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What is Known About N-Alpha Terminal Acetylation in Kinetoplastida Organisms

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ABSTRACT

Nα-terminal acetylation (Nt-acetylation) is a conserved post-translational modification that plays an important role in stabilizing, localizing, and performing protein functions in general. Among kinetoplastid parasites such as species of Trypanosoma and Leishmania however, little work has been done to understand the role that this type of modification plays despite possessing an Nα-terminal acetylation machinery with unique N-terminal acetyltransferases. Particularly, since Nt-acetylation is important in stabilizing several key proteins that participate in various cellular processes, Nt-acetylation could be influencing parasite biology such as host-parasite interactions, establishment of different ecological niches, and general pathogenicity. The diverse roles that Nt-acetylation could be playing within kinetoplastids, given their complex life cycles and interactions with hosts, could be important. It is possible that the acetylation machinery, because of such kintoplastid-specific adaptations, represents an attractive target for therapeutic intervention. The unique aspects of Nt-acetylation in kinetoplastids can allow for further understanding of their biology along with innovative approaches for the treatment of pathogenic protozoans. Future studies should address the substrates, functional relevance, and druggability of Nt-acetylation in kinetoplastids to fill knowledge gaps, while also paving the path towards target-oriented drug development.

KEYWORDS: N-terminal acetylation, Acetyl transferases, Drug targets, Trypanosomes, Leishmania

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1. INTRODUCTION

Nα-terminal acetylation (Nt-acetylation) is the most common and evolutionarily conserved post-translational modification (PTM) (1)(2). It is emerging as a subject of significant interest in the study of kinetoplastids (3)(4) and pathogenic protozoan species of the genera Trypanosoma and Leishmania. These parasites are responsible for numerous human diseases such as African trypanosomiasis, Chagas disease and different forms of Leishmaniasis (4)(3)(5).

Acetylation is a PTM in which an acetyl group is attached to the amino-terminal residues of proteins, and has been shown to be important in many cellular processes, affecting protein localization, functionality, stability, and interactions (6)(7). In kinetoplastids, the specificity of unusual cellular processes and unconventional biology adds new dimensions to Nt-acetylation. In contrast to the classical eukaryotic strategy for transcriptional control of gene expression, kinetoplastids lack a canonical mechanism for transcriptional regulation and instead utilize post-transcriptional processing (8)(9). This peculiarity may extend to the regulation of Nt-acetylation, where the absence of certain components seen in other organisms raises questions about the precise machinery involved (10)(11)(12)(13).

The intricacy of the life cycle of kinetoplastids, characterized by transmission via insect vectors and by stages within their mammalian hosts, can only mean precise molecular processes are involved in driving the life cycle of these parasites (schematic in Figure 1) (14)(15). For instance, Nt-acetylation

may be necessary for protein modulations that allow parasites to evade the host immune challenges and adapt to different environmental cues. A recent identification of some kinetoplastid-specific N-alpha terminal acetyltransferases (NATs) (3)(4), enzymes that undergo acetylation reaction, adds another layer of complexity to this intricacy. If examined closely, these observations suggest that kinetoplastids may have special traits that make them different from those in other eukaryotes. To better understand Nt-acetylation in these parasites in this context therefore, it is important to understand substrate specificity, regulatory actions, and effects of these kinetoplastid-specific NATs on cellular functions. In addition, the use of Nt-acetylation as a target for treatment is increasingly becoming important in different fields (16). With few treatment choices for kinetoplastid diseases and a desire for new drug targets, it is important to examine how the acetylation system can be targeted. Treatments aimed at this modification could interfere with vital cellular processes in parasites, while reducing the potential effects on host cells. In this context, the investigation of N-terminal acetylation (Nt-acetylation) in kinetoplastids extends beyond fundamental molecular biology, offering novel avenues for therapeutic intervention against parasitic infections. This review endeavors to comprehensively synthesize current knowledge on Nt-acetylation, emphasizing its distinctive characteristics, functional consequences, and prospective applications as a target for anti-kinetoplastid drug development.

Finally, this review highlights valuable opportunities, issues, and future paths for Nt-acetylation studies. First, we discuss the basic biology of kinetoplastids, their impact worldwide and therapeutic strategies for kinetoplastid control. This is followed by a discussion on the roles that PTM may be playing in molecular interactions in kinetoplastid, and a comparison of kinetoplastid NATs across species. We conclude by offering future research directions.

1. Kinetoplastids (*T. brucei*, *T. cruzi,* and Leishmania).

Kinetoplastids are a mixed group of tiny single-cell organisms known for their tails and kinetoplast, a unique and special cellular feature found near the base of the flagellum. The feature contains a complex system of mitochondrial DNA referred to as kinetoplast DNA (kDNA) inside their one mitochondrion (17) (18). The two main groups in kinetoplastids are Trypanosoma and Leishmania, which cause severe illnesses in humans and animals (18). For Trypanosoma, African Trypanosome (*T. brucei*), with a complex life cycle involving mammalian hosts and tsetse flies (19)(20) (Figure 1) and transmitted by tsetse flies, do cause sleeping sickness in humans and Nagana in cattle (19). The disease progresses through various stages in both humans and animals. Also belonging to Trypanosoma, *T. cruzi* causes Chagas disease (American trypanosomiasis), is transmitted by reduviid bugs, Figure 1 and is most prevalent in Latin America (21). Severe cardiac and digestive complications can occur in patients with chronic infections.

For the Leishmania, a group of parasites known to be responsible for a spectrum of diseases collectively known as Leishmaniasis, transmission is through the bite of infected sandflies and can cause cutaneous, mucocutaneous, and visceral forms of the disease (22). Leishmania species exhibit various adaptations to different hosts and environments, contributing to the diverse clinical manifestations of Leishmaniasis (23) (see life cycle in Figure 1).

