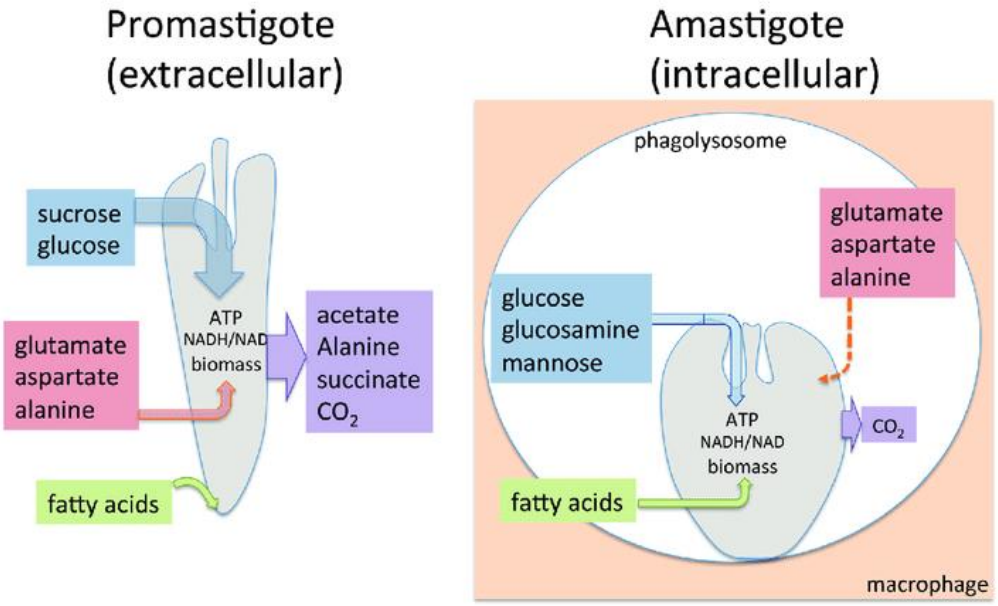


Figure 1.5: Central carbon metabolism of Leishmania promastigotes (insect stage) and amastigote (macrophage host). Promastigotes exhibit high absorption rates of glucose and amino acids, which are co-catabolized through the major pathways of central metabolism. Promastigotes also absorb fatty acids, which are not used as carbon sources, although primarily incorporated into membrane lipids (downward arrows). Amastigotes also preferentially use glucose as a carbon source but exhibit significantly reduced rates of uptake of sugar and amino acids as well as excess metabolism. Amastigotes also actively catabolize fatty acids in the TCA cycle as they absorb less glucose. (**Adopted from McConville MJ et al., F1000Research, 2015, 4, 938.)**

Aside from that, this highly dynamic compartment takes in a range of host macromolecules through fusion with vesicles from the phagocytic, autophagic, endocytic, and endoplasmic reticulum pathways, which are degraded by luminal hydrolases to release free sugars, lipids, and amino acids, that are then able to take up by amastigote plasma membrane transporters. Amastigotes can directly absorb a variety of host macromolecules and break many of them down using their own lysosomes. Leishmania is auxotrophic for several vital elements such as purines, vitamins, heme, and various amino acids that must be scavenged from the lysosome [McConville et al., 2015]. However, the studies show that when the host immune response strengthens, the number of parasites increases during the early stages of infection, plateaus, and then falls to a low level [Michel et al., 2011].

The metabolite profiling and 13C-stable isotope labeling studies suggested that the amastigote enters a metabolically quiescent state to shield itself from changes in pH, temperature, and other physiological changes. Compared to fast replicating or non-dividing promastigotes, the lesion amastigote started utilizing its resources more efficiently and drastically reduced its intake of glucose and amino acids. The stringent response is increased in lesion amastigotes as compared to axenic amastigotes. The sublethal quantity of reactive nitrogen species (RNS), which can inactivate many enzymes in the mitochondrial TCA cycle and respiration chain comprising iron-sulfur cluster, may impede amastigote proliferation in emerging lesions [Auger et al., 2011].

The amastigote may be shielded from an overabundance of nutrients by the stringent response. The excess nutrients cause metabolic stress, which increases the synthesis of NADH in the mitochondria, allowing electrons to escape the respiratory chain of the mitochondria and increasing the generation of endogenous reactive oxygen species (ROS). Leishmania is highly vulnerable to reductive stress because it cannot transcriptionally downregulate the TCA cycle enzymes that generate NADH [Teodoro et al., 2013].

Detailed 13C-tracer studies have shown that, although the lesion amastigotes have much lower uptake rates, they preferentially use carbohydrates as like promastigotes. Furthermore, amastigote uses fatty acids also as a significant source of carbon through β- oxidation of fatty acid, resulting in the production of acetyl-CoA, which is mainly used in the TCA cycle and gives glutamate, glutamine, and aspartate, which are intermediates for amino acid synthesis. These amino acids are necessary for synthesizing thiols, amino sugars, and nucleotides. The pharmacological inhibition of enzymes involved in the TCA cycle-mediated synthesis of non-essential amino acids totally inhibits amastigote growth and survival [Saunders et al., 2014].

