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Evaluation of PEN2-ATP6AP1 axis as an antiparasitic target for metformin based on phylogeny analysis and molecular docking

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ABSTRACT

Background: Metformin (Met), the first-line drug used in the treatment for type 2 diabetes mellitus, is effective against a variety of parasites. However, the molecular target of Met at clinical dose against various parasites remains unclear. Recently, low-dose Met (clinical dose) has been reported to directly bind PEN2 (presenilin enhancer protein 2) and initiate the lysosomal glucose-sensing pathway for AMPK activation *via* ATP6AP1 (V-type proton ATPase subunit S1), rather than perturbing AMP/ATP levels.
Methods: To explore the possibility of PEN2-ATP6AP1 axis as a drug target of Met for the treatment of parasitic diseases, we identified and characterized orthologs of PEN2 and ATP6AP1 genes in parasites, by constructing phylogenetic trees, analyzing protein sequences and predicting interactions between Met and parasite PEN2.
Results: The results showed that PEN2 and ATP6AP1 genes are only found together in a few of parasite species in the cestoda and nematoda groups. Indicated by molecular simulation, Met might function by interacting with PEN2 on V37/W38/E5 (*Trichinella spiralis*) with similar binding energy, and on F35/S39 (*Caenorhabditis elegans*) with higher binding energy, comparing to human PEN2. Hence, these results indicated that only the *T. spiralis* PEN2-ATP6AP1 axis has the potential to be the direct target of low-concentration Met. Together with contribution of host cells including immune cells *in vivo*, *T. spiralis* PEN2-ATP6AP1 axis might play roles in reducing parasite load at low-concentration Met. However, the mechanisms of low-concentration Met on other parasitic infections might be mainly achieved by regulating host cells, rather than directly targeting PEN2-ATP6AP1 axis.
Conclusions: These findings revealed the potential mechanisms by which Met treats various parasitic diseases, and shed new light on the development of antiparasitic drugs.

1. Introduction

Metformin (Met, N,N-dimethylbiguanide) derived from galegine, a natural product from the plant *Galega officinalis*, belonging to the biguanide class of antidiabetics, is the first-line drug used in the treatment for type 2 diabetes mellitus with average oral bioavailability and safety profile [1,2]. Moreover, Met can reduce body weight, hepatic fat content as well as the cancer incidence, and improve cardiovascular outcomes [3–5]. Other benefits, such as extending lifespan and healthspan, and alleviating fibrosis, have been demonstrated in *Caenorhabditis elegans* and mice models [6–8]. Importantly, Met was reported to be effective against a variety of pathogens, including bacteria (*Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*) [9–11], virus (SARS-CoV-2, Zika virus, dengue virus, hepatitis B virus, hepatitis C virus and human immunodeficiency virus) [12–16] and parasites [17–25].

Because Met was introduced before current target-based drug development, molecular mechanistic details have not been fully understood prior to its clinical use. Current evidences suggest that, beyond the field of diabetes, the beneficial effects of Met involve improving the function of multiple organs through various putative mechanisms [1, 26–28]. Among them, the accumulation of Met in mitochondria and lysosomes *via* AMPK-dependent and independent pathways was considered the most classical mechanism [2,29]. Moreover, other mechanisms have been proposed, for instance, Met can stimulate the secretion of the glucose-lowering hormone glucagon-like peptide 1 (GLP1) from enteroendocrine L cells, thereby switching human intestine to glycolysis and increasing glucose uptake from the circulation

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[30–32]. In addition, Met has been shown to have direct effects on inflammation by affecting monocyte differentiation into macrophages, pro-inflammatory cytokine secretion [33,34], and the gut microbiome [35]. However, most of these mechanisms can only explain the effects of Met at high concentration (mM range), which cannot be reached to under clinical dose.

Similarly, Met has been reported to reduce parasite growth or activity at high concentration (1–10 mM) *in vitro*, but alleviate parasitic infections at much lower concentrations in rodents even after oral administration with dose (10–500 mg/kg) comparable to or lower than the maximum daily human dose (2 g) [17–25,36] (Table 1). However, there were some exceptions, for example, no anti-schistosomal activity but a reduction in egg deposition and an anti-fibrotic effect on granulomatous development was found when Met was used alone or in combination with praziquantel (PZQ) treatment [37]. Meanwhile, it was found that Met increased *T. vaginalis* and *L. braziliensi* viability *in vitro* and parasite loads *in vivo* [38,39]. Notably, Met concentrations (1–10 mM) used in these *in vitro* studies were much higher than those in plasma (5–18 μM) and liver (50–100 μM) *in vivo* [17–25,40]. At high concentration (mM range), it is reported that Met could impair ATP synthesis

Table 1
The known effects and mechanisms of metformin on parasites.

