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MOLECULAR PHARMACOLOGY, 34:139-147

Specific Inactivation by 17β-Substituted Steroids of Rabbit and Rat Liver Cytochromes P-450 Responsible for Progesterone 21-Hydroxylation

JAMES HALPERT, JOHN-YAN JAW, and CELIA BALFOUR

Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona, Tucson, Arizona 85721

Received August 3, 1988; Accepted September 27, 1988

SUMMARY

The selective inactivation by 17β-substituted steroids of rabbit and rat liver cytochromes P-450 involved in the 21-hydroxylation of progesterone has been investigated. Five derivatives each of pregnenolone and progesterone were prepared, in which the methylketo substituent in the 17β-position was replaced by a dichloromethylketo, chlorofluoromethylketo, difluoromethylketo, vinyl, or ethynyl group. The ability of the compounds to cause time-dependent (inactivation) and time-independent (inhibition) decreases in progesterone hydroxylase activity was assessed *in vitro* using intact liver microsomes as well as reconstituted systems containing the major forms of hepatic cytochrome P-450 responsible for progesterone 21-hydroxylation, P-450 1 in the rabbit and PB-C in the rat. In each species, one compound was identified that specifically inactivated the 21-hydroxylase, namely 21-chloro-21-fluoropregnenolone in the rabbit and pregn-4,20-diene-3-one in the rat, although both compounds inhibited several other hydroxylases as well. Moreover, the most effective and specific 21-hydroxylase inactivators were not necessarily the most effective or specific inhibitors. These results suggest that conversion of the enzyme-inhibitor complex to metabolites that inactivate the enzyme, rather than complex formation, is the crucial factor in determining the specificity of the compounds as cytochrome P-450 inactivators. The results indicate the feasibility of designing specific inactivators of hepatic cytochromes P-450 by utilizing the normal regioselectivity of the target enzyme towards steroids.

The cytochrome P-450-dependent monooxygenase system plays a key role in the metabolism of a wide variety of xenobiotics and endogenous compounds. Cytochromes P-450 in experimental animals and in humans exist in multiple forms,[1] the relative amounts and activities of which may largely govern the balance between bioactivation and detoxification of a particular compound (3, 4). Many questions remain to be answered, however, about the roles of various forms of cytochrome P-450 in the metabolism of particular compounds *in vivo*. Furthermore, the idea of manipulating the activities of individual human cytochromes P-450 for therapeutic purposes, although an attractive one, remains largely speculation (3). A major reason for the slow progress in these areas has been the lack of specific chemical inhibitors that could be used to probe and modulate the function of the different forms of cytochrome P-450 *in vivo*.

Among the potentially most specific enzyme inhibitors are the mechanism-based inactivators, also known as suicide substrates. These are substrate molecules for the target enzyme, which in the process of catalytic conversion, are changed into intermediates or products that inactivate the enzyme (5). The specificity resides both in the binding and in the catalytic specificity of the enzyme, in contrast to reversible inhibitors, which rely solely on binding. The high degree of selectivity often obtainable with mechanism-based inactivators makes them particularly attractive for *in vivo* applications (6, 7).

To date, the most successful efforts to design selective mechanism-based inactivators of cytochromes P-450 have involved the introduction of acetylenic moieties into substrates such as

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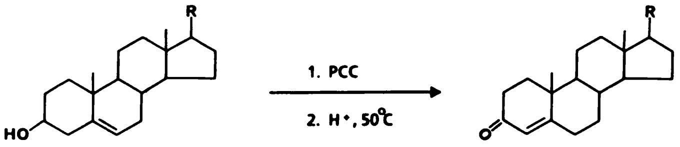
This work was supported by National Institutes of Health Grant ES03619. J. H. is the recipient of Research Career Development Award ES00151.