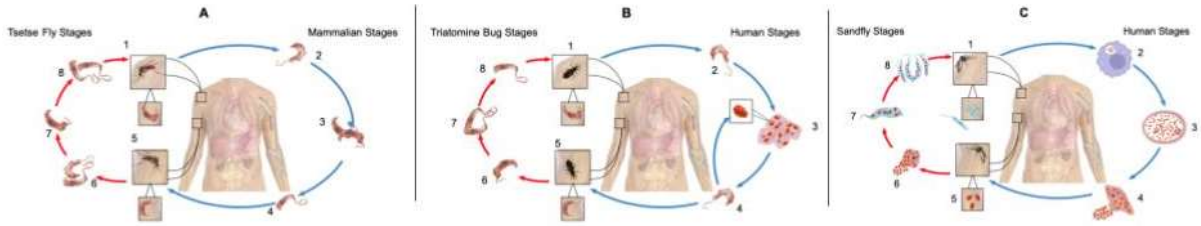


Figure 1. Life Cycles of Trypanosomatid Parasites

This figure illustrates the complex life cycles of three important trypanosomatid parasites: Trypanosoma brucei, Trypanosoma cruzi, and Leishmania spp. A. Trypanosoma brucei life cycle: (1) Tsetse fly injects metacyclic trypomastigotes during a blood meal. (2) Metacyclic trypomastigotes transform into bloodstream trypomastigotes. (3) Trypomastigotes multiply by binary fission in various body fluids. (4) Trypomastigotes circulate in the bloodstream. (5) Tsetse fly ingests trypomastigotes during a blood meal. (6) In the fly's midgut, trypomastigotes transform into procyclic trypomastigotes and divide. (7) Procyclic trypomastigotes transform into epimastigotes. (8) In the salivary glands, epimastigotes transform into infective metacyclic trypomastigotes. B. Trypanosoma cruzi life cycle: (1) Triatomine bug defecates near the bite site, releasing infective metacyclic trypomastigotes. (2) Metacyclic trypomastigotes enter cells and transform into amastigotes. (3) Amastigotes multiply inside cells. (4) Amastigotes transform into trypomastigotes, which are released into the bloodstream. (5) Triatomine bug ingests trypomastigotes during a blood meal. (6) In the bug's midgut, trypomastigotes transform into epimastigotes. (7) Epimastigotes multiply by binary fission in the midgut. (8) In the hindgut, epimastigotes transform into infective metacyclic trypomastigotes. C. Leishmania spp. life cycle: (1) Promastigotes are introduced into the host through a sandfly bite. (2) Macrophages and other mononuclear phagocytes engulf promastigotes. (3) Promastigotes transform into amastigotes within phagocytes. (4) Amastigotes multiply in various tissues and infect other cells. (5) Sandfly ingests macrophages infected with amastigotes during a blood meal. (6) Parasitized cells are ingested by the sandfly. (7) Amastigotes transform into promastigotes in the sandfly gut. (8) Promastigotes divide in the gut and migrate to the proboscis.

Kinetoplast, the special traits of kinetoplastida organisms, has an exclusive design that is a prerequisite for kinetoplastids, which coordinates the regulatory mechanism which impacts in direct ways, the processes of energy creation and replication. One other structure that is unique to kinetoplastida organisms is the flagellum, important in parasite mobility and survival (24). The flagellum is the result of cellular dysmorphogenesis, a morphological oddity,

termed the flagellar pocket that is unique to kinetoplastids (24). Simply put, the pocket functions as an arena for transporting materials across the cell membrane and as a device for regulating surface proteins. The flagellum is, therefore, a key player in host-parasite interactions (24)(25).

Kinetoplastids have additionally a plethora of unique features such as glycosomes and specialized peroxisome-like organelles (26). These organelles play a significant role in glycolysis and metabolism of different compounds, for example, fatty acids (26). Moreover, glycosomes also take part and are responsible for the adaptation of kinetoplastids to different host environments. The glycosomes are therefore potential drug targets (27).

At the molecular level, kinetoplastids also show an array of compound structures, particularly at the surface, that are important for host–parasite interactions and immune evasion (4). Variant Surface Glycoproteins (VSGs), a collection of proteins found on the surface of African trypanosomes, are antigenically variable. Because of this variability, VSGs enable African trypanosomes, including *T. brucei*, to gain a competitive advantage through selection and host immune responses (25). In Leishmania species, surface molecules like lipophosphoglycan (LPG) and glycoprotein 63 (gp63) show that the pathogens are immune modulated and infective (28)(29). For *T. cruzi*, the causing agent of Chagas disease, Trypomastigote surface antigen (TSA) and mucin-like glycoproteins are some of the typical molecules enabling the parasite to escape the host (4) (30)(31). Altogether, these molecules are actors in kinetoplastid pathogenesis and are therefore the most probable targets for treatment and vaccine development (31) (32). It is also likely that many of the proteins associated with the molecules are N-terminally acetylated, thus providing a basis for studying the mechanism of N-terminal acetylation as potential drug targets.

One of the ways by which kinetoplastids, such as Trypanosoma and Leishmania, differ from other eukaryotes is that their gene regulation mechanism operates at the post-transcriptional routes in the production of polycistronic RNA (genomic materials) (9) (33). Instead of going through the usual mode of control at the transcription level, kinetoplastids employ RNA processing and stability for gene expression (9), while preserving mRNA processing, including trans-splicing of 5' capped mini-exon sequences and polyadenylation, essential to basic downward processes in eukaryotes (9) (34). In this context, the bio editing of RNA involving insertion and deletion of uridine residues into the DNA to create diversity in protein coding, offers yet another way of regulating cellular activity at the RNA level (35)(36).

The simplified and non-conical mechanism of kinetoplastid gene regulation mediates expedited acclamatory responses to physiological changes or environmental stimuli. This effectively eliminates the requirement for an elaborate transcriptional apparatus for gene expression. This approach to post-translational regulation facilitates efficient protein modulation in kinetoplastids as they respond to external cues. An informed understanding of these unique regulatory processes is essential to the development of therapeutic strategies, which can target kinetoplastid-borne diseases with a high degree of specificity. Current hypotheses state that a significant portion of kinetoplastid proteins exhibit N-terminal acetyltransferases (NATs)-mediated N-terminal acetylation. It is probable that this kind of modification contributes to the maintenance of parasite infectivity and pathogenicity.

2. IMPACT OF KINETOPLASTIDS ON GLOBAL HEALTH.

Kinetoplastids such as the Trypanosoma and Leishmania species have a massive impact on global health as they cause diseases such as African Trypanosomiasis, Chagas disease, and Leishmaniasis (14)(15). In tropical areas, such infectious diseases commonly affect populations in low-resource areas, which in turn brings about a cycle of poverty (37). Kinetoplastid infections particularly cause a large number of morbidities and mortalities, which in turn hampers economic development and overloads healthcare systems (38). In this regard, the WHO (World Health Organization) ranks the need to address kinetoplastid infections as one of the required means of achieving global health and promoting efforts to manage neglected tropical diseases.

Collectively, the known diseases caused by kinetoplastida organisms, kinetoplastids complex life cycles, and challenges in diagnosis and treatment, as well as the fact that they infect a range of organisms, currently make the infection problematic (38).

3. RESEARCH, DRUG DEVELOPMENT, AND THERAPEUTIC STRATEGIES FOR KINETOPLASTID CONTROL.

Kinetoplastid research has concentrated on understanding parasites that have a different biology from the hosts and on the relationships between the parasites and their hosts. The focus has also been on the development of powerful therapeutic drugs (14) (39)(40)(41). In finding solutions to controlling kinetoplastid, the occurrence of drug resistance and parasites with complex life cycles continually demonstrates the vital need for innovative methods for drug development. Diseases such as African trypanosomiasis, Chagas disease, and Leishmaniasis are those that can become a pandemic if not adequately addressed (14).

Kinetoplastid biology, which is unique in nature and characterized by unconventional gene regulation and life cycle patterns, present both challenges and opportunities (39). As an example of an opportunity, some undertakings currently are deeply into the molecular mechanisms of host-parasite interactions (42). In this approach, the goal is to probe specific parasite vulnerabilities as targets for novel drugs. The incorporation of genomics, proteomics, and high-throughput screening into the process of target identification

and validation is among the most promising developments (43).

The development of revolutionary therapeutic strategies, such as vaccines and small molecules that target essential pathways, represents the main goal (4) (44). Drug discovery is critical for establishing treatment regimens that overcome challenges such as drug resistance and maximize efficiency in killing the pathogen (45). In summary, problems such as drug resistance and the elaborate life cycle of these pathogens may justify new approaches to drug development, which would require both international cooperation and interdisciplinary approaches.

