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CHARACTERIZATION OF CANNABIDIOL-MEDIATED CYTOCHROME P450 INACTIVATION

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Abstract—Cannibidiol (CBD) has been shown to impair hepatic drug metabolism in several animal species and to markedly inhibit mouse hepatic microsomal Δ^1-tetrahydrocannabinol (THC) metabolism by inactivating specific cytochrome P450s (P450) belonging to the 2C and 3A subfamilies. Elucidation of the mechanism of CBD-mediated P450 inhibition would be clinically very important for predicting its effect on metabolism of THC and the many other clinically important drugs known to be metabolized by P450s 2C and 3A. CBD-mediated inactivation of mouse hepatic microsomal P450s did not decrease hepatic microsomal heme content. However, [^{14}C]CBD was found covalently bound to microsomal protein in an approximately equimolar ratio to P450 loss. Immunoprecipitation of microsomal protein with antibodies raised against either P450 2C or 3A revealed that approximately equal amounts of [^{14}C]-CBD were bound to each of these P450s after CBD-mediated inactivation. Furthermore, this specific P450 binding was equivalent to P450 loss and accounted for nearly all of the microsomal [^{14}C]CBD irreversible binding. Although >80% of the enzyme activities attributed to P450s 2C and 3A were inactivated by CBD at the anticonvulsant dose of 120 mg/kg, P450 2C was approximately 3-fold more sensitive than P450 3A at the lower CBD doses tested. CBD analogs were synthesized in order to elucidate the chemical pathways for CBD-mediated P450 inactivation *in vivo*. Oxidations at allylic carbon positions or saturation of either the exocyclic double bond or both double bonds of the terpene moiety did not markedly affect the inhibitory properties of the analogs. Methylation of both phenolic groups of the resorcinol moiety completely blocked the P450-inhibitory properties of this analog, revealing the involvement of a free hydroxyl group in the inactivation process. Rotation of the resorcinol moiety in *abnormal*-CBD did not impair the inhibitory properties of the analog, suggesting that the position of the hydroxyl group relative to the terpene ring is unimportant. Further studies are required to fully understand the chemical basis of CBD-mediated P450 inactivation.

Cannabidiol (CBD)† is known to impair hepatic drug metabolism in several animal species [1–6] and to markedly inhibit mouse hepatic microsomal Δ^1-tetrahydrocannabinol (THC) metabolism [7, 8]. In the mouse, inhibition of drug metabolism results from CBD-mediated inactivation of specific cytochrome P450s (P450) belonging to the 2C and 3A subfamilies‡ [8, 10], which also catalyze most of the hepatic microsomal THC metabolism. Orthologs of these P450s are also found in human liver microsomes, and immunoinhibition studies reveal that their metabolic profiles are qualitatively similar to those of their mouse counterparts [11]. CBD-mediated inhibition of THC metabolism in humans has not been directly demonstrated, but CBD has been shown to modulate the clinical psychotropic effects of THC [12–15], possibly by altering the formation of THC metabolites that are known to be pharmacologically active [12–15]. In addition, clinically, CBD treatment apparently inhibits hexobarbital metabolism [16], suggesting that CBD can affect drug metabolism in humans. Elucidation of the mechanism of CBD-mediated P450 inhibition would be of great clinical importance not only in assessing the contribution of THC metabolites to its psychotropic effects, but also in predicting the effect of CBD on the metabolism of various other clinically important drugs known to be catalyzed by P450s 2C and 3A [17–22]. The present work characterizes the CBD-mediated inactivation of these mouse hepatic P450s in an attempt to elucidate the molecular basis for such inactivation.

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† Abbreviations: CBD, cannabidiol; and THC, Δ^1-tetrahydrocannabinol.

‡ The specific mouse P450s have been tentatively classifed into the P450 2C and 3A subfamilies based on limited amino-terminal sequence data. The sequences of individual subfamily members in the mouse have not been identified [9].

MATERIALS AND METHODS

Materials. CBD and THC were supplied by the National Institute on Drug Abuse. All cannabinoids were prepared for intraperitoneal injection in a Tween 80 suspension as previously described [2]. [^{14}C]CBD (7.68 mCi/mmol) was prepared from the condensation of [^{14}C]olivetol and *p*-mentha-2,8-dien-1-ol (Firmenich Corp., Princeton, NJ) as previously described [23, 24]. [^{14}C]Olivetol was prepared from the condensation of [^{14}C]diethyl malonate (8.11 mCi/mmol, Research Products International Corp., Mount Prospect, IL) and 3-nonen-2-one (Aldrich Chemical Co., Milwaukee, WI) as previously described [25].

