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

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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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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.

2.3. *In vitro* infections

Vero cells (20,000 in 0,5 ml of MEM 4% FBS) were plated onto round coverslips 24 h before infection. Infections were performed for 4 h with 2×10^6 trypomastigotes per coverslip, pretreated as described before with the corresponding compound or DMSO (or ethanol in TSA assays) as controls. After infection, cells were washed twice in PBS1x, and incubated in fresh medium for additional 48 h to allow amastigotes replication. Then, coverslips were washed twice in PBS1x, fixed with paraformaldehyde 4% for 20 min, washed again and mounted in 5 μl of FluorSave Reagent (Calbiochem) and 5 μl of DAPI (100 μg/ml final concentration) for nucleus and kinetoplastid staining and observed and photographed using a Nikon Y-FL fluorescence microscope. Each infection was performed in duplicate and the percentage of infected cells and the number of intracellular amastigotes was calculated using the cell counter plugin from ImageJ software. For this, 60 fields, each containing a mean of 20 cells, were photographed with the 40× magnification objective for each experiment.

2.4. Western blots

Protein extracts were prepared by incubation of parasite pellets in PBS-0.5% NP40, with PMSF 0.1 mM and EDTA 0.5 mM for 15 min on ice and then collected by centrifugation at 1300g for 10 min. Pellets were dissolved directly in cracking buffer 5× (0.2 M Tris-HCl pH 6.8, 10% SDS, 0.1% bromophenol blue, 5% NP40, 5% TritonX100 and 10% glycerol) to a parasite concentration of 2×10^6 cell/μl, and then DNAse I was added to a final concentration of 100 μg/ml and incubated at 4 °C until DNA was dissolved. SDS-PAGE was performed by loading 20×10^6 parasites per well of each treatment and the corresponding control in a 12% polyacrylamide gel and transferring to nitrocellulose membranes. Filters were blocked for 1 h in TBS-5% milk and incubated for 16 h at 4 °C with anti-acetyl histone H4 rabbit polyclonal antiserum directed to a KLH-conjugated peptide [AGGAcKGGAcKGMGAcKVGAAcKRHS-C] corresponding to amino acids 2–19 of *Tetrahymena* histone H4 acetylated in all the lysine residues (06–866 Millipore), in a 1:2000 dilution. After incubation, filters were washed twice in TBS1x for 5 min and incubated with anti-rabbit HRP-conjugated serum in a 1:10,000 dilution for 1 h, washed twice in TBS-Tween 0.2% for 5 min and once in TBS1x for another 10 min and developed with Super Signal pico (Pierce). Western blot signals were normalized with the total amount of histones proteins observed by coomassie blue staining quantified using the gel analyzer tool of ImageJ software. Coomassie staining was used as loading control because the effects on the transcription rates due to the treatment with these compounds might affect the expression levels of proteins commonly used for normalization like tubulin or GAPDH.

2.5. Indirect immunofluorescence

A drop of parasites (5×10^6/ml in PBS1X) was layered onto Poly-L-Lysine (Sigma–Aldrich) coated coverslips and let stand for 20 min at room temperature. Parasites were fixed with 4% paraformaldehyde and washed with PBS. Blocking and antibody solutions were prepared in PBS containing 2% BSA, 5% normal goat serum and 0.5% saponin. To test the expression changes in trypomastigote surface proteins, the coverslips were incubated for 16 h at 4 °C with: rabbit antiserum directed to mucin proteins from the TcMUCII family (anti-TcMUCII) (Buscaglia et al., 2004) or TcMUCIII (anti-TSSA) (Di Noia et al., 2002) at 1:200 dilutions or the repetitive element SAPA (for Shed Acute Phase Antigen) of the trans-sialidase superfamily (anti-SAPA) (Buschiazzo et al., 2012), diluted at 1:8000 or mouse antiserum directed toward the Mucin Associated Proteins family (anti-MASP) at a 1:250 dilution. Anti-MASP serum was raised against the MASPEP peptide (NH2-PDDDDPAADGAGC-COOH) (GenScript), coupled through its C-terminal Cys residue to maleimide-activated KLH (Pierce). This peptide corresponds to an internal sequence from the MASP member TcCLB.511173.64 and can cross react with the following other members from this protein family on the CL Brener strain: TcCLB.412419.10, TcCLB.506131.84, TcCLB.506703.110, TcCLB.508759.60, TcCLB.511081.60, TcCLB.511089.140, TcCLB.506187.50, TcCLB.506245.270, TcCLB.508309.10, TcCLB.510583.130, TcCLB.511089.19, TcCLB.511089.30, TcCLB.507523.80, TcCLB.508293.44. Then after PBS washings, Alexa 488-conjugated goat anti-mouse immunoglobulins G (H + L) or Alexa 568-conjugated goat anti-rabbit (1:10,000, Molecular Probes) were added for 60 min at room temperature and washed as before. Coverslips were mounted and photographed as described before. Fluorescence signals were quantified by image analysis using the ImageJ software. For this, 30 photographs, containing an average of 15 parasites each, were taken for each coverslip with the 100× magnification objective.

2.6. Quantitative PCR

Total RNA from control and treated trypomastigotes was purified from 200×10^6 parasites scattered in Trizol reagent (Invitrogen) using DirectZol RNA miniprep columns (Zymo Research). cDNA was synthesized with the SuperScript II system (Invitrogen) and oligo dT and used for real time PCR assays. Control cDNA from untreated parasites was diluted in water and used to obtain calibration curves for all genes tested. Only curves showing a percentage of efficiency between 90 and 110% were used for quantification. Specific primers for each gene were designed with Primer Express software (Table 1). PCR was carried out in a final volume of 10 μl reaction mixture containing 0.1 μM of each primer, 0.1 μM of ROX high normalization dye, 5 μl of SYBR Green reaction mix (Kapa SYBR Fast qPCR kit) and 4 μl of cDNA template. cDNA was quantified and analyzed using the 7500 software from Applied Biosystems. qPCR quality was evaluated analyzing the melting curves to ensure that only one product was amplified. Data were normalized by the levels detected for 18S rRNA, which bears a region containing 11 adenines (region between positions 479–488 in GeneBank acc. N° ×53917.1) that is recognized by the oligo (dT) primer, allowing the subsequent detection after retro-transcription assays. Quantifications were performed for three independent experiments.