1.10 Tricarboxylic acid cycle (TCA cycle): An antileishmanial drug target

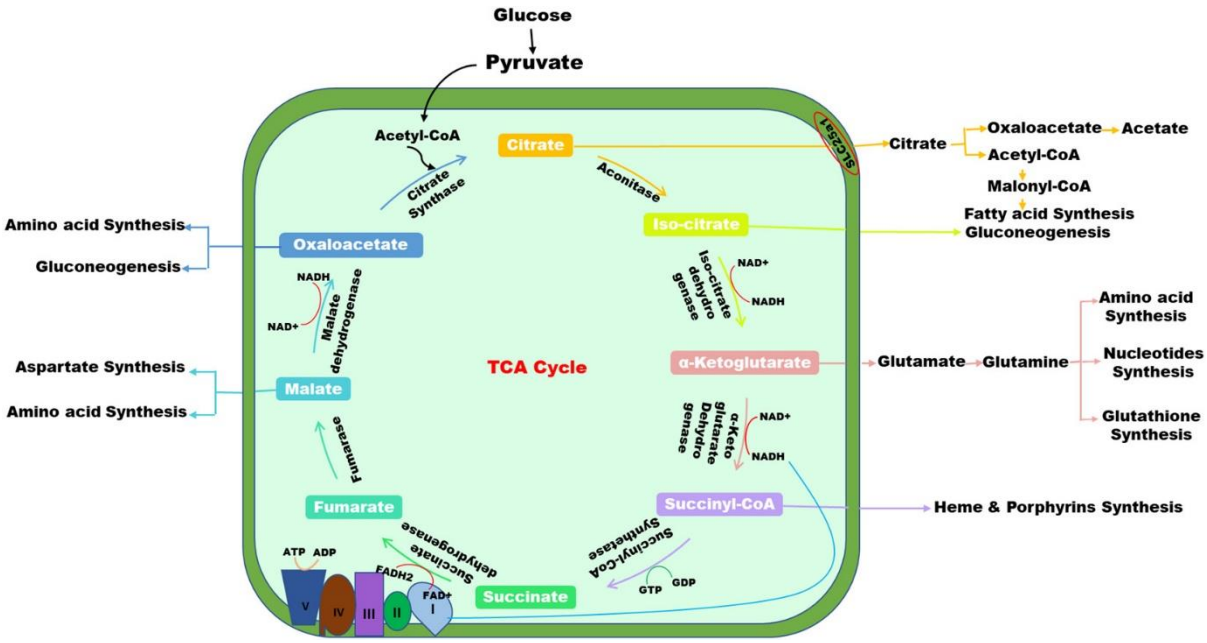


Figure 1.6: Biochemical role of the TCA cycle intermediates. (Adopted from Ranjan et al., Life Sci., 2023, 322, 121314.)

Most of the ATP generated in the metabolic processes is formed from the complete oxidation of fuel molecules, which take place inside the mitochondrial matrix and are known as Tricarboxylic acid (TCA), Krebs, or Citric acid cycles. It is a fantastic process that starts with sunlight's energy, which in the form of photons get captured in plants during photosynthesis and from where in the form of electrons get captured in the Krebs cycle and finally involved in

ATP generation by creating a proton gradient [Ryan and O'Neill, 2020]. It is the pathway where oxidation of all carbon sources such as carbohydrates, protein, and lipids occur, and mostly they enter inside the cycle in the form of acetyl-coenzyme A (acetyl-CoA). It is a central metabolic hub of the cells where both the cataplerosis and anaplerosis processes occur, which is essential for the functionality of the cycle [Owen et al., 2002]. In the Krebs cycle, acetyl-CoA oxidation to CO_2 occurs, which is a critical process in energy metabolism and is required for the generation of nicotinamide adenine dinucleotide (NADH) and Flavin adenine dinucleotide (FADH2). It acts as a shuttle to transfer a high-energy electron to O_2 in the ETC cycle [Owen et al., 2002]. In this cycle, as shown in Figure 1.6, a total of eight reactions occur. The cycle starts with the condensation of oxaloacetate and acetyl-CoA catalyzed by citrate synthase that forms citryl-CoA as an intermediate, further hydrolyzed into citrate and CoA [Remington, 1992]. The citrate isomerizes to isocitrate by aconitase with the formation of intermediate cis-aconitate [Lloyd et al., 2008]. The isocitrate is converted into α-ketoglutarate by isocitrate dehydrogenase (IDH) via an oxalosuccinate intermediate formation. In this step, the first molecule of NADH is formed with the loss of one carbon unit in the form of CO_2. The α-ketoglutarate is converted into succinyl-CoA with the help of the enzyme α-ketoglutarate/oxoglutarate dehydrogenase complex (OGDC). In this step, the second molecule of NADH is formed by deducting one carbon unit as CO_2. Succinyl-CoA synthetase (SCS) is involved in the cleavage of the thioester bond of succinyl-CoA and the formation of succinate. This step is connected with the phosphorylation of GDP [Johnson et al., 1998]. The succinate further converts into fumarate with the help of the succinate dehydrogenase enzyme and forms FADH2. The fumarate, with the enzyme fumarate hydratase, is converted into L-malate [Rose, 1998], which is further oxidized into oxaloacetate by the enzyme malate dehydrogenase, and finally, the third molecule of NADH is formed [Chapman et al., 1999]. Thus, the total output of this cycle is 3 NADH, 1 FADH2, and 1 GTP/ATP molecule.

However, there are several controversies related to this cycle in Leishmania. All the enzymes of this cycle are present in the genome and show good expression but have deplorable enzyme activities [Tielens and van Hellemond, 2009]. Some studies have even revealed that the TCA cycle does not occur in a cyclic but non-cyclic manner [Saunders et al., 2010]. However, the recent studies based on metabolite profiling and 13C isotope labeling experiments provide direct evidence for the complete operation of a TCA cycle in Leishmania promastigotes and suggest that the glycosomal and the mitochondrial metabolism are tightly coupled [Saunders et al., 2011]. A detailed study based on the 13C-isotope metabolic labeling experiment also shows that the TCA cycle is essential for the growth and survival of the amastigote stage of Leishmania [Saunders et al., 2014].

1.11 Citrate synthase

Citrate synthase is a component of nearly all living cells and plays a key role in the central metabolic pathway of aerobic organisms, the citric acid cycle, which functions as a source of two reducing equivalents for the electron transport chain and as a source of intermediates for biosynthesis of amino acid required for ketogenesis, lipogenesis, and gluconeogenesis.

Citrate synthase is an enzyme involved in the first step of the tricarboxylic acid cycle. It catalyzes the condensation of acetyl-coenzyme-A (CoA) and oxaloacetic acid to form citrate and CoA via an intermediate citryl-coenzyme A through an ordered bi-bi mechanism. It

oxaloacetate + acetyl-CoA + H_2O → citryl CoA → citrate + CoA

is the only enzyme in the TCA cycle that catalyzes a carbon-carbon bond formation. Citrate acts as a substrate for the citric acid cycle within the mitochondria and as an acetyl donor for synthesizing acetyl-Coenzyme A by ATP-citrate lyase outside the mitochondria after transport through the mitochondrial membrane. Citrate synthase occurs inside eukaryotes exclusively in the mitochondria; the exception is the germinating plants found in glyoxysomes and involved in the glyoxylate cycle. The mitochondrial citrate synthase is coded by nuclear DNA, which is

translated into the cytoplasm as a precursor and transported into the mitochondria, localized on the inner membrane [Wiegand and Remington, 1986]. Based on their oligomeric condition and an extra β-sheet at the N-terminal region, citrate synthase is classified into type-I and type-II. The type-I CS are homo-dimeric and are present in gram-positive bacteria, archaea, and eukaryotes, while the type-II CS are hexameric with an extra β-sheet on N-terminal region and are present in gram-negative bacteria [Lee et al., 2019]; [Ge et al., 2019].

