Chapter 11

Silent Information Regulator 2 from *Trypanosoma cruzi* Is a Potential Target to Infection Control

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Abstract

Human trypanosomiasis is a neglected tropical disease caused by protozoan parasites of the genus *Trypanosoma*. *Trypanosoma brucei* is responsible for sleeping sickness, also called African trypanosomiasis, while *Trypanosoma cruzi* causes Chagas disease, or American trypanosomiasis. Together, these diseases are responsible for significant mortality, morbidity and lost productivity in the endemic regions. There are no vaccines and treatments rely on drugs with limited efficacy, high cost, serious side effects and long administration periods. Since these diseases affect mostly the poor, there is no economic interest in the development of new drugs by pharmaceutical companies, and hopes for new treatments rely on public initiatives, public-private partnerships or philanthropic programs. The first step in the discovery of new drugs involves the identification of active molecules as starting points for further development, by either employing whole cells or by specific molecular target screenings. Research efforts undertaken by the authors' groups have focused on exploiting both strategies in the search for new molecules for trypanosomiasis drug discovery. In this chapter, we focus on Chagas disease and the recently uncovered potential of using sirtuins as targets for infection control.

Keywords: *Trypanosoma cruzi*, Chagas disease, sirtuins, drug discovery, chemotherapy

1. Introduction

Despite the efforts of many individuals and organizations over the years, human trypanosomiasis remains one of the most neglected diseases in the world. Chagas disease in particular, is a leading cause of disease and disability in Latin America, with thousands of deaths every year [1]. The negligence is particularly patented by the lack of new drugs. Indeed, the available treatment

options, benznidazole and nifurtimox (**Figure 1**), were discovered more than 40 years ago. Different strategies have been employed to control the disease, but the most impactful so far has been the control of the transmitting vector led by the World Health Organization (WHO) and Pan American Health Organization (PAHO). Vector control has caused the reduction of cases from a staggering 24 million in the 1980s to about 6 million nowadays [1, 2], with some countries considered to be free of domestic vectorial transmission. Continued and rigorous implementation of the disinfestation programs in the remaining zones should decrease even further the global numbers of *Trypanosoma cruzi* vectorial transmission. Also, the screening of donor blood and transplant organs in endemic regions and other parts of the world has greatly reduced the

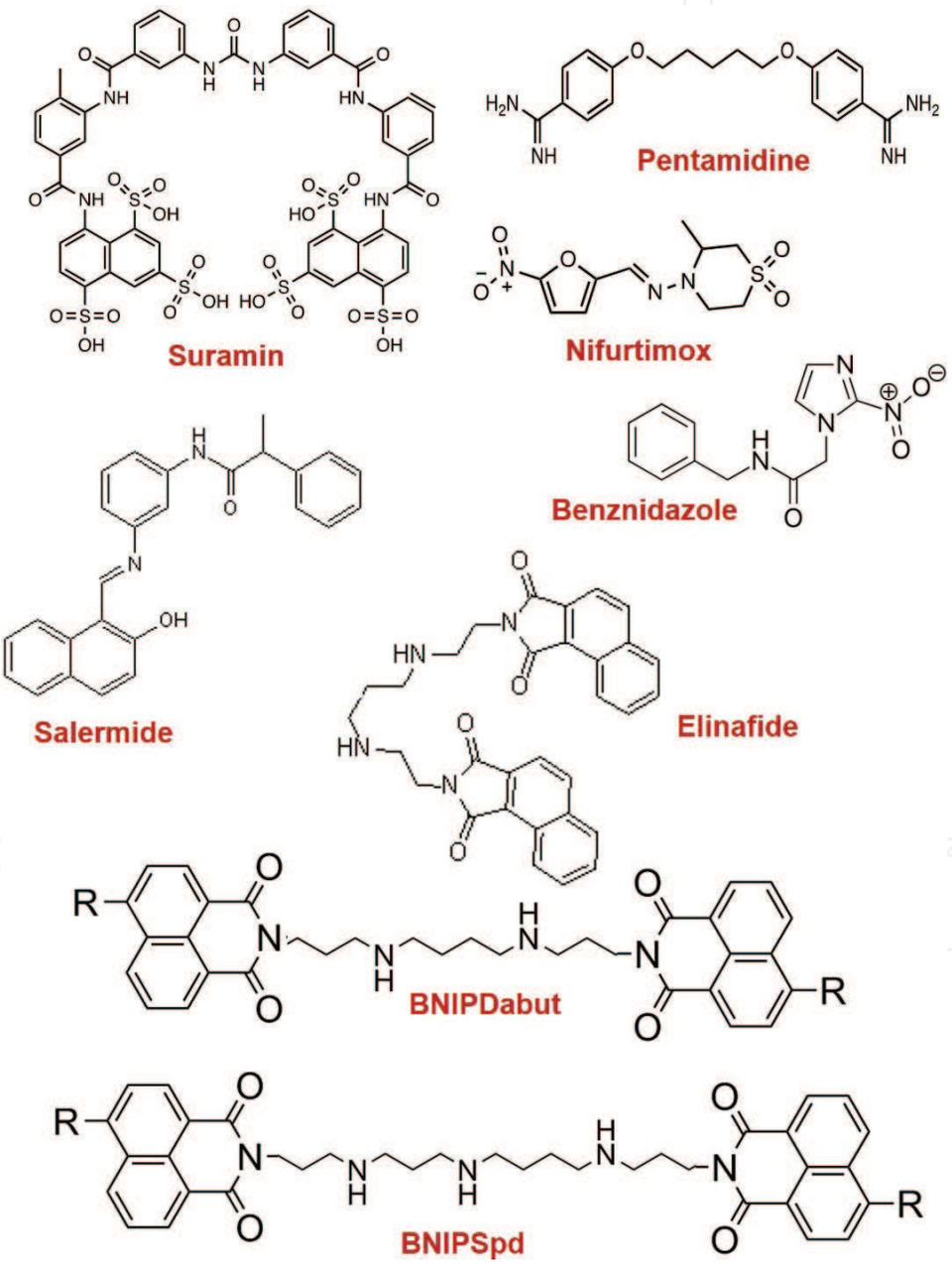

Figure 1. Various trypanocidal agents.

number of cases transmitted by this route. Whereas the global interruption of the domestic cycle will be a major breakthrough and reduce to a minimum the number of new cases of Chagas disease, complete eradication of the parasite, however, is unlikely to be achieved due to the huge natural reservoir of *T. cruzi* and the many species of triatomines capable of its transmission in the sylvatic environment [3–5].

What then, stands in the way of disease control for Chagas? First, no major technological advances are required to interrupt vectorial transmission responsible for the majority of new cases; second, decades of research in the molecular understanding of *T. cruzi* biology, the particularities of pathogenesis of the disease or the dynamics of immune response against the parasite have failed to translate into therapeutic alternatives; and finally, vaccination, either preventive or therapeutic, has remained an elusive achievement. The answer lies in new therapeutics. New, safer, cheap, easy to administer and efficacious drugs that are able to treat not only new cases, but also the millions of people already affected by the chronic stage and indeterminate form of the disease. There is growing evidence that chronic manifestations are ultimately related with inflammation resulting from parasite persistence [6, 7] and effective treatment of these cases would be highly beneficial to stop the development of symptomatology.