[1] In this paper, preparations of cytochrome P-450 from different laboratories are referred to according to the nomenclature in use in each respective laboratory. A recently recommended nomenclature for some of the cytochromes P-450 mentioned in this paper based on inferred amino acid sequences and a discussion of the trivial nomenclatures in use in different laboratories can be found in Ref. 1 and 2, respectively. The major cytochromes P-450 referred to in this report and their associated diagnostic steroid hydroxylase activities are as follows: P-450 1, a progesterone 21-hydroxylase encoded by the rabbit P-450IIC5 gene; P-450 3b, a progesterone 6β- and 16α-hydroxylase encoded by the rabbit P-450IIC3 gene; P-450 3c, a progesterone 6β-hydroxylase encoded by the rabbit p-450 IIIA4 gene; UT-F (P-450a), a steroid 7α-hydroxylase encoded by the rat P-450IIA1 gene; PB-B (P-450b), an androstenedione 16β-hydroxylase encoded by the rat P-450IIB1 gene; PB-C (P-450k), a progesterone 21-hydroxylase encoded by the rat P-450IIC6 gene; P-450p, a steroid 6β-hydroxylase encoded by the rat P-450IIIA1 gene; and UT-A (P-450h), a steroid 2α-hydroxylase.

ABBREVIATIONS: PCC, pyridinium chlorochromate; PB, phenobarbital; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NZW, New Zealand White; 21H and 21L, phenotypes of rabbits expressing high and low hepatic progesterone 21-hydroxylase activity (>1 and <1 nmol/min/mg of microsomal protein, respectively); NCS, *N*-chlorosuccinimide; THF, tetrahydrofuran.



1 a	$R = -CO-CHCl_2$	1 b
2 a	$R = -CO-CHF_2$	2 b
3 a	$R = -CO-CHFCl$	3 b
4 a	$R = -CH = CH_2$	4 b
5 a	$R = -C \equiv CH$	5 b

Fig. 1. Structure of 17β-substituted steroids. The general procedure for oxidizing pregnenolone to progesterone derivatives is indicated.

fatty acids (8) or steroids (9) that are normally metabolized in a specific fashion by the target enzymes. Our own recent work suggests that the dichloromethyl moiety, by virtue of its conversion to an acyl chloride, may also confer upon a molecule the ability to inactivate cytochromes P-450 (10, 11). This finding led to the hypothesis that the judicious incorporation of a dichloromethyl group into particular substrates might prove to be an alternative means of designing selective inactivators of cytochromes P-450.

As model target systems for testing this hypothesis, the inactivation by 21,21-dichloropregnenolone and 21,21-dichloroprogesterone of the rabbit and rat liver microsomal cytochromes P-450 involved in progesterone metabolism (12, 13) was recently examined, because previous work had shown that, in liver microsomes from both species, the 21-methyl group of progesterone is specifically hydroxylated by a single major form of cytochrome P-450, termed P-450 1 in the rabbit (14, 15) and P-450k in the rat (16). In each species, one of the dichloromethyl compounds was found to preferentially inactivate the target enzyme, but inactivation of a cytochrome P-450 that does not normally participate in 21-hydroxylation of the parent compound was observed as well. These results suggested that the important question of the retention of the regioselectivity of the target and non-target enzymes towards the putative inactivator, compared with the underivatized substrate, would have to be approached on an empirical basis. In the present investigation, four additional derivatives each of pregnenolone and progesterone have been prepared, in which the methylketo group in the 17β-position has been replaced by a chlorofluoromethylketo, difluoromethylketo, vinyl, or ethynyl group (Fig. 1). The results suggest that the judicious combination of steroid nucleus and 17β functional group results in specific hepatic progesterone 21-hydroxylase inactivators.

Experimental Procedures

Materials. [4-^{14}C]Progesterone was purchased from NEN Research Products (Boston, MA). Unlabeled progesterone, 16α-OH-progesterone, dilauryl-L-3-phosphatidylcholine, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). Deoxycorticosterone (21-OH-progesterone), 2α-OH-progesterone, and 6β-OH-progesterone were purchased from Steraloids (Wilton, NH). Pregnenolone, PCC, and NCS were purchased from Aldrich Chemical Company (Milwaukee, WI). Perchloryl fluoride gas ($FClO_3$) was purchased from Ozark-Mahoning Company (Tulsa, OK). THF was distilled from sodium benzophenone ketyl before use. PB-C (P-450k) (17), rat liver cytochrome b_5 (13), and rat liver NADPH-cytochrome P-450 reductase (18) were purified as described previously.

21,21-Dichloropregnenolone (**1a**) and 21,21-dichloroprogesterone (**1b**) were synthesized as described previously (12). 21,21-Difluoropregnenolone (**2a**) was synthesized according to a published procedure with some modification (19). 21-Chloro-21-fluoropregnenolone (**3a**) was synthesized as described below. Pregn-5,20-diene-3β-ol (**4a**) and pregn-5-en-20-yne-3β-ol (**5a**) were synthesized according to known procedures (20). Progesterone derivatives (**2b, 3b, 4b,** and **5b**) were synthesized from the corresponding pregnenolone derivatives by PCC oxidation as described below.