6α-, 6β, and 10-Hydroxy-CBD and 6- and 10-

keto-CBD were prepared according to the method of Lander *et al.* [25]. Dihydro-CBD [26], tetrahydro-CBD [27], mono- and dimethyl-CBD [4, 28], and *abnormal*-CBD [23] were prepared as described. Spectra obtained after gas chromatography/mass spectrometry and NMR analyses of synthesized compounds were similar to those previously reported [4, 23, 25, 28]. Olivetol was obtained from the Sigma Chemical Co. (St. Louis, MO).

Animals were treated with cannabinoids (120 mg/kg for 2 hr, except where otherwise stated) before being killed by cervical dislocation. Liver microsomes were prepared as described [5].

Analytical procedures. Cytochrome P450 concentrations were determined with an SLM Aminco DW2000 spectrophotometer according to the method of Omura and Sato [29]. Protein concentrations were determined by the method of Lowry *et al.* [30], using bovine serum albumin as the standard.

Determination of [^{14}C]*CBD covalent binding.* Hepatic microsomal protein (10 mg) from mice treated with [^{14}C]CBD was precipitated with 25% trichloroacetic acid and washed repetitively as described previously [31, 32] until the radioactivity in the washes was reduced to background levels. Functionally reconstituted [8] purified P450 2C (0.2 nmol) was incubated with [^{14}C]CBD (32 μM) and NADPH (1 mM) at 37° for 30 min and precipitated with 5% sulfuric acid in methanol. Mouse hepatic microsomes (5 mg protein) were added as carrier protein and the pellet was washed twice more with 5% sulfuric acid in methanol, three times with diethyl ether:ethanol (1:1, v/v), and once with 80% aqueous methanol. Non-radiolabeled CBD (100-fold excess) was added at the end of the 30-min incubation, and at all washing steps to decrease the non-specific binding of [^{14}C]CBD. Precipitated protein was dissolved in 1 N sodium hydroxide and the amount of [^{14}C]CBD covalently bound quantitated by liquid scintillation counting (Beckman LS-7000).

Immunochemical procedures. Polyclonal antibodies (IgG) were raised against purified mouse P450s 2C [8] and 3A [10] as described. Solubilized microsomes were immunoprecipitated as described previously [33], in the presence of 5 or 15 mg of antibody/mg of microsomal protein using anti-P450 2C or 3A IgG, respectively. These antibody:microsomal protein ratios have been found to maximally inhibit the corresponding P450 functional activities [8]. Pre-immune IgG (obtained from animals before exposure to the purified P450) was first added to the solubilized microsomes to determine the extent of non-specific [^{14}C]CBD binding in the immunoprecipitate.

Determination of THC hydroxylase activity. Microsomal protein (0.1 mg) was preincubated at 37° for 3 min, in a buffer containing 0.1 M potassium phosphate, pH 7.4, diethylenetriaminepentaacetic acid (1 mM) and THC (130 μM, final concentrations, added in 3 μL of ethanol) in a total volume of 1 mL. The incubation, metabolite extraction and quantitation were performed as described previously [8]. THC hydroxylase activity of purified P450 2C after CBD-mediated inhibition *in vitro* was determined as follows: Functionally reconstituted [8] purified P450 2C (200 pmol) was preincubated in the presence or absence of CBD (32.5 μM) and NADPH (1 mM) for 0–12 min. Aliquots (10 pmol of purified P450 2C) were diluted 10-fold in buffer containing THC (130 μM) and NADPH (1 mM), and incubated for an additional 5 min before metabolites were extracted and quantitated as above.

Statistical analysis. Statistical significance was determined by Student's *t*-test.

RESULTS

CBD-mediated microsomal P450 loss and covalent binding. CBD (120 mg/kg for 2 hr) destroyed approximately 25% of the spectrally detectable hepatic microsomal P450 content in the mouse. In contrast to the heme loss sometimes observed after treatment with other P450 inactivators [34–36], CBD-mediated P450 inactivation was not accompanied by decreased hepatic microsomal heme content, in confirmation of an earlier study [4]. Because CBD treatment did not result in heme loss, the possibility that CBD-mediated P450 inactivation involved apoprotein alkylation was examined. To determine if such apoP450 alkylation involved covalent binding of CBD, hepatic microsomes from mice treated with [^{14}C]CBD (120 mg/kg for 2 hr) were acid precipitated and washed extensively with organic solvents to remove non-covalently bound CBD. CBD was found covalently bound to the microsomal protein in an approximately 1:1 stoichiometry to P450 loss (Table 1). Accordingly, in untreated mice, CBD treatment resulted in the loss of 0.28 nmol of P450/mg of microsomal protein as well as in the covalent binding of 0.33 nmol of CBD/mg of microsomal protein (Table 1).