2.7. Statistical analysis

All data obtained in each experiment were first analyzed for normal distribution of the corresponding residues using the Shapiro-Wilk test. If this was true ($p > 0.20$), then statistical differences between treatments and the corresponding control were analyzed using ANOVA or two-tailed Student-t tests. If distribution of the residues analyzed was not normal, then the non-parametric Kruskal-Wallis test was used. To ensure the accuracy of the results obtained with these tests all the experimental data analysis was also evaluated using a “Lineal general mixed model (LGMM)” and the statistical differences between each treatment and the corresponding control were analyzed applying the Less Significant Difference (LSD) Fisher test, with a global level of signification of 5% ($p < 0,05$). Values were expressed as the mean of three independent experiments ± standard deviation. Differences between the experimental groups were considered significant as follows: $p < 0.05$ (*), $p < 0.005$ (**). All these tests were applied using the software InfoStat (version 2016, http://www.infostat.com.ar).

Table 1
List of transcripts selected for quantification by Real Time PCR.

Primer name	Sequence (5′ to 3′)	Gene name	Gene Description on TriTryp	TriTryp gene ID
18SrRNA	CGGAATGGCACCACAAGAC	18S	ribosomal RNA small subunit	TcCLB.419325.10
18SrRNA	TGGTAAAGTTCCCGTGTTGA	18S	ribosomal RNA small subunit	TcCLB.419325.10
873.20aFw	AACATGGTGGGATCGAAAGAAT	DP873	cell differentiation prot	TcCLB.507873.20
873.20aRev	CTCGGAAAGTCGCAAATAACAA	DP873	cell differentiation prot	TcCLB.507873.20
099.50bFw	CAGGCATCACCGTATTTTCCA	PCD6	programmed cell death 6 prot like	TcCLB.507099.50
099.50bRev	CTCTTGTTCCGTGCCAAACA	PCD6	programmed cell death 6 prot like	TcCLB.507099.50
405.10aFw	CACTTTTAACGCGCCTTTCC	CC405	cell cycle division protein	TcCLB.508405.10
405.10aRev	TTTCAATGCAACTTAGTCGTTCCT	CC405	cell cycle division protein	TcCLB.508405.10
907.260bRev	TCCAAATCTCCAGAGAACA	CC907	cell cycle division protein	TcCLB.511907.260
907.260bFw	GCCAACGACCTCCCAATG	CC907	cell cycle division protein	TcCLB.511907.260
913.30aFw	CACGTCGCCCTTGTCCAT	PCNA	Proliferative cell nuclear antigen (PCNA)	TcCLB.507913.30
913.30aRev	GTTCCGCTCACACTGGTACTTG	PCNA	Proliferative cell nuclear antigen (PCNA)	TcCLB.507913.30
TcBDF2Fw	GGCGAGGGTTGATTTGGATA	BDF2	Hyphotetical protein	TcCLB.507769.30
TcBDF2Rev	GCCCAATCGAAAGTGGTAGTG	BDF2	Hyphotetical protein	TcCLB.507769.30
HAT_120Fw	GGAGGAGAACGGAAAGATGACA	HAT120	Histone acetyl transferase	TcCLB.506743.120
HAT_120Rev	GCACCTCGCGGTCTTTCA	HAT120	Histone acetyl transferase	TcCLB.506743.120
HAT_60Fw	CAGCCTCCGCCTTGTGAT	HAT60	Histone acetyl transferase	TcCLB.509203.60
HAT_60Rev	TCCCTCGCCCTTTCGTACT	HAT60	Histone acetyl transferase	TcCLB.509203.60
Sir2RPIFw	CGAGAGAGTTATGGTGCTTGTGA	SIR2RP1	Histone deacetylase	TcCLB.508207.150
Sir2RPIRev	TGTCTGTGTGTACATGTGTGTGTGA	SIR2RP1	Histone deacetylase	TcCLB.508207.150
HDAC_9Fw	CACGGTGCTGCACGATGA	HDAC9	Histone deacetylase	TcCLB.507805.9
HDAC_9Rev	GTGGCCCACAAACAAGAACA	HDAC9	Histone deacetylase	TcCLB.507805.9
HDAC50Fw	AGCGGGTACGCCAAACAC	HDAC50	Histone deacetylase	TcCLB.503653.50
HDAC50Rev	CATTCAGGGTGGGAAAAGCA	HDAC50	Histone deacetylase	TcCLB.503653.50
HDAC59Fw	CTCCTTGCAACCCCCAGAT	HDAC59	Histone deacetylase	TcCLB.507803.59
HDAC59Rev	GAGCAACATGCGTGAATCGT	HDAC59	Histone deacetylase	TcCLB.507803.59
HDAC80Fw	CTCCCCACTGTTTATGCGTATG	HDAC80	Histone deacetylase	TcCLB.504159.80
HDAC80Rev	GACGGCGCCGCAAAG	HDAC80	Histone deacetylase	TcCLB.504159.80
HDAC159Fw	TTTGAAGGTTCGACCCAGTTG	HDAC159	Histone deacetylase	TcCLB.511911.159
HDAC159Rev	CACCGGGAAGTGGTTTGTTT	HDAC159	Histone deacetylase	TcCLB.511911.159
CAF1BFw	CGGAGGAGCCGCAGAAG	CAF1B	chromatin assembly factor 1 subunit B	TcCLB.510181.60
CAF1BRev	GCGGTATCGCCCCAATAGTT	CAF1B	chromatin assembly factor 1 subunit B	TcCLB.510181.60

