The protective effects of demethoxyviridine and 1-α-hydroxy-demethoxyviridine in the livers of male rats treated with diethylnitrosamine and 2-acetylaminflourene

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ABSTRACT

الأهداف: لتحديد الآثار الوقائية للاستقلاب البؤري لعقار ديميثوكسيفيريدين (DMV) ومشتقه، (1-α-hydroxy-DMV) في الكبد لدى الجرذان الذكور نوع سبراكيو- داولي والبالغة من العمر شهرين، والذين تمت معالجتهم بعقار (DEN) و (2-AAF).

الطريقة: أجريت الدراسة بقسم الأحياء الطبية – كلية الطب – جامعة عثمان غازي – تركيا – في مايو 2006م. قُسمت حيوانات الدراسة إلى عشر مجموعات وهي: مجموعة التحكم، مجموعة زيت الزيتون، مجموعة DMSO، مجموعة DMV، مجموعة 1-α-hydroxy-DMV، مجموعة DEN، مجموعة 2-AAF، مجموعة DEN+2-AAF، مجموعة DEN+2-AAF+DMV، ومجموعة DEN+2-AAF+1-α-hydroxy-DMV. تم إعداد الكبد من الجرذان وتم تحديد مستوى ظهور أنزيمات (CYP1A2) بواسطة تقنية اللطخة الغربية. تم تقييم شرائح نسيج الكبد من الناحية النسيجية المرضية مع صبغة (H&E) من الناحية الكيمائية المناعية من اجل الحصول على (Ha-Ras, GST-p)، وبروتينات (Cx32).

النتائج: من الملاحظ أنه لم يكن هنالك فروقات ظاهرة في مستويات (CYP1A2) بين مجموعة التحكم، ومجموعة زيت الزيتون، ومجموعة DMSO في الجرذان المعالج. وكان مستوى (CYP1A2) منخفضا بشكل ملحوظ لدى مجموعة 2-AAF، ومجموعة DEN+2-AAF، ومجموعة DEN، ومجموعة DEN+2-AAF+DMV، ومجموعة DEN+2-AAF+1-α-hydroxy-DMV، ومجموعة 1-α-hydroxy-DMV، ومجموعة DMV، بالمقارنة مع مجموعة التحكم. تبين وجود معظم البؤر قبل الإصابة بالورم في مجموعة DEN+2-AAF. في مجموعة DEN+2-AAF، كان تحول (Ha-Ras) وجلوتاثيون-S إلى (GST-p) أكثر من مجموعة DEN+2-AAF+DMV ومجموعة DEN+2-AAF+1-α-hydroxy-DMV المعالجة.

خاتمة: لدى (DMV) و (1-α-hydroxy-DMV) أثر وقائي في الكبد من (DEN) و (2-AAF) و (DEN+2-AAF) في الجرذان المحرضة.

Objectives: To determine the protective effects of a fungal metabolite of demethoxyviridine (DMV) and its derivative, 1-α-hydroxy-DMV in the livers of 2-month-old male Spraque-Dawley rats treated with diethylnitrosamine (DEN) and 2-acetylaminflourene (2-AAF).

Methods: This study was performed in the Department of Medical Biology, Faculty of Medicine, Eskisehir Osmangazi University, Eskisehir, Turkey from May 2006. Animals were divided into 10 groups. Those were the control, olive oil, dimethyl sulfoxide (DMSO), DMV, 1-α-hydroxy-DMV, DEN, 2-AAF, DEN+2-AAF, DEN+2-AAF+DMV, and DEN+2-AAF+1-α-hydroxy-DMV-treated animal groups. The liver microsomes were prepared from rats and the levels of expression of cytochrome P450 1A2 (CYP1A2) enzymes were determined with western blot technique. The liver tissue slides were evaluated histopathologically with hematoxylin and eosin staining and immunohistochemically for Harvey-retrovirus associated DNA sequences (Ha-Ras), glutathione S- transferase (GST-p), and connexion-32 (Cx32) proteins.

