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Research Article

In vitro and *in silico* insights into moniliformin induced global DNA methylation in liver cells

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ABSTRACT

Moniliformin (MON) is a mycotoxin that is not routinely monitored in foods; however, it is increasingly recognized as an emerging food safety concern. Although some clastogenic effects were observed, non-genotoxic mechanisms may also contribute to the toxic effects of MON. Therefore, this study aims to investigate the effects of global DNA methylation, the most studied non-genotoxic mechanism, on MON toxicity in human hepatocarcinoma (HepG2) cells. HepG2 cells were exposed to MON at 5, 10, and 50 μM concentrations for 24 hours. Global DNA methylation levels and gene expression of *DNMT1*, *DNMT3A*, *DNMT3B, TET1, TET2*, and *TET3* were analyzed. Interactions among B-DNA, DNMT1, DNMT3A, DNMT3B, and MON were investigated using molecular docking. MTT cytotoxicity assay revealed that the IC_{50} value of MON in HepG2 cells was 253±8.41 μM. MON at 10 and 50 μM resulted in at least a 2.18-fold increase ($P<0.05$) in global DNA methylation levels, accompanied by upregulated expression of *DNMT1* and *DNMT3A*. Moreover, 5, 10, and 50 μM of MON resulted in at least 2.37-fold ($P<0.05$) increase in *TET1* genes. In contrast, *TET2* expression was significantly downregulated by at least 37% ($P<0.05$) and 42% ($P<0.05$) at 10 and 50 μM of MON. However, *TET3* expression showed no significant change in any treatment group. MON formed hydrogen bonds with B-DNA, DNMT1, DNMT3A, and DNMT3B. However, the binding energy calculations indicate that the interactions are not biologically active. This study suggests that alterations in global DNA methylation might have a role in MON-induced toxicity.

Keywords: Epigenetics, Global DNA methylation, HepG2 cells, Molecular docking, Moniliformin

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Introduction

Mycotoxins, classified as secondary metabolites, are produced by several filamentous fungi, primarily by species of *Fusarium*, *Penicillium*, and *Aspergillus*, under suitable environmental conditions (18, 29). Mycotoxins can contaminate food and feed (23) and are major health concerns for humans and livestock because they may cause severe toxic effects (29, 31). Moniliformin (MON, 3-hydroxycyclobut-3-ene-1,2-dione) is a mycotoxin produced by many *Fusarium* species, mainly *Fusarium proliferatum* (9, 18, 29, 31), and has recently been found to be a metabolite of *Penicillium melanoconidium* (27). In nature, MON is generally present as a potassium or sodium salt of semisquaric acid and exhibits high solubility in polar solvents (9). MON was detected in many cereals, such as rice, barley, corn, wheat, oats, triticale, and rye (9, 29). The formation, toxicity, and contamination levels of MON are poorly understood globally, so MON is classified as an emerging mycotoxin (30). Although emerging mycotoxins, including MON, are not regularly assessed or regulated, there is growing evidence of their prevalence (29).

To date, few studies reporting conflicting results on the genotoxicity of MON have been published. Çelik et al. (2009) reported that MON was clastogenic in human lymphocytes and caused dose-dependent chromosomal aberrations, increasing sister chromatid exchanges and micronucleus frequency (14). On the other hand, MON

does not exhibit mutagenicity in the Ames test and reactivity in bacterial genotoxicity assays. Therefore, although some clastogenic effects are observed, it is considered non-genotoxic because of the lack of activity in standard genotoxicity assays (41). Even though cytotoxicity was generally low in most cell lines exposed to MON, significant cytotoxic effects were observed in certain cell types, including lymphocytes, skeletal myocytes, and cardiomyocytes (9, 41). While the heart is the primary target organ of MON in animals (23, 31), MON also has toxic effects on the liver and kidney (28).

In a 28-day repeated oral-dose toxicity study in male mice, MON at 40 mg/kg b.w. induced centrilobular hepatocellular hypertrophy with increased liver weight (40). A reduction in liver weight and increases in serum creatinine concentration, creatine kinase, aspartate aminotransferase, and alanine aminotransferase levels were observed in chickens in response to diets containing 50 and 100 mg of MON per kg for 420 days (34), indicating impaired liver function. Similarly, hepatotoxic effects were observed in turkeys (≥37.5 mg/kg) and broiler chickens (≥25 mg/kg) after 14 weeks of dietary exposure (6).

Pyruvate dehydrogenase and other enzymes requiring thiamine as a cofactor appear to be the primary targets of MON, resulting in energy deficiency, respiratory stress, and myocardial dysfunction (4). MON can disrupt carbohydrate metabolism by inhibiting aldose reductase and gluconeogenesis (9). Glutathione reductase and glutathione peroxidase are inhibited by MON, resulting in the formation of free radicals, which may contribute to toxicity (9, 11). MON induces mitochondrial dysfunction (8), which may result in reduced ATP production. ATP is required to produce S-adenosylmethionine (SAM), the major methyl donor in DNA methylation. Thus, disruption of energy metabolism can lead to changes in the capacity for DNA methylation. Although the number of studies on the epigenetic effects of MON, particularly in relation to DNA methylation, is limited, the fact that MON can alter the intracellular energy balance, induce oxidative stress, and potential damage DNA suggest that it may indirectly affect DNA methylation.