The present therapy ascribed to kinetoplastida organisms is essentially chemotherapy that exploits drugs such as Suramin, Pentamidine, Benznidazole, and Amphotericin B, but these drugs have the problems of toxicity and drug resistance (46). Moreover, antimonials and miltefosine are recommended for Leishmaniasis (47), whereas nifurtimox and benznidazole are used for the treatment of Chagas disease (46). Although the search for novel and more effective therapeutics has been ongoing for many years, this remains a challenge. Projects are still being conducted to develop new drugs whose targets are kinetoplastid enzymes characterized by species-specificity and essentiality at the same time (41)(45). The huge demand for drug target identification has placed kinetoplastid research in a nice position for offering solutions. Therefore, using kinase inhibitors to inhibit the cell division mechanisms of trypanosomes and prevent infection is one of the top research areas (41).

Immunotherapeutic modalities and vaccine technologies are among the most widely used methods for preventing the proliferation of infections (14). However, although increased resistance to infection is expected to be achieved using this approach, improving the healthcare infrastructure should also be a goal. Efficacy and safety are also two key elements to be addressed in this context. Regarding the fact that it is so important to promote the reform of therapeutic methods for kinetoplastid diseases, a critical emphasis here must be placed on research which can in turn help to find effective therapeutics. Consequently, one key focus of future research is the study of kinetoplastid infections and their interactions with their respective hosts, and the Nt-acetylation mechanism is an obvious target in it. In this context, we give an account of what is known about N-alpha acetyltransferases, the main enzymes involved in Nt-acetylation.

2. Overview of N-alpha terminal acetylation.

Nt-acetylation is a ubiquitous post-translational modification that is evolutionarily conserved in eukaryotic cells (1) (2). This modification plays such a significant role in several cellular processes as influencing protein stability, subcellular localization of proteins, and protein interactions with other molecules, which has been established for the last several decades (5)(6). Although not the focus of this review, N-epsilon terminal acetylation provides a section of protein – the N-terminus – with the highest level of acetyltransferase in the presence of acetylated lysine protein in a process that is reversible (48)(49) (Figure 2).

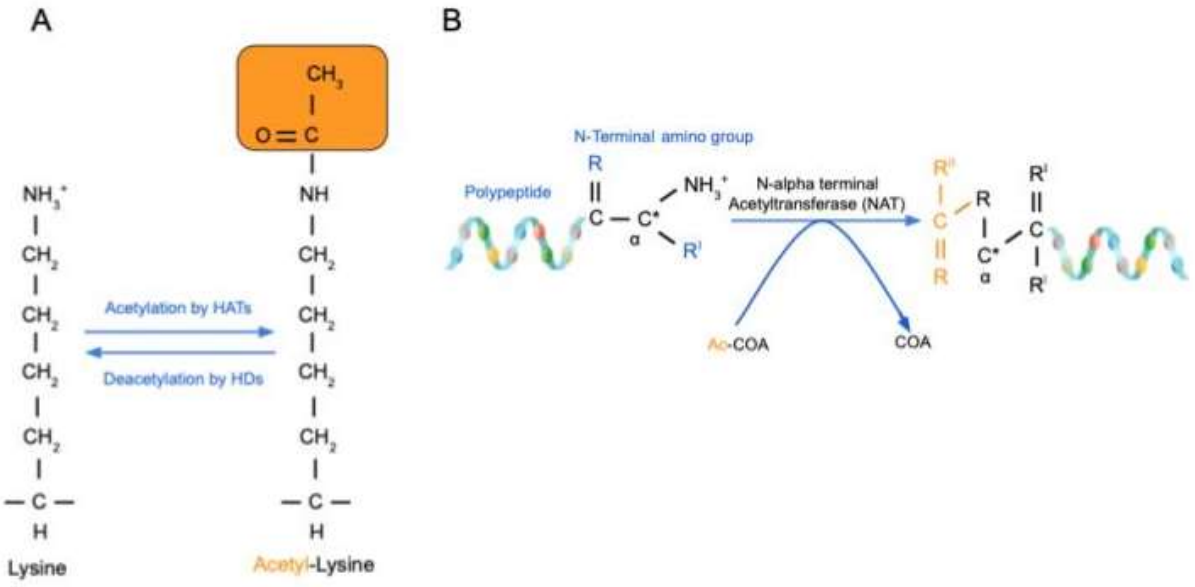


Figure 2.

Protein acetylation. A. Lysine/histone acetyltransferase (HATs), a reversible process by histone deacetylases (HDs/HDACs). B. N-alpha terminal acetyltransferase (NAT), a putative non-reversible process.

1. Enzymatic Machinery

N-alpha terminal acetyltransferases (NATs) are involved in addition of acetyl groups to amino termini of proteins (1), (Figure 2). Acetylation of proteins in certain amino acid sequences or protein structures is a NAT attribute (1). The machinery taking part in the process of Nt-acetylation has several components and as a consequence can offer the same or different patterns of Nt-acetyltransferase activities in organisms from different evolutionary stages (50)(51)(52).

2. Functional implications and conservation across species

The N-terminal acetylation directly affects protein structure and biochemical properties (53). This modification is known to participate in various cellular processes such as chromatin organization, vesicular trafficking, and cellular stress responses (13)(54). It is frequently used as a label for protein degradation and is related to the half-life of proteins in cells (10) (55). Furthermore, Nt-acetylation can interfere with the interactions of proteins with other molecules, thus affecting the assembly of complexes and the communication of extracellular signals (5) (13)(54). It is also able to actuate protein conformation (56) and plays a central role in many cellular activities and hampers human health (5).

Nt-acetylation is a conserved event across eukaryotes, from bacteria and yeast to mammals (57)(58). Conservation is the most basic of biological processes. The core of NAT and its functional outcomes are universal, notwithstanding variations observed in specificity and acetylation prevalence among organisms (51).

3. Disease associations, emerging roles, and research advances of Nt-acetylation

Recent research has shed light on the new roles of Nt-acetylation in various cell settings, thus unveiling its impact beyond classical functions (13). Indeed, Nt-acetylation has been associated with diverse diseases ranging from cancer to neurodegenerative disorders (16). Changes in the acetylation pattern has also been shown to be one reason for the malfunctioning of cells; thus, increasing chances of disease pathogenesis (59). Understanding the intricacies of Nt-acetylation in diseases will therefore pave the way for the development of novel therapeutic targets. Advances in mass spectrometry and proteomics have increased our ability to identify and quantify acetylated proteins, thereby providing a comprehensive view of the acetylome under various cellular conditions (60).

In summary, Nt-acetylation represents a fundamental form of post-translational modification associated with a myriad of events in cellular physiology and pathology. The progression and functionality of the enzyme machinery, substrate specificity, and the resulting effects signal a paradoxical relationship between the molecules involved in the process. As research continues, the next step in the elucidation of the regulatory mechanisms and significance of Nt-acetylation may be a new area in our understanding of cellular processes, as well as in the therapeutic medication of various pathological conditions. Kinetoplastids are not well characterized in terms of Nt-acetylation and this is one more reason to delve into summarizing what is known about this post-translational protein modification in Kinetoplastids in this review.