	Model	Dose	Low (L) or High (H) Met	Mechanism	Effects	Study
Protozoa	*P. berghei*	500 mg/kg/d *in vivo*, or 10 mg/kg daily combined with 15 mg/kg PQ	H	NA	Met has a potent activity against liver stage malaria *in vivo*	[17]
	P. falciparum	50 and 200 μM *in vitro*	L	NA	$IC_{50} = 45.17 \pm 0.05$ μM (*P. falciparum* in human hepatocytes) $IC_{50} = 3.7 \pm 1.1$ mM (Plasmodium asexual erythrocytic stages)	
	P. yoeli	*ad libitum* with water containing 5 mg/ml *in vitro*	L	γδ T cell expansion	Levels of parasitemia were reduced in treated mice	[21]
	P. falciparum	0.0064–40 mM *in vitro*	H	NA	$IC_{50} = 1.32$ mM (Intraerythrocytic stage)	[18]
	T. cruzi		H		$IC_{50} = 18.5$ mM (intracelular amastigotes)	
	T. brucei		H		$IC_{50} = 17.3$ mM (bloodstream stage)	
	L. infantum		H		NA (intracelular amastigotes)	
	L. infantum		H		NA (infected macrophages)	
	A. castellanii	5 and 10 μM *in vitro*	L	NA	Conjugation of Met with silver nanoparticles was found to enhance its antiamoebic effects against *A. castellanii*	[19]
	T. vaginalis	2% Met in drinking water	L	Lead to specific population changes of innate immune cells and their impact on the *T. vaginalis* viability.	Increased viability of *T. vaginalis* by 43%	[39]
	L. braziliensi	500 mg/kg *in vivo*, 2 mM *in vitro*	H	Immunomodulation, reducing intracellular ROS and enabling parasitic growth inside the parasitophorous vacuole	1. 2 mM Met in *L. braziliensi* culture allow for the maintenance of stationary parasite growth phase. *L. braziliensi*-infected cells 2. Met treatment interfered with lesion kinetics, increased parasite load and reduced macrophage proliferation *in vivo*.	[38]
Helminth	*E. multilocularis*	50 mg/kg of body weight/day for 8 weeks *in vivo* 10 mM *in vitro*	H *in vitro* and L *in vivo*	High dose Met *in vitro* leads to mitochondrial membrane depolarization, activation of Em-AMPK, suppression of Em-TOR, and overexpression of Em-Atg8 (AMPK-TOR-autophagy pathway)	1. Met has effect on viability of primary stem cells of E. multilocularis and on the dedifferentiation process of protoscoleces to metacestodes at 10 mM 2. Oral administration of Met was effective in achieving a significant reduction of parasite weight in a secondary murine AE model	[20]
		100 mg/kg+ 5 mg/kg ABZ *in vivo*	L	Decrease parasite glucose availability	Combination treatment led to a significant reduction in parasite weight	[23]
	E. granulosus	1, 5, 10 mM *in vitro*	H	AMPK-TOR-autophagy	NA	[22]
		1, 5, 10 mM *in vitro*	H	Activation of AMP-Activated Protein Kinase led to carbohydrate starvation and increased glucogenolysis and homolactic fermentation, and decreased transcription of inter mediary metabolism genes	Met alone or combined with ABZ led to a dose-dependent decrease in the viability of protoscoleces and metacestodes	[24]
		50 mg/kg/day or combination with 5 mg/kg ABZ *in vivo*, 60 days 1, 5 mM Met alone or combined with 2.5 μM ABZ *in vitro*	L	NA	1. Met or combined with ABZ showed significant dose- and time-dependent killing effects on *in vitro* cultured protoscoleces and metacestodes 2. Met or combined with ABZ was highly effective in reducing the weight and number of parasite cysts *in vivo*	[25]
	S. mansoni	PZQ (500 mg/kg) combined with Met (150 mg/kg), 15 days, *in vitro*	L	Met lead to an anti-fibrotic effect	Met has no anti-schistosomal activity but led to a reduction in egg deposition and showed an anti-fibrotic effect on granulomatous development	[37]
	T. spiralis	50 mg/kg *in vivo*	L	Anti-inflammatory and anti-angiogenic effects that effect the host biochemial environment that can affect the parasite	Marked reduction in the inflammatory cellular infiltration, cyclooxygenase-2 (COX-2) expression, and oxidative stress was noted in the small intestines	[41]

and in turn increase AMP/ATP and ADP/ATP ratios in parasites, resulting in parasite glucose reduction and AMPK activation [23,24]. However, the mechanism of low-concentration Met in parasites control remains unknown. Hence, if low-concentration Met has a direct effect on parasite viability, there must be other mechanisms to explain how clinical low-concentration Met realize its function on these parasites.

Recently, it was revealed that low-dose Met can directly bind presenilin enhancer protein 2 (PEN2) and initiate the lysosomal glucose-sensing pathway for AMPK activation (v-ATPase–AXIN–AMPK axis) *via* ATP6AP1 (V-type proton ATPase subunit S1), without perturbing AMP/ATP and ADP/ATP levels [2], and this PEN2–ATP6AP1 axis was also required for reducing hepatic fat in mice and extending lifespan in *C. elegans* [2]. Inspirited by this newly released mechanism, in the present study, we explored the possibility of PEN2-ATP6AP1 axis as a target of Met for the treatment of parasitic diseases, from the evolutionary perspective based on sequences and structures characteristics of parasite PEN2 and ATP6AP1, and the possible binding sites in PEN2 for interaction with Met and ATP6AP1. These results were helpful for deeper understanding the mechanisms of Met in the treatment for various parasitic diseases.

2. Results

2.1. Genes of PEN2 and ATP6AP1 in parasites genomes

The parasites that reported to be sensitive to Met (list in Table 1) were searched for orthologs of PEN2 and ATP6AP1 (Fig. 1A) in their genomes. Results showed that PEN2 only presented in nematoda, trypanosomatidae and cestoda, while was absent in plasmodiidae, trichomonadidae and trematoda (Fig. 1B and C). Moreover, orthologs of ATP6AP1, the downstream effector of PEN2, were only identified in platyhelminthes, rather than protozoa (Fig. 1B and D). In plasmodiidae, trichomonadidae and bacteria, both of *PEN2 and ATP6AP1 were absent.*

The distribution of PEN2 and ATP6AP1 in a variety of parasite species revealed the evolution history of these proteins. PEN2 was a conserved protein originated from the last eukaryotic ancestor (LECA), and retained in the genome of nematoda, cestoda and trypanosomatida individually during the subsequent evolution (Fig. 2). The lost of PEN2 was also found in some parasites, for instance, *Plasmodium* and *Schistosoma* (Fig. 2). It was inferred from the evolutionary tree that unlike PEN2, ATP6AP1 in platyhelminthes (cestoda and trematoda) and nematoda might have been acquired from the ancestor of metazoa, because it was absent in trypanosomatidae and plasmodiidae in the early evolutionary branch, and in archaeplastida as well (Fig. 2).

