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CYP1 induction, binding to the hepatic aromatic hydrocarbon receptor and mutagenicity of a series of 11-alkoxy cyclopenta[*a*]phenanthren-17-ones: a structure activity relationship

Gary W. Boyd[a], Maurice M. Coombs[b], Costas Ioannides*[a]

[a]*Molecular Toxicology Group, School of Biological Sciences, Guildford, Surrey, GU2 5XH, UK*
[b]*Department of Chemistry, University of Surrey, Guildford, Surrey, GU2 5XH, UK*

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Abstract

A series of four 11-alkoxy cyclopenta[*a*]phenanthren-17-ones, ranging from the methoxy to the butoxy derivative, has been synthesised in order to investigate the effect of the size of the 11-substituent on the mutagenicity and ability of these compounds to induce hepatic CYP1 activity in rats. The latter was monitored by using as diagnostic probes methoxy and ethoxy-resorufin, and immunologically in Western blots employing anti-CYP1A1 antibodies. All four members of the series induced both CYP1A1 and CYP1A2 activities and apoprotein levels, but the methoxy- and ethoxy-CPP-17-ones were clearly the most potent. Of the four isomers, only 11-methoxy-CPP-17-one displaced ^{3}H-TCDD from the cytosolic Ah receptor. Similarly only 11-methoxy-CPP-17-one elicited a positive mutagenic response in the Ames test in the presence of an Aroclor 1254-induced activation system. The relevance of these findings to the carcinogenicity of these compounds in the mouse skin painting model is discussed.

Keywords: Polycyclic aromatic hydrocarbons; Cyclopenta[*a*]phenanthrenes; Cytochrome *P*-450; Ah receptor; Enzyme induction

1. Introduction

The polycyclic aromatic hydrocarbons (PAH) comprise a major group of environmental chemical carcinogens to which man is continuously exposed primarily through the air he breathes and through the diet. Many members of this group are carcinogenic, the major targets being the lung and skin. The carcinogenicity of PAH is mediated through metabolic products, the dihydrodiol-epoxides (Gelboin, 1980). Three metabolic steps are required to generate these, two oxidations catalysed by the cytochrome *P*-450 system, specifically the CYP1A1 protein, and a hydrolysis catalysed by the epoxide hydrolase.

* Corresponding author.

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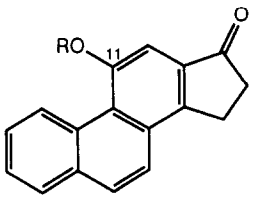
Insertion of a methyl group at the non-benzo bay-region site of a PAH enhances its carcinogenic activity. For example of all the isomers of chrysene, the most potent carcinogen is the bay-region-substituted 5-methyl compound (Hecht et al., 1974; Hoffmann et al., 1974). Similarly, methyl substitution at the bay-region potentiates the carcinogenicity of benz[*a*]anthacene (Wislocki et al., 1982). In order to rationalise these marked differences in carcinogenic potential we have studied in detail the non-carcinogenic 15,16-dihydrocyclopenta[*a*]phenanthren-17-one (CPP-17-one) and its carcinogenic 11-methyl derivative (11-CH_3-CPP-17-one) (Boyd et al., 1993a,b). Our findings indicated that two factors which contribute to this difference in carcinogenic potential are: (a) the 11-methyl derivative is more extensively converted to A-ring metabolites, such as the 3,4-dihydrodiol, leading to the production of dihydrodiol-epoxides and (b) the 11-methyl analogue is a morepotent inducer, than the unsubstituted compound, of the CYP1 family which is responsible for their activation (Ayrton et al., 1990; Boyd et al., 1993a,b). Synthesis of the dihydrodiol epoxides of the hydrocarbon 16,17-dihydro-15*H*-cyclopenta[*a*]phenanthrene(CPP) and of its carcinogenic 11-methyl analogue (11-CH_3-CPP), and investigation of their mutagenicity in the Ames test revealed that both displayed the same high degree of genotoxicity which led us to conclude that 11-methyl substitution does not confer enhanced genotoxic activity on the dihydrodiol-epoxide itself (Papaparaskeva-Petrides et al., 1993).

