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SAHA, histone deacetylase inhibitor causes reduction of aflatoxin production and conidiation in the *Aspergillus flavus*

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Abstract

Transcriptional regulation in eukaryotes occurs within a chromatin setting and is strongly influenced by posttranslational modification of histones.The regulation of histone acetyltransferases (HATs) and histone deacetylases(HDACs) is one of important post translational modification (PTMs).*Aspergillus flavus* is the main source of aflatoxin,the most important mycotoxins in the world and food supplies.HDACs can play an important role in secondary metabolites production and normal vegetative growth.The focus of our work was on underastanding the possible effect of HDAC inhibitors ,SAHA, on growth, aflatoxin synthesis, conidiation and morphology in A.flavus.The result indicate that SAHA inhibits the ability to produce aflatoxin B1 and also causes a “fluffy” phenotype.This change was dose-dependent and not hereditary.Three HDACs genes were founded in this fungi ,*HosA*,*HosB* and *Rpd1* that have conserved domains like other fungi .This results suggest that HDACs have important roles in A. flavus and HDACi could prove to be a valid approach for selective treatment of infection cause by *Aspergillus* species and other fungi and be a good subject for epigenetic remodeling in fungi .

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Introduction

The basic elements of chromatin is the nucleosome .Histones H4,H3,H2A and H2B form the core histone octamer by protein-protein intraction of their folded domains.The free, flexible N-terminal extension of the histones protrude from the nucleosome, they contain conserved Lysines undergoing posttranslation alacetylation. Histone acetyltransferases (HATs) and histone deacetylases(HDACs) is one of important modification for epigenetic control, protein activity and protein stability (Izawa *et al.,* 2009; Grassle *et al.*, 2001; Houllier *et al.*, 2009). In eukaryotes, developmentally regulated genes are frequently controlled at the epigenetic level by several classes of HDAC.A wide array of information has been made available on these enzymes in fungi and this knowledge had led to the synthesis of HDAC inhibitors(HDACi). HDACi are promising new class of anticancer drugs,however,their mechanism of action has not been completely elucidated. Most studies have investigated the effect of HDACi on regulation of gene transcription. In fungi, important cellular function were shown to be controlled by different HDACs and HDACi were shown to effect a number of cellular function in these organisms.Trichostatin A(TSA) ,a well characterized HDACi,was shown a significantly affect in fungi ceels. Recently, more data have been reported that, Suberoylanilide hydroxamic acid(SAHA) can also significantly affects like TSA (Simonetti *et al.*, 2007). Treatment with HDACi was also used for investigation of gene regulation in fungi like *Saccharomyces* sp.,*Neurospora crassa*, *Magnoporth oryzea*, *Cochliobolus carbonum, Aspergillus* species and indicat that HDACi causes change in germination, secondary metabolite synthesis,pathogenecity and normal growth in fungi (Simonetti *et al.*, 2007; Fisch *et al.*, 2009; Graessle *et al.*,2000; Zacchi *et al.*, 2010; Shwab *etal.*, 2007; Izawa *et al.*, 2009; Graessle *et al.*, 2001).

Aspergillus flavus is saprophytic soil fungus that infects and contaminates preharvest and postharvest seed crops with the carcinogenic secondary metabolite Aflatoxin.The fungus is also an opportunistic animal ana human pathogen causing aspergillosis diseases. *A.flavus* has been known as an asexual species that only produces asexual spores,conidia and the overwintering fruiting bodies,sclerotia and causes diseases on several important agricultural crops such as Maize(Ear rot), Pea Nut (Yellow mold), pistachio and Cotton seed,before and after harvest. The fungus also causes animal and human diseases through consumption of contamination feed , causing aflatoxicosis or through invasive growth, aspergilosis (Amaike & Keller, 2011). The pathogen can produce the polyketide derived carcinogenic and mutagenic secondary metabolite Aflatoxin (Lin *et al.*, 2012; De Costa *et al.*, 2010). HDACs may function in the regulation of secondary metabolism among abroad rang of fungal genera (Shwab *et al.*, 2007) or involved in regulation of normal germination and vegetative growth (Lee *et al.*, 2009).The effect of HDAC is on *A. nidulans* were summarized by inhibition of HDACs targets primarily the class II enzyme HdaA and Rpd3-type that leads to a pronounced reduction in growth and sporulation.