3. Results

3.1. Resveratrol reduced parasite infection and proliferation whereas TSA and sirtinol blocked metacyclogenesis

To assess the effect of the compounds on parasite replication, differentiation and infection, both epimastigotes and cell-derived trypomastigotes were incubated with different concentrations of each HDAC inhibitor and the activator resveratrol. As a control, to test whether these compounds were actually affecting HDAC activity, changes in the total amount of acetylated histones were evaluated by western blot analysis using anti-acetylated histone H4 serum. Consistent with the specificity previously described for this serum (Respuela et al., 2008), cross-reaction with *T. cruzi* acetylated histones H4, H2B and H3 was observed (supplementary Fig. 1). The two inhibitors caused enrichment in the acetylated histones H4 and H2B in a concentration-dependent manner in both epimastigotes and trypomastigotes, without affecting the total amount of histones, as judged by the Coomassie blue-stained SDS-PAGE (supplementary Fig. 1). For sirtinol, enrichment in acetylated histone H3 was also observed, although global hyperacetylation of histones was always more notorious in the infective forms. The opposite effect was observed in both parasite forms after treatment with the activator resveratrol. Overall, these results show that the compounds tested were able to alter histones acetylation state probably by modulating parasite HDAC activity.

Compounds modulating HDACs activity have shown to either delay or inhibit the growth of the proliferative forms of several protozoan parasites, including *T. cruzi* strains other than the CL Brener strain (Engel et al., 2015; Soares et al., 2012; Valera Vera et al., 2016; Vergnes et al., 2005; Patil et al., 2010). To test this in the CL Brener strain, epimastigote cultures were incubated with different concentrations of the HDAC inhibitors for three days, monitoring parasite growth daily. Both TSA and sirtinol had no effect on parasite growth or duplication rates comparing to controls (Fig. 1A and data not shown). On the other hand, parasites treatment with resveratrol strongly inhibited replication with an IC_{50} of 250 μM estimated from the growth curves of epimastigote cultures incubated for 48 h with resveratrol concentrations ranging from 4 to 800 μM (Fig. 1B).

It has been previously reported that HDAC inhibitors can affect the life cycle of other parasites, either by promoting or inhibiting their differentiation process (Bougdour et al., 2009; Sonda et al., 2010). Given that in the present study parasite replication was not affected by the treatment with the two HDAC inhibitors, these compounds were used to test their effect on *T. cruzi* differentiation. For this, epimastigote cultures were incubated with two different concentrations of each inhibitor or the corresponding vehicle as control, until spontaneous metacyclogenesis on control parasite cultures was observed. Then, the number of living epimastigotes and metacyclic trypomastigotes present in all incubations was recorded. The results showed an average of 20% of inhibition of the spontaneous emergence of metacyclic trypomastigotes in the cultures treated with either of the two HDAC inhibitors at the highest concentration tested (Fig. 1C). Consistent with the inhibition of metacyclogenesis, a concomitant increase in the percentages of replicating epimastigotes was observed in these cultures. The opposite result was obtained with the parasite cultures treated with resveratrol, since this compound caused an increase from 10 to 30% in the number of metacyclic trypomastigotes comparing to control at a concentration of 4 and 40 μM (Fig. 1C).

Next, the effect of HDAC inhibitors and resveratrol on the parasite capacity for infection was tested by incubating cell-derived trypomastigotes for 18 h with different concentrations of each compound previous to infection of Vero cells. After each treatment, trypomastigote viability was monitored by direct microscopy

