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METHYLENEDIOXYPHENYL SUBSTITUTED COMPOUNDS FROM *PIPER* SPECIES AS INHIBITORS OF LIVER MICROSOME-MEDIATED AFLATOXIN B_1-DNA BINDING *IN VITRO*

Hanumantharao G. Raj[+]*, Sangita Gupta[+++], Ashok K. Prasad[++], Per M. Boll[++], Jesper Wengel[++], Gopa Biswas[+], Sanjay K. Singh[+++], Nawal K. Sharma,[+++] Kirpal S. Bisht[+++] and Virinder S. Parmar[+++]*

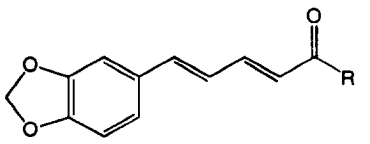
[+] *Department of Biochemistry, V.P. Chest Institute, University of Delhi, Delhi- 110 007, India*

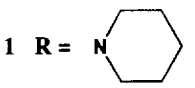
[++] *Department of Chemistry, Odense University, DK 5230 Odense M, Denmark*

[+++] *Department of Chemistry, University of Delhi, Delhi-110 007, India*

Abstract: Several compounds from *Piper* species bearing methylenedioxyphenyl moieties are reported for the first time to effectively inhibit the rat liver microsome-mediated aflatoxin B_1-DNA binding *in vitro*. From the preliminary results obtained, a structure-activity relationship is proposed.

Aflatoxin B_1 (AFB_1), a secondary metabolite of *Aspergillus flavus* has been recognised as a potent hepatocarcinogen for several animal species.[1] It is also a suspected human carcinogen.[2] AFB_1 exerts its biological effect after conversion to a reactive metabolite, i.e. AFB_1-8,9-epoxide by cytochrome P-450 dependent monooxygenase. The AFB_1 epoxide can form adduct at nucleophilic sites of proteins and nucleic acids.[3,4] Suppression of AFB_1-epoxide formation by suitable chemicals, synthetic as well as of dietary origin, is expected to inhibit AFB_1 carcinogenesis. Several synthetic compounds, such as butylated hydroxyanisole, butylated hydroxytoluene[5] and oltipraz[6] have acquired significance in chemoprevention of AFB_1-induced neoplasia. Many natural products, such as indole-3-carbinol,[7] flavonoids, vitamin A,[8] etc. have been examined for their efficacy to modulate AFB_1 epoxidation.[9] Lignans from the *Schizandra* fruit, a Chinese medicinal material have been found to ameliorate the harmful effect of toxic drugs and to facilitate liver functions.[10] Our earlier study on piperine, a methylenedioxyphenyl substituted compound from *Piper* species revealed the capability of this alkaloid to enhance the bioavailability of AFB_1 in rat tissues.[11] *Piper* species are known for their biological importance as the lignans and neolignans occurring in this genus have been found to possess antimitotic,[12] antitumour,[13] antiviral[14] and enzyme-inhibitory activities[14]. We have isolated[15-17] several compounds possessing methylenedioxyphenyl moiety (alkaloids, esters, lignans, neolignans, etc.) from *Piper acutisleginum*, *Piper betle*, *Piper schmidtii*, *Piper argyrophyllum* and *Piper longum*. In the present study, we have examined the capability of this type of compounds isolated from the above species, i.e. piperine (**1**), methyl piperate (**2**), (+)-asarinin (**3**), kadsurin-B (**4**), kadsurin-A (**5**) and isodihydrofutoquinol-A (**6**) against liver microsome-mediated AFB_1-DNA binding for the first time.



1 R = 

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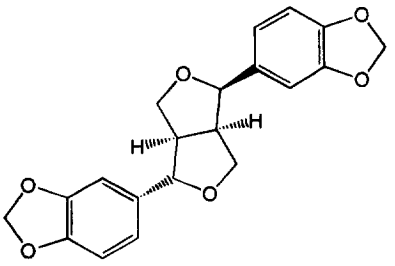

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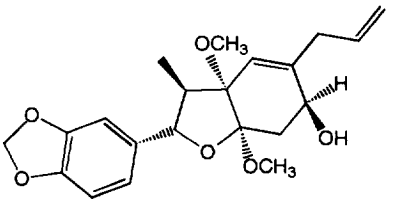

