ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at: www.jgpt.co.in

RESEARCH ARTICLE

Sulforaphane and Bardoxolone (CDDO)-Induced Inhibition of Aflatoxin B1-Mediated Genotoxicity in Human Lymphocytes: Role of CYP3A4 and CYP1A1 Gene Expression

Safa M. Abdulateef [1, 2], Nemat J. Al-Judy[2]

[1.] *Department of Medical Lab. Techniques, Dijlah University College, Baghdad, Iraq.*

[2.] *Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.*

Abstract

Real-time PCR used to investigate the ability of sulforaphane (SFN) and Bardoxolone (CDDO) on inhibition of aflatoxin B1 genotoxicity in Human lymphocytes *in vitro*. Real time PCR analysis carried out for AFB1 treated lymphocytes with/without SFN and CDDO separately to assess its effects on global transcription through monitoring gene expression variation among genes responsible for AFB1 biotransformation including those involving in AFB1 bio activation like *CYP*1A1 and *CYP3A4*, calibrated with *B-actin* gene. Lymphocytes incubated with 10 and 100ng/ml of AFB1 separately and simultaneously with two different anti-proliferative, anti-inflammatory agents (SFN and CDDO). Protective effect of SFN and CDDO required co-treatments with AFB1. Human lymphocytes incubated with 10 and 100ng/ml AFB1 mixed with 10 and 50µM SFN respectively for 2 hr., on the other hand lymphocytes incubated with 10 and 100ng/ml AFB1 mixed with 10 and 50µ M CDDO respectively for 2hr. *CYP1A1* expressed more than *CYP3A4* in human lymphocytes; Transcriptional repression for genes involved in AFB1 bio activation was showed after treating with SFN and CDDO. SFN able to inhibit *CYP1A1* expression more than CDDO, SFN inhibit *CYP1A1* to (~4.32) fold comparing with separately AFB1 treated cells (P<0.05*).

Introduction

Mycotoxins are secondary metabolites produced by toxigenic strains of different species of fungi. Aflatoxin B1 (AFB1) is one of the most important mycotoxins due to its hepatotoxic and carcinogenic effects on certain animal models and humans [1, 2]. *Aspergillus flavus* and *Aspergillus parasiticus* are the most important fungi responsible for its production [3, 4]. Aflatoxins (AFs) undergo biotransformation, this process aimed to converting the original molecules into more hydrophilic compounds readily excreted in the urine. This process occurs in two phases known as Phase I and Phase II [5].

AFB1 is bioactivated by Phase I [Cytochrome P450 (*CYP450*)], producing reactive metabolite, known as aflatoxin-8, 9-epoxide (AFBO). AFs undergo Phase I metabolism by oxidation reactions including epoxidation, hydration, hydroxylation and O-demethylation reactions involving the CYP 450 mainly in the liver to produce AFB1-exo-8,9-epoxide (AFBO), AFB2a, AFM1, AFQ1 and AFP1 that are excreted in bile and urine after conjugation[6]. *CYP450s* are a large superfamily of heme binding enzymes involved in the synthesis and metabolism of endogenous substrates as well as in the biotransformation of xenobiotics like aflatoxins [7]. The AFB1 is metabolized in the body by group one and three of CYP gene mainly *CYP1A1, CYP1A2, CYP3A4, CYP3A5,* and *CYP3A7* [8]. *CYP* families play key roles in metabolic pathways of AFB1 in the body. Main role in AFB1 activation is played by *CYP3A4, CYP1A2* and *CYP1A1*, *CYP1B1, CYP2A1* and *CYP2A6* can form AFBO. *CYP3A4* and *CYP1A2* can also change AFB1 to less toxic forms, AFQ1 and AFM1, respectively [9].