Proton magnetic resonance spectra were recorded at 90 MHz on a Jeol-FX90Q FTNMR or at 250 MHz on a Bruker WM-250 NMR spectrometer as indicated. Carbon magnetic resonance spectra were recorded at 62.9 MHz on a Bruker WM-250 NMR spectrometer. Mass spectral analysis was performed on a MAT-90 mass spectrometer (electron impact mode, 70 eV).

21,21-Difluoropregnenolone (2a). A solution of 4.0 g (9.2 mmol) of sodium salt of 21-ethoxalylpregnenolone (21) in methanol (135 ml) and methanolic sodium methoxide (18.2 ml, 1 N) was cooled to −10°. Perchloryl fluoride gas was slowly bubbled into the above solution. When the solution was neutral (about 25 min), the mixture was thoroughly flushed with nitrogen to remove excess $FClO_3$. The same procedures were repeated twice more (18 ml of 1 N CH_3ONa/CH_3OH, $FClO_3$ gas, flushed with nitrogen). The reaction mixture was poured into 100 ml of water. The product was extracted with chloroform twice, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was dissolved in a solution of methanol (110 ml) containing 3.7 g of potassium acetate. The mixture was refluxed for 90 min, and then the methanol was removed *in vacuo*. The residue was treated with water (100 ml), and the product was collected by filtration. The crude products were dried overnight and were separated by column chromatography (silica gel; ethyl acetate/hexanes, 1:5 and 1:3, v/v) to give 1.75 g of pure **2a**, m.p. 133–134° [recrystallized from acetone/hexane; literature m.p. 135–136° (19)] and 0.81 g of pure 21-fluoropregnenolone.

Physical data of **2a**: ^{1}H NMR ($CDCl_3$, 90 MHz) δ 0.70 (s, 3H, C18), 1.01 (s, 3H, C19), 2.97 (t, 1H, —CHCO-), 3.54 (m, 1H, —CHOH−), 5.35 (d, 1H, =CH), 5.64 (t, 1H, J = 54.2 Hz, —CHF_2); ^{13}C NMR ($CDCl_3$) δ 13.69, 19.37, 21.0, 23.60, 24.68, 31.58, 31.75, 31.99, 36.48, 37.21, 38.32, 42.21, 46.13, 49.77, 56.78, 57.12, 71.65, 109.82 (t, J = 253.4 Hz, C21), 121.23, 140.78, 201.21 (t, J = 25.2 Hz, C20); mass spectrum, m/z (relative intensity) 353.3 (16), 352.3 (69), 350.3 (4), 335.3 (19), 334.3 (87), 320.3 (18), 319.3 (100), 301.3 (7), 295.3 (12), 293.2 (13), 292.2 (13), 268.2 (12), 267.2 (61), 241.2 (69), 240.2 (21), 223.2 (15), 213.2 (18), 173.2 (23), 167.1 (29), 161.2 (19), 159.2 (25), 149.1 (72), 147.2 (21), 145.2 (55), 133.2 (33), 131.2 (24), 129.1 (16), 121.2 (34), 120.2 (21), 119.1 (35), 117.1 (19), 107.1 (49), 105.1 (46), 95.1 (26), 93.1 (34), 91.1 (63), 81.1 (35), 79.1 (51), 77.1 (23), 55.1 (37).

Synthesis of 21-chloro-21-fluoropregnenolone (3a). To a well stirred solution of lithium diisopropylamide (2.4 mmol), freshly prepared from diisopropylamine (0.34 ml) and n-butyllithium (1.5 ml, 1.6 M) in 15 ml of THF at −78° for 10–15 min, was added via a cannula a cold (0°) solution of 334 mg (1.0 mmol) of 21-fluoropregnenolone (22) in 15 ml of THF. After the reaction was stirred at −78° for 15 min, 147 mg (1.1 mmol) of NCS was added, and the reaction was stirred at −78° for 20 min. At this time another 40 mg (0.3 mmol) of NCS was added, and the reaction was continued for 60 min. By that time the temperature of the dry ice/acetone bath had risen to −50°. The solution was poured into 30 ml of water and was extracted with ethyl acetate (2 × 25 ml). The organic extracts were dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was chromatographed over silica gel (ethyl acetate/hexanes, 1:3, v/v) to obtain 99 mg (27%) of 21-chloro-21-fluoropregnenolone and 120 mg (36% recovery) of 21-fluoropregnenolone.

