Review Article

Recent advances in identification of potential drug targets and development of novel drugs in parasitic diseases. Part II: Parasite targets

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ABSTRACT

Understanding the parasite biology on molecular basis is the starting point for identification of novel parasite drug targets. The interpretation of gene regulatory networks is a "blueprint" for discovery of new interactions among system biological events that lead to identification of novel potential drug targets and/or vaccine candidates. The aim of the present review is to simplify the molecular bases of the mandatory biological processes involved in the parasite survival, growth, replication, pathogenesis, and virulence. Growth and replication include nucleic acid synthesis, DNA replication and gene expression (topoisomerase, histone variants, and histone modification enzymes), and translation process for protein synthesis (initiation and elongation factors). Parasite survival includes signaling pathways (protein kinases, and protein lipidadtion), regulated cell death machinery, mitochondrial respiratory electron chain, and transmembrane transporters. Parasite pathogenesis and virulence include proteases, endogenous protease inhibitors (cysteine and serine protease inhibitors), heat shock proteins, glycoproteins, and tetraspanins. This publication is part II in a series of reviews dealing with identification of potential drug targets and development of novel drugs in parasitic diseases published in PUJ as part I[1].

Keywords: cytochrome complex; myristoylation; protein kinases; proteases; regulated cell death; salvage pathway; sirtuins; topoisomerase; transporters.

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Abbreviations: ABC: ATP-binding cassette, **ATP:** Adenosine triphosphate; **CP:** Cysteine protease; **Cyt c:** Cytochrome c; **HDAC:** Histone deacetylase; **HAT:** Histone acetyltransferase; **MCAs:** Metacaspases; **MDR:** Multi-drug resistance; **MP:** Metalloprotease; **PCD:** Programmed cell death; **PK:** Protein kinase; **PTMs:** Post-transitional modifications; **SIR:** Sirtuin; **SP:** Serine protease; **Top:** Topoisomerase.

[I] Growth and replication (DNA synthesis and gene expression)

1. Nucleic acids synthesis

Nucleotides serve as monomeric units of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). They are formed of a nitrogenous base (nucleoside), five-carbon sugars (ribose or deoxyribose), and a phosphate group (1-3 phosphates). Accordingly, they are termed monophosphate (MP), diphosphate (DP), and triphosphate (TP), respectively. Symthesis of DNA and RNA copies requires supply of two nitrogenous bases (purines and pyrimidines) in similar quantities. As long as both bases are available, synthesis is self-activated, and when there are no more bases, the synthesis is self-inhibited. While purines include adenine (A), and guanine (G), pyrimidines include cytosine (C), thymine (T), and uracil (U). Notably, DNA is formed of two purines (A and G), and two pyrimidines (C and T), while (U) replaces (T) in RNA. Nucleotides have several cellular functions including growth and replication *via* synthesis of DNA and RNA signaling pathways since they are involved in synthesis of intracellular cyclic second messengers (cAMP, and cGMP). They are energy sources due to involvement of nitrogenous bases in the components of nucleoside TPs (ATP, GTP, TTP, CTP, and UTD). Energy is required for protein and cell membrane syntheses, cell division, and motility. Moreover, adenine contributes in formation of several cofactors required for enzymatic reactions such as coenzyme A, nicotinamide adenine dinucleotide (NAD), NAD phosphate (NADP), flavine adenine dinucleotide (FAD) and flavine mononucleotide (FMN). Lastly, uracil diphosphate (UDP) is utilized in glucogenesis; a ubiquitous metabolic pathway required for production of glucose from non-carbohydrate carbon substrates[2].

Synthesis of purine and pyrimidine nucleotides is either *de novo* or through a salvage pathway. In such a pathway, nucleoside transporters (NTs), known also as permeases, carry salvaged bases and nucleosides across parasite plasma membrane to synthesize nucleotides. Therefore, nucleoside transporters and enzymes involved in purine or pyrimidine synthesis, either *de novo* or *via* salvage pathways contributing to nucleic acid processing, are potential drug targets.

Transporters: There are four balanced NTs (ENTs). While ENT1 and ENT2 are the major

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plasma membrane transporters for both purines and pyrimidines import, ENT3 is expressed in intracellular membranes, and ENT4 is characterized as a plasma membrane monoamine transporter (PMAT). All ENTs possess a cytoplasmic N-terminus and an extracellular C-terminus as binding sites[3].

Purine salvage pathway: Due to inability of almost all intracellular protozoa to synthesize purines *de novo*, they salvage bases and nucleosides formed during degradation of host' DNA and RNA, with a subsequent six-step process for synthesizing purines. Salvaged adenosine undergoes either phosphorolysis by adenosine kinase **(AK)** forming adenosine MP (AMP) or hydrolysis by adenosine deaminase **(ADA)** forming inosine. Purine nucleoside phosphorylase **(PNP)** converts inosine to hypoxanthine that is converted to inosine MP (IMP) by hypoxanthine-guanine-xanthine phosphoribosyltransferase **(HGXPRT)**. Then, IMP is metabolized to either AMP, or xanthine MP (XMP) that is further metabolized to guanosine MP (GMP). The last step is the conversion of the nucleotides (AMP and GMP) to deoxynucleotides by ribonucleotide diphosphate reductase **(RNR)**. Accordingly, five potential targets are recognized in intracellular protozoa; AK, ADA, PNP, HGXPRT, and RNR[4].

2. Topoisomerases

During DNA replication and transcription, DNA topoisomerases (Tops) prevent and correct the topological problems that may happen due to tangled DNA double-helical structure. They are enzymes that prevent DNA tangled torsion, allowing DNA polymerases to continue splitting the double-helical strands. In fact, Tops are enzymes acting on genomic stability and DNA topology. According to their structures and mechanism of actions, Tops are classified into type I and II families. Members of type I (Top I) are ATP-independent enzymes and involved in DNA single strand cleavage and rotation, and are divided into two subfamilies (IA and IB) with different structure (amino acids sequence) and mechanism of action (ablating DNA binding site). In eukaryotes, there are two genes encoding subfamily IA Tops for expression of two isoforms (3α and 3β) or (III α and III β). In addition, two putative isomers were identified for subfamily Top IB, i.e., 4α and 4β or IVα and IVβ. It is worth mentioning that Top 3α (subfamily IA) is involved in genomic stability *via* homologous recombination accomplishment[5].

On the other hand, members of type II Tops (Top II) require divalent metal ions for their cleavage activity. Only a single Top II is identified in prokaryotes (bacteria and archaea) and lower eukaryotes (Protists), whereas higher eukaryotes (Metazoans) express two types (IIA and IIB). They have high sequence similarities, but with different expression patterns, and cellular functions. While type IIA is involved in DNA replication and chromosome segregation, type IIB elaborates only in neural development[6]. In contrast to members of Top I, those of Top II need ATP hydrolysis to break both DNA strands. To achieve its catalytic activities, it requires Mg^{2+} facilitating interaction with its substrate. Similarly, there are putative isoforms for Top IIA (5α, and 5β or Vα and Vβ), and Top IIB (6α, and 6β or VIα and VIβ)[7].

In kinetoplastids, kinetoplast DNA (KDNA) consists of several mini-circles and few maxi-circles organized as a massive chain network to be connected within the flagellar basal body. Prior to DNA replication, KDNA network is condensed into disk-shaped structure. To stabilize its architecture, a mitochondrial kinetoplast-associated protein (KAP) is expressed to promote Top II mediating KDNA de-concatenation within the disk-shaped network. A mitochondrial high mobility group (HMG) box-containing protein, termed KAP6 was identified and characterized in *T. brucei*. Results confirmed the essential role played by trypanosomatids KAPs in KDNA repair and maintenance, and subsequently in parasite survival. Due to lack of a similar DNA network in the mammalian hosts, the investigators proposed KAPs as promising drug targets[8]. On the other hand, gyrase, a type II Top previously recognized in prokaryotes, was identified in apicomplexans playing an essential role in apicoplast DNA topology. Due to its absence in human, it is a promising drug target in *Plasmodium* spp.[9], and *T. gondii*[10].