In previous studies by one of us (Coombs, 1979a; Coombs and Bhatt, 1989), it was observed that the effect of the substitution at the bay-region was size-limited. Although the 11-methyl substitution conferred strong carcinogenicity on CPP-17-one, the ethyl analogue was a very weak carcinogen and the n-butyl derivative was inactive. Similar observations were made using the more synthetically accessible 11-alkoxy derivatives (Bhatt, 1982).

Using the 11-alkoxy derivatives of CPP-17-one as a model series (Fig. 1), we investigated the effect of size of the 11-substituent on the mutagenicity of these compounds and their ability to induce the CYP1 family and related enzymes involved in



R= CH_3, C_2H_5, C_3H_7 or C_4H_9

Fig. 1. General structure of the 11-alkoxy-CPP-17-ones.

PAH metabolism. The CYP1 induction by PAH is mediated by a cytosolic protein, the Ah receptor (Bigelow and Nebert, 1982; Nebert, 1989) and it was considered pertinent to study also the binding of the 11-alkoxy-CPP-17-ones to this receptor. The objective of this work is to establish a structure-activity relationship between the size of the 11-substituent on one hand, and mutagenicity and CYP1 induction on the other, in an attempt to provide a rationale for the marked difference in carcinogenic potential between these 11-alkoxy-CPP-17-ones.

2. Materials and methods

Methoxy- and ethoxy-resorufin (Molecular Probes, Eugene, OR, USA), Aroclor 1254 (Robens Institute, Guildford, Surrey, UK), benzo[*a*]pyrene-4,5-oxide (NCI Chemical Carcinogen Repository, Kansas, USA) tetrachloro-dibenzo-*p*-dioxin (TCDD) (Chemsyn Science Laboratories, Kansas, USA), tetrachloro-dibenzofuran (TCDF) (Promochem, St Albans, Herts, UK), 3,4-dichloronitrobenzene (DCNB) and 1-chloro-3,4-dinitrobenzene (CDNB) (Aldrich Chemical Co, Dorset, UK), were all purchased. The *Salmonella tryphimurium* strain TA100 was a kind gift from Professor B.N. Ames, Berkeley, CA, USA. The purification of rat CYP1A1 and production of polyclonal antibodies has already been described (Rodrigues et al., 1987). The antibodies detect both the CYP1A1 and CYP1A2 proteins.

2.1. Synthesis of 11-alkoxy-CPP-17-ones

The 11-alkoxy compounds were prepared from 1-acetoxy-15,16-dihydrocyclopenta[*a*]phenanth-

rene-17-one (Robinson, 1983), via the phenol by the following improved procedure. The phenol (1 mmol) was mixed with anhydrous potassium carbonate (1.5 mmol) and acetonitrile (21 ml). The mixture was heated under reflux, the appropriate alkyl iodide (3 mmol) was added to the flask, and refluxing was allowed to continue for 24 h. The reaction mixture was filtered through a hot sintered funnel, the filtrate was cooled on ice and the precipitated 11-alkoxy-CPP-17-ones were collected, washed with acetonitrile, exhaustively with water and finally dried over anhydrous calcium chloride. The identity of each product was verified by melting point determination, UV and IR spectroscopy, and ^{1}H-NMR and 70 eV electron impact mass spectra (Table 1).

2.2. Treatment of animals

Male Wistar albino rats (about 200 g) received single daily intraperitoneal doses of the appropriate 11-alkoxy-CPP-17-one (25 mg/kg) for 3 days, whereas control animals received the corresponding volume of the vehicle, corn oil. All animals were killed 24 h after the last administration and hepatic postmitochondrial, microsomal and cytosolic fractions were prepared as previously described (Ioannides and Parke, 1975). For the preparation of the activation systems in the Ames test, rats were treated with a single intraperitoneal injection of Aroclor 1254 and killed on the 5th day following administration.

