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Chemoprevention of Carcinogen–DNA Binding: the Relative Role of Different Oxygenated Substituents on 4-Methylcoumarins in the Inhibition of Aflatoxin B_1–DNA Binding in vitro†

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Abstract—Eighteen 4-methylcoumarins bearing methoxy/hydroxy/acetoxy functionalities have been reported to effectively inhibit the rat liver microsome-mediated aflatoxin B_1–DNA binding in vitro. The contribution of functionality on coumarin nucleus towards the inhibition of AFB_1–DNA binding is in the order acetoxy > hydroxy > methoxy. The results illustrate the structure–activity relationship. Copyright © 1996 Elsevier Science Ltd

Introduction

Epidemiological data suggest that aflatoxin B_1 (AFB_1) may be an important etiological factor in human liver cancer in several parts of Asia and Africa.[1] Prevention of AFB_1 exposure can be accomplished primarily by proper crop storage and handling which is a costly proposition and unaffordable for populations in different parts of the world. Therefore chemoprevention, preferably through dietary sources may prove an effective measure against the lethality of AFB_1. Cellular cytochrome P-450-dependent monooxygenases catalyze the conversion of AFB_1 to reactive intermediate, i.e. AFB_1–8,9-epoxide which forms adducts with DNA, primarily as AFB_1-N(7)-guanine. Suppression of AFB_1–epoxide formation by chemicals of dietary origin forms the basis of chemopreventive intervention. Accordingly, several polyphenolic compounds have been found to be promising in the modulation of AFB_1-induced neoplasia in experimental animals.[2] We herein report the effect of a large number of substituted 4-methylcoumarins, a few of which occur in the edible plant fenugreek (*Trigonella foenumgraecum*)[3–5] and other plants[6–8] on liver microsome-mediated AFB_1–DNA binding in vitro.

Materials and Methods

Animals

Male albino wistar rats (185–215 g body weight) were used for the preparation of liver microsomes.

†Part of the results have been presented at the Sixth Annual Research Conference of the American Cancer Research Institute on the theme 'Dietary Phytochemicals in Cancer Prevention and Treatment' held in Washington D.C., U.S.A. on 31 August–1 September 1995.

Chemicals

[^{3}H] AFB_1 was purchased from Moravek Biochemicals Inc. (Brea, CA, U.S.A.); AFB_1, calf thymus DNA and bovine serum albumin were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.); NADPH was procured from Sisco Research Laboratory Pvt. Ltd. (Bombay, India). All other chemicals were of analytical grade.

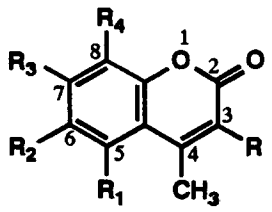
Test compounds

The 4-methylcoumarins **1–18** (Table 1) were synthesized by the well known Pechmann condensation of the corresponding di/trihydroxyphenol (resorcinol, phloroglucinol, pyrogallol or 1,2,4-trihydroxybenzene) with ethyl acetoacetate or its analogue having ethoxycarbonylmethyl or ethoxycarbonylethyl substituent at the methylene carbon, followed by methylation and acetylation by the standard procedures.[9,10] The physical and spectral data of compounds **1–3**, **5–9**, **11**, **13** and **15–18**, assayed for activity in this communication have been published previously.[5,10–13] The IR, UV and ^{1}H NMR spectral data of the compounds **4**, **10**, **12** and **14** not reported earlier is given below; their ^{13}C NMR and mass spectral data has already been published.[12,13]

5,7-Dihydroxy-4-methylcoumarin (4). Mp 285–286 °C (lit.[14] mp 285 °C). UV λ_{max}^{MeOH} nm: 268 and 325; IR ν_{max}^{KBr} cm^{-1}: 3435, 1660, 1612, 1540, 1450, 1370, 1290 and 1050; ^{1}H NMR (90 MHz, DMSO-d_6): δ 2.50 (s, 3H, C-4CH_3), 5.85 (s, 1H, C-3H), 6.16 (d, J=2 Hz, 1H, C-6H), 6.28 (d, J=2 Hz, 1H, C-8H).

