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Schistosoma mansoni: Developmental arrest of miracidia treated with histone-deacetylase inhibitors

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Abstract

In the present study we examined the effect of the histone deacetylase (HDAC) inhibitors Trichostatin A (TSA), valproic acid (VA) and sodium-butyrate on the metamorphosis of larvae of the human blood-fluke *Schistosoma mansoni* from the free-swimming miracidia into the intramolluskal sporocyst. We show that HDAC inhibitors block transformation in concentration dependant manner. TSA reversibly blocks this developmental process: only 13±11% of TSA treated miracidia transform into sporocysts *in-vitro*, compared to 92±8% in the mock-treated control. Other enzyme inhibitors such as cycloheximide or hydroxyurea had no effect on metamorphosis. For treatment of up to 4 hours, the effect of TSA was completely reversible. Our data indicates that HDAC activity is necessary for the transformation of *S.mansoni* miracidia during infection of the snail host.

Keywords

Platyhelminth, *Schistosoma mansoni;* histone deacetylase; Trichostatin A; metamorphosis; development

Schistosoma mansoni is a parasitic helminth whose life-cycle goes through two obligatory sequential hosts: the fresh-water snail *Biomphalaria glabrata*, and man or rodent as hosts for the sexual part of the life-cycle. After mating, the eggs of the parasite are emitted with the faeces into the water, and pre-formed free-swimming miracidia hatch out of the egg. After penetration into the snail host, the parasite develops via a primary (mother) sporocyst and a daughter sporocyst generation into the cercaria and these are released into the water and infect the vertebrate host. The life-cycle is well-described but the complex cascade of biochemical events that govern the metamorphosis into the morphological very different miracidia, cercaria and finally adults, are far from being apprehended. In many organisms embryonic development is associated with changes of chromatin structure. It is becoming increasingly clear that these chromatin modifications serve to establish transcriptionally active and inactive regions of the genome. Information about the appropriate chromatin modification is stored in epigenetic information carriers such as DNA methylation, covalent modifications of histones, non-messenger RNA and nuclear topography. In mammals, insects and plants, it was shown that modification of the epigenetic information can lead to dramatic developmental changes. De-novo DNA methyltransferases DNMT3a and DNMT3b for instance are essential for normal embryonic development of mammals. Homozygous mouse knock-outs of DNMT3a survive only a few days after birth and Dnmt3b-/- embryos die early during embryogenesis (Okano et al., 1999). Indeed, chromatin-remodeling enzymes are ubiquitous and shape the development from *C. elegans* (reviewed by (Cui and Han, 2007) to *A. thaliana* (reviewed by (Pfluger and Wagner, 2007). Histone deacetylase (HDAC) are a specific class of chromatin-remodeling enzymes that remove acetyl moieties from histones. Acetylated histone isoforms are in general associated with the open, transcription-permissive form of the chromatin: the euchromatin. Deacetylation of histones allows for local or regional chromatin compactation, *i.e.* heterochromatization, and leads to repression of gene expression. If the enzyme activity of HDAC is impaired, histone deacetylation cannot take place and the chromatin modification is shifted towards acetylation through the continuing action of histone acetyltransferases. HDAC activity can be inhibited by specific drugs such as Trichostatin A (TSA). Trichostatin A reversibly inhibits the histone deacetylases types I and II (Yoshida et al., 1995). Microarray experiments have shown that the treatment of human cell lines with TSA induces over-expression of 0.1 – 0.9% of