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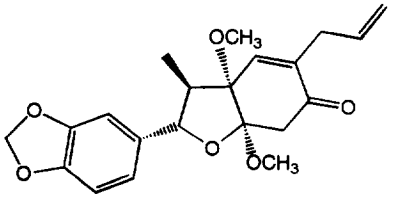

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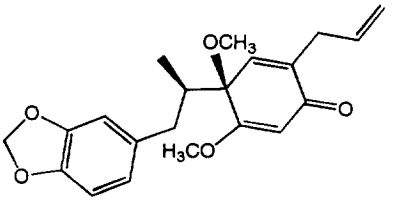

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MATERIALS AND METHODS

Animals

Male albino Wistar rats (185-215 g body weight) were obtained from the animal house of our laboratories and were maintained on a commercial diet supplied by Hindustan Lever Ltd. (Bombay, India) and provided water *ad libitum.*

Chemicals

[^{3}H] AFB_1 was purchased from Moravek Biochemicals Inc. (Brea, California, USA). AFB_1, calf thymus DNA, bovine serum albumin (BSA) and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Piperine was isolated from *Piper acutisleginum*[15] and *Piper betle*.[15] Methyl piperate, kadsurin-A, kadsurin-B and isodihydrofutoquinol-A were isolated from *F.schmidtii*.[16,17] (+)-Asarinin was isolated from *P.longum.* Other chemicals were of analytical grade.

Processing of Tissue

Rats were sacrificed by decapitation and liver was quickly removed and homogenised in 0.01 M potassium phosphate buffer (pH 7.0) having 0.25 M sucrose and 0.014 M ß-mercaptoethanol. The homogenate was centrifugated in a Sorvall centrifuge (RC-5B) at 10,000xg for 30 min to remove mitochondria. The post-mitochondrial supernatant was centrifuged at 100,000xg for 1 hour using Beckmann ultracentrifuge (L-7). The pellet was suspended in 0.25 M sucrose solution and kept at 0°C. Microsomal protein was determined according to the method of Lowry *et al.*[18] using bovine serum albumin as a standard.

Microsome-mediated AFB_1-DNA Binding

Microsome-mediated AFB_1-DNA binding was quantitated as described earlier.[19] DNA (0.1 mg) was reacted with AFB_1 (2nmol) containing [^{3}H] AFB_1 (specific activity 250µci/µmol) in 0.03 ml DMSO in a reaction mixture containing 0.01 M phosphate buffer (pH 7.4), 2mM NADPH, liver microsome equivalent to 1 mg of protein and distilled water to make total volume of 1 ml. The reaction mixture was incubated at 37°C for 30 min, followed by the addition of two volumes of phenol:chloroform:isoamyl alcohol (50:50:1 v/v/v) and DNA (0.9 mg) as carrier. The DNA was isolated and estimated by Burton's method[20] and counted for radioactivity according to the procedure described earlier.[19] The compounds **1-6** were dissolved separately in DMSO (0.01 ml) at required concentration and added to reaction mixture before addition of the microsomes. AFB_1-DNA binding is expressed as pmol AFB_1 bound/mg DNA/30 min.

RESULTS AND DISCUSSION

The microsome-mediated adduct formation between AFB_1 and DNA can be modulated by a variety of natural products,[7-9] such as flavonoids, coumarins, riboflavins, ß-carotene, etc. Most of these compounds were found to inhibit metabolic activation of AFB_1 as measured by liver microsome-catalysed AFB_1-DNA binding *in vitro*. In the present investigation, we have examined structural analogs of piperine, *i.e.* methyl piperate and lignans (another class of natural products often bearing methylenedioxyphenyl moiety) to inhibit liver microsome-mediated AFB_1 epoxidation *in vitro*. We have also investigated the structural relationship in the inhibitory action of liver microsome-mediated AFB_1-DNA binding. It is apparent from Table 1 that piperine (**1**) and methyl piperate (**2**) have similar effects on AFB_1-DNA binding, although they are functionally different compounds, *i.e.* piperine and methyl piperate are amide and ester, respectively of piperic acid. It seems that the piperidine ring and the methoxy group in piperine and methyl piperate, respectively are not playing any role for their inhibitory action. This indicates that some other structural unit in these compounds (most probably the methylenedioxyphenyl group with substituted side chain) is the active moiety for the inhibition of AFB_1-DNA binding. In order to examine whether the methylenedioxyphenyl group is an active group (or not) in these compounds, we have tested four different lignans having this structural moiety in the *in vitro* AFB_1-DNA binding assay.