It is worth mentioning that Top inhibitors are classified into two categories, I and II including Top poisons, and catalytic inhibitors, respectively. Both TopIB and Top II inhibitors are validated anti-neoplastic drugs in human medicine[7].

3. Histone variants

All eukaryotes have a DNA complex, known as chromatin; and histone with its variants (H2A, H2B, H3, and H4) are essential components of the nuclear chromatin. The latter components prevent DNA damage during cell division, and regulates DNA replication, and gene expression. Notably, histone variants are substitutes for the established core histone in nucleosomes. Two main differences were characterized. First, established histones are replication-dependent expressed during the S-phase, while variants are replication-independent expressed molecules during the whole cell cycle. Second, genes encoding the established histones are present in multiple copies with conserved gene sequencing among species. In contrast, single-copy genes, showing high degree of variation among species, encode histone variants. Therefore, multicellular organisms possess a large number of histone variants that confer different specific structural and functional features[11]. Maintained by the National Center for Biotechnology Information, Histone DB 2.0 with variants (www.ncbi.nlm.nih.gov/research/HistoneDB2.0/) is an online database. The histone variant that has the highest number of variants is H2A,

in which H2A.Z is the variant involved in DNA repair and transcription. In contrast, H2B has a limited number of variants and they are involved in spermatogenesis. The most common H3 variant in all eukaryotes is H3.3 that plays an essential role in maintaining genome integrity during DNA replication. In addition, H4 is one of the slowest evolving proteins with no functional variants in the majority of species[12]. Identification of histone variants that substitute established core histone in parasites during stressful conditions definitely lead to recognition of potential novel drug targets[13].

4. Histone modification enzymes

Acetylation and deacetylation enzymes using histone acetyltransferase (HAT), and histone deacetylase (HDAC), respectively regulate DNA expression. Notably, HDACs remove acetyl group from histone permitting histone to tightly wrap DNA and inhibit DNA expression. In such process (acetylation-deacetylation), histone-modifying enzymes change gene' expression activity during cell divisions and differentiation without altering gene sequence. Accordingly, genes encoding HATs and HADCs are epigenetic markers utilized for gene therapy utilized in cancer therapy[14], while their inhibitors were proposed as promising anti-parasitic drugs[15].

According to their subcellular localization, HATs have two categories. Class (A) HATs are nucleolarly located to regulate gene expression *via* classical acetylation of nucleosomal histones. Families of this class contain bromodomains that bind the acetylated lysine residues to the histone substrates. Activation and increasing gene expression is the single role assigned for class (A) HATs. The most common families reported in intracellular protozoa are MYST, Gcn5-related N-acetyltransferases (GNATs), and transcription initiation factor II (TAFII250). Interestingly, MYST family is named after its four founding members MOZ, Ybf2 (Sas3), Sas2, and Tip60. They are characterized by the presence of zinc fingers and chromodomains employed in acetylation of lysine residues on H2A, H3, and H4 histones[16,17] (discussd later). In contrast, class B are cytoplasmic HATs employed in acetylation of newly synthesized histones before conjugation into nucleosomes. Once the latter enter the nucleus, the acetyl group is removed by HDACs to be incorporated into chromatin[18].

On the other hand, HDACs are grouped into four classes according to their sequence. Classical HDACs (1-11) includes classes I, II and IV with similar catalytic domains having zinc as cofactor, whereas class III include only sirtuins (SIRs). Interestingly, SIR (silent information regulator) is a cell nucleus enzyme with the essential function of deacetylation of transcription factors that contribute in cellular regulation required for survival under stress conditions. It is also known as SIR2-like genes that encode SIR2 described in *T. brucei, T. cruzi, L. infantum* and *P. falciparum*. They are involved in DNA repair (cellular responses to DNA-damaging agents), and parasite proliferation with life cycle stages differentiation[19].

In addition to schistosomes' HATs, HDACs, and SIR2, several histone demethylases (HDMs) were recently identified with a crucial role in cercarial transition to schistosomula, and in sexual differentiation in adult worms. Utilizing molecular docking studies, the investigators selected one of them (*Sm*p_034000) and identified its predictive specific site. A specific *Sm*HDM inhibitor significantly exhibited *in vitro* potency against adult worms and schistosomulae. Ultrastructural results revealed marked reduction in adult motility and viability as well as loss of muscle fibers architecture. It was concluded that *Sm*p_34000 inhibition should undergo further evaluation and validation as a novel anti-schistosomal drug[20].

Histone post-translational modifications (PTMs): A wide range of enzymes have an essential role in the epigenetic (i.e., non-genetic influences on gene expression) regulation of gene expression including alteration of histone PTMs or nucleosomes reconfiguration. The complexity of life cycle, i.e., multiple stages and different host tissues, obligates parasites relying on epigenetic control strategies to fine-tune gene expression facilitating rapid adaptation, survival, and growth in different stressful environments. This simply means that PTMs work together to elicit changes in gene expression in response to stress. While parasite PTMs recognize acetylated lysine residues *via* bromodomains (amino acid domains that recognize the N-terminal tails of histones), they recognize methylated lysines utilizing chromodomains (amino acid residues associated with chromatin' remodeling and manipulation). Moreover, they recognize arginine on histone through zinc fingers (fingerlike loop of peptides enclosing a bound zinc ion at one end to regulate transcription)[21].

5. Translation for protein synthesis

The translation process describes how a protein is synthesized from the information carried by messenger RNA (mRNA). The mRNA sequence is recognized by its genetic code, and it is translated into the 20-letter code of amino acids, i.e., the building blocks of the target synthesized protein. The genetic code is known to be a set of three-letter combinations of nucleotides; adenosine (A), uracil (U) and guanosine (G) called codons, e.g., AUG. The translation occurs in ribosomes, which are the machine for proteins synthesis composed of several ribosomal RNA molecules with small and large subunits. Translation process occurs in three stages: initiation, elongation, and termination. To initiate translation, both the small and large ribosomal subunits as well as the transfer RNA (tRNA) bind to the start codon (AUG) of the mRNA sequence forming the complete initiation complex. Elongation stage starts when ribosomes translate each codon,

add each corresponding amino acid to the growing chain, and link them *via* a peptide bond. Elongation process continues until all codons are translated. The last stage is termination when the ribosome reaches a stop codon, i.e., codons not recognized by tRNA. At that point, the ribosome recognizes that translation is complete[22]. Therefore, initiation and elongation factors are promising drug targets.

Initiation factors: In eukaryotes, synthesis of DNA and RNA copies is a multi-step process that requires tight regulation of translation initiation. It is carried out through either cap-dependent or cap-independent processes, i.e., in stress conditions. Notably, mRNA has its 5` cap ending with a methyl guanosine cap, while its other end has a polyA tail that interacts with initiation factors. These factors also bind with the 5` cap ending recruiting eukaryote initiation factor 4 family (eIF4s; A, E, and G). In the cap-dependent process, eIF4E in synergy with helicase eIF4A and the scaffolding protein eIF4G binds to mRNA. Once assembled on mRNA, they allow recruitment of ribosomes for translation initiation, i.e., transfer RNA (tRNA). In PTMs, phosphorylation regulates and controls eIF4s function. On the other hand, the cap dependent process is blocked during stress, and two major pathways either upstream open reading frame (UORF) or internal ribosome entry site (IRES) are identified. To initiate gene expression in response to stress, the 40S ribosomal subunit is recruited instead of the cap, to recognize the first codon to be bound at the mRNA 5′ end. Both pathways recruit the ribosomal subunit either directly (UORF), or inside an IRES. The IRES pathway, regulated by conserved IRES trans-acting factors (ITAF), is observed in translation of circular RNA (circRNA). The latter is a recently identified class of mRNA recognized for post-transcriptional regulation[23].

