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Review

Genomic Insight of Leishmania Parasite: In-Depth Review of Drug Resistance Mechanisms and Genetic Mutations

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Cite This: *ACS Omega* 2024, 9, 12500−12514

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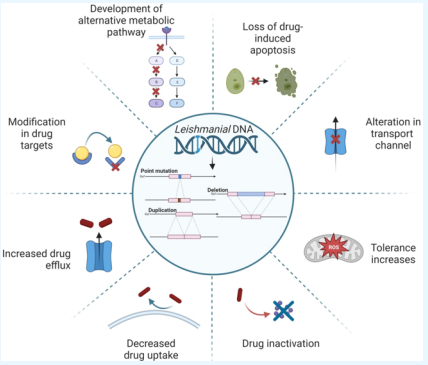
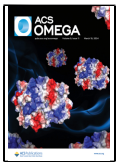
ABSTRACT: Leishmaniasis, which is caused by a parasitic protozoan of the genus *Leishmania*, is still a major threat to global health, impacting millions of individuals worldwide in endemic areas. Chemotherapy has been the principal method for managing leishmaniasis; nevertheless, the evolution of drug resistance offers a significant obstacle to therapeutic success. Drug-resistant behavior in these parasites is a complex phenomenon including both innate and acquired mechanisms. Resistance is frequently related to changes in drug transportation, drug target alterations, and enhanced efflux of the drug from the pathogen. This review has revealed specific genetic mutations in *Leishmania* parasites that are associated with resistance to commonly used antileishmanial drugs such as pentavalent antimonials, miltefosine, amphotericin B, and paromomycin, resulting in changes in gene expression along with the functioning of various proteins involved in drug uptake, metabolism, and efflux. Understanding the genetic changes linked to drug resistance in *Leishmania* parasites is essential for creating approaches for tackling and avoiding the spread of drug-resistant variants. Based on which specific treatments focus on mutations and pathways could potentially improve treatment efficacy and help long-term leishmaniasis control. More study is needed to uncover the complete range of genetic changes generating medication resistance and to develop new therapies based on available information.



1. INTRODUCTION

Leishmania is a parasitic eukaryotic protozoan that belongs to the class *Trypansomatidae* (order *Kinetoplastida*). These species are distinguished by the presence of a prominent Feulgen stain-positive kinetoplast. All of the species of this class are pathogenic from invertebrates to vertebrates, and their morphology changes as they progress through their life cycle. Over fourteen *Leishmania* species are harmful to mammals, nine of which are identified as human parasites.[1,2] Based on the morphological characterization, *Leishmania* exists in two different forms: a promastigote stage that penetrates inside the host's phagocytic cell and later transforms into an obligatory intracellular amastigote stage. This parasite performs a digenetic life cycle; each time the parasite shuttles between the host and the carrier, it undergoes morphological differentiation.[3,4] The female sandfly (Old World: genus *Phlebotomus* and New World: *Lutzomyia* and *Psychodopygus*) becomes infected after consuming blood from a diseased host. Once the parasite is inside the sandfly, it goes through the first stage of differentiation and becomes a procyclic promastigote. Promastigotes are flagellated and migratory parasites with thin bodies that range in length from 15 to 20 μm and 0.5−3.5 μm in width. The flagellar size is about 5−14 μm, which aids the parasite in adhering to the sandfly's gut.[5] The procyclic forms divide in the sandfly's abdominal midgut, culminating in the formation of indivisible nectomonad promastigotes. These nectomonad promastigotes pass through the midgut of the abdomen to the anterior midgut, where they change into leptomonad promastigotes. The leptomonad promastigotes then mature into metacyclic promastigotes and travel to the insect's proboscis, where they can be transferred to mammalian hosts. The following phase in the lifecycle is known as metacyclogenesis, and the promastigotes are introduced into the host species via a sandfly bite. Through the proboscis by biting, metacyclic promastigotes transmit to the host organism, where they initiate a phagocytic procedure by attaching to the cell membrane. In this way, the promastigotes infiltrate the phagocytes and attack the parasitophorous vacuole. Here, promastigotes convert into oval-shaped amastigotes two and four micrometers in width. It is crucial to highlight how the parasitic organism survives both the macrophages' acidic

Received: November 25, 2023
Revised: February 7, 2024
Accepted: February 14, 2024
Published: March 8, 2024