3. N-alpha Terminal Acetyltransferases (NATs) in kinetoplastids.

The study of Nt-acetylation in kinetoplastids, a group of protozoan parasites like Trypanosoma and Leishmania species, has just begun (3) (4) (61)(62). It is an important post-translational modification that most likely influences various aspects of protein functions and cellular processes in these organisms. Specifically, PTM may be impacting protein stability, localization, interactions, and crucial biological pathways in kinetoplastids, thus are important for their survival and pathogenicity (4). That said, investigators have not found evidence that Nt-acetylation in kinetoplastids is the main actor in regulating basic cellular functions of proteins that allow these parasites to go through the different stages of their life cycle. It is likely though that, Nt-acetylation impacts the movement of proteins between different cellular parts and may help kinetoplastids communicate with human cells and stay safe from immune reactions (4). These hypotheticals suggest the lack of clarity about the significance of Nt-acetylation in kinetoplastids. In terms of public health, Nt-acetylation, which is thought to be an important process in kinetoplastids, is more than a simple biological marker; it has the potential to be targeted for therapeutic purposes. A test of this concept would be to develop drugs that block this modification with the hope that they mitigate infections by these parasites.

1. Identification and classification of NATs in kinetoplastids

The identification and classification of NATs are the primary steps in research designed to comprehend protein post-translational modifications (PTMs) and diversity in organisms. The identification process, both at the cellular and molecular level, is a mix of molecular, biochemical, and bioinformatics solutions. At the initial step, molecular identification of NATs involves the isolation and characterization of proteins for Nt-acetylation (3). This step has been greatly aided by proteomic techniques such as mass spectrometry which monitors changes in protein mass after acetylation (60). The enzymes involved in modification can then be located by comparing acetylated and non-acetylated protein samples (13) (63). Bioinformatic instruments and datasets play a significant role in distinguishing and characterizing NATs (63).

Kinetoplastid NATs are identified by aligning a known NAT sequence from a model organism with the genome of interest and searching for homologous sequences (3). The acetyltransferase enzyme, along with its conserved and yielded domains, is proximal to NATs. However, other studies have shown that NATs display a plethora of different structural and substrate specificity profiles (64). NATs consist of catalytic subunits, which can be assigned to different families that are able to perform a specific cellular function, and each one of the catalytic subunit families is involved in a specific cellular function (56) (64) (65). The NAT families in humans are NatA, NatB, NatC, NatD, NatE, NatF, and NatH (56). Furthermore, NatG has been reported to be present in plants (66).

The connection between NATs and preferred substrates is shown in (56) and (67). These studies showed that each NAT

family had distinct substrate preferences: the proteins that are specifically targeted have a certain sequence of amino acids that are acetylated through the NAT family. Here, we annotated the catalytic subunits of kinetoplastid NATs using a BLAST search, Table 1.

2. Substrate Specificity

Nt-acetylation shows substrate specificity for NATs recognition by distinct amino acid sequences or structural correlations preceding the N-terminus of a protein (64). Specifically, (i) the NatA complex prefers N-termini of protein where the first amino acid is either a small uncharged one or the initiator, methionine (64), (ii) NatB generally accepts N-terminal methionine followed by a basic residue (64) (68), and (iii) NatC identifies substrates containing a hydrophobic residue at the second position (3)(64). These preferences give rise to the diversity of Nt-acetylated proteins and highlight the importance of sequence context in substrate recognition.

NatA, which contains the catalytic subunit Naa10 (Ard1) and the auxiliary subunit Naa15 (Nat1), is among the best-characterized NAT complexes (69). Naa10 possesses acetyltransferase activity and Naa15 stabilizes the complex and promotes substrate specificity. NatA commonly catalyzes the acetylation of proteins containing amino-terminal sequences that begin with a small, neutral amino acid (64) (69). The initial indication showed that NatA is present in kinetoplastids (3), Table 1. It should be noted that in the case of *T. cruzi*, the CL-Brener strain, a hybrid comprising the Esmeraldo and None-Esmeraldo strains, the TcNatA displayed is Esmeraldo and non-Esmeraldo homologous sequences merged during the assembly.

The NatB contains the catalytic subunit Naa20 (Nat3) and the auxiliary subunit Naa25 (Mdm20). NatB favors acetylation of proteins containing methionine-glutamic acid, methionine-aspartic acid, methionine-glutamine, and methionine-asparagine-N-termini (70) (71). To date, there have been no regular experimental substrate screenings for NatB in kinetoplastids. However, in our hands, predicted NatB is present, Table 1. In *T. cruzi* CL-Brener, both haplotypes were detected, but only one is shown, Table 1.

NatC complex is a mixture of the catalytic subunit Naa30 (Mak3) joined by Naa35 (Mak10) and Naa38 (Mak31) (72) (73). NatC generally operates on proteins with amino-terminal residues, starting with methionine-leucine, methionine-isoleucine, methionine-tryptophan, or methionine-phenylalanine N-termini (72) (73). Indeed, sequence analysis and experimental data, especially for *T. cruzi*, show that NatC is present in kinetoplastids (3), Table 1. Two haplotypes were detected in *T. cruzi* Cl-Brener strain.

NatD consists of the catalytic subunit Naa40 (Nat4) (74). Naa40 is different from other NATs. To date, two substrates, H2A and H4, have been detected in yeasts and humans. H2A acetylates S-G-R N-temini and for H4, it acetylates S-G-G N-termini (74)(75). No NatD was found within kinetoplastids (61), Table 1.

NatE and NatF: Other NATs are NatE and NatF, with Naa50 (Nat5) as their catalytic subunit for NatE and Naa60 (Nat6) for NatF (1). These complexes have distinct substrate specificities and contribute to acetylation of protein products at the N-terminus (1). Furthermore, NatG (Naa70), found in plants, acetylates M-, A-, S-, and T- N-termini substrates (4)(66). NatH (Naa80) has been detected only in animals and acetylates ß- and γ-actin substrates, which contain acidic N-termini DDDI and EEEI, respectively (4)(76). However, in this review, an attempt to identify NatF, G, and H in kinetoplastids was not successful. Human NatF and H are known to acetylate membrane and actin proteins, respectively (67). It would be of interest to determine whether membrane and actin proteins are acetylated by NATs in kinetoplastida organisms as well. In the *T. cruzi* CL-Brener strain, the identified catalytic subunits contained both the haplotypes. This raises the question as to whether in biological function, one of the haplotypes NATs is shut down or both are active at the same time, and what it means in terms of parasite infectivity. Experimental evidence will provide these answers in the distant future.

As described, the catalytic subunits of these NATs play a central role in influencing various cellular activities, including stabilization or destabilization of proteins, shifting the subcellular localization of macromolecules, and modulation of protein interactions (10). Nt-acetylation plays important roles in modulating protein folding, degradation, and functions, which in turn influences diverse cellular pathways (10) (54) (77), and moreover N-terminal acetylation shields proteins from degradation (78). Taken together, these functional N-terminal roles may also be present in kinetoplastids (4).