Results: Notably, there were no appreciable differences in CYP1A2 level among control, olive oil, and DMSO-treated animals. The CYP1A2 level was significantly decreased in 2-AAF, DEN+2-AAF, DEN, DEN+2-AAF+DMV, DEN+2-AAF+1-α-hydroxy-DMV, 1-α-hydroxy-DMV, and DMV-treated animals as compared to the control. Most prenoplastic focus was found in DEN+2-AAF treated group.

Conclusion: Demethoxyviridine and 1-α-hidroksi-DMV had protective effect in the livers of DEN, 2-AAF and DEN+2-AAF induced rats.

Saudi Med J 2008; Vol. 29 (9): 1241-1246

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Received 25th June 2008. Accepted 12th August 2008.

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Hepatocarcinogenesis is one of the most frequent cancers in the world and associated with exposure to environmental factors including diethylnitrosamine (DEN), 2-acetylaminofluorene (2-AAF), aflatoxin B1 metabolite, alcohol consumption, phenoparbital and hepatitis B viruses. Diethylnitrosamine initiates preneoplastic liver lesions while 2-AAF promotes it in hepatocyte.[1-4] The tumors induced by DEN almost exclusively cause harbor mutations in codon 61 of the Ha-Ras proto-oncogene and decrease connexin 32 (Cx32) expression.[5,6] Direct interaction with guanosine triphosphate (GTP)-complexed p21Ras has also been demonstrated for some proteins including members of the Raf kinase family and PI3 kinase, suggesting that these proteins represent downstream effectors of Ras. The activation of Ras is involved in the activation of mitogen-activated protein kinase (MAP kinase), which can play a pivotal role in cell proliferation.[6,7] Wortmannin was shown to inhibit phosphoinositide 3-kinase (PI-3 kinase activity) in human MCF-7 mammary tumors in vivo.[8] A fungal metabolite of demethoxyviridine (DMV) and 1-α-hydroxy-DMV are structural analogues of wortmannin. Also, DMV was discovered as an specific inhibitor of IP-3 kinase.[9] Glutathione S- transferase (GST-p) is one of the best markers for detection of preneoplastic cells in the chemical hepatocarcinogenesis models. Expression appears very early in initiated hepatocytes and defined GST-p stained focuses in the early stage hepatocarcinogenesis in DEN+2-AAF treated rats.[10] Diethylnitrosamine is defined as a widely occurring carcinogenic nitrosamine that requires oxygenation of a-carbon catalyzed by cytochrome P450 (CYP) for its DNA-damaging activity. A single injection of DEN might result in more than 2-AAF being transformed to N-hydroxy-2-acetylaminofluorene (N-OH-AAF). Based on this proposal, it could be concluded that alteration of expression of CYP1A2 might contribute to the hepatocytes within the preneoplastic focus. The acquisition of resistance is against 2-AAF in hepatocyte whose CYP1A2 could be over expressed in the hepatocytes of DEN-2-AAF treated rats. The relationship between CYP1A2 and defined preneoplastic changes in the early stage of hepatocarcinogenesis is still unclear. The expression level of CYP1A2 was decreased in DEN and 2-AAF induced Spraque-Dawley rats via western blot technique.[11] The aim of this study was to determine the protective effects of DMV and 1-α-hydroxy-DMV on the livers of 2-month-old male Spraque-Dawley rats treated with DEN and 2-AAF.

