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Trypanocidal activity, cytotoxicity and histone modifications induced by malformin A_1 isolated from the marine-derived fungus *Aspergillus tubingensis* IFM 63452

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Abstract

Malformin A_1, a cyclic pentapeptide, was isolated from the marine-derived fungus *Aspergillus tubingensis* IFM 63452. The identity of the compound was established based on TOF-MS and ^{1}H NMR data. Malformin A_1 exhibited trypanocidal activity against *Trypanosoma congolense* (IC_{50}: 15.08 ng/mL). Interestingly, the compound was selective for *T. congolense* rendering a selectivity index value that ranged from 3.33 to 4.67. It also demonstrated cytotoxicity against HeLa (IC_{50}: 50.15 ng/mL) and P388 (IC_{50}: 70.38 ng/mL) cell lines. To further identify the possible mechanism of its cytotoxic effect, immunofluorescence staining was conducted to follow the epigenetic changes induced by the compound in the amino acid lysine of histone H3 and H4 in HeLa. The compound induced repressive levels of H3K27me3, H3K27ac and H4K5ac, and enhanced levels of H3K9me2, H3K9me3 and H4K16ac supporting the compound's chemotherapeutic potential.

Key words – bioactivities – epigenetics – fungal natural products – marine fungi

Introduction

Protozoan diseases are considered to be among the world's most widespread human health problems, with the greatest morbidity attributed to trypanosomatid and apicomplexan parasite infections (Martin et al. 2001). However, studies on the mode of action of commercially available antiprotozoal drugs have linked their antiparasitic activity with mammalian host intoxication (Urbina 2002). Moreover, drug resistance among protozoan-causing diseases, such as *Plasmodium falciparum* causing malaria and *Trypanosoma congolense* causing animal trypanosomiasis, are widely reported (Afewerk et al. 2000). Thus, this calls for finding new pipeline of antiprotozoal

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chemicals to overcome drug resistance and unwanted toxicity of available chemotherapeutic agents (Tempone et al. 2005). Of equal importance is combatting cancer which has been linked recently with epigenetic alterations such as modification of histone protein and methylation of the DNA (Kelly et al. 2010). Histone modifications, in particular, are important epigenetic changes that alter gene expression and modify cancer risk (Gibbons 2005). Interestingly, many cytotoxic chemicals derived from natural resources induced epigenetic changes and thus facilitated inhibition of tumor growth (Li et al. 2010, De Silva et al. 2012, 2013). In this study, the cyclic pentapeptide malformin A_1 derived from the marine fungus *Aspergillus tubingensis* IFM 63452 isolated from the seagrass *Enhalus acoroides* was reported to have trypanocidal activity against *Trypanosoma congolense* and cytotoxicity against HeLa and P388 cancer cells. The pure compound also induced epigenetic change by histone modification in HeLa cells. Malformin A_1 was previously isolated from the terrestrial fungus *A. niger* (Kim et al. 1993). Other study reported the cytotoxicity of malformin A_1 from *A. tubingensis* against human lung, pancreatic, breast, and central nervous system cancer cell lines (Zhan et al. 2007), antiprotozoal activity against *Plasmodium falciparum* and *T. brucei brucei* (Kojima et al. 2009), and antiviral activity against tobacco mosaic virus (Tan et al. 2015). The strains of *A. tubingensis* in these previous reports were isolated from terrestrial sources as opposed to the marine-derived isolate in this paper. This study also reports for the first time the trypanocidal activity of malformin A_1 against *T. congolense* and its cytotoxicity achieved through histone modification.