Based on the genes distribution patterns in different parasites, it can

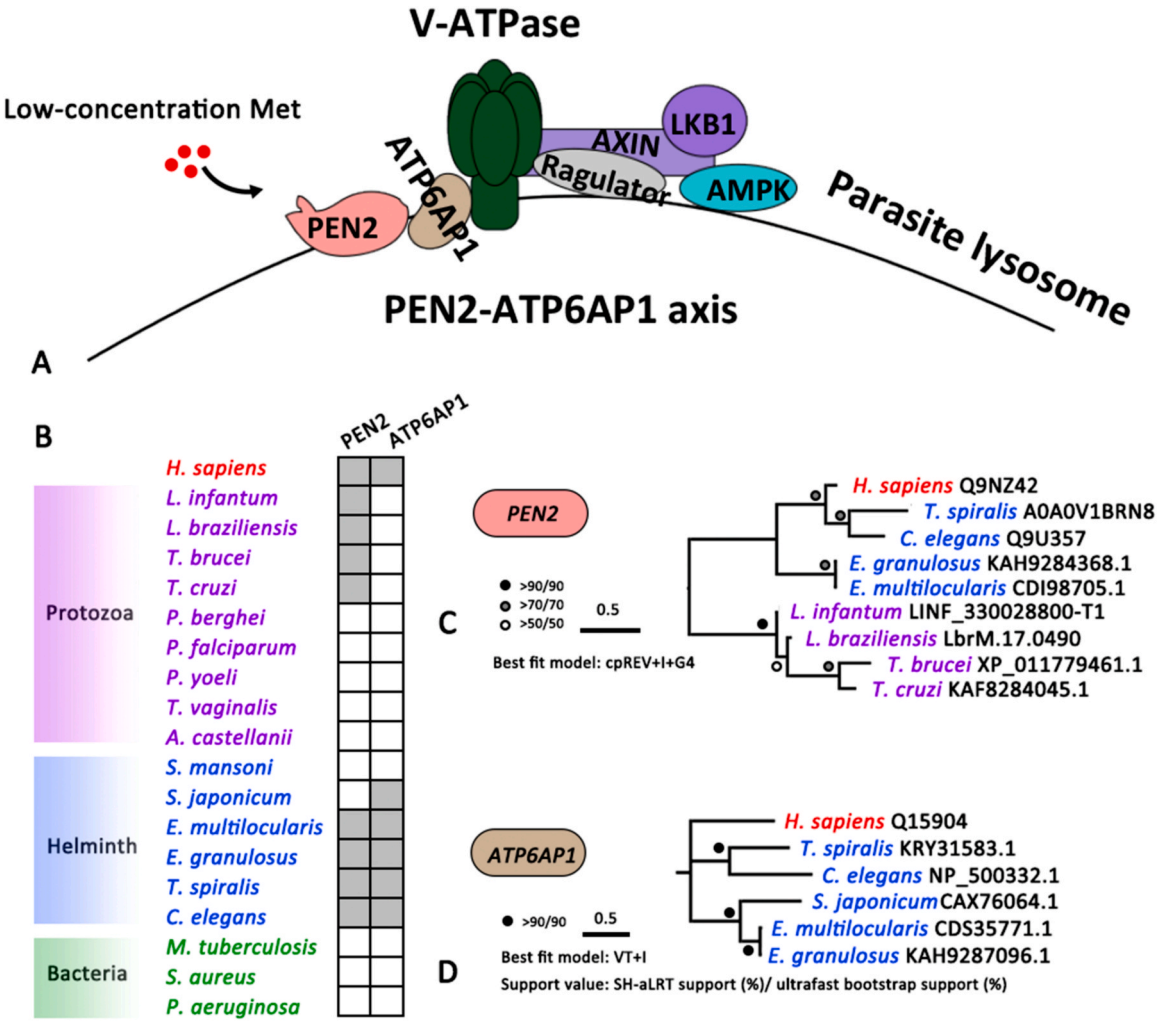

Fig. 1. The distribution and phylogenetic relationship of PEN2 and ATP6AP1 in parasites sensitive to metformin. (A) The schematic diagram of Met interfering with PEN2-ATP6AP1 axis. (B) Distribution of PEN2 and ATP6AP1 genes in parasites and bacteria sensitive to metformin. (C) Phylogenetic relationship of parasites PEN2 genes. (D) Phylogenetic relationship of parasites ATP6AP1 genes.