DNA methylation, a key epigenetic mechanism, plays an important role in modulating functional pathways, such as cell cycle regulation, DNA repair, apoptosis, and chromatin remodeling. DNA methylation is the chemical modification of the fifth carbon of cytosine to 5-methylcytosine (5-mC) at CpG dinucleotides, catalyzed by DNA methyltransferases (DNMTs) that use SAM as the methyl donor (16, 30). Ten-eleven translocation (TET) enzymes play a role in oxidizing methyl groups, reversing cytosine methylation, and forming hydroxymethylcytosine (45). DNA lesions caused by oxidative stress can disrupt interactions between DNA and the epigenetic machinery, leading to changes in DNA methylation and subsequent cell dysfunction implicated in many types of chemical-induced toxicity (19, 48) The widespread presence of MON in feeds and foods, as well as its potential to affect multiple organ systems, requires further investigation into the cellular effects of this toxin. The present study therefore aims to analyze global DNA methylation and expression of the *DNMT* and *TET* genes in HepG2 cells after 24 hours of MON treatment. The data obtained in this study are expected to contribute to a critical understanding of the role of DNA methylation in the toxic effects of MON.

Materials and Methods

For Prediction of MON Toxicity: The SMILES identifier of MON was obtained from PubChem (39). The SMILES format represents the atoms and chemical bonds present in a molecular structure. The SMILES identifier was submitted to the SwissADME (15) and ADMETlab 3.0 (20) tools to obtain *in silico* predictions of the pharmacokinetic parameters and toxicological properties of MON.

Chemicals and Cell Culture: MON (sodium salt, ≥95% purity) was obtained from Cayman Chemical (Ann Arbor, Michigan, USA). A 1000 µM stock of MON was prepared by dissolving it in sterile, double-distilled water (ddH2O) and was stored at -20°C. Cell culture media and supplementary reagents were obtained from Wisent Bioproducts (Saint-Jean- Baptiste, QC, Canada). The human hepatocellular carcinoma (HepG2) cells were obtained from American Type Culture Collection (ATCC® HB-8065TM). HepG2 cells were maintained in Eagle's Minimum Essential Medium (EMEM) cell culture medium supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (100 U-100 µg/mL) and incubated at 37°C in a humidified environment with 5% CO_2. Subculturing was conducted using trypsinization when the cells achieved 70-80% confluence. Cells with passage numbers between 7 and 20 were used to ensure reproducibility and prevent contamination. Good laboratory practices were followed to ensure mycoplasma-free culturing.

Cytotoxicity: For the cytotoxicity assay, sterile ddH2O was used as the solvent control, and 1×10^4 cells in 100 µL of medium were cultured in 96-well plates. The cells were then exposed to MON at doses ranging between 6.25 and 400 µM for 24 hours. After a 24 hours of MON treatment, cytotoxicity was assessed using the MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. The MTT assay is based on the principle that yellow MTT is converted into purple formazan by succinate dehydrogenase in mitochondria of viable cells, provided mitochondria are functioning properly. This test is used to assess cellular metabolic activity as an indicator

of cell viability and cytotoxicity (37). A microplate spectrophotometer was used to measure the absorbance of the resulting colored solution at 590 nm (Biotek-Epoch, Winooski, USA). The findings of the cytotoxicity calculations were reported as the half-maximal inhibitory concentration (IC_{50}) of the substance, defined as the concentration that resulted in 50% inhibition of enzyme activity in the cells, and as a percentage relative to control cells.

MON Exposure to Cell Culture: For DNA methylation and gene expression analyses, cells (1×10^6) were cultured in a flask for 24 hours in a CO_2 incubator before exposure. Cells were exposed to MON at the concentrations of 5, 10, and 50 µM, and sterile ddH_2O was used as solvent control for 24 hours. The exposure concentrations used in this study were determined based on cytotoxicity results and previously reported dose ranges in the literature (22). Dead cells were stained blue with the trypan blue test, and live cells were counted with the Luna cell counter (Virginia, USA). Later, cell suspensions were split into two groups to isolate total RNA and genomic DNA. Each concentration group was evaluated in three independent biological replicates, with the second replicate performed on a different day. Thus, a total of 24 samples were studied, with six replicates in each of the four groups.