Active drug discovery efforts for Chagas disease have been restricted, until some years ago, to just a very limited number of groups, mostly based in academia. As a consequence, results have been sporadic, slow, ineffective and highly dependent upon intermittent funding, failing to deliver an alternative treatment. Chagas disease is as much neglected by the pharmaceutical industry as it is by research funding organizations, whose majority of funds are directed to developed world diseases.

Only recently has drug discovery for Chagas been met with concerted, focused efforts. While still not privately embraced by pharmaceutical companies, public-private partnerships have been set up with the objective of bringing together the biology expertise from academia and the technical expertise, facilities and resources from pharmaceutical industries. One organization that is leading the efforts to find new therapies for neglected diseases, including human trypanosomiasis, is the Drugs for Neglected Diseases Initiative (DND*i*), that has been involved in coordinating activities from early drug discovery to the launch and conclusion of clinical trials for some candidates like inhibitors of ergosterol biosynthesis of *T. cruzi*. The Bill and Melinda Gates Foundation, a non-governmental organization devoted to human development in underdeveloped countries, has committed to help control neglected diseases by signing The London Declaration on Neglected Diseases together with the WHO, the World Bank and 13 leading pharmaceutical industries. The Declaration states that by 2020 the signers will achieve, among other ambitious milestones, the eradication of Human African trypanosomiasis (HAT) and the control of American trypanosomiasis. Since then, other initiatives have been launched with the objective of boosting the research in new drugs for *Trypanosomal* diseases, like the European Commission Seventh Framework Program (FP7) consortia NMTrypI and KINDReD. The recent award of the Nobel Prize in Physiology or Medicine 2015 to William C. Campbell, Satoshi Omura and Youyou Tu is a recognition of the importance of drug discovery for parasitology and should further increase the awareness of neglected diseases by the international community.

Pharmaceutical research and drug discovery for infectious disease have historically began with what would be classified today as phenotypic assays, and can be traced to the pioneering work of Paul Ehrlich in the nineteenth century, while testing the effect of different dyes in trypanosomes [8]. Cultures of the microorganism of interest, bacteria or parasites, were incubated with a compound of interest, and the selective staining of the dyes was monitored by microscopy. Products of such "dye therapy" approaches were in the origin of well-known chemicals like the crystal violet dye that was proposed to be used in blood banks of endemic areas to kill *T. cruzi* parasites present in transfusion blood as a way to reduce transmission by this route [9]. Another example is trypan blue, that is still widely used as a cell biology reagent and that was the starting point for the design of the colorless analogue Suramin, a drug still in use today for the treatment of HAT and infected animals as well [10]. Such early whole cells drug screening principles were also central to the development of nifurtimox and benznidazole (**Figure 1**) in the 1960s and 1970s, by the pharmaceutical companies Bayer and Roche, respectively [11].

With the genomic era there was a dramatic shift in the way new drugs are discovered. The past 20 years have witnessed incredible advancements in genomics, proteomics, structural biology, computational chemistry and structure based drug-design, that coupled with high-throughput screening and combinatorial chemistry have helped to shape the reductionist mentality "one gene—one protein—one drug" [12]. However, the complexity of many diseases and the capacity of adaptation to adverse conditions, like the presence of a xenobiotic, evidenced by many living cells, have brought the more naïve phenotypic whole-cell screening strategies back to the spotlight. With modern phenotypic approaches, the effect of a pure molecule in a fully intact whole living organism, bacteria, parasites or human-derived cell lines, results in the identification of hit compounds that are potentially useful as scaffolds for further medicinal chemistry optimization.

While early phenotypic screenings for *T. cruzi* sometimes used the insect-specific epimastigote stage due of its extracellular nature and ease to culture, the use of reporter genes expressed during the clinically relevant stage of the disease, the intracellular amastigotes, has met a widespread application. The first of such assays was based upon the β-galactosidase-expressing parasites that made possible the detection of anti-*T. cruzi* activity by a colorimetric reaction [13]. Later, tdt-tomato and luciferase genes were also constitutively expressed in parasites, allowing more sensitive measurement of a fluorescent or luminescent signals, respectively [14].

However, the use of genetically unmodified parasites has always been an attractive pursuit, made available only recently due to technologic advancements. Such cell-based assays were developed by researchers at Institut Pasteur Korea and have met widespread use [15–18]. This assay employed the use of wild type parasites of *T. cruzi* infecting a non-modified cell line and the imaging of the resulting infection (in the amastigote stage) by high-content analysis (HCA) microscopy. Furthermore, the assay was developed in the 384-well format, allowing a high throughput testing of compounds. Preliminary cell toxicity is concomitantly determined by quantifying the ratio of host cell nuclei, a clear advantage since it reduces the need of an independent assay to assess this parameter. Using this screening assay, the authors of this chapter have also screened a library of 4000 kinase/phosphatase-like inhibitors that allowed

the identification of 11 compounds with strong anti-parasitic activity and selectivity, suitable for follow up hit-to-lead optimization (unpublished results). In addition, a complementary assay developed for phenotypic profiling also allowed the identification of several compounds that interfered with the development and intracellular differentiation of *T. cruzi*. Compounds that hindered the differentiation from trypomastigotes to amastigote and the replication of amastigotes inside host cells are among the examples of "phenotypic" hits discovered (unpublished results). Due to the complex genetics and still many unknown aspects of *T. cruzi* biology, these types of compounds have the potential to constitute important chemical genomic tools that may help answering fundamental questions like: what triggers stage differentiation and what are the pathways involved? What factors are responsible for parasite persistence? How are amastigotes kept dormant for years to decades in host cells, hidden from the immune system? Due to the nature of the chemical library, it is likely that the compounds target kinases, of which the *T. cruzi* genome has 190 annotated potential members [19]. *T. cruzi* and other Trypanosomatids have a relatively big kinome when compared with other parasites that undergo several stage differentiations and contact with distinct environments, like *Plasmodium* spp. [20, 21]. One hypothesis is that while in metazoa and yeast the ultimate targets of many signaling cascades are transcription factors, which then trigger the expression of new sets of genes, Trypanosomatids have constitutive transcription of a majority of genes in large polycistronic units, hinting at a greater role of post-translational modifications (PTMs) like phosphorylation and acetylation.

2. Sirtuins: family and functions

Post-Translational Modifications (PTMs) represent one of the major mechanisms in regulating protein function in all life forms. Through phosphorylation, acetylation, methylation, glycosylation and ubiquitination, cells greatly extend the possibilities beyond the coding genome [22]. PTMs can change the enzymatic activity of a protein, change its subcellular localization, interfere with protein complexes assembly, increase or decrease its stability and induce interactions with DNA and RNA [22, 23].

Discovered half a century ago and largely ignored for the following years, lysine acetylation has re-emerged in the last two decades as a highly important PTM [24, 25]. Initial studies had focused in the role of lysine acetylation in the regulation of chromatin structure and gene expression, but with the advances in proteomic approaches, it was possible to begin to explore the function of lysine acetylation in non-histone proteins [24, 25].

Studies based on proteomic analysis to describe the lysine-acetylated proteins repertoire of an organism, called acetylome, have shown the presence of lysine acetylation in proteins from different cellular compartments and involved in different biological processes in several organisms [26]. Because of that, lysine acetylation has been placed by some authors in the same level of biological relevance as phosphorylation [24, 25]. In fact, studies of the acetylome of mammalian cells revealed acetylation sites in 1750 different proteins [27], a number close to the about 2000 proteins found to be phosphorylated [28].