The following determinations were carried out on the microsomal fractions: *O*-dealkylations of

Table 1
11-Alkoxycyclopenta[*a*]phenanthrenes: melting points and NMR data

Compound	Mol. formula (mol. wt.)	Melting points (lit. mp.,) [11]	NMR data (300 MHz, $CDCl_3$ solutions, δ = chemical shift in ppm from Me_4Si)
11-Methoxy	$C_{18}H_{14}O_2$ (262)	176–180°C (179°C)	δ9.75 (1H, d,d 1-H), 7.9–7.66 (5H, m, aromatic), 7.38 (1H, s, 12-H), 4.16 (3H, s, methyl), 3.40 (2H, m, 15-CH_2), 2.85 (2H, m, 16-CH_2)
11-Ethoxy	$C_{19}H_{16}O_2$ (276)	189–192°C (191–193°C)	δ9.86 (1H, dd, 1-H), 7.90–7.69 (5H, m, aromatic), 7.36 (1H, s, 12-H), 4.36 (2H, q, ethyl CH_2), 3.40 (2H, m, 15-CH_2), 2.85 (2H, m, 16-CH_2), 1.70 (3H, t, methyl).
11-*n*-Propoxy	$C_{20}H_{18}O_2$ (290)	178–180°C (181–182°C)	δ9.86 (1H, dd, 1-H), 7.94–7.66 (5H, m, aromatic), 7.37 (1H, s. 12-H), 4.27(2H, t, side chain α-CH_2), 3.41 (2H, m, 15-CH_2), 2.85 (2H, m, 16-CH_2), 2.08 (2H, sext., side chain β-CH_2), 1.22 (3H, t, methyl)
11-*n*-Butoxy	$C_{21}H_{20}O_2$ (304)	153–156°C (156–157°C)	δ9.85 (1H, dd, 1-H), 7.90–7.67 (5H, m, aromatic), 7.38 (1H, s, 12-H), 4.31 (2H, t, side chain α-CH_2), 3.42 (2H, m, 15-CH_2), 2.86 (2H, m, 16-CH_2), 2.08 (2H, quint, side chain β-CH_2), 1.66 (2H, sext., side chain γ-CH_2), 1.06 (3H, t, methyl).

The molecular weights were in all instances confirmed by strong molecular ions in the mass spectra. UV spectra were as previously reported (Bhatt et al., 1982).

Table 2
Induction of rat hepatic microsomal and cytosolic xenobiotic-metabolising enzymes by a series of four 11-alkoxy-CPP-17-ones

Parameter	Control	11-Methoxy-CPP-17-one	11-Ethoxy-CPP-17-one	11-Propoxy-CPP-17-one	11-Butoxy-CPP-17-one
Methoxyresorufin *O*-demethylase (pmol/min per nmol *P*-450)	49 ± 3	247 ± 19***	308 ± 42***	125 ± 29***	186 ± 12***
Ethoxyresorufin *O*-deethylase (pmol/min per nmol *P*-450)	157 ± 28	1468 ± 157	1894 ± 241***	425 ± 110*	459 ± 38***
Total cytochrome *P*-450 (nmol/mg protein)	0.51 ± 0.05	0.64 ± 0.04	0.56 ± 0.05	0.33 ± 0.06	0.28 ± 0.04*
Epoxide hydrolase (nmol/min per mg protein)	3.71 ± 0.42	3.20 ± 0.13	3.35 ± 0.28	2.21 ± 0.11**	3.21 ± 0.06
Glutathione *S*-transferase (DCNB) (nmol/min per mg protein)	48.6 ± 4.3	51.3 ± 3.7	54.9 ± 2.3	47.9 ± 3.3	60.3 ± 0.7
Glutathione *S*-transferase (CDNB) (nmol/min per mg protein)	906 ± 67	928 ± 63	954 ± 28	864 ± 45	1025 ± 41
Microsomal protein (mg/g liver)	27.9 ± 1.2	32.3 ± 2.5	32.3 ± 1.8	31.6 ± 1.4	31.3 ± 2.9
Cytosolic protein (mg/g liver)	92.4 ± 3.9	97.4 ± 2.6	92.2 ± 3.3	93.1 ± 3.5	87.4 ± 4.2