(+)-Asarinin (**3**) having two methylenedioxyphenyl groups attached to furan rings inhibited AFB_1-DNA binding as effectively as piperine and methyl piperate. Kadsurin-B (**4**) and kadsurin-A (**5**) have similar structures in that **4** is the reduced form of **5** and the two compounds gave interesting results (Table 1). Compound **4** significantly lowered the AFB_1-DNA binding as compared to **5**, *i.e.* **4** inhibits 31,64 and 81% AFB_1-DNA binding (as compared

Table 1. CONCENTRATION-RELATED EFFECT OF METHYLENEDIOXYPHENYL SUBSTITUTED ANALOGS ON MICROSOME-MEDIATED [^{3}H]AFB$_1$-DNA BINDING *IN VITRO*

COMPOUND	[^{3}H] AFB$_1$-DNA BINDING (pmol/mg DNA/30 min) *IN VITRO*			
	CONCENTRATION OF COMPOUND (μM)			
	Control	10	50	100
PIPERINE (**1**)	130.8	96.6 (27)	70.0 (47)	56.6 (57)
METHYL PIPERATE (**2**)	130.8	110.0 (16)	76.0 (42)	50.7 (61)
(+)-ASARININ (**3**)	132.0	105.0 (21)	76.5 (42)	68.2 (48)
KADSURIN-B (**4**)	132.0	91.0 (31)	47.96 (64)	24.8 (81)
KADSURIN-A (**5**)	139.2	126.8 (9)	110.0 (21)	107.5 (23)
ISODIHYDRO-FUTOQUINOL-A (**6**)	139.3	123.8 (11)	123.0 (12)	121.8 (13)

The values are an average of 4 analysis with variation < 5%. The numbers in parenthesis indicate % inhibition of AFB$_1$-DNA binding compared to control.

to control) at 10, 50 and 100 μM concentrations, respectively, while **5** gives only 9,21 and 23% inhibition at the same concentrations. Thus, we may conclude that the carbonyl group is probably not playing any significant role, whereas the hydroxyl group could be an active functional group. Isodihydrofutoquinol-A (**6**) differs from (+)-asarinin (**3**), kadsurin-B (**4**) and kadsurin-A (**5**) in not possessing a furan ring. From the AFB$_1$-DNA inhibition point of view, it shows very low activity as compared to the other five compounds. As can be seen from Table-1, isodihydrofutoquinol-A gives only 11,12 and 13% inhibition at 10, 50 and 100 μM concentrations, respectively.

It is possible to conclude that the methylenedioxyphenyl group is the active moiety for the observed microsome-mediated AFB$_1$-DNA binding inhibition *in vitro*, while presence of other groups like the furan ring and the hydroxyl group can enhance the inhibitory activity. These two groups are present in kadsurin-B, which showed the highest activity among the compounds tested. Earlier studies have suggested that polyhydroxy flavonoids, such as quercetin, naringenin and fisetin are good inhibitors, while their methoxy derivatives lack the inhibitory activity.[21] A similar observation is valid for compounds **4, 5** and **6**. The presence of a carbonyl group at C-6' in **5** compared to the hydroxyl group at C-6' in **4** reduces the inhibitory potential of **5**. In **6**, the presence of a carbonyl group at C-6' and the absence of a furan moiety further reduces the inhibitory action against the liver microsome-mediated AFB$_1$-

DNA binding and as a result, it is least active. These compounds were examined for dose-related inhibitory potential of AFB_1-DNA binding. It is interesting to note that kadsurin-B is found to be an exceptionally active compound.

The action of compounds described above is mediated through the modulation of enzymatic activation of AFB_1 leading to suppression of covalent interaction of AFB_1-8,9-epoxide with DNA. Cytochrome (CY)P-450-2B1 is reported to be specific for catalysing epoxidation of AFB_1.[22,23] Piperine was found to reduce metabolism of AFB_1 considerably by marked inhibiton of (CY)P-450-2B1.[24] We do not have a clear picture of the biotransformation of these naturally occurring compounds. The inhibition of AFB_1-DNA binding by these compounds may be due to competition for the active site of the participating isozyme of microsomal cytochrome P-450, similar to piperine.[24] These naturally occurring compounds can play a useful role in modulating the biotransformation of AFB_1 and subsequent acute and chronic toxicity of the carcinogen.

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