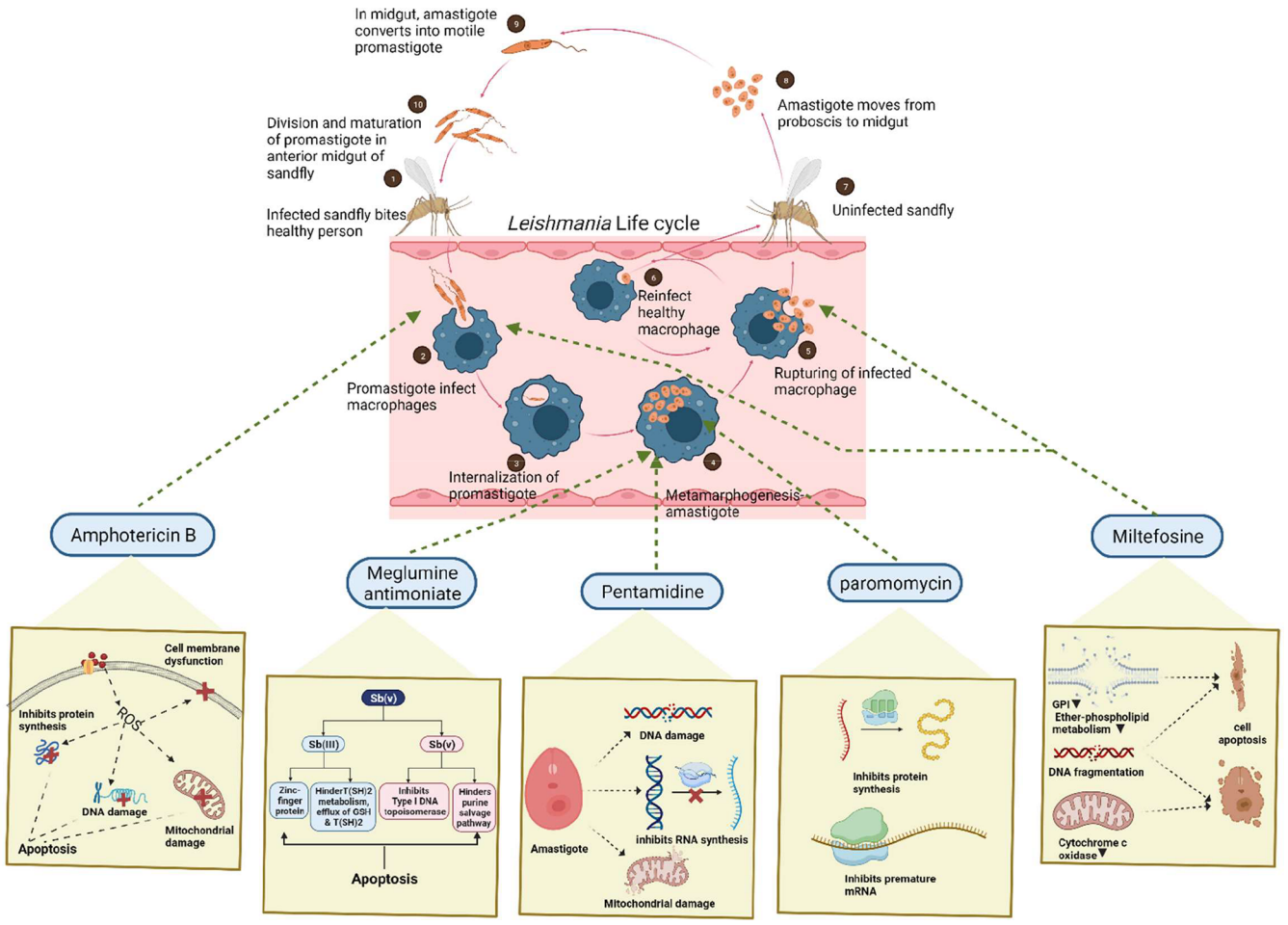


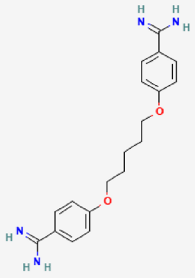
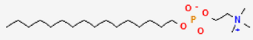
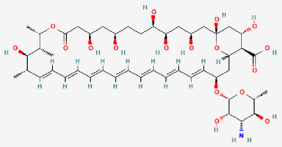
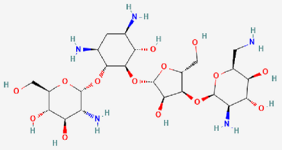
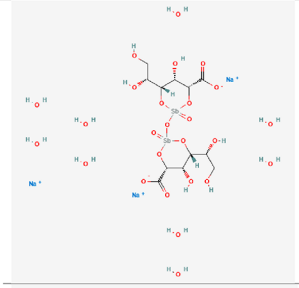
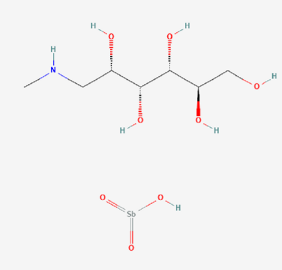
Figure 1. Life cycle of the *Leishmania* parasite along with its mode of action.

circumstances and the stomach's acidic environment. The amastigotes grow and multiply within the parasitophorous vacuole until the macrophage breaks off, secreting all matured amastigotes. These released amastigotes set off a chain reaction that finally develop into leishmaniasis as shown in Figure 1.[3,4]

According to the World Health Organization (WHO), leishmaniasis is a parasitic infection that causes tropical and subtropical illnesses that are spread in over 89 countries including Africa, Asia, America, and the Mediterranean, thereby creating a global health crisis. About 10 to 15 million individuals worldwide are affected, with an annual incidence of new infections exceeding 0.7−1 million.[6] This prevailing epidemiology shows a wide range of clinical symptoms ranging from minor skin lesions to life-threatening systemic infections, in the case of cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) respectively.[7] There are medications available for such illnesses, including antimonial, pentamidine, amphotericin B, miltefosine, paromomycin, and others, as shown in Figure 1. The drug structure (PubChem) and its mode of action is described in Table 1.[8] Despite continued attempts to manage and treat leishmaniasis, the emergence of drug-resistant strains presents a serious problem, weakening the effectiveness of current treatment strategies. Drug resistance in leishmaniasis is a complex problem driven by genetic, environmental, and clinical factors that could be considered innate and acquired mechanisms. The innate mechanism in clinically relevant *Leishmania* species shows that the presence of different molecular and biochemical components generates variances in *in vitro* drug susceptibility. Although pharmacokinetics and host immune responses play a role in the infected host, these species-specific changes often result in distinct *in vivo* medication efficacy. On the contrary, due to the defensive acquired mechanisms in the *Leishmania* parasite, it exhibits amazing genomic plasticity; genetic alterations can rapidly occur, allowing it to survive under pharmacological strain. The development of such resistance mechanisms can be linked to changes in drug uptake, drug efflux or sequestration, enzymatic drug inactivation, improved cellular responses to deal with drug-induced stress or cell damage, and/or changes in the expression, abundance, or drug binding affinity of the primary therapeutic target.[9,10] The convoluted life cycle of *Leishmania*, which involves both mammalian hosts and sandfly vectors, adds to the complication of drug resistance development. Furthermore, socioeconomic factors, inadequate healthcare infrastructure, and restricted access to effective treatment choices have led to the proliferation of drug-resistant parasites. The goal of this study is to provide a complete overview of the mechanisms causing drug resistance in leishmaniasis, the factors that contribute to its emergence, and the strategies used to tackle this challenge. This review focuses on investigating the genetic basis of drug resistance and looking at how changes in critical genes can lead to resistance to antileishmanial medicines. In addition, it also focuses on the influence of treatment practices, such as monotherapy and incomplete treatment sessions, on the selection of drug-resistant strains. To address this critical

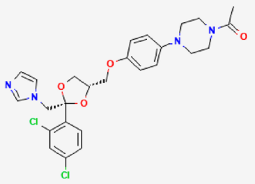
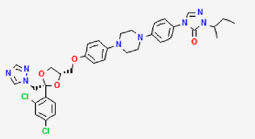
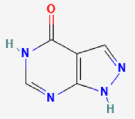
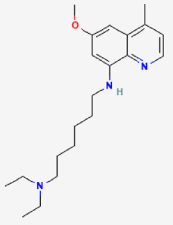
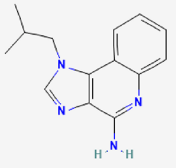
https://doi.org/10.1021/acsomega.3c09400
ACS Omega 2024, 9, 12500−12514