Global DNA Methylation Analysis: Genomic DNA was isolated from HepG2 cells using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in accordance with the manufacturer's protocol. DNA concentration (optical density, OD_{260}) and quality ($OD_{260/280}$) were measured by spectrophotometric analysis, and the OD260/280 ratio of the isolated DNA was 1.8±0.1, indicating acceptable purity. The 5-mC DNA ELISA kit (Zymo Research, Irvine, CA, USA) was used to analyze 100 ng of genomic DNA per sample, in accordance with the manufacturer's protocol. A microplate spectrophotometer system was utilized to measure absorbance at 405 nm (Biotek-Epoch, Winooski, USA). The percentage of 5-mC was calculated using the standard calibration curve (with a range of 1–50% 5-mC), which was prepared using positive control (DNA containing 5-mC at a concentration of 100 ng/µL) and the negative control (unmethylated DNA at a concentration of 100 ng/µL) provided in the kit. The correlation coefficient was 0.9937. The calculated 5-mC% is expressed as 5-mC/total DNA (A + G + C + T). Each DNA sample was analyzed in duplicate to ensure accuracy.

Gene Expression Analysis: Expression of *DNMT* genes (*DNMT1*, *DNMT3A*, and *DNMT3B*) and *TET* genes (*TET1*, *TET2*, and *TET3*) was assessed by real-time PCR. Total RNA was isolated from HepG2 cells using the PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). RNA concentration (optical density, OD_{260}) and quality ($OD_{260/280}$) were assessed by spectrophotometric analysis. The OD260/280 ratios of the isolated RNA were 1.95±0.10, indicating acceptable purity. Reverse transcription was performed using the iScript cDNA Synthesis Kit (Bio-Rad, California, USA) with 1000 ng of total RNA and a mixture of anchored-oligo (dT) and random hexamer primers. The synthesized cDNA was stored at -20°C for subsequent PCR analyses. Real-time PCR reactions were performed using BioLine SensiFast™ SYBR® No-ROX kit (London, UK) and LightCycler® 480 Instrument II (Roche Life Science), and the cycling conditions for the amplification reaction were determined in line with the study carried out by Guler et al. (2023) (26). Table 1 displays the primer sequences and annealing temperatures of the studied genes and the reference gene *(β-actin)*. The primers were validated using the National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (38). Melting curve analysis showed that no non-specific products or primer dimers were present in the samples.

Table 1. Primer sequences and annealing temperatures of reference gene and genes related to DNA methylation.

Gene Name	Primer sequence (5'-3')	Temperature (°C)	Product length (bp)	Reference
DNMT1	*F: CCTCCAAAAACCCAGCCAAC* *R: TCCAGGACCCTGGGGATTTC*	60	101	(1)
DNMT3A	*F:* GGTCACGCAAAACAGAACCC *R:* CCTTGGTGAAACCCTTTGCG	60	293	(24)
DNMT3B	*F:* CTCTTCCTCAGCTGTGTGGG *R:* CTGTCGGCACTGTGGTTTTG	60	277	(24)
TET1	F: CCCTTGGAAATGCCATAGGAA R: GAGAGCCTGCTGGAACTGTTG	58	81	(2)
TET2	*F: GGCTGTTGGCCAGAGACTTA* *R:ATACCTGTAGGTGTTTGCCTGTTTA*	54	117	(2)
TET3	*F: GCCAACTTCAACATACCCTGGAC* *R: CACCTGGATGTGGGACTGTGTAA*	58	81	(2)
β-actin	*F: AACTACCTTCAACTCCAT* *R: TGATCTTGATCTTCATTGTG*	48	161	(43)

Additionally, no-template controls were included in each reaction to confirm the absence of non-specific amplification in RT-PCR. Ct values for *DNMT* genes, *TET* genes, and *β-actin* were obtained by real-time PCR and relative gene expression levels were calculated using the comparative Ct (ΔΔCt) method (38). Each PCR reaction was performed in duplicate to ensure accuracy.

Molecular Docking: 3D crystal structures of B-DNA and DNMT enzymes were obtained from the RCSB Protein Data Bank (5). The 3D model of MON was obtained from PubChem (28). MON (PubChem CID: 40452) was docked into B-DNA (PDB ID: 1BNA (17)), DNMT1 (PDB ID: 4WXX (51)), DNMT3A (PDB ID: 6W8J (3)), and DNMT3B (PDB ID: 6U8V (21)). Macromolecular resolutions were ≤3 Å. The docking protocol was previously optimized by Cakir and Ozden (7). Avogadro 1.2.0 was utilized to add missing hydrogen atoms and optimize the geometry of the ligand. Macromolecules were processed using UCSF ChimeraX 1.7.1, MGL Tools 1.5.7, and AutoDock 4.2. MGL Tools 1.5.7 and AutoDock 4.2 were used for docking calculations. Bonds in the ligand were set as rotatable. Macromolecules were kept rigid. The Lamarckian genetic algorithm was used as the search method. The lowest estimated binding free energies of MON were reported. Binding free energy estimates more negative than -6.0 kcal/mol are accepted as indicative of biologically active interactions (44). 2D visualizations of macromolecule-ligand interactions were prepared using BIOVIA Discovery Studio 2025, while 3D visualizations of ligands in their macromolecular binding sites were prepared using ChimeraX 1.7.1.