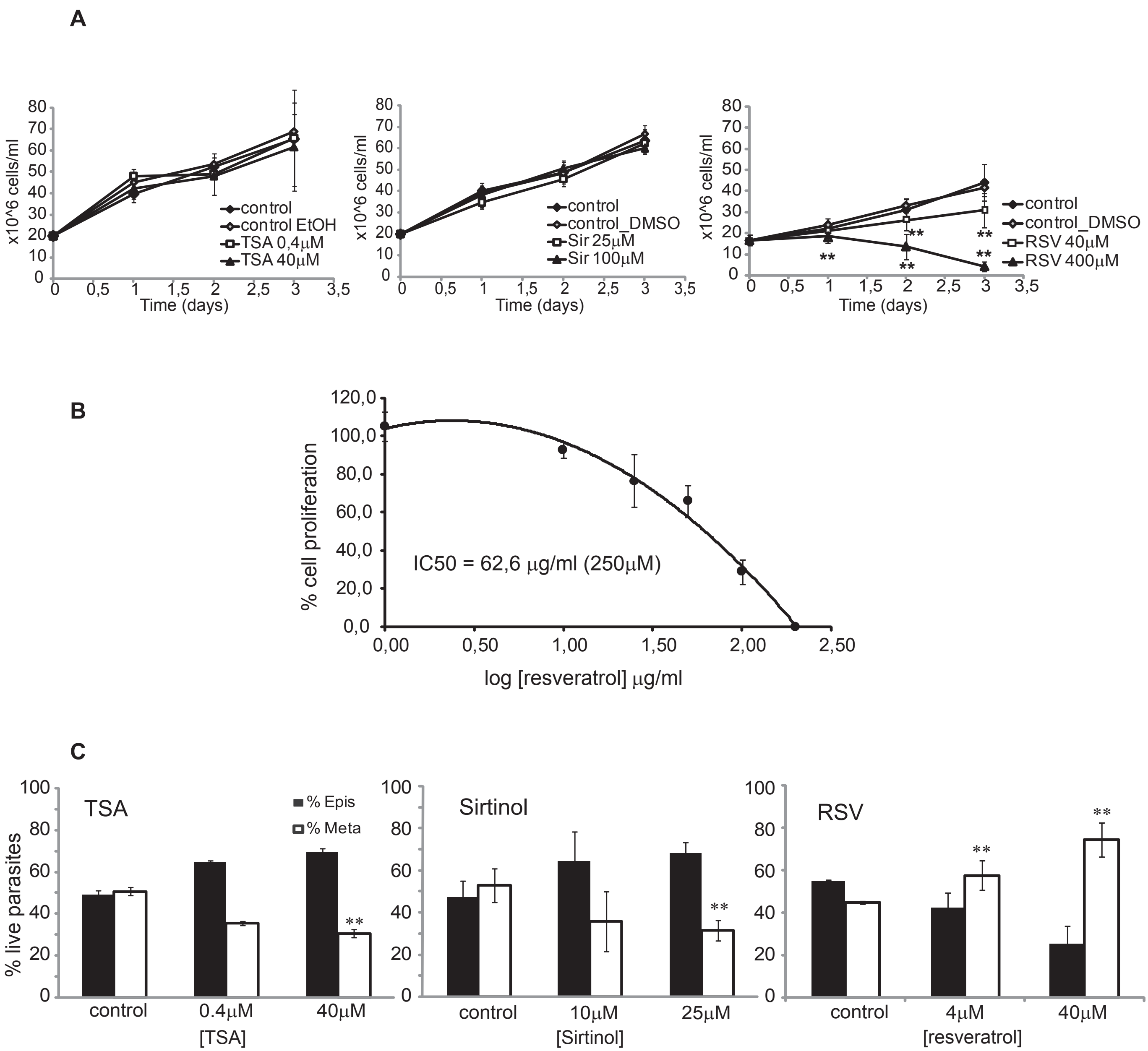


Fig. 1. Epimastigotes growth and differentiation in the presence of HDACis and resveratrol. Panel A: Growth curves of epimastigotes incubated for three days with the indicated concentrations of Trichostatin A, sirtinol and resveratrol, or equivalent amount of the corresponding vehicle. Also control curves corresponding to parasites without treatment are shown. Values are expressed as the mean between three independent experiments, with the corresponding standard deviation bars. **Panel B**: Epimastigotes cultures were treated with different concentrations of RSV (1–200 μg/ml or 4–800 μM) during 48 h s when cell proliferation (%) was recorded by direct counting of living parasites. The graphic shows the mean values from three independent experiments ± standard deviation bars. The IC_{50} value indicated was estimated by non-linear regression. **Panel C**: Percentages of living metacyclic trypomasigotes and epimastigotes observed after incubations of starved epimastigotes cultures with different concentrations of each compound or the corresponding vehicle as control. Values are expressed as the mean between three independent experiments with the corresponding standard deviation bars. For all panels asterisks indicate only the values for treated parasite cultures showing statistical significance comparing to control parasites, according to the ANOVA and LGMM tests analysis (*p < 0.05, **p < 0.005).

examination and only resveratrol showed to reduce the percentage of living parasites with an estimated IC50 of 50.3 μM (Fig. 2A). Nevertheless, the surviving parasites were still motile and viable. Thus, an equal number of the remaining trypomastigotes obtained after each treatment was used for *in vitro* infection assays. To evaluate only the effects on the parasites, all infections were performed in the absence of the compounds. Surprisingly, pre-incubation of the parasites with high concentrations of TSA or sirtinol caused an increase from 10 to 20% in the number of infected cells comparing to control infections (Fig. 2B), without affecting the differentiation and/or proliferation of the intracellular amastigotes (data not shown). Conversely, infections using trypomastigotes pretreated with resveratrol reduced the percentage of infected cells by 50% compared to the DMSO control (Fig. 2B). Moreover, the number of intracellular amastigotes was also reduced when infection was performed with parasites pretreated with the two concentrations tested (Fig. 2C). These results suggest that resveratrol affects not only the attachment and/or invasion of trypomastigotes to the host cells but also the differentiation and/or replication of amastigotes.