Rats were treated with single daily intraperitoneal doses of the 11-alkoxy-CPP-17-one (25 mg/kg) for 3 days whilst control animals received the corresponding volume of the vehicle, corn oil. All animals were killed 24 h after the last administration. Figures are presented as mean ± S.E.M. for at least three animals.
*$P < 0.05$; **$P < 0.01$; ***$P. < 0.001$

methoxyresorufin (Burke and Mayer, 1983) and ethoxyresorufin (Burke and Mayer, 1984), epoxide hydrolase activity using benzo[*a*]pyrene-4,5-oxide as substrate (Dansette et al., 1979), and total cytochrome *P*-450 levels (Omura and Sato, 1964). On the cytosolic fraction: glutathione-*S*-transferase activity using CDNB and DCNB as substrates (Habig et al., 1974). Protein levels were determined on both hepatic fractions (Lowry et al., 1981).

Immunoblot analysis of microsomal proteins employing anti-CYP1A1 antibodies, following resolution by electrophoresis (Laemmli, 1970) was carried out essentially as described by Towbin et al. (1979). Displacement of ^{3}H-TCDD from the rat hepatic cytosolic Ah receptor was determined as described by Gasiewicz and Neal (1982), employing seven concentrations of the displacer, each performed in triplicate.

The mutagenic potential of the various 11-alkoxy-CPP-17-ones was evaluated in the Ames mutagenicity test (Maron and Ames, 1988), using the *Salmonella typhimurium* strain TA100 and an activation system containing 10% (v/v) hepatic postmitochondrial preparation (25% w/v) from Aroclor 1254-treated rats.

Statistical evaluation was carried out using the Student's *t*-test.

3. Results

All 11-alkoxy derivatives of CPP-17-one enhanced the *O*-dealkylations of ethoxyresorufin and methoxyresorufin, with the methoxy- and ethoxy-CPP-17-ones being clearly the most potent (Table 2). None of the compounds increased microsomal epoxide hydrolase, cytosolic glutathione *S*-transferase or total cytochrome *P*-450 levels. However, treatment with the two longer-chain 11-alkoxy-CPP-17-ones decreased total cytochrome *P*-450 levels and the effect was statistically significant in the case of the butoxy derivative. The 11-propoxy-CPP-17-one also inhibited epoxide hydrolase activity (Table 2).

Immunoblot analysis using anti-CYP1A1 antibodies revealed that treatment of rats with 11-ethoxy-CPP-17-one, and to a lesser extent 11-methoxy-CPP-17-one, resulted in increased CYP1A1 and CYP1A2 apoproteins (Fig. 2). Treatment with the butoxy- and propoxy analogues showed only modest increases in the apoprotein levels.

11-Methoxy-CPP-17-one displaced ^{3}H-TCDD from the cytosolic Ah receptor with an EC_{50} of about 10^{-7}M (Fig. 3). The ethoxy-analogue was a very poor displacer whereas the propoxy- and butoxy-CPP-17-ones were unable to displace ^{3}H-TCDD, even at concentrations of 5×10^{-4} M.

None of the alkoxy-CPP-17-ones exhibited a mutagenic response in the Ames test when isolated microsomes derived from the liver of Aroclor 1254-induced rats served as the activation system (results not shown). When the activation system employed hepatic post mitochondrial (S9) preparations from the same source, 11-methoxy-CPP-17-one displayed a clear, but weak mutagenic response (Fig. 4). None of the remaining three