1.11.1 Structure of citrate synthase

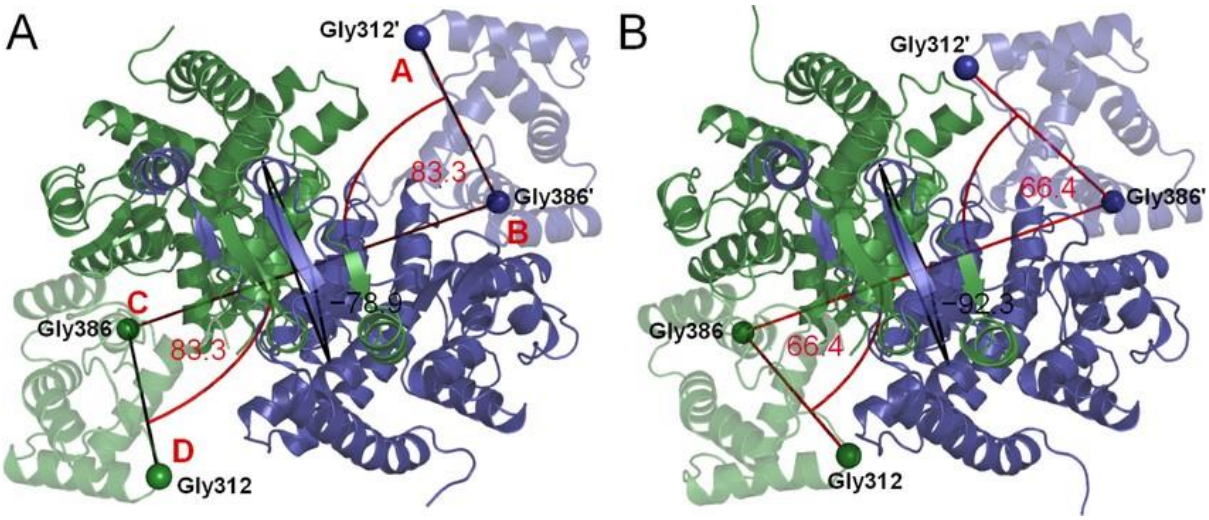


Figure 1.7: Crystal structure of Pig heart citrate synthase with labelled angle and dihedral measurements. Chain A is depicted in dark blue, and Chain B is depicted in dark green, whereas the small domain is depicted transparent. **A)** Open structure, **PDB ID: 3ENJ B)** Closed structure, **PDB ID: 2CTS (Adopted from Wells et al., PLOS One, 2015, 10(8): e0133372.)**

Animals, plants, gram-positive bacteria, fungi, and archaebacteria all include a homodimeric form of citrate synthase consisting of two identical monomeric subunits of 437 amino acid residues each. Each subunit comprises 20 alpha helices, which collectively form 75% of the protein structure and contain 13 residues long, one beta-sheet. Aside from that, the remaining residues are unstructured. Each subunit is subdivided into a large and a small domain. There are fifteen alpha helices in the large domain, while the remaining five are in the small domain.

Both subunits of citrate synthase have an active site that functions separately. The entire enzyme packs together to form dense globular molecules; the two subunits of the dimer interface with one another via four pairs of helices that twist together to produce a shape resembling a beta-sheet [Murakami and Kouyama, 2016]. Citrate synthase was subjected to crystallographic refinement by Remington et al., who identified two distinct conformation forms of the protein crystal structure based on differences in domain arrangement. The one termed “Open,” as shown in Figure 1.7 (A), has a tetragonal shape with a large cleft between the small and large domains that permit the substrate to reach the active site. The other, monoclinic and referred to as “closed,” is depicted in Figure 1.7 (B). The small domain's 18° inward rotation, which closes the fissure around the substrate, gives it the name "closed." According to Roycki and Cieplak (2014), oxaloacetic acid binding to the active site in the tetragonal form results in a conformational transition to the monoclinic form, which exposes the acetyl-CoA binding site and shields the substrate from the solvent. After the substrates were bonded, the active site residues catalyzed the condensation reaction, producing two molecules of citrate from each protein dimer.

X-ray crystallographic studies of CS from psychrophilic to thermophilic bacteria have shown that the enzyme exists in a dimeric form akin to that of higher animals. Each monomer has 16 helices, which are crucial for catalytic reactions. As the high-resolution protein structures of the ligand-free and ligand-bound states are not available from the same species, the mechanism behind the conformational change between the open and closed states is unknown [Kanamori, 2015]. Although both psychrophilic and thermophilic bacteria have similar structures, but their molecular makeup is different. The proteins found in thermophilic species have smaller cavities, more ionic bonds, higher surface polarity, more tryptophan and charged residues, and fewer asparagine, methionine, and phenylalanine residues than psychrophilic bacteria.

The crystal structure of Leishmania citrate synthase is not yet known. It has a 50% similarity with human citrate synthase. Proteomic profiling of *Leishmania donovani* shows that citrate synthase consists of two isoforms with 80% sequence similarity [Jardim et al., 2018].

1.11.2 Stereochemistry and Mechanism of Enzyme

The mechanism of citrate synthase is illustrated in Figure 1.8. The process begins when oxaloacetate binds to binding site residues like His 274, His 320, and Asp 375. This converts the enzyme from an open to a closed state. The other residue, Arg 329, reduces the energy barrier for the conformational transition by forming a salt bridge with oxaloacetate. After the subunit is triggered, its large and small domains close inward on a hinge, allowing acetyl-CoA to access the binding site. Asp 375 removes a proton from acetyl-CoA, activating the alpha carbon for nucleophilic attack and acetyl-CoA to transform into an enol. The enol is stabilized by the hydrogen bond that forms between His 274 and the acetyl-CoA oxygen molecules. The active acetyl-CoA enol can nucleophilically attack the carbonyl carbon on oxaloacetate and produce the intermediate citryl-CoA through the same process in which His 320 acts as an acid and extracts the proton from the carbonyl oxygen of oxaloacetate. Following the formation of citryl-CoA, the hydrolysis of its thioester bond occurs when a water molecule is deprotonated using His 320, followed by an attack on the hydroxide ion that remains at the thioester carbon site. After the CoA is released, His 274 loses a proton. When citrate is produced and released, the citrate synthase conformationally shifts to its open state. As citrate synthase molecules have two active sites per molecule, two citrate molecules can be produced at once [Bloxham et al., 1981]; [Wiegand and Remington, 1986].