Methods. A fungal metabolite of DMV was biosynthesized with Nodulisporium hinnuleum and its derivative, 1-α-hydroxy DMV was synthesized from DMV by following literature methods.[9] Two-month-old 84 male Spraque-Dawley rats were used, which were trained in the Department of Medical Biology, Faculty of Medicine, Eskisehir Osmangazi University, Eskisehir, Turkey from May 2006. Approval from Ethics Committee was taken in the same University. Rats were fed with commercial standard diet and tap water ad libitum. The studied animals were divided into 10 groups (**Table 1**). Diethylnitrosamine (175 mg/kg) and 2-AAF (20 mg/kg), both dissolved in 0.1 ml dimethyl sulfoxide (DMSO), were administrated to the rats together with 0.1 ml olive oil through intraperitonal (IP) injection and gavage, respectively. Demethoxyviridine or 1-α-hydroxy-DMV (1.5 mg/kg) was dissolved in DMSO and administrated by IP. The rat liver tissue were removed under ether anesthesia in the 5th week of the study. The removed livers were fixed in formalin and processed routinely for paraffin embedding followed by preparing liver slides in 4 mm thickness. Tissues sections were then mounted on poly-L-lysine-coated slides and stained with hematoxylin and eosin for routine prenoplastic focus histology. For immunohistochemical study, tissue sections were fixed and treated with a mixture of methanol and hydrogen peroxide. The nonspecific binding was blocked with goat serum. Sections were incubated with antibody per Ha-Ras, GST-p and Cx32

Table 1 - Administration protocols of male Spraque-Dawley rats.

Groups	n	Initiation	Promotion (7, 8, and 9 days)	Treatment (16, 23, and 30 days)
Control	8	-	-	-
Olive oil	8		Olive oil	-
DMSO	8	DMSO	-	-
DMV	8	-	-	DMV
1-α-hydroxy DMV	8	-	-	1-α–hydroxy DMV
DEN	8	DEN	-	-
2-AAF	8	-	2-AAF	-
DEN+2-AAF	14	DEN	2-AAF	-
DEN+2-AAF+DMV	8	DEN	2-AAF	DMV
DEN+2-AAF+1-α-hydroxy-DMV	8	DEN	2-AAF	1-α-hydroxy DMV

DMSO- Dimethyl sulfoxide, DMV- demethoxyviridine, 1-α-hydroxy DMV-1-α-hydroxyl demethoxyviridine, DEN-Diethylnitrosamine, 2-AAF-2-acetylaminflourene

Disclosure: This study was supported by a grant of the Research Foundation of University of Eskisehir Osmangazi, Eskisehir, Turkey (Grant No. 200411012).

and then incubated with horseradish peroxidase (HRP) conjugated with antigoat IgG. After each section treated with streptavidin-peroxidase conjugate, color was developed with aminoethyl carbazole (AEC) chromate. Quantitative Sterology was supplied with Leice Image Analysis system using software Leica Qwin 500 for all sections. All images were captured with microscope attached with cooled camera device (CCD) camera and images were transferred to the computer. Each slide was then examined with fields. The pathologic scores were counted for the histological and immunohistochemical studies of the liver.

Briefly, frozen tissues were sliced very thinly and thawed in radio immuno precipitation assay (RIPA) buffer using 3 ml of ice-cold RIPA buffer per gram of tissue. The tissue was further disrupted and homogenized with sonicator, maintaining the temperature at 4°C throughout the study period and incubated on ice for 30 minutes. It was then transferred to a micro centrifuge tube, centrifuged at 10,000xg for 10 minutes at 4°C. The supernatant was removed and centrifuged again. The supernatant fluid was total cell lysate, which then centrifuged for 3 hours in order to obtain a clear lysate. The expression of CYP1A2 enzymes was determined with Western blot technique. The total protein was measured at lysate using the bicinchonic acid assay kit. Twenty nanogram purified protein with equal volume of 2x electrophoresis buffer was boiled for 4 minutes and unused samples were stored at -20°C. The loading buffer and sample combination were loaded up inside of 1mm together with molecular weight marker after the stacking and resolving gel. The standard protocol was used for the application of electrophoresis and proteins were transferred from gel to a nitrocellulose membrane using mini trans-blot. Protein that transferred to membrane was blocked in blotto. The blocked membrane was incubated in primary antibody diluted in blotto for one hour at room temperature, and then the membrane was washed with tris-Buffered sodium chloride solution with tween 20 (TBST) 3 times for 5 minutes followed by incubating the membrane with HRP conjugated secondary antibody for 45 minutes at room temperature. The membrane was again washed 3 times with TBS for 5 minutes and incubated in Chemiluminescence's Luminol Reagent for one minute in dark medium. Signals were transferred to a film and evaluated for CYP1A2 expression.