Only exo-AFBO, produced by *CYPs*, can bind to N7-guanine thus causing mutation [10]. Since the discovery of sulforaphane (SFN) in 1992 and the recognition of the bioactivity of this phytochemical, many studies have examined its mode of action in cells, animals and humans. Broccoli, especially as young

©2009-2019, JGPT. All Rights Reserved

sprouts, is a rich source of SFN and broccoli-based preparations are now used in clinical studies probing efficacy in health preservation and disease mitigation. On another hand synthetic triterpenoid analogues of oleanolic acid, bardoxolone [cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO)] act as anti-inflammatory, antioxidant, anti-proliferative, anticancer, and anticarcinogeni compounds , CDDO have been used for medicinal purposes because of their properties. Are potent inducers of the phase II response as well as inhibitors of inflammation? Triterpenoid is a highly potent chemo preventive agent that inhibits aflatoxin-induced tumorigenesis [11, 12].

Materials and Methods

SFN, CDDO and AFB1

SFN and CDDO were purchased from Cayman chemical Company while AFB1 was purchased from Enzo life science Company.

Preparation, Culturing, and Treatment of Human Lymphocytes

Blood samples were taken from several volunteers with no history of using any of known inducer/ inhibitor of (*CYP450s*) drugs and almost homogenous (18–21 years old). From those volunteers, heparinized blood samples were applied to lymphocyte isolation [13, 14]. Heparinized blood samples separately by syringe take 2ml of blood and diluted with 2ml of Roswell Park Memorial Institute medium(RPMI1640) or phosphate buffered saline (PBS) and then gently layered above 4ml of Ficoll (Lymph prep lymphocyte separation media) and finally centrifuged at 400xg or (2500rpm) for (30min).

The cloudy white layer (creamy web like layer) arising between the lymph prep. Layer and plasma layer was transferred to sterile test tube with one ml of RPMI1640. If there is a drop or tiny drop of blood must be lysed by adding 500µl of lysis buffer then set for 5min then centrifuge again at 2500rpm for 10min by adding 1ml of RPMI1640. Discard the supernatant and re suspended the precipitate with 1ml of RPMI and centrifuge at 2500rpm for 10min. Repeat the previous step two times for washing lymphocyte from any debris. After final washing step remove the supernatant and re suspended the precipitate (lymphocyte) with 1ml of RPMI1640. The isolated lymphocyte from each sample were transferred and seeded in polystyrene 24-well tissue culture plates containing RPMI medium with 10% heat-inactivated FBS, final volume in each well 250µl, then incubated for 24 h in a 37 °C incubator containing 95% humidity and 5% CO2. Cells were treated, in duplicate, with two doses of AFB1 (final concentration of 10 and 100 ng/ml) separately and simultaneously and two doses SFN or CDDO (final concentrations of 50 and 10 µM) with final reaction volume 500µl (250µl cell+250µl treatment). After 2 h incubation with treatments, the lymphocytes in the wells were separately collected by centrifugation (3000_g, 4 min). After elapsing incubation period, the contents of each well were collected and used for molecular analyses.

Molecular Analysis of Human Lymphocyte RNA

RNA isolation accomplished by using TRI-ZOL kit provided from Ambion life Technologies Company. Quantus Florometer was used to detect the concentration of extracted RNA in order to detect the goodness of samples for downstream applications. Real time PCR was used for measuring *CYP1A1 and CYP3A4* expression calibrating with *B-Actin* gene for treated and control cells. Total RNA from treated cultures and untreated culture were extracted and purified using TRIZOL reagent. The expression of genes was quantified using the Syber Green reagent (1-Step RT-qPCR Kit). Primers were designed according to Gross-steinmeyer *et al*., (2005) and checked according to http://www.incbi.com. The real time was performed using MIC System. Primers were obtained from Alpha DNA Company [15].

Table (1) showed the primers and their sequences are used in Real time PCR analysis. Fluorescence signals were measured over 40 PCR cycles. The cycle number (Ct) at which the signals crossed a threshold set within the logarithmic phase was recorded. Expression levels were quantified using relative quantitation, the difference in cycle threshold (ΔCt) and fold difference evaluated between the treated group and control of each gene. Each real time PCR reaction was done in a 10 µl final volume containing 1µl of specific forward and reverses primers, 5 µlGo Taq 1-Steps RT-qPCR, 2 µl template and 0.25µl Reverse transcriptase mixture, then completing volume with nuclease free water.