1.11.3 Function of Citrate synthase

1.11.3.1 In Cellular Metabolism

Citrate synthase catalyzes the condensation of acetyl-CoA and oxaloacetate to form citrate in the mitochondrial matrix, which aids in the continuation of the TCA cycle. However, when

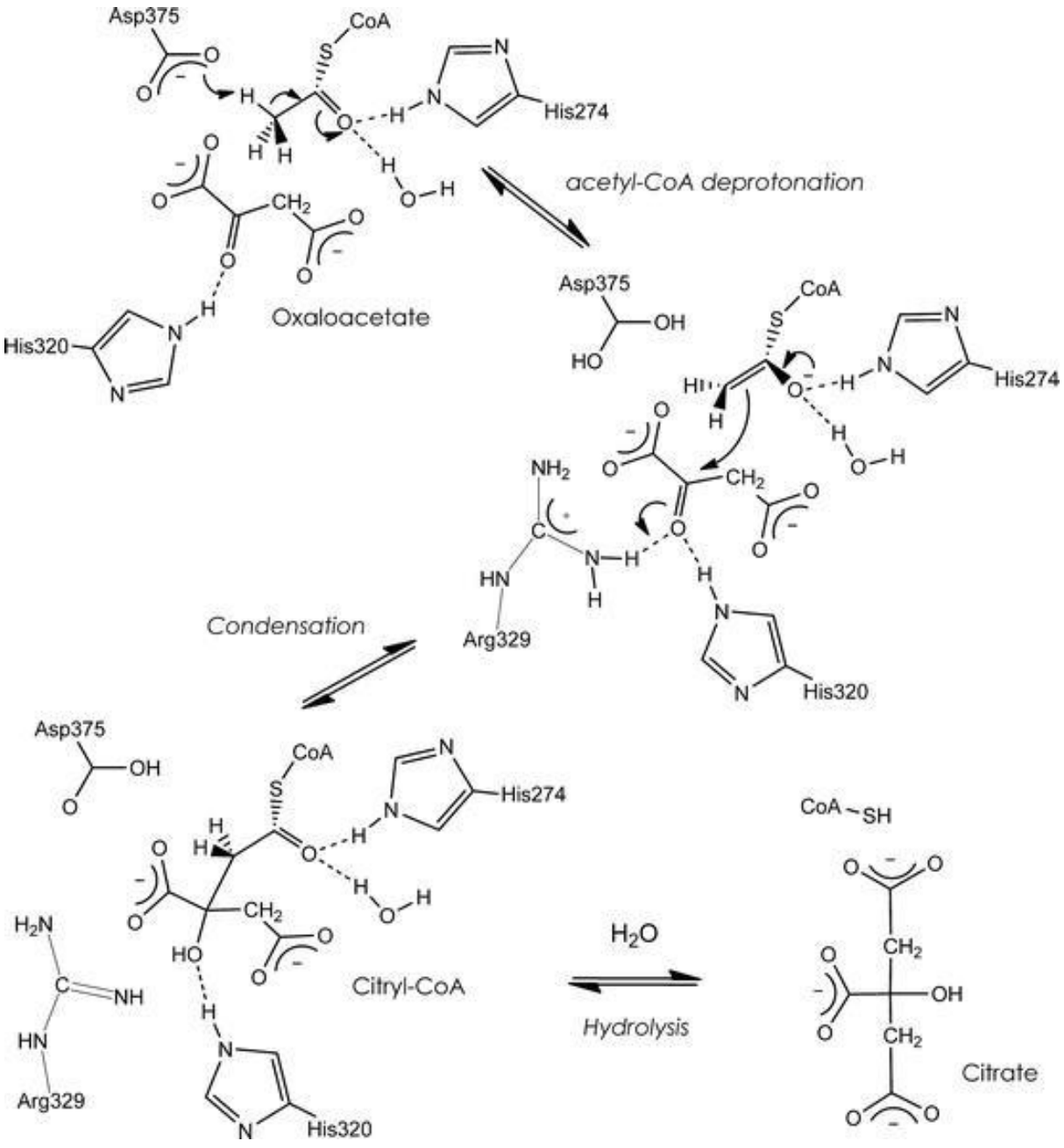


Figure 1.8: Mechanism of Citrate synthase enzyme. (Adopted from Mulholland et al., Encyclopedia of Biophysics, Springer, 2013)

ATP and NADH concentrations rise, this cycle is halted, and citrate is transported outside of the mitochondria via the mitochondrial citrate carrier (CIC), where it is exchanged with other negatively charged cytosolic molecules across the inner mitochondrial membrane. Outside mitochondria, the enzyme ATP-citrate lyase converted citrate into oxaloacetate and acetyl-CoA. Acetyl-CoA carboxylase (ACC) transforms acetyl-CoA into malonyl-CoA, which is then integrated into the production of fatty acids and cholesterol. However, the biochemical and genomics studies revealed that fatty acid synthesis is most unusual in Trypanosomatids. There is no evidence of cytosolic type-I fatty acid synthase, like in mammals and other higher organisms. Instead, in *T.brucei* and *L.major,* three elongase genes are present. Even in *L.major,* 12-tandemly linked homologous genes are present, which causes a more prolonged fatty acid synthesis in *Leishmania* [Opperdoes and Michels, 2008]. In *Leishmania*, Fatty acid desaturases are also present, which causes triglycerides formation for energy storage and phosphoglycerides for the cell membrane. Oxaloacetate is reduced into malate through cytosolic malate dehydrogenase, which then regenerates the cytosolic NAD^+ needed for the continuation of glycolysis. The cytosolic malate is then transferred to the mitochondrial matrix in exchange for citrate, where malate oxidation generates NADH and donates its electron to the respiratory chain, thereby stimulating oxidative phosphorylation (OXPHOS) [Zara et al., 2022]. Alternatively, the cytosolic malate acts as the substrate for the malic enzyme, producing pyruvate that is then re-entering the mitochondria through the pyruvate/citrate shuttle. NADPH is then used outside of mitochondria as a source of reducing equivalents for cholesterol and fatty acid biosynthesis [Oeggl et al., 2018].