The data were presented as means±SEM and analyzed using the Statistical Package for Social Science for windows version 10.01 software. If data passed the normality test, the significance of the differences between groups was determined by Tukey's test, after significant analysis of variance. $P<0.05$ was considered statistically significant.

Results. Histological studies showed that no preneoplastic focus was found in the control, the olive oil, DMSO, DMV, and 1-a-hydroxy DMV groups, but DEN, 2-AAF, DEN+2-AAF, DEN+2-AAF+DMV, and DEN+2-AAF +1-α-hydroxy-DMV treated groups contained different degree of pathological effects. Diethylnitrosamine and 2-acetylaminofluorene treated group (**Figure 1**) had more preneoplastic focus than both DEN+2-AAF+DMV (**Figure 2**) and DEN+2-AAF +1-α-hydroxy-DMV treated groups (**Figure 3**).

Immunohistochemical studies indicated that there were no differences between the control and the olive oil, DMSO, DMV, 1-α-hydroxy-DMV, DEN, 2-AAF induced groups for Ha-Ras, GST-p, and Cx32 proteins. However, Ha-Ras and GST-p proteins counted in the stained areas were found to be higher in DEN+2-AAF induced group, however, they were lower in DEN+2-AAF+DMV and DEN+2-AAF+1-α-hydroxy-DMV induced groups (**Figures 4a & 4b**). In addition, the counted Cx32 proteins were the same in all studied groups (**Figure 4c**). The pathologic scores of both histological and immunohistochemical studies of the liver tissue sections were evaluated with the corresponding preparates and the counting was listed in **Table 2**. The amount of the CYP1A2 expressions were evaluated with western blot technique images and sample image was summarized in **Figure 5**.

Notably, there were no appreciable differences in CYP1A2 level among control, olive oil ($p=0.991$), and DMSO ($p=0.662$) treated animals. Cytochrome P450 1A2 level was significantly decreased in 2-AAF ($p=0.0000$), DEN+2-AAF ($p=0.000$), DEN ($p<0.000$), DEN+2-AAF+DMV ($p<0.000$), DEN+2-AAF+1-α-hydroxy-DMV ($p<0.000$), 1-α-hydroxy-DMV ($p<0.01$), and DMV ($p<0.085$)-treated animals as compared to the control (**Table 3**).

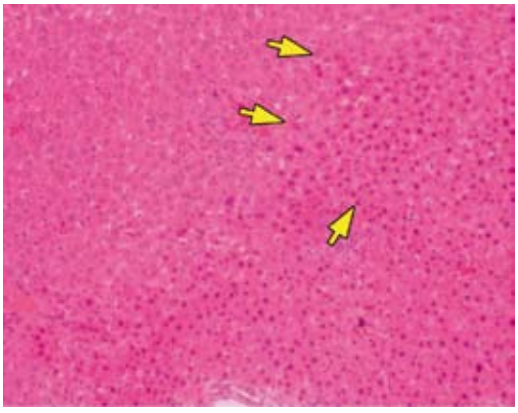

Figure 1 - The section of diethylnitrosamine+2-acetylaminflourene induced rats, which increased preneoplastic focus (yellow arrows) had found hematoxylin and eosin, original magnificationx100.