probe sets but also leads to repression of about the same number of the probe sets (Dannenberg and Edenberg, 2006; Heller et al., 2008). This latter effect might be an indirect one through the activation of repressor transcription, or it might simply be the result of a global change in the distribution of heterochromatin and euchromatin. Valproic acid (VPA) is another HDAC inhibitor (Phiel et al., 2001), but has also a variety of effects that are not fully understood, including increase in levels of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) and effects on the extracellular signal-regulated kinase (ERK) pathway (reviewed by (Rosenberg, 2007). VPA is used as an anticonvulsant in epilepsy treatment. Sodium-butyrate (BA) is an HDAC inhibitor and has long been known as an unspecific gene activator (Candido et al., 1978). Given the importance of chromatin structure changes during development in many model organisms, we hypothesized that the development of *S. mansoni* will also be under epigenetic control. Since DNA methylation has not been found in *S. mansoni* (Fantappie et al., 2001), we focused on histone modifications as epigenetic information carrier. We show here for the first time that HDAC inhibitors can arrest the metamorphosis of the free-swimming miracidia into primary (mother) sporocysts *in-vitro*. Our findings indicate that HDAC action is essential for the miracidia-sporocyst transition.

S. mansoni eggs were recovered from infected hamsters (*Mesocrecetus auratus*) 8 weeks post-infection. Livers were collected and kept in sterile saline 0.85% containing an antibiotic / antimycotic mixture (penicillin 100 units/ml, streptomycin 0.1 mg/ml, amphotericin 0.025 µg/ml; Sigma 057K2402). Livers were homogenized and eggs were filtered and washed. Miracidia were allowed to hatch and were concentrated by sedimentation on ice for 15 minutes. For initiation of *in-vitro* transformation into primary sporocysts miracidia were transferred into Chernin's balanced salt solution (CBSS) (Chernin, 1963). Trichostatin A (TSA) (invivoGen met-tsa-5) was dissolved in ethanol, cycloheximide (CX) (Sigma C7698) and Hydroxyurea (HU) (Sigma H8627) were dissolved in 10 mM Tris/Cl pH 7.5, Sodium Dodecyl Sulfate (SDS) (Sigma L5750), sodium-butyrate (BA) (Sigma B5887) and sodium-valproate (VPA) (Sigma P4543) were prepared in water. Products were added to the culture to achieve appropriate final concentrations every two hours. If necessary, an identical volume of ethanol was added to the negative controls (mock treatment). For each experiment, 25 - 100 miracidia were incubated at 25°C in a final

volume of 3 ml CBSS in sterile 24 well tissue culture plates (Becton Dickinson 3047). Transformation was determined by visual inspection under an inverted microscope. Metamorphosis into sporocysts was considered as accomplished when the miracidia had lost the major part of ciliated epidermal plates (TSA, VPA, BA), and explorative movement had ceased (TSA). For western blots, 300 miracidia were re-suspended in denaturation buffer (0.2 % bromophenol, 10% sucrose, 3% SDS, and 0.2 M DTT, 62.5 mM Tris/Cl, pH 6.8) treated by sonication (Vibra CellT.M. 75185) (60% intensity, 6 times 15 sec, with cooling intervals on ice) and boiled 10 min at 95°C. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Amersham RPN203D) by the semi-dry method (SEMI-PHOR Bio-Rad). The membrane was blocked overnight at 4°C in blocking buffer (150 mM NaCl, 0.05% v/v Tween 20, 5% w/v fat-free dry milk, 20 mM Tris/Cl, pH 7.5) and incubated with Anti-hyperacetylated histone H4(Penta) (Upstate #06-946 Lot#29860) or Anti-histone H3 (abcam #ab1791-100 Lot#455351) for 2 hours in blocking buffer, washed and incubated with peroxydase-coupled anti-rabbit (Pierce 31460). Bands were revealed by Enhanced Chemical Luminescence (ECL Pierce) and direct exposure to x-ray film (Amersham EmNo.27304). Statistical analysis (Mann-Whitney test) was done with SPSS 13.0. Sample preparation and microscopy was done as described previously (Roger et al., 2008).