Elongation factors: Eukaryotic elongation factor 2 (eEF2) is an essential member of GTP-binding translation elongation factor family encoded by *ef2* gene. Its crystal structure identification enabled the investigators to identify its functional properties[24]. It was reported that EF2 catalyzed the translocation of tRNAs and the mRNA after its peptidyl transfer on the 80 S ribosome; i.e., continuation of translation process for protein synthesis. The process is terminated by EF-2 kinase phosphorylation, while EF-2 inactivation of EF-2 by inhibitors or toxins terminates protein production[25].

[II] Survival

1. Protein kinases (PKs)

They are enzymes essentially required in protein phosphorylation involved in several signaling pathways that transmit cellular signals in all eukaryotes. In such a process, PKs regulate protein functions involved in parasite growth, differentiation, stress response, and apoptosis. They are two types; eukaryotic (ePKs) and atypical (aPKs) with the main difference of lacking the kinase motifs in all ePKs members, and the presence of two motifs in aPKs. Eukaryotic type is classified into several groups including tyrosine kinase (TK), mitogen activated PK (MAPK), calcium/calmodulin-regulated kinase (CAMK), calcium-dependent PK (CDPK), cell kinase I (CK1), NimA-related kinase (NEK), TK-like (TKL), and cyclin-dependent kinases (AGC group). The AGC group is termed so for including cyclic adenosine, guanine, and cytosine MP-dependent PK (PKA, PKG, and PKC, respectively). However, PKB is related to both PKA and PKC and it was termed AKT. In addition, there is a classified group termed "others" including other PKs that do not fit in the previously classified groups[26].

Severity of *falciparum* malaria is attributed, partially, to its ability to export several proteins into infected RBCs causing major structural and functional changes. Atypical kinases (FIKKs) family is unique in *P. falciparum* with no orthologues in humans and utilizes gene knocking out techniques. Eight FIKK members were identified as potential drug targets. Several roles were assigned for FIKKs including growth and survival *via* mitotic nuclear division, merozoites egress and de novo RBC invasion, as well as their role in RBC adhesive properties and knob morphology[27]. In addition, atypical PK (Riok-2) involvement was essentially identified in RNA biogenesis and cell cycle processes in schistosomes. Its transcription level was localized in the vitellarium and ovary, and its bioinformatics analysis revealed possession of glycosylation sites. The investigators recommended that application of CRISPR technology combined with virtual screening would accelerate development of novel anti-schistosomiasis drugs[28].

Recently, a study observed that AKT has three conserved domains, among them was the N-terminal pleckstrin homology (PH) domain which is a protein domain (~120 amino acids) involved in intracellular signaling through binding with phosphatidylinositol lipids within the plasma membrane. By such interaction, it recruits glycogen synthase kinase-3 (GSK-3), as a substrate, required for cellular signal transduction pathways. In all eukaryotes, phosphoinositide 3-kinase/AKT (PI3K/AKT) pathway is a tightly regulated process with essential roles in cell cycle survival, growth and proliferation. However, PI3K/AKT pathway is conservative in pathogenic kinetoplastids, i.e., controlling cell growth in stressful conditions. Utilizing molecular docking studies with virtual screening of compounds that have high inhibitory efficacy against PI3K/AKT pathway, a recent study concluded that GSK-3 inhibitors could serve as a novel anti-leishmanial drug[29].

2. Protein lipidation (protein-lipid interaction enzymes)

In eukaryotes, protein lipidation or fatty acids acetylation is the process in which lipids and lipid metabolites, the major component of cell membrane, are generated to modify proteins required for signaling

transduction pathways. It includes N-myristoylation and S-palmitoylation utilizing N-myristoltransferase (NMT) or palmitoyl acyltransferase (PAT), respectively. Both types are dynamic enzymatic reactions in which fatty acids, either myristate or palmate, is covalently linked to the N-terminal glycine residue, i.e., cysteine, or less frequently serine and threonine, of the cell membrane protein. Both enzymes are involved in PTMs essentially necessary for target protein configuration and subsequent signal transduction pathways in the cell membrane. Configuration includes conformation, stability, trafficking, localization, and binding affinity for cofactors[30]. Accordingly, the major role of NMTs and PATs is facilitating the initial transient interaction with membrane lipoproteins. Subsequently, they influence protein-protein interactions, and enhance cross talk (signaling transduction) between cell membrane and other cellular organelles (Golgi apparatus, endoplasmic reticulum, mitochondrion, and endosome). Both enzymes also trigger and enrich the activity of PKs required for signaling pathways, e.g., PKC, MAPK and AKT[31].

A British review[32] discussed NMTs as potential drug targets in intracellular parasites. Like other protozoa, *Plasmodium* spp. possess a single NMT isoform for N-myristoylation of several proteins involved in PTMs. These included glutamate receptor' associated protein-1 (GRASP1) for Golgi functions, ADP-ribosylation factor-1 (ARF1) for trafficking, glideosome associated protein (GAP45) for host cell invasion, CDPK1 and calpain for membrane localization during life cycle stages regulation and progression, and adenosine kinase (AK) for energy metabolism. Notably, GAP45 was reported to be involved in parasite egress, motility, and invasion in *T. gondii*. Similarly, D-ribosylation factor like protein-1 (ARL1), and ARF1 were N-myristoylated for trafficking in *Leishmania* spp., and *Trypanosoma* spp., respectively. Other targeted proteins included calpain-related cytoskeleton associated protein (CAP5.5), flagellar calcium binding protein (FCaBP), and hydrophilic surface protein (HASP) involved in morphogenesis, motility, and host cell invasion, respectively. In addition, cleavage of trypanosomatids metacaspases (MCAs) involved in programmed cell death (PCD) were also N-myrisolyated. Remarkably these described proteins are localized at the plasma membrane, mainly of the infective stages[32]. Later, a recent review concluded that NMT inhibitors serve as efficient drugs in cancer and diseases caused by proliferating intracellular protozoa, i.e., *Plasmodium* spp., pathogenic trypanosomatids, and *T. gondii*[33].

Glycosylphosphatidylinositol (GPI) anchoring is another form of N-myristoylation in pathogenic trypanosomatids, but without utilizing NMTs. Kinetoplastids possess several GPIs with a highly conserved GPI-anchor motif to be conjugated in the surface membrane complex. *Leishmania* GPIs are dynamically changed during stage transformation facilitating host cell immunoevasion. Therefore, N-myristoylation is involved during GPI-anchor synthesis by fatty acid remodeling, i.e. myristate replaces the existing fatty acids[34].

Unlike N-myristoylation, S-palmitoylation is a reversible process, and less common in intracellular protozoa. In spite of that, several PATs were identified. The majority of PATs are localized in the Golgi apparatus and endoplasmic reticulum however, some were identified in the plasma membrane, and endosomes[34]. Notably, PATs play a significant role in trafficking of virulence factors between membrane compartments. In this concept, they were identified in *T. brucei* (*Tb*DHHC 1–11), *P. falciparum* (*Pf*DHHC 1–12), T. gondii (*Tg*DHHC 1–18), and *C. parvum* (*Cp*DHHC 1–10). Apicomplexan PATs were mainly localized in the Golgi apparatus and endoplasmic reticulum. However, those localized in rhoptries were involved in secretion of factors necessary for parasite invasion[35].