Examination the antifungal activity MGCD209, a novel Hos2 fungus- specific HDACi, incombination with fluconazole, voriconazole and posaconazole in *Candida* spp. showed that MCGD209 was synergistic with these antifungal. In Candida spp. TSA and SAHA also caused a 90% reduaction in adherence to human cultured pneumocytes and enhanced Azole susceptibility (Chen *et al.*, 2011). According to above studies ,HDACs can play an important role in secondary metabolites production and normal vegetative growth in fungi and Our work was aimed at identification of HDACs genes and demonstraiting that HDACi ,SAHA, actually inhibit HDACs of *A.flavus* and consequently elucidate the possible effects SAHA on growth, aflatoxin synthesis, conidiation and morphology in this fungus.

Materials and methods

Chemicales and organisms

Toxigenic isolate of *Aspergillus flavus* Link(PTCC 5004) was used as a single culture throughout this study obtained from Iranian Research Organization for science & Technology(IROST) for effect of HDACi

and also two isolates of *A. flavus* ,toxigenic and non-toxigenic (tox-, tox+)were used For PCR and HDAC genes identification .Then conidia were inculated on Potato Dextrose Agar (PDA) medium and the plates incubated at 25 °C . Histone deacetylase inhibitore ,SAHA,was purchase from Sigma-Aldrich Biotechnology (St. Louis, MO, USA).

Phenotypic characterization analysis

Colony growth, spore production and phenotype of colony were measured by cultured in PDA media with HDACi in 2,10 and 50 µM concentration.SAHA was suspended in DMSO and added to molten medium after it cooled to 45°C. For each concentration we had 3 replicates. Spores (10^6) were inoculated on media and were kept at 25°C and away from light to avoid its effect on AF biosynthesis or SAHA stability. After 7 days spore production, colony colore and other macroscopic characters evaluated as described previously (Jin *et al.*, 2002).

Detection of aflatoxin production

Aflatoxin production was assessed by thin layer chromatography (TLC). One ml of chloroform was added into a 1.5-ml centrifuge tube containing 400 µl of *A. flavus* mycelium, shaken at 200 rpm for 30 min,and centrifuged at 2000 rpm for 10 min. Four hundred µl of organic phase was transferred into a new tube, dried an 65°C water bath,and resuspended in 100 µl of choloroform. Extracts were loaded onto silica TLC plates and metabolites were separated in the developing solvent choloroform: acetone:water (28:4:0.06). Photographs were taken following exposure to UV radiation at 365 nm wavelengths (Lin *etal.*,2013).

Preparation of fungal total DNA

Test tube liquid medium culture of *A. flavus* was shaken in 200 rpm for 5 days. Fungal mycelia were harvested for DNA extraction by milipore filter. The harvested mycelia were pulverized to a fine powder in the presence of liquid nitrogen.Total DNA was prepared using phenol: chloroform: isoamyl alcohol. DNA was precipitated with isopropanol and ammonium acetate (3M), sampling washed with ethanol and resuspended in 100µl distilled water (Sambrook & Russell , 2001 ; Doyle & Doyle, 1987) and kept in -20°C for PCR. Extracted DNA samples quality were evaluated by gel electrophoresis.

Gene amplification by polymerase chain reaction (PCR)

The DNA extracted from the different strains of *A.flavus* was used as PCR template for evaluating the specificity of the fragment of the gene encoding the HDACs protein.Three pairs primers amplifying fragments of HDACs genes.

The primers are: Two mixed degenareted oligodeoxynucleotide primers were (OA): GGNCAYCCNATGAARCC and (A2R): ACNCCRTCNCCRTGRTG,were N is an equal mixture of all four nucleotides,Y of C and T and R of A and G(Grassle *etal.*,2000).