7-Acetoxy-3-ethoxycarbonylmethyl-4-methylcoumarin (10). Mp 116–119 °C (lit.[15] mp 98 °C). UV λ_{max}^{MeOH} nm: 206, 266 and 296 (sh); IR ν_{max}^{KBr} cm^{-1}: 3060, 1770, 1720,

Table 1. Inhibition of liver-microsome-catalyzed AFB_1–DNA binding in vitro by various substituted coumarins



	Percent inhibition of AFB_1–DNA binding[a] compared to control[b] Concentration of inhibitor (μM)		
	10	50	100
1 $R=R_1=R_2=R_4=H$, $R_3=OCOCH_3$	41	56	68
2 $R=R_1=R_2=H$, $R_3=R_4=OH$	14	25	51
3 $R=R_1=R_2=H$, $R_3=R_4=OCOCH_3$	16	50	64
4 $R=R_2=R_4=H$, $R_1=R_3=OH$	52	60	69
5 $R=R_2=R_4=H$, $R_1=R_3=OCH_3$	22	36	40
6 $R=R_2=R_4=H$, $R_1=R_3=OCOCH_3$	56	60	84
7 $R=R_1=R_4=H$, $R_2=R_3=OCH_3$	27	35	45
8 $R=R_1=R_4=H$, $R_2=R_3=OCOCH_3$	35	52	82
9 $R=CH_2COOC_2H_5$, $R_1=R_2=R_4=H$, $R_3=OH$	9	21	59
10 $R=CH_2COOC_2H_5$, $R_1=R_2=R_4=H$, $R_3=OCOCH_3$	48	58	68
11 $R=CH_2COOC_2H_5$, $R_1=R_2=H$, $R_3=R_4=OCH_3$	15	30	37
12 $R=CH_2COOC_2H_5$, $R_1=R_3=OCOCH_3$, $R_2=R_4=H$	39	59	82
13 $R=CH_2COOC_2H_5$, $R_1=R_4=H$, $R_2=R_3=OCH_3$	43	39	34
14 $R=CH_2CH_2COOC_2H_5$, $R_1=R_2=R_4=H$, $R_3=OCH_3$	16	28	47
15 $R=CH_2CH_2COOC_2H_5$, $R_1=R_2=H$, $R_3=R_4=OCOCH_3$	15	52	64
16 $R=CH_2CH_2COOC_2H_5$, $R_1=R_3=OH$, $R_2=R_4=H$	18	30	51
17 $R=CH_2CH_2COOC_2H_5$, $R_1=R_3=OCOCH_3$, $R_2=R_4=H$	33	52	81
18 $R=CH_2CH_2COOC_2H_5$, $R_1=R_4=H$, $R_2=R_3=OH$	38	48	52

[a]The values are an average of four analyses with variation less than 10%.
[b]Control value of AFB_1–DNA binding: 125 ± 11 (pmol/mg DNA/30 min).

1690, 1625, 1620, 1580, 1520, 1450, 1390, 1375, 1335, 1270, 1180, 1140, 1120, 1090, 1060, 1010, 980, 900, 880, 840, 810, 780, 640 and 570; ^{1}H NMR (90 MHz, $CDCl_3$): δ 1.30 (t, $J=7$ Hz, 3H, $-CH_2COOCH_2CH_3$), 2.36 (s, 3H, $-OCOCH_3$), 2.43 (s, 3H, C-4CH_3), 3.75 (s, 2H, $CH_2COOCH_2CH_3$), 4.20 (q, $J=7$ Hz, 2H, $-CH_2COOCH_2CH_3$), 7.15 (m, 2H, C-6H and C-8H), 7.65 (d, $J=9$Hz, 1H, C-5H).

5,7-Diacetoxy-3-ethoxycarbonylmethyl-4-methylcoumarin (12). Mp 115–117 °C (lit.[16] mp 114–115 °C). UV λ_{max}^{MeOH} nm: 210 and 278; IR ν_{max}^{KBr} cm^{-1}: 3030, 3010, 1785, 1770, 1740, 1720, 1625, 1580, 1490, 1440, 1380, 1340, 1300, 1250, 1190, 1135, 1110, 1080, 1050, 1020, 900, 870, 840, 790, 750, 700 and 600; ^{1}H NMR (90 MHz, $CDCl_3$): δ 1.26 (t, $J=7$ Hz, 3H, $-CH_2COOCH_2CH_3$), 2.22 (s, 3H, $-OCOCH_3$), 2.28 (s, 3H, $-OCOCH_3$), 2.40 (s, 3H, C-4CH_3), 3.65 (s, 2H, $-CH_2COOCH_2CH_3$), 4.10 (q, $J=7$ Hz, 2H, $-CH_2COOCH_2CH_3$), 6.75 (d, $J=2$ Hz, 1H, C-6H), 6.95 (d, $J=2$ Hz, 1H, C-8H).