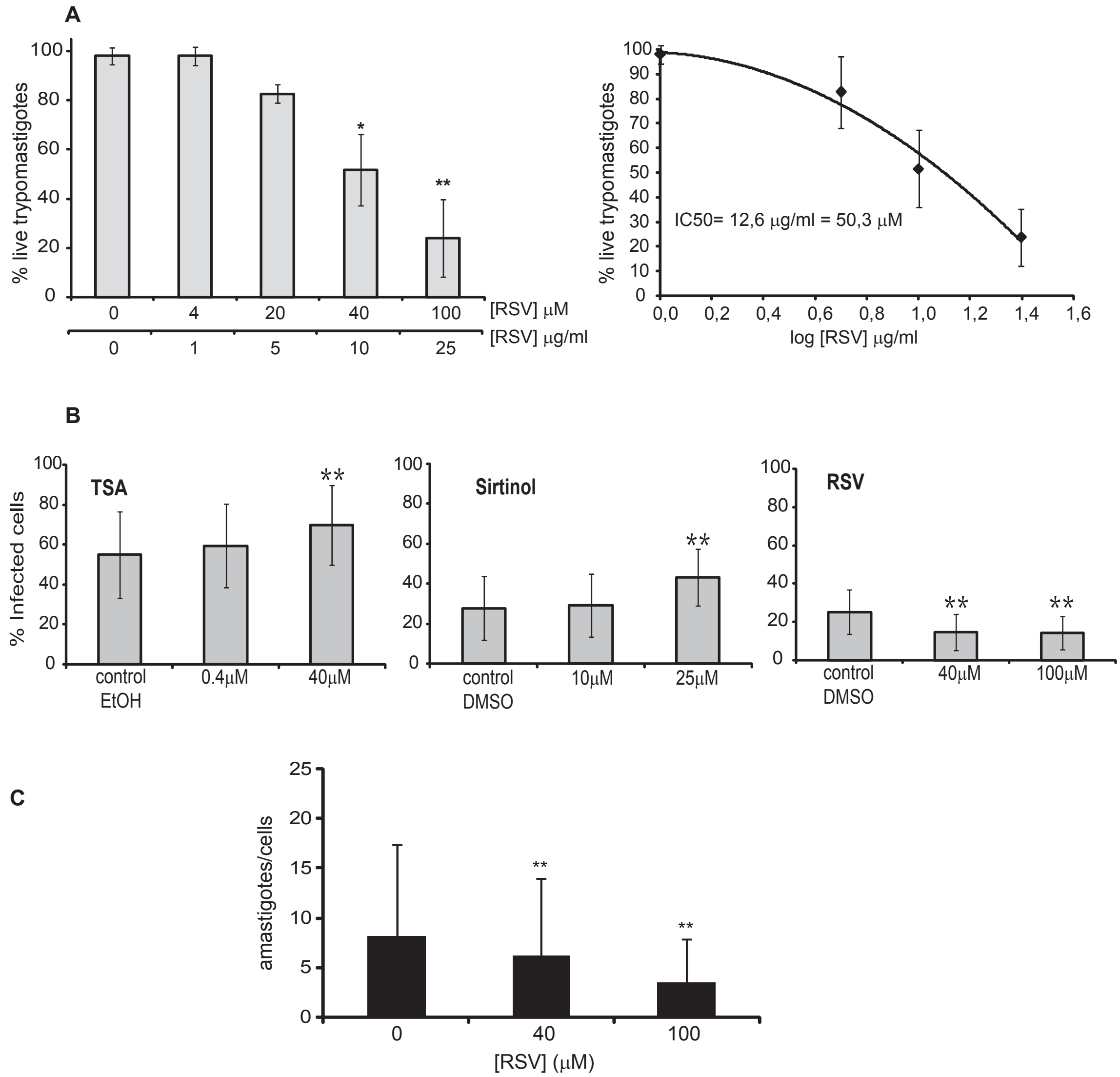


Fig. 2. ***In vitro*** **infections with trypomastigotes pretreated with HDACis and resveratrol. Panel A**. Trypomastigotes were treated with different concentrations of RSV (1–25 µg/ml or 4–100 µM) during 18 h s when the remaining living parasites (%) were recorded by direct counting. The graphic shows the mean values from three independent experiments with the corresponding standard deviation bars. The right panel shows the curve plotted with these values to estimate the IC$_{50}$ value by non-linear regression. **Panel B**. Percentages of infected Vero cells after incubation with trypomastigotes pretreated during 18 h s with the indicated concentration of TSA, sirtinol and resveratrol with the corresponding control infections obtained with trypomastigotes pretreated with the same amount each compound vehicle. All infections were performed during 4 h s in the absence of the compounds. Values are expressed as means of three independent experiments with the corresponding standard deviation bars. **Panel C**. Percentages of intracellular amastigotes from infection assays shown in panel B. Values are expressed as the mean of three independent experiments with the corresponding standard deviation bars. In all panels asterisks indicate those values showing differences with statistical significance comparing to control according to the analysis by ANOVA and LGMM tests (*$p < 0.05$, **$p < 0.005$).

3.2. HDAC inhibitors and resveratrol selectively altered trypomastigote transcript levels

Studies describing alterations of the cell cycle and differentiation induced by HDAC inhibitors in other organisms (Chen et al., 2013; Turgeon et al., 2013) led to examine the possibility that these compounds might affect the levels of transcripts coding for proteins involved in these processes in *T. cruzi*. Gene sequences coding for such proteins were obtained from the TriTryp database and used to design primers that allowed their quantification by real-time PCR. In addition, other two groups of transcripts were analyzed: one comprised of genes coding for HATs and HDACs and the other comprised of genes coding for chromatin-associated proteins and the bromodomain factor 1. Given that trypomastigote infectivity was affected after short times of incubation with the HDAC inhibitors tested, this parasite life stage was selected for the qPCR assays. Treatment of trypomastigotes with TSA increased the levels of transcripts coding for proteins involved in cell cycling, cell division and cell differentiation, when comparing with control parasites (Fig. 3). Surprisingly, sirtinol treatment led to a global down-regulation for the same transcripts. The second group of transcripts analyzed included genes coding for chromatin-associated proteins like the Chromatin Assembly Factor 1b (CAF1b) previously reported as involved in epigenetic gene silencing in *T. brucei* (Alsford and Horn, 2012), the Proliferative Cell Nuclear Antigen (PCNA) and the Bromo Domain Factor 2 (BDF2), known to bind to acetylated histones (Villanova et al., 2009). While parasite treatment with TSA caused mild changes in the levels of these transcripts, sirtinol down-regulated all three of them (Fig. 3). Transcripts coding for HATs and HDACs were also down-regulated by sirtinol, but up-regulated by TSA. Moreover, for three of these transcripts (HDAC159, HDAC80 and Sir2), the change resulted two-