Remarkably, in addition to being the precursor of acetyl units, which are crucial for lipid synthesis, citrate is a potent regulator of the activity of multiple enzymes involved in cellular metabolism [Petillo et al., 2020]. As fructose 1,6-bisphosphate is an allosteric activator of pyruvate kinase (PK), another glycolytic enzyme, high citrate levels inhibit

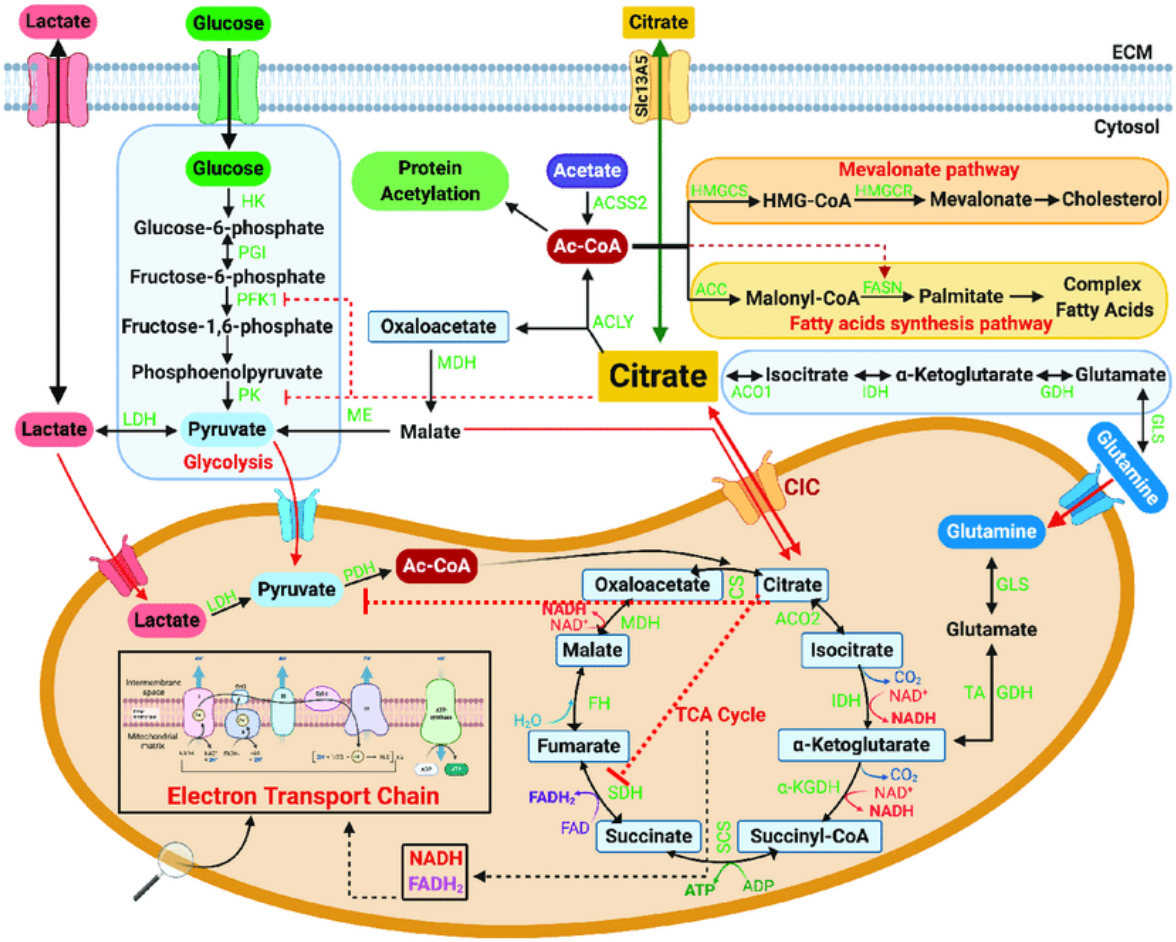


Figure 1.9: Role of Citrate in cellular metabolism. Glucose-derived citrate is synthesized by converting glucose to pyruvate with a series of enzymatic reactions. Pyruvate is reduced to lactate via lactate dehydrogenase (LDH) or transported into mitochondria, producing acetyl-Coenzyme A via pyruvate dehydrogenase (PDH). Citrate synthase then catalyzes the condensation of acetyl-CoA and oxaloacetic acid to form citrate, which is exported through CIC into the cytosol. In the cytosol, citrate is converted into oxaloacetic acid and acetyl-CoA via ATP citrate lyase (ACLY). Oxaloacetic acid is converted into malate through malate dehydrogenase (MDH) and imported to mitochondria through CIC, which is involved in the electron transport chain. Acetyl-CoA is involved in protein acetylation and also enters into the mevalonate pathway for cholesterol synthesis, which is mediated through hydroxy-methylglutaryl-CoA synthase (HMGCS) and hydroxy-3-methyl-CoA reductase (HMGCR). It is also involved in the fatty acid synthesis pathway via acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN). Cytosolic citrate inhibits phosphofructokinase 1(PFK1) and pyruvate kinase (PK), thus controlling the glycolytic pathway. Mitochondrial citrate inhibits enzymes such as pyruvate dehydrogenase (PDH) and succinate dehydrogenase (SDH). Cytosolic acetyl-CoA can also be generated from acetate through a deacetylation reaction by acetyl-CoA synthetase 2 (ACSS2). An alternative source of mitochondrial and cytosolic citrate occurs through reductive carboxylation of alpha-ketoglutarate to isocitrate via isocitrate dehydrogenase 1(IDH1) in cytosol and IDH2 in mitochondria.
Additional Abbreviations: HK—hexokinase; G6PD—glucose-6-phosphate dehydrogenase; 6PGL—6-phosphogluconolactonase; 6PGD—6-phosphogluconate dehydrogenase; Rpi—ribose-5-phosphate isomerase; PGI—phosphoglucose isomerase; ME—malic enzyme; MDH—malate dehydrogenase; CS—citrate synthase; ACO2—aconitase 2; IDH—isocitrate dehydrogenase; α-KGDH—α-Ketoglutarate dehydrogenase; SCS—succinyl coenzyme A synthetase; SDH—succinate dehydrogenase; FH—fumarase; ACO1—aconitase 1; GHD—glutamate dehydrogenase; GLS—glutaminase. **(Adopted from Masaoa et al., biomolecules, 2021, 11, 141.)**