Table 1. Antileishmanial Drugs along with Their Mode of Action

Drugs	Structure	Mode of action	Diseases	Reference
Pentamidine		Inhibits RNA polymerase, active transport system, mitochondrial topoisomerase II that eventually leads to parasitic death, inhibits DNA synthesis and cell-cycle arrest in the G2/M phase	Visceral leishmaniasis Cutaneous leishmaniasis	[11]
Miltefosine		Alteration in alkyl-lipid metabolism and phospholipid biosynthesis inhibits cytochrome-c oxidase leading to mitochondrial dysfunction	Cutaneous leishmaniasis Mucocutaneous leishmaniasis Visceral leishmaniasis	[12]
Amphotericin B		Apoptotic death binds with ergosterol of the cell membrane of the parasite forming microspores and leakage of cellular content	Visceral leishmaniasis	[13]
Paromomycin		Inhibition of protein synthesis, decreasing of mitochondrial membrane potential, alteration in membrane fluidity and lipid metabolism, respiratory dysfunction	Cutaneous leishmaniasis	[14]
Pentavalent antimonial (Sodium stibogluconate & Meglumine antimonate		Trypanothione reductase Inhibition	Cutaneous leishmaniasis Mucocutaneous leishmaniasis Visceral leishmaniasis	[15]
		inhibit DNA topoisomerase glycolytic enzymes and fatty acid beta-oxidation	Cutaneous leishmaniasis Mucocutaneous leishmaniasis Visceral leishmaniasis	[16]

https://doi.org/10.1021/acsomega.3c09400
ACS Omega 2024, 9, 12500−12514

Table 1. continued

Drugs	Structure	Mode of action	Diseases	Reference
ketoconazole		Inhibits synthesis of ergosterol causing cell leakage, causes alternation in the ultrastructure of promastigotes, mitochondrial dysfunction, and induces autophagic vacuole. Formation	Cutaneous leishmaniasis	[17]
Itraconazole		Leishmanial ergosterol membrane disruption	Cutaneous leishmaniasis	[18]
Allopurinol		Used in combination with other drugs Inhibits RNA synthesis	Cutaneous leishmaniasis, Visceral leishmaniasis	[19,20]
Sitamaquine		Mitochondrial inner membrane damage	Visceral leishmaniasis	[21]
Imiquimod		Act via stimulation of interferon-gamma by CD4 T helper-1 cells by activation of phagocytic cell which destroys amastigotes cells	Cutaneous leishmaniasis	[22]

issue, researchers and healthcare practitioners are implementing a variety of strategies to reduce the impact of drug resistance. Combination therapy, which involves the administration of numerous medications with diverse mechanisms of action, has the potential to postpone or overcome resistance. Understanding the complicated host-parasite interactions in leishmaniasis reveals new targets for innovative drug development. This review aims to shed light on the complex interplay between drug resistance and leishmaniasis by an examination of current research and clinical experiences, emphasizing the significance of a multidisciplinary approach to address this public health concern. We can pave the road for more tailored therapies and better treatment results in leishmaniasis by identifying the mechanisms causing drug resistance and evaluating the effectiveness of various strategies.[10]

2. THE GENOME OF *LEISHMANIA*

Leishmania species have a wide range of chromosomes and gene sets. A recent genome assembly of *L. major* reported 32.8 Megabase genomic with 11,238 genes scattered among thirty-six chromosomes.[23] Initially, *Leishmania* species were assumed to possess atypically diploid genomes; however, mosaic aneuploidy has since been determined to be the norm in these parasites' genomes, with aneuploidy varied depending on strain or species.[24] Almost all *trypanosomatid* protein-coding genes lack introns and organize themselves in unidirectional polycistronic transcriptional zones that lack functional gene associations. Polycistronic transcription yields pre-mRNAs, which are later processed to form mature mRNAs. Every gene is continuously produced, mainly by RNA polymerase II, but

https://doi.org/10.1021/acsomega.3c09400
ACS Omega 2024, 9, 12500−12514

the timing of the initial stages of the gene transcriptional process is unknown due to canonical promoter sites not yet being identified in these parasitic organisms.[25] Epigenetic processes that influence DNA accessibility seem to have an essential function in *Leishmania* transcription initiation. Having the base J results in transcriptional completion at the final point of each polycistronic transcriptional region.[26] However, due to the lack of transcriptional regulation, it is hypothesized in the literature that protein production in these species is modulated via post-transcriptional mechanisms such as ribonucleic acid deterioration, translational management, and protein breakdown. Numerous investigations have discovered a link between the chromosome copy number and transcription levels, lending support to the theory that expression regulation occurs after transcription. However, transcript and protein levels are not always related.[27]

3. GENOME VARIATION AND PLASTICITY

Mutations within the parasite gene have been connected to geographic location and illness symptoms, which may impact leishmaniasis treatment. A surprisingly extensive study incorporating an enormous number of isolates from many different regions indicates that genetic variation is considerably greater than earlier stated.[28] Single-cell sequencing recently showed the existence of multiple distinct karyotypes inside the same Leishmanial clone, implying that multigenotype infections can occur within the same host's cells as well as in tissue.[29,30]

Aside from the function of mutations in parasite variety, these species' genomes are exceptionally flexible and constantly reorganize, which leads to changes in gene copy values, sets of DNAs, as well as the entire chromosome makeup. As a result, mosaic aneuploidy is not common within several species, but it also serves as a key adaptation process, allowing a certain genomic pattern to be rapidly selected during times of difficulty.[31,32] Ploidy alterations are not entirely random but appear to follow an identical sequence in specimens exposed to a variety of stressors, and each strain often follows the same pattern, indicating the presence of selecting processes.[33] In this ploidy, a gene's copy number could either be altered by inserting or removing genes in tandem, as well as through making extrachromosomal replicas of genetic material, that are either circular or linear.[34,38] These genes are generally detected under wild-type conditions but are expressed through alterations in *Leishmania* populations under stress conditions. In *Leishmania*, the genome contains sets of recurring regions enclosing several genes, and double-stranded DNA breakages close to or inside the repetitive regions can promote homologous recombination, and this is linked to an increase in gene reorganizations.[35−37]

3.1. TXNPx. TXNPx is a member of the 2-cysteine peroxiredoxin group and is classified according to as it is found in the cytosol or the mitochondria.[39] Enzymes such as these have been extensively conserved and found in a variety of *Leishmania* species. Most parasitic organisms, involving *Leishmania spp.*, tend to be more vulnerable to reactive oxygen compounds than their hosts. Organisms have evolved several antioxidant defense systems to counteract cell damage caused by reactive oxygen species (ROS).[40] Compared to other eukaryotes that utilize glutathione and catalase, trypanosomatid parasites possess trypanothione [N1, N8-bis(glutathionyl)-spermidine] (TS2) that acts as the main detoxifying agent against oxidative damage. Trypanothione synthetase (TryS) plays a role in this dithiol synthesis which is later reduced to $T(SH)_2$ by the trypanothione reductase (TR). The tryparedoxin/tryparedoxin peroxidase I (TXN/TXNPx) complex uses this $T(SH)_2$ to reduce the negative effect produced by macrophages through hydrogen peroxide neutralization. By catalyzing the reduction of H_2O_2, and small-chain organic hydroperoxides to alcohol and water, respectively, these antioxidant enzymes protect against chemical and oxidative stress.[41] The cooperative function of trypanothione reductase, tryparedoxin, and tryparedoxin peroxidase is thus critical for maintaining a low hydrogen peroxide (H_2O_2) content.[42] This enzyme is essential for *Leishmania's* survival during oxidative stress caused by macrophages and medications.[43]