Physical data of **3a**: ^{1}H NMR ($CDCl_3$, 250MHz) δ 0.74 (d, 3H, J = 2.4Hz two stereoisomers at C18), 1.01 (s, 3H, C19), 2.95 (m, 1H, —

CHCO), 3.53 (m, 1H, —CHOH), 5.34 (m, 1H, =CH), 6.09 (d, $J = 51.6$ Hz) and 6.16 (d, $J = 51.6$Hz) (1H, two stereoisomers at —CHFCl); ^{13}C NMR ($CDCl_3$, two stereoisomers) δ 13.50, 13.55, 19.28. 20.9, 23.97, 24.53, 24.81, 25.52, 30.84, 31.46, 31.64, 31.87, 36.38, 37.11, 38.25, 38.40, 42.08, 45.84, 46.10, 49.68, 55.96, 56.78, 56.99, 71.54, 95.7, (d, $J = 102.6$ Hz) and 97.0 (d, $J = 102.6$ Hz) (two stereoisomers at C21), 121.13, 140.69, 199.8 (d, $J = 9.5$ Hz) and 200.6 (d, $J = 9.5$ Hz) (two stereoisomers at C20); mass spectrum, m/z (relative intensity) 371.3 (3.4), 370.3 (16, M^+), 369.3 (10.8), 368.3 (48, M^+), 353.3 (17), 352.3 (23), 351.3 (17), 350.3 (63), 337.3 (37), 336.3 (23), 335.3 (100), 311.2 (17), 301.3 (16), 285.2 (19), 283.2 (55), 259.2 (22), 257.2 (61), 256.2 (20), 255.3 (31), 213.2 (19), 189.1 (17), 173.2 (18), 161.2 (32), 159.2 (38), 157.2 (16), 147.2 (33), 145.2 (68), 143.1 (21), 133.2 (43), 131.2 (33), 129.1 (16), 121.2 (40), 120.2 (32), 119.2 (43), 117.1 (22), 107.2 (67), 105.1 (62), 95.2 (28), 93.1 (46), 91.1 (57), 81.1 (37), 79.1 (44), 77.1 (25), 67.1 (27), 55.1 (33), 40.6 (46).

Representative procedure for oxidation of pregnenolone derivatives to progesterone derivatives: synthesis of 21,21-difluoroprogesterone (2b). To a suspension of 905 mg (4.2 mmol) of PCC and 50 ml of methylene chloride was added a solution of 740 mg (2.1 mmol) of 21,21-difluoropregnenolone (**2a**) in 15 ml of methylene chloride. After the solution was stirred at room temperature for 3 hr, it was concentrated *in vacuo* to about 20 ml. The dark-colored solution was treated with 100 ml of anhydrous ether and was then filtered over a pad of $MgSO_4/SiO_2$. The filtrate was concentrated *in vacuo* and was treated with a solution of 20 ml of methanol and 2 ml of HCl (2 N). The reaction was stirred at 50–55° for 15 min followed by concentration *in vacuo* and was extracted with 50 ml of methylene chloride. The extract was dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was chromatographed over silica gel and eluted with ethyl acetate/hexane (1:4), yielding 440 mg (59%) of **2b**, m.p. 148–149° [acetone/hexane; literature m.p. 140–143° (19)]. ^{1}H NMR ($CDCl_3$, 90MHz) δ 0.74 (s, 3H, C18), 1.19 (s, 3H, C19), 2.97 (t, 1H, —CH—CO), 5.64 (t, 1H, $J = 54.1$ Hz, —CHF_2), 5.73 (s, 1H, =CH); ^{13}C NMR ($CDCl_3$) δ 13.69, 17.27, 20.86, 23.53, 24.45, 31.78, 32.63, 33.85, 35.56, 35.63, 38.04, 38.45, 45.97, 53.36, 56.16, 56.49, 109.74 (t, $J = 253.2$ Hz, C21), 123.91, 170.57, 199.27 (s, C4), 200.98 (t, $J = 25.3$ Hz, C20); mass spectrum m/z (relative intensity) 352.2 (2), 351.2 (17), 350.3 (68, M^+), 335.2 (13), 309.2 (24), 308.2 (100), 299.2 (20), 293.2 (12), 266.2 (14), 265.2 (39), 229.2 (14), 173.1 (11), 159.1 (10), 149.1 (12), 147.2 (25), 145.1 (13), 135.2 (19), 133.2 (21), 124.1 (86), 123.1 (21), 121.1 (16), 119.1 (18) 109.1 (21), 107.1 (31), 105.1 (25), 95.1 (23), 93.1 (28), 91.1 (42), 81.1 (21), 79.1 (37), 77.1 (18), 67.1 (22), 55.1 (20).