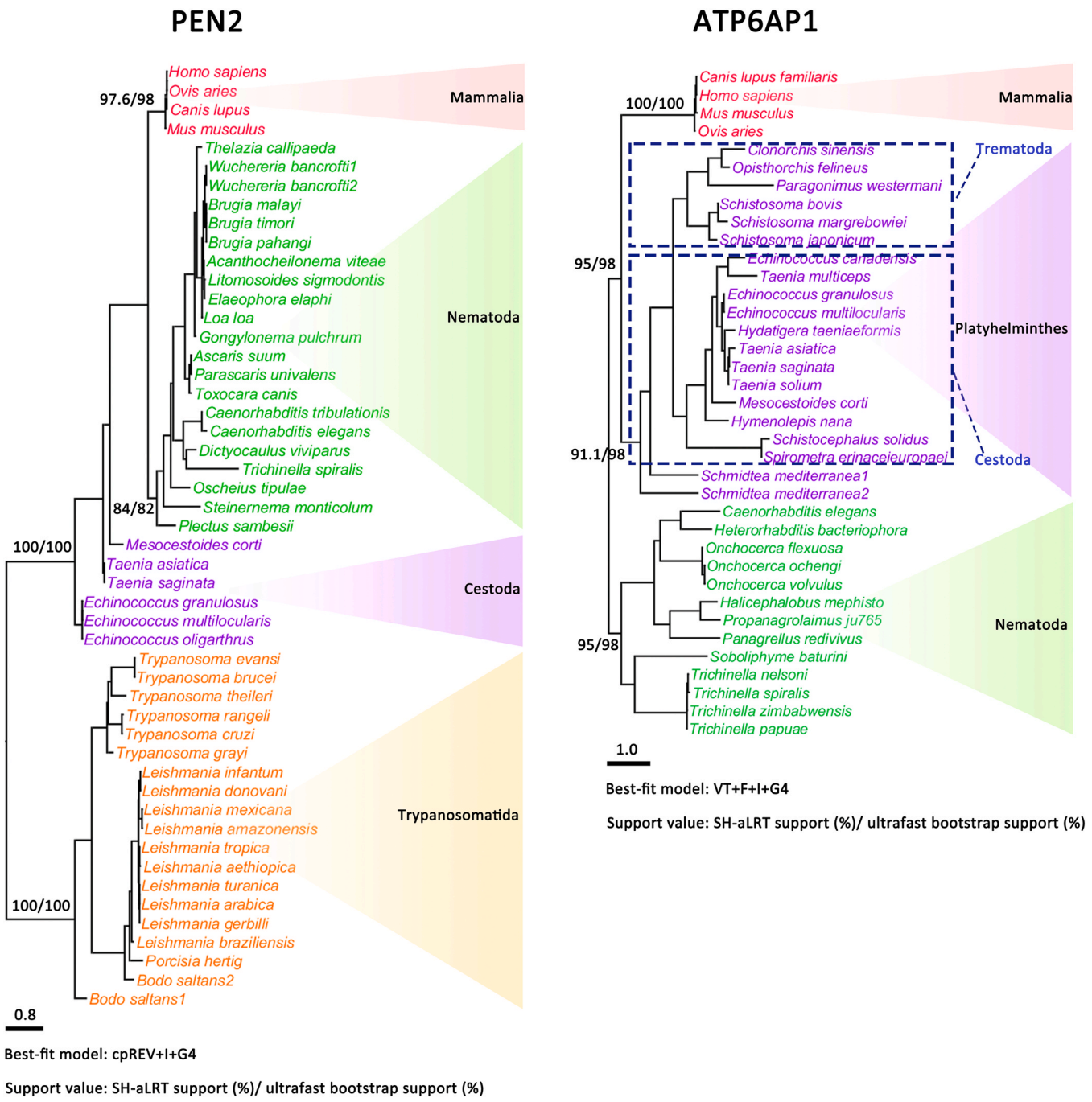

Fig. 2. The phylogenetic tree of PEN2 and ATP6AP1 in various parasites.

be confirmed that Met was unable to realize anti-parasitic effects on parasites such as *Plasmodium* spp., *T. vaginalis*, *A. castellanii* and trypanosomatida, *via* the PEN2-ATP6AP1 axis pathway since these parasites do not contain clear PEN2 or ATP6AP1 orthologs. However, some cestoda (*Echinonoccus* spp.) and nematoda (*T. spiralis*) which retained both of these two proteins could be potentially affected by low-concentration Met through the PEN2-ATP6AP1 and v-ATPase axis (Fig. 2).

2.2. Sequence and topology characteristics of parasites PEN2

To explore the possibility of PEN2 as the drug target of Met in parasites, their amino acid sequences and topology structure were further analyzed (Fig. 3). The similar sequences and conserved domain presented in both human and helminth PEN2 indicated their conserved functions (Fig. 3A). However, PEN2 sequences in protozoa were notablely diverse. Hence, together with the unidentified ATP6AP1 in protozoa, the sequence differences of protozoa PEN2 further suggested that the functional defect of PEN2-ATP6AP1 axis in protozoa.

As the subunit of γ-secretase complex, human PEN2 is an integral membrane protein with 101-amino acids containing two transmembrane domains. Previous studies have shown that PEN2 spans membrane twice, with both the N- and C-termini facing the lumen of the endoplasmic reticulum [42,43]. However, the structures of human PEN2 have been resolved using the high-resolution electron microscopy and indicated that its N-termini is cytoplasmic, followed by two short helices that dip into the membrane but do not cross it [44]. According to the putative binding site of PEN2 to Met in resolved protein structure, the PEN2 topological features were further clarified in the present study