3.2. ABC Transporters. ABC transporters make up a well-known family of proteins that perform important physiological tasks. These molecules observed in organisms such as prokaryotes as well as in humans utilize ATP hydrolysis for the elimination of numerous kinds of substances throughout the cell membranes.[44] ABC proteins have an important activity in resistance toward drugs via 2 modes of action. The initial phase is when ABC-carrying genes are amplified or expressed more, increasing carriers at the cell surface membrane and have been associated with a large amount of drug efflux through cells.[45] The subsequent phase is a mutation in the gene, which alters the biochemical characteristics of ABC transporters and, thus, their carrier capacity. ABC protein is a multidrug-resistant protein (MRP) in *Leishmania* that contributes to metal resistance via thiol metabolic processes and drug elimination pathways. And, the P-glycoprotein A (PGPA) is a member of the MRP ABC transporter family that has been linked to arsenite and antimoniate resistance. The Leishmanial parasite genome has forty-two ABC genes, designated ABCA to ABCH. In *L. major* infection, some investigations suggest that there is a link between nonresponding to glucantime and alleles such as ABCC7, ABCC3, ABCG2, and ABCI4.[46,47] The ABCI4 transporter, which is present in parasite mitochondria and cell membranes, is involved in heavy metal transfer. The ABCI4 gene is found on chromosome no. 33.[48] The ABCG2 transporter is in intracellular vesicles and the plasma membrane and is expressed with the help of the ABCG2 gene on the sixth chromosome. It transports the conjugated thiol-antimony complex outside the amastigote cell. The ABCC7 (PRP1) transporter is also in intracellular vesicles and is expressed by ABCC7 on chromosome 31.[49] Similarly, the ABCC3 transporter (MRPA), encoded by the ABCC3 gene on chromosome 23, is present in vesicles across the nuclei and the flagellar pocket of the cell. As these are related to tubulin vesicles, ABCC7 and MRPA proteins bind in both endocytosis and exocytosis processes. These protein carriers are important in drug response.[50]

3.3. Glycoprotein. The zinc-dependent metalloprotease glycoprotein 63 (GP63) or leishmanolysin, which was discovered on the surface of the *Leishmania* parasite in the 1980s, has been identified genetically as well as biochemically as a crucial surface antigen presented on *Leishmania* promastigotes from different species and to have an array of materials including casein, gelatin, albumin, hemoglobin, and fibrinogen.[51,52] This protease is included in the metzincin class, which includes the HExxHxxGxxH sequence motif and the N-terminus pro-peptide that suppresses the pro-enzyme during translation and is eliminated during maturation and activation.[53,54] GP63 is prevalent in promastigotes, but not in amastigotes. As it is found in both phases, it is expected to

https://doi.org/10.1021/acsomega.3c09400
ACS Omega 2024, 9, 12500−12514

serve various functions based on the parasitic stage. GP63 was discovered to cleave C3b into iC3b in promastigotes of *L. amazonensis* and *L. major*, hence assisting the parasite to evade complement-mediated lysis. Alongside, iC3b production can operate as an opsonin, allowing the parasite to engage with macrophages via complement receptors 1 and 3, thereby promoting parasite internalization. GP63 has also been shown to have interactions with the fibronectin receptor (FR), which may aid parasite adhesion to macrophages.[55] Another significant discovery was that when *L. mexicana* promastigotes encounter the outermost layer of subcutaneous tissue, GP63 could break down extracellular components, allowing for faster migration over matrix gel *in vitro*. According to these findings, GP63 shows a drastic impact on macrophage processes that favor *Leishmania* survival by cleaving and/or degrading different proteins.[56]

3.4. Aquaglyceroporins. The AQPs are a superfamily of aquaporins as well as aquaglyceroporins, which are channels in animals ranging from prokaryotes to eukaryotes that aid in the submissive pathway of water and tiny neutral molecules through the plasma membranes. Despite these channels not being found in many microorganisms, most eukaryotic genomes encode a minimum of one channel; plants have more than 30, some vertebrates have more than ten, and *Plasmodium spp*. and other Apicomplexa generally have one.[57] There are three types such as AQP1, AQP2, and AQP3 out of which AQP1 is a flagellar membrane protein, and the AQP3 is a cell membrane protein that carries water, glycerol, urea, dihydroxyacetone, and ammonia. Along with *in vitro* analysis, it was found that the deletion of these genes leads to resistance to antimonial drugs.[58]

3.5. L-Asparaginase. L-Asparagine synthase is essential for *Leishmania* survival and was recently recognized as a potential therapeutic candidate. A whole proteome BLAST revealed that among numerous protozoan diseases, only *Leishmania*, *Giardia*, and *Trichomonas spp*. have a unique genomic region coding for suspected L-asparaginase.[59] L-Asparaginases are amidohydrolase enzymes that have been employed as efficient antileukemic medicines. Also, some studies suggest that this L-asparagine (substrate for L-asparaginase) has an inhibitory influence on the Leishmanial autophagosomal pathway. According to a recent study, *Mycobacterium tuberculosis* L-asparaginase (MyAnsA) provides survival advantages to the pathogen by decreasing the acidic conditions inside the host cell. Each *M. tuberculosis* and *Leishmania donovani* uses a phagolysosome fusion process to establish infection. Transaminases and synthases may collaborate in a particular subcellular region to restore important chemical byproducts, as it was hypothesized that LdAI might not be the only one contributing to the metabolism of nitrogen. The STRING database added to the evidence by anticipating a functional relationship between LdAI and important enzymes that govern the metabolism of parasite aspartate, arginine, and purine metabolism. Some of the major enzymes, arginosuccinate synthase engaged in arginine biosynthesis and adenylate synthetase involved in purine biosynthesis salvage pathway, were predicted to interact strongly with LdAI.[60,61] These two enzymes were demonstrated to produce arginosuccinate (ATP dependent) and adenylate (GTP dependent) using ASP as a source. It has been demonstrated that functionally inactivating these enzymes causes deficiencies in parasite development and contagiousness. The knowledge that could be combined to successfully target the N2 metabolic processes of parasites does, therefore, indicate the synergistic interdependence of these pathways. Fortunately, utilizing an overexpression system, this undesirable potential was eliminated; by overexpression of LdAI in parasites, it was found that longevity increased by 20 percent, by having the ability to withstand detrimental impact at all doses of both inhibiting agents. The findings conclusively show L-asparaginase of *L. donovani* is one of the essential enzymes involved in metabolism for early cautious reaction to Amphotericin B used to treat visceral leishmaniasis.[62]

3.6. Cysteine Synthase. Cysteine is a sulfur-containing amino acid that plays a crucial role in cellular redox homeostasis; cysteine synthase plays an important role in the synthesis of cysteine, which is a precursor for the antioxidant molecules glutathione, and trypanothione, which is crucial for maintaining cellular redox balance and protecting against oxidative stress.[63] An upregulation of cysteine synthase could contribute to increased levels of cysteine and, subsequently, glutathione, enhancing the pathogen's antioxidant defense mechanisms and aiding in the detoxification of drugs.[64] The de novo or assimilatory and reverse transsulfuration (RTS) processes are 2 pathways for cysteine production. RTS has been established in fungi and mammals, and it comprises the entire process leading to cysteine from methionine via cystathionine synthesis. Cystathionine β-synthase (CS), which synthesizes cystathionine from homocysteine and serine, and cystathionine γ-lyase (CGL), which produces cysteine from cystathionine, catalyze these processes. The de novo process is likewise a two-step catalytic reaction that begins with serine acetyltransferase (SAT) to make O-acetyl serine (OAS) from L-serine and acetyl coenzyme A, followed by an alanyl transfer reaction driven by cysteine synthase (CS). This de novo pathway for cysteine biosynthesis is found in plants, bacteria, and several protozoan parasites such as *Entamoeba histolytica*, *Entamoeba dispar*, *Leishmania major*, and *Leishmania donovani*.[65,66]