The "acetyl code" is maintained by three different protein types: the "writers", lysine acetyltransferases (KATs) that add acetyl groups to proteins, the "erasers", lysine deacetylase (KDACs) that remove acetyl groups, and "readers", proteins that specifically recognize and bind acetyl-lysine groups [26].

KDACs in particular have been the focus of great interest by the scientific community due to their many roles in cell function and disease. KDACs are interchangeably called histone deacetylases (HDACs), because the first discovered reactions catalyzed by these proteins were the removal of acetyl groups in histone tails [24, 29].

HDACs are separated into four different classes based upon sequence homology (class I, II, III and IV) and two different families: the histone deacetylase family and the sirtuin family, the latter being all class III HDACs. While the first family has a limited set of molecular targets, mainly composed of histones, sirtuins have a variety of substrates ranging from metabolic enzymes to structural proteins, as well as histones [30–32]. The sirtuin family seems to be ubiquitous throughout all kingdoms of life. The number of genes coding for sirtuins within an organism ranges from as little as one in bacteria, to seven in vertebrates [33]. The sirtuin family is further classified in 5 subclasses (I, II, III, IV and V) [34].

The most common reaction catalyzed by sirtuins is deacetylation. This reaction is of upmost biological importance as there is a clear relation between the acetylation status of several proteins and their cellular functions [35–38]. The deacetylase reaction requires (nicotinamide adenine dinucleotide) NAD^+, an acetylated lysine residue and produces deacetylated lysine, nicotinamide and 2′-O-acetyl-ADP-ribose (OAADPR) [39]. Studies on the kinetics and biochemical properties of the enzymes revealed binding to the acetyl-lysine substrate prior to NAD^+. This is followed by nicotinamide cleavage from NAD^+, that is the first product released, followed by deacetylated lysine and OAADPR [40] (**Figure 2A**). All sirtuins are strictly NAD^+ dependent, a distinct characteristic that distinguishes them from other deacetylases. In fact, SIRT6 is a sirtuin capable of tightly binding to NAD^+ without the requirement of an acetylated substrate, indicating that it may function as a NAD^+ sensor [41].

Besides being an endogenous product of the deacetylation reaction, nicotinamide is also a well-known inhibitor of sirtuins. Nicotinamide is an amide of nicotinic acid (vitamin B3) and is part of common enzyme co-factors like NAD^+ and NADP (nicotinamide adenine dinucleotide phosphate) [42]. Intracellular physiological levels of nicotinamide in some mammalian cells seem to be in the range similar to the IC_{50}'s of some sirtuins reinforcing the hypothesis that some sirtuins may act as NAD^+ and nicotinamide sensors [43, 44].

OAADPR is another product of the deacetylation reaction [45] (**Figure 2A**). Early studies characterizing this molecule found that quantitative microinjection into starfish oocytes led to a blockage of oocyte maturation, indicating for the first time that OAADPR can evoke a biological activity [46]. There is now mounting evidence that OAADPR can elicit downstream responses that might synergize or antagonize the biological functions of sirtuin genes. So far, OAADPR has demonstrated to be related with functions in gene silencing, ion channel modulation and cell redox state maintenance [45].

Another reaction catalyzed by sirtuins is ADP-ribosylation. Although sirtuins were firstly described as ADP-ribosyltransferases (**Figure 2B**), their deacetylase activity has quickly

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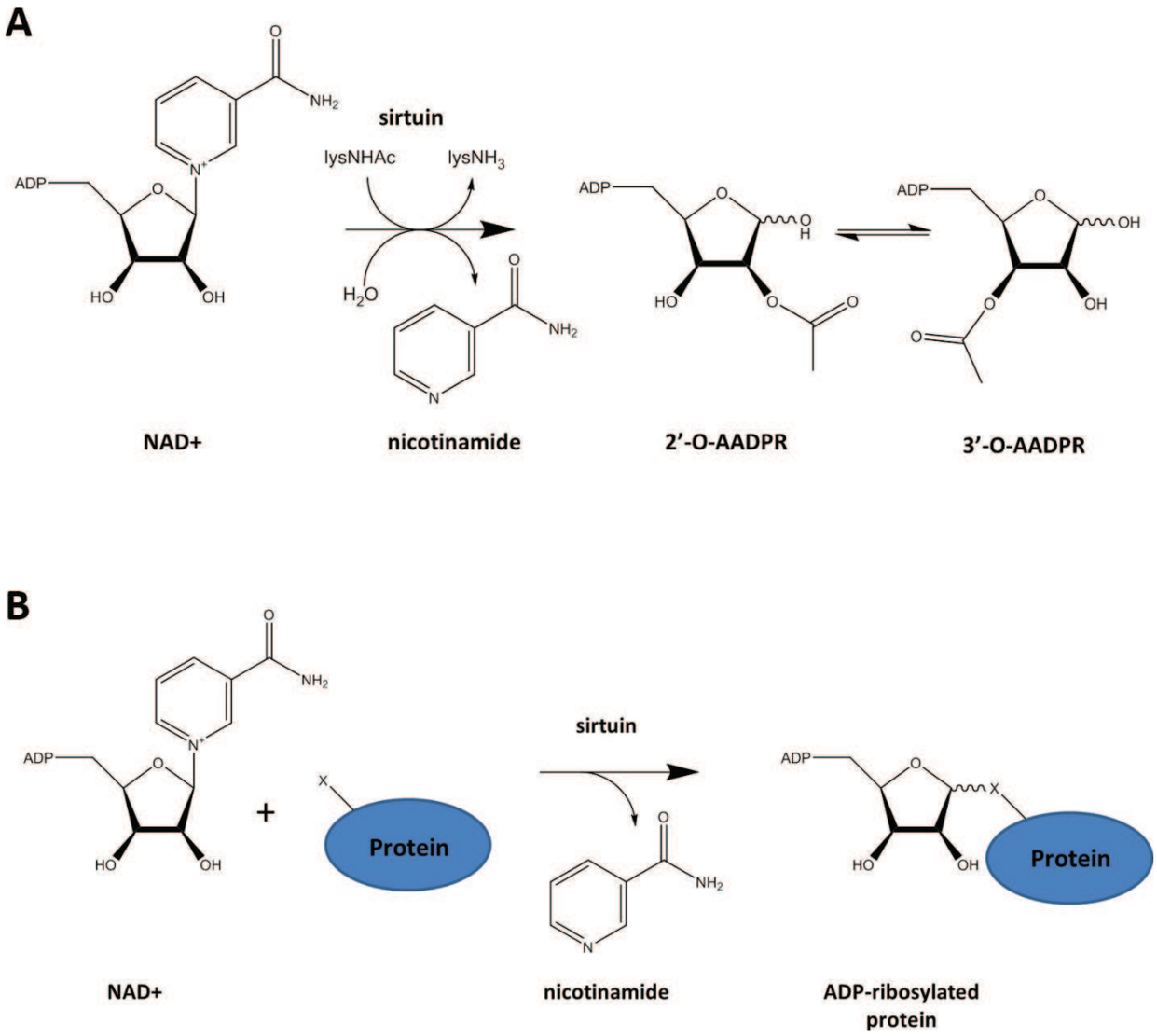