In addition, there is a possibility of crosstalk involving PTMs in kinetoplastids, which may entail complex interactions among various protein modifications. Investigations about the interplay between N-terminal acetylation and other modifications, such as phosphorylation and ubiquitination, has revealed a sophisticated regulatory framework that governs cellular processes, particularly phase separation within cells (79). This aspect, however, remains unexplored in kinetoplastids and yet the interaction between acetylation and phosphorylation could affect protein stability and functionality in kinetoplastid organisms, potentially aligning with the findings of other studies (13). Moreover, ubiquitination affects protein degradation pathways (77) (79), which may also be applicable to kinetoplastids. Understanding these dynamic interactions could shed light on the precise control of cellular activities and present possible therapeutic avenues.

The intricacies highlighted above points to the importance of PTMs for managing diverse biological functions. Importantly, blocking modifications, such as N-terminal methylation or acetylation can influence the subsequent N-terminal acetylation of proteins (80). Previous studies have indicated that early modifications at the N-terminus can either

inhibit or facilitate acetylation by NATs (1) (80), showing profound complexity regarding how PTMs regulate protein functionality. However, no detailed research exists on N-terminal acetylation, specifically in kinetoplastids. Additionally, when examining signal transduction pathways within kinetoplastids (81), it is clear that N-terminal acetylation may play a pivotal role in shaping these pathways and affecting key cellular responses, a phenomenon documented in other investigations (10). Analysis of this intricate web of relationships holds great promise for advancing our understanding of cellular mechanisms specific to kinetoplastid organisms.

With regard to the disease-related effects, dysregulation of NATs and aberrant Nt-acetylation were found to be the cause of several diseases such as cancer and neurological disorders (82). Injuries to the catalytic subunits and their independence of the NAT complexes can provide a way for the theoretical prediction of new therapeutic targets for these conditions and even kinetoplastid infections in the future. Briefly, both the catalytic subunits and the residues of NATs, Nt-acetylating act as a core of the control of Nt-acetylation, a significant PTM with many functional implications in cell biology and diseases. The finding of the target selectivity of these catalytic units and their specific roles in cells improves our understanding of the complicated regulatory mechanism of acetylation, thereby offering such interventions for individuals with related pathologies.

4. COMPARATIVE ANALYSIS OF NATS WITHIN KINETOPLASTID SPECIES AND OTHER ORGANISMS.

Comparing the NATs of organisms, both parasitic and non-parasitic, not only helps to elucidate the peculiarities of the NATs in kinetoplastids but also within kinetoplastida species. Through this practice, researchers can become cognizant of similarities and divergences in things such as the NAT machinery the organisms share, which is one of the building blocks of the overall classification.

1. Primary sequences.

We performed PSI-BLAST analysis using different human NATs catalytic units as query sequences against different kinetoplastid proteomes. The identified catalytic subunits of the different NATs are shown in Table 1.

Table 1. Selected kinetoplastid catalytic subunits and their other characteristics.

Catalytic subunits	Protein	Length (aa)	Identity (%)	Localization	Source
Tc NatA	XP_817467.1	258	60	Cytoplasm/Nucleus	(3)
Tb NatA	XP_828529.1	239	61	Cytoplasm/Nucleus	Predicted (83)
Lm NatA	XP_001681770.1	260	56	Cytoplasm/Nucleus	Predicted (83)
Tc NatB	XP_808666.1. Es	186	51	Mainly cytoplasm	Predicted (83)
Tb NatB	XP_024498417.1	239	31	Mainly cytoplasm	Predicted (83)
Lm NatB	CAG9575008.1	186	53	Mainly cytoplasm	Predicted (3)
Tc NatC	XP_813656.1 Non-Es	300	45	Mainly cytoplasm	(3)
Tb NatC	XP_845859.1	308	45	Mainly cytoplasm	Predicted (83)
Lm NatC	XP_001683197.1	289	45	Cytoplasm/Nucleus	Predicted (83)
Tc NatD *	-	-	-	-	-
Tb NatD *	-	-	-	-	-
Lm NatD *	-	-	-	-	-
Tc NatE	XP_812778.1 Es	203	35	Mainly cytoplasm	Predicted (83)
Tb NatE	XP_822567.1	205	32	Mainly cytoplasm	Predicted (83)
Lm NatE	XP_003721691.1	182	32	Mainly cytoplasm	Predicted (83)
Tc NatF	XP_812778.1 **	-	-	-	-
Tb NatF	XP_822567.1 **	-	-	-	-
Lm NatF	XP_003721691.1 **	-	-	-	-
Tc NatG *	-	-	-	-	-
Tb NatG *	-	-	-	-	-
Lm NatG *	-	-	-	-	-
Tc NatH *	-	-	-	-	-
Tb NatH *	-	-	-	-	-
Lm NatH*	-	-	-	-	-

* No significant sequence similarity was found. **: PSI-BLAST e-values worse than threshold. **aa**: amino acid.

Note that in Table 1, *T. cruzi*, Cl-Brener strain was used and one haplotype, either Esmeraldo-like (Es) or Non-Esmeraldo-like (Non-Es) with the highest hit was chosen. However, for TcNatA, the catalytic subunit displayed is Esmeraldo and non-Esmeraldo homologous sequences merged during assembly. For *T. brucei*, *T. brucei brucei* TREU927 strain was chosen and for Leishmania, *L. major* Friedlin strain was used.

The amino acid lengths of human NatA, B, C, D, E, F, and H is 235, 179, 362, 237, 169, 242, and 286, respectively.

For NatA, the amino acid length of the human catalytic subunit is similar to that of kinetoplastids, with approximately 60% identity, Table 1. TbNatB has a much longer amino acid compared to its human counterpart, and only 31% identity, Table 1. Human NatC contains much more amino acid than *T. cruzi* Cl-Brner, *T. brucei brucei* and *Leishmania major* counterparts, Table 1.

For kinetoplastids NatF, when the human sequence was PSI-BLAST analyzed against *T. cruzi*, *T. brucei,* and Leishmania proteome, no sequence with an e-value better than the threshold was observed. Only sequences with e-values worse than the threshold were detected, corresponding to kinetoplastid NatF represented in Table 1 with two stars. This suggests that NatF might be absent in kinetoplastids. In our hands, NatD, NatG and NatH in *T. brucei*, Leishmania, and *T. cruzi* were absent as no significant sequence similarity was found, Table 1.

When similar analysis was carried out with human NatH protein sequence against kinetoplastid (taxid:5653), a *Trypanosoma vivax* protein (KAG8345080.1) with a significant e-value, 147 amino acids in length, and 43% identity was observed. This protein contains a GNAT motif with a coenzyme-A binding pocket and belongs to the N-acyltransferase superfamily. If experimentally confirmed, the information herein indicates that NatH is present in *T. vivax,* the subspecies, causing Ngana disease in cattle and wild mammals (84), but not in those associated with human diseases.

Of all the NATs in kinetoplastida organisms, thus far, only NatA and NatC have been somehow characterized in *T. brucei* and *T. cruzi* (3), suggesting that, much more experimental data about NATs in kinetoplastids are warranted to avail their substrate profiles and significance of parasite physiology. However, understanding the protein secondary structures may partially aid the characterization of NATs in kinetoplastids.