phosphofructokinase-1 (PFK1), the rate-limiting enzyme of glycolysis, which lowers the amount of fructose 1,6-bisphosphate and inhibits pyruvate kinase (PK) [Iacobazzi and Infantino, 2014]. On the other hand, high levels of cytosolic citrate activate fructose 1,6-bisphosphatase (FBPase1), an essential enzyme of gluconeogenesis [Taketa et al., 1971]. Citrate synthase is referred to as a bifunctional enzyme in the cytosol because it has two different enzymatic activities within the same polypeptide chain: phosphofructokinase-2 (PFK-2) and fructose 2,6-bisphosphatase (FBPase 2). High citrate concentrations inhibit PFK-2, which decreases fructose 2,6-bisphosphate production, an allosteric activator of PFK1 and an allosteric inhibitor of FBPase1. As a result, high citrate levels inhibit glycolysis and activate gluconeogenesis. It's interesting to note that the citrate molecule can change the activity of several enzymes like pyruvate dehydrogenase complex (PDH) [Taylor and Halperin, 1973], citrate synthase (CS) [Dashty, 2013], and succinate dehydrogenase (SDH) [Hillar et al., 1975] in the mitochondrial matrix in addition to the cytosol.

As a result, it is clear that the citrate molecule plays a significant role in cellular metabolism since it is a source of carbon units for anabolic pathways and regulates various biochemical processes. In this regard, we may state that the citrate levels cause a coordinated modification of the metabolic pathways involved in the production and consumption of energy. Elevated citrate levels inhibit the glycolysis and Krebs cycle, which reduces the amount of ATP and NADH. On the other hand, it stimulates the anabolic pathways of lipogenesis and gluconeogenesis, which need ATP and NADPH to complete [Zara et al., 2022].

1.11.3.2 In inflammation as an inflammatory signal

Infection of murine macrophages with *L. donovani* elevated the expression of glucose transporters, which enhanced aerobic glycolysis. Even 24 hours after infection, there was an increase in mitochondrial respiration and a shift towards the M2-polarized phenotype, which is associated with increased mitochondrial biogenesis and full functioning of the TCA cycle

that supports amastigote proliferation [Moreira et al., 2015]. On the other hand, IFNγ- activated M1 macrophages effectively channel the absorbed glucose into glycolysis and the pentose phosphate pathway, which produces ATP and NADPH, necessary for generating ROS mediated by NADPH oxidase 2. The mitochondria catabolize glycolytically produced pyruvate, but there is a disruption in the tricarboxylic acid cycle that prevents flux through isocitrate dehydrogenase and succinate dehydrogenase. This results in citrate/isocitrate and succinate accumulation and increased mitochondrial ROS through reverse electron transport [Saunders and McConville, 2020]. The increase in citrate also causes NF-κβ dependent upregulation of CIC and ACLY, resulting in the export of citrate from mitochondria to cytosol and the production of oxaloacetate and acetyl-CoA. The acetyl-CoA is involved in the production of pro-inflammatory prostaglandin E2 (PGE2), NO, and ROS, while oxaloacetate, via cytosolic malate dehydrogenase and malic enzymes, produces NADPH, which is required for the generation of NO and ROS. Moreover, the elevated succinate levels stabilize the HIF-1α, which stimulates the transcription of inflammatory cytokines (IL-1β) and glycolytic enzymes, which means collectively, both are detrimental to leishmania infection [Williams and O'Neill, 2018]. The cytosolic build-up of acetyl-CoA also triggers the fatty acid synthase (FASN) enzyme, which yields fatty acids. Even the condensation of acetyl-CoA and malonyl-CoA results in aceto-acetyl-CoA synthesis, which aids in the synthesis of cholesterol and draws TLR4 to lipid rafts [Ranjan and Dubey, 2023a]. In leishmania, cyclooxygenase 2 produces leukotriene B4 (LTB4), and arachidonic acid produces prostaglandin E2 (PGE2) from stored lipids. The PGE2 is involved in the production of pro-IL1β and LTB4 in NO and ROS [Saunders and McConville, 2020].

Moreover, Citrate is a precursor for synthesizing itaconate (an α, β- unsaturated dicarboxylic acid), which is crucial for mammals and mediates the interaction between

infection, immunity, and metabolism [Zara et al., 2022]. It is a potent inhibitor of isocitrate lyase, an essential glyoxylate cycle enzyme, and helps in survival.