Figure 2. Sirtuin deacetylation and ADP-ribosylation mechanisms. (A) Sirtuins carry out protein deacetylation by removing acyl chains from protein lysine residues. This activity requires the cofactor nicotinamide adenine dinucleotide (NAD^+), such that nicotinamide is released with concomitant production of O-acetyl-ADP-ribose (O-AADPR). There is a sirtuin-independent equilibrium between 2′ and 3′-O-AADPR isomers. The unspecified stereochemistry at the Cl′ position of O-AADPR reflects the fast epimerization observed in solution between α and β-anomers. (B) A number of sirtuins also exhibit ADP-ribosylating functionality. This reaction is also NAD^+-dependent, as the cofactor acts as the source of ADP-ribose. 'X' represents the nucleophilic side-chain of a protein residue.

overshadowed this activity, and as a consequence the biological processes associated to this reaction remains poorly understood [47]. In truth, sirtuins may have just deacetylase activity, both, or be mostly ADP-ribosyltransferases. An example is SIRT4 that is an efficient *in vitro* ADP-ribosyltransferase of histones that only recently was discovered to possess deacetylase activity [48, 49]. There is an active debate on whether ADP-ribosylation is in fact a biologically relevant function of sirtuins, or just an irrelevant side reaction/non-enzymatic artifact [50]. Nevertheless, some of the players in the dynamics of intracellular ADP-ribosylation have only been recently identified and it is becoming apparent that this PTM might be relevant for the modulation of important cell processes and signaling pathways like signal transduction mechanisms, transcription and DNA repair [51]. The ADP-ribose hydrolysis in Trypanosomatids

has recently been studied in both *Trypanosoma brucei* and *Trypanosoma cruzi* and found to be mediated by a macrodomain with a conserved catalytic site [52].

Although (de)acetylation is the most common PTM, (de)acylation of other groups can be targeted, like succinyl and malonyl groups. SIRT5 and SIRT6 are some examples of proteins that perform deacylations of lysines other than acetyl groups, and their activities are important regulators of cell functions [53–55].

There is a wealth of information regarding the structural features of sirtuins. To date, some 83 structures of sirtuins are available in the protein databank, many of them co-crystalized with natural ligands or inhibitors. Though the available structures range from bacterial to mammalian sirtuins, the majority of the structures originate from the human genome.

Although the sequence homology can vary significantly between sirtuins, especially between prokaryotic and eukaryotic proteins, there is a conserved catalytic core of about 250 amino acids common to all members in the family [56]. The structure similarity of the *Plasmodium falciparum* PfSir2A with the mammalian SIRT5, despite a sequence homology of just 33% is a clear illustration [56]. This core contains a Rossman fold domain that is a NAD^+ binding site, and a Zn^{2+} binding domain containing four highly conserved cysteine residues arranged in the motif ($CX_2CX_{20}CX_2$). The catalytic site is located inside a hydrophobic channel formed at the interface of these two domains [56].

Whereas in HDACs from class I, II and IV, Zn^{2+} is an active participant in catalysis by producing free acetate and deacetylated lysine, in sirtuins it does not participate in reaction. However, the metal is essential for structural integrity, as was elucidated by the reversible loss of activity in a *P. falciparum* sirtuin where the zinc ion was removed [57]. Interestingly, an exception to the conservation of the $CX_2CX_{20}CX_2$ motif is found on some sirtuins of Trypanosomatids, where one of the cysteines is not present [58]. However, deacetylase activity does not seem to be affected [59]. The molecular mechanism of deacetylation is still not completely elucidated, but it is generally accepted that the first step in the reaction involves the nucleophilic addition of the acetyl oxygen to nicotinamide ribose by a mechanism of S_N2 substitution to produce O-alkylamine intermediate and nicotinamide. Then the acetyl group is transferred to ADP-ribose to form O-acetyl-ADP-ribose and deacetylated lysine [60].

The founding member of the sirtuin family is Sir2 from the budding yeast and was initially identified as part of a protein complex necessary to silence the expression of the mating-type-loci [61, 62]. Subsequently, it was also implicated in transcriptional silencing at telomere proximal sites [63] and ribosomal repeats at the ribosomal DNA (rDNA) locus [64–66]. Sir2 can be associated in distinct protein complexes that vary according to target site. For instance, at telomeres and the mating-type-loci, Sir2 forms a complex with two other homologs, Sir3 and Sir4 [63], while at rDNA locus Sir2 associates with Net1 and Cdc14 to form the regulator of nucleolar silencing and telophase exit—RENT complex [67, 68]. Yeast cells lacking Sir2 present a reduced lifespan that has been correlated with the accumulation of extrachromosomal ribosomal DNA circles originating from illegitimate recombination that are toxic to the cell and have been associated with aging [66, 69, 70].

In mammals, the nuclear SIRT1 is the most extensively studied member among the sirtuin family. The TATA box binding protein-associated factor RNA polymerase I subunit B (TAF_I68) was the first substrate to be identified for SIRT1 in mouse cells. It is a transcription factor necessary for regulating the RNA polymerase I transcriptional complex, where it was shown that deacetylation inhibits transcriptional initiation *in vitro* [71]. Studies on p53, a non-histone substrate, demonstrated that acetylation activates the DNA-binding activity and target gene expression, also increasing its stability [72]. Consistent with this proposed SIRT1 inhibition of p53 function, SIRT1 knockout mice exhibit p53 hyper-acetylation and increased radiation-induced apoptosis, suggesting that SIRT1 can facilitate tumor growth by antagonism of p53 [73]. Still, the fact that SIRT1 can be found either overexpressed or underexpressed in different tumor types, and the finding that it can also function as a tumor suppressor [74] has hindered the clarification of its role in tumorigenesis. SIRT1 also plays an important role in metabolism, and its relation with caloric restriction and life-span extension has received much attention (reviewed in [75–77]). The beneficial effects of caloric restriction have been focused on the insulin-like growth factor-1 (IGF-1) and the target of rapamycin (TOR) pathways [78, 79], but increasing evidence suggest a role of SIRT1 in caloric restriction in mammals as well. For instance, SIRT1 expression was found to be elevated in models of caloric restriction, like fasting mice, low calorie diet in rat, or humans on a 25% caloric restriction diet [80–82]. On the other hand, mice lacking SIRT1 lost the life-span extension benefits of a 40% reduced calorie diet [83]. Despite many studies, the exact molecular mechanisms of SIRT1 in caloric restriction are still to be unraveled.

In vitro studies attribute a role to human SIRT2 in cell cycle regulation through the deacetylation of both tubulin and histone H4 [32, 84, 85]. In particular, it has been found that SIRT2 overexpressing cells were significantly delayed in cell cycle progression through mitosis [86]. Some links with age-related diseases have been described for SIRT2, such has neurodegenerative diseases [87–89], and different kinds of cancer. Mice lacking SIRT2 are prone to the appearance of tumors, an effect believed to be mediated by SIRT2 negative regulation of the anaphase-promoting complex [90]. It was demonstrated that SIRT2 expression is reduced in human gliomas, some of the most frequent malignant tumors in the brain [32, 91].

SIRT3 is the major mitochondrial deacetylase and studies with double knockout mice have revealed high levels of acetylation in protein targets [92, 93]. In addition, it was observed that these mice have impaired production of ATP [92]. When fasting or fed with a high-fat diet, the mice display atypical phenotypes that include cold intolerance and decreased ketone body formation [94, 95]. This strengthened the link with thermogenesis that had been previously demonstrated [96, 97]. In fact, SIRT3 expression is induced in mice in both white and brown adipose tissue upon caloric restriction and exposure of brown adipose tissue to cold temperatures [98]. In addition, SIRT3 also has a role in the deacetylation and activation of fatty acid β-oxidation, amino acid metabolism, electron transport chain and antioxidant defenses [99, 100].