Statistical Analysis: Cytotoxicity and global methylation results are presented as mean ±standard deviation (SD). Statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 21.0 (IBM Analytics, New York, USA). Data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test to compare each treatment group with the control. Statistical significance was set at $P<0.05$ and $P<0.001$.

Results

MON Toxicity Predictions: The physicochemical and pharmacokinetic parameters of MON were calculated using SwissADME. The molecular weight of MON is 98.06 g/mol. MON has three hydrogen-bond acceptors and one hydrogen-bond donor. The mlogP of MON was calculated as -1.80. According to Lipinski's rule (35), molecules with MW≤500, hydrogen-bond acceptors≤5, hydrogen-bond donors≤10, and mlogP ≤ 4.15 are orally bioactive. Exposure to MON occurs predominantly via the oral route. These findings indicate that MON may be readily absorbed through the gastrointestinal tract and induce toxicity.

The toxicological properties of MON were predicted using ADMETlab 3.0. The probabilities of human hepatotoxicity, genotoxicity, and carcinogenicity were calculated to be 0.476, 0.581, and 0.612, respectively. These results suggest the possibility of moderate risks associated with the endpoints of interest. Preliminary predictive findings indicate that MON exposure via food consumption may induce liver toxicity, genotoxicity, and carcinogenicity.

Cytotoxicity: Figure 1 illustrates the effects of MON (6.25–400 µM) on cell viability in HepG2 cells after 24 hours of exposure, as assessed using the MTT assay. Given the MTT results, the IC_{50} value of MON in HepG2 cells was calculated to be 253±8.41 µM. Compared to the control group, treatments with MON at concentrations of 6.25, 12.5, 25, 50, 100, 200, and 400 µM reduced cell viability by 8.30%±0.15 to 60.16%±9.61.

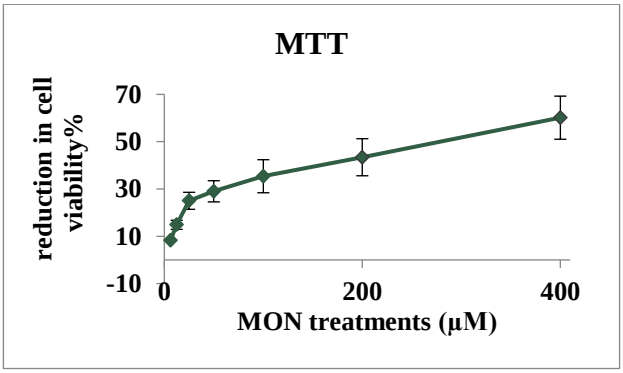


Figure 1. Effects of MON (0-400 µM) on cell viability, assessed using the MTT assay, in HepG2 cells following 24 hours of exposure.
Data are expressed as mean ±SD. (Statistical analysis was conducted using ANOVA, followed by Dunnett's post hoc test. Significant differences are denoted by * P< 0.001)

Table 2. 5-mC% levels in HepG2 cells exposed to 0–50 µM MON for 24 hours.

MON treatments (µM)	5-mC%±SD	P
Control	2.54±0.7	
5	5.81±1.6	0.211
10	9.49±1.2*	0.024
50	9.78±2.01*	0.021

SD: Standard deviation

Global DNA Methylation: Figure 2 illustrates the effects of MON treatment on 5-mC% levels in HepG2 cells after 24 hours of exposure to MON. 5-mC% levels were found to be 2.53±0.7 for control group and 5.81±1.6; 9.48±1.19; 9.78±2.01 for 5 µM, 10 µM, and 50 µM of MON treatments, respectively. A concentration-dependent increase in global DNA methylation was observed, with 5 µM, 10 µM, and 50 µM of MON treatments resulting in 2.28-fold, 3.73-fold (P<0.05), and 3.85-fold (P<0.05), respectively, compared to the control group.

Levels of Gene Expression: HepG2 cells treated with MON at doses of 5, 10, and 50 µM showed a dose-dependent increase in *DNMT1* and *DNMT3A* gene expression (Figure 3). Significant upregulations were observed at 10 and 50 µM of MON with expression levels increasing by 2.18-fold (P<0.05) and 3.47-fold (P<0.001), respectively (Figure 3a). However, at 5 µM of MON, *DNMT1* expression exhibited a non-significant 1.7-fold increase in comparison to the control group. Similarly, significant increases of 2.22-fold (P<0.05) and 2.78-fold (P<0.001) were observed at 10 and 50 µM of MON, respectively, while *DNMT3A* gene expression demonstrated a non-significant 1.42-fold increase at 5 µM of MON (Figure 3b). In contrast, *DNMT3B* expression showed non-significant changes, with decreases of 39% and 11% at concentrations of 5 and 10 µM of MON, respectively, and a 1.04-fold increase at 50 µM of MON. (Figure 3c).