Materials & Methods

Isolation and identification of *Aspergillus tubingensis* IFM 63452

The seagrass, *Enhalus acoroides* (Linnaeus) Royle, was collected from the intertidal zone of Piapi Beach, Dumaguete City, Negros Oriental, Philippines. The leaf samples of *E. acoroides* were then cut into 3-5 mm explants, washed initially with sterile artificial seawater (ASW), then immersed in 70% ethanol (EtOH) for 60 seconds for surface sterilization, and followed by rinsing three times with ASW for 3 minutes. Afterwards, the explants were placed onto malt extract agar plates (MEAS) containing 33 g/L marine salt and 450 µg/mL streptomycin. The fungus that grew out of the edges of the explants were isolated and purified on MEAS. The isolated fungus was identified by morphological methods following the taxonomic keys of Klinch (2002) and Raper and Fennell (1977) and confirmed by sequence analysis of β-tubulin gene using forward Bt2a (5'-ggtaaccaaatcggtgctgctttc-3') and reverse Bt2b (5'-accctcagttgagtgacccttggc-3') primer pair (Glass & Donaldson 1995). The β-tubulin gene sequence of *A. tubingensis* was deposited at the DNA Data Bank of Japan (DDBJ) with the accession number LC168474. Pure culture of *A. tubingensis* was deposited at the Chiba Medical Mycology Research Center with strain number IFM 63452. Furthermore, to test if the isolated fungus was adapted to the marine environment, the colony extension rate (CER) for *A. tubingensis* in MEA with or without salt was computed using the formula: [mean colony radial growth (day 7) — mean colony radial growth (day 3)] / number of days of incubation (4 days). A paired t-test was computed for the CER on MEA and MEAS to determine if the presence or absence of marine salt in the medium significantly affected the colony extension rate of *A. tubingensis*.

Mass production and extraction of crude secondary metabolites

Aspergillus tubingensis IFM 63452 was mass produced in malt extract broth supplemented with 33 g/L marine salt (MEBS). After 4-week incubation under stationary condition, the culture broth was extracted with ethyl acetate in 1:1 proportion (v/v). The fungal crude extract was then concentrated *in vacuo* and freeze-dried in pre-weighed vials.

Isolation, purification and structure elucidation of malformin A_1

The crude ethyl acetate extract (162.4 mg) of *A. tubingensis* was subjected to ODS flash chromatography using gradient elution starting with pure H_2O, followed by MeOH: H2O (1:1, 7:3), then MeCN:H_2O (7.5:2.5, 8.5:1.5), then pure MeOH, and lastly $CHCl_3$:MeOH:H_2O (6:4:1) solvent

systems that afforded seven fractions (Fraction 1-1 to 1-7). The most bioactive Fraction 1-3 was further purified with HPLC using a filtered 40% MeCN (HPLC grade), then 100% MeOH for final washing of the column (FR: 2 mL/min, 0.08 AUFS, UV 220 nm). HPLC was carried out using a Shimadzu LC-10 liquid chromatograph equipped with a Shimadzu SPD-10A UVevis detector (Shimazu, Japan). Reversed phase HPLC afforded ten fractions (Fraction 2-1 to 2-10). Among these fractions, Fraction 2-8 (4.7 mg), a pure compound that rendered the highest activity in the bioassays, was later chosen for spectroscopic analyses.

To determine the molecular weight of the bioactive compound, Fraction 2-8 was dissolved in 40% MeCN and analyzed with LC-MS using a Shimadzu Nexera UHPLC combined with LCMS-8040 (Shimazu, Japan). For the elucidation of the compound's chemical structure, Fraction 2-8 was dissolved in DMSO-d_6 for ^{1}H NMR analysis at 400 MHz using a JEOL JNM-ECP500 spectrometer (JEOL, USA). The obtained spectra were then compared with published literature to find possible match.

ATP-based luciferase viability assay for *Trypanosoma congolense*

As previously described by Suganuma et al. (2014), the blood stream form of *Trypanosoma congolense* IL 3000 was propagated at 33 °C in air using IMDM (Wako Pure Chemical Industries, Osaka, Japan), containing 20% heat inactivated-fetal bovine serum, 60 mM HEPES, 1 mM pyruvic acid sodium salt, 0.1 mM bathocuproine, 1 mM hypoxanthine, 16 µM thymidine, 10 µg/L insulin, 5.5 µg/L transferrin, 6.7 ng/L sodium selenite, 0.0001% 2-β-mercaptoethanol, 0.4 g/L BSA, and 2 mM L-cysteine. Following culture, 50 µL suspension of *T. congolense* (2×10^5 protozoans/mL) was added to each well of the 96-well microplate. This was followed by the addition of the test compound to each of the wells. The reference antiprotozoal drug was pentamidine. The microplates were incubated for 72 hours, and subsequently, 50 µL of CellTiter-Glo® Luminescent Cell Viability Assay reagent (Promega Japan, Tokyo, Japan) was added to each well to evaluate ATP concentration. The microplates were incubated for another 10 minutes at room temperature and luminescence was read using a GloMax®-Multi Detection System plate reader (Promega Japan). A dose-dependent curve was plotted in GraphPad PRISM 5 software (GraphPad Software Inc., CA, USA) to calculate the IC_{50}. The selectivity index for the trypanocidal activity was calculated using the formula of Koch et al. (2005) with slight modification: IC_{50} HeLa or P388 cells / IC_{50} *Trypanosoma congolense*.