SIRT4 was originally thought to be an unusual sirtuin due to the lack of deacetylase activity [101]. However, it was shown to ADP-ribosylate and down-regulate glutamate dehydrogenase production of ATP and has been implicated in insulin regulation of β-cells [48, 101]. Moreover, SIRT4 has recently been attributed a tumor suppressive function due to its involvement in

DNA damage protection mediated by inhibition of mitochondrial glutamine metabolism, suggesting it might have therapeutic potential for treating glutamine-dependent cancers [102]. This mechanism is inhibited by mammalian target of rapamycin complex 1 (mTORC1) pathway [103]. SIRT4 also coordinates the balance between lipid synthesis and their catabolism by repressing malonyl-CoA decarboxylase, proving that it has, in fact, deacetylase activity [49].

SIRT5 is a NAD^+-dependent protein lysine demalonylase and desuccinylase [104] and also has a deglutarylase activity [105]. It has a deacetylase activity [30], but has preference for acyl-carboxyl negatively charged groups [104, 105]. Some of its functions are related to glycolysis modulation [55]. The succinylome of mammalian cells has revealed many points of succinylation that are possible targets of SIRT5, mostly concentrated on mitochondrial metabolism [54]. SIRT5 also promotes urea cycle function via the regulation of carbamoyl-phosphate synthase [30, 35], and purine metabolism via urate oxidase [106]. Although a global protein hypersuccinylation and elevated serum ammonia during fasting were observed in SIRT5 knockout mouse model, the enzyme deficiency did not lead to any major metabolic abnormalities under either low or high fat diet conditions. These observations suggest that SIRT5 is likely dispensable for metabolic homeostasis under the basal conditions. It remains to be evaluated the role of SIRT5 in extreme conditions [107].

While most mammalian sirtuins have been implicated with metabolism, SIRT6 seems to be the only one with a direct link supporting a defined role in mammalian aging [108]. In fact, mice lacking SIRT6 gene develop a progeroid-like symptom with loss of subcutaneous fat, curved spine and lymphopenia. They develop normally for 2 weeks after birth, but then suffer from acute degeneration processes, ending up dying at 1 month of age [109]. At first considered to not possess deacetylase activity, but solely an ADP-ribosyltransferase activity [110], it was later found that SIRT6 removes both acetyl and long chain acyl groups from target molecules [53, 111]. It is localized in the nucleus, associated with chromatin, where it promotes the specific NAD^+-dependent deacetylation of H3K9 and H3K56 [111–113]. SIRT6 is involved in genome protection by assuring correct telomere maintenance [111, 112], as well as DNA repair by double-strand break repair machinery [114, 115]. Like other sirtuins it also has a role in metabolism by influencing both glycolysis and gluconeogenesis [116–118] and lipid metabolism, by regulating triglyceride synthesis [108, 119]. Conditions like inflammation, heart disease and cancer all seem to be linked with SIRT6 function [120].

SIRT7 is the least studied sirtuin of all the mammalian sirtuins, but recent findings have established new functions and roles for this protein. It is a nucleolar sirtuin [121] and its localization is associated with the main process happening at this sub-nuclear structure, namely rDNA transcription [122]. SIRT7 does not possess a very strong deacetylate activity toward common synthetic and natural peptides [121], which is in agreement with the fact that SIRT7 depletion in mice did not change the global acetylation levels of either nucleus or nucleolus proteome [123], indicating that SIRT7 deacetylase activity is specific to a limited set of proteins. One example is specific deacetylation of H3K18 [31, 124] that underlies its role in chromatin remodeling. Also, SIRT7 has been found to be closely associated with B-WICH complex, a chromatin-remodeling complex [125]. It also has a role in protein synthesis [123, 126]

and contributes to cell survival, namely by protecting against genomic insult [127, 128], hypoxia [129] and low glucose induced stress [130]. All the functions described characterize SIRT7 as a pro-survival protein. Indeed, it is currently considered to be an oncogene in all the cancer types studied so far [126, 131, 132].

3. Parasitic sirtuins

Various genome-sequencing projects demonstrated the presence of genes coding for sirtuins in most protozoan parasites of medical importance. An interesting finding was that depending on the protozoan parasite species the number of sirtuins varied (**Table 1**).

Organism	Number of sirtuins	GeneID	Subcellular Localization	Data Source
Trypanosomatids				
Trypanosoma cruzi	2	TcCLB.508207.150 (TcSir2rp1); TcCLB.447255.20 (TcSir2rp3)	cytoplasm (TcSir2rp1); mitochondria (TcSir2rp3)	TriTrypDB (v.34)
Trypanosoma brucei	3	Tb927.7.1690 (TbSir2rp1); Tb927.8.3140 (TbSir2rp2); Tb927.4.2520 (TbSir2rp3)	nucleus (TbSir2rp1); mitochondria (TbSir2rp2, TbSir2rp3)	TriTrypDB (v.34)
Leishmania spp.	3	LinJ.26.0200 (LiSir2rp1); LinJ.23.1450 (LiSir2rp2); LinJ.34.1900 (LiSir2rp3)	cytoplasm (LiSir2rp1); mitochondria (LiSir2rp2, LiSir2rp3)	TriTrypDB (v.34)
Apicomplexa				
Plasmodium spp.	2	PF13_0152 (PfSir2A); PF14_0489 (Sir2B)	nucleus; cytoplasm	PlasmoDB (v34)
Toxoplasma gondii	2	TGVEG_068040; TGVEG_108780	n.d	ToxoDB (v34)
Babesia bovis	1	BBOV_I003070	n.d	PiroplasmaDB (v34)
Others				
Giardia lamblia	5	GL50803_10708; GL50803_10707; GL50803_16569; GL50803_11676; GL50803_6942	n.d	GiardiaDB (v34)
Trichomonas vaginalis	10	TVAG_549940; TVAG_409810; TVAG_026260; TVAG_319320; TVAG_480900; TVAG_190210; TVAG 362260; TVAG_413390; TVAG_146810; TVAG_016210; TVAG_146820; TVAG 256040	n.d	TrichDB (v34)

n.d=not determined

Table 1. Sirtuin genes identified in genome-sequencing programs for parasitic protozoa.

Plasmodium spp. have been shown to contain two sirtuin orthologues, called Sir2A and Sir2B. Sir2A is the most extensively studied homolog, mainly located at the nucleus [133] although it can also shuttle to cytoplasm after posttranslational SUMOylation [134]. Sir2A has been characterized as a mediator of transcriptional silencing at the telomeric regions of chromosomes [133, 135]. The telomeres of *P. falciparum* are rich in gene families involved in antigenic variation such as the *var.* family of genes. These genes are responsible for the expression of parasite-derived *P. falciparum* erythrocyte membrane protein, PfEMP1, responsible for immune evasion in humans [136]. The family of *var.* genes is tightly regulated by sirtuins, with the expression of its members being mutually exclusive [137, 138]. The switch of active *var.* is controlled exclusively at the epigenetic level [137, 139].

PfSir2B is a larger homolog with a molecular weight more than four times the size of Sir2A and is involved in the transcriptional silencing of a complementary subset of *var.* genes with distinct promoter types [140].

Sirtuins from *Leishmania* spp. parasites were among the first to be identified in Trypanosomatids, when a complementary DNA (cDNA) isolated and sequenced from *Leishmania major* showed a high homology with yeast Sir2 [141]. Antibodies raised against this LmSir2 later showed to be reactive against different life cycle stages of *L. major*, but also to *Leishmania amazonensis* and even to the serum of a patient infected with *Leishmania infantum* [142]. Furthermore, the protein was found to be among the secreted material of *L. major* [142].