Physical data of 21-chloro-21-fluoroprogesterone (**3b**): ^{1}H NMR (90 MHz, $CDCl_3$) δ 0.74 (s, 3H, C18), 1.19 (s, 3H, C19), 3.0 (m, 1H, —CH—CO), 5.73 (s, 1H, =CH), 6.10 (d, $J = 51.8$Hz) and 6.16 (d, $J = 51.3$Hz) (1H, two stereoisomers at —CHFCl); mass spectrum, m/z (relative intensity) 369 (3), 368 (12.6), 367 (9), 366 (36.1), 326 (19), 324 (64), 300.1 (29), 299.1 (100), 281 (27), 271.1 (56), 253.1 (29) 244.1 (18), 229.1 (18), 149.1 (16), 147.1 (33), 133.1 (23), 124 (81), 123 (23), 121 (17), 119 (19), 109 (21), 107 (26), 105 (30), 95 (27), 93 (32), 91 (46), 79 (42), 77 (23), 69 (23), 67 (23), 57 (34), 55 (42).

Physical data of pregn-4,20-diene-3-one (**4b**): m.p. 121–123° [(recrystallized from ethyl acetate/hexanes, literature m.p. 123.5–126.5 (23)]; ^{1}H NMR ($CDCl_3$, 250 MHz) δ 0.64 (s, 3H, C18), 1.19 (s, 3H, C19), 4.95 (d, 1H, $J = 5.1$ Hz, C21), 5.01 (s, 1H, C21), 5.69–5.83 (m, 1H, C20), 5.73 (s, 1H, C_4; ^{13}C NMR ($CDCl_3$) δ 12.76, 17.34, 20.57, 24.66, 27.08, 32.01, 32.86, 33.91, 35.63, 35.69, 37.13, 38.63, 43.34, 54.02, 54.95, 55.15, 114.73, 123.73, 139.43, 171.45, 199.30; mass spectrum, m/z (relative intensity) 300.1 (3), 299.1 (24), 298.1 (100), 284.1 (14), 283.1 (56), 270.1 (11), 256.1 (14), 231.1 (13), 229.1 (39), 211.1 (12), 201.1 (14), 187.1 (23), 175.1 (23), 174.1 (19), 149.1 (20), 148.1 (26), 133.1 (20), 124.1 (31), 123.1 (16), 121.1 (29), 120.1 (13), 119.1 (24), 107.1 (27), 106 (12), 105 (31), 95.1 (20), 93 (25), 91 (41), 81 (22), 79 (41), 77 (22), 67 (22), 55 (26).

Physical data of pregn-4-en-20-yne-3-one (**5b**): m.p. 142–146° [literature m.p. 143–145.5° (20)]; ^{1}H NMR ($CDCl_3$, 250 MHz) δ 0.85 (s, 3H, C18), 1.20 (s, 3H, C19), 2.10 (s, 1H, —C≡CH), 5.73 (s, 1H, =CH); ^{13}C NMR ($CDCl_3$) δ 13.43, 17.36, 20.77, 24.51, 28.98, 31.92, 32.77, 33.92, 35.69, 36.07, 36.90, 38.61, 41.80, 43.68, 53.76, 54.04, 57.0, 70.08, 123.86, 171.10, 199.52; mass spectrum, m/z (relative intensity) 298.1 (3), 297.1 (19), 296.1 (63), 282.1 (13), 281.1 (59), 255.1 (10), 254.1 (34), 239.1 (14), 229.1 (29), 211.1 (23), 201.1 (15), 187.1 (23), 185.1 (11), 174.1 (18), 173.1 (39), 172.1 (16), 159.1 (23), 149.1 (18), 145.1 (24), 143.1 (14), 133.1 (22), 131.1 (30), 124.1 (100), 123.1 (19), 121.1 (25) 119.1 (29), 117.1 (23), 109.1 (20), 107.1 (25), 105.0 (47), 95.1 (31), 93 (31), 91 (60), 81 (29), 79 (44), 77 (36), 69.1 (25), 67 (29), 57.1 (26), 55 (43).