Other primers were *HosA*-forward: ACGCTGCTCGGAAGCTCTGC, HosAreverse: TGGAGAACGATTGCGCCGGG, *HosB*-forward: TCGAGCCCCAAAACGGCACC and *HosB*-reverse:CGTGGCTCCCGGCGTAACAG.Amplificationr eactionswere carried out in volumes of 50 µL containing 3µL of template DNA, 0.5 µL of each primer (10 pM), 5 µLo 10× PCR buffer, 1.5 µL of MgCl (50 mM), 1µL of dNTPs (10 mM) and 0.5 µL of Taq DNA polymerase (5 U/AL). The PCR amplification protocol as fol lows: 1 cycle of 30 s at 95°C, 30 cycles of 30 s at 95 °C (denaturalization), 30 s at 62°C (annealing), 1 min at 72°C (extension) and finally 1 cycle of 3 min at 72°C. PCR products were detected in 1.2% agarose ethidium bromide gels in TAE 1× buffer (Tris–acetate 40 mM and EDTA 1.0 mM). The DNA ladder was used as molecular size marker (Bele *etal.*,2005; Sambrook & Russell ,2001). The amplification products required for sequencing were purified with ethanol. All the strains were sequenced in both directions. The resultant Sequences were aligned to the other organisms detailed earlier by a sequence software analysis in NCBI. BLASTN was used for identification of DNA sequences by database

similarity search. Multiple alignments were produced using MultAlin online software.

Results

Effect of SAHA on morphology of A. flavus

After conidia of *A. flavus* were inoculated on PDA plates with or without SAHA and kept at 25 °C for 7 days , nearly all the SAHA treated mycelia converted to a "fluffy" phenotype lighter green conidia (Fig. 1). This fluffy phenotype remained under extended incubating but could not be heritable changes and after transferred onto a fresh PDA medium without SAHA and green colour of colony reappeared (Fig. 2). Microscopy showed that the fluffy form having smaller conidiophores heads and number of conidiophores were reduced (fig. 3). Data showed that diameter of colony were not significant reduced. The dry weight of fungal mycelia of 5-days culture with SAHA had 33,35 and 42 % reduction in 2,10 and 50 µM of SAHA (data not shown) . SAHA decreased the sporulation dependent on concentration (fig.4).

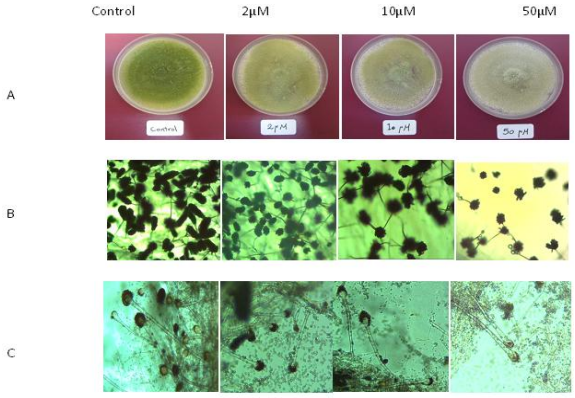

Fig. 1. Morphologic characteristics of A.flavus.(A)Growth with or without SAHA on PDA plates in different concentration of SAHA.(B)Light microscopy views of conidiophores of the cultures.(C) Close-up views of conidiophores heads.

SAHA prevents aflatoxin production

We found that SAHA reduced AFB1 production compared to the control through detection by TLC.Similare resulrs were obtained in each of three replicates experiments. SAHA inhibited dose-dependent production of AFB1. The migration pattern of TLC fluorescence samples reached the same Rf(0.71) as the positive control in total aflatoxin standard (fig. 5)The *A. flavus* strain that we examed in this study had just AFB1 .

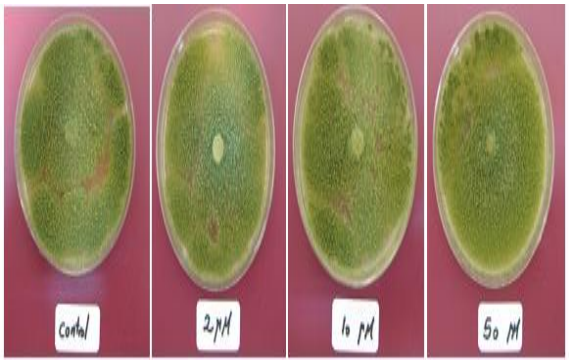

Fig. 2. Green colour of colony reappeared after transferred onto a fresh PDA medium without SAHA.