Overexpression of the Sir2 protein in *L. infantum*, sharing 93% homology to the *L. major* protein, led to an increased survival of amastigotes under axenic conditions [143]. Also, when the overexpression was performed in mammalian fibroblasts, host cells became more permissive to infection by *Leishmania* infection in comparison with wild type cells, hinting at a modulation of host cell by the parasite [144]. Genetic knockouts in *L. infantum* of the Sir2 related protein 1 (Sir2rp1) gene also highlighted the importance of this protein in the parasite. While single knockouts were readily obtainable, double deletion of the alleles was only possible after the rescue by an ectopical copy of the gene, suggesting an essential role for parasite survival [145]. When single-knockouts of *L. infantum* Sir2rp1 were used to infect a macrophage cell line, *in vitro*, it was noted that although they had the same invasive capacity than wild-type parasites, they showed a hindered replication rate leading to diminished infections over-time. Furthermore, the mutant parasite also failed to establish an infection in an *in vivo* mouse model of Leishmaniasis [145]. Cellular and biochemical studies later established LiSir2rp1 has NAD^+-dependent deacetylase with ADP-ribosylation activity that co-localized to the cytoskeleton and potentially interacted with tubulin as well as with HSP83, an orthologue of mammalian HSP90 [146, 147]. The association with cytoskeleton is a characteristic feature of both SIRT2 and HDAC6 in mammalian cells [32, 148]. In addition, an orthologous from *L. amazonensis*, LaSir2rp1 was found to be a glycosylated protein, but whether this is the case for other species remains to be seen [149]. Although the Sir2 related protein 1 has received much attention, no studies have been made for the other two proteins codified by the *Leishmania* species; Sir2 related protein 2 and Sir2 related protein 3.

Sir2rp1 from *L. donovani* has also been implicated in the resistance of amphotericin B, a reference drug in the treatment of visceral Leishmaniasis. When clinical isolates were targeted for gene knockout of the protein, parasites showed a lower level of multi-drug resistant

transporter MDR1, lower drug efflux, increased ROS production and increased sensitivity to amphotericin B [150]. On the contrary, overexpression led to a resistant phenotype, thereby suggesting Sir2 as a new resistant marker for visceral Leishmaniasis [150]. Comparative transcriptomic analysis also implicates Sir2 in resistance to miltefosine, another drug used to treat the disease [151]. In addition to its potential as a novel drug target, Sir2rp1 from *Leishmania* spp. has also been suggested as a vaccine [152, 153].

Recently, LiSir2rp2 and LiSir2rp3, the others *Leishmania* sirtuins, were characterized as mitochondrial proteins, and while LiSir2rp3 was demonstrated to not be essential (**Table 1**), attempts to generate LiSir2rp2 knockout cells failed. LiSir2rp2 was implicated in parasite proliferation depending on NAD^+ availability [154].

Trypanosoma brucei, like *Leishmania* spp., has 3 sirtuins annotated in its genome (**Table 1**). The first enzyme to be characterized in the parasite was TbSir2rp1 [155]. The enzyme is localized mainly in the nucleus in association with chromosomes. The protein was shown to possess both deacetylase activity toward endogenous histones while being also able to ADP-ribosylate calf thymus histones and, to a lesser extent, bovine serum albumin (BSA). Up to that time, no ADP-ribosylation had been detected in common members of the sirtuin family like yeast Sir2 or HST2, hence TbSir2rp1 was one of the first enzymes to exhibit this dual activity [156–159]. Furthermore, mutation of a catalytic histidine essential for deacetylase activity also affected ADP-ribosylation activity, suggesting that the two activities were occurring simultaneously. Because of the increased or decreased resistance to DNA damage caused by the alkylating drug methyl methanesulfonate (MMS) in overexpressing or RNAi-induced knockdown *T. brucei* cell lines, respectively, TbSir2rp1 was also considered to have a role in DNA repair in this organism [155].

A later study performed with bloodstream forms (as opposed to insect stage forms in the previous works) characterized TbSir2rp1 and also the other two sirtuins, TbSir2rp2 and TbSir2rp3 that both with mitochondrial localization [160]. TbSir2rp1 was found in the nucleus, but when overexpressed to high levels in *T. brucei* cells, it localizes to the cytoplasm, with toxic effects to the parasite [160]. Besides, gene knockouts for the three proteins caused no growth in parasites maintained in standard conditions [160]. TbSir2rp1 mutants, however, did show an increased sensitivity to MMS damage, confirming the previous results performed with RNA interference (RNAi). The particular localization of TbSir2rp1 led to the investigation of Sir2 mediated telomere gene silencing like the one that occurs in yeast and *Plasmodium* spp., as discussed earlier [161, 162]. Although TbSir2rp1 was found to have a role in telomere DNA repair and telomere silence, it was not required for antigenic variation [160] as described for *Plasmodium* Sir2 [133, 135].

TbSir2rp1 has also been studied as a model sirtuin, with both deacetylase and ADP-ribosylation activity. Biochemical experiments revealed that ADP-ribosylation is 5-fold less active than the deacetylation reaction, and occurs only in the presence of an acetylated substrate by two distinct biochemical mechanisms [163]. Another research group demonstrated that ADP-ribosylation can also occur in arginine, independent of the presence of an acetylated substrate, as supported by mass spectrometry and molecular dynamics simulations [164].

Additional information about parasitic sirtuins can be found in a recent review by Hailu et al. [165].

4. *Trypanosoma cruzi sirtuins*

Although the draft of the *T. cruzi* genome has been published a decade ago [19], it was not until recently that the first experimental studies involving the sirtuins of this parasite have been published. Unlike *Leishmania* spp. and *T. brucei* that possess three Sir2-like proteins, *T. cruzi* only has two coding sequences annotated in its genome, TcSir2rp1 and TcSir2rp3 (**Table 1**).

One study employing parasites overexpressing TcSir2rp1 and TcSir2rp3 by a tetracycline inducible vector characterized some of the features of both proteins [58]. Localization studies employing both wild type parasites and polyclonal sera raised against both proteins, as well as localization of tagged copies in the overexpression mutants with monoclonal antibodies, attributed a cytoplasmic localization to TcSir2rp1 and a mitochondrial to TcSir2rp3 [58]. Both of the proteins' levels are regulated throughout the life cycle of the parasite, with a significant decrease in both at the trypomastigote stage [58]. Overexpression of TcSir2rp1 was responsible for higher metacyclogenesis and higher infectivity of Vero cells. Since metacyclogenesis occurs under nutrient deprivation, it is hypothesized that TcSir2rp1 may function like sirtuins from other organisms that respond to starvation [101, 166, 167]. On the other hand, overexpression of TcSir2rp3 led to a decrease in epimastigote replication time, lower infectivity in Vero cells, increased amastigote replication and normal metacyclogenesis [58]. Due to the oxidizing environment in which amastigotes replicate, it has been suggested that TcSir2rp3 performs protecting functions against oxidative stress like SIRT3 [36]. Both of the overexpressing cell lines reduced the levels of acetylation for particular proteins, as well as protected against the effect of specific sirtuin inhibitors [58].