©2009-2019, JGPT. All Rights Reserved

Real time PCR conditions for all genes were carried out (in duplicate) using a MIC system (Mic -4- /Australia) with a cycling program including holding for 15 min at 37°C for Reverse transcriptase and 5 min at 95°C as Hot start, followed by cycling 45-times at 95, 58, and 72 °C (20 s for each temperature) with melting curve analyses (72°C to 95°C at 0.3°C/s).

Table 1: Primer sequences were ordered for this study form Alpha DNA Company

Primers Name	Primer sequence (5′ – 3′)	Position
Beta-actin	AACCCCAAGGCCAACCG	372–388
	AGGGATAGCACAGCCTGGA	466–448
CYP1A1	ACCTTCCGACACTCTTCCTTCG	1245–1266
	CAGATGGGTTGACCCATAGCTTCT	1298–1275
CYP3A4	CACAGATCCCCCTGAAATTAAGCTTA	1516–1541
	AAAATTCAGGCTCCACTTACGGTG	1621–1598

Gene expression was calculated according to livak method [2^ΔΔCT], the following equation summarized the best way used to find folding for each gene and compared with controlled:

Folding =2^-ΔΔCT

ΔΔCT =ΔCT Treated - ΔCT Control

ΔCT =CT gene - CT House Keeping gene

Statistics

Comparisons of the means between the AFB1-treated and control were performed using a student's t-test. All real time assay data were analyzed using a one-way analysis of variance (ANOVA). A p-value≤0.05 was accepted as significant.

Results and Discussion

Real time PCR analysis was assessed to determine gene expression of xenobiotic metabolizing genes including *CYP1A1* and *CYP3A4*, real time- PCR then caliberate with *β-Actin* gene expression in human lymphocytes *in vitro* (Figure 1). In this study *CYP1A1* folding changes in human lymphocytes was examined after various treatments from AFB1, SFN and CDDO saparately, as well as *CYP1A1* folding changes studied again in AFB1 treated cells simultanously with SFN and CDDO at different doses to assess its effect on global transcription (Figure 2).

Comparing with control over expression of *CYP1A1* was showed in cells treated with 10ng/ml AFB1 as (~ 454.76±215.70) fold while the expression decreased to (~24.28±8.82) fold when treated with 100 ng/ml AFB1(P<0.05**), inhibition of *CYP1A1*gene expression was observed in AFB1 treated cells simultanously with CDDO and SFN separately. SFN able to inhibit *CYP1A1*expression more than CDDO, whereas SFN able to inhibit the expression to (~4.32±0.10) fold comparing with separately AFB1 treated cells (P<0.05*), (Table 2).Real time PCR was carried again to determine the ability of *CYP3A4* gene for metabolizing AFB1 in human lymphocytes *in vitro* and determined whether its activity could be diminsh with antiproliferative agent using or not. Minimal percentage of changing was showed in *CYP3A4* expression on cultured human lymphocyte when treated with AFB1, SFN and CDDO separately *in vitro*.

Effective antiproliferative drug CDDO able to induce *CYP3A4* expression on 100ng/ml AFB1 treated cells,whereas the expression upregulated to (~4.44±1.85) fold compared with control(P<0.05*), SFN act as inhibiter for *CYP3A4* in AFB1 treated cells, whereas 100ng/ml AFB1 mixed with 50μM SFN treatment able to inhibit *CYP3A4* expression in cells to (~0.61±0.04) fold less than control, (P>0.05), (Table2). Kim *et al.*, (2004) were tested the ability of CDDO to modulate the activities of several CYP450 enzymes using human liver microsomes[16].

Alena Vanduchova *et al.*, (2016) were studied the influence of SFN metabolites, natural

©2009-2019, JGPT. All Rights Reserved

compounds present in broccoli, on drug-metabolizing CYP450 enzymes in human liver. Their possible effect on four drug-metabolizing genes, *CYP3A4*, *CYP2D6*, *CYP1A2* and *CYP2B6*, was tested. Their result represented as Inhibition of four prototypical *CYP* activities by SFN metabolites[17].

Table 2: gene expression values for *B-actin*, *CYP1A1*and *CYP3A4* after treating with 10, 100 ng/ml AFB1 with and without inducers (SFN and CDDO) *in vitro*.