Identification of HDAC genes in A. flavus

After DNA exteraction, quality of DNA were evaluated by gel electrophoresis. To identify HDAC homologs of *A. flavus* a PCR strategy with degenerate primers ,AO and A2R(Grassle *etal.*,2000) and two pair primers that we design in this study based on highly conserved regions of this core domain was used. One fragment ,560 bp in length,were amplified from frist primer. Two fragmentes , *HosA* by 410 bp and *HosB* by 500 bp in length,were amplified from other primers (Fig. 6). Blast searches with genomic database in (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that *Rpd1* gene in *A. flavus* that very similar to *RPD3 Aspergillus oryzae* RIB40 (NCBI Reference Sequence: XM_001727142.2), *HosA* similar to *HOS2* in *Aspergillus oryzae* RIB40(NCBI Reference Sequence: XM_003189254.1) and *HosA* in *Aspergillus flavus* NRRL3357 (NCBI Reference Sequence: XM_002373613.1). *HosB* in *A. flavus* also similar to *HosB* in *Aspergillus oryzae* RIB40 (NCBI Reference Sequence: XM_001826514.1) and *HosB* in *Aspergillus flavus* NRRL3357(NCBI Reference Sequence: XM_002385504.1). *Rpd1* , *HosA* and *HosB* are orthologs of *Rpd3* ,*Hos2* and *Hos3* in *Saccharomyces cerevisiae*.

Discussion

Recently, HDACs were identified as proteins involved in the regulation of transcription. Different members of HDAC family have been sequenced in a number of species including mammals, insect, plant and fungi (Grassel *et al.*, 2001; Brocsh *et al.*, 2008).

Investigators have identified several classes and subclasses of HDACs in fungi (class I enzymes *RPD3* and *HosA* , the class II enzymes *Hda1* and *HosB*). We found 3 HDACs genes in *A. flavus* that homologous with HDACs in *S. cerevisiae* and other fungi. Comparision of our sequences and homologous genes revealed that most of them share significant sequence similarities located in larg N-terminal core domain. Blast N search results revealed no significant similarities of C-terminal extension to any known protein in the database and these results are similar to other sequences in fungi like *S.cerevisiae, A. nidulans, Cochliobolus carbonum* and other *Aspergillus* species (Simonetti *et al.*, 2007; Fisch *et al.*, 2009; Graessle *et al.*, 2000; Zacchi *et al.*, 2010; Shwab *et al.*, 2007; Izawa *et al.*, 2009; Graessle *et al.*, 2001).

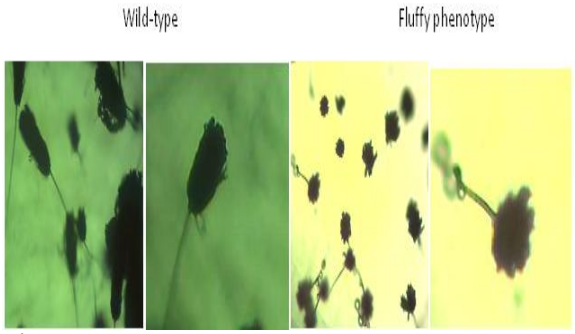

Fig. 3. Close-up views of conidiophores heads showing the conidial chains in Wild-type and Fluffy phenotype.

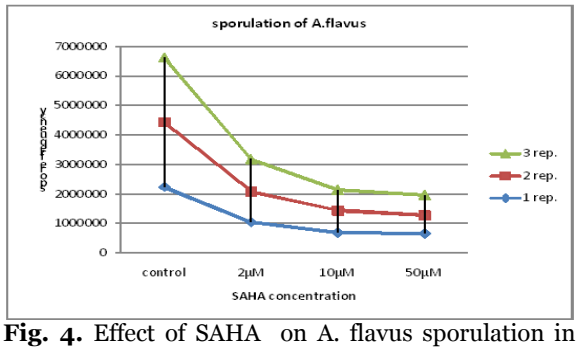

Fig. 4. Effect of SAHA on A. flavus sporulation in PDA medium.