2. Secondary structure.

One of the critical features of a protein may be its secondary structure post-Nt-acetylation. Parasite NATs, as do all enzymes, probably have certain structural requirements in terms of their substrates. At the very least, these requirements may include binding to helices or unstructured regions. The secondary structural effects might be added as an extra element to determine the specificity, which might be the general folding and conformation of proteins in the cellular milieu. This can be the way host cells regulate their metabolic pathways and other cellular processes by means of functional proteins, and if so, can serve as a reflection of the ability of kinetoplastids to respond to several different host environments.

To assess the protein secondary structure, we used I-TASSER (Iterative Threading ASSEmbly Refinement) (85), where the number of alpha helices and beta strands were examined. Although proteins with more alpha helices tend to be more stable and resistant to mutations compared to those with more beta strands, proteins with more alpha helices can evolve more rapidly while maintaining their overall structure and function by adapting to a new environment (86).

The specific arrangement of beta strands can create active sites for enzymatic reactions; moreover, proteins that are related share the same beta strand, suggesting common ancestry and functional similarities (86). Although the predicted secondary structure is not shown, as indicated in Table 2, all the different NATs have similar numbers of beta strands, suggesting evolutionary conservation. However, there are more alpha helices for kinetoplastids, especially for NatA, B and E than for their human counterparts, in contrast to NatC, Table 2. Diverse functional capabilities are possible because of more proportion of alpha helices, which in this case, also reflects more predicted active sites, except for human NatB, Table 2.

Table 2. Human and kinetoplastids selected NATs showing alpha helix, beta strand and active sites.

NATs	α-Helix (H)	β-Strand (E)	Active site
HNatA. sp|P41227|	6	7	112-**R**, 118-**A**, 126-**L**, 128-**F**
TcNatA. XP_817467.1	7	7	76-**I,** 78-**V,** 113-**R,** 119-**A**,125-**E**,127-**L**,129-**F**
TbNatA XP_828529.1	7	7	76-**I**, 78-**V**, 90- **R**,113-**R**,119-**A**,127-**L**,129-**F**
LmNatA XP_001681770.1	7	7	78-**V**,113-**R**,119-**A**,127-**L**,129-**F**
HNatB p|P61599|	5	7	94-**E**,113-**R**,119-**A**,126-**L**,155-**A**
TcNatB XP_808666.1	9	6	93-**V**,128-**R**,134-**A**,141-**L**
TbNatB XP_024498417.1	7	6	78-**V**,113-**R**,119-**A**,126-**L**
LmNatB CAG9575008.1	7	7	78-**V**,113-**R**,119-**A**,126-**L**
HNatC sp|Q147X3|	15	7	223-**V**, 291-**I**, 301-**R**, 304-**G**, 315-**Y**, 339-**L**
TcNatC XP_813656.1	11	8	207-**V**, 239-**E**, 247-**A**, 254-**L**, 256-**F**
TbNatC XP_845859.1	12	7	205-**V**, 237-**E**, 245-**A**, 252-**L**, 254-**F**
LmNatC XP_001683197.1	10	8	197-**V**, 229-**E**, 237-**A**, 244-**L**, 246-**F**
HNatE sp|Q9GZZ1|	5	7	120-**A**, 129-**F**
TcNatE XP_812778.1	6	7	101-**I**, 103-**V**, 145-**A**,154-**F**
TbNatE XP_822567.1	5	7	101- **I**, 103-**V**, 118-**E**,154-**F**

LmNatE XP_003721691.1	6	7	92-**I**, 94-**V**,136-,145-**F**,164-**I**

3. Three-dimensional structure.

Structural insights into NATs in kinetoplastids could be important for understanding the peculiarities of these enzymes in protozoan parasites. Kinetoplastids may have specific structural features in their NATs that are important for the regulation of PTMs. These are critical for their biology, thereby providing the demand for crystallographic studies to yield valuable information on the three-dimensional architecture of NATs in kinetoplastids.

Based on Nt-acetylation prediction in kinetoplastids, one can hypothesize that the amino acid context surrounding the N-terminus is an important factor in the regulation of Nt-acetylation. If identified, substrate specificity may underlie the cellular regulation of Nt-acetylation in kinetoplastids by the sophisticated control mechanisms that govern this PTM. Considering the interplay of sequence context, structural preferences, and eventual cellular localization, one can imagine that NATs may contribute to the unique protein modification landscape in these parasitic organisms, providing insights into their adaptation strategies and potential avenues for therapeutic interventions against kinetoplastid infections.

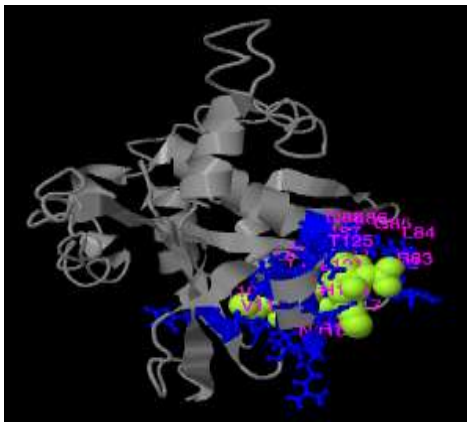
Moreover, the enzymatic complex responsible for this modification, NATs, shows conservation of core catalytic domains across diverse organisms, indicating evolutionary preservation of this modification process and suggesting essential roles in core cellular processes, such as protein stability, cellular localization, and interactions, emphasizing its functional importance from unicellular organisms to higher eukaryotes. Although the core enzymatic machinery is conserved, species-specific adaptations should be pursued, because kinetoplastids may contain unique NAT isoforms that diverge from those in their hosts, reflecting adaptations to their particular cellular environments and lifestyles.

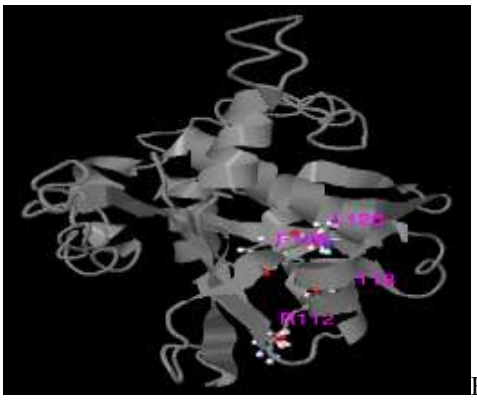
A comparative analysis may also indicate substrate specificity, which could provide insights into the functional diversification of Nt-acetylation in different biological contexts. It is likely that kinetoplastids, as parasitic organisms, may adapt to their Nt-acetylation machinery, which fulfills the specialized requirements for their survival within the host environment. These adaptations may provide insight into host-parasite interactions and the pathogenicity of these organisms.

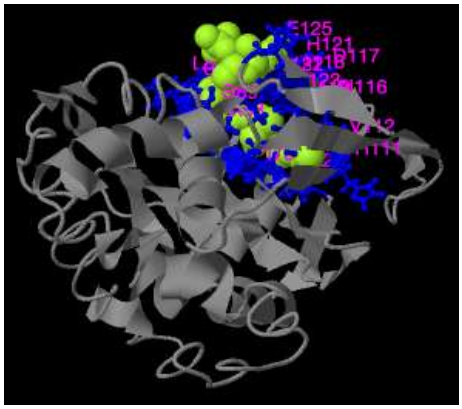
In the context of 3D structure and active site, that is, the enzyme parts where molecules bind and where reaction of chemical takes place, we used I-TASSER (Iterative Threading ASSEmbly Refinement), as conducted elsewhere (85), to generate 3D structure and predict active site, see Figure 3 and Table 2. Active sites can influence substrate specificity, catalytic function, binding affinity, protein function and flexibility, evolution, and adaptation, because different active sites reflect the evolutionary adaptations of proteins to perform specific functions in various biological contexts (87).