Initial experiments with TSA concentrations ranging from 2 nM to 200 µM and incubation times from 2 to 16 hours established 200 µM and incubation time of 4 hours as appropriate experimental conditions. Under these conditions, 67 - 98% (87±11%, n=190) miracidia did not develop into sporocysts, while in the mock-treated control only 5 - 13% (8±3%, n=160) did not undergo metamorphosis (figure 1). The experiments were repeated five times. The transformation rates are significantly different ($p = 0.004$). Western blots with an antibody against acetylated histone H4 confirms that the portion of this histone isoform increases (figure 1). Histone H3 served as loading control. Visual inspection by light microscopy shows that with TSA, miracidia are in movement, but ‘cork-screw’ like motion of the miracidia increases. The morphology of these arrested miracidia is unaffected (figure 2). These data suggest that histone-deacetylases activity is necessary for the miracidia-sporocyts transition. The experiments were repeated with sodium-valproate (VPA) at concentrations between 0.1 and 100 mM. With 50 mM VPA 80±13% of

miracidia did not transform into sporocysts, and at 100 mM almost all larvae remained miracidia (98±2%, n=2). However, in contrast to TSA, movement of the cilia ceased rapidly and completely. Low concentrations of sodium-butyrate (BA) (1.25 – 5 mM) increased the percentage of untransformed miracidia to roughly 25%. At higher concentrations, no transformation into sporocysts was observed. However, concentrations ≥20 mM appear to be toxic since the miracidia did not show visible flame-cell activity. To investigate whether the TSA-induced metamorphosis arrest is transient or whether an alternative dead-end developmental pathway had been activated, we incubated miracidia for 2, 3, 4 and 16 hours with 200 µM TSA. After microscopic observation, the TSA containing medium was replaced by fresh CBSS and the behavior of the larvae was observed in 10 min intervals. For incubation of up to 4 hours, metamorphosis into sporocysts started about 30 min after removal of TSA and continued without visible impairment of the sporocysts. However, no miracidum survived after 16 h incubation with TSA. We then wondered whether other essential enzyme activities such as protein synthesis or DNA replication are necessary for the metamorphosis. Miracidia were incubated with decreasing concentrations of cycloheximide (3 g/l – 30 µg/l, 10fold dilution steps), hydroxyurea (200mM – 2µM, 10fold dilution steps) and SDS (20 mg/l – 0.2 ng/l, 100fold dilution steps). Cycloheximide inhibits protein synthesis by competition with the binding of ATP to the 60S ribosomal subunit. By blocking the translation of cyclins it arrests cell cycle in G1 and/or G2 (Hung et al. 1996). Hydroxyurea acts probably as an inhibitor of ribonucleotide reductase and blocks DNA synthesis. It prevents the dNTP pool expansion that normally occurs at G1/S interface and arrests cell cycle at this point (Koc et al., 2004). SDS disrupts membranes and non-covalent bonds in proteins, and served as toxic control without specific enzyme-inhibiting function. Under none of the tested conditions, was a metamorphosis arrest similar to the one observed with HDAC inhibitors, detected.

In-vitro transformation of *S.mansoni* miracidia into sporocysts is a well-established technique that allows for simple and rapid analysis of this part of the life cycle that occurs *in-vivo* during infection of the mollusk host. Transformation rates in CBSS are typically around 90% (e.g. (Walker and Rollinson, 2008)), and our data (92±3 %) corresponds to these findings. Addition of the HDAC inhibitor TSA blocked metamorphosis from miracidia into sporocysts almost entirely (87±11%). Miracidia