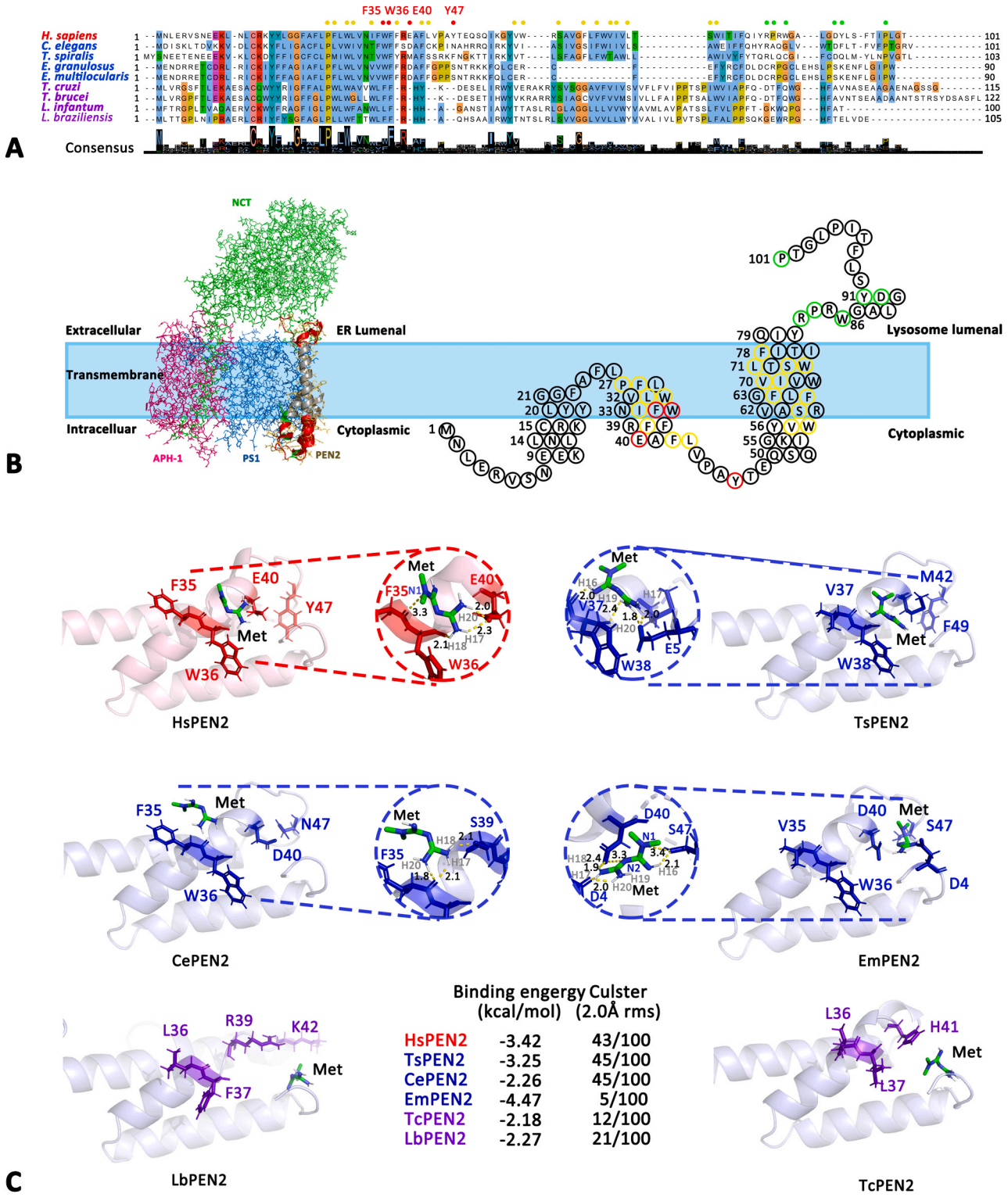

Fig. 3. Sequences and structures of parasites PEN2. (A) Multiple-sequence alignment of PEN2 from parasites sensitive to Met. The background of alignment was colored by Clustalx model. Red dots: amino acids potentially interact with Met at cytoplasm; green dots: amino acids potentially interact with Met at lysosome lumen; yellow dots: amino acids potentially interact with ATP6AP1. In the alignments, the background of hydrophobic amino acids are blue, positive charged ones are red, negative charged ones are magenta, polar ones are green, cysteines are pink, glycines are orange, prolines are yellow and aromatic ones are cyan. (B) A membrane topology diagram of human PEN2. The N-termini and middle amino acids face cytoplasm, while the C-termini face the lumen of lysosome. Red circles: amino acids potentially interact with Met at cytoplasm; green circles: amino acids potentially interact with Met at lysosome lumen; yellow circles: amino acids potentially interact with ATP6AP1. (C) *In silico* modeling of Met bound to the N-terminal, cytosolic face of PEN2. The center of grid box was set in the pocket containing residues corresponding to F35, W36, E40 and Y47 of human PEN2. The hydrogen bonds were labeled with yellow dotted line. Hs= *Homo sapiens*, Ce= *C. elegans*, Ts= *T. spiralis*, Em= *E. multilocularis*, Lb= *L. braziliensi*, Tc= *T. cruzi.*

(Fig. 3B). It is speculated that the N-termini and parts of middle amino acids faced cytoplasm on lysosome membrane, while the C-termini faced the lumen of lysosome (Fig. 3B). Although both N-termini and C-termmini were predicted to bind Met [2], our study found that only the pocket formed by E35, W36, E40 and Y47 (red circle, Fig. 3B) was most likely to interact with Met, from the topology perspective. In addition, the residues of PEN2 associated with ATP6AP1 binding located at the trans-membrane region (yellow circle, Fig. 3B), which was consistent with the fact that ATP6AP1 was also a trans-membrane protein.

The N-termini sequences of parasites PEN2 were similar to human PEN2, while the key residues related to ATP6AP1 binding (red circles in Fig. 3B) and C-termini sequences were diverse, especially in protozoan PEN2 (such as PEN2 of *L. braziliensi* and *T. cruzi*), indicating their different responses to low concentration Met. Moreover, helminthic PEN2 showed a higher similarity compared to human PEN2, but its residues involved in interacting with ATP6AP1 were different from that in human PEN2 (yellow circle, Fig. 3B). Hence, whether the PEN2-ATP6AP1 axis is the direct target of low-concentration Met in these parasites was further analyzed by *in silico* molecular simulation.

2.3. *In silico modeling of Met bound to parasites PEN2*

In silico modeling of Met binding to the cytosolic face of human PEN2 (HsPEN2) indicated that Met could dock into the pocket formed by F35, W36, E40 and Y47 with potential hydrogen bonds (yellow dotted lines in Fig. 3C) between these residues and Met (binding energy = −3.42 kcal/mol, 43 conformations out of 100 genetic algorithm runs). When docking Met into parasitic nematoda *T. spiralis* PEN2 (TsPEN2) at the same pocket, interactions can be identified with V37, W38 and E5 (binding energy = −3.25 kcal/mol, 45 conformations out of 100 genetic algorithm runs) (Fig. 3C). However, the docking pose of Met in nematode *C. elegans* (CePEN2) was deviated from that of Met in HsPEN2 and TsPENs, in which Met interacted with F35 and S39 by hydrogen bonds (binding energy = −2.26 kcal/mol, 45 conformations out of 100 genetic algorithm runs). In addition, the conservation of putative residues in TsPEN2 and CePEN2 to HsPEN2 for interacting with ATP6AP1 indicated that Met can interfere the PEN2-ATP6AP1 axis in nematoda.

Comparing to nematoda PEN2, the overall sequences of *Echinococcus* spp. PEN2 are more different from that of HsPEN2, especially the residues responsible for Met and ATP6AP1 binding, as well as C-termini sequences (Fig. 3A). These diverse residues formed a different binding pocket accommodating Met that was consisted of D4, S47 and D40 in *Echinococcus multilocularis* PEN2 (EmPEN2) (Fig. 3C). Although the binding energy between Met and EmPEN2 is low (−4.47 kcal/mol), the few conformation (5 conformations) returned from 100 GA runs raised concerns on accuracy of the binding pose. In addition, the lack of amino acids responsible for binding ATP6AP1 in *Echinococcus* spp. PEN2 strongly indicated that the possibility of PEN2-ATP6AP1 axis as the target of low-concentration Met in *Echinococcus* spp should be carefully evaluated in future.