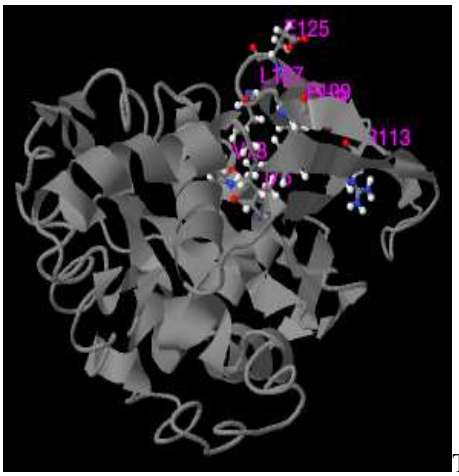
In this study, although we did not examine in detail as presented elsewhere (87), NatA in kinetoplastids has more active sites than its human counterparts, (Figure 3) and Table 2. Furthermore, valine, an active site observed in kinetoplastids, is missing in human NatA. For NatB, there were almost an equal number of active sites, as presented in Table 2. Similar to NatA, human NatB does not contain valine; moreover, the active site contains glutamic acid, which is absent in kinetoplastids, Table 2. For NatC, the human active site appears different from the kinetoplastids, except for valine, Table 3. NatE however, has only two predicted active sites for humans in contrast to kinetoplastids, which have more, Table 2.

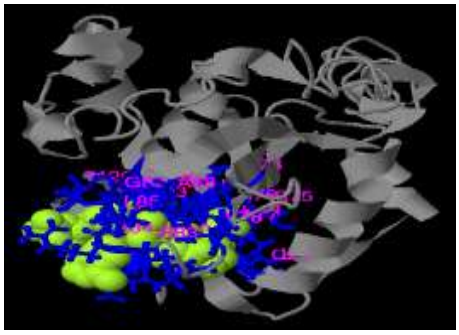
In all the predicted NATs (i.e., NatA, B, C, and NatE), there are 22 ligand-binding site residues on average, except for human NatC which has only 18 residues. As an example, predicted NatA with a binding ligand is shown in (Figure 3). It is known that molecular processes guiding cell behavior primarily are linked to protein-binding partners, and co-factors, including substrates where ligands bind to the active site are fundamental. According to the predictions herein, it seems that the active sites in kinetoplastids are similar in contrast to their human counterparts, raising the possibility that specific drug design may target the three pathogens.

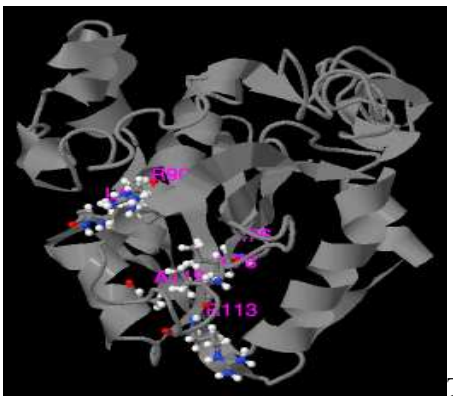

HNatA

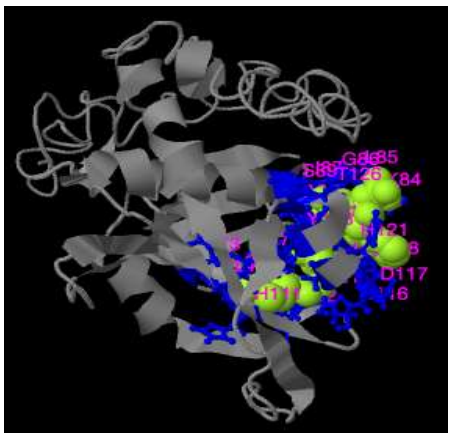

HNatA active site


TcNatA


TcNatA active site


TbNatA


TbNatA active site


LmNatA

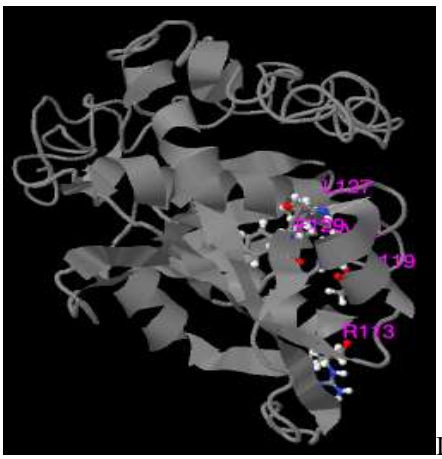

LmNatA active site

Figure 3. Generated 3D structure of human and kinetoplastids showing predicted coenzyme A (CoA) binding ligands on the left and active sites on the right. On the left, the green/yellow indicates predicted binding ligand and blue indicates binding residues in ball and stick. On the right, colored ball stick is the predicted active site residues, and pink is the actual predicted amino acid. Not that, only NatA is shown.

5. OPPORTUNITIES, CHALLENGES, AND FUTURE DIRECTIONS FOR NT-ACETYLATION STUDIES IN KINETOPLASTIDS.

1. Opportunities

N-acetylation is important for proteins to remain stable, function properly, and be in the right place in cells. By targeting this process, important cellular activities can be disrupted, which may help eliminate harmful parasites. It appears that kinetoplastids, a type of parasite, may have different N-acetyltransferases (NATs) than human cells. For example, kinetoplastids do not contain NatD (61), which is present in the human cells.

Scientists have developed small-molecule inhibitors that target acetylated microtubules for cancer treatment (99). We believe that similar inhibitors that specifically target kinetoplastid NATs can be designed to reduce the chances of affecting human cells. Additionally, small inhibitors that target the helper parts of kinetoplastid NATs can be created because these parts are very different in terms of similarity from those in human NATs. These small inhibitors can function by attaching to the active sites of NATs and blocking their function.

Thirdly, small-molecule inhibitors that target Nt-acetylation, as mentioned in another study (100), can offer a new strategy that does not rely on existing resistance mechanisms. This could help to address issues with drug-resistant strains. Looking at it in another angle, combining these inhibitors with current anti-parasitic drugs might also create stronger effects. This approach can improve treatment, lower the risk of resistance, and provide a more effective way to fight kinetoplastid infections. Additionally, the knowledge gained from studying Nt-acetylation in these parasites could be applied to other protozoan infections, making this strategy more widely applicable.