Methyl thiol tetrazolium (MTT) cytotoxicity assay

HeLa cervical cancer and P388 murine leukemia cancer cell lines were cultivated in DMEM (Wako Pure Chemical Industries, Osaka, Japan), containing 10% fetal bovine serum, 2 mg/mL gentamicine, and 10 mg/mL antibiotics adjusted to pH 7.0–7.4 by 1 M HCl, and RPMI-1640 (Wako Pure Chemical Industries), containing 10% fetal bovine serum, 100 mg/mL kanamycine, and 10 mM 2-hydroxyethyldisulfide, respectively. The cell lines were maintained at 37 °C under an atmosphere of 5% CO_2. Following the termination of cell culture, an aliquot of 200 µL tumor cell suspension (1×10^4 cells/mL) was dispensed unto each well of the 96-well microplate and pre-incubated for 24 hours. After pre-incubation, the test compound was added to each of these wells. The cells were further incubated for 72 hours and 50 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) saline solution (1 mg/mL) was added to the wells, followed by incubation for 3 hours under the same condition to stain live cells. Finally, the culture medium was withdrawn and 150 µL of DMSO was dispensed to dissolve cells. Absorbance was finally read by Fusion^{TM}α microplate reader (Packard Bioscience Company, CT, USA). A dose-dependent curve was plotted in GraphPad PRISM 5 software (GraphPad Software Inc., CA, USA) to calculate the IC_{50}.

Immunofluorescence staining for detection of H3 and H4 modifications in HeLa

As previously described by Kimura et al. (2008), HeLa cells were cultivated in DMEM (Wako Pure Chemical Industries, Osaka, Japan) and maintained at 37 °C under an atmosphere of 5% CO_2. Following the termination of cell culture, an aliquot of 200 µL tumor cell suspension (2×10^4 cells/mL) was dispensed unto each well of the 96-well microplate and pre-incubated for 24 hours.

HeLa cells were treated with malformin A_1 dissolved in DMSO at a reaction concentration of 10 and 100 ng/mL, followed by incubation for 20 h. HeLa treated with DMSO served as the control. After incubation, an aliquot of 100 µL fixing agent, containing milli-Q water, 0.25 M HEPES adjusted to pH 7.4, 4% paraformaldehyde and 0.1% Triton X-100, was added to each well and was withdrawn after 10 minutes. The cells in each well were washed two times with 200 µL PBS, then maintained in agitation for 20 minutes with 100 µL 1% Triton X-100, and re-washed twice with 200 µL PBS. After fixation, the cells were incubated with 50 µL blocking one-p (Nakalai Tesque, Kyoto, Japan) for 20 minutes while shaking and re-washed twice with PBS. Immunolabeling was performed by incubating the cells in each well with 50 µL fluorescent dye (Hoechst 33342, Alexa fluor 488-congugated mouse monoclonal antibodies specific for H3K9me3, H3K27me3 and H3K9me2, or Cy3-congugated antibodies specific for H4K5ac, H3K27ac and H4K16ac) followed by shaking for another 2 hours and washing with PBS three times. Hoechst 33342 and Alexa fluor- and Cy3-conjugated antibodies' signals were acquired by sequential scanning of treated cells using an Olympus IX71 inverted microscope (Olympus America, Central Valley, Pennsylvania). Fluorescence intensities in nuclei were measured using CellProfiler (http://www.cellprofiler.org). The net intensities were calculated by subtracting the background. Paired t-test was then computed to determine if there was significant difference in the level of fluorescence intensity between DMSO and the test compound.

Results

Identification of the marine-derived fungus

The marine-derived fungus from the seagrass *Enhalus acoroides* formed black colony with globose to radiate conidial heads, globose vesicles, and smooth, long and coarse conidiophores. In addition to the macro- and micro-morphology examination, molecular characterization was also performed by sequencing the β-tubulin gene of the isolated marine *Aspergillus*. Comparison of the isolate's 509 bp sequence by Blast search showed 100% similarities to *Aspergillus tubingensis*. The combined morphological and gene sequence analysis identified strain IFM 63452 as *A. tubingensis* (Fig. 1). Furthermore, to assess the adaptability of the isolate to the marine environment, *A. tubingensis* was cultured in MEA with or without salt (Fig. 2). Statistical analysis by one-tailed paired T-test confirmed that the mean colony extension rate in MEAS was greater than MEA and that the difference was highly significant (p-value $0.0047 < \alpha = 0.05$).