HDAC inhibitors are members of an interesting family compounds that actively studied for various application in organisms. SAHA is the well-known HDACi inhibited class I and class II HDACs. A number of study indicated that SAHA may be a regulator in fungal biology and secondary metabolites(SM). Ten out of 12 fungi studied by Williams (Williams *et al.*, 2008) , including *A. flavus* are responsive to SAHA and produce new or changed levels of SM. Our finding in this paper show that SAHA has the ability to reduce AFB1 production in *A. flavus* which is similar to corresponding studies (Williams *et al.*, 2008; Fisch *et al.*, 2009, Grassle *et al.*, 2000). Another study found that HDACis inhibited germ tube formation, growth and conidiation. In *Candida albicans* SAHA caused a 90% reduction in fungi adherence to human cultured Pneumocytes (Simonetti *et al.*, 2007). In *A. nidulans* HDACis inhibited the class II enzyme Hda1 and Rpd3-type HDAC RpdA ,leads to pronounced reduction in growth and sporolation of this species (Tribus *et al.*, 2010; Lee *et al.*, 2009). Similare data were reported in the Rice blast fungus *Magnaporth oryzae* that treated with TSA ,another HDACis, that causes reduction of appressorium formation (Izawa *etal.*,2009). Our outcomes in this paper are similar to above results. Relationship exist between a fluffy colony phenotype and secondary metabolism in the *Aspergillus* family *A. niger, A. nidulans* and *A. fumigates,*when treated with 5-Azacytidine,well-known DNA methylation inhibitor, developed an inheritable fluffy phenotype (Tamame *et al.*, 1983a,b). In *Aspergillus* , several observation linked a fluffy form to loss of AF productin (Teniente *et al.*, 2011). We observed that treatment of A. flavus with SAHA induced fluffy phenotype and reduction in SM production but this chang were not inheritable and after transferred to PDA without SAHA this phenotype were reappear similar results was reported in *A. flavus* treatmented with 5-Azacytidine(Lin *et al.*, 2012).

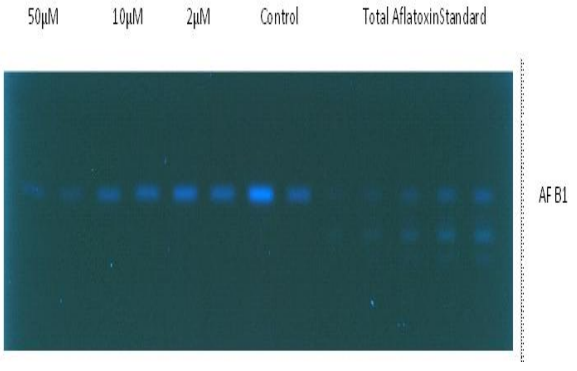

Fig. 5. Inhibitory effect of AF production in A. flavus by SAHA on TLC plate.

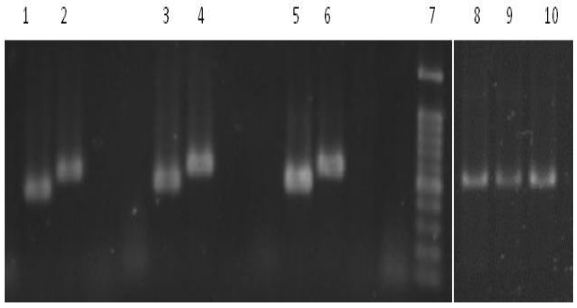

Fig. 6. PCR products of HDAC genes in A. flavus. 1,3 and 5: *Hos A* genes(410 bp) in tox-, tox+ and PTCC 5004. 2,4 and 6*: Hos B* genes(500bp) in tox-, tox+ and PTCC 5004.8,9 and 10 : *Rpd 1* genes(560 bp) in tox-, tox+ and PTCC 5004 and 7 is ladder.

Consistent with our results, treatment with HDACis resulted in transcriptional regulation of HDACs and were able to affect several changes in organisms. These experimental data suggest that HDACis could prove to be instrumental in developing new therapies for fungal infection . These compounds could be developed to inhance specificity for HDACs enzymes and the biochemical and structural affect of this enzymes. HDACis are one of the good subject for study about Epigenetic remodeling and histone modification in fungi and other organisms. In fact much work is still needed to understand the role of HDACs in fungi and suggesting that histone modification might have a role in A. flavus biology like AF production . With regard to the regulation AF ,it seems that genetic study of AF genes experesion is necessary bean it remains unclear what factors are involved in determining which some gene clusteres to be affected by this HDACi .

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