TREATMENT	B-ACTIN	CYP1A1	CY3A4
Control	19.61	1.00	1.00
AFB1 10ng/ml	23.54	454.76	1.09
AFB1 100ng/ml	22.41	24.28	0.55
SFN 10µM	22.88	31.31	2.57
SFN 50µM	16.1	22.76	0.05
AFB1 10ng/ml SFN 10µM	15.82	4.32	0.63
AFB1 100ng/ml SFN 50µM	21.65	29.32	0.61
CDDO 10µM	19.06	18.12	0.07
CDDO 50µM	18.69	70.25	1.05
AFB1 10ng/ml CDDO 10µM	18.66	58.07	1.50
AFB1 100ng/ml CDDO 50µM	19.96	34.45	4.44

Figure (2) summarized the expression of all studied genes in response to AFB1 separately or simultaneously with SFN or CDDO. *CYP1A1* highly expressed than *CYP3A4* in cultured human lymphocytes when treated with AFB1 *in vitro* comparing with control and calibrate with β *—Actin* gene.

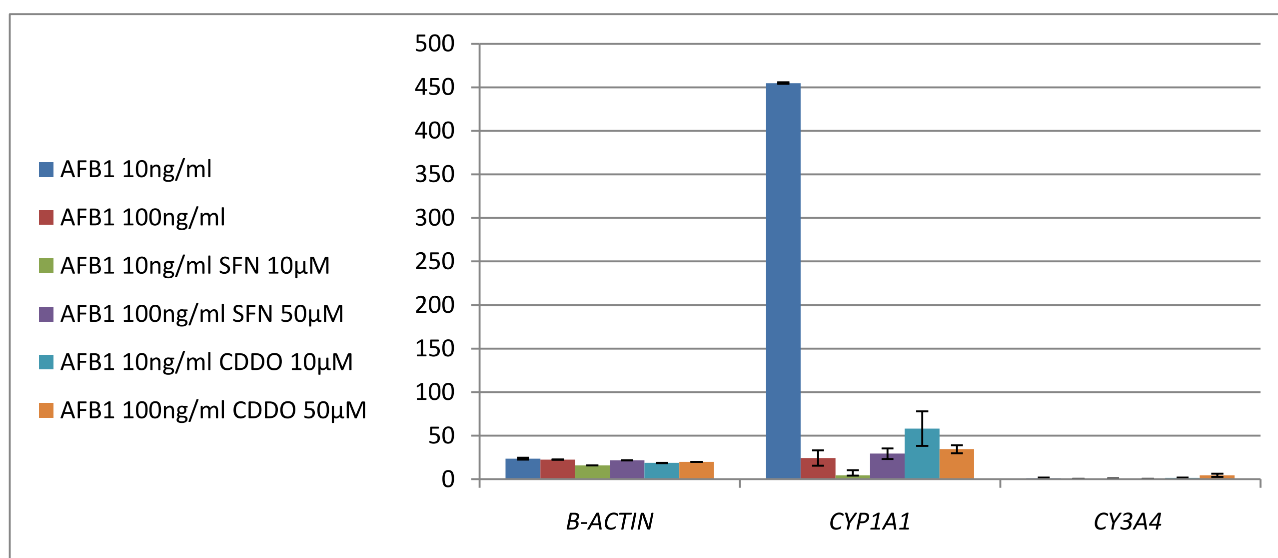

Figure 2: Gene expression quantification of CYPs isoforms including (*CYP1A1* and *CYP3A4*) in human lymphocytes exposed to different doses of AFB1 with/without SFN and CDDO *in vitro*

*CYP1A1*expression was up-regulated to (~454.76)fold when treated with 10ng/ml AFB1, while the expression was inhibited as (~4.32) fold in lymphocytes after co-treatment with 10ng/ml AFB1and 10µM SFN. In particular, cytochromes P450 (CYPs) are involved in the activation of AFB to the genotoxic aflatoxin B1-8, 9-oxide (AFBO) [18, 7, 19]. SFN may act as modulators of expression and/ or catalytic activity of phase I and II biotransformation enzymes that play key roles in the bioactivation of the hepatocarcinogenic mycotoxin aflatoxinB1 (AFB).