Trypanosomatida PEN2 was lack of not only the conserved residues corresponding to E40 and Y47 in human PEN2, but also the interaction between L36 or L37 with Met. Moreover, the diversity of PEN2 residues associated with ATP6AP1 binding, together with the unidentified ATP6AP1 in these parasites (Fig. 1), supported the possibility that PEN2-ATP6AP1 axis is not a direct target for Met in these protozoa (Fig. 3C).

3. Discussion

Met, a first-line drug used in the treatment for type 2 diabetes mellitus, has been shown to be effective against a variety of parasites, but the direct molecular target at clinical dose was unclear [1,26–28]. Until recently, the mechanism that low-dose Met directly binds to PEN2 and initiates AMPK-activated lysosomal glucose-sensing pathway *via* ATP6AP1, rather than perturbing AMP/ATP levels, has been revealed [2]. These findings have led to interests in deciphering the mechanism of low-dose Met acting on parasites. In the present study, we comprehensively explored the possibility of the PEN2-ATP6AP1 axis as a drug target of Met for the treatment of parasitic diseases, using bioinformatics, such as phylogenetic analysis and molecular simulation. Firstly, we identified orthologs of PEN2 and ATP6AP1 in various parasites through homology searching, domain filtering and phylogenetic trees construction. Then, we analyzed the amino acid sequences and three-dimension structures of parasites PEN2, especially the key residues that interact with Met and ATP6AP1. Finally, we predicted interactions between Met and parasite PEN2 using molecular docking. The results suggested the functional gains and losses of PEN2-ATP6AP1 axis during parasite evolution and a possible role for this pathway in repression of parasites by Met.

3.1. *The phylogenetic analysis of parasites PEN2 and ATP6AP1 delineated the gains and losses of these proteins during parasite evolution*

The distribution of PEN2 and ATP6AP1 in selected parasites indicated that PEN2 might originate from LECA, ahead of ATP6AP1, which originated from the ancestor of metazoa (Fig. 2). PEN2 is a regulatory component of γ-secretase complex, a protease complex that plays a role in the regulation of Notch and Wnt signaling cascades and downstream processes [42,45]. As an accessory subunit of the proton-transporting vacuolar (V) -ATPase protein pump [46], ATP6AP1 exerts various functions, such as guiding the V-type ATPase into specialized subcellular compartments and regulating intracellular iron homeostasis [47–49]. However, so far, no studies have showed when these two proteins act together to affect lysosomal system. Deduced from the similarity of PEN2 residues responsible for binding ATP6AP1 in human and nematoda (free-living *C. elegans* and parasitic *T. spiralis*) (Fig. 3A), the present study suggested that the interactions between PEN2 and ATP6AP1 could be traced back to the ancestor of metazoa. However, the lost of PEN2 or ATP6AP1, or both of the two proteins in many metazoa parasites were found during evolution. For example, *Conchocerca* spp. lost PEN2 while *Wuchereria bancrofti* and *Brugia malayi* lost ATP6AP1. In addition, although *Echinococcus* spp. retained these two proteins, the unidentified residues in PEN2 for binding its partner-ATP6AP1 indicated the weak or no binding possibility between these two proteins.

The gene gains and losses enchance an organism's capacity to evolve and adapt [50,51]. For parasites, the gene gains and losses involved in adaptive genome evolution are likely to be associated with host-parasite coevolution [51]. However, the understanding of the evolution of PEN2 and APT6AP1 genes, and their roles in parasite development and adaption to the parasitic environments is still in the infancy.

3.2. *Met as the direct target of PEN2-ATP6AP1 axis*

Results returned from phylogenetic tree, amino acid sequences comparison and molecular docking indicated that Met might interact with PEN2-ATP6AP1 axis in nematoda *T. spiralis* and *C. elegans*. Notably, Met can bind to *T. spiralis* PEN2 at the same pocket as human PEN2, with the similar binding energy and reliable conformations. Furthermore, the conserved residues in *T. spiralis* PEN2 for binding ATP6AP1 indicated that this PEN2-ATP6AP1 axis can play an important role in maintaining physiological homeostasis of this helminth under low-concentration Met, as their function in human. However, the evidence for the direct role of PEN2-ATP6AP1 axis participating in the low-dose metformin effect on reduction of number of larvae needs to be explored in future, and the involvement of host cells including immune cells also can not be excluded. For *C. elegans*, the deviation of docking poses and the higher binding energy suggested that a higher Met concentration should be needed. These findings are consistent with previous reports that lifespan extension of *C. elegans* could be induced by Met through PEN2-ATP6AP1 axis and lysosomal pathway [2,52] only at high concentration (50 mM).

As mentioned above, the *Echinococcus* spp. retained both of PEN2 and ATP6AP1, but there might be no interaction between these two

proteins due to lacking of residues for binding ATP6AP1 in PEN2. However, a low binding energy with low-reliable conformations was found in the molecular docking. Hence, it was speculated that PEN2-ATP6AP1 axis is not the target of Met on this parasite, but the potential that Met binds PEN2 and plays the corresponding functions through other pathways cannot be excluded.

3.3. Other potential mechanisms of Met for the treatment of parasitic diseases

For other parasites without clear PEN2-ATP6AP1 pathway but were broadly inhibited at high concentration range *in vitro* and low concentration range *in vivo*, there must be multiple mechanisms of Met on different parasitic infections (Fig. 4). Firstly, high-concentration Met can mediate parasite glucose reduction, AMPK activation by inhibiting the complex I of the mitochondrial electron transport chain [1,2,26,27,53,54], then a cascade of signaling events will be initiated, such as mimicking energy and nutrient acquisition, reducing protein synthesis and inducing autophagy (recycling of existing intracellular metabolites) by antagonizing mTOR kinase [55]. Moreover, Met can lead to carbohydrate starvation, increased glucogenolysis and homolactic fermentation, and decreased transcription of intermediary metabolism genes at the larval stages of *E. granulosus in vitro*, by inducing activation of Eg-AMPK and Em-AMPK [20,22,24]. In addition, anti-plasmodial activity of Met was proposed to disrupt pyrimidine biosynthesis dependent on the complex I-free *Plasmodium* respiratory chain [18,56]. Conversely, Met has been reported to have some positive effects on the viability of *L. braziliensis*, such as interfering with lesion kinetics and increasing parasite load in *L. braziliensis*-infected mice, presumably by reducing macrophage proliferation with altered reactive oxygen species (ROS) at mM range [38].