Assay of progesterone hydroxylase activity. Microsomal protein (20–50 μg) was incubated with 25 μM progesterone and 1 mM NADPH in a final volume of 0.1 ml of 0.05 M HEPES buffer (pH 7.6) containing 15 mM $MgCl_2$ and 0.1 mM EDTA. Incubations were carried out at 37° for various times ranging from 0 to 5 min and were quenched by the addition of 0.05 ml of THF. Aliquots of 0.05 ml were spotted on the preadsorbent loading zone of a thin layer chromatography plate [Baker silica gel, 250 μm, Si 250F (19c)], and the plate was developed three times in benzene/ethyl acetate/acetone (10:1:1, v/v). Metabolites were localized by autoradiography and identified by comparison with unlabeled standards. The R_F values of the standards were 16α-OH, 0.10; 6β-OH, 0.22; 21-OH, 0.32; 2α-OH, 0.40; and progesterone, 0.69. The radioactive areas from the plate were scraped into scintillation vials, and the metabolites were quantified by liquid scintillation counting. In the case of incubations with purified cytochromes P-450, the microsomes were replaced with 4–10 pmol of cytochrome P-450, 6 units of NADPH-cytochrome P-450 reductase/nmol of P-450, and 3 μg of sonicated dilauryl-L-3-phosphatidylcholine. The reconstituted proteins were incubated at room temperature for 5 min before addition of the remaining ingredients of the incubation mixture. When present, cytochrome b_5 was added to the final incubation mixture at a ratio of 2 nmol/nmol of cytochrome P-450.

Results

Progesterone hydroxylases in rabbit liver microsomes. Extensive work by Johnson and collaborators (14, 15) has shown that, in liver microsomes from outbred NZW rabbits, the 21-methyl group of progesterone is specifically hydroxylated by cytochrome P-450 1. P-450 1 exhibits high 21-hydroxylase activity towards pregnenolone as well as progesterone (24) and may constitute up to 20% of the total microsomal cytochrome P-450 in liver microsomes from certain individual rabbits of the phenotype termed 21H (14). The other two major pathways of progesterone metabolism in rabbit liver microsomes, 16α- and 6β-hydroxylation, are not catalyzed by P-450 1 but largely by P-450 3b and 3c (25, 26). Outbred NZW rabbits contain two subforms of cytochrome P-450 3b, one of which is a high affinity 16α- and 6β-hydroxylase, whereas the other is solely a low affinity 16α-hydroxylase. Only the latter subform is found in inbred rabbits of the B/J strain (27). After treatment with rifampicin, 6β-hydroxylation of progesterone in liver microsomes from B/J rabbits is catalyzed primarily by cytochrome P-450 3c (25, 26).

Inactivation and inhibition of rabbit liver progesterone hydroxylases by pregnenolone and progesterone derivatives. In our previous study, 21,21-dichloropregnenolone (**1a**) was shown to be a selective 21-hydroxylase inactivator, causing little or no inactivation of the progesterone 6β-hydroxylase or 16α-hydroxylase in microsomes from the NZW rabbits or of the progesterone 6β-hydroxylase in microsomes from control or rifampicin-treated rabbits of the B/J strain. However, **1a** did cause some inactivation of the 16α-hydroxylase in microsomes from the B/J rabbits, indicating an effect on the low-affinity subform of cytochrome P-450 3b. The strategy followed in the present investigation was to use liver microsomes from the outbred NZW rabbits, as well as a reconstituted system containing P-450 1, to identify compounds equally effective and selective as **1a** in inactivating P-450 1. Any compounds so identified would then be tested further using liver microsomes representing the other phenotypes of progesterone hydroxylase activities. Initially attention was focused on 21,21-difluoro- (**2a**) and 21-chloro-21-fluoropregnenolone (**3a**), the idea being that either the steric or electronic properties of fluorine relative to chlorine might render these compounds more similar to the parent compound pregnenolone, such that the normal regioselectivity of the rabbit liver progesterone hydroxylases might be retained. Subsequent experiments examined the corresponding progesterone derivatives **1b, 2b,** and **3b**, as well as two pregnenolone (**4a** and **5a**) and two progesterone (**4b** and **5b**) derivatives bearing a vinyl or ethynyl substituent in the 17β-position. These substituents retain the two-carbon side chain of the parent and halogenated steroids and have been used extensively to prepare selective mechanism-based inactivators of a number of different enzymes including cytochromes P-450 (5–9).