To determine if the covalent CBD binding was associated with the particular P450s inactivated after *in vivo* CBD exposure [8, 10], hepatic microsomes from mice treated with [^{14}C]CBD (*in vivo*) were solubilized and immunoprecipitated with antibodies raised against mouse P450 2C or 3A. Approximately equal amounts of CBD were found tightly bound to each of these P450s (Table 1), even after extensive washing of the immunoprecipitates which effectively removed exogenously added [^{14}C]CBD. The total amount of CBD associated with P450s 2C and 3A (0.14 and 0.19 nmol/mg of microsomal protein, respectively) was roughly equivalent to the loss of P450 as well as to the total amount of CBD covalently bound to microsomal protein (Table 1). Pretreatment of mice with phenobarbital induces P450s 2C [37] and 3A [10, 38] and enhances CBD-mediated P450 loss [10]. [^{14}C]CBD treatment of phenobarbital-pretreated mice also resulted in increased covalent binding, which could be largely attributed to increased specific binding to P450s 2C and 3A (Table 1). Although the total amount of [^{14}C]CBD covalently bound to hepatic microsomal protein from phenobarbital-treated mice was somewhat greater than either the P450 loss or selective binding to immunoprecipitated P450s 2C and 3A, this increase was not statistically significant ($p > 0.10$), and may reflect limited binding to other phenobarbital-inducible P450s. However, the finding that [^{14}C]-CBD covalent binding, P450 loss and selective

Table 1. Effect of phenobarbital pretreatment on P450 loss and [^{14}C]CBD binding after CBD treatment

Treatment	P450 (nmol/mg microsomal protein)		[^{14}C]CBD binding (nmol/mg microsomal protein)			
			Total covalently bound	Immunoprecipitated P450s		
	Content	Loss		2C	3A	2C + 3A
None/CBD	0.81 ± 0.04	0.28 ± 0.04	0.33 ± 0.06	0.14 ± 0.03	0.19 ± 0.08	0.34
Phenobarbital/CBD	1.34 ± 0.11	0.56 ± 0.08	0.77 ± 0.20	0.24 ± 0.09	0.27 ± 0.12	0.51

Mice either previously untreated or pretreated with phenobarbital (100 mg/kg, i.p., daily for 4 days) were given [^{14}C]CBD (7.68 mCi/mmol; 120 mg/kg, i.p.) and killed 2 hr later; then hepatic microsomes were prepared. P450 content was determined as described in Materials and Methods. The amount of [^{14}C]-CBD covalently bound to microsomal protein after acid precipitation and extensive washing with organic solvents, or the amount of [^{14}C]CBD bound to immunoprecipitated P450 after incubation of microsomal protein with antibodies raised against purified mouse P450s 2C or 3A was also determined. Values are the means ± SD of three different microsomal preparations. Basal P450 values before CBD treatment were 1.09 ± 0.08 and 1.90 ± 0.14 nmol/mg of microsomal protein for untreated and phenobarbital-pretreated mice, respectively. P450 loss was determined as the difference between the P450 content before and after CBD treatment.

binding to immunoprecipitated P450s 2C and 3A were approximately equal and that phenobarbital pretreatment comparably increased each of these parameters further suggest that these processes are related.

The extent of covalent binding of [^{14}C]CBD to purified and functionally reconstituted P450 2C was also examined, since P450 2C was found previously to be resistant to CBD-mediated destruction as assessed by the loss of spectrally detected chromophore [8]. [^{14}C]CBD covalently bound to functionally reconstituted purified P450 2C in an approximately 1:1 stoichiometry (1.05 nmol of [^{14}C]-CBD bound/nmol of P450 2C) when incubated in

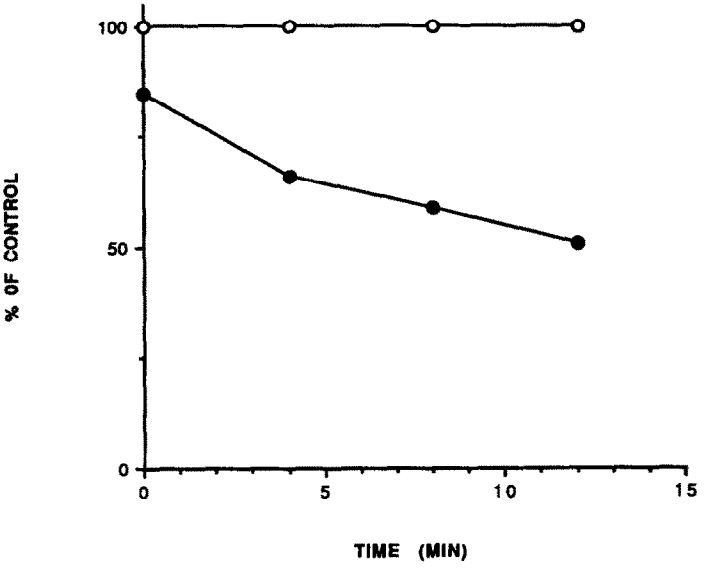

Fig. 1. Effect of *in vitro* CBD preincubation on purified P450 2C-mediated activity. THC hydroxylase activity of purified P450 2C after CBC-mediated inhibition *in vitro* was determined as follows: Functionally reconstituted [8] purified P450 2C (200 pmol) was preincubated in the presence (●) or absence (○) of CBD (32.5 μM) and NADPH (1 mM) for 0–12 min. Aliquots (10 pmol of purified P450 2C) were diluted 10-fold in buffer containing THC (130 μM) and NADPH (1 mM), and incubated for an additional 5 min before metabolites were extracted and 7-hydroxy-THC activity was quantitated as described previously [8]. Values are expressed as percent of control values (samples incubated in the absence of CBD) and represent the average of two determinations. Control values (7.62 nmol 7-hydroxy-THC formed/nmol P450 2C/min at zero incubation time) decreased <15% during the incubation period.