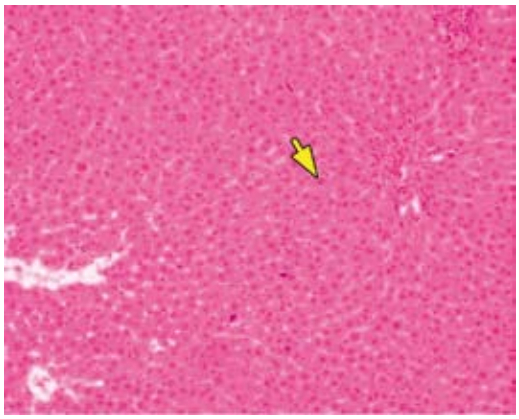

Figure 2 - The section of diethylnitrosamine+2-acetylaminflourene+de methoxyviridine induced rats, which decreased preneoplastic focus (yellow arrows) had found hematoxylin and eosin, original magnificationx100.

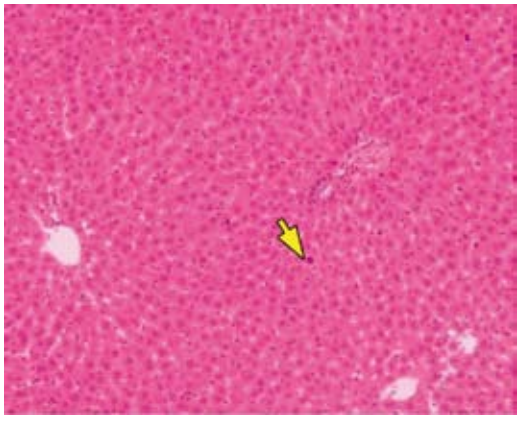

Figure 3 - The section of diethylnitrosamine+2-acetylaminflourene+1-α-hydroxy demethoxyviridine induced rats, which decreased preneoplastic focus (yellow arrows) had found hematoxylin and eosin, original magnificationx100.

Discussion. Initiation using DEN followed by promotion with the 2-AAF is well-established model of detecting early liver carcinogenesis in rats consistently showing to produce many lesions. The results obtained from this study were discussed below: Hyper basophilic focal lesions, basophilic foci, clear cell, eosinophilic cell, and sinusoid are lesions found in the hepatocytes of 2-AAF treated rats.[12] It is known that GST-p is one of the best markers for detection of preneoplastic cells in the chemical hepatocarcinogenesis models in DEN+2-AAF treated rats.[4,10] The tumor induced by DEN almost exclusively results in mutations in codon 61 of the Ha-Ras proto-oncogene and decreases Cx32 expression.[5,6] Direct interaction with GTP-complexed p21Ras is demonstrated for some proteins including members of