3.7. Ascorbate Peroxidase. Ascorbate peroxidase (APx) is an enzyme that is essential for the glutathione-ascorbate cycle. Glutathione, which keeps cells in a reducing environment, is likely to be responsible for the reduction of many cellular components.[67] A single copy of the *Leishmania major* ascorbate peroxidase gene has been shown to possess a crucial role in the H_2O_2 detoxification, which is produced by both endogenous and exogenous processes such as an oxidative rupture of diseased host macrophages and *Leishmania* parasite medication mechanism.[68] Aminotriazole or sodium azide, which blocks heme-containing enzymes like catalase and peroxidase, dramatically slowed the elimination of H_2O_2 from amastigotes. Overexpressing ascorbate peroxidase in *L. major* promastigotes enhanced adaptability toward oxidative stress-induced apoptosis. Overexpression of APx (LmAPx) in *L. major* mitochondria protects cells from the harmful effects of oxidative damage, such as mitochondrial dysfunction and apoptosis.[69]

3.8. Silent Information Regulator 2. The silent information regulator 2 (SIR2) includes a family of NAD^+-dependent protein deacetylases and is conserved in microbial to eukaryotic organisms. These genes regulate a wide range of functions in eukaryotic cells, including transcriptional repression, recombination, the division of cells, cellular responses to DNA-damaging substances, and spindle organization.[70] Sirtuins are currently studied in parasitic protozoa such as *Plasmodium* and *trypanosomes* as they are required for proper cellular functioning and proliferation. Recently it was

https://doi.org/10.1021/acsomega.3c09400
ACS Omega 2024, 9, 12500−12514

Table 2. Gene Mutation along with Its Associated Drug

associated drug	gene name	protein	function	type of mutation	mutation (a.a.)	clinical relevance	references
Pentamidine	MRPA	Multidrug resistance protein	Role in detoxification	Amplification, overexpression	F360S, gene duplication, increased expression	Increased drug efflux	77
	AQP1	Aquaglyceroporin 1	Maintains osmoregulation, drug uptake	Deletion	Protein truncation, G126E	Decreased drug uptake Altered channel role	78
	TDR1	Thiol-dependent reductase I	Redox regulation	Point mutation	G146S	Reduced drug accumulation	79
	PTR1	Pteridine reductase 1	Increases resistance against oxidative stress	Point mutation	R30L	Altered drug metabolism	80
	DHFR-TS	Dihydrofolate reductase, thymidylate synthase	DNA synthesis	Overexpression	S91F, F185Y, I164L	Reduced drug binding	81
				Deletion	Gene deletion	Impaired drug-target interaction	
Miltefosine	LdMT	Miltefosine transporter	ATP-dependent transport of NBD-labeled phosphatidylethanolamine (PE) and phosphatidylcholine	Deletion point mutation	Gene deletion T245I, L832F, T420N, L856P	Impaired drug uptake	82
	LdRos3	Miltefosine transporter beta subunit	Maintains phospholipid symmetry	Point mutation	L262P	Reduced drug influx	83
Paromomycin	PMP3, PMP24	Phospholipid-Methyltransferase 3 and 24	Lipid metabolism	Point mutation	L42S, L55P	Altered drug susceptibility	84
				Gene deletion	L62P		
	HSP23	Heat shock protein 23	Maintains cellular integrity during stress condition	Deletion	Gene deletion	Reduced drug uptake	85
Amphotericin-B	ERG1	Ergosterol biosynthesis 1	Sterol biosynthesis	Point Mutation	G487S, S405A	Altered ergosterol biosynthesis	86
	LmABCB3	ABC transporter protein	Transportation	Deletion	Gene deletion	Impaired drug transport	87
	ERG11	Ergosterol Biosynthesis 11	Encodes the enzyme lanosterol 14-alpha demethylase	Point Mutation	Y306F G117V	Reduced drug binding	88
Antimony	AQP1	Aquaglyceroporin 1	Transports water, other small molecules across the membrane, maintains osmotic balance & metabolic process	Point mutation	SL75L, N146D, G245A	Altered water channel function	89
	TDR1	Tryparedoxin peroxidase 1	Protecting cells against oxidative stress	Amplification	Gene duplication	Increased drug efflux	90
	MRPA (ABCG2-like)	Multi-drug resistance protein A1	Role in drug efflux	Amplification	Gene duplication	Increased drug efflux	91, 92
	PGPA (ABCB1-like)	P-Glycoprotein A	Role in drug efflux	Amplification	Gene duplication	Increased drug efflux	93

https://doi.org/10.1021/acsomega.3c09400
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discovered to have both conserved and unique functions that regulate a wide range of biological processes; parasitic sirtuins are promising therapeutic options for treatment. SIR2RP1, SIR2RP2, and SIR2RP3 are SIR2 homologues found in the *Leishmania* genome.[71] The cytosolic sirtuin SIR2RP1 is known to be critical for parasite infectivity and survival, making it an interesting therapeutic target for antileishmanial treatment. The role of SIR2RP2 in *L. donovani* was explored. LdSIR2RP2 is linked to the human orthologue HsSIRT4, which corresponds to Sirtuin class II, according to phylogenetic and sequencing analyses. Some studies show that LdSIR2RP2, like HsSIRT4, exclusively exhibits NAD+-dependent ADP-ribosyltransferase activity. This protein, like HsSIRT4, was discovered to be localized in the mitochondria of the parasite. LdSIR2RP2 was not proven to be needed for parasite survival, but null variants showed delayed growth and lower infectivity. A G2/M block was discovered in the null mutant protozoa cell cycle, which could explain a mutant line growth issue. Thus, in *Leishmania*, deletion of the mitochondrial sirtuin LdSIR2RP2 affects mitochondrial activity, leading to lower ATP content and thus slower growth kinetics.[72]