The modulatory effects of SFN on various DMEs have been studied in numerous *in vitro* and *in vivo* models, e.g., in human volunteers, laboratory animals, various cell lines and subcellular fractions. The modulation of DMEs is probably caused by the inhibition of the catalytic activity of various *CYP450* enzymes, which can activate certain carcinogens, and by the induction of some antioxidant and phase II enzymes' expression. Little is known about the effect of SFN in combination with *CYP* inducer [20]. On the xenobiotics are not reactive themselves, but exert toxicity only after

©2009-2019, JGPT. All Rights Reserved

metabolic activation by a variety of enzymes responsible for drug metabolism. *CYPs* are among the major enzymes involved in the activation of carcinogenesis [19].

Therefore, one target of the chemo-preventive effect of CDDO could be the inhibition of the metabolizing activity of *CYPs*. Taking these facts into account, inhibition by CDDO of the metabolic activation of pro-carcinogens and the toxic metabolite formation catalyzed by *CYPs* may be one of the mechanisms of carcinogenesis inhibition and hepatoprotective effects. Although reduction in the gene expression level of *CYPs* by these substances has been demonstrated [21], a detailed mechanism for the inhibition by CDDO of human *CYPs* has not yet been elucidated.

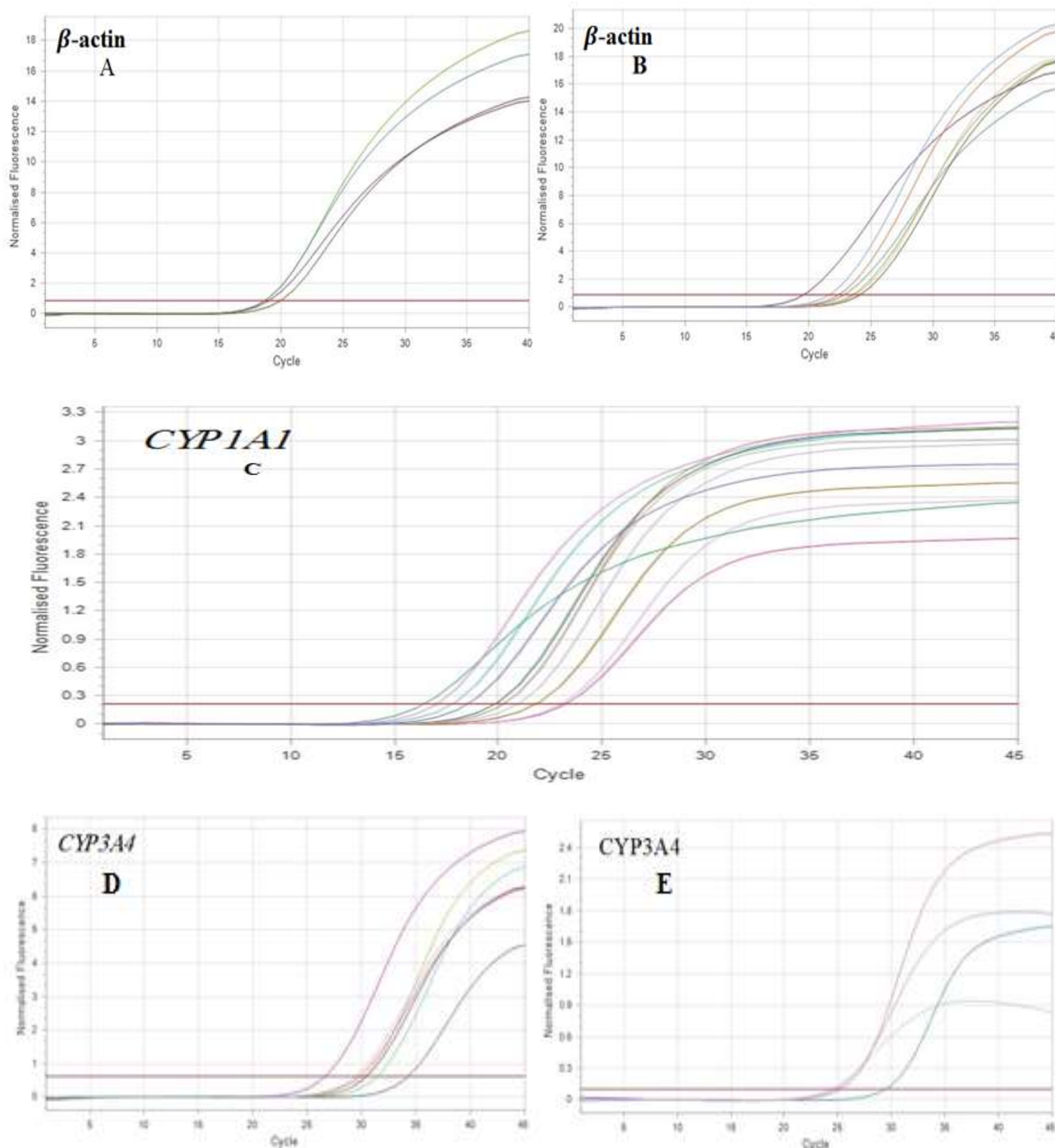