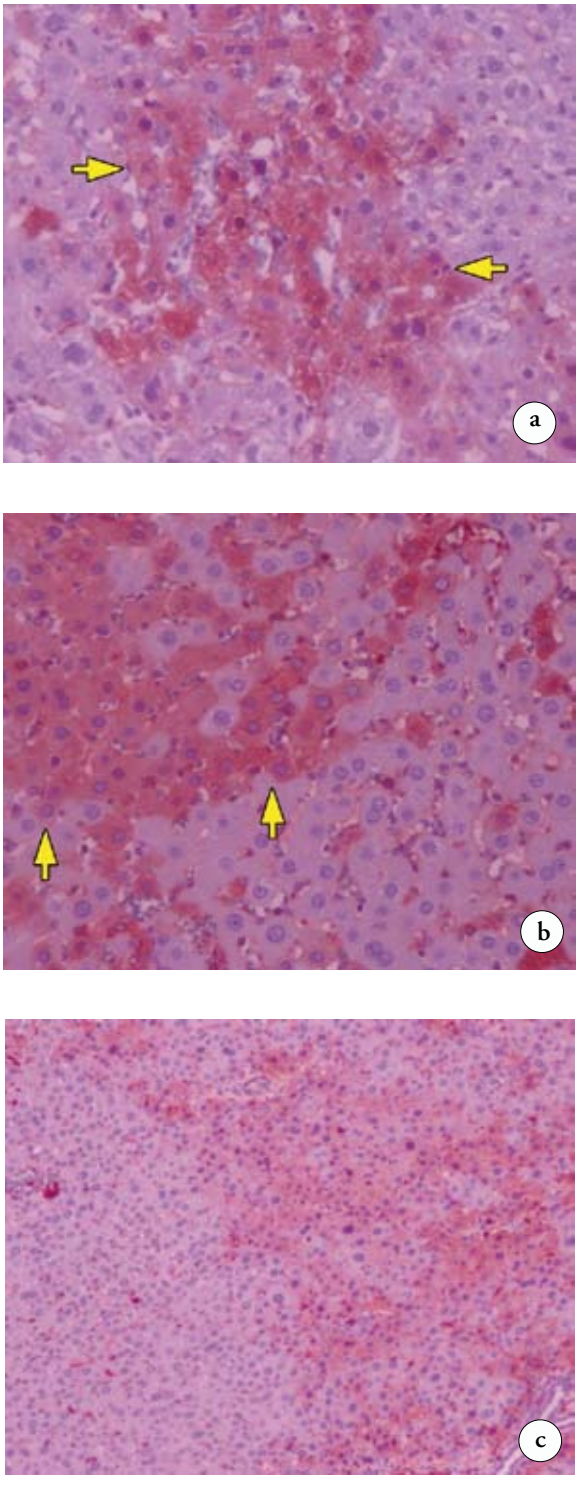

Figure 4 - Immunohistochemically, Ha-Ras (yellow arrows) and **a**) glutathione S- transferase-p (yellow arrows) **b**) increased in diethylnitrosamine (DEN)+2-acetylaminflourene (2-AAF) induced group, when the compare that in control, DEN+2-AAF+demethoxyviridine (DMV) and DEN+2-AAF+1-α-hydroxy DMV treated groups, but unchanged connexion-32 protein expression **c**) in all groups, original magnification x200.

Table 2 - The pathologic scores of the immunohistochemical study with corresponding preparation.

Groups	Ha-Ras	GST-p	Cx32
Control	0	0	0
Olive oil	0	0	0
DMSO	0	0	0
DMV	0	0	0
1-α-Hydroxy DMV	0	0	0
DEN	0	0	0
2-AAF	0	0	0
DEN + 2-AAF	54	48	0
DEN + 2-AAF + DMV	4	4	0
DEN + 2-AAF + 1-α hydroxy DMV	4	4	0

DMSO - dimethyl sulfoxide, DMV - demethoxyviridine, 1-α-hydroxy DMV - 1-α-hydroxyl demethoxyviridine, DEN -diethylnitrosamine, 2-AAF - 2-acetylaminflourene, GST-p - glutathione S- transferase, Cx32 - connexion-32

Table 3 - The CYP1A2 expression of groups and comparison according to control group.

Groups	n	CYP1A2 (ng/mg microsomal protein)	*P*-value
Control	8	53.28±2.85	
Olive oil	8	46.71±2.43	0.991
DMSO	8	40.85±1.22	0.662
DMV	8	33.28±2.43	0.085
1-α-Hydroxy DMV	8	28.42±1.89	0.11
DEN	8	19.14±3.67	0.000
2-AAF	8	11.28±2.77	0.000
DEN + 2-AAF	14	15.57±4.48	0.000
DEN + 2-AAF + DMV	8	20.14±3.82	0.000
DEN + 2-AAF + 1-α-hydroxy DMV	8	28.14±2.44	0.010

DMSO - dimethyl sulfoxide, DMV - demethoxyviridine, 1-α-hydroxy DMV - 1-α-hydroxyl demethoxyviridine, DEN - diethylnitrosamine, 2-AAF - 2-acetylaminflourene, CYP1A2 - cytochrome P450 1A2