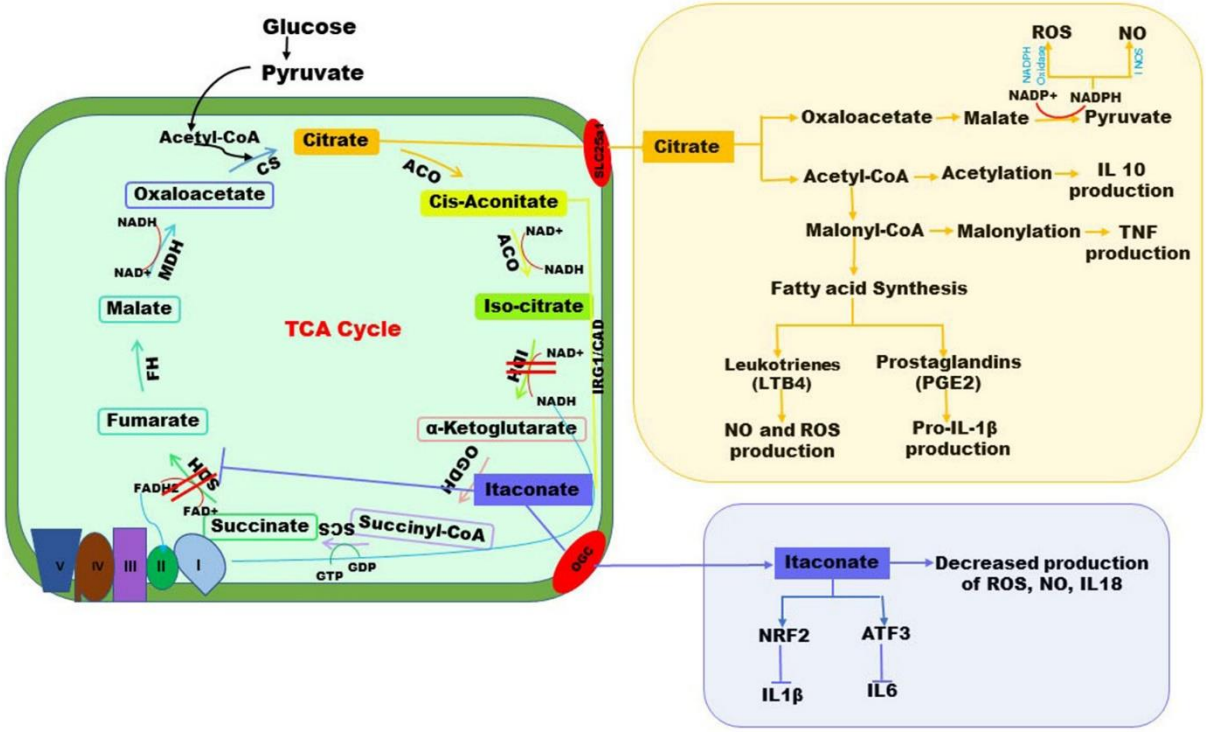


Figure 1.10: Role of Citrate in inflammation. Cytosolic citrate undergoes breakdown into acetyl-CoA and oxaloacetate. Oxaloacetate is further metabolized to generate NADPH, which is essential for the activity of NADPH oxidase and iNOS in producing reactive oxygen species (ROS) and nitric oxide (NO). Acetyl-CoA is pivotal for protein acetylation and is subsequently converted into malonyl-CoA, participating in fatty acid synthesis, which in turn influences cytokine and PGE-2 production. Citrate is also a precursor for itaconate synthesis, an α, β-unsaturated dicarboxylic acid. Upon transfer to the cytosol, itaconate activates the NRF-2 pathway and ATF3 inflammatory axis, thereby suppressing the expression of IL-1β and IL-6. Moreover, citrate exhibits suppressive effects on ROS, TNF-α, IL-6, and IFN-β activities while also inhibiting succinate dehydrogenase (SDH) and dampening immune activation by inhibiting the hypoxia-induced factor (HIF)-1α-IL-1β axis. **(Adopted from Ranjan et al., Life Sci., 2023, 322, 121314.)**

of many parasitic pathogens. It is exported from the mitochondria to the cytosol, where it depletes intracellular glutathione (GSH) levels, alkylates the Kelech-like ECH-associated protein 1 (KEAP 1), and activates the anti-inflammatory nuclear factor erythroid 2-related factor 2 (NRF2) pathway, which regulates the 1L-1β expression. Its electrophilic derivatives

also activated the NF-Kb inhibitor zeta (Ikb)-ATF3 inflammatory axis, which regulates the expression of IL-6. The cell-permeable itaconate derivatives dimethyl itaconate reduces the activity of ROS, TNF-α, IL-6, and IFN-β. It is a positive modulator of pro-inflammatory cytokine production and a negative regulator of inflammation. Additionally, it suppresses the succinate dehydrogenase (SDH) and reduces the immunological activation through inhibition of the hypoxia-induced factor (HIF)-1α-1L-1β axis [Ranjan and Dubey, 2023a].

1.11.3.3 Acetylation of Histones and Non-histone Proteins

Apart from being a substrate for de novo lipogenesis, acetyl-CoA plays a vital role as a cofactor in the acetylation of proteins, including histones and non-histones. This mechanism acts either co-translationally, influencing the α-amino group of a protein's N-terminal residue, or post-translationally, changing the ε-amino group of lysine residues. Because lysine acetylation is reversible, it offers an incredibly helpful strategy for controlling the expression of genes and the function of proteins [P. Tu, 2016]. Because acetyl-CoA cannot cross the cell membrane, it needs to be synthesized throughout many cellular compartments in order to have an effect. Acetyl-CoA is produced in the mitochondria by the β-oxidation of fatty acids and from pyruvate through the PDH complex. In the cytosol, it is formed by ACSS from acetate or, as was previously mentioned, from citrate [Pietrocola et al., 2015]. The enzyme ATP-citrate lyase, which is present in both the nucleus and the cytoplasm, converts glucose-derived citrate to Acetyl-CoA, thereby establishing a connection between metabolism and histone acetylation. Citrate allows acetyl-CoA to be produced in both cellular compartments because it is small enough to pass through nuclear pores. Although histone acetylation is essential for DC differentiation and macrophage activation, no studies have been done on the precise connection between citrate-derived acetylation and M1 macrophages or DCs. The acetylation of histone and non-histone proteins controlled the generation of IL-6 and IL-10 [Wang et al., 2014]. Zhong et al. (2018) reported that acetylation of the RelA/p65 subunit was involved in the

activation of NF-κβ. In non-immune cells, it has been found that many enzymes involved in metabolic processes are acetylated. Therefore, it is likely that the regulation of immune cell metabolism depends heavily on the acetylation process [Williams and O'Neill, 2018].

1.12 Drug Repurposing: An Emerging Approach in Drug Discovery

"The real voyage of discovery consists not in seeking new landscapes but in having new eyes."

Marcel Proust

Traditional drug discovery has historically been a protracted and costly endeavor, often spanning years or even decades before a new medication reaches the market, as depicted in Figure 1.11. The journey from initial target selection to clinical approval is fraught with challenges, including insufficient efficacy, toxicity concerns, and unforeseen side effects (Cressey, 2011). These hurdles inflate the costs of research and development, rendering the creation of new drugs economically unfeasible for both patients and pharmaceutical companies. This issue is particularly pronounced in the case of neglected tropical diseases such as leishmaniasis, where patients typically earn less than $2 per day. Consequently, there is an urgent call for innovative approaches to expedite the drug development process and enhance the likelihood of success, addressing a pressing need (Charlton et al., 2018).