3. Molecular machinery of PCD (apoptosis and autophagy)

Apoptosis is an essential phenomenon for normal development and survival in multicellular parasites, whereas its occurrence in unicellular protozoa seems strange since they have to evolve strategies to increase their replication, not death. Intracellular protozoa have to self-regulate their intensity of infection. This simply means that cell death is either a passive or active process. The first occurs due to extrinsic factors (e.g., treatment), leading to rapid irreversible necrosis and damage. In contrast, an active process occurs due to intrinsic factors (sense of infection intensity), leading to PCD, i.e., a regulated step-manner reversible process. If the parasite colonies are high enough to cause host or vector survival at risk, the best strategy for intracellular protozoa is to undergo PCD to maintain a sub-lethal density. In fact, theory of "let us commit suicide to proliferate" is a beneficial strategy since a higher number of apoptotic parasite cells lead to bigger benefits to survivors[36]. It was previously reported that *P. falciparum*[37], *Leishmania* spp.[38] and *T. brucei*[39] can determine the genetic diversity of their infections suggesting their ability to estimate the density or proliferation rate of their clone-mates.

Although cathepsins, the major cysteine protease (CP), contribute with MCAs in PCD in protozoa, MCAs were proposed as promising drug targets because their amino sequences showed low similarity to human caspases. Those of pathogenic trypanosomatids are the most common MCAs studied. Involvement of *Leishmania* MCAs in the signaling initiation for apoptosis pathway and activation of caspase-like proteins leading to PCD was reported[40,41]. Later, a study proposed the essential role of *T. gondii* MCAs in PCD[42].

On the other hand, autophagy or self-devouring is a natural, conserved degradation process to

remove dysfunctional cellular components through a lysosome-dependent regulated mechanism, allowing recycling of these components. In macroautophagy (the most common form of autophagy), cytoplasmic components (e.g. mitochondria) are targeted and isolated within a double-membrane vesicle, known as autophagosome. The latter fuses with an available lysosome to form autolysosomes that are degraded and recycled. Autophagy is an adaptive response to stress conditions promoting cell survival. Since autophagy promotes cell death, breakdown of cellular components leads to cell survival through maintaining energy levels. Autophagy is an essential process during parasite differentiation and transformation in intracellular protozoa. Expressed casthpsins have an essential role in activation of PCD machinery pathways, e.g., autophagosomes were demonstrated during stage differentiation in *Leishmania* spp.[43], as well as in chronic toxoplasmosis[44]. Regarding the last-mentioned study, the investigators observed that expression of *T. gondii* cathepsins in bradyzoites contributed in autophagosomes turnover in the vacuolar compartment leading to tissue cyst survival. Since death of bradyzoites lacking cathepsins was preceded by accumulation of undigested autophagosomes, the investigators concluded that longer parasite persistence in chronic toxoplasmosis was attributed to upregulation of cathepsins expression for significant proteolysis[44].

In addition, *Leishmania* spp. possess two autophagy related genes (*atg*) expressing ATG4.1 and ATG4.2 that were essential for stage differentiation. It was demonstrated that ATG4.2 expression, not ATG4.1, increased parasite virulence through its significant role in autophagy, without evidence of involvement in PCD[46].

4. Mitochondrial electron transport chains (cytochrome complex)

In eukaryotes, mitochondrial protein complexes (MPCs) transfer electrons from electron donors, i.e., nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH), to electron acceptors through oxidative phosphorylation processes. Electrons transfer through MPCs provides oxygen required for energy that creates an electrochemical proton gradient and initiates ATP synthesis by coupling the oxidative phosphorylation with ATP synthase. Accordingly, mitochondrial electron transport chains are an enzymatic series of electron donors and acceptors; each donor passes electrons to an acceptor of higher redox potential, releasing energy from each enzymatic reaction. Subsequently, energy generates a proton gradient across the mitochondrial inner membrane[46]. On the other hand, a proton pump is an integral membrane protein pump that generates a proton concentration gradient across cell membrane. In cell respiration, the proton pump uses energy to transport protons from the mitochondrial matrix to its inter-membrane space. Proton pump forms a gradient to store energy for later use. While electron transfer is terminated with oxygen transfer from donors to acceptors in aerobic respiration, other lower-energy electron acceptors, e.g. nitrate, nitrite, ferric iron, sulfate, and carbon dioxide, are used in anaerobic respiration[47].

The MPCs, as proton pumps, are classified into four complexes. However, only complex III (cytochrome bc1 complex), and IV (cytochrome c-oxidase) comprise water-soluble proteins containing iron. Both hemeproteins belong to cytochrome c (Cyt c) family of proteins that play an essential role in the respiratory mitochondrial electron transport chain and cell apoptosis. While complex III is present in the inner mitochondrial membrane of all aerobic eukaryotes and the inner membranes of most eubacteria, complex IV is a large transmembrane protein complex found in bacteria and inner mitochondrial membrane of eukaryotes. In eukaryotes, Cyt c is a highly conserved small protein (~ 100 amino acids) with a motif that binds heme. The heme group of Cyt c accepts electrons from complex III and transports them to complex IV. Since iron oxides are converted from ferrous (Fe^{+2}) to ferric (Fe^{+3}) forms (and *vice versa*), Cyt c is able to process oxidation and reduction without binding oxygen. Although Cyt c is commonly associated within the mitochondrial inner membrane, it may be released into the cytosol to be involved in cell apoptosis. It initiates cell apoptosis through activation of caspases and MCAs[48].

Recently, Japanese investigators suggested mitochondrial cytochrome bc1 complex (Cyt c III) as a promising drug target for development of new therapeutic drugs against alveolar hydatid cysts. It was observed that the mitochondria of *E. multilocularis* had NADH-cytochrome c reductase (mixed complexes I and III) and succinate-cytochrome c reductase (mixed complexes II and III). Therefore, the investigators tested combined atovaquone (complex III inhibitor) and atpenin (complex II inhibitor) that showed complete eradication of protoscoleces *in vitro*. In addition, atovaquone significantly reduced primary alveolar hydatid cyst development *in vivo*[49].

5. Transporters

Active transport of molecules across the cell membrane is either primary or secondary. Primary type uses cell energy, powered by ATP, to transfer molecules passively against their concentration gradient. The common example is the Na^+/K^+ pump used in the transport of molecules with charged ions to be easily diffused. In contrast, secondary active transport utilizes an electrochemical gradient, i.e., not using the cell energy. Accordingly, membrane transporters are proteins involved in molecules' movements across the cell membrane either by primary through passive diffusion (i.e. pores, or channels) or secondary *via*

active transfer (i.e., carrier). While pores and channels are open to the extra- and intracellular environments at the same time allowing molecules diffusion without interruption, the inner and outer gates of the active transfer carriers do not open simultaneously to both environments. Carriers have receptors with specific binding sites, while pores and channels do not. Therefore, each carrier transporter is characterized by a designed structure to recognize only molecules with a high affinity to its binding site, while primary active transporters are pore forming proteins or water/ion channels. Millions of ions can pass/sec through pores and channels, while few (100-1000) molecules pass through a molecule' carrier. Defects mainly in carriers, less commonly in pores and water/ion channels, are associated with human diseases[50].

In facilitated diffusion, some molecules dissolved in water are not able to diffuse across cell membranes due to the hydrophobic nature of phospholipids. Notably, transmembrane carrier proteins used in primary transport are different from those used in secondary active transport. They are gated transmembrane channels; i.e. substrates are released into the cell without utilizing ATP energy. On the other hand, reverse transport is a phenomenon through which substrates of the membrane transporters are moved in the opposite direction. The common example is molecule' transfer after being phosphorylated by a particular PK for signaling pathway[50].

In this concept, the role and functions of aquaporins (AQPs), P-, V-type ATPases, and ATP-binding cassette (ABC) are simplified as examples of water and ion channels, proton pumps, and secondary active transporters respectively.

