International Journal for Parasitology: Drugs and Drug Resistance 7 (2017) 23–33

Contents lists available at ScienceDirect

International Journal for Parasitology: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijpddr

Comparative effects of histone deacetylases inhibitors and resveratrol on *Trypanosoma cruzi* replication, differentiation, infectivity and gene expression

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ARTICLE INFO

Article history:
Received 24 June 2016
Received in revised form
7 December 2016
Accepted 7 December 2016
Available online 21 December 2016

Keywords:
Trypanosoma cruzi
Histone acetylation
Histone deacetylases inhibitors
Resveratrol

ABSTRACT

Histone post-translational modification, mediated by histone acetyltransferases and deacetylases, is one of the most studied factors affecting gene expression. Recent data showing differential histone acetylation states during the *Trypanosoma cruzi* cell cycle suggest a role for epigenetics in the control of this process. As a starting point to study the role of histone deacetylases in the control of gene expression and the consequences of their inhibition and activation in the biology of *T. cruzi*, two inhibitors for different histone deacetylases: trichostatin A for class I/II and sirtinol for class III and the activator resveratrol for class III, were tested on proliferative and infective forms of this parasite. The two inhibitors tested caused histone hyperacetylation whereas resveratrol showed the opposite effect on both parasite forms, indicating that a biologically active *in vivo* level of these compounds was achieved. Histone deacetylase inhibitors caused life stage-specific effects, increasing trypomastigotes infectivity and blocking metacyclogenesis. Moreover, these inhibitors affected specific transcript levels, with sirtinol causing the most pronounced change. On the other hand, resveratrol showed strong anti-parasitic effects. This compound diminished epimastigotes growth, promoted metacyclogenesis, reduced *in vitro* infection and blocked differentiation and/or replication of intracellular amastigotes. In conclusion, the data presented here supports the notion that these compounds can modulate *T. cruzi* gene expression, differentiation, infection and histones deacetylase activity. Furthermore, among the compounds tested in this study, the results point to Resveratrol as promising trypanocidal drug candidate.

1. Introduction

Trypanosomatids are microorganisms that cause serious health problems in humans and domestic animals. *Trypanosoma cruzi* is a protozoan parasite that causes American Trypanosomiasis or Chagas' disease, an endemic illness in Latin America (Rodrigues Coura, 2013). This parasite has a complex life cycle, alternating between two different hosts, an insect vector and a mammalian reservoir. In each host, the parasite develops into two main life stages: a proliferative form (named epimastigote within the insect and amastigote within mammalian cells) and an infective form (named metacyclic trypomastigote in the insect vector and cell-derived trypomastigote in the mammalian host). In addition to its medical relevance, this pathogen represents an interesting study model due to its structural and biological particularities. For example, RNA pol II (RNAPII) transcription is polycistronic. This means that groups of genes, named Polycistronic Transcriptional Units (PTUs), are transcribed at the same time. Also, there are no classical signals for transcription initiation. The intergenic regions, named Strand Switch Regions (SSRs), flanking two divergent (arranged head to head) or convergent (arranged tail to tail) PTUs have been associated with the initiation and termination of transcription, respectively. According to this, distinctive histone types are associated to trypanosomatid SSRs (Martinez-Calvillo et al., 2010). Specifically, enrichment in acetylated H4K10 and H3 at divergent SSRs has been found in *T. brucei* and *Leishmania major*, respectively (Siegel et al., 2009; Thomas et al., 2009), whereas acetylated H3K9/H3K14, H4K10 and methylated H3K4 mark the bidirectional transcription initiation sites in *T. cruzi* (Respuela et al., 2008).

In normal cells, chromatin structure can switch between an open transcriptionally active and a compact silenced conformation.

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http://dx.doi.org/10.1016/j.ijpddr.2016.12.003

One of the main epigenetic mechanisms regulating this shift is the acetylation of histone lysine residues at the N-terminal tail, which results in destabilization of the nucleosome and activation of transcription (Eberharter and Becker, 2002). This epigenetic event is involved in the gene regulation of important pathways such as cell cycle and differentiation in parasites (Chaal et al., 2010; Sonda et al., 2010; Dubois et al., 2009). Although *T. cruzi* chromatin is not condensed into chromosomes during cell division, a differential degree of condensation and different acetylation levels of histone H4 have been described during cell cycle, after exposure to DNA damage and during differentiation between proliferative and infective forms of the parasite (Nardelli et al., 2009). This suggests that besides the post-transcriptional mechanisms, the epigenetic events modulating the chromatin structure might play a role in the regulation of gene expression.

Histone acetylation is mediated by Histone Acetyltransferases (HATs), which cancel the positive charge on lysine residues thus reducing chromatin compression, while deacetylation is mediated by Histone Deacetylases (HDACs), which have the opposite effect (Shahbazian and Grunstein, 2007). HDACs form a family that can be divided into four main distinct classes based on their structure described in humans (Gray and Ekstrom, 2001). HDACs I, II and IV share a similar catalytic core that uses zinc as a cofactor, but differ in size and structural organization, whereas HDACs III, also called sirtuins (from Sir2-related proteins), use nicotinamide adenine dinucleotide (NAD^+) as a cofactor. In protozoan parasites, genome *in silico* analysis has shown the presence of coding sequences for several of these enzymes (Ivens et al., 2005). In *T. brucei*, two class I HDACs (HDAC 1 and 2) and two class II HDACs (HDAC 3 and 4) have been characterized. HDAC1 and 3 are essential for viability, while HDAC4 is required for normal cell cycle progression (Ingram and Horn, 2002). Coding sequences for HDACs have also been found in *T. cruzi* (El-Sayed et al., 2005), but only sirtuins deacetylases have been recently characterized (Ritagliati et al., 2015; Moretti et al., 2015).