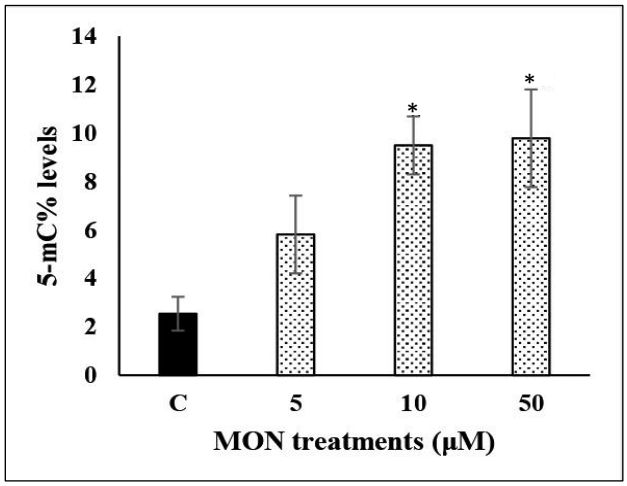


Figure 2. Effects of MON (5, 10 and 50 µM) on 5-mC% levels in HepG2 cells after 24 hours of exposure.

Data are expressed as mean ±SD. Statistical analysis was conducted using ANOVA, followed by Dunnett's post hoc test. Significant differences are denoted by * P< 0.05. (The P values are 0.211, 0.024, 0.021for 5-mC%, respectively, at 5, 10 and 50 µM of MON).

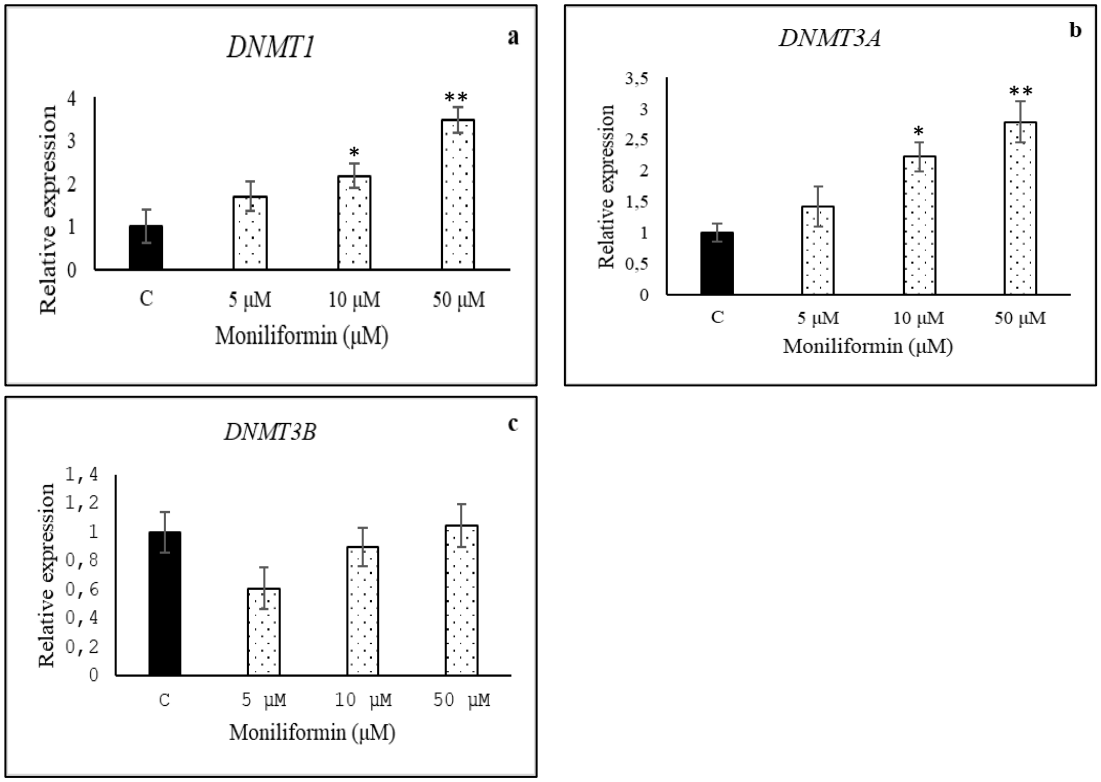


Figure 3. Effects of MON (5, 10 and 50 µM) on relative gene expression levels of *DNMT1* (a), *DNMT3A* (b) and *DNMT3B* (c) in HepG2 cells after 24 h exposure.

Data are expressed as mean ±SD. Statistical analysis was conducted using ANOVA followed by Dunnett's post hoc test. Significant differences are denoted by *P< 0.05; **P< 0.001. (The P values are 0.121, 0.008 and 0.000 for *DNMT1*; 0.260, 0.002 and 0.000 for *DNMT3A*; and 0.051, 0.735 and 0.957 for *DNMT3B*, respectively, at 5, 10 and 50 µM of MON).