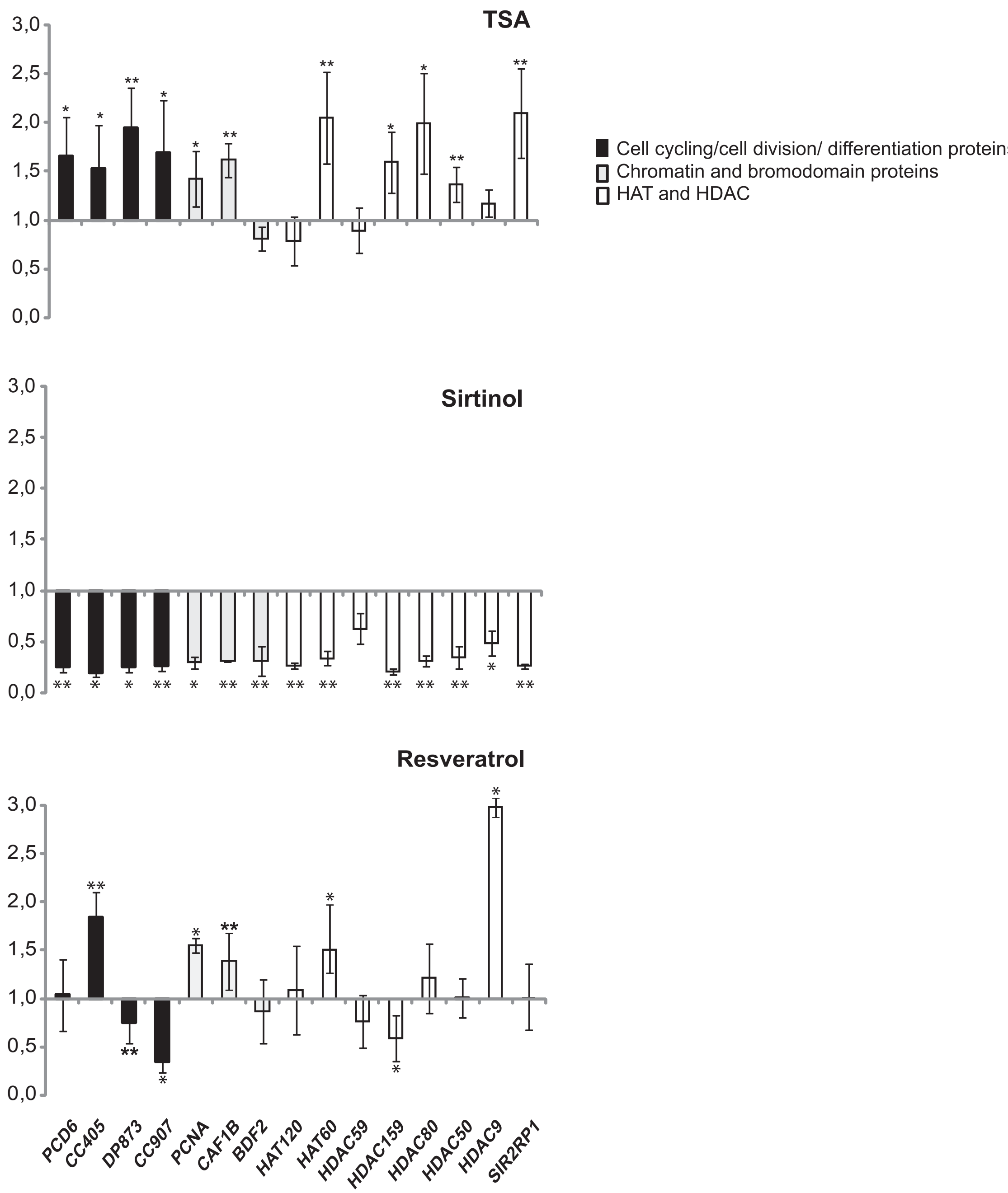
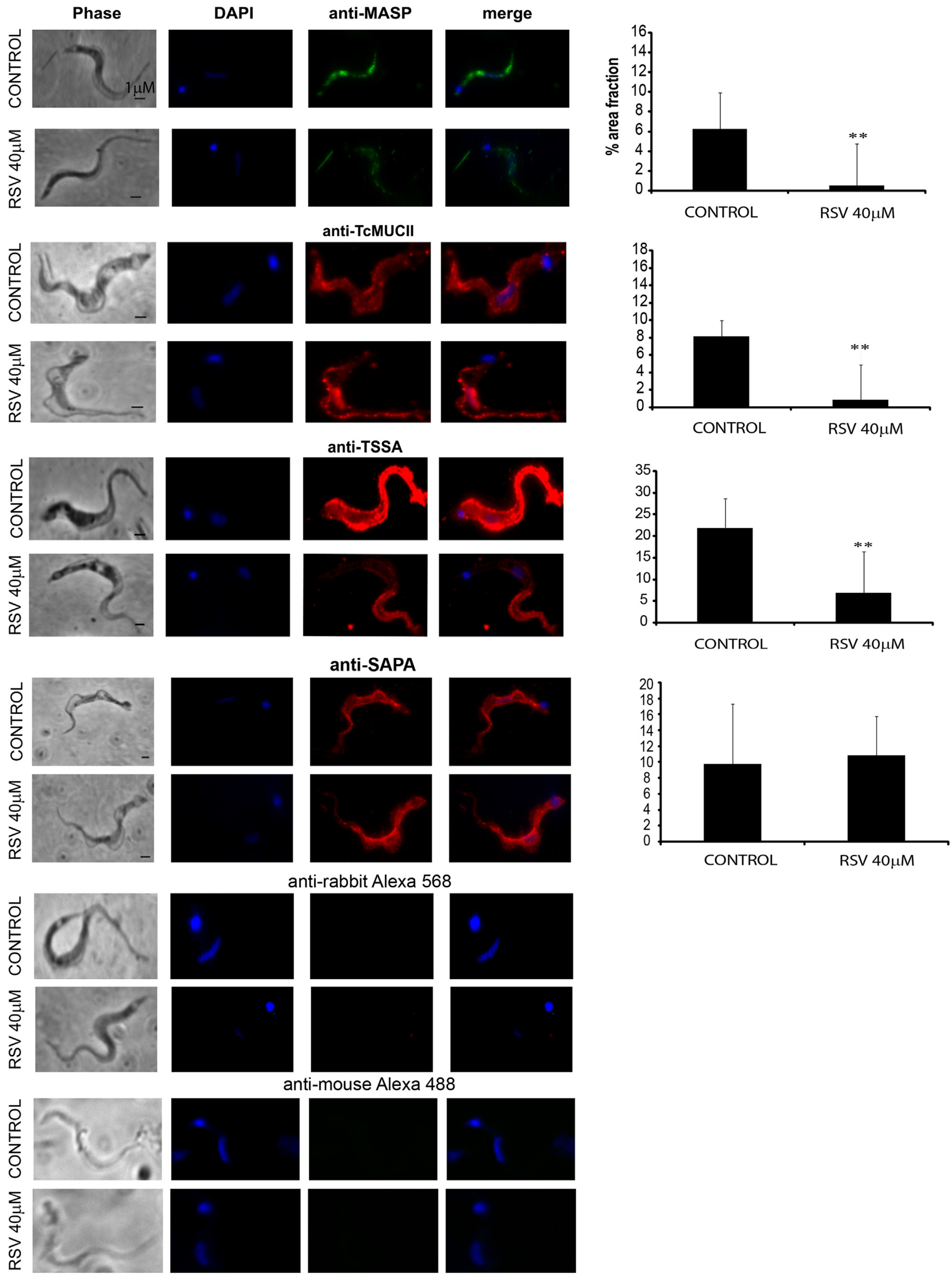