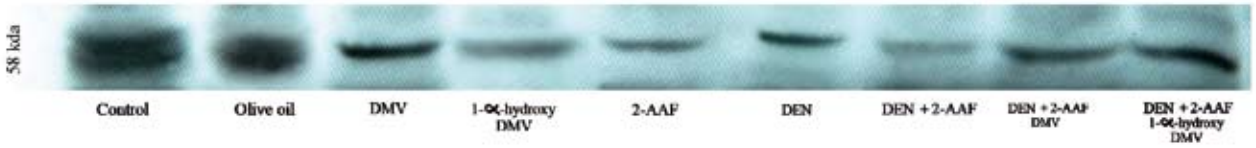

Figure 5 - Shown differences of the expression levels of cytochrome P450 1A2 protein (58 kDa) in control, olive oil, demethoxyviridine (DMV), 1-α-hydroxyl, 2-acetylaminflourene (2-AAF), diethylnitrosamine (DEN), DEN+2-AAF, DEN+2-AAF+DMV, DEN+2-AAF+1-α-hydroxy-DMV administrated groups.

the Raf kinase family and PI3 kinase, which suggested that these proteins represent downstream effects of Ras. The activation of Ras oncoproteins may lead to an increase in the cell proliferation.[6] It is thought that, if PI3 kinase was inhibited, MAP kinase pathway would be inactive, and thus, cell proliferation would have been prevented by DMV and 1-α-hydroxy-DMV. In this study, preneoplastic focus changes were observed mostly in DEN+2-AAF. Whereas, they were less observed in DMV and 1-α-hydroxy-DMV-treated groups. Immunohistochemical studies showed that Ha-Ras and GST-p increased in DEN+2-AAF induced group, but not in DEN+2-AAF+DMV and DEN+2-AAF+1-α-hydroxy-DMV induced groups. The DEN, 2-AAF, DMV and 1-α-hydroxy-DMV were administrated at the correct amount and time since the early stage of hepatocarcinogenesis formed with preneoplastic focus and Ha-Ras, GST-p activation area and other degenerations in DEN+2-AAF induced rats were observed, but also DMV and its analogue, 1-α-hydroxy-DMV, prevented more pathological degenerations in the early stage of hepatocarcinogenesis of rats among induced groups. It is possible that a decrease observed in degenerations could be due to the inhibition of PI3 kinase activities by DMV and 1-a-hydroxy-DMV, resulting in inactivation of Ha-Ras and MAPK proteins in preneoplastic period. Decreased Cx32 protein level in Centro lobular hepatocyte in PB treated rats is a sign of the degeneration of fatty acids in Centro lobular hepatocyte.[13,14]

In our study, Cx32 protein levels did not change in any groups, confirming that the early stage of hepatocarcinogenesis was formed. In some studies, it is reported that there might be a relationship between preneoplastic altering and cytochrome p450 enzymes levels such as over expressed CYP1A2 in the hepatocyte of DEN+2-AAF treated rats, however, defined preneoplastic changes in the early stage of hepatocarcinogenesis are still unclear. A decrease in CYP1A2 levels determined by western blot technique on the DEN+2-AAF treated Spraque-Dawley rat's microsomal hepatocytes was also observed. Diethylnitrosamine is a widely occurring

carcinogenic nitrosamine that requires oxygenation of a carbon catalyzed by CYP for its DNA-damaging activity. A single administration of DEN might result in more 2-AAF being transformed to N-OH-AAF.[11] In this study, CYP1A2 expressions were compared with that of the control group and found that no differences were observed in olive oil and DMSO treated groups. The CYP1A2 level was decreased in 2-AAF, DEN+2-AAF, DEN, DEN+2-AAF+DMV, DEN+2-AAF+1-α-hydroxy DMV, 1-α-hydroxy DMV, and DMV-treated animals as compared to control. The possible reason for this could be the decreasing effect of lipid peroxidation possibly caused by DEN and 2-AAF. On the contrary, DMV and 1-α-hydroxy DMV increased to become less of CYP1A2 expression in DEN and 2-AAF-treated animals.

In conclusion, our result suggests that DMV and 1-α-hydroxy DMV may prevent to lower CYP1A2 expression during the early type hepatocarcinogenesis or in preneoplastic period.

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