Significant upregulation of *TET1* gene expression was observed at concentrations of 5, 10, and 50 µM of MON, with 2.37-fold (P<0.05), 2.92-fold (P<0.05) and 3.21-fold (P<0.001) changes, respectively (Figure 4a). In contrast, *TET2* expression was significantly downregulated with decreases by 37% (P<0.05) and 42% (P<0.05) at 10 and 50 µM of MON, while no significant change (23%, decrease) was observed at 5 µM of MON (Figure 4b). *TET3* expression non-significantly decreased by 24% at 5 µM, and increased slightly but dose-dependently by 1.02-fold and 1.44-fold at 10 and 50 µM, respectively. (Figure 4c).

Molecular Docking Results: MON formed hydrogen bonds with guanine 10 (DG10) and cytosine 11 (DC11) of chain A, as well as with adenine 17 (DA17) of chain B in B-DNA. The estimated binding free energy of the chosen conformation was -3.83 kcal/mol. Phenylalanine 1145 (PHE1145) and asparagine 1190 (ASP1190) residues were involved in hydrogen bonds between MON and DNMT1. The free binding energy of the chosen conformation was estimated at -4.6 kcal/mol. ASP641, threonine 645 (THR645), and tryptophan 893 (TRP893) residues of DNMT3A formed hydrogen bonds with MON. The free binding energy of this interaction was calculated to be -4.31. Leucine 580 (LEU580), PHE 581, THR 586, and Glycine 587 (GLY587) residues of DNMT3B formed hydrogen bonds with MON. The binding free energy of the selected conformation was estimated to be −4.3 kcal/mol. Additional interactions between MON and the macromolecules, together with 2D and 3D visualizations, are presented in Figure 5.

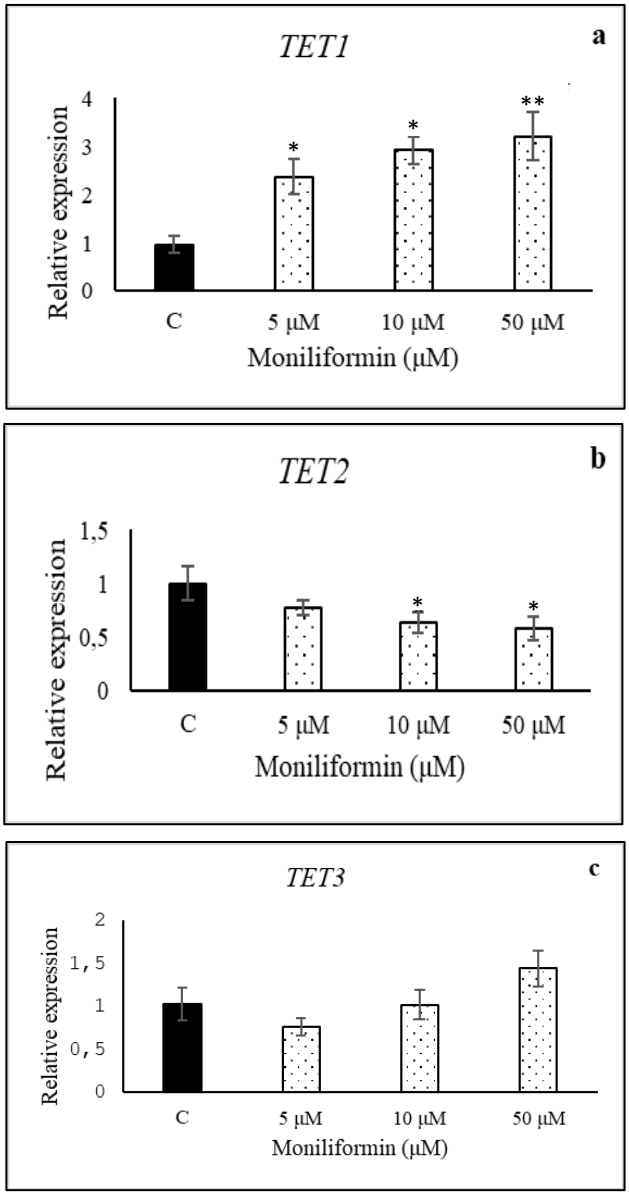


Figure 4. Effects of MON (5, 10 and 50 µM) on relative gene expression levels of *TET1* (a), *TET2* (b) and *TET3* (c) in HepG2 cells after 24 h exposure.

Data are expressed as mean ±SD. Statistical analysis was conducted using ANOVA followed by Dunnett's post hoc test. Significant differences are denoted by *P< 0.05; **P< 0.001. (The P values are 0.009, 0.001 and 0.000 for *TET1*; 0.187, 0.031 and 0.028 for *TET2*; and 0.332, 1.000 and 0.065 for *TET3*, respectively, at 5, 10 and 50 µM of MON).