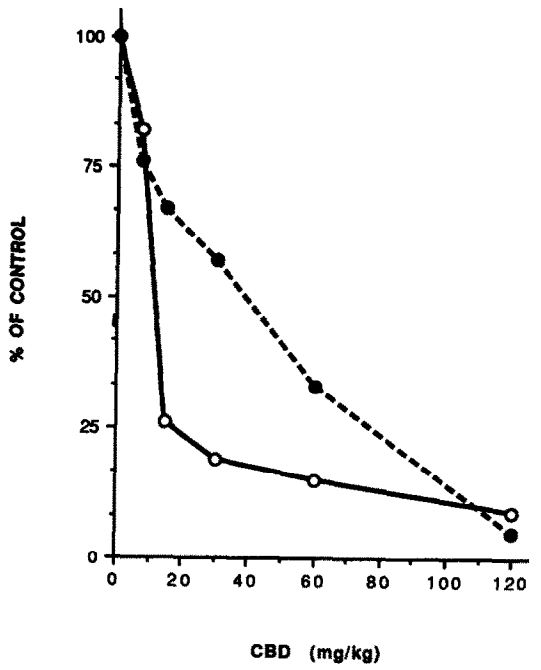

Fig. 2. Relative effects of CBD dose on P450 2C- and 3A-mediated activities. Mice were treated with CBD for 2 hr at the indicated doses. Hepatic microsomes were prepared and assayed for 7-hydroxy-THC (——) and 6-keto-THC (– – –) formation. Values are expressed as percent of control values and were determined from the average of three different microsomal preparations. The mean ± SD of the control microsomal preparations was 2.11 ± 0.16 and 0.21 ± 0.03 nmol/mg/min for 7-hydroxy-THC and 6-keto-THC formation, respectively.

the presence of NADPH for 30 min. Non-specific binding (determined in the absence of NADPH) accounted for only 0.25 nmol of [^{14}C]CBD bound/nmol of P450 2C, suggesting that most of the binding in the NADPH-supplemented incubation was metabolism dependent. In addition, purified P450 2C-mediated THC hydroxylase activity was inhibited in a time-dependent manner after preincubation with CBD (Fig. 1). Thus, although the incubation of functionally reconstituted P450 2C with CBD did not result in any loss of P450 chromophore [8], the demonstration of CBD-mediated loss of P450 2C functional activity as well as the stoichiometric covalent binding of [^{14}C]CBD suggest that P450 2C inactivation does occur.

Comparison of hepatic microsomal P450s 2C and 3A sensitivity to CBD-mediated inactivation. To determine the relative susceptibility of P450s 2C and 3A to CBD-mediated inactivation *in vivo*, 7-hydroxy- and 6-keto-THC formation were used as selective functional markers for P450s 2C and 3A, respectively, since antibodies prepared against these P450s selectively inhibit >80% of their respective microsomal activity [8]. P450 2C appeared to be much more susceptible than P450 3A, with 75% inactivated at a 15 mg/kg CBD dose (Fig. 2). However, both isozymes were inactivated to a comparable extent (>80%) at a 120 mg/kg dose of CBD, which is a pharmacologically effective anticonvulsant dose in mice [39].

CBD-mediated inhibition: Structural features. To elucidate the chemical pathways involved in the inactivation process, several CBD analogs were synthesized in order to determine the structural features necessary for inactivation (Fig. 3). To assess analog-mediated inhibition, hepatic microsomal 7-hydroxy- or 6-keto-THC formation was used as a selective marker for P450 2C or 3A, respectively [8]. To minimize competitive inhibitor effects of CBD analogs on THC hydroxylase activity, the effect of each analog was assessed after *in vivo* treatment using hepatic microsomes washed with 1.15% potassium chloride. The amount of residual analog typically found in washed microsomes was 1–3 μM in the microsomal incubations used in the THC hydroxylase assay, a concentration found to exert little effect (<20% inhibition) on THC hydroxylase activity, which was determined in the presence of 130 μM THC.