3.9. Sterols. Lanosterol 14-demethylase (CYP51) is a cytochrome P450 (CYP) monooxygenase that stimulates the elimination of the 14-methyl group from various sterol substrates. This elimination process includes an essential stage within the cholesterol synthesis process, which produces cholesterol in mammals and ergosterol and ergosterol-like sterols in plants, fungi, and protozoa. As a result, CYP51 is being identified as an option for drugs since human cells possess sterol in their outermost layer, whereas parasitic organisms need ergosterol. *Leishmania* CYP51 is a prime example of a naturally occurring plant-like sterol 14-demethylase that can be addressed specifically to produce potent antileishmanial medications.[73,74] Cell surface sterols are crucial biological elements that help to produce functional cell membranes. Sterol 14-demethylase inhibition prevents sterol production, which is fatal in the affected organism. Sterol methyltransferase (SMT) catalyzes the conversion of a methyl group from adenosine to methionine into the sterol end chain of the C24 position. This enzyme is necessary for the protection of mitochondrial membrane potential, ROS generation, and parasite pathogenicity in *Leishmania major*.[75]

4. DRUG RESISTANCE LINKED TO MUTATION

As drug resistance is frequent in these parasites, gene variation is postulated as the primary factor in the evolution of various drug-resistant phenotypes. There is no single indicator for evaluating tolerance in clinical specimens since a variety of alterations may cause resistance to existing medicines. It is critical to understand the genetic basis of medication resistance to create successful treatment techniques.[76] By studying the specific genetic variations that contribute to drug resistance, researchers can identify potential targets for new drugs or therapies. Additionally, investigating the correlation between genetic variation and drug susceptibility in clinical specimens and *in vitro* tests can provide valuable insights into the mechanisms underlying drug resistance and guide future research efforts. Some of the examples of drugs associated with the mutation leading to its resistance are given in Table 2.

4.1. Pentamidine. Pentamidine, an aromatic diamidine, was initially used for treating insomnia in 1937. The first use of this drug in the treatment of VL in antimony-resistant patients in India was in 1949. PMD is utilized to treat *L. guyanensis* and *L. panamensis*-caused systemic CL. However, growing resistance to PMD and unexpected consequences like low blood sugar, low blood pressure, a cold, myocarditis, and renal toxicity were the main reasons for the drug's discontinuance throughout India in the nineties. Although the precise mechanism of PMD activity in *Leishmania* is unknown, a few studies suggest that it alters the parasite organism's mitochondrial internal membrane potential.[94] Pentamidine buildup in mitochondria may cause death by apoptosis in *L. donovani* by blocking respiratory cycle complexes I to III, producing reactive oxygen species, and elevating cytosolic Ca^{2+}. It can also act on DNA topoisomerases (TOPs), an enzyme that is required for the topology of DNA modulation during transcription, replication, recombination, and reparation. TOPI and TOPII of the parasite *Leishmania* differ significantly in structure and biochemistry by comparing with the human enzymes, as they play an important part within the parasite's kinetoplast DNA network organization.[95] PMD has been demonstrated as an arginine transport competitor in *L. donovani* as well as a noncompetitive antagonist of spermidine along with putrescine transportation in *L. infantum*, *L. donovani*, as well as in *L. mexicana*. The medication can enter both forms, promastigote and amastigote of *L. mexicana*, through a transporter-mediated process by identifying the medicine. Amastigotes, on the other hand, have a far higher absorption rate than promastigotes.[96]

Mutations in several transporters have been associated with PMD tolerance in the *Leishmania* parasites. ABC transporters were recently discovered in a variety of *Leishmania* species, and several of these species have been widely investigated and linked to treatment resistance.[97] The ABC transporters superfamily (ABCC7) comprises the P-glycoprotein (PGP), which includes the pentamidine resistance protein 1 (PRP1). Aquaglyceroporin 2 (AQP2) is a transporter in trypanosomes that regulates the resistance to pentamidine and melarsoprol. AQP2 is a surface channel protein that aids in the passive movement of liquids and tiny noncharged substances through the plasma membrane. More research is required, but an AQP2 mutation may be the cause of pentamidine resistance in *Leishmania* parasites.[98,99] Verapamil, a Ca^{2+} channel and P-glycoprotein inhibitor, has been demonstrated to restrict its efflux, resulting in PMD buildup in resistant parasites.[100] Flavonoid dimers were synthesized to avoid pentamidine resistance in *Leishmania* parasites, and they demonstrated much stronger reversal activity to pentamidine tolerance in *L. enriettii*, because of increased drug accumulation in mitochondria. These synthesized flavonoids work as reversal inhibitors in parasite *Leishmania* to overcome PMD resistance. The identical dimers functioned in tandem with quinacrine also used to reverse PMD resistance in the *Leishmania* parasite.[101]

4.2. Antimonial. Sodium stibogluconate (SSG), as well as meglumine antimoniate (MA), is currently the only viable therapy for all types of leishmaniasis with positive clinical and scientific outcomes. Because there are no oral medicines available, these therapies have serious side effects such as pancreatitis and renal and cardiac toxicity and can only be administered via an injection. Both SSG and MA have been shown to inhibit trypanothione reductase (TR), which is thought to be important for the survival of parasites in the host organism. TR degrades trypanothione, which is employed for neutralizing ROS generated by macrophages during infection.[102] In contrast to glutathione (GSH), which is the principal redox defense molecule in mammals, trypanothione is

https://doi.org/10.1021/acsomega.3c09400
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the primary detoxifying pathway for oxidative damage in *Trypanosomatid* parasites. Pentavalent antimonials are considered prodrugs because their *in vivo* transformation results in the generation of active as well as toxic trivalent antimonials Sb(III), which causes *Leishmania* apoptotic death. Acidic pH values and high temperatures aid in Sb(V) reduction.[103] Both macrophages and parasites can experience a decrease in antimonial levels. The ability of *Leishmania* to convert Sb(V) to Sb(III) changes with each stage. Because amastigotes can transform Sb(V) to Sb(III), they are more sensitive to Sb(V), while promastigotes are unable to. Many patients' incorrect antimonial therapy causes pharmacological stress on the parasite organisms, resulting in adaptation and, finally, persistence against Sb(V).[103,104]

The gradual evolution of antimony resistance raises the notion that numerous mutations need to occur to produce a resistant phenotype. Several *in vitro* processes may clarify the reported antimonial resistance, albeit it should be noted that the *in vitro* response is not always translated into clinical resistance. The reduction in the concentration of drugs in the parasitic protozoan may be due to reduced absorption or by higher outflow of medicine; drug activity suppression; deactivation of the active drug; and gene amplification, all possible explanations for resistance evolution.[105] Sb(III) resistance relates to TXNPx overexpression and greater intracellular thiol levels. *In vivo*, antimonial resistance was demonstrated by inhibiting Sb(V) activity and decreasing amastigotes' absorption of active Sb(III) in thiol production. The gene encoding aquaglyceroporin 1, gamma-glutamylcysteine synthetase, and ornithine decarboxylase, which are involved in Sb(III) uptake and glutathione and trypanothione digestion, was lowered throughout the procedure.[106,107] The overexpression of membrane ABC transporters on the surface parasites is also the reason for the resistance. This transport route influences drug efflux and intracellular accumulation, which contribute to resistance development. Elimination of the drug as a metal−thiol in combination with ABC carriers such as ABCI4 and ABCG2 may improve antimony resistance. Sb-resistant *L. donovani* parasites also boost host cell production of the MRP, and the P-gp, reducing antimony influx and hence suppressing drug accumulation inside the cell. For the binding of parasites, the cholesterol membrane of the host is required as well to invade phagocytes.[108] Cholesterol is an important lipid membrane component in eukaryotic organisms, where it aids in the structure, dynamics, and activity of its constituents. Statins, such as lovastatin, work by inhibiting HMG-CoA reductase, which is the rate-limiting enzyme in the cholesterol production pathway. Lovastatin inhibits the proteins MRP1 and P- glycoprotein in *L. donovani*, enabling antimony to accumulate and decreasing Leishmanial cell growth, macrophage infection, and causing its death.[109] As a result, the statin class mitigates the Sb resistance. Flavonoids naturally inhibit P-glycoprotein and its related ABC transporters in *Leishmania*. Synthetic flavonoid dimers were employed to overcome antimony drug resistance in *L. donovani*, enhancing intracellular drug accumulation.[110,111] Heat shock proteins (HSP70) were found to play a function in antimony tolerance by employing functional cloning to extract drug-resistance genes. Antimony and Sb(III)-resistant variant cells were found to have high levels of HSP70 proteins.