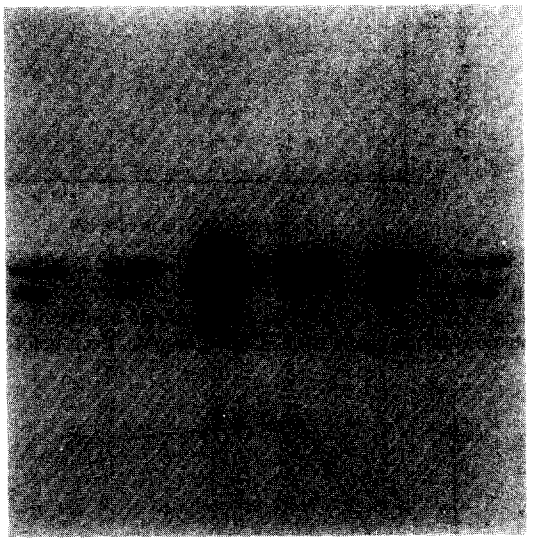


Fig. 2. Immunoblot analysis of hepatic microsomes from 11-alkoxy-CPP-17-one-treated animals. Microsomal proteins (40 μg) were resolved by electrophoresis in a 10% (w/v) sodium dodecyl sulphate-polyacrylamide gel and transferred electrophoretically to nitrocellulose. The immunoblot was carried out with sheep anti-CYP1A1 (diluted 1:10 000) followed by peroxidase — labelled anti-sheep IgG (diluted 1:2500). Bu, 11-butoxy-CPP-17-one; Pr, 11-propoxy-CPP-17-one; Ar, Aroclor 1254; Et, 11-ethoxy-CPP-17-one; Me, 11-methoxy-CPP-17-one; and Co, control.

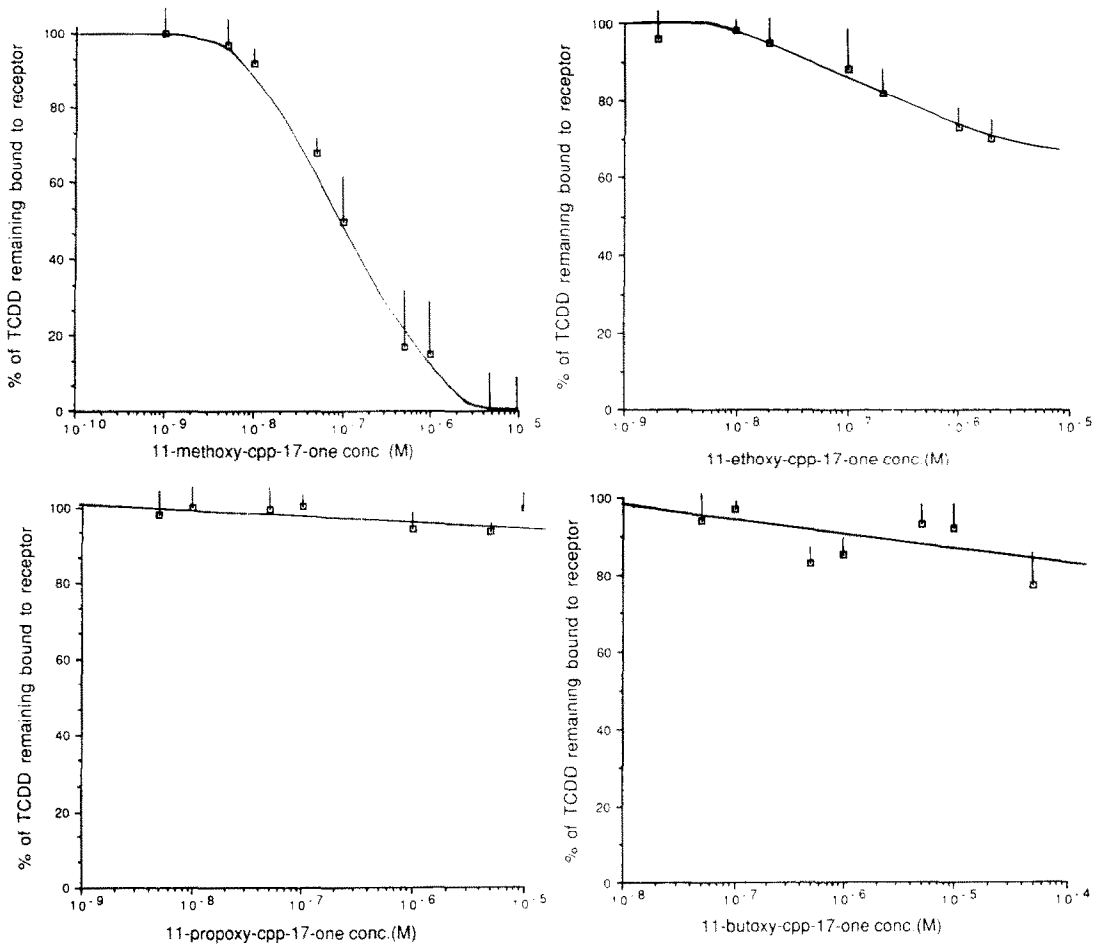
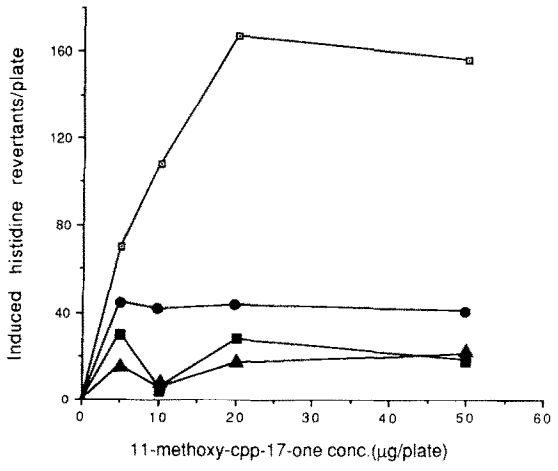