Water and ion channels

A family of small (~30 kDa) proteins, the aquaporins (AQPs) are expressed in all plasma membranes involved in fluid transport. They form pores facilitating water transport between cells by passive diffusion through the membrane phospholipid bilayer. There are ten characterized mammalian AQPs composed of water specific channels (e.g., AQP1, AQP2, AQP4, AQP5, AQP6, and AQP8) or aquaglyceroporins (GLPs) (e.g., AQP3, AQP7, AQP9, and AQP10). The latter transport water and other small- uncharged dissolved molecules including glycerol, ammonia, reactive oxygen species (H2O2), urea, gases (CO2, ammonia, and nitric oxide). It was observed that the overall structure of AQPs and GLPs is conserved over 2-3 billion years of evolution. Interestingly, genetic defects involving genes encoding AQPs was associated with several human diseases including nephrogenic diabetes insipidus and neuromyelitis optica[51]. A German report that reviewed structure, and function of water and solutes transport concluded that understanding the mechanism of AQPs, and GLPs would facilitate development of channel-modulating agents for therapy[52].

Structurally, classical AQPs consist of six α-helical transmembrane domains and five connecting loops. The N- and C-terminal regions of AQPs, as well as B and D loops, are within the cytoplasm. In contrast to mammalian AQPs, those in protozoa have a low number of isoforms, i.e., a self-protection parasite strategy reducing excessive antigen exposure, and avoiding host immune response system. Based on AQPs sequence, Chinese reviewers constructed a phylogenetic tree for AQPs in parasites. Results revealed 1) two distinct clusters, orthodox or classical AQPs, and GLPs, 2) the latter is divided into 4 subgroups; protozoa, trematode, cestode, and nematode, 3) each parasite possesses at least one multifunctional AQP, 4) *T. gondii* AQP showed 47% similarity to that observed in plants, with both water and glycerol permeability. The reviewers proposed AQPs and GLPs as promising drug targets for treatment of malignant malaria, leishmaniasis, and schistosomiasis. Therefore, they recommended further studies on parasitic AQPs to understand their mechanism of action, and to identify their crystal structure aiming to design new selective inhibitors[53].

An American review claimed that intracellular protozoa possess variable numbers of AQPs, *Plasmodium* and *Cryptosporidium* spp. (1), *T. gondii* (2), *T. brucei* (3), and *Leishmania* spp. (5). Except for *Leishmania* spp., all protozoan water channels are GPLs, i.e. bifunctional conducting both water and glycerol. While *Leishmania* AQP1 was involved in water, glycerol and other neutral solutes, the other four AQPs are classical water channels[54]. Since antimony and arsenic behave as inorganic molecular mimics of glycerol, a property that allows their passage through AQPs, drug resistance in leishmaniasis and African trypanosomiasis was attributed to mutations in the genes encoding AQPs, and/or GLPs, as previously reviewed[1].

On the other hand, neuromuscular function in helminths is mainly dependent on networks of ion channels. They are highly conserved proteins, with representatives in both parasites and their hosts. In 2014, an American scientist reviewed the use of helminth ion channels as drug targets with subsequent development of several anti-helminthic drugs, e.g., Ivermectin targeting glutamate-gated chloride channels, and Praziquantel targeting voltage-gated calcium channels. Ion channels form a gated pathway for charged ions to flow passively across the impermeable lipophilic cell membrane. Therefore, they are potential drug targets due to their role in fast neurotransmission[55]. Later, genomic studies of 258 eukaryotic pathogens revealed identification of gamma-aminobutyric acid (GABA) transporter in *S. mansoni, S. haematobium, T. spiralis,* and *Eimeria mitis*. The investigators demonstrated that drugs targeting GAPA transporter acted on parasite neurotransmission *via* binding to glutamate-gated chloride channels. The investigators proposed GABA transporters as potential

drug targets for development of novel alternative drugs against schistosomiasis[56].

P-type ATPases

This type is termed also ENA ATPase from Greek words "exitus natru" i.e., exit of Na^+. They are a large group of evolutionarily related ion and lipid pumps found in the plasma membrane of all creatures. The most common examples in eukaryotic cells are Na^+/K^+, and Ca^+ pumps, while plasma membrane proton pump (H^+-ATPase) is common in plants and fungi. They create the gradients required for uptake of energy sources and precursors for biosynthesis, and release of waste metabolites, i.e., primary active transporters[57]. All P-type ATPases have a core of six transmembrane-spanning segments (TSSs), termed transport domain. However, some P-type ATPases have an additional number of TSSs, termed support domain for specialized functions. According to their ion transport, P-type ATPases are classified into four subfamilies (P1-P4), while members of P5 subfamily are with unknown ion specificity. Notably, P5-ATPases are only found in eukaryotes, and divided into PVA, and PVB involved in regulation of homeostasis in the endoplasmic reticulum, and lysosomal membrane, respectively[58].

An American reviewer claimed that identification of the genes responsible for P-ATPases recognized in pathogenic trypanosomatids would encourage further studies to understand their structures and functions. The reviewer concluded that P-type ATPases require much advanced research for development of new therapeutic interventions against leishmaniasis and trypanosomiasis[59]. In this concept, due to similarity of the initially described P2 (Ca^+ pump) to Ca^{2+}-ATPases of the sarco/endoplasmic reticulum Ca^+ pump (SERCA) it is now termed ATP4. The latter was identified in plasma membrane of the endoplasm reticulum of intracellular protozoa. In fact, Na^+ extrusion from the intracellular protozoa is a challenge and a prerequisite for their survival and growth. Due to the essential role played by ATP4 in maintaining low cytosolic Na^+ concentration in apicomplexans and pathogenic trypanosomatids and its absence in mammals, two recent reviews proposed it as a promising drug target[60,61].

Sarco/endoplasmic reticulum membrane Ca^+ ATPases (SERCA): It is a Ca^+ P-type ATPase transporting Ca^+ from the cytosol into the endoplasmic reticulum. It is named after its first discovery as a membrane-bound structure found in the sarcoplasmic reticulum within muscle cells. In addition to its major role, proton pump for Ca^+ transfer during muscle relaxation, SERCA1 generates thermogenesis in adipose tissue and skeletal muscles. Notably, SERCA has three major isoforms (1, 2, and 3) that are expressed at various levels in different cell types. Additional post-translational isoforms of SERCA2 and SERCA3 were identified to increase the overall complexity of the Ca^{2+} signaling mechanism. It was demonstrated that SERCA contributed to *T. cruzi* invasion in cardiomyocytes. The efficacy of Thapsigargin, a drug that binds with SERCA ATPases causing removal of intracellular Ca^+ stores, was investigated. Results revealed 75% inhibition of cardiomyocytes' invasion[62].

V-type ATPases

To acidify intracellular compartments, vacuolar ATPases, a family of ATP-dependent proton pumps, are localized within the membranes of endosomes, lysosomes, and Golgi-derived vesicles. Notably, pH hemostasis is crucial in several essential processes such as proteases' activation, apoptosis, intracellular membrane trafficking, and toxin delivery. Moreover, the ability of several pathogens to invade cells is dependent upon V-ATPase activity[63].

ATP-binding cassette (ABC)

They are multi-domain membrane proteins utilizing the energy of ATP hydrolysis to transfer molecules across cellular membranes in all creatures. Prokaryotes and eukaryotes possess an additional group of ABC non-transporter proteins, located in the cytosol for gene regulation and mRNA translation control. It is the largest transport system superfamily attracting much attention in medical researches. They play a crucial role in several cellular processes including maintenance of osmotic homeostasis, nutrient uptake, resistance to cancers and pathogenic agents, antigen processing, cell division, cholesterol and lipid trafficking, and developmental stem cell biology resistance. The most common example of ABC system is P-glycoprotein multidrug resistance transporter (MDR1, or ABCB1). Mutations in the genes encoding human ABC transporter cause a number of genetic diseases, cystic fibrosis is the most well- known[64].