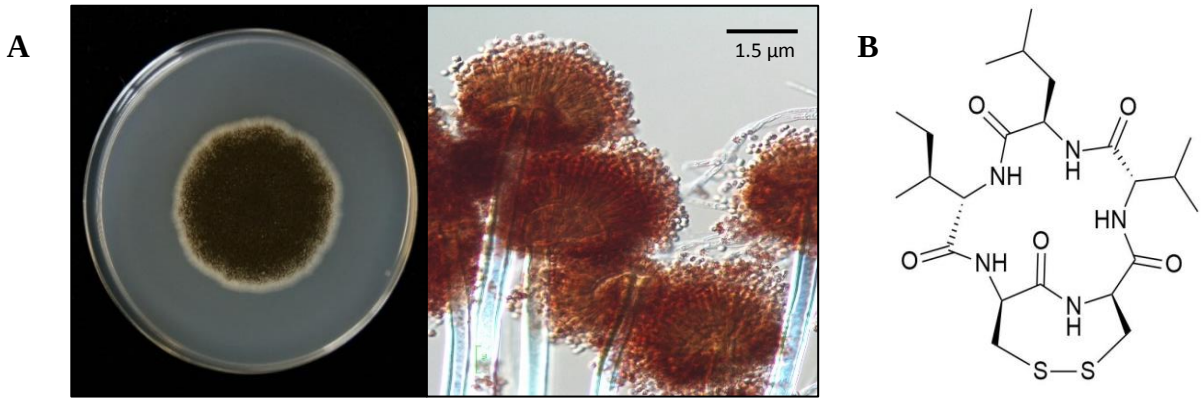

Figure 1 – (A) Seagrass-derived *Aspergillus tubingensis* IFM 63452 grown on MEAS and (B) the isolated cyclic pentapeptide malformin A_1.

Bioactivity-guided isolation of malformin A_1

Aspergillus tubingensis IFM 63452 was cultivated for three weeks in malt extract broth supplemented with salt. The 500-mL culture broth of the fungus was extracted exhaustively with EtOAc in 1:1 (v/v) proportion and the resulting crude extract was screened for its cytotoxicity against HeLa (IC_{50}: 1,301 ng/mL) and P388 (IC_{50}: 1,028 ng/mL), and its trypanocidal activity against

Trypanosoma congolense (IC_{50}: 485.58 ng/mL). Following crude extraction, ODS flash chromatography was performed to afford seven fractions (Fractions 1-1 to 1-7). Fraction 1-3 (31.9 mg) showed the greatest bioactivity against HeLa and P388 rendering an IC_{50} of 169.60 ng/mL and 413.30 ng/mL, respectively. Moreover, trypanocidal assay against *T. congolense* rendered an IC_{50} of 82.60 ng/mL. Further purification of Fraction 1-3 was then performed by reversed phase HPLC that generated ten more fractions (Fractions 2-1 to 2-10). Of these fractions, Fraction 2-8 (4.7 mg) showed the highest trypanocidal activity (IC_{50}: 15.08 ng/mL) and cytotoxicity against HeLa (IC_{50}: 50.15 ng/mL) and P388 (IC_{50}: 70.38 ng/mL). Furthermore, Fraction 2-8 was also found to be selective for the protozoan *T. congolense* rendering a selectivity index (SI) value that ranged from 3.33 to 4.67. Based from the HPLC chromatogram, Fraction 2-8, affording a single peak with an elution time after 36 minutes, was regarded as a pure compound and subjected to spectroscopic analyses.

The compound was isolated as a white amorphous powder. The protonated molecular ion peak at *m/z* 530.2456 $[M + H]^+$ corroborated to the molecular formula $C_{23}H_{39}N_5O_5S_2$ (530.2456 amu) with TOF-MS spectral data. Meanwhile, the ^1H NMR spectrum showed five N-H proton signals ($^{\delta}H$ 7.42 (d), 8.57 (d), 8.89 (d), 7.19 (d), 7.83 (d)) for Leu (1), Ile (1), Cys (2) and Val (1) characteristic of amide linkages in malformin A_1, a known cyclic pentapeptide (Table 1).