Fig. 2 shows the effect of preincubation with **1a, 2a,** and **3a** on progesterone 21-hydroxylase activity in liver microsomes from NZW 21H rabbits, as well as in a reconstituted system containing purified P-450 1. In this type of experiment, residual unmetabolized inactivator is present during the assays of substrate metabolism. Therefore, any time-dependent loss of activity (inactivation) is superimposed upon a time-independent component (inhibition). The extent of inhibition is evident from the decrease in the extrapolated activity at zero preincubation time (y intercept) compared with the controls, whereas the rate constant for the inactivation process is derived from the decrease in activity as a function of preincubation time. From these experiments, it is clear that all three compounds inhibited P-450 1, whereas only the chlorofluoro compound (**3a**) retained the ability of the dichloropregnenolone (**1a**) to inactivate the enzyme. It should be noted that, although ineffective as a P-450 inactivator, the difluoro compound (**2a**) was the most effective of the halogenated compounds as an inhibitor of the enzyme, and a comparable extent of inhibition of purified P-450 1 was obtained with 4 μM[2] **2a** as with 20 μM **1a** and **3a** (Fig. 2B).

Similar experiments were performed with the other seven compounds at concentrations ranging from 1 to 100 μM. Although all the compounds preferentially inhibited the 21-hydroxylase in liver microsomes from NZW 21H rabbits, they were inferior to **1a** and **3a** as selective P-450 1 inactivators. Thus, like **2a**, four of the compounds (**4a, 1b, 2b,** and **3b**) showed poor ability to inactivate the target enzyme in NZW 21H microsomes or a reconstituted system (data not shown), whereas the other three compounds (**5a, 4b,** and **5b**) caused considerable time-dependent decreases in 6β- and 16α-hydroxylase activities in microsomes from NZW rabbits. An example of the lack of selectivity of the vinyl compound **4b** is shown in Fig. 3. With regard to inhibition of the 21-hydroxylase in both the intact NZW 21H microsomes and a reconstituted system, a simple pattern was observed. For example, at a concentration of 20 μM inhibitor and 25 μM progesterone, the per cent inhibition caused by the pregnenolone derivatives **1a, 3a, 2a, 4a,** and **5a** was 25, 54, 86, 94, and 98%, respectively whereas, at a concentration of 4 μM inhibitor and 25 μM progesterone in the reconstituted system, the same compounds caused 35, 52, 83, 93, and 98% inhibition. The same pattern $(\mathrm{Cl_2} < \mathrm{ClF} < \mathrm{F_2} < {=} < {\equiv})$ was observed for the inhibition caused by the progesterone derivatives (data not shown).