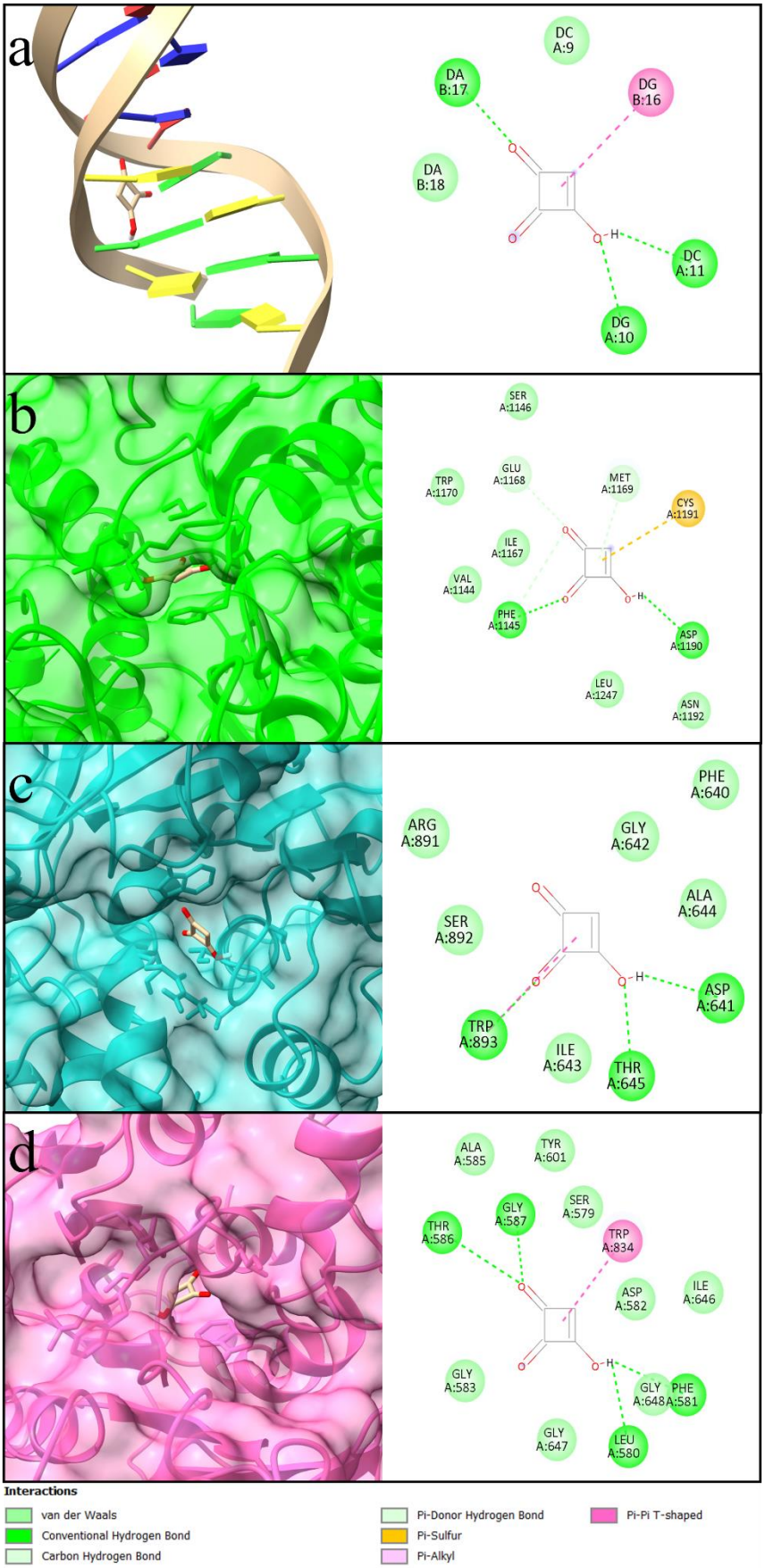


Figure 5. 2D and 3D illustrations of docking results. B-DNA (a), DNMT-1 (b), DNMT3A (c), DNMT3B (d) interactions with MON are provided.

Interactions in 2D diagrams are shown with dotted lines.

Discussion and Conclusion

The toxic effects of MON in the liver are related to mitochondrial stress (29) and oxidative damage (11), and possible epigenetic mechanisms may also contribute to its toxicity. In the present study, the effect of MON on DNA methylation, a key molecular epigenetic mechanism, was investigated in HepG2 cells. IC_{50} values of MON were indicated as 39.5 μM in HepG2 for 48 h, 26.8 μM in HepG2 for 72 h (8), 22 μM in human lymphocytes, >100 μM in human leukemia cell line K-562 (47), and 4.1 μM in progenitors of red blood (18). In the present study, the IC_{50} of MON was 253±8.41 μM in HepG2 cells after 24 hours The cytotoxic effects of MON depended on cell type and exposure duration.