Structurally, they have two highly conserved cytosolic ATP binding domains, known also as nucleotide-binding domains, and two less conserved core transmembrane domains. Based on sequence homology, and gene structure of both domains, mammalian ABC transporters are classified into seven classes termed ABCA to ABCG. It is worth mentioning that classes ABCE and ABCF are non-transporters, without nucleotide-binding domains. Except for the latter, all ABC transporters are involved in cellular import of the required nutrients and molecules, and export of waste metabolites, toxins, and drugs. To achieve their function, they utilize their phosphate-binding loop (Walker A), and magnesium binding site (Walker B) of the nucleotide-binding domains. Besides, there are three conserved motifs; a signature motif specific to the ABC transporter, a histidine loop or switch region, and a Q-loop that interacts with the phosphate-binding protein through a water bond. Two additional loops are also observed in some ABC transporters, A-loop containing a conserved aromatic residue (tyrosine) to stack ATP with the adenine ring, and a D-loop to form the site for ATP hydrolysis. A

correlation between their functional characterization and phylogenetic classification was documented, hence, more than 50 subfamilies were described for ABC importers and exporters. In their review, Beek *et al.*[65] claimed that identification of the crystal structures of ABC transporters revealed a remarkable diversity with suggestive significant mechanistic diversity. Understanding the mechanism involved in the active transport played by ABCs system accompanied by identification of their binding site would definitely lead to development of specific selective inhibitors[65]. A classification based on sequence homology of only the nucleotide binding domains was recently proposed[66].

In 2014, ABC multidrug transporters in helminths were reviewed, and it was suggested that combination of anti-helminthic drugs with inhibitors of ABC transporters, for short term, could overcome resistance. The reviewers observed that P-glycoprotein (Pgp), classified as ABCB1, is implicated in drug resistance in mammalian cells, and ivermectin is a substrate for mammalian and nematodes Pgps. Notably, helminth ABC transporters play a major role in modulating drug susceptibility in addition to other functions, e.g. excretion, reproduction, and modulation of host responses[55]. Later, utilizing *in vitro* resistance selection studies, validated by CRISPR/Cas9, and conditional knockdown assays, the investigators identified point mutations in the *P. falciparum* ABCI3 as a pleiotropic mediator of anti-malarial drug resistance. This was followed by investigating the efficacy of ABC inhibitors, but unfortunately, none showed promising efficacy. Accordingly, the investigators recommended further studies identifying ABC13 structure and its potential endogenous functions for better understanding the mechanism underlying mediation of ABC13 point mutation in drug resistance[67].

Apicoplast transporters: Except for *Cryptosporidium* spp., all apicomplexans have an apicoplast, a stunted derived chloroplast from their ancestors. Since *Plasmodium* spp., and *T. gondii* store their energy in granules located in their cytoplasm, apicoplast transporters transfer nutrients, and other required molecules for trafficking *via* intra-vacuolar membranous network (IVMN). This means that the driven chloroplast has an essential function other than photosynthesis. Besides, apicoplast is involved in isopentenyl pyrophosphate synthesis. This explains three facts; 1) drugs targeting apicoplast for treatment of malaria and toxoplasmosis are not effective in cryptosporidiosis, 2) drug resistance is linked to gene mutation in the genes encoding apicoplast transporters, as previously reviewed[1], and 3) both apicomplexans are *in vitro* grown in isopentenyl pyrophosphate-rich medium[68]. A Swiss review was published recently discussing the role of apicoplast transporters and their potentiality as drug targets in *Plasmodium* spp. and *T. gondii*. It is worth mentioning that parasitophorous vacuole membrane (PVM) functions as a molecular sieve for proteins and metabolites transport to be distributed *via* IVMN to parasite cytosol, endoplasmic reticulum, Golgi apparatus, and mitochondrion. Metabolic pathways within apicoplast, targeted by several antibiotics such as clindamycin and doxycycline, confirmed its suitability as a potential drug target. Moreover, PV possesses two drug resistance transporters termed chloroquine resistance (CRT), and MDR[69].

[III] Pathogenesis and virulence

1. Proteases

Proteases or peptide hydrolases are enzymes involved in proteins catabolism through cleavage of their peptide bonds. According to their capability of formation of transient covalent bond, two broad types are known. Metalloproteases (MPs) and aspartyl-proteases catalyze proteins without formation of covalent bond through use of the activated oxygen of a water molecule with the aid of a metal cation or without it, respectively. Proteases that form a transient covalent bond use the catalytic oxygen of serine (SPs) or threonine, or the sulfur of an essential cysteine (CPs). The Welcome trust foundation, MEROPS is an online updated database for classification, characterization and structural properties of all identified proteases and their inhibitors[70].

Among proteases, CPs attracted much attention in several publications during the last two decades. They include several clans; however, clan CA includes the major CP (cathepsins) belonging to family C1. Among clan CD, two families are assigned for legumains (C13), and caspases (C14). Family C13 includes two CPs that play important roles in parasite-host interactions: Asparaginyl endopeptidase (AEP) or true legumains for tissue degradation, and GPI–protein transamidases. Pathogenic trypanosomatids possess surface-bound GPI-anchored proteins, and through their proteolytic activity, the latter CPs bind to host cell-surface receptors facilitating host cell invasion in addition to their role in immunoevasion[71]. On the other hand, C14 members are included in C14A (helminth caspases) and C14B (protozoal MCAs)[72].

All intracellular protozoa utilize proteases proteolytic activity for nutrition, biosynthesis and differentiation of life cycle stages, migration and host cell invasion, egress and *de novo* invasion cascade, apoptosis and autophagy, and immunomodulation of host immune responses[73]. Proteases established as virulence factors, and hence potential drug targets were reviewed. Several proteases were identified in *E. histolytica* (CPs 2, 5 and 7; and one leishmanolysin homologue as MP), *Leishmania* spp. [(CPs (A-C), MPs (leishmanolysins 1 and 2), and a SP (prolyl oligopeptidase)], *Trypanosoma* spp. (oligopeptidase B and prolyl oligopeptidase as SPs), and *T. vaginalis* [(CPs (CP65 and legumain-1), and MPs (gp63-like MP and aminopeptidase P)]. Only one CP was established

as virulence factor in each of *N. fowleri* (cathepsin B), *G. lamblia* (giardipain-1), *Cryptosporidium* spp. (cryptopain), and *Blastocystis* spp. amoebic forms (cathepsin B)[74]. Although no protease was established as a virulence factor in *Plasmodium* spp., Mishra *et al.*[75] reviewed proteases arsenal and proposed 12 proteases as potential drug targets. They included falcipains 1 and 2, serine repeat antigens 5 and 6, and dipeptidyl aminopeptidase I (CPs), plasmepsins II and V (aspartyl), alanyl and leucyl aminopeptidases, and DPAP III (MPs), HsIV (threonine), and a subtilisin-like protease-1 (SUB1). The reviewers assigned several biological processes for the proposed proteases including hemoglobin degradation, synthesis of proteins required for stage growth, survival and differentiation, and egress-invasion cascade. On the other hand, SUB1 has an essential role in processing proteases required for egress-invasion cascade. It is worth mentioning that serine repeat antigens 5 and 6 possess CP motifs, but its cysteine catalytic site is replaced by a serine. Interestingly, the crystal structure of plasmepsins II, and V showed conserved unique features with relative evolutionary divergence from host' aspartyl proteases that increased their potentiality for being drug targets. *Plasmodium* HsIV also showed high similarity and conservancy in all species with unknown sequence analog in humans. Besides, the crystal structure of *Plasmodium* SUB1 showed scissile bond allowing unusual interaction of its active site with substrate residues. Later, Sojka *et al.*[76] proposed plasmepsin III, previously known as histo-aspartyl protease (HAP), a potential drug target due to its high expression in merozoites' digestive vacuole.