Drug repurposing, also known as drug repositioning or reprofiling, offers a promising solution to this dilemma. It involves identifying new therapeutic applications for existing drugs already licensed for other indications or undergoing clinical trials for different purposes. Leveraging the knowledge and safety profiles of these drugs enables researchers to bypass several steps of preclinical testing and early clinical trials, resulting in significant time and cost savings (Pushpakom et al., 2018). The rationale behind drug repurposing stems from the extensive database of existing drugs, encompassing pharmacokinetics, pharmacodynamics, and adverse event profiles (GNS et al., 2019). This wealth of information provides insights into drug interactions with the human body, potential side effects, and recommended dosage

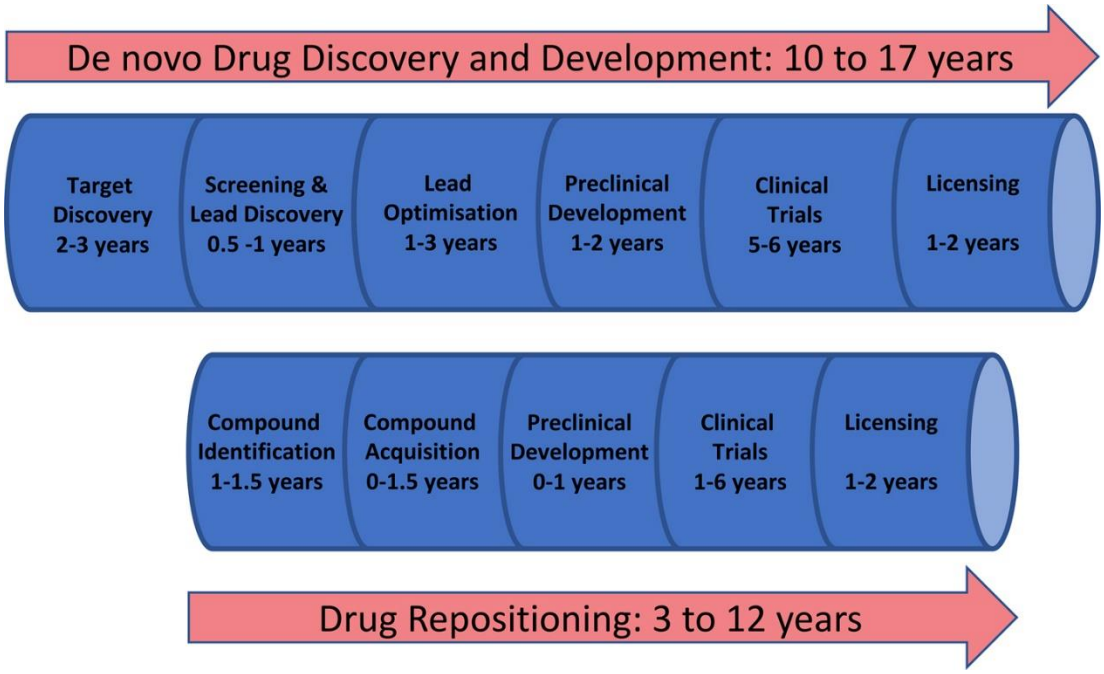


Figure 1.11: Drug discovery and repositioning pathways (adapted from Charlton et al., Parasitol., 2017, 145(2), 1-18)

regimens. Leveraging this data accelerates the identification of novel therapeutic targets and elucidates mechanisms of action, making drug repurposing a promising approach to address unmet medical needs (Parvathaneni et al., 2019). Furthermore, drug repurposing opens avenues to explore alternative therapeutic options and potential uses of medications beyond their original indications. The evolution of the drug repurposing field has the potential to transform the drug development landscape, offering patients new hope through alternative therapies and encouraging pharmaceutical companies to invest in areas where repurposing opportunities exist (Zubair Ahmad, Abdur Rauf, 2024).

1.13 Scope of current approach and objective

Globally, neglected tropical diseases (NTDs) affect over one billion people, with leishmaniasis standing as one of the prominent ones and ranking as the second largest parasitic killer worldwide, following malaria (eBioMedicine, 2023). Annually, the disease accounts for between 700,000 to 1 million new cases and 25,000 to 26,000 deaths worldwide. Its prevalence

was traditionally confined to tropical and subtropical regions, notably Latin America, Southeast Asia, East Africa, and North Africa. However, shifts in climate patterns, co-infections, population movements, and malnutrition have altered its distribution, heightening susceptibility (Sasidharan and Saudagar, 2021). Another pressing concern is the escalating resistance of parasites to conventional chemotherapy drugs, attributed to climate adaptations and vector-host co-evolution (Butt et al., 2020). Compounding the issue is the disease's prevalence among economically disadvantaged populations, who lack influence on governmental authorities and face limited healthcare access. Additionally, the pharmaceutical industry's minimal attention to neglected diseases due to limited commercial returns inhibits research and development of innovative therapeutics (Ullah et al., 2016). In light of these challenges, the quest for novel therapeutic targets becomes imperative. The discovery of novel drug targets is pivotal for advancing new drug development efforts. Repurposing existing drugs with novel targets holds promise in combating leishmania strains resistant to current treatments, thereby enhancing the quality of life for low-income populations and incentivizing pharmaceutical investment in developing repurposed chemotherapeutics. Our current project focuses on identifying a structure-based specific inhibitor of *L. donovani* citrate synthase (LdCS), aiming to establish a novel lead candidate for affordable leishmaniasis treatment. Notably, *L. donovani* citrate synthase exhibits significant structural and sequence disparities from its human counterpart and plays a crucial role in Leishmania biology, rendering it a promising drug target. Thus, the work embodied in this thesis is divided into the following objectives:

1) Modeling of *Leishmania donovani* citrate synthase and virtual screening of compounds using different databases.
2) Characterization of *Leishmania donovani* citrate synthase through biophysical studies.

3) Evaluation of selected compound as a potential inhibitor of citrate synthase: an alternative chemotherapeutic option against leishmaniasis.
4) Deciphering molecular mechanism of anticancer compound abemaciclib on underlying antileishmanial activities.