DNA methylation is one of the most common epigenetic modifications, playing an important role in the regulation of gene expression, genomic stability, and cellular differentiation (36). It also plays a critical role in liver toxicity and carcinogenesis associated with chemical exposures (52). In the present study, MON exposure increased 5-mC% and the expression of the *DNMT1* and *DNMT3A* genes in a dose-dependent manner. To the best of our knowledge, no study has specifically addressed the effects of MON on global DNA methylation. However, DNA methylation changes have been well documented for other *Fusarium* toxins, such as fumonisin B1 (FB1). 200 μM FB1 exposure in HepG2 cells causes a decrease in global DNA methylation and the levels of *DNMT1*, *DNMT3A*, and *DNMT3B* (12). In a study on human colorectal adenocarcinoma cells (Caco-2), an increase in global DNA methylation was detected after 10 μM FB1 exposure for 72 hours (33). In another study, it was reported that global DNA methylation was significantly increased in human embryonic kidney (HEK293) cells due to 100 μM FB1 exposure for 24 hours (46). In a recent study on human kidney cells (HK-2), increases in *DNMT3A* and *DNMT3B* levels were observed after 100 μM FB1 exposure for 24 hours, while decreases in *DNMT1* levels were observed after 50 μM and 100 μM exposure (32). As seen in FB1, which affects epigenetic changes, MON's effect on epigenetic mechanisms may be important. In this study, a dose-dependent increase in *TET1* gene expression was observed in HepG2 cells after MON treatment. *TET1* is known to play roles in active DNA demethylation, developmental processes, maintenance of embryonic stem cell pluripotency, maturation of the intestinal epithelium, liver regeneration, and neuronal reprogramming (50). It has also been associated with tumour-suppressing effects (42) and with the regulation of inflammation and lipid metabolism (10). An increase in *TET1* in liver cells may reflect reprogramming, a stress response, or the need to reverse DNA methylation following MON exposure. In the present study, MON treatment decreased *TET2* gene expression in HepG2 cells, suggesting that MON may have an epigenetic suppressive effect via *TET2*. *TET2* is associated with hepatocyte differentiation, metabolic regulation, and anti-inflammatory responses in the liver (13). Decreased *TET2* expression may trigger processes such as liver dysfunction or carcinogenesis through increased oxidative stress or chronic inflammation (13, 42, 49). *TET3* plays a critical role in DNA demethylation during embryonic development and zygote formation (25) and is linked to metabolic balance and to the functions of the immune and nervous systems (50). In the present study, the slight increase in *TET3* may represent a compensatory response to the balance of DNA methylation. Docking studies between xenobiotics and the B-DNA dodecamer are performed to predict and evaluate the binding modes of genotoxicants. In the docking results obtained in this study, MON formed three hydrogen bonds with DNA. However, the estimated free binding energy was less negative (i.e., higher) than the commonly accepted threshold of -6.0 kcal/mol, suggesting that MON may not be genotoxic. However, it should be noted that genotoxicity may be observed at higher MON concentrations *in vitro* and *in vivo*. This finding is consistent with predictions obtained from ADMETlab 3.0. Similar to the B-DNA docking results, MON also formed hydrogen bonds with DNMT1, DNMT3A, and DNMT3B, but the binding energies were less negative than the generally accepted bioactivity threshold of -6.0 kcal/mol. This suggests that MON may not disrupt the enzymatic activity of DNMTs, which could contribute to the observed DNA hypermethylation. As shown, MON caused changes in the expression of *DNMT* and *TET* genes, resulting in an imbalance of DNA methylation. These findings may indicate the onset of disease processes in liver tissue. Further studies should be conducted to determine whether this epigenetic response is protective or pathological following MON exposure. The present study introduces a novel mechanism underlying MON induced liver injury. The HepG2 cell line is commonly used as an alternative to animal models in toxicity testing due to its ease of handling, low cost, genetic similarity to humans, and absence of ethical concerns. However, findings from *in vitro* and *in silico* analyses should be further verified by *in vivo* studies and supported by epidemiological data for a more robust risk assessment. Data obtained from more complex liver *in vitro* models and cell lines of different origins could enhance our understanding of MON toxicity at the organismal level. Mechanistically, additional gene-specific methylation analyses may provide further insights into the toxicological outcomes associated with the global DNA methylation alterations observed in this study.

In conclusion, epigenetic alterations serve as valuable biomarkers for assessing the toxicity of

environmental toxicants. The findings of this study suggest that MON may influence global DNA methylation by altering the expression of the *DNMT1*, *DNMT3A*, *TET1*, and *TET2* genes. The interactions between DNMTs and MON are unlikely to disrupt DNMTs' enzymatic activity, consistent with the observed global DNA hypermethylation. These findings suggest that MON exposure may exert long-term biological effects through epigenetic mechanisms and highlight the importance of incorporating epigenetic endpoints into future risk assessment frameworks for this mycotoxin.

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Ethical Statement

Ethical approval was not required.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Author Contributions

ZC and SO designed the study; ZC, ZRG and MCC performed the experiments; *in silico* molecular docking studies were performed by MCC; ZC and SO analyzed the data and performed the computational analyses; ZC wrote the manuscript. SO reviewed a draft of this manuscript and made comments. All authors read and approved the final manuscript.

Data Availability Statement

Upon reasonable request, the corresponding author will provide the data that support the conclusions of this study.

Animal Welfare

No animals were used in the experiments.

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