Fig. 3. Quantitative PCR analysis of specific groups of transcripts after trypomastigotes treatment with HDACis and resveratrol. The genes analyzed were grouped in three categories: black bars for cell cycling (CC405, CC907), programmed cell death protein 6(PCD6) and differentiation putative protein (DP873); gray bars for chromatin putative proteins (PCNA, CAF1b) and bromodomain factor 2 protein (BDF2); and white bars for HAT enzymes (HAT120, HAT60) and HDAC enzymes (HDAC49, HDAC159, HDAC80, HDAC50, HDAC9, SIR2RP1). Values are expressed as fold-change ratios obtained from three independent experiments between transcript levels from parasites treated during 18 h s with each compound at a fixed concentration (25 μM for sirtinol, 20 μM for TSA and 40 μM for RSV) and the corresponding controls incubated with the compound vehicle (1% DMSO for sirtinol and RSV and 1% ethanol for TSA). Asterisks indicate those values showing differences with statistical significance with the corresponding control using ANOVA test analysis (*$p < 0.05$, **$p < 0.005$).

fold comparing to control. All together, the results showed that inhibition of different classes of HDACs (class I and II by TSA, and class III by sirtinol) causes opposite effects on the expression of the same groups of genes.

The next issue analyzed whether resveratrol, as a HDAC activator, was able to induce changes in the same transcripts analyzed before with the HDAC inhibitors. Surprisingly, the qPCR results showed that this compound affected only a few of the transcripts analyzed (Fig. 3). Moreover, contrary to the results obtained with the inhibitors, resveratrol caused both up- and down-regulation of the transcript levels within the same group. This might be due either to deacetylation of other non-histone targets contributing to modulating the abundance of these transcripts or to the fact that resveratrol affected the expression of other genes not included in the groups selected for qPCR analysis. This last possibility was next analyzed.


Phase
DAPI
anti-MASP
merge
CONTROL
RSV 40μM
1μM
% area fraction
16
14
12
10
8
6
4
2
0
**
CONTROL
RSV 40μM
anti-TcMUCII
18
16
14
12
10
8
6
4
2
0
**
anti-TSSA
35
30
25
20
15
10
5
0
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anti-SAPA
20
18
16
14
12
10
8
6
4
2
0
anti-rabbit Alexa 568
anti-mouse Alexa 488

3.3. Resveratrol reduced the expression of trypomastigote surface proteins

Given that treatment of trypomastigotes with resveratrol reduced infection, it became interesting to examine whether there was a correlation with changes in the expression of proteins that are relevant for parasite invasion. The surface of *T. cruzi* trypomastigotes is covered by a thick coat of glycoproteins that are important for parasite attachment to host cells, protection from the immune response, and the establishment of a chronic infection. The main components of this coat are mucin-like proteins grouped in a large family named TcMUC. It has been previously shown that the TcMUC II group shares a common C-terminus that elicits strong antibody responses in patients with Chagas' disease and infected animals (Buscaglia et al., 2004). TcMUC III or Trypomastigote Small Surface Antigen (TSSA) is a mucin-like protein that also elicits strong antibody response in infected patients and is a lineage marker for *T. cruzi* (Di Noia et al., 2002). Other families of proteins anchored to the parasite surface are the trans-sialidase enzyme family (Frasch, 1994) and the Mucin-Associated Surface Proteins (MASPs) (Ivens et al., 2005). Because these surface proteins are encoded by large gene families, a fact that precludes the molecular approach by quantitative PCR, protein expression analysis by indirect immunofluorescence was used. For this, trypomastigotes were treated with resveratrol and incubated with specific antibodies directed toward conserved regions of each surface protein family. As shown in Fig. 4, a reduced fluorescence signal was observed for TcMUC II, MASPs and TSSA in parasites treated with resveratrol but without affecting the expression of trans-sialidase proteins (Fig. 4, anti-SAPA signal). These results suggest a partial correlation between the reduction observed in parasite infection and the expression of proteins that are important for the attachment to the host cell (mucins and MASPs) but not for the invasion process and establishment of the infection such as the trans-sialidase enzyme. Nevertheless, the same assays performed on trypomastigotes treated with the two HDAC inhibitors showed no differences as compared to controls (data not shown), suggesting that other targets for resveratrol might be contributing to this effect.

4. Discussion

Histone acetylation is emerging as a major regulatory mechanism thought to modulate gene expression by altering the access of the transcription machinery to DNA. In eukaryotes, transcription initiation is essential for the regulation of gene expression. However, previous studies in trypanosomes have shown that gene expression is regulated by post-transcriptional mechanisms (Clayton and Shapira, 2007). The clustering of functionally unrelated genes in polycistronic units and the absence of classical promoters established the paradigm that transcription initiation was not relevant in these organisms. On the other hand, data showing changes in the histone acetylation state during the *T. cruzi* cell cycle (Nardelli et al., 2009) and the presence of modified histones at the SSRs (Respuela et al., 2008) suggest some role for epigenetics in the control of gene expression. Moreover, recent reports have shown that the expression of non-acetylable mutated versions of histone H4 leads to a diminished transcription rate (Prata Ramos et al., 2015). However, whether acetyltransferases and deacetylases function to globally or selectively affect gene expression has not yet been well explored. The most common approach to study the function of chromatin acetylation is the use of HDAC inhibitors. Studies using different pharmacological variations of natural HDAC inhibitors and different parasite species have concluded that HDACs play a critical role in the life cycle of these organisms (Dubois et al., 2009; Ingram and Horn, 2002; Bougdour et al., 2009; Andrews et al., 2012a). Also, the transcription of genes coding for proteins involved in cell cycle and differentiation have been previously reported as especially affected by changes in the histone acetylation state (Bougdour et al., 2009; Chen et al., 2013; Turgeon et al., 2013; Andrews et al., 2000). The common statement is that HDAC inhibition causes hyperacetylation of histones, which should lead to transcriptional activation. In agreement with this, qPCR results showed a global up-regulation of all genes tested when parasites were incubated with TSA (inhibitor of HDACs I and II). However, down-regulation of the same genes was observed with treatment with sirtinol (inhibitor of HDACs III) (Fig. 3). Although this might appear to be contradictory, similar findings have been previously reported (Chaal et al., 2010; Glaser et al., 2003). The opposite effects observed on transcript levels with these compounds might indicate that more than one HDAC class is involved in the acetylation of the histones affecting chromatin remodeling. Although the identification of all HDAC-regulated genes remains undefined, this was a first attempt to evaluate whether compounds modulating HDAC activity were capable of affecting gene expression in this parasite. Even though histones seem to be substrate for these enzymes, it is also possible that this effect might be caused by the action of HDACs on other non-histone targets. In fact, reversible acetylation of non-histone proteins by HDACs and HATs has been previously described in mammals and includes transcription factors like p53, STAT3, *c*-MYC and NF-κB and cellular proteins like α-tubulin and Hsp90 among others (Glozak et al., 2005). However, no specific non-histone targets for protozoan HDACs have been identified yet.