were alive and could be maintained for up to 4 hours in the untransformed state in the presence of TSA. TSA is not simply toxic, since removal of TSA released the metamorphosis arrest and miracidia transformed into sporocysts. Our data indicates that HDAC inhibition leads to a true transient developmental arrest and not an alternative dead-end developmental pathway such as apoptosis. TSA is known to arrest the cell cycle in G1/G2 (Hung et al., 1996) and it could be argued that the observed effect is due to this cell cycle arrest. We therefore used other enzyme inhibitors that block either protein synthesis (CX) or DNA replication (HU) and that also block cell cycle progression. Similar to TSA, CX stops cell cycle in the G1/G2 phase. HU blocks cells at the G1/S interface and is routinely used for synchronization of cell cultures. At none of the tested concentrations did any of the two drugs have an effect on the metamorphosis of the miracidia. TSA is a specific inhibitor of HDAC types I and II. Other protein deacetylases are apparently not affected and it is reasonable to assume that the observed effect on metamorphosis is due exclusively to its action on HDAC and consequently acts on chromatin level. Other HDAC inhibitors such as VPA and BA showed transformation blocking activity similar to TSA. However, both have pleiotrophic effects that make these reagents difficult to use. We attribute the complete loss of cilia movement, after addition of VPA, to the known anticonvulsive action of the drug (reviewed by (Rosenberg, 2007). Elevation of neuronal GABA concentration and inhibition of voltage-gated Na^+channels have been proposed as a mechanism of action for VPA (reviewed by (Rosenberg, 2007) but remain controversial. BA is often used in millimolar concentrations, and the observed toxic effect on comparable low dose is surprising. However, the drug is known to induce apoptosis and to stimulate the generation of reactive oxygen species (Jeng et al., 2006). Nevertheless, both HDAC inhibitors had the same transformation blocking effect as TSA. The used concentration of TSA is relatively high. However, TSA is known to act at a large range of concentrations depending on the target cells. While many laboratories use concentrations of around 1 nM to several hundred nM, treatment with up to 20 µM was reported for cell cultures. Miracidia are free-living larvae that must resist a variety of potentially hazardous environmental factors including those that affect HDAC. It is plausible to assume that their resistance to TSA is higher than those of naked culture cells. Walker and Rollinson observed a comparable however less pronounced effect on miracidia transformation after incubation of the larvae with genistein (Walker and Rollinson, 2008). There,

miracidia were cultured for 48 h with 50 µM genistein and 63% of the larvae did not transform into sporocysts. The authors assign this to the tyrosine kinase inhibiting action of genistein. However, the precise molecular mechanism of genistein action is still not known. Treatment of cancer cells with genistein can reactivate the expression of tumor suppressor genes like p21 (*WAF1/CIP1/KIP1*) and p16 (*INK4a*) and TSA has the same effect (Majid et al., 2008). Indeed, genistein upregulates the expression of histone acetyltransferase (HAT) two to threefold, and chromatin immunoprecipitation experiments have shown that genistein treatment (25 µM, 96 h) results in enrichment of acetylated histones H3 and H4 close to the transcription start site of the *p21WAF1* and *p16INK4a* genes (Majid, Kikuno et al. 2008). Since HDAC activity and location is dependent on phosphorylation (Tsai and Seto, 2002), genistein could also act on this level. Both, TSA as HDAC inhibitor and genistein as HAT inducer or potential modulator of HDAC activity via phosphorylation, have therefore the same effect: changes in the chromatin structure. In the light of these recent findings it appears to be plausible that the observed inhibition of transformation from miracidia to sporocysts is at least in part due to the indirect chromatin remodeling action of genistein. Our results clearly indicate that HDAC activity is necessary for the metamorphosis of miracidia of *S.mansoni*. Chromatin remodelling via histone (de)acetylation has been observed in a number of developmental or metamorphosis processes. In *Toxoplasma gondii* for instance promotor regions of tachyzoite-specific genes are acetylated in tachyzoites but hypoacetylated in quiescent bradyzoites, and *vice-versa* (Sullivan and Hakimi, 2006). TSA was shown to have an impact on development of *Drosophila melanogaster* (Pile et al., 2001) and frogs (Almouzni et al., 1994), and HDAC is necessary for normal formation of zebrafish fins (Pillai et al., 2004), for instance. Histone deacetylation is an essential step in heterochromatization. It remains to be investigated whether local or regional heterochromatization is a prerequisite for initiation of metamorphosis of the larvae of *S. mansoni*, or whether HDAC inhibitor treatment prevents certain genes from being inactivated and the presence of their gene products prevents transformation from being executed.

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