Secondly, low-concentration Met can mediate host to affect parasites. For example, no anti-schistosomal activity but an anti-fibrotic effect was observed in host liver using Met at clinically low concentration [37], and Met was only effective against liver-stage *Plasmodium* rather than asexual erythrocytic stages [17], and against *Echinococcus* spp. infected mice in which the lesions are mainly located in livers [20,23,25]. These specific effects of Met on different parasitic infections strongly indicated that Met may exert its anti-parasitic effect through host regulation. Moreover, low-dose metformin could effect on immune cells [57,58], and many reports have confirmed that Met could increase the viability of parasites by immunomodulatory and anti-inflammatory effects. For instance, in *T. spiralis* infected mice, Met can altered the changes in the host environment, such as significantly reduced inflammatory cellular infiltration, cyclooxygenase-2 (COX-2) expression, oxidative stress in the small intestines [41]. In addition, as a neutrophil extracellular traps (NETs) inhibitor, Met can inhibit NADPH oxidase in NETs to increase *T. vaginalis* viability *in vivo* [39,59]. For *Plasmodium* infection, Met was shown to reduce *Plasmodium* parasitemia through adaptive immune responses, such as dramatically increase the number of Vγ2 + γδ T cells in the spleen of treated mice during the late phase of infection [21].

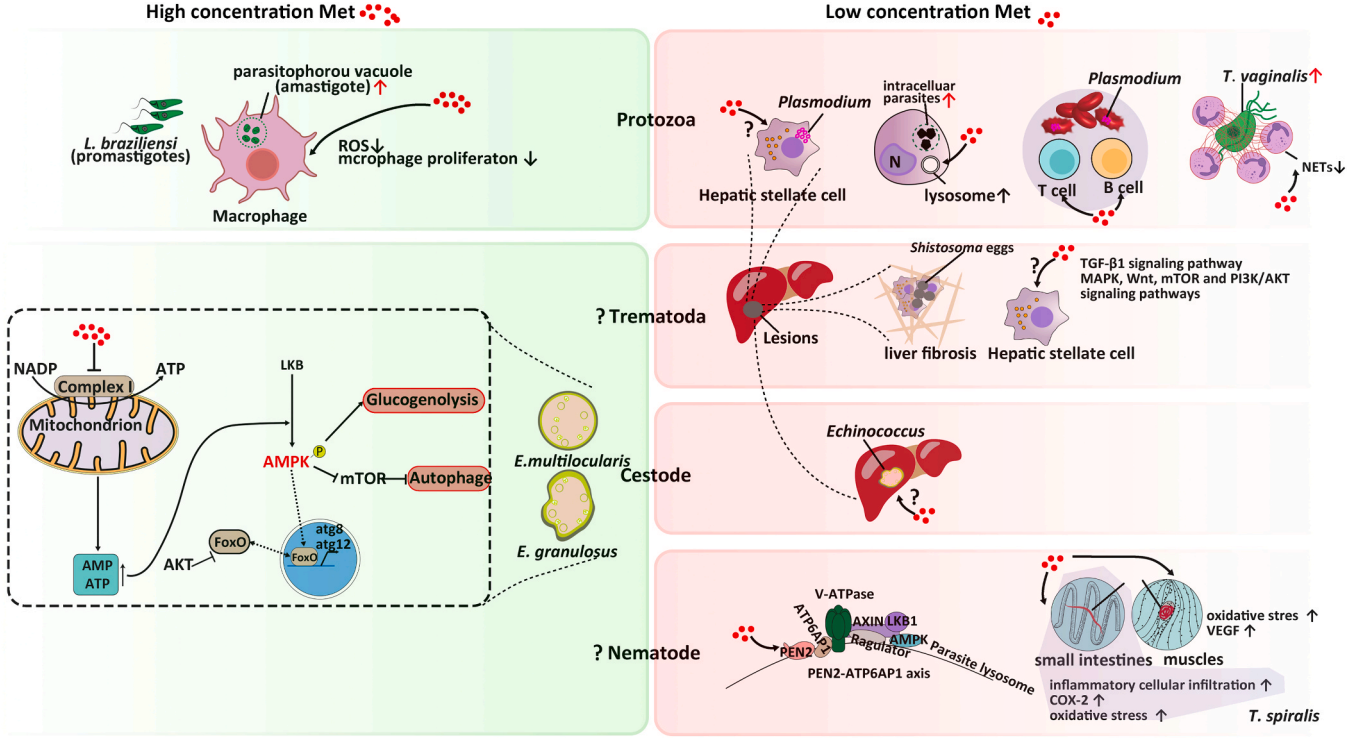

Fig. 4. Mechanisms of low- and high-concentration Met for the treatment of parasitic diseases. High-concentration Met can promote the viability of *L. braziliensis* (red arrow) by reducing macrophage proliferation with the altered ROS; High-concentration Met affects the viability of *Echinococcus*. spp by inhibiting the complex I of the mitochondrial electron transport chain, through mediating parasite glucose reduction, AMPK activation and subsequent TOR suppression and autophagy pathway induction. Low-concentration Met might alleviate parasitic infections by regulating host cells, especially hepatocytes where higher Met accumulations. Met has been reported to be effective on hepatic pathogens, such as *Plasmodium* spp., *Schstosoma* spp. and *Echinococcus* spp., but mechanism is still unknown. Low concentration-Met may trigger autophagy and other processes in the pathogen-infected host cell, providing them with nutrients for their survival and parasite replication (red arrow); Met induced parasitemia is potentially dependent on α/β-T cells and B cells; Met inhibits NADPH oxidase in NETs to increase *T. vaginalis* viability *in vivo* (red arrow); Met may directly target to the PEN2-ATP6AP1 axis of *T. spiralis*, or alter the host environment. ROS, reactive oxygen species; NADP, nicotinamide adenine dinucleotide phosphate; ATP, adenosine triphosphate; AMP, adenosine monophosphate; LKB, AKT, protein kinase B; FoxO, transcription factor forkhead box O-3; atg8, autophagy-related protein 8; atg12, autophagy-related protein 12; mTOR, mammalian target of rapamycin; NETs, neutrophil extracellular traps; VEGF, vascular endothelial growth factor; COX-2, Cyclooxygenase 2.