Transfected *Leishmania* species developed antimony resistance, most likely due to improved cell tolerance to metals; this enabled the cell to become a more specialized and more efficient defense activity. HSP90 has been related to the development of resistance in *Leishmania* in recent research. In summary, antimonial resistance is a difficult issue. Several antimonial resistance mechanisms in experimental *Leishmania* isolates have been identified.[112,113]

4.3. Miltefosine. An alkyl phospholipid called miltefosine (also known as hexadecyl phosphocholine, or MT) was first created as an anticancer medication. As MT received approval as an initial oral medication for VL in India in 2002. MT is now used for treating VL as well as CL diseases, as it is the first-line treatment for CL. It is easier to acquire and has lesser toxicities when compared to antimonials.[114] Hepatotoxicity and nephrotoxicity are two of the medication's side effects. The main disadvantages of MT involve its teratogenicity, the possibility of resistance resulting from its extended half-life (7 days), the occurrence of subtherapeutic dosages over time, and the high price.[115] Phosphatidylcholine formation is inhibited by MT, changing the phospholipid biosynthesis. The proposed mechanism of action begins with adhesion to the plasma membrane and then proceeds to internalization via 2 protein membranes: the miltefosine transporter (LdMT), as Ld stands for the species *L. donovani*; this transporter is a member of the P4-ATPase subfamily, as well as possessing LdRos3, a potential noncatalytic component of LdMT. These 2 proteins reside mostly in the cell membrane of *Leishmania* and present as an important factor for the quick intracellular absorption of alkyl phosphocholine medications. LdMT and LdRos3 combined to form a stable protein assembly, allowing phospholipids to migrate across the cell membrane from the exoplasmic to the cytoplasmic sections.[116,117] It is also shown that MT inhibits mitochondrial cytochrome c oxidase, resulting in a decrease in the *L. donovani* oxygen utilization rate and ATP levels. In addition to other immunologic and inflammatory effects on macrophages, MT was discovered to cause the death of cells during the promastigote phase of *L. donovani* through programmed cell death. Considering the most recent emergence of miltefosine use, therapeutically resistant parasites were observed in Nepal in the occurrence of VL. In the lab, mutants were generated to forecast the development of MT resistance and define the mutants that resulted.[118]

In the Indian subcontinent, miltefosine-resistant parasites have been discovered. In the laboratory, 2 strains of visceral leishmaniasis with evolved miltefosine resistance (*L. donovani*) were morphologically as well as genotypically characterized.[119] Even though there are just a few MT-resistant clinical strains, their genetic and biological characteristics are strikingly similar to those of laboratory-selected strains. Although the precise mechanism of MT resistance is unknown, every miltefosine-resistant *Leishmania* strain examined demonstrated a reduction in drug accumulation. This could be because of decreased drug absorption, increased efflux, faster metabolic rate, and altered cell membrane permeability. A single locus variation within the LdMT gene is two different alleles rendering the transporter protein LdMT inactive. The LdMT mutations in genes L832F, T420N, or L856P increased susceptibility (includes *in vitro* and *in vivo*), as well as decreased absorption, greater emission, quicker metabolic processes, and alterations in the lipid makeup of parasitic cell membranes. Other LdMT gene variants such as V176D, W210, the lately found Y354F, and F1078Y, M1 mutation of LdRos3.[120] Overexpression of ABC carriers has been identified as another avenue for MT resistance. The first molecule associated with experimental MT resistance was LMDR1/ABCB4, a P-glycoprotein-like

https://doi.org/10.1021/acsomega.3c09400
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transporter found among ABC transporters. *Leishmania* is more resistant to MT when the ABC transporters such as ABCB4 (MDR1), ABCG4, and ABCG6 are overexpressed, resulting in a decrease in their accumulation inside the cell because of increased drug efflux across a cell membrane.[121,122] Changes in MT-resistant promastigotes' membrane lipid content sterol production may also alter drug-membrane interactions. Recently, it was revealed that the use of cosmid-based functional cloning in conjunction with next-generation sequencing genes implicated in ergosterol production and translocation of phospholipid could result in *L. infantum* resistance.[120]

To fight MT resistance, several substances have been produced. Sesquiterpenes have been found to overcome multidrug resistance with the help of ABC transporters and by boosting intracellular drug concentration. An additional flavonoid analogue is effective in reversing LMDR1 over-expression at modest dosages. Sitamaquine, an 8-aminoquinoline, reverses LMDR1-mediated MT resistance by elevating intracellular drug accumulation, resulting in an effective LMDR1-mediated miltefosine resistance reversal agent in this parasitic organism.[123]

4.4. Amphotericin B. As the second-line therapy for VL, amphotericin B (AMB) is a polyene medication produced by the *Streptomyces nodosus* filamentous bacterium. It is the most effective drug for pentavalent antimonial resistance.[124] Although it has substantial adverse reactions, including severe renal toxicity, which necessitated inpatient treatment and 4 weeks of patient monitoring. Another significant downside is the expensive price of AMB. To overcome these limitations, liposomal AMB (LAMB), a lipid-associated composition with less harmful effects and a prolonged plasma lifespan that allows for a single infusion, was developed. To treat leishmaniasis, oral formulations of AMB are being developed. AMB's mechanism of activity could include interacting with surface sterols, resulting in membrane disarray and increased accessibility of protons and monovalent cations.[125] AMB may influence cells due to oxidation along with the following creation of reactive oxygen species. Cell damage produced by AMB may be linked to ion mobility, oxidative effects, and the formation of ROS. Because of the advent of drug resistance to past therapies, amphotericin B has become a more important therapy for leishmaniasis. Because there are few options, the emergence of AMB resistance is a real possibility. As a result, figuring out how AMB resistance develops is a primary priority, which led to the development of laboratory-based amphotericin B, species that are resistant to it.[126] A recurrence incidence of about 3.7% following therapy with liposomal amphotericin demonstrates that resistance to LAMB can also emerge. Regardless of this minimal risk, recurrence is important in transmission dynamics because it increases the worldwide population of parasitic organisms in the host that are set to be transmitted to the vector; in persons with HIV who are not receiving antiretroviral medication, VL recurrence raises the possibility of spreading by suppressing the immune system, raising the parasite burden, along with a lack of responsiveness to treatment, and there is a chance that parasites may become resistant to antileishmanial drugs.[127]