Figure 1: Real time PCR cycling for all genes after 2hour exposure to different concentration of AFB1, SFN and CDDO, (A)*B-actin* cycling of 7 samples including: control, AFB1 10ng/ml, AFB1 100ng/ml, SFN 10µM, SFN 50µM, AFB1 10ng/ml + SFN 10µM and AFB1 100ng/ml + SFN 50µM), (B)*B-actin* cycling of 4 samples including CDDO 10µM, CDDO 50µM, AFB1 10ng/ml + CDDO 10µM and AFB1 100ng/ml + CDDO 50µM. (C) *CYP1A1*cycling of 11 samples including: control, AFB1 10ng/ml, AFB1 100ng/ml, SFN 10µM, SFN 50µM, AFB1 10ng/ml + SFN 10µM, AFB1 100ng/ml + SFN 50µM, CDDO 10µM, CDDO 50µM, AFB1 10ng/ml + CDDO 10µM and AFB1 100ng/ml + CDDO 50µM. (D) *CYP3A4* cycling of 7 samples including: control, AFB1 10ng/ml, AFB1 100ng/ml, SFN 10µM, SFN 50µM, AFB1 10ng/ml + SFN 10µM and AFB1 100ng/ml + SFN 50µM, (E) *CYP3A4* cycling of 4 samples including CDDO 10µM, CDDO 50µM, AFB1 10ng/ml + CDDO 10µM and AFB1 100ng/ml + CDDO 50µM

©2009-2019, JGPT. All Rights Reserved

Conclusion

- Human lymphocytes showed different model of expression after treating with AFB1, SFN and CDDO. The diversity of expression results from the differences mechanism of action for each compound on human cells.
- *CYP1A1* was highly expressed comparing with *CYP3A4* on human lymphocytes when treated with different doses of aflatoxinB1.
- Excellent inhibition was observed for *CYP1A1* expression after co-treatment with AFB1 and SFN or CDDO, SFN able to inhibit *CYP1A1* activity more than CDDO, whereas when 10ng/ml AFB1 treated lymphocytes incubated with 10μM SFN for 2hr., expression down regulated to (~4.32).