5.2 Challenges

Studying Nt-acetylation in kinetoplastids is difficult and has potential for future research. Understanding how this process occurs in these parasites is important for identifying new drug targets and developing better treatments. However, there are challenges and exciting opportunities to study Nt-acetylation in kinetoplastids. These parasites have different life cycles (15), ways of interacting with their hosts, and cell structures (4). This variety makes it difficult to use the same methods for all species; therefore, each method requires its own approach. Another challenge is that some antibodies can specifically detect Nt-acetylation in kinetoplastids. Antibodies are important tools for finding and studying acetylated proteins, but creating these antibodies for kinetoplastids is still difficult, or little interest from the scientific communities to carry out the tasks. Third, the manner in which Nt-acetylation probably changes in response to different environments and life stages in kinetoplastids makes its study more difficult. Observing and understanding of these changes is a major technical challenge. Fourth, kinetoplastids probably have several NATs, and the fact that these enzymes can perform similar functions may make it difficult to determine how each affects protein acetylation. Determining the exact role of each NAT in different cellular activities is challenging.

In addition, it may be difficult to create inhibitors that specifically target kinetoplastid-specific NATs without affecting host cells. Sufficient selectivity is important to ensure the safety and effectiveness of any potential treatment. In addition, knowing more about how NATs work differently will help to create better inhibitors for the many types of parasites that cause different diseases. However, solving problems by getting drugs to the right place is key to making these targeted treatments effective. Moreover, as with any medicine, we need to monitor resistance to these small-molecule inhibitors, find ways to prevent it, and keep the treatments effective in the long run.

2. Future directions

There are many directions that studies geared towards understanding protein modification in kinetoplastida organisms can take, a few are suggested below. Tools that could be helpful in the process are those based on recent advances in mass spectrometry technology such as high-resolution mass spectrometry and tandem mass spectrometry (MS/MS) (88), and its powerful application in different aspects of protein analysis including PTM (89). These tools can make it possible to detect much more accurately and sensitively Nt-acetylated proteins in complex kinetoplastid proteomes (61) (90)(91). Indeed, it has been shown that new methods, such as top-down proteomics based on mass spectrometry, can allow an examination of an intact acetylated protein for a better understanding of protein modification (101).

First, specific genes encoding NATs or proteins of interest can be genetically knocked out or downregulated to deepen our understanding of the role of Nt-acetylation in protein stability, cellular localization, and cellular processes. For example, target knockdown using RNA interference (RNAi) has been performed in African trypanosomes (41). In the past, researchers have used CRISPR/Cas9 (102) to make precise changes to the DNA of kinetoplastids to understand how the changes in Nt-acetylation affect these organisms. CRISPR/Cas9-based genome editing has revolutionized the precision of genetic studies in many organisms (92), and the preclinical and clinical benefits of CRISPR/Cas-9 have been recently documented (93). This technology has been applied in part to investigate gene function in kinetoplastids (94) (95). It could be used to disrupt or adjust the specific NATs.

Second, by combining information from transcriptomics, proteomics, and metabolomics, we can obtain a complete picture of how Nt-acetylation occurs or how it functions in kinetoplastids. This approach helps us to understand how acetylation affects many cellular processes. Based on initial research on *T. cruz*i using NatC (3), it is clear that Nt-

acetylation plays an important role in kinetoplastid biology. Therefore, NATs are promising targets for novel drug development. Future studies should identify small molecules that specifically block NATs, which may lead to new treatments for diseases caused by kinetoplastids.

Third, future kinetoplastids research could focus on identifying specific targets, understanding the roles of Nt-acetylation at various stages of their life cycle, and exploring how acetylation affects processes, such as differentiation, transmission from one host to another, and adaptation to different environmental conditions.

Fourth, understanding the features and roles of kinetoplastid-specific NATs is important because there are many questions regarding these enzymes, such as what they act on, how they are controlled, and how they affect parasite strength. By learning more about these unique NATs, better drugs can be developed. It would also be interesting to study how Nt-acetylation affects the relationship between the parasite and its host and how it may influence and change the host's immune system. Examining how these parasites have changed their acetylation systems over time can help us to better understand their biology, showing what has remained the same and what has changed. Studying the structure of these enzymes can provide a closer look at how they function, how they bind to their targets, and how they are regulated.

Fifth, site-directed mutagenesis, that is, the introduction of mutations at the N-terminus to prevent or mimic acetylation, allows for functional characterization and can unravel the nuanced roles of individual acetylation sites in the regulation of protein function (13).

Sixth, overexpression studies can also be performed to examine the consequences of elevated Nt-acetylation, providing grounds for assessing its impact on protein stability, cellular localization, and cellular functions, as suggested elsewhere (77).

Seventh, in vitro acetylation assays can be used to examine NAT enzymatic activity and substrate specificity, where purified NATs are incubated with target proteins or peptides, and acetylation reactions monitored (96)(97)(78). This has been performed for NtaC in *T. cruzi* as well (3).

And eight, by deleting NATs in kinetoplastids, next-generation sequencing can be performed to detect, for example, methylation, as suggested elsewhere (98). Subsequently, comparative genomics can also be performed (39).

Overall, combining genetic and biochemical approaches helps us obtain a complete picture of Nt-acetylation and its roles in cellular processes and molecular interactions, providing a better understanding of how cells function and are regulated. Above all, interrogation of Nt-acetylation, its activities, and mode of action can be a powerful tool in kinetoplastid drug discovery, as presented, for example, in a study of protein kinases and proteasomes in Trypanosomes and Leishmania (41). Collectively, these approaches provide insight into the function, evolution, and potential drug targets of NATs in kinetoplastids.

This information is important for the development of small-molecule drugs that can block kinetoplastid NATs. Therefore, more research should be conducted to determine whether the acetylation system can be targeted by drugs and to develop specific drugs that could be used to treat diseases. Using computer simulations and combining acetylation data with methods from systems biology, such as studying genes, RNA, and proteins, can provide a complete picture of how the acetylation system functions in kinetoplastids. This big-picture view will help us to understand how acetylation affects many different cellular activities.

The study of Nt-acetylation in kinetoplastids has great potential to help us better understand how these parasites work and find new ways to treat the diseases they cause. By answering important questions and filling gaps in our knowledge, we can gain a deeper and more detailed understanding of how Nt-acetylation is controlled in these important parasites. To overcome the challenges of studying Nt-acetylation in kinetoplastids, we need to collaborate across different fields, use new technologies, and collaborate with other researchers.

To sum up, the effects of Nt-acetylation on future research and treatment of kinetoplastids are very important, and this area of study is still in its early stages. This evolutionarily conserved PTM likely controls crucial aspects of parasite biology. Kinetoplastid-specific NATs, the enzymes that are essential for Nt-acetylation and affect protein stability and function, have been identified. Their potential impact on the relationship between the parasite and its host, as well as on the parasite's life cycle, is currently being explored. Understanding the structural features, substrate preferences, and functional roles of NATs can help us better understand kinetoplastid biology and may lead to new targets for developing treatments against parasitic infections. Future studies should aim to answer these unresolved questions, use new technologies, and investigate how this modification has changed over time. The constantly changing field of Nt-acetylation in kinetoplastids offers exciting opportunities to improve our knowledge and create new ways to fight diseases caused by these parasitic protozoans and other parasitic infections.

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AUTHOR'S CONTRIBUTION

SO: Conceptualization, wrote the first draft, performed the prediction analysis, critical analysis, and refinement of the draft.

RA: Participated in the manuscript draft and helped with the refinement of the figures.

OO: Edited the manuscript draft and fine-tuned the text.
BA: Participated in editing of the manuscript and text refinement.

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