Besides their function in transcription, HDAC inhibitors are being pursued as new drugs for the treatment of a wide range of diseases, including infections caused by parasites, like malaria, leishmaniasis (Andrews et al., 2012b), schistosomiasis (Heimburg et al., 2016) and African and American trypanosomiasis [reviewed in Wang et al., 2015]. In the present study, the effect on the biology of *T. cruzi* CL Brener strain of two different inhibitors and the activator of sirtuin deacetylases resveratrol was analyzed. The HDAC inhibitors tested caused histone hyperacetylation, whereas resveratrol had the opposite effect in both proliferative and infective forms, consistent with the attenuation and activation of parasite HDAC activity, respectively. Although, there is one report showing that resveratrol can inhibit the recombinant human zinc-dependent HDACs *in vitro* (Venturelli et al., 2013), the changes on histone acetylation observed here support the originally described action for resveratrol as a deacetylation activator [reviewed in Villalba and Alcaín, 2012 and in Hubbard and Sinclair, 2014]. Despite the reports on the anti-parasitic activity of HDAC inhibitors attenuating growth of *Plasmodium*, *Toxoplasma*, *Leishmania*, *Trypanosoma brucei* and other strains of *T. cruzi* (Andrews et al., 2012b; Carrillo et al., 2015; Engel et al., 2015; Murray et al., 2001; Veiga-

Fig. 4. Indirect immunofluorescence on trypomastigotes pretreated with RSV. Representative photographs of immunofluorescence assays performed on CL Brener trypomastigotes treated during 18 h s with the indicated concentrations of RSV and incubated with antibodies directed toward four different surface protein families: mucin-associated proteins (anti-MASP), mucins (anti-TcMUCII), trypomastigote small antigen (anti-TSSA) and trans-sialidase (anti-SAPA). Control assays performed with incubations only with the secondary antibodies anti-mouse Alexa 488-conjugated or anti-rabbit Alexa 568-conjugated are also shown. Bright field photographs (phase) and DAPI staining of the nucleus and kinetoplast are shown (magnification x 180). Next to each IIF panel the quantification of the signals obtained by analysis with ImageJ software is shown. Values are expressed as the median of the percentage of fraction area with fluorescence obtained for three independent experiments for each treatment with the corresponding IQR. Asterisks indicate those values with statistical significance according to T-Student test ($^{**}p < 0.001$).

Santos et al., 2014; Soares et al., 2012; Wang et al., 2015; Sereno et al., 2005), the inhibitors tested had little effect on CL Brener epimastigote growth (Fig. 1A), but blocked differentiation to metacyclic trypomastigotes (Fig. 1B). Nevertheless, HDAC inhibitors slightly increased the ability of trypomastigotes for *in vitro* infection (Fig. 2B). These stage-specific effects of HDAC inhibitors have been previously reported, for example with sirtinol in *Leishmania* (Patil et al., 2010) and with TSA in *T. gondii* (Strobl et al., 2007) and *Schistosoma mansoni* (Dubois et al., 2009). Along with the action of HDAC inhibitors, this study describes the effects of the sirtuin activator resveratrol. Although the localization of the two *T. cruzi* sirtuins has been described as cytoplasmic and mitochondrial for TcSIR2rp1 and TcSIR2rp3, respectively (Ritagliati et al., 2015), nuclear transport cannot be ruled out. The changes in the amount of acetylated histones observed after parasite treatment with both the sirtuin inhibitor sirtinol and the activator resveratrol support this last notion (supplementary Fig. 1). However, the activity of other HDACs targeted by these compounds cannot be ruled out. The effects of resveratrol on *T. cruzi* replication, differentiation and infection were also stage-specific, but opposite to the action of HDAC inhibitors. This compound reduced epimastigote growth, promoting metacyclogenesis (Fig. 1), markedly reduced cell-derived trypomastigote infectivity (Fig. 2B), and inhibited differentiation and/or replication of intracellular amastigotes (Fig. 2C). Moreover, a diminished expression of trypomastigote surface proteins important for parasite attachment to the host cell was also observed (Fig. 4). These data are in accordance with previous reports describing the anti-parasite effects of resveratrol on *Leishmania major* (Kedzierski et al., 2007) and other *T. cruzi* strains (Valera Vera et al., 2016). Resveratrol can stimulate yeast Sir2 and its mammalian ortholog Sirt1 (Howitz et al., 2003) but can also affect other targets that might be contributing to the effects observed (Valera Vera et al., 2016; Harikumar and Aggarwal, 2008). Moreover, the effect of resveratrol described in this study can be only partially compared with the effects observed in parasites overexpressing sirtuins (Ritagliati et al., 2015; Moretti et al., 2015), further supporting the notion that targets other than sirtuins might be also responsible for its trypanocidal action. Resveratrol is a natural occurring phytoalexin found in the skin of red grapes, originally reported as a potential anticancer agent (Jang et al., 1997; Boocock et al., 2007). Since this compound is now widely used in humans and has been shown to be completely non-toxic (Boocock et al., 2007), the data presented here point to resveratrol as a very attractive anti-parasite drug candidate for further testing.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijpddr.2016.12.003.

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