Oxidations at the C-6 and C-10 allylic carbon positions of the terpene moiety (6α-, 6β-, and 10-hydroxy-CBD, and 6- and 10-keto-CBD) did not affect markedly the inhibitory properties of the analogs (Fig. 4). Analogs saturated at either the exocyclic double bond alone (dihydro-CBD) or at both double bonds of the terpene moiety (tetrahydro-CBD) were also capable of significant inhibition. Although tetrahydro-CBD was not as effective a P450 inhibitor as CBD, it still caused significant inhibition, thereby revealing that the double bond between either C_{1-2} or C_{8-9} is not an absolute requirement for inactivation. Pharmacokinetic differences between tetrahydro-CBD and the other analogs may exist, since at a higher tetrahydro-CBD dose (240 mg/kg for 4 hr), P450 2C and 3A inhibition increased to >50%, comparable to that observed with the other analogs. Thus, structural/chemical alterations of the terpene moiety do not appear to influence the P450 inhibitory properties of CBD.

To assess the role of the resorcinol moiety in the inactivation process, CBD methyl ethers of one or both phenolic groups (mono- or dimethyl-CBD) were prepared. Methylation of one of these groups impaired the ability of the monomethyl-CBD to inhibit P450 at the 120 mg/kg dose, although significant inhibition was observed at the 240 mg/kg dose. However, methylation of both phenolic groups (dimethyl-CBD) prevented significant P450 inhibition, even at the higher dose tested, revealing the requirement for at least one free hydroxyl group in the inactivation process.

The positional isomer of the resorcinol moiety of CBD (*abnormal*-CBD), in which the position of a phenolic group and the pentyl side-chain are interchanged, was, however, as effective as CBD in inhibiting both P450s. Finally, olivetol, a resorcinol analog containing both the phenolic groups and the pentyl side-chain present in CBD but not its terpene moiety, was ineffective as a P450 inhibitor. Although this implies a requirement for the terpene ring in the inactivation process, the markedly different chemical nature and lipophilicity of olivetol from