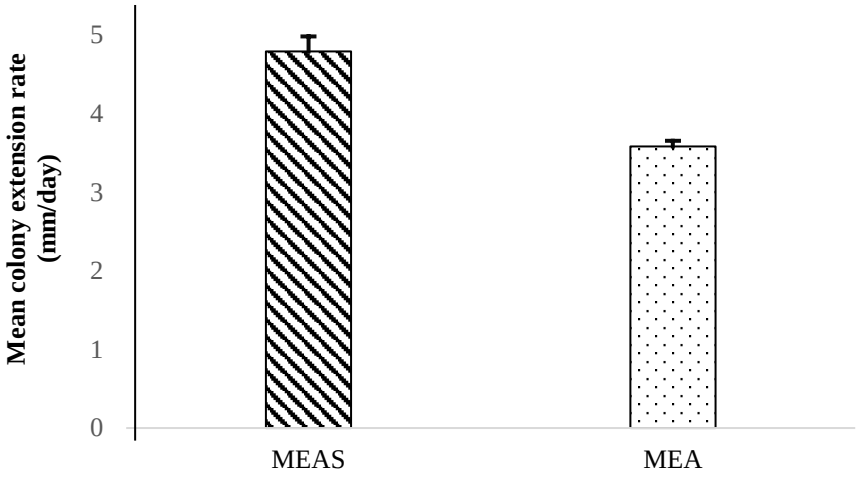

Figure 2 – Colony extension rate of *Aspergillus tubingensis* IFM 63452 grown on MEA with or without marine salt (n=3). Mean standard deviation is expressed in error bar.

Histone modifications induced by malformin A_1

Immunofluorescence staining was performed to follow the epigenetic changes induced by the compound in HeLa cancer cells. In the current study, six histone modification markers (H3K9me2, H3K9me3, H3K27me3, H3K27ac, H4K5ac, and H4K16ac) were monitored in HeLa after treatment with malformin A_1 (Fig. 3). Interestingly, both the 10 and 100 ng/mL concentrations of malformin A_1 significantly enhanced H3K9me2/me3 (corresponding p-values 0.0001, 0.002, 0.01 and $0.02 < \alpha$-level 0.05) and H4K16ac (corresponding p-values 0.001 and $0.004 < \alpha = 0.05$) when compared to HeLa treated with DMSO. Meanwhile, of the two concentrations of malformin A_1 tested, only the 10 ng/mL concentration significantly repressed the level of H3K27me3 (p-value $0.002 < \alpha = 0.01$), while the 100 ng/mL concentration induced significant downregulation in the levels of H3K27ac (p-value $0.002 < \alpha = 0.01$) and H4K5ac (p-value $0.002 < \alpha = 0.01$) in comparison with the DMSO control.

Discussion

The isolated fungus in this study was typically of terrestrial in origin. However, it is possible for terrestrial fungi to be introduced into the marine ecosystem and subsequently evolved or adapt as a result of selective pressure to this new habitat (Jones 1994). As shown in Fig. 2, the colony extension rate of the isolated fungus was greater in MEAS as opposed to MEA. This demonstrates the adaptability of *A. tubingensis* to the salinity in the marine environment. Kohlmeyer & Kohlmeyer (1979) noted that there are two types of marine fungi: the obligate marine fungi that exclusively grow

and sporulate in seawater and the facultative ones that originate from freshwater or terrestrial environment that have undergone physiological adaptations for them to thrive and sporulate in the marine ecosystem. In this study, in particular, *A. tubingensis* was found to be facultative to have grown both in MEA with and without salt as similarly observed in other marine fungi, e.g. species of *Dendryphiella* (dela Cruz et al. 2006) and also *Aspergillus* (Solis et al. 2010).

Table 1 ^{1}H NMR data of malformin A_1 recorded on a 400 MHz spectrometer (DMSO-d_6 solvent).