Fig. 3. Displacement of [^{3}H]-TCDD from the hepatic cytosolic Ah receptor by 11-alkoxy-CPP-17-ones. The assay was conducted using [^{3}H]-TCDD (0.3 nM) as the ligand and a protein concentration of 2.0 mg/ml. Non-specific binding was calculated from incubations of the ligand with TCDF (200 nM). Each point represents the mean of three determinations.



alkoxy analogues elicited a mutagenic response under the same conditions.

4. Discussion

For PAH to be converted to their ultimate carcinogens, the dihydrodiol epoxides, the presence

Fig. 4. Activation of 11-alkoxy-CPP-17-ones by Aroclor 1254-induced rat hepatic S9 preparations. The test was carried out using a 10% (v/v) S9 activation system and the *Salmonella typhimurium* strain TA 100. The spontaneous reversion rate of 90 ± 4 has already been subtracted. Each point represents the mean of triplicates. (□) 11-methoxy-; (●) 11-ethoxy-; (■) 11-propoxy- and (▲) 11-butoxy-CPP-17-one.

of two enzymes is indispensable. The CYP1 family of haemoproteins catalyses the two oxidative steps, and the microsomal epoxide hydrolase hydrolyses the epoxide resulting from the first oxidation to form the dihydrodiol. This then undergoes a second oxidation to form the dihydrodiol epoxide (Gelboin, 1980). In the present study total cytochrome *P*-450 levels decreased following the treatment of the animals with the 11-propoxy- and 11-butoxy-CPP-17-ones, but only in the latter case was statistical significance attained. CYP1 activity was determined using two specific probes, methoxyresorufin *O*-demethylase, a marker for CYP1A2, and ethoxyresorufin *O*-deethylase, a marker for CYP1A1 (Namkung et al., 1988). All four analogues induced both CYP1 proteins, as exemplified by increases in the activities of the above two enzymes, expressed in terms of nmol *P*-450 to account for the differences in cytochrome *P*-450 content. This is not surprising since these are largely planar compounds and consequently may readily interact with the CYP1 family (Lewis et al., 1986). X-ray crystallographic studies have demonstrated the presence of a strong interaction between the ether oxygen and 1-H in 11-methoxy-CPP-17-one rendering the ring system essentially flat (Kashino et al., 1986). This is confirmed by the NMR chemical shift of this proton which at δ9.8 is about 1 ppm higher than that of the corresponding 1-proton in the 11-methyl analogue. Since similar chemical shifts are shown by the other 11-alkoxy compounds (Table 1), a similar close interaction exists also in these compounds, and therefore their ring systems are essentially planar. The 11-ethoxy and 11-methoxy-CPP-17-ones were the most potent inducers of both enzyme activities. Immunoblot analysis confirmed that the ethoxy- and to a lesser extent the methoxy-CPP-17-one, enhanced the apoprotein levels of both isoforms comprising the CYP1 family. In the case of the propoxy- and butoxy-derivatives the increases in apoprotein levels were modest. It must be pointed out, however, that the total cytochrome *P*-450 levels in the animals treated with the two longer chain analogues were lower and, since the immunoblot was loaded on the basis of protein, the CYP1A1 and CYP1A2 levels may be underestimated.