7-Methoxy-3-ethoxycarbonylethyl-4-methylcoumarin (14). Mp 174–175 °C (lit.[17,18] mp 74–75.5 °C). UV λ_{max}^{MeOH} nm: 220 and 320; IR ν_{max}^{KBr} cm^{-1}: 1725, 1700, 1610, 1505, 1440, 1385, 1280, 1200, 1155, 1072, 1020 and 825; ^{1}H NMR (90 MHz, $CDCl_3$): δ 1.25 (t, $J=7$ Hz, 3H, $-CH_2CH_2COOCH_2CH_3$), 2.40 (s, 3H, C-4CH_3), 2.60 (m, 2H, $-CH_2CH_2COOCH_2CH_3$), 2.75 (m, 2H, $CH_2CH_2COOCH_2CH_3$), 3.81 (s, 3H, $-OCH_3$), 4.02 (q, $J=7$ Hz, 2H, $-CH_2CH_2COOCH_2CH_3$), 6.70 (dd, $J=8$, 2 Hz, 1H, C-6H), 6.81 (d, $J=2$ Hz, 1H, C-8H), 7.40 (d, $J=8$ Hz, 1H, C-5H).

Preparation of liver microsomes and AFB_1–DNA binding assay

Details of the procedures were outlined previously.[19] Compounds **1–18** were dissolved separately in DMSO (0.01 ml) at the required concn and added to the reaction mixture principally containing ^{3}H-AFB_1 (specific activity 250 μCi/μmol), 2 mM NADPH and DNA (0.1 mg) before addition of the microsomes (equivalent to 1 mg protein). AFB_1–DNA binding is expressed as pmol AFB_1 bound/mg DNA/30 min.

Results and Discussion

Plant-derived food materials consumed by man everyday contain an impressive variety of chemicals.[20] These chemicals are now receiving greater attention as they have been shown to modulate the kinetics and metabolism of xenobiotics in experimental animals and man. The minor dietary constituents influence not only the effect of drugs but also the carcinogenic or the toxic properties of environmental pollutants.[21,22] Coumarins belong to the class of widely occurring polyphenolics in nature. In the present investigation, we have evaluated the inhibitory activity of 4-methylcoumarins towards AFB_1–DNA binding. We have chosen derivatives of 4-methylcoumarins since they are metabolized faster and show enhanced biological activities than their analogues lacking the C-4 methyl substituent.[23–25] It may be added here that coumarins are known to undergo liver microsome mediated epoxidation forming coumarin 3,4-epoxides, which in the

presence of liver cytosol and NADPH are converted to *ortho*-hydroxyphenyl acetaldehyde, an active metabolite responsible for hepatotoxicity of coumarin.[26,27] This suggests that the presence of a methyl group at the C-4 position would possibly inhibit the formation of coumarin 3,4-epoxide, thus making 4-methylcoumarins less toxic.

We have examined the 4-methylcoumarins **1–18**, (Table 1) bearing a combination of three functional groups, i.e. methoxy, hydroxy and acetoxy and an aliphatic side chain of 0–5 carbon atoms at the C-3 position for their ability to inhibit rat liver microsome mediated AFB_1 adduction to DNA in vitro. The number of carbon atoms in the aliphatic side chain at the C-3 position does not seem to make any difference in the activity of coumarins in inhibiting the AFB_1–DNA binding as the 5,7-diacetoxy-4-methylcoumarins **6**, **12** and **17** having 0, 4 and 5 carbon atom long moieties produce 84, 82 and 81% inhibition of AFB_1–DNA binding at the 100 μM level, respectively (Table 1). Similar observations are consistent with the two pairs of identically substituted coumarins in the benzenoid ring, i.e. **1** and **10**, and **3** and **15** lacking a C-3 substituent or carrying an ethoxycarbonylmethyl or ethoxycarbonylethyl substituent at the C-3 position; both **1** and **10** inhibit the AFB_1–DNA binding by 68%, whereas both 3 and 15 inhibit the binding by 64% at 100 μM concentration (Table 1). Since the aliphatic side chain has neither any oxidizable functionality to modify cytochrome P-450 heme or the apoprotein, nor has any oxidizable radical to be acted upon by the mixed function oxidase, there is no effect of the aliphatic side chain in altering the liver microsome-catalyzed AFB_1–DNA binding.