Moretti and colleagues also independently characterized both of the sirtuins in a simultaneous study [59]. In their study, they show that salermide, a sirtuin inhibitor analogue of sirtinol found to be a strong anticancer molecule, is active against both *in vitro* cultures of epimastigotes, and against an *in vivo* model of infection by *T. cruzi*, albeit at moderate levels [59, 168]. Salermide was also found to be a strong inhibitor of TcSir2rp3 recombinant protein [59]. The authors report the same localization for both proteins, as well as the interference in epimastigote growth, metacyclogenesis, infectivity of host cells and amastigote replication in lines overexpressing the sirtuins. Differently from Ritagliati work [58], in their studies, the overexpression of the cytosolic TcSir2rp1 caused a decreased in the epimastigotes proliferation while TcSir2pr3 increased the growth rate. These differences might be due to the amount of overexpression achieved and parasite strains used [59].

5. Potential of *Trypanosoma cruzi* sirtuins as targets for infection control

A strategy that has been traditionally employed in Chagas disease drug discovery is the target-based approach. One such molecular target that has gained increasing interest as a potential drug targets against parasitic diseases is that of sirtuins [169, 170]. The hypothesis arose by the time that it was demonstrated that sirtuins are life-span regulators in organisms like yeast, flies and worms [70, 171, 172]. Therefore, many groups promptly investigated whether sirtuin

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orthologues present in parasites could have important functions that could be exploited for novel therapeutic applications.

One important aspect for the viability of targeting sirtuins in parasites is the homology between the protein of interest and other proteins present in the host organisms. Although sirtuins are conserved through evolution [33], significant difference at the sequence level can be found between Trypanosomatid and human homologs. For instance, *T. cruzi* Sir2rp1 shares only 33% identity with mammalian SIRT2, its closest homolog (Multi-way protein alignment, BLOSUM 62) [173].

Another argument that has led to the consideration of *T. cruzi* sirtuins as a drug target is that this family of proteins is considered to possess structural properties adequate to inhibition by small-molecule compounds. In particular, the catalytic site is located inside a hydrophobic channel formed at the interface between the two constituting domains, the Rossman fold containing the NAD^+ binding domain and the Zn^{2+} ion binding domain [56]. Catalytic pockets buried inside the protein are considered an essential feature for target druggability [174].

One last fact that prompted the evaluation of TcSir2rp1 as a drug target was the previous evidence that a class of experimental compounds preferentially inhibited LiSir2rp1 over the human homolog SIRT1 [175]. The possibility to synthetize selective sirtuin inhibitors has been successfully achieved for human homologs, based upon structural knowledge of the catalytic site as has been demonstrated for human SIRT2 [176, 177].

Enzymatic inhibition by small molecule compounds is an essential step in the druggability assessment of novel therapeutic targets [174]. Biochemical studies performed by our research groups evaluated the effect of nicotinamide, a classic non-competitive inhibitor of sirtuins in TcSir2rp1 and TcSir2rp3 (to be published). TcSir2rp1 was shown to be inhibited by nicotinamide, albeit at a relatively high IC_{50} when compared with other sirtuins (4-fold higher for hSIRT1 and 11-fold higher for LiSir2rp1) [175]. Different nicotinamide sensitivities are found among distinct sirtuins, and may explain the differences described [177]. Nicotinamide inhibits deacetylation by binding to a conserved C pocket present in sirtuins that participates in NAD^+ binding and catalysis, where it promotes a base-exchange reaction at the expense of deacetylation [178]. A hypothesis for the high IC_{50} for nicotinamide in TcSir2rp1 could be related with structural characteristics of this conserved C pocket. Structural determination of TcSir2rp1 by X-ray crystallography currently ongoing in our group will certainly highlight these differences. Contrary to previous studies [179], we could not observe any antiparasitic activity of nicotinamide against *T. cruzi* amastigotes for up to a concentration of 2 mM. Several studies report the activity of nicotinamide against parasitic protozoa [180–182], but to our knowledge, none clearly establishes a relation between antiparasitic activity and sirtuin inhibition.

Other biochemical functions and protein interactions have been attributed to Sir2rp1 in related Trypanosomatids, and future experiments should shed light whether it applies to TcSir2rp1. One of the biochemical functions that has been characterized for both TbSir2rp1 and LiSir2rp1 is ADP-ribosylation. Both orthologous showed to ADP-ribosylate calf thymus histones and BSA [155, 183]. Later studies involving TbSir2rp1 demonstrated that this biochemical function is dependent upon acetylated histones, is coupled to the deacetylase activity of the sirtuin, but occurs at a much lower rate than the latter [163]. In fact, even though ADP-ribosylation has clear functions in both physiological and pathogenic situations when catalyzed by other

ADP-ribosyltransferases [183, 184], the reaction catalyzed by sirtuins is currently challenged to be an unspecific side-reaction [159].

Like the human SIRT2, *L. infantum* Sir2rp1 was also found to be associated with tubulin [146], the major component of Trypanosomatids cytoskeleton formed by an array of subpellicular microtubules that span the whole cell of the parasite [185]. SIRT2 is a tubulin deacetylase that displays a higher affinity for tubulin than for histones [32], and has been found to be linked to regulation of mitotic progression [86], chromatin condensation [186] and cell migration [187]. TcSir2rp1 overexpression in *T. cruzi* was found to increase the deacetylation level of endogenous tubulin [58]. It is interesting to note that Sir2rp1 from *T. cruzi* is a cytoplasmic protein like LiSir2rp1 and not nuclear like TbSir2rp1. Since *T. cruzi* shares some characteristics with *L. infantum* like the amastigote intracellular stage, it should not be ruled out that Sir2rp1 may have functions in the cytoskeleton remodeling necessary for stage differentiation. Several proteins, sirtuins included, have demonstrated the ability to shuttle from the nucleus to the cytoplasm and vice-versa [188, 189]. SIRT2, the closest sirtuin homolog of mammalian cells, is actively exported to the cytoplasm during interphase, but is accumulated in the nucleus from prophase until cytokinesis where it co-localizes with important mitotic structures like centrosomes and the mitotic spindle [190]. Curiously, analysis of TcSir2rp1 by Wregex and cNLS Mapper, bio-computational tools that identify nuclear export signals (NES) and nuclear localization signals (NLS), respectively, indicate the presence of non-canonical NES/NLS in the sequence of this sirtuin [191, 192]. Whether TcSir2rp1 does shuttle to the nucleus during specific phases of *T. cruzi* life cycle, for instance to repair DNA damage like the *T. brucei* orthologue, remains to be reported.

The mitochondrial TcSir2rp3 was found less expressed in *T. cruzi* forms proliferating in mammalian cells. Its expression increased when the parasite transformed in trypomastigotes [59]. The fact that cells overexpressing only the active form, but not the inactive form of TcSir2rp3 showed an increased intracellular growth and failed to transform in to extracellular trypomastigotes [59], suggested that it could also be a drug target to control the infection, although further experiments to generate knockout cell lines need to be performed. We recently identified several compounds that prevented intracellular growth of *T. cruzi* some of them inhibiting specifically TcSir2rp1 or TcSir2rp3 which might indicate the requirement of both enzymes for the parasite (to be published).