Amino Acid	Position	$^{\delta}$H (mult.)	$^{\delta}$H (mult.) [a]
Leu	C = O	–	–
	NH	7.42 (d)	7.38 (d)
	α	4.47 (dt)	4.47 (dt)
	β	1.35 (m)	1.37 (m)
	γ	1.55 (m)	1.56 (m)
	δ	0.89 (d); 0.85 (d)	0.89 (d); 0.85 (d)
Ile	C = O	–	–
	NH	8.57 (d)	8.59 (d)
	α	3.90 (dd)	3.87 (dd)
	β	1.74—1.87 (m)	1.69 (m)
	γ	1.5 (m); 0.77 (d)	1.5 (m); 1.13 (m); 0.77 (d)
	δ	0.81 (t)	0.81 (t)
Cys	C = O	–	–
	NH	8.89 (d)	8.84 (d)
	α	3.98 (dd)	3.98 (dd)
	β	3.58 (dd); 3.2 (m)	3.51 (dd); 3.14 (m)
Cys	C = O	–	–
	NH	7.19 (d)	7.11 (d)
	α	4.73 (dt)	4.71 (dt)
	β	3.35 (m); 3.19 (m)	3.24 (m); 3.19 (m)
Val	C = O	–	–
	NH	7.83 (d)	7.94 (d)
	α	3.93 (m)	3.92 (m)
	β	2.02 (m)	2.05 (m)
	γ	0.82—0.84 (d)	0.82 (d); 0.82 (d)

Multiplicities of the protons in malformin A_1: d (doublet); dd (doublet of doublet); dt (doublet of triplet) m (multiplet). [a]Proton signals for malformin A_1 as previously described by Tan et al. (2015).

Interestingly, a cyclic pentapeptide malformin A_1 was isolated for the first time from the marine-derived fungus *A. tubingensis* IFM 63452. Terrestrial strains of *A. tubingensis* were previously reported capable of producing such compound. This shows that production of malformin

A_1 is an inherent genotypic trait of *A. tubingensis*, and not produced only in response to culture conditions. Biological profiling revealed that malformin A_1 was active against *T. congolense* (IC_{50}: 82.60 ng/mL). This is supported by the standard of Koch et al. (2005) wherein extracts or pure compounds exhibiting an IC_{50} less than 10 µg/mL are considered as having antiprotozoal activity. In addition to this finding, the compound was also found to be selective for *T. congolense* with a selectivity index (SI) value ranging from 3.33 to 4.67. As previously described by Koch et al. (2005), an SI value greater than 2.0 indicates that the sample is not a general toxin and therefore can be recommended as an antiprotozoal agent. The potency of the said compound as an antiprotozoal agent is supported by Kojima et al. (2009) wherein malformin A_1 synthesized in the laboratory showed to be bioactive against *P. falciparum* and *T. brucei brucei*. In addition to these findings, the study also reported that malformin A_1 exhibited promising cytotoxicity against HeLa (IC_{50}: 50.15 ng/mL) and P388 (IC_{50}: 70.38 ng/mL). This biological profile demonstrates the potency of malformin A_1 since the standard for extracts or pure compounds exhibiting anticancer property is an IC_{50} less than 30 µg/mL (Suffness and Pezzuto 1990). In addition to the findings of the present study, Zhan et al. (2007) also reported that malformin A_1 is cytotoxic against human non-small cell lung cancer (NCI-H460), human pancreatic cancer (MIA Pa Ca-2), human breast cancer (MCF-7), and human CNS cancer (SF-268) cell lines.