To predict the emergence of resistance, several experimental approaches have been developed. Many *Leishmania* species are resistant to AMB. Promastigotes were selected and studied through elevated drug pressure. Many alterations were detected when the biological properties of these resistant varieties were examined in the wild-type parent strain. Variation within sterol biosynthesis, enzyme lanosterol 14-demethylase (CYP51) was shown to have a function in *L. mexicana*. Several AMB-resistant *Leishmania* strains exhibited genetic changes in other sterol biosynthesis enzymes, including sterol C24-methyltransferase (SMT), which adds the C24-methyl group to the ergosterol side chain, and sterol C5-desaturase (SC5D), which is required for sterol 5(6)-7(8) double- bond pairing.[128] Overall, resistance is associated with decreased AMB adherence to a cell membrane due to sterol modifications (declination of SMT gene expression). MDR1 effluxes AMB from the membrane, whereas the rest of the intracellular AMB auto-oxidizes and generates ROS. The thiol metabolic pathway's tryparedoxin cascade may be able to counteract the adverse effects of this ROS. The cumulative effects of transformed membrane characteristics, including MDR1 and the tryparedoxin cascade, might be the cause of AMB-resistance.[129]

4.5. Paromomycin (PMM). PMM is an aminoglycoside medication that was approved for the therapy of VL in 2006. PMM tends to be tolerated effectively, can be given orally or intramuscularly, and has few side effects.[130] PMM inhibits the production of proteins by interfering with the ribosomal subunits and enhances the ribosomal subunit interaction in bacterial infections. *Escherichia coli* was discovered to connect to the major cleft at the A-site of the 16S rRNA sequence and to activate mRNA misreading. However, the method through which it works in *Leishmania* is uncertain. PMM may inhibit protein production in *Leishmania* parasites by binding to the 16S ribosomal unit and creating a specific structural alteration in the 16S rRNA's A site. Alteration at the N1 sites of A1492 and A1493 on the minor groove regions of the A-site RNA showed modal activity during the translational process. Changes in membrane fluidity and lipid metabolism, a decrease in mitochondrial membrane potential, and respiratory failure have all been proposed as possible mechanisms of action.[131,132] Endocytosis increases PMM intake by binding PMM to parasitic membrane proteins like PFR 1D and 2D, inhibiting, as well as a P-type H^+ ATPase, the main purpose of which is to induce endocytosis thus allowing the drug within vacuoles.[133] PMM provides several benefits, including affordable prices, quick administration, good protection characteristics, and convenience of use. Paromomycin's physicochemical nature, on the other hand, prevents appropriate accumulation at the location of infection. The application of solid nanoparticles of lipids as a PMM transport vehicle increased drug absorption into macrophages, raised immunological response, and hence improved PMM efficacy.[134] To target *Leishmania* parasites in monocytes, a combination of albumin microspheres infused with PMM was created for VL therapy. This formulation has the advantage of preferentially targeting macrophages, which means less toxicity and an easier procedure over an intramuscular injection.[135] PMM is additionally combined with stearyl amine (SA)-containing phosphatidylcholine (PC) liposomes. PMM-SA-PM revealed enhanced immune protection, antileishmanial activity, and no toxicity.[136]

5. CONCLUSIONS

Owing to the variation in the parasitic class, it is extremely difficult to develop drugs that can be useful in curing diseases triggered by distinct species or subspecies. Certain-omics-based studies (metabolomics, genomics, proteomics, and transcriptomics) have also focused on how the resistivity is

https://doi.org/10.1021/acsomega.3c09400
ACS Omega 2024, 9, 12500−12514

enhanced and demonstrated in the *Leishmania* genus due to the synthesis of specific proteins that provide such traits. Furthermore, because these interactions are crucial for the success of treatments, it is necessary to explore the host-parasite interactions. Gene editing in parasites is an intriguing method for learning more about proteins that aid in drug metabolism. There are now various techniques for genetically modifying *Leishmania*, including deletion via allelic substitution, overexpression, and heterologous expression. In addition, parasite genome sequencing can work as a novel and crucial method, since the parasitic genes could be a potential therapeutic target for drug discovery, and the data collected could act as a beneficiary repository for TritrypDB-like databases focused on *Leishmania*.

Despite all of the tools available, studying the resistant effect exhibited by parasites needs in vitro and artificial laboratory-based genetic manipulation like the ones that are exhibited by the parasite inside the host organism owing to their genetic plasticity and vulnerable environment tolerance capacity. Because parasites can undergo various modifications *in vitro* to survive, it was proposed that genetic changes be undertaken directly in clinical specimens to avoid experimental artifacts. Similarly, gene deletion can lead to the selection of parasitic organisms with aneuploidies and altered phenotypes that are not found in nature. Unfortunately, inducible strategies for modulating gene expression in *Leishmania* between active and inactive states are currently being researched, and little is known about their use. The diversity of the *Leishmania* genus and the factors that determine diversity are the subject of this review. We emphasize the need to apply genetic manipulation methods to learn more about leishmaniasis drug resistance mechanisms and chemotherapeutic targets. Single-cell sequencing will allow the discovery of numerous new parasite species that were previously unknown. An integrated study that combines data from these many approaches and aspects of host-parasite linkages would improve understanding of the intricacies of medication, resistance mechanisms, therapeutic failure, and the sly parasites' incredible durability. This understanding will pave the path for the development of more effective treatment choices as well as the discovery of new therapeutic targets. It will also help to create preventive techniques to stop the spread of drug-resistant strains, lowering the burden of leishmaniasis on affected people.

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K.B. and R.S.K. conceived and designed the study. K.B. and R.S.K. conducted the literature search and data collection. R.S.K. and T.K.U. performed data analysis and compilation. K.B. and R.S.K. wrote the initial draft of the manuscript. R.S.K. and T.K.U. provided critical revisions and intellectual input. All authors have read and approved the final manuscript.

Funding

This research was funded by the Deanship of Scientific Research at King Khalid University for funding this work through the large Research Group Project under grant number RGP.02/317/44.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors are thankful to the Deanship of Scientific Research, King Khalid University, Abha, Saudi Arabia, for financially supporting this work through the Large Research Group Project under grant no. R.G.P.2/557/44. We are also thankful to Department of Life Sciences, Parul Institute of Applied Sciences and Research & Development Cell, Parul University, Vadodara, Gujarat, India 391760.

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https://doi.org/10.1021/acsomega.3c09400
ACS Omega 2024, 9, 12500−12514