References

1. Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D (2004) Human aflatoxicosis in developing countries: A review of toxicology, exposure, potential health consequences, and interventions. Am. J. Clin. Nutr., 80: 1106-1122.
2. Zain ME (2011) Impact of mycotoxins on humans and animals. Journal of Saudi Chemical Society, 15(2):129-144.
3. Sargeant K, Sheridan A, O'Kelly, J Carnaghan RBA (1961) Toxicity associated with certain samples of groundnuts. Nature 192:1096-1097.
4. Abdulateef SM (2013) Genetic variation among Aspergillus flavus isolates by using microsatellite marker. M.Sc. thesis in Mycology, Baghdad University/Iraq.
5. Lee SE, Campbell BC, Molyneux RJ, Hasegawa S, Lee HS (2001) Inhibitory effects of naturally occurring compounds on aflatoxin B1 biotransformation. Journal of agricultural and food chemistry, 49(11): 5171-5177.
6. Omar HE (2013) Mycotoxins-induced oxidative stress and disease. Intech, 3.
7. Gallagher EP, Wienkers LC, Stapleton PL, Kunze KL, Eaton DL (1994) Bioactivation of aflatoxin B1DNA-expressed cytochromes P4501A2 and P4503A4 in the role of human. Cancer Research, 54: 101-108.
8. Gross-Steinmeyer K, Eaton DL (2012) Dietary modulation of the biotransformation and genotoxicity of aflatoxin B (1). Toxicology, 299: 69-79.
9. Kensler TW, Roebuck BD, Wogan GN, Groopman JD (2011) Aflatoxin: a 50-year odyssey of mechanistic and translational toxicology. Toxicological sciences, 120(1): S28-S48.
10. Langouët S, Coles B, Morel F, Becquemont L, Beaune P, Guengerich FP, Ketterer B, Guillouzo A(1995) Inhibition of CYP1A2 and CYP3A4 by oltipraz results in reduction of aflatoxin B1 metabolism in human hepatocytes in primary culture. Cancer Research, 55(23): 5574-5579.
11. Yates MS, Kwak MK, Egner PA, Groopman JD, Bodreddigari S, Sutter TR, Baumgartner KJ, Roebuck BD, Liby KT, Yore MM, Honda T (2006) Potent protection against aflatoxin-induced tumorigenesis through induction of Nrf2-regulated pathways by the triterpenoid 1-[2-cyano-3-, 12-dioxooleana-1, 9 (11)-dien-28-oyl] imidazole. Cancer research, 66(4): 2488-2494.
12. Wang YY, Yang YX, Zhe H, He ZX, Zhou SF (2014) Bardoxolone methyl (CDDO-Me) as a therapeutic agent: an update on its pharmacokinetic and pharmacodynamic properties. Drug design, development and therapy, 8: 2075.
13. Boyum A (1968) Separation of lymphocytes from blood and bone marrow. Scand. J. Clin. Lab. Invent, 21:97.
14. Rafael F, Vaclav V (2000) Advanced Methods in Cellular Immunology, 3.CRC-Press LLC.U.S.A.
15. Gross-Steinmeyer K, Stapleton PL, Tracy JH, Bammler TK, Lehman T, Strom SC, Eaton DL (2005) Influence of Matrigel-overlay on constitutive and inducible expression of nine genes encoding drug-metabolizing enzymes in primary human hepatocytes. Xenobiotica, 35(5): 419-438.
16. Kim KA, Lee JS, Park HJ, Kim JW, Kim CJ, Shim IS, Kim NJ, Han SM, Lim S (2004) Inhibition of cytochrome P450 activities by oleanolic acid and ursolic acid in human liver microsomes. Life sciences, 74(22): 2769-2779.
17. Vanduchova A, Tomankova V, Anzenbacher P, Anzenbacherova E (2016) Influence of Sulforaphane Metabolites on

©2009-2019, JGPT. All Rights Reserved

Activities of Human Drug-Metabolizing Cytochrome P450 and Determination of Sulforaphane in Human Liver Cells. Journal of medicinal food, 19(12):1141-1146.

18. Eaton DL, Gallagher EP (1994) Mechanisms of aflatoxin carcinogenesis. Annual review of pharmacology and toxicology, 34(1): 135-172.
19. Guengerich FP, Shimada T (1991) Oxidation of toxic and carcinogenic chemicals by human cytochrome P-450 enzymes. Chemical Research in Toxicology, 4: 391-407.
20. Lnenickova K, Dymáková A, Szotáková B, Boušová I (2017) Sulforaphane Alters ß-Naphthoflavone-Induced Changes in Activity and Expression of Drug-Metabolizing Enzymes in Rat Hepatocytes. Molecules, 22(11): 1983.
21. Jeong HG (1999) Inhibition of cytochrome P450 2E1 expression by oleanolic acid: hepatoprotective effects against carbon tetrachloride-induced hepatic injury. Toxicology Letters, 105.

©2009-2019, JGPT. All Rights Reserved