Cruzipain (CRZ), previously known as a glycoprotein antigen (GP57/51), is the major cathepsin in *T. cruzi*. It displays dual cathepsin specificity (L and B) with several encoding genes (up to 130) giving rise to isoforms with varying degrees of similarity[77]. Due to its significant role in *T. cruzi* survival and virulence in both the host and vector, CRZ was suggested as a promising drug target and vaccine candidate. Its crystal structure was identified and several studies were conducted for development of selective CRZ inhibitor (reviewed by Abaza[78]). A combination of two synthesized oxyguanidine analogs (WRR-483, and WRR-669) was established as an approved novel drug for chronic Chagas disease[79,80].

2. Endogenous protease inhibitors

(a) Cystatins

Cystatins (CYSs) are a family of intracellular CP inhibitors, extensively expressed in all creatures. They share a sequence homology and a common tertiary structure. They are reversible, tight-binding ICPs that inhibit parasite CPs belonging to protease families C1 (papains) and C13 (legumains). Besides, CYSs have other functions including inhibitory effect on host's CPs, immunoevasion of host immune response, and regulation of hemoglobin degradation. According to MEROPS, three types are assigned and belong to clan IH, family 125 (A-C). Type I, stefins are intracellular CYSs with a single-chain of polypeptides that have neither disulfide bonds nor carbohydrate side-chains. Type II, cystatins are single-chain proteins containing two conserved disulfide bridges. They are expressed in body fluids and human tissues. There are seven types however; CYS-C attracts much attention due to its importance in assessing kidney functions. In addition, they are expressed in all helminths, but they are identified in extracellular protozoa, e.g., free-living amoeba, *G. lamblia*, and *T. vaginalis*. Type III CYSs, kininogens are the most complex type because it contains three type 2 domains. They are secretory proteins involved in the systemic protection against leaking endolysosomal CPs, as well as both innate and adaptive immune responses. Type IV, chagasins (Clan JL, family 142) are CYS-like proteins, and are expressed in apicomplexan, pathogenic trypanosomatids and *E. histolytica*[70].

In addition to their main role, i.e., regulation of CPs expression, CYSs interfere with host immune responses including antigen processing and presentation, immune cells migration, T-cell proliferation, toll-like receptors' activation, cytokines secretion, and nitric oxide production. Type II CYSs are expressed in nematodes, with emphasis in filarial nematodes, to establish active long parasitism due to their significant contribution towards immunosuppression of their hosts[81]. Later, a Chinese study demonstrated high expression of type II CYS on the surface of male and female *D. immitis* adults using an immunolocalization study. The investigators recorded that *Di*CYS inhibited canine T-cell proliferation, increased IL-10, and reduced IL-12 and TNF-α expression. It was concluded that *Di*CYS was an immunoevasion molecule that modulated inflammatory responses, and had an essential role in host–parasite interactions[82].

Interestingly, a recent American review concluded that helminth' CYSs are immunomodulatory molecules. The experimental and clinical studies were reviewed, and it was documented that developed countries with high hygienic environment reported increased cases with irritable bowel diseases, rheumatoid arthritis, and allergic diseases. In contrast, few cases were reported in developing countries with low sanitation facilities. Hence, the therapeutic strategy of using helminth' CYSs as immunomodulatory agents against aggressive inflammatory immune responses in chronic inflammation associated diseases[83].

(b) Serpins (Srps)

According to MEROPS classification, Srps belong to family 14 (Clan ID)[70]. They are structurally related proteins of SPs inhibitors endogenously expressed in parasites to regulate SPs expression purposing three functions. They have an essential role against host SPs and immune response, i.e. immunoevasion.

They increase SPs involved in proteolytic degradation during invasion and migration and are expressed to avoid own proteins degradation. Several Srps were characterized in helminths with a separate function in each. In the free living nematode *C. elegans*, Srps have a vital role for the adult' survival through preventing host lysosomal protease' injury[84]. In the whipworm *T. muris*, it was observed that SP identified in *Trichuris* spp. degraded mucin in the mucus barrier. The investigators demonstrated that endogenous SPI (*Tm*Srp) was highly expressed in adults to suppress host Th2 immune response responsible for protecting mucin barrier *via* increased expression of host Srps[85]. Recently, a study was conducted to elucidate the role played by *T. spiralis* Srps, highly expressed in the excretory/secretory products, in host immune response immunomodulation during early trichinosis. The investigators demonstrated that *Ts*Srps initiated suppression of host immune response in early trichinosis to maintain adult worms' survival during intestinal infection. Application of T. spiralis-related proteins as therapeutic drugs against acute inflammatory disease was recommended[86]. Similarly, Srps identified from *H. polygyrus*, a mice nematode, was found exhibiting potent inhibitory activity against IL-33 responses in a murine model of asthma. The investigators also recommended used of nematodes' Srps as promising drugs in treated allergic diseases mediated by human IL-33[87].

In *C. sinensis*, Srps were highly expressed in metacercaria for invasion and migration[88]. In *S. mansoni*, high expression was detected in adults to avoid own degradation. Lopez Quezada *et al.*[89] demonstrated *Sm*Srp role in preventing internal damage of adult' proteins in accidental release of cercarial elastase (CE) in adult acetabular glands. It is worth mentioning that CE is an essential enzyme involved in host skin penetration. Besides, an Australian study identified the gene encoding *S. japonicum* Srp (*Sj*B6), and had it cloned and expressed. It was reported that *Sj*B6 amino acid sequence shared low homology with other known Sers and was highly expressed in eggs. The investigators suggest a potential role in disease pathogenesis[90]. Later, the investigators demonstrated that *S. japonicum* Srp had essential role in decreasing the trans-endothelial permeability in either *Sj*Srp transfected or treated endothelial cells *in vitro*. It was concluded that Srp exhibited inhibitory activity against host' tight junction proteins for migration of schistosomula and juvenile flukes[91]. Recently, a study identified seven different Srps in *F. hepatica*, and succeeded to characterize *Fh*Srp1 and *Fh*Srp2, highly expressed on the surface of the invasive newly excysted juveniles. The investigators hypothesized that their main role was protection of the own proteins from the proteolytic effects of the highly SPs expression during migration and invasion. Moreover, they demonstrated that both recombinant *Fh*Srp1, and *Fh*Srp2 exhibited potent inhibitory activity against kallikreins, and chymotrypsin, respectively. It is worth mentioning that kallikreins are human SPs, and chymotrypsin is a pancreatic enzyme involved in proteolytic degradation. Based on the obtained results, the investigators suggested their role in regulating host immune response favorable for the parasite survival during migration and invasion. Using *Fasciola* Srp as a vaccine candidate to control fasciolosis was suggested[92].

3. Heat shock proteins (HSPs)

They are stress proteins, also known as chaperon proteins, expressed upon exposure to several stress conditions such as temperature increase, oxygen deprivation, pH extremes and nutrient deprivation. They are categorized according to their molecular weight (MW) and/or their location, e.g. cytosolic, mitochondrial, and endoplasmic reticulum. While HSPs with low MW (10-60) are involved in classical protein folding, HSP40-HSP70 system stabilizes peptides in a linear, unfolded state and delivers them to the HSP10-HSP60 system. Members of HSP90 family are found predominantly in the cytoplasm mediating the folding of specialized proteins such as steroid receptors and protein kinases. The larger HSP100 is engaged in thermal tolerance, disaggregation and unfolding of aggregated proteins for enzymatic digestion[93].

The majority of HSPs are fundamental for parasite survival, viability, growth, and life cycle stages differentiation, as well as pathogenesis and virulence. Therefore, they are promising drug targets. On the other hand, those involved in elicitation of humoral and/or cellular immune response are proposed as adjuvants with a vaccine candidate or as a serodiagnostic marker. There was a significant association between HSP70 and HSP100 expression and the severity of toxoplasmosis[94] and leishmaniasis[95], respectivly. Both encoding genes were established as virulence factors, hence potential drug targets. Notably, HSPs 70 and 90 deserved much attention due to their essential role in diseases pathogenesis of intracellular protozoan. In a study conducted in 2015, South African investigators performed sequence and motif analyses of more than one humdred HSP90s of different protozoa, helminths, vectors, and human using large-scale bioinformatics. It was concluded that cytosolic HSP90s showed the highest conservative sequence and motif in all intracellular protozoa[96].