An unexpected observation was that 11-ethoxy-CPP-17-one failed to bind to the Ah receptor, despite the fact that of the four compounds it was the most potent CYP1 inducing agent. In contrast the 11-methoxy-analogue interacted with the receptor as evidenced by the displacement of ^{3}H-TCDD. The two longer-chain 11-alkoxy-CPP-17-ones did not displace ^{3}H-TCDD. Interestingly, in another study where the interaction of a series of trichlorodibenzofurans with the receptor was studied, the ethyl analogue was anomalous in that it did not bind as expected (Astroff and Safe, 1989). It is worthwhile to note that other CYP1 inducing agents, such as omeprazole, failed to bind to the Ah receptor (Daujat et al.,1992). The possibility that the Ah receptor is not a single entity but may comprise more than one, or even a family of receptors with different substrate preferences cannot be excluded. Experimental evidence supporting such a possibilityhas been reported (Landers et al., 1991). An alternative explanation is that the observed increase in CYP1 levels is not the result of transcriptional activation but might represent mRNA or protein stabilisation, as reported previously with respect to other cytochrome *P*-450 proteins (Song et al., 1987).

Of the four 11-alkoxy-CPP-17-one derivatives, only the methoxy compound elicited a clear, albeit weak, mutagenic response in the Ames test, in the presence of an Aroclor 1254-induced activation system. In the case of the 11-methyl analogue, of all the metabolites only the 3,4-dihydrodiol derivatives could be converted to mutagens by further metabolism (Coombs et al., 1979b). Therefore it is likely that the mutagenicity of 11-methoxy-CPP-17-one is also due to the formation of its corresponding dihydrodiol epoxide. On the basis of the present studies it is not possible to discern whether the lack of mutagenicity of the other three 11-alkoxy-CPP-17-ones is due to their poor genotoxicity, and/or lower rates of formation of their corresponding dihydodriol-epoxides. It is conceivable that they are poor substrates of the CYP1 family and/or their primary epoxides are poor substrates of epoxide hydrolase.

The carcinogenicity of the 11-alkoxy-CPP-17-ones in the mouse skin painting model was found to decrease with increasing alkoxy chain length

(Bhatt et al., 1982). The carcinogenicity of the 11-methoxy derivative can be rationalised in terms of its mutagenic potential and its ability to induce CYP1 activity, one of the key enzymes in the activation of CPP-17-ones (Boyd et al., 1993, 1993). However, the ethoxy-derivative also induced a good carcinogenic response, yet it was not mutagenic in the Ames test under the present conditions, but was nevertheless a good CYP1 inducer. This discrepancy cannot be explained at present. It may be argued that the skin may be capable of metabolising this 11-alkoxy-CPP-17-one to other mutagenic metabolites, not generated by the liver, but examination of the structure of this molecule makes this possibility very unlikely. An alternative explanation is that species differences between the mouse and rat CYP1 proteins may exist, so that the mouse CYP1, in contrast to the orthologous rat protein, can readily activate 11-ethoxy-CPP-17-one.

In summary the present studies have shown that increasing the size of the 11-alkoxy chain of CPP-17-ones beyond the ethoxy group, markedly reduces their ability to induce rat hepatic CYP1 proteins. Moreover, 11-methoxy-CPP-17-one provokes a positive mutagenic response in the Ames test in the presence of an activation system, but increasing the size of the 11-alkoxy group abolishes mutagenicity.

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