4. Conclusions

Met has a variety of functions, including treating type 2 diabetes, cancer and pathogenic infections, and prolonging life. It is worth noting that its therapeutic effects on pathogenic infections might be mainly achieved by influencing the host, rather than directly acting on the pathogens. These findings highlight the important role of controlling parasitic diseases by manipulating host cells to inactivate parasites, which is potentially an alternative way for searching broad-spectrum anti-parasitic drugs.

The repurposing of old drugs is considered to be an effective way to develop anti-parasitic drugs by saving R&D time and investments and, more importantly, with known drug targets. However, as showed in the present study, the challenge of old drug repurposing is to identify the drug target in different parasites, because that the modes of action of these old drugs against other nonparasitic diseases are not necessarily identical to those against parasites. Hence, in view of the performance of Met in the treatment of parasitic diseases, more research on the underlying mechanisms of Met for the treatment of parasitic infections/diseases is needed in the future.

5. Materials and methods

5.1. Orthologs searching

The human PEN2 (Gamma-secretase subunit PEN-2, Presenilin enhancer protein 2) and ATP6AP1 (V-type proton ATPase subunit S1) sequences were used as initial queries to search orthologs in 13 parasites genomes. We choose these parasites because they were reported to be sensitive to Met. Moreover, the genomes of *C. elegans*, *M. tuberculosis*, *S. aureus* and *P. aeruginosa* were also included in this study (Fig. 1A). Web-based PSI-BLASTP was performed to search among NCBI nonredundant protein sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 16 March 2022) with reciprocal BLAST method. Proteins were considered as orthologs as the top hit in the reciprocal BLAST search with an E-value cut-off less than 0.05. The conserved domains were identified by CDD (https://www.ncbi.nlm.nih.gov/cdd/, accessed on 26 March 2022). Only the sequences on the presence of the characteristic domains (PEN2 should include PEN-2 superfaminly domain, ATP6AP1 should include vacuolar ATP synthase subunit S1 domain) were used in the following studies [60].

In order to explore the gene distribution of PEN2 and ATP6AP1 in other parasites genomes, TriTrypDB (https://tritrypdb.org/tritrypdb, accessed on 17 March 2022), PlasmoDB (https://plasmodb.org/plasmo/, accessed on 17 March 2022) and WormBase (https://parasite.wormbase.org/index.html, accessed on 17 March 2022) were also used to search orthologs with on an E-value cut-off less than 0.05, and only the sequences with characteristic domains were retained for the next step analysis.

In addition, to explore the evolution history of PEN2 and ATP6AP1, some mammalian (*ovis aries*, *canis lupus* and *mus musculus*) and achaeplastida (https://mycocosm.jgi.doe.gov/archaeplastida/archaeplastida.info.html, accessed on 17 March 2022) were also included and analyzed in this study, with the same method.

5.2. Alignment and phylogenetic analysis

The amino acid residues were visualized with Jalview Version 2 [61] and aligned using MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/, accessed on 29 March 2022) integrated in Jalview platform, with the default parameters. These alignments were trimmed with TrimAI using the heuristic automated1 method [62]. Maximum likelihood (ml) analyses were performed by online IQ-TREE platform [63]. The best fitting model was defined by IQ-TREE with Bayesian Information Criterion. The tree branches were tested with ultrafast bootstrapping (1000) and SH-like approximate likelihood ratio test (SH-aLRT, 1000 replicates). The final trees were visualized and presented using TreeGraph2 [64].

5.3. In silico modeling of parasite PEN2 and characterize the topology structure

Data for other structures without resolved crystal structures were modeled using SIWSS-MODEL [65]. The PDB proteins with highest sequence similarity were chosen as the template for homology modeling. All the protein structures were visualized using Open-Source PyMoL [66]. For better understanding the topology structure of PEN2, the precise amino acid position of human PEN2 locating in the membrane were delineate based on the released 3D structure. Human PEN2 structure (6iyc_D) was retrieved from the PDB database (https://www.rcsb.org, accessed on 25 March 2022).

5.4. Docking metformin to parasites PEN2

As the ligand, Met was downloaded from the PubChem (https://pubchem.ncbi.nlm.nih.gov/, accessed on 25 March 2022). The molecular docking for these PEN2 proteins with Met were carried out to predict their interactions by Autodock tool with default parameters [67]. PEN2 and Met in the pdbqt format were prepared by plugin using scripts from the Autodock Tools package. As a trans-membrane protein, only the N-termini facing to the cytoplasm was considered as the interface with Met in this study, hence the center of grid box (x: 40 points, y: 40 points, z = 64 points) with a default step size of 0.375 Å was set in the pocket containing F35, W36, E40 and Y47 of these proteins corresponding to human PEN2 [2]. For PEN2 proteins of human, *C. elegans*, *T. spiralis* and *E. multilocularis*, set the Coordinates of Central Grid Point of Maps as 183.363, 198.012, 146.697; for PEN2 proteins of *L. braziliensi* and *T. cruzi*, set the Coordinates of Central Grid Point of Maps as 148.608, 100.135, 105.032. The 100 docking poses were returned using Genetic Algorithm and clustered using an RMSD-tolerance of 2.0 Å. The other docking parameters were the default sets, such as Population size of 150, Maximum number of evals of 2500000, maximum number of generations of 27000 and so on.

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CRediT authorship contribution statement

Congshan Liu: Writing – review & editing, Writing – original draft, Visualization, Methodology, Funding acquisition, Formal analysis. **Shangrui Zhang**: Methodology, Formal analysis. **Jian Xue**: Formal analysis. **Haobing Zhang**: Methodology, Supervision. **Jianhai Yin**: Writing – review & editing, Validation, Supervision, Methodology, Conceptualization.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data Availability Statement

The data is all available for free access.

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