An important approach to study the function of chromatin acetylation is the use of histone deacetylase inhibitors (HDACis). These compounds have been used to study the role of histone acetylation in gene regulation in a wide variety of parasites. For instance, in *Entamoeba histolytica*, microarray analysis has shown that the HDAC inhibitor Trichostatin A (TSA) produce differential expression of genes involved in the regulation of the stage conversion pathway (Ehrenkaufer et al., 2007). In *Toxoplasma gondii*, stage-specific expressed genes are influenced by HDAC3 (a class I HDAC) inhibitors (Bougdour et al., 2009). Also, incubation of *Plasmodium falciparum* parasites with three hydroxamate-based compounds: Trichostatin A, Suberoyl Anilide Hydroxamic Acid (SAHA) and a 2-AminoSuberic Acid derivative (2-ASA-9), has shown to cause profound transcriptional effects (Andrews et al., 2012a). These and many other examples support the idea that enzymes involved in chromatin modification may be targeted to create effective new therapies against protozoan pathogens. In fact, HDACi's originally targeted for cancer use are now being investigated as compound leads for parasitic diseases (Andrews et al., 2012b). For instance in a recent study, HDACis that are currently in clinical trials for oncology were evaluated for treatment of the human African trypanosomiasis (Carrillo et al., 2015). These inhibitors were found to block proliferation of blood-stage in culture; however, none were lethal to cultured parasites when tested at human tolerated doses. Other studies also evaluated the *in vitro* activity of anti-cancer HDACis against this *T. brucei* and *Plasmodium*. These compounds were found to have some selectivity for malaria parasites compared with mammalian cells, but not for trypanosome parasites (Engel et al., 2015). However, little is known about the action of HDACis on *T. cruzi* biology, and only the effects of apicidin derivatives (targeted to mammalian HDACs II) and nicotinamide (unspecific inhibitor of mammalian sirtuins) on other parasite strains have been reported (Murray et al., 2001; Veiga-Santos et al., 2014; Soares et al., 2012). On the other hand, HDAC activators, especially for sirtuins, are now being considered for anti-parasite use (Kedzierski et al., 2007; Valera Vera et al., 2016). With this in mind, two inhibitors for different HDAC classes (TSA for HDAC I and II and sirtinol for HDAC III) and one activator (resveratrol) for HDAC III were used to evaluate the effects on *T. cruzi* replication, differentiation, infectivity and gene expression.

2. Materials and methods

2.1. Histone deacetylase inhibitors and resveratrol treatment

The HDACis tested during this study were: the hydroxamic acid type inhibitor of HDAC class I and II, trichostatin A (TSA, [R-(E,E)]-7-[4-(Dimethylamino)phenyl]-N-hydroxy-4,6-dimethyl-7-oxo-2,4-heptadienamide) (#9950, Cell Signaling) and the HDAC III inhibitor sirtinol (2-[(2-Hydroxynaphthalen-1-ylmethylene)amino]-N-(1-phenethyl)benzamide) (S7942, Sigma). The HDAC III activator used was 3, 4′, 5-trihydroxystilbene or resveratrol (ab120726, Abcam). All assays were performed with the corresponding control of parasites incubated with equal amounts of the vehicle, dimethylsulfoxide (1% DMSO final concentration), or ethanol (1% ethanol final concentration) for TSA incubations. The starting concentrations for each compound were set according to the manufacturer's indications. The incubation time was 18 h s for all treatments.

2.2. Parasites

Parasites of the *T. cruzi* CL-Brener strain, the genome project reference clone (El-Sayed et al., 2005), were used throughout this study. Growth curves of epimastigotes were performed in duplicates by incubation of parasites cultures at 28 °C until exponential phase, in Brain-Heart-Tryptosa media (BHT: 33 g/L brain heart infusion, 3 g/L bacto-tryptona, 5.4 mM KCl, 22.5 mM Na_2HPO_4, 0.3% (w/v) glucose and 0.1% (w/v) hemin) supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin and 100 mg/L streptomycin. Then cultures were diluted again in fresh medium with the corresponding compounds and monitored daily by counting live parasites in Neubauer chamber. Cell viability was assessed by direct microscopic examination.

To evaluate the effect of these compounds over the differentiation process to metacyclic trypomastigotes, epimastigote cultures were grown until stationary phase (70×10^6 cells/ml) and starved until parasites attached to the bottom of the bottles. Then, cultures were diluted until a parasite concentration of 20×10^6 cell/ml and maintained in BHT with 4% FBS with different concentrations of each compound or the equivalent amount of the corresponding vehicle (DMSO or ethanol to a final concentration of 1%) as control. Incubations were maintained for three days at 28 °C. After this time, percentages of epimastigotes and metacyclic trypomastigotes were recorded by counting in Neubauer chamber. The viability of each parasite form was assessed by direct microscopic examination.

Cell-derived trypomastigotes were purified from infection supernatants by centrifugation at 5200*g* for 10 min and allowing trypomastigotes to swim for 4 h at 37 °C. Then trypomastigotes were collected from the supernatant, concentrated by centrifugation and incubated for 18 h at 37 °C in MEM 4% FBS with the compound or the corresponding vehicle for control. After incubation, and washed once with PBS1x, living parasites were counted for infection assays.