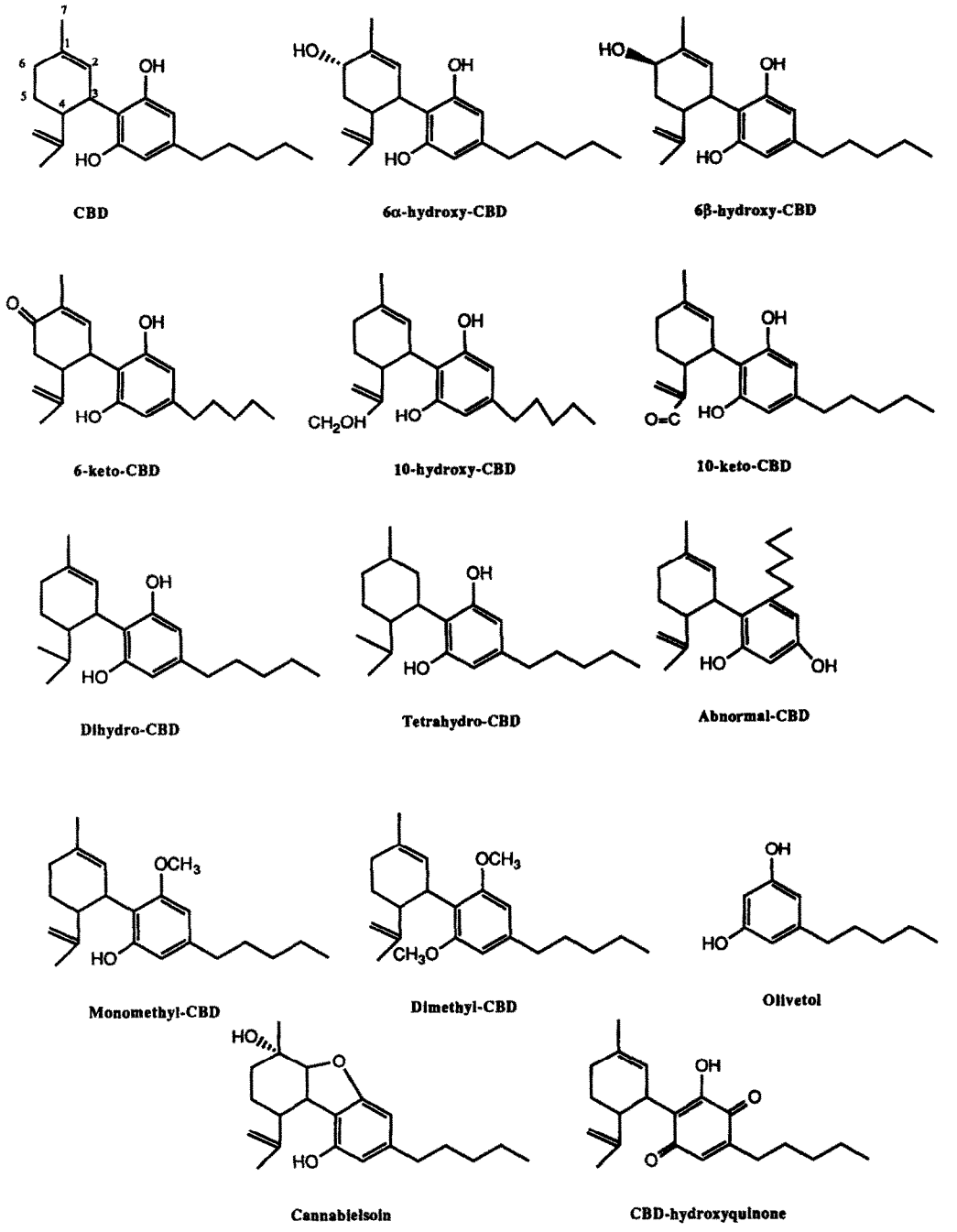

Fig. 3. Structures of CBD and other analogs.

that of CBD or the other analogs make any interpretation difficult.

DISCUSSION

P450 inactivation can result from the binding of a substrate or its metabolite to the apoprotein [40, 41], and/or to the heme moiety [34–36] of the enzyme. Because CBD treatment did not result in heme loss as expected after heme modification, the possibility of apoprotein alkylation by CBD was investigated. Apoprotein alkylation would result in enzyme inactivation, only if the alkylation prevented subsequent enzyme turnover. Although P450-mediated metabolism of certain compounds results in their alkylation of the apoP450, an inordinately large ratio of covalent binding to P450 loss is sometimes observed, indicating that the binding does not necessarily impede further catalytic turnover and activation [40, 42]. After CBD treatment, however,

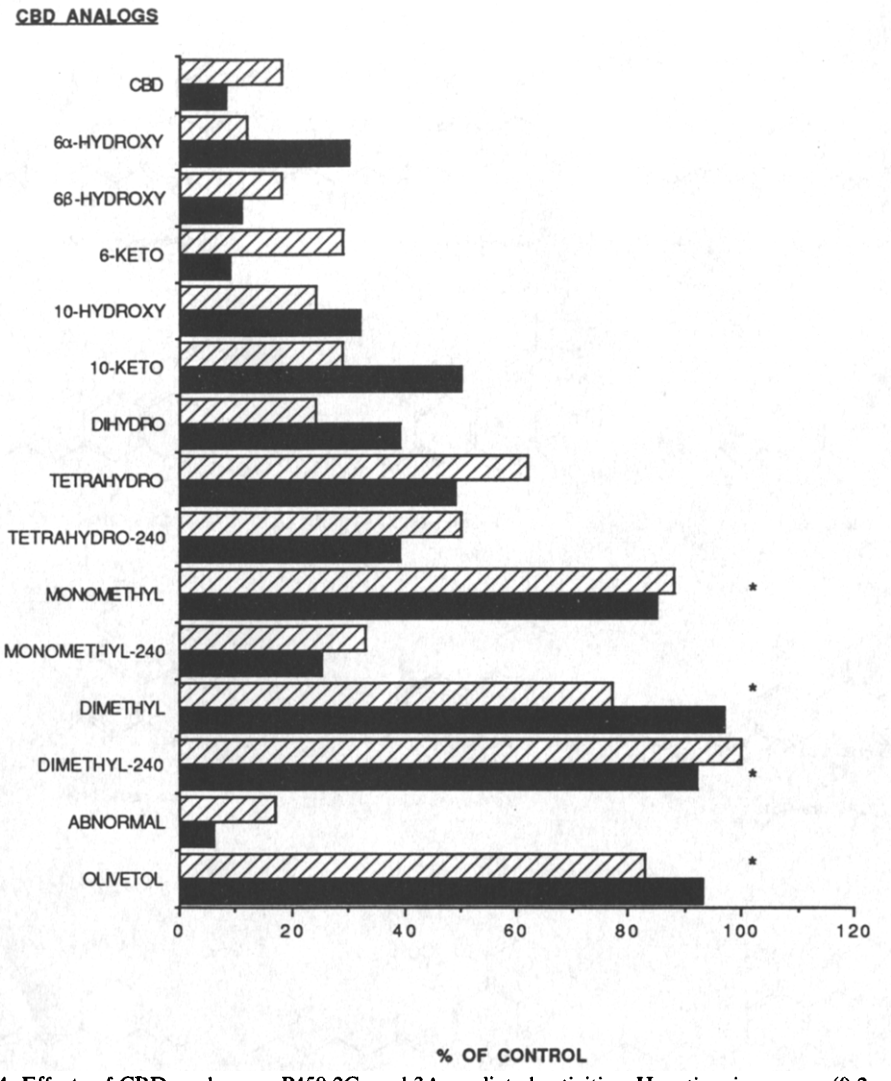

Fig. 4. Effects of CBD analogs on P450 2C- and 3A-mediated activities. Hepatic microsomes (0.2 mg) from animals treated with various analogs (120 mg/kg for 2 hr, except where otherwise indicated) were assayed for 6-keto-THC (▨) and 7-hydroxy-THC (■) formation, selective markers for P450 3A and 2C, respectively, in the presence of THC (130 μM). Values are expressed as percent of control values and were determined from the average of three different microsomal preparations. The mean ± SD of ten different control microsomal preparations was 1.86 ± 0.74 and 0.14 ± 0.03 nmol/mg/min for 7-hydroxy-THC and 6-keto-THC formation, respectively. All values were significantly different ($P < 0.05$) from control mean values *except* those marked by an asterisk (*).