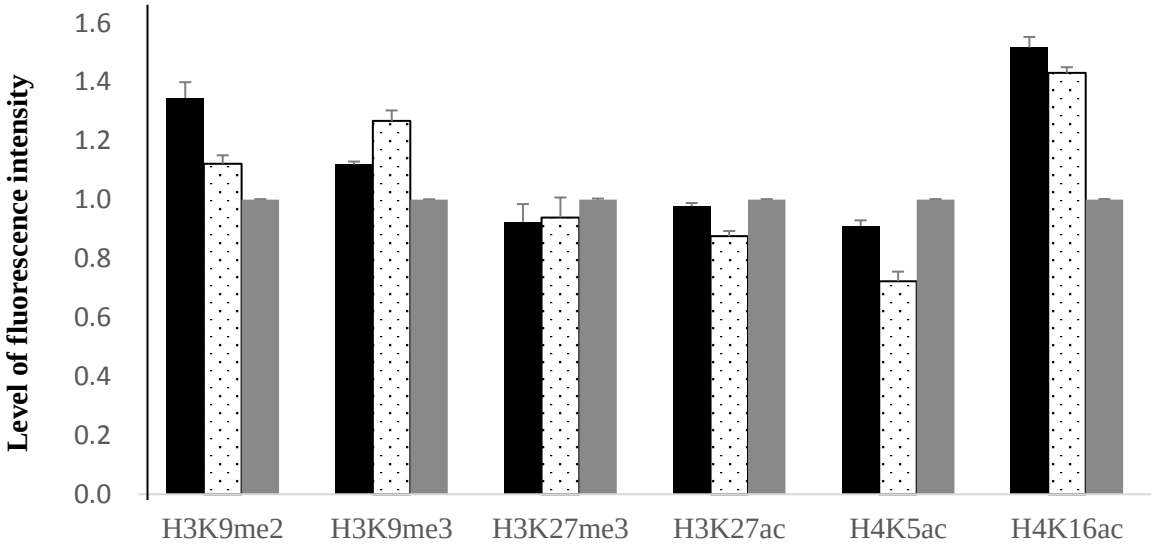

Figure 3 - Acetylation and methylation of lysine in histone H3 and H4 of HeLa cells induced by malformin A_1 (n=3). The black bar represents the effect of malformin A_1 at 10 ng/ml, the dotted bar for 100 ng/ml, and the gray bar for the DMSO control.

Given that malformin A_1 showed cytotoxicity against cancer cells, the compound's mechanism of action was further investigated. This was achieved by determining the ability of the compound to induce epigenetic change in histone protein. It is known that the alteration in the structure of chromatin can influence gene expression by either inactivating genes, which takes place when the chromatin is closed (heterochromatin), or by activating genes when the chromatin is open (euchromatin) (Rodenhiser and Mann 2006). The nucleosome, as the fundamental repeating unit of chromatin, is composed of DNA wrapped around a histone octamer consists of an H3-H4 tetramer and two H2A-H2B dimers (Reuter et al. 2011). Each successive nucleosomal core is then joined together by the DNA linker associated with histone H1. Post-translational modifications in the chromatin occur at the amino acids of the *N*-terminal tails of histones which can either facilitate or prevent the association of DNA repair proteins and transcription factors with the chromatin. Consequently, this mechanism either enhances or hinders the accessibility of genes for expression (Reuter et al. 2011).

In this study, both the 10 and 100 ng/mL concentrations of malformin A_1 upregulated H3K9me2/me3 and H4K16ac when compared to HeLa treated with DMSO. This observation might be correlated with the ability of the compound to induce cytotoxicity in HeLa cancer cells. For instance, it was demonstrated that the downregulation of H3K9me2/me3 may trigger the promoters to become hypermethylated that consequently causes the inactivation of tumor suppressor genes leading to cancer development (Lennartsson & Ekwall 2009; Seligson et al. 2009). On the other hand, low levels of H4K16ac were found to be associated with an invasive cancer pathogenicity, as in the case of human breast cancer (Elsheikh et al. 2009) and mouse model multistage skin carcinogenesis (Fraga et al. 2005). The loss of H4K16ac is implicated to reduce DNA damage response and enhance genomic instability and, therefore, facilitates mutations and chromosomal rearrangements increasing cancer risk (Gupta et al. 2008). The upregulated level of H3K9me2/me3 and H4K16ac following treatment with malformin A_1 in HeLa may indicate that the compound can influence the suppression of the transcription of oncogenes that are linked to carcinogenesis.

On the other hand, of the two concentrations of malformin A_1 tested, only the 10 ng/mL concentration was able to downregulate the level of H3K27me3, while the 100 ng/mL concentration induced significant repression in the levels of H3K27ac and H4K5ac in comparison with the DMSO control. The downregulation of H3K27me3, H3K27ac, and H4K5ac supports the compound's chemotherapeutic activity. H3K27me3, in particular, has been associated with PolyComb (PcG) protein complexes that repress certain genes essential to maintain the pluripotency of developing cells (Lee et al. 2006). As previously revealed by Kleer et al. (2003), the upregulated level of PcG complex proteins is associated with the metastasis of cancer. As shown in this study, malformin A_1 was able to downregulate H3K27me3 which is suggestive of its potential in inhibiting the proliferation of cancerous cells. In addition, the compound was also able to repress H3K27ac which has been linked to the severity of lung adenocarcinomas, squamous cell carcinomas, and colon cancer (Roche et al. 2013; Karczmarski et al. 2014) and H4K5ac that has been associated with faulty DNA replication and DNA damage control in the late S phase of the cell cycle and in the reduction of chromatin compaction and heterochromatin content (Bhaskara et al. 2010). Having these results, it is implicated that the cytotoxicity of malformin A_1 is caused by its ability to induce epigenetic changes in HeLa by modifying lysine of histone H3 and H4 either through acetylation or methylation.

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