Coumarins substituted with one or more methoxy groups appear to be least effective in causing inhibition of AFB_1–DNA binding in vitro. It was observed that presence of hydroxyl group(s) on the coumarin nucleus results in marginally higher inhibition of AFB_1–DNA binding as compared to those having methoxy group(s) at the same positions (Table 1) as revealed by comparison of compounds **9** and **14**, and **13** and **18**. The number and position of hydroxyl groups appear to have no significant effect. Furthermore, it was observed that acetylation of the hydroxyl groups results in considerably higher inhibition of AFB_1–DNA binding. This is evident (Table 1) by comparison of compounds **16** and **17**, **9** and **1**, **18** and **8**, and **2** and **3**. In addition, it was noticed that coumarins carrying two acetoxy groups possess much higher inhibitory activity as compared to compounds bearing one acetoxy group as revealed by comparison of compounds **1** and **6**, **1** and **8**, and **10** and **12** (Table 1). It was further noted that coumarins carrying two acetoxy groups at C-5 and C-7, and C-6 and C-7 positions, were significantly more active as compared to those having acetoxy groups at the C-7 and C-8 positions (Table 2). Thus the compounds **6** (and **12**), having the two acetoxy groups at the C-5 and C-7 positions, had the best activity: **6** (and **12**) inhibited the AFB_1–DNA binding to the extent of 56% (39%), 60% (59%) and 84% (82%) at 10, 50 and 100 μM concentration, respectively (Table 1). In order to clarify whether the coumarin nucleus per se was essential for inhibiting microsome-mediated AFB_1–DNA binding, a series of acetoxybenzenes (such as monoacetoxybenzene, 1,3-diacetoxybenzene, 1,2,3-triacetoxybenzene and 1,3,5-triacetoxybenzene) having acetoxy groups at positions similar to those in the acetoxycoumarins were examined. It was found that none of the four acetoxybenzenes were effective in inhibiting the microsome-mediated AFB_1–DNA binding. This observation showed that the coumarin nucleus is essential for the inhibitory activity under study.

Table 2. Relative effects of methoxy, hydroxy, and acetoxy substituents of 4-methylcoumarins on rat liver microsome-mediated AFB_1–DNA binding in vitro

Substituents	Percent inhibition of AFB_1–DNA binding in vitro[a]
Monomethoxy	40
Monohydroxy	56
Monoacetoxy	68
C-7, C-8 Diacetoxy	64
C-5, C-7 Diacetoxy	82
C-6, C-7 Diacetoxy	82

*These estimates are calculated from the data in Table 1 at 100 μM concentration of the inhibitor based on the conclusions that (a) position and number of methoxy and/or hydroxy groups did not affect the AFB_1–DNA binding, and (b) the side chain at the C-3 position made no difference in binding.

We can conclude from the above observations that the contribution of functionality on the coumarin nucleus towards the inhibition of AFB_1–DNA binding in vitro is in the order acetoxy > hydroxy > methoxy. It is possible that acetoxycoumarins, such as compounds **6** and **12**, are good candidates to effect mechanism-based inhibition. The acetoxy group(s) of 4-methylcoumarins may acylate the lysine residue in the cytochrome-P-450 active centre similar to chloramphenicol,[28,29] thereby causing irreversible inhibition of AFB_1–DNA binding. These studies have a bearing on AFB_1 activation and can prove useful in the designing of useful chemopreventive agents, effective against AFB_1 carcinogenesis. It is interesting to note that the compounds **16** and **17** have been tested in vitro at the National Cancer Institute (NIH, USA) as anti-HIV and anticancer agents (against leukemia, non-small cell lung and small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer and other miscellaneous cancer cell lines), the compound **16** was found to be a methylation inhibitor. Furthermore, compounds **1**, **3** and **8** have been shown previously[10] to be active against the poliomyelitis virus, and an analogue of compound **17** has been shown to be strongly active against the herpes simplex virus. In addition, compounds **9**, **11** and **15** have been shown to possess remarkable herbicidal activity, and analogues of compounds **14** and **18** have exhibited significant fungicidal and herbicidal activities, respectively (unpublished results from our laboratories).

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