we found that CBD covalently bound to the microsomal protein in an approximately 1:1 stoichiometry to P450 loss (Table 1). A stoichiometric ratio of covalent binding to P450 inactivation has also been found after chloramphenicol metabolism [41]. However, in contrast to the P450 loss observed after CBD treatment, spectrally detectable microsomal P450 content was not affected after chloramphenicol-mediated inactivation, presumably because the reactive chloramphenicol metabolite-binding only interferes with the electron transfer from NADPH-P450 reductase [43].

Although the correlation of P450 loss and covalent binding of CBD to microsomal protein suggests that these processes are related, such binding could have occurred independently of P450 inactivation and entailed microsomal proteins other than P450. The demonstration that the specific binding of [^{14}C]CBD to P450s 2C and 3A was approximately equal to the total [^{14}C]CBD bound to microsomal protein (Table 1), however, presents additional support that the covalent binding of CBD to microsomal protein reflects binding to the inactivated P450s rather than to non-P450 microsomal proteins. Indeed, the stoichiometry of P450 loss, covalent binding, and specific binding to P450s 2C and 3A in both phenobarbital-induced and non-induced animals suggests that these processes are related. The

identification of the precise alkylating species and the site of P450 alkylation are currently under investigation.

Previous studies with purified P450s revealed that although P450 3A is destroyed by CBD *in vitro* [10], as determined by the loss of the chromophore, P450 2C appears resistant to such destruction [8], despite the functional inactivation of both P450s by CBD after *in vivo* treatment. Chloramphenicol also inactivates P450 (2B1) without causing loss of its chromophore, and such inactivation is reportedly due to altered P450 reducibility after covalent binding of a chloramphenicol metabolite to the P450 apoprotein [41, 43]. We have therefore examined the effect of CBD on purified, functionally reconstituted P450 2C by determining both the extent of covalent binding of [^{14}C]CBD to the purified enzyme as well as its functional inhibition during *in vitro* CBD metabolism. The results suggest that P450 2C is indeed inactivated by CBD *in vitro* without loss of its chromophore. [^{14}C]CBD was found to bind covalently to functionally reconstituted purified P450 2C in equimolar amounts and when CBD was preincubated with P450 2C, a time-dependent loss of its functional activity was observed (Fig. 1). In such experiments, the time-dependent loss of enzyme activity (inactivation) is often superimposed on time-independent (competitive) enzyme inhibition, resulting from the presence of any unmetabolized CBD during the functional (THC hydroxylation) assay. P450 2C-mediated 7-hydroxy-THC formation was inhibited in a time-dependent manner, exhibiting a 50% loss of activity after 12 min of preincubation with CBD. Incubation of reconstituted purified P450 2C for longer periods resulted in a CBD-independent loss of >20% of activity and was therefore not included. Thus, although *in vitro* incubation of reconstituted purified P450 2C with CBD does not result in loss of its spectrally detectable content [8], the above data demonstrate the functional inactivation of purified P450 2C by CBD, probably resulting from [^{14}C]-CBD covalent binding.

To determine the structural features required for inactivation, a series of CBD analogs were synthesized. The relative *in vivo* effectiveness of these inhibitors, which would be influenced by differential rates of analog absorption, metabolism and excretion was not compared. In addition, the maximally inhibitory dose or time of treatment for each analog was not determined. The treatment chosen (120 mg/kg for 2 hr) was found to cause maximal inhibition of P450s 2C and 3A after CBD administration. However, several analogs that failed to significantly inhibit P450s 2C and 3A at the above dose were also administered at a higher dose (240 mg/kg for 4 hr), in order to circumvent differential distribution.