6. Naphtalimide derivatives as potential drugs for Chagas disease control

Naphthalimides are a class of compounds that have generated intense interest as active molecules with potential to treat a range of conditions [193]. A naphthalene ring linked to an imide group that forms a third heterocycle composes the basic chemical scaffold of the naphthalimide derivatives. This moiety has a planar nature and is considered to be responsible for the pharmacological activities attributed to compounds derived from this structure, that can be as distinct as anticancer, antibacterial, antiprotozoal, antiviral, analgesic, and anesthetic [193]. Their potential as anticancer compounds has received particular attention, mostly because of their DNA intercalating properties and also to their reported activity as topoisomerase inhibitors [194–196]. Compounds like amonafide and bisnafide have been proposed as anticancer agents and have inclusively reached clinical

trials in the past [197, 198]. Elinafide is another derivative with two naphthalimide moieties that has been evaluated in preclinical studies and demonstrated potential against various mouse xenograft models [199]. This last compound was in the origin of the synthesis of the first BNIPs that differed in the alkyl chain linking the naphthalimide and amine group, i.e. a propyl instead of an ethyl chain [200]. These derivatives showed potential activity against breast cancer MCF-7 cell line and actively bound DNA as demonstrated by thermal denaturation measurements, ethidium bromide displacement and DNA gel mobility [200]. Later derivatives that varied in the length of the chain linking the two amines of bisnaphthalimidopropyl groups were also evaluated against cancer cell lines and promastigotes of the parasite *L. infantum* [201]. While screening for enzymatic inhibitors of the recently characterized LiSir2rp1, BNIPs were identified as inhibitors of its deacetylase activity [175]. Furthermore, they were active against intracellular amastigotes, the clinically relevant stage of the parasite present in humans, at concentrations in the single micromolar range [175].

Our groups' previous results demonstrating activity toward *L. infantum* led to the testing of BNIPs as inhibitors of the related Trypanosomatid *T. brucei* and its Sir2rp1 orthologue, TbSir2rp1 [202]. BNIPs revealed to be very potent inhibitors of *in vitro* parasite growth, with one of the compounds, BNIPDabut (**Figure 1**) with an EC_{50} in the range of the reference drug pentamidine. However, when tested against the TbSir2rp1 recombinant enzyme, BNIPDabut had an IC_{50} more than 10^4 times superior to the EC_{50} against the whole cell parasite, indicating that Sir2rp1 inhibition is probably not a major mechanism of action for the compound. Whether BNIPDabut inhibits other *T. brucei* sirtuin enzymes remains to be elucidated. It should be noted that RNAi and gene knockout experiments of the three sirtuins did not led to a deleterious effect, and unlike LiSir2rp1, there is no indication of that these proteins may be essential [155, 160]. The optimal *in vitro* properties of BNIPDabut led to the testing with an *in vivo* model of trypanosomiasis by bioluminescence imaging. Although BNIPDabut maintained a strong trypanocidal activity *in vivo*, as assessed by the decrease in bioluminescent signals to levels similar to those of the reference drug control pentamidine, it was not sufficient for infection clearance, as animals' parasitaemia relapsed shortly after treatment interruption. Nevertheless, BNIPDabut should constitute a scaffold for further consideration in HAT drug discovery [202].

In a different study, BNIP derivatives were also tested against TcSir2rp1 and *in vitro* cultures of *T. cruzi* amastigotes (unpublished results). BNIPs demonstrated to inhibit the deacetylase activity of this enzyme, with BNIPSpd (**Figure 1**) as the most potent compound, showing a dose-dependent effect on inhibition. BNIPSpd also proved to be active and selective against amastigotes of *T. cruzi* in a high content screening (HCS) assay – an assay that takes advantage of computer-assisted image processing of hundreds of microscopic images originating in automated microscopes able to simultaneously capture multiple conditions or drug compounds. In this work, a new set of derivatives was also synthetized in order to improve both solubility and binding to cellular targets, mostly by including cyclic structures and heteroatoms in the carbon chain linking the two naphthalimide groups. The most active compounds were BCNIPP, also a TcSir2rp1 inhibitor, and trans BNIP-1,4-Dacyhex, a derivative of BNIPDabut that weakly inhibited the enzyme. In turn, BNIPDabut had some inhibitory activity on *T. cruzi* amastigotes, with low selectivity, but also did not inhibit TcSir2rp1 at 10 μM. The activity of BNIPSpd in a mice model of Chagas disease using bioluminescent parasites was also determined and found to be absent at the doses tested. An explanation might be the poor pharmacokinetic profile of the compounds, which fails to ever achieve at least the *in vitro* IC_{50} against *T. cruzi* amastigotes.

Altogether, our data indicate that BNIP derivatives may not be acting only by a mechanism of Sir2rp1 inhibition, with other targets contributing to the activity detected. BNIPs were originally designed and developed as anti-cancer agents [203, 204] through DNA intercalation. This property might explain some of the cytotoxic effects verified against host cells, but may also be an important mechanism of activity toward the parasite, especially since trypanosomes are highly susceptible to intercalating agents [205].

Confirmation of the mechanism of action can be undertaken by appropriate target deconvolution experiments [206]. The most common type of such experiments are biochemical methods that employ some variation of biochemical affinity purification, where the compounds are immobilized in a column, and allowed to interact with protein extracts, preferably previously fractionated. After stringent washing steps, the bound proteins are eluted and identified. Such strategy has been employed in the identification of small molecule activators of cryptochrome of mammalian cells [207]. A disadvantage is that there is a bias toward high affinity ligands, and when the target is relatively less abundant or has less affinity, important targets may not be detected. Furthermore, the washing steps may eliminate protein complexes that may be important for appropriate drug activity.

Genetic methods can also be valuable for target deconvolution. Gene knockouts and RNAi screens can be used to try to phenotype a compound's effect [208]. Furthermore, if the mutant is hypersensitive to the compound in question, the evidence that the protein could be the target for the compounds would be strengthened. The validation of trypanothione synthetase and N-myristoyltransferase as drug targets against trypanosomes are examples where the differential sensitivity of an inhibitor in wild type, overexpression, and knockout mutants is clearly illustrated [209, 210]. An additional genetic strategy is based on the generation of resistant cell lines by culturing the parasites in increasing sub-lethal drug concentrations that are posteriorly sequenced to find mutated genes related to the mechanism of action for the compound [208]. Genetic methods have recently been employed in the search of the mechanism of action of oxaboroles [211], a class of compounds in development for HAT, but also active against *T. cruzi* [212, 213].

Chemical genomics can also be applied to the discovery of novel drug targets, as exemplified by the recent characterization of cytochrome b from *T. cruzi*. This enzyme was demonstrated to be selectively targeted in relation to the human homolog by a hit compound coming from a phenotypic screening [214].

7. Perspective

Here we highlighted the potential of sirtuins, particularly in the *T. cruzi* as possible targets of drug development for Chagas disease for the following reasons:

1. The parasite contains only two sirtuins, instead of three present in *Leishmania* spp. and *T. brucei* species, and seven in the human host, which facilitates a more precise design and avoid redundant effects.

2. Several experiments demonstrate the requirement of these enzymes for growth and survival, particularly in intracellular forms of the parasite, which are clinically relevant.
3. There are several compounds already designed to be selective sirtuin inhibitors that could be modified to provide increased specificity and selectivity to the parasite enzyme. Some of these compounds have already defined druggability and specific derivatives can be repurposed more easily.
4. Both sirtuins were produced to perform enzymatic screenings and crystals of TcSir2rp1 have been obtained to be the basis of further medicinal chemistry.

Finally, several lead compounds were identified for *T. cruzi* sirtuin, which can provide the basis for future development. It is also important to note that these possible inhibitors could act synergistically with drugs already in use for treatment, as a novel combinational therapy, opening new avenues to eliminate Chagas disease.

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