It is worth mentioning that *P. falciparum* has a proteome with a majority of asparagine (Asn) residues in its amino acid repeats. With increased temperature in malaria, Asn-rich sequences leads to increased aggregate formation that recquires HSPs funtion. Besides, the capacity of *P. falciparum* to grow and thrive depends on its ability to export ~5% of its encoded genome. Membrane' trafficking requires an additional element, known as a *Plasmodium* translocon of exported proteins (PTEX), and HSP101 is one of its components[97].

Interestingly, HSP70 was utilized for diagnosis and species identification of several parasitic diseases. To identify *C. parvum* in clinical samples, a nanotechnology technique was developed using gold nanoparticles targeting *C. parvum* oocysts HSP70[98]. Similarly, gold nanoparticles functionalized with anti-HSP70 monoclonal antibodies were bound to labeled recombinant *P. falciparum* HSP70 and showed significant results in detection of malaria antigen[99]. Furthermore, using the sequence analysis of hsp70 gene in different *Leishmania* species, a study succeeded to identify primers designed as restriction enzymes for PCR-RFLP protocol that was validated and recommended[100]. Phylogenetic analysis utilizing *hsp70* gene sequencing received much attention and was investigated in several protozoa such as *T. vaginalis*[101], *Trypanosoma* spp.[102], and *Leishmania* spp.[103]. Functions and applications of HSPs in diagnosis, species identification, treatment and protection against several parasitic diseases were previously reviewed[104,105].

4. Glycoproteins (GPs)

These are proteins that contain oligosaccharide chains (glycans) covalently attached to amino acid side-chains. Usually, they are important membrane proteins since they play a role in cell-cell interactions. It is important to distinguish endoplasmic reticulum-based glycosylation of the secretory system from cytosolic-nuclear glycosylation. Functionally, glycosylation that occurs in the latter is reversible, and GPs are utilized as an additional regulatory mechanism controlling phosphorylation-based signaling. In contrast, classical secretory glycosylation that occurrs in endoplasmic reticulum and Golgi apparatus is an irreversible process essentially required for specific function, i.e. most likely directd to its role in host-pathogen interactions. Although different types of GPs are known, the most common are those involved in the composition of antibodies and hormones[106].

Surface GPs are established virulence factors in all species of *Leishmania* and *Trypanosoma*. Pathogenic trypanosomatids possess a highly conserved glycosylphosphatidylinositol (GPI)-anchor motif. The dense glycocalyx membrane on their surface is composed of lipid-anchored-GPs and polysaccharides. *Leishmania* spp. utilizes different GPIs, e.g., lipophosphoglycan (LPG), glycosylinositol phospholipids (GIPLs), proteophosphoglycans (PPGs) and gp63, for conjugation in the surface membrane complex. Notably, the composition of the *Leishmania* glycocalyx membrane dynamically changes during stage transformation. This strategy of immunoevasion is essentially utilized by promastigotes because their surface constitutes the first interface with host immune response(s). Since LPGs implication occurs in multiple activities for the sake of parasite survival and virulence, they received much attention than other GPs[107]. For amastigotes survival inside macrophages, both GIPLs and PPGs contribute in host immunoevasion, but with two different mechanisms: *via* inhibition of nitric oxide synthase and protein kinase C, or via binding to macrophage receptors, respectively[108].

5. Tetraspanins

Tetraspanins (TSPs), also known as transmembrane 4 superfamily proteins, are membrane or surface proteins involved in the formation of cell membrane protein complex, and intercellular signaling, as well as virulence and pathogenesis in some pathogens. Structurally, TSPs have four transmembrane α-helices, two extracellular domains, and three short intracellular regions. One of the extracellular domains (EC2) possesses a well-conserved disulfide bond (CCG motif). Functionally, they are involved in signaling pathways through controlling cell-to-cell interactions, immune cells migration through integration to the membranes of extracellular vesicles such as CD9, CD63, CD81, CD82, and CD151. In addition, they display several properties suggestive of involvement in cell adhesion, motility, activation of migration, and proliferation. To achieve their functions, TSPs act as scaffolding proteins, attaching integrins to one area of the cell membrane. This means that TSP extracellular domains link to the integrins, while the cytoplasmic domains link to intracellular signaling enzymes (e.g. PKs). Notably, integrins are certain membrane proteins that function mechanically by attaching the cell cytoskeleton to the extracellular matrix, and biochemically by sensing adhesion occurrence[109].

Helminths

Since they are present in all multicellular eukaryotes, TSPs organize the TSPs web in helminths. The web is an association of other transmembrane proteins (itegrins) stabilizing cell membranes and coordinating intracellular processes, i.e., signal transduction, cell proliferation, adhesion, migration, fusion, and host-pathogen interactions. In fact, a *S. mansoni* transcriptomic study revealed several TSPs encoding genes with different expression profiles[110]. Among them, two major TSPs (TSP1 and TSP2) attracted much attention in the last decade. Since *Sm*TSP2 proved to have essential roles in tegument creation and maturation, it exhibited high levels of protection as a recombinant vaccine in animal models[111,112]. Later, American investigators assessed its safety and immunogenicity in phase I, double-blind trial on healthy adults from a non-endemic area[113].

Utilizing a gene-silencing approach, the investigators identified and characterized the structure of TSP1 from *O. viverrini*. An immunolocalization study revealed its distribution throughout adults' tegumental membrane, as well as in the eggs. Its expression was observed in all developmental life cycle stages with the higher expression in metacercaria. The investigators confirmed *Ov*TSP-1' role in biogenesis of the cell membrane and maintenance of its structural integrity, and proposed it as a potential drug target[114]. Later,

two genes encoding *Ov*TSP2 and *Ov*TSP2 were characterized, and their expression was silenced. Similar to *Ov*TSP1, their expression was detected in all developmental stages, but with higher levels in eggs. Silencing of both genes showed impaired tegument formation in adults demonstrated by membrane vacuolation. Interestingly, both TSPs were detected inside the cholangiocytes lining the bile ducts of infected hamsters, indicating their role in cholangiocarcinoma. Therefore, the investigators proposed them as potential antigens for protection against opisthorchiasis[115].

Protozoa

In the unicellular protozoa, *T. vaginalis*, TSPs act as modulators of host-pathogen interactions. Eight TSPs were identified, among them *Tv*TSP6 with flagellar localization was involved in sensory reception and migration during invasion. The investigators demonstrated that *Tv*TSP6 redistributed to trophozoite' plasma membrane and intracellular vesicles upon initial binding to host cell[116]. Later, it was demonstrated that *Tv*TSP8 expression was upregulated upon contact with vaginal ectocervical cells. The investigators suggested its involvement in parasite aggregation[117]. On the other hand, Japanese investigators conducted an *E. histolytica* genome-wide study and compared their results to the eight TSPs characterized in *T. vaginalis*. Based on sequence similarities to *T. vaginalis* TSPs, the investigators identified new nine potential TSPs in *E. histolytica*. Accordingly, the investigators hypothesized that differences in the amino acids and nucleotides sequences might create characteristic features that had implications on the function and evolution of *E. histolytica* TSPs. They recommended further studies to identify their crystal structures aiming to design selective inhibitor, as novel drug[118].

CONCLUDING REMARK

Parasite targets are divided into three categories. Those involved in parasite growth, replication, and survival, as well as proteases and TSPs of protozoa are promising drug targets. Those involved in pathogenesis and survival including GPs, endogenous protease inhibitors (CPIs, and eSPIs), and TSPs of helminths are utilized as vaccine candidates. Only HSPs have dual applications, i.e. drug target or an adjuvant with a candidate vaccine.

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