The CBD molecule contains a terpene and a resorcinol moiety. The terpene moiety contains endo- and exocyclic double bonds that may be bioactivated to alkylating functions [44]. CBD metabolites formed by rodent liver [45] or isolated from human urine after CBD treatment [46] include the expected products hydroxylated at C-6 and C-7 (allylic to the endocyclic C_{1-2} double bond) and in the pentyl side-chain, as well as those hydroxylated at the exocyclic C_{8-9} double bond or at the allylic C_{10} position. Oxidation at the endocyclic C_{1-2} double bond of CBD has not been demonstrated, but 1,2-epoxyhexahydrocannabinol has been reported as a THC metabolite [11, 47]. Analogs structurally modified at many of these predictable sites of oxidation were therefore prepared (Fig. 3). All modifications made in the terpene moiety failed to influence the inhibitory properties of the analogs. Only the analog dimethylated in the resorcinol moiety failed to inhibit P450s 2C and 3A. The lack of P450 inhibition by this analog was not due to insufficient hepatic uptake or resistance to P450-mediated metabolism, since residual amounts of dimethyl-CBD were detected in hepatic microsomes at levels comparable to those found after *in vivo* CBD treatment, and *in vitro* microsomal metabolite profiles of dimethyl-CBD were also very similar to that of CBD. Although at least one free phenolic group appears to be involved in the inactivation process, the effectiveness of *abnormal*-CBD as a P450 inactivator suggests that the relative position of the hydroxyl group to the terpene ring is not critical for inactivation. In addition, since the rotation of the resorcinol moiety with respect to the terpene ring would be sterically hindered in *abnormal*-CBD, this feature apparently is also not essential for inactivation.

Although these limited structure–activity studies reveal the importance of a free phenolic group in the resorcinol moiety, the active CBD species reponsible for P450 inactivation is yet to be identified. A free phenolic hydroxyl group of CBD has been implicated in the intramolecular rearrangement of CBD to cannabielsoin (Fig. 3). A plausible mechanism [48, 49] entails an initial P450-mediated oxidation at the C_{1-2} position to yield 1*S*, 2*R*-epoxy-CBD, which is attacked by a phenoxy anion to yield cannabielsoin. This reaction failed to proceed when both phenolic groups were methylated, but could occur after monomethylation of one of the phenolic groups. The CBD-mediated P450 inactivation process probably does not involve the same cannabielsoin cyclization mechanism, since epoxide formation at the C_{1-2} position would not occur in tetrahydro-CBD, an analog capable of inactivating P450. Nevertheless, the cannabielsoin cyclization mechanism demonstrates the potential reactivity of the phenolic groups with oxygenated CBD intermediates and this reactivity also may be recruited in P450 inactivation, albeit in a different reaction pathway.

CBD-hydroxyquinone has been shown to inactivate hepatic P450 and inhibit drug-metabolizing enzymes in mice [50]. The mechanism of the inhibitory effects of this compound was not determined but appeared to differ from that of CBD since, in contrast to CBD, CBD-hydroxyquinone decreased hepatic microsomal heme content and did not require NADPH for its effects. Since glutathione and cysteine attenuated the effect of CBD-hydroxyquinone on P450, the mechanism of inactivation by this compound was hypothesized [50] to involve the generation of radical oxygen species by the quinone. In contrast, glutathione and cysteine

were ineffective in preventing P450 inactivation by CBD [4], again implying different inactivation mechanisms by CBD and CBD-hydroxyquinone. Nevertheless, CBD-hydroxyquinone appears to be reactive and although probably not involved in CBD-mediated P450 inactivation, demonstrates the reactivity of a CBD oxidation product.

The involvement of the free phenolic groups of CBD in the generation of carbon monoxide during *in vitro* CBD metabolism [2, 51] has also been demonstrated [52]. Although the source and the biochemical basis for the carbon monoxide generation are not presently understood, a recent study revealed that the structural features required for carbon monoxide generation are similar to those required for P450 inactivation after *in vivo* treatment, i.e. a role for free hydroxyl groups of the resorcinol moiety. It is unclear, however, whether the CBD-mediated P450 inactivation is mechanistically related to the carbon monoxide generation observed during *in vitro* CBD metabolism. We and others [51] have found that although the carbon monoxide generated during *in vitro* CBD metabolism can complex more than 60% of the P450 present, the loss of hepatic microsomal P450 content was minimal and much lower than that observed after *in vivo* CBD treatment. Thus, CBD-mediated carbon monoxide generation cannot directly result from P450-heme destruction but may involve the aforementioned potential reactivity of the phenolic groups with other cellular constituents such as lipids, known to produce carbon monoxide during peroxidative degradation [53]. Additional studies are required to determine the molecular basis of CBD-mediated P450 inactivation and carbon monoxide generation.

In summary, the results discussed above indicate that the CBD-mediated inhibition of drug metabolism is probably a result of the covalent binding of a reactive CBD metabolite to hepatic microsomal P450. This binding correlated well with the decreased hepatic P450 content and the specific binding of CBD to P450s 2C and 3A observed after [^{14}C]CBD treatment. P450 2C is more susceptible than P450 3A to CBD-mediated inhibition *in vivo*, and appears to be alkylated and inactivated after CBD-mediated incubation *in vitro*, albeit by a mechanism independent of P450 heme destruction. Structural studies reveal the involvement of one of the two free phenolic groups of CBD in the inactivation process, but the mechanism of inactivation remains to be elucidated.

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