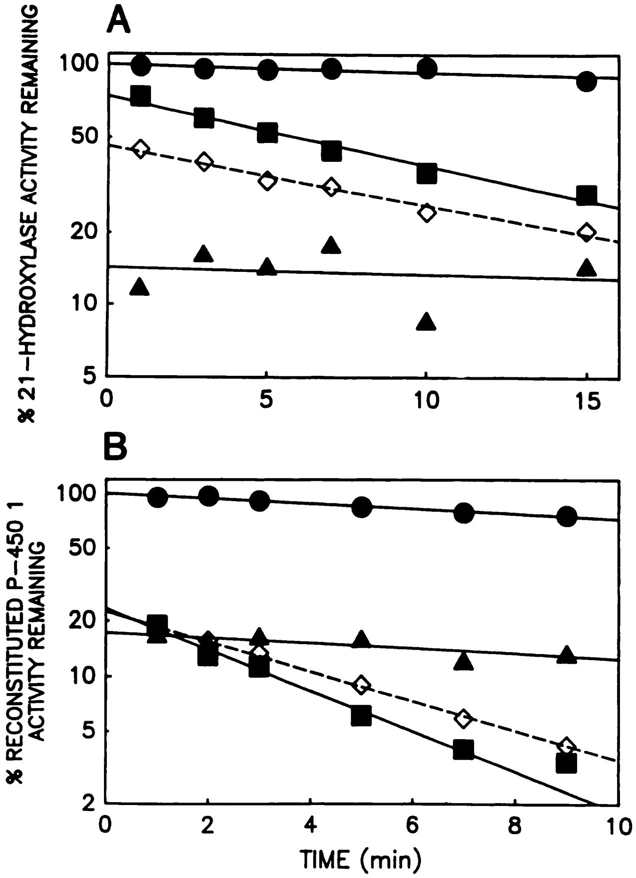


Fig. 2. Effect of preincubation with 21,21-dihalo pregnenolone derivatives on progesterone 21-hydroxylation. A, Liver microsomes from NZW 21H rabbits. ●, Methanol controls; ■, 25 μM 21,21-dichloropregnenolone, **1a**; ◇, 25 μM 21-chloro-21-fluoropregnenolone, **3a**; ▲, 25 μM 21,21-difluoropregnenolone, **2a**. B, Purified P-450 1 in a reconstituted system. ●, Methanol controls; ■, 25 μM **1a**; ◇, 25 μM **3a**; ▲, 5 μM **2a**. The *lines* shown were drawn by linear regression analysis of the natural logarithm of the residual activity as a function of time. Rate constants for inactivation are derived from the negative slope of the lines. Samples were incubated with or without inhibitor for 2 min at 37°. Reactions were started by the addition of NADPH and were allowed to proceed for the times indicated, at which point 80-μl aliquots were removed and added to 20-μl of [^{14}C]progesterone in buffer. The reactions were allowed to proceed for an additional 1.5 min and were quenched with 50 μl of THF. The concentrations of the various components of the incubation mixture after addition of the progesterone were 200 μg/ml microsomal protein (or 40 pmol/ml of reconstituted P-450 1), 25 μM progesterone, 1 mM NADPH, 50 mM HEPES buffer (pH 7.6), 15 mM $MgCl_2$, and 0.1 mM EDTA. The inhibitors were added from methanol stock solutions, and the concentrations indicated refer to the preincubation period before addition of the progesterone. The 100% value for the 21-hydroxylase activity was 3.12 nmol of product formed/min/mg of protein in the microsomes and 16.2 nmol of product formed/min/nmol of P-450 1 in the reconstituted system.

Further characterization of 21-chloro-21-fluoropregnenolone. As the only compound potentially superior to **1a** as a selective P-450 1 inactivator, the properties and selectivity of **3a** were examined further. As illustrated in Fig. 4, the time-

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[2] Due to the dilution of the inhibitor upon addition of substrate, preincubation at an inhibitor concentration of 5 or 25 μM results in a concentration of 4 or 20 μM, respectively, during the assays of progesterone hydroxylase activity.

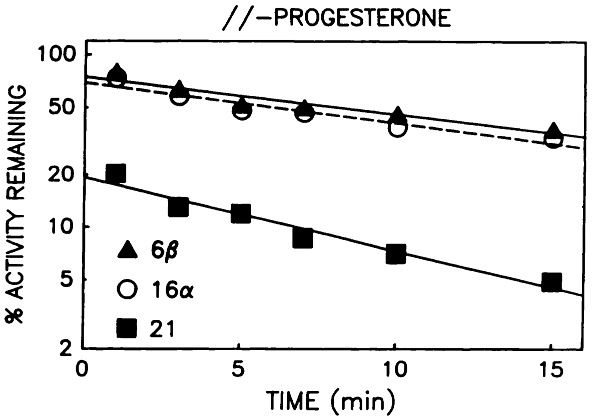

Fig. 3. Effect of preincubation with 5 μM pregn-4,20-diene-3-one (//-progesterone, **4b**) on progesterone hydroxylation catalyzed by liver microsomes from outbred NZW rabbits. The experiments were conducted essentially as described in the legend to Fig. 2. The 100% activities, in nmol of product/min/mg of protein, were 6β-OH, 1.00; 16α-OH, 0.52; and 21-OH, 2.18. The rate constants derived from the negative slopes of the lines were 0.05 min^{-1} for the 6β- and 16α-hydroxylases and 0.10 min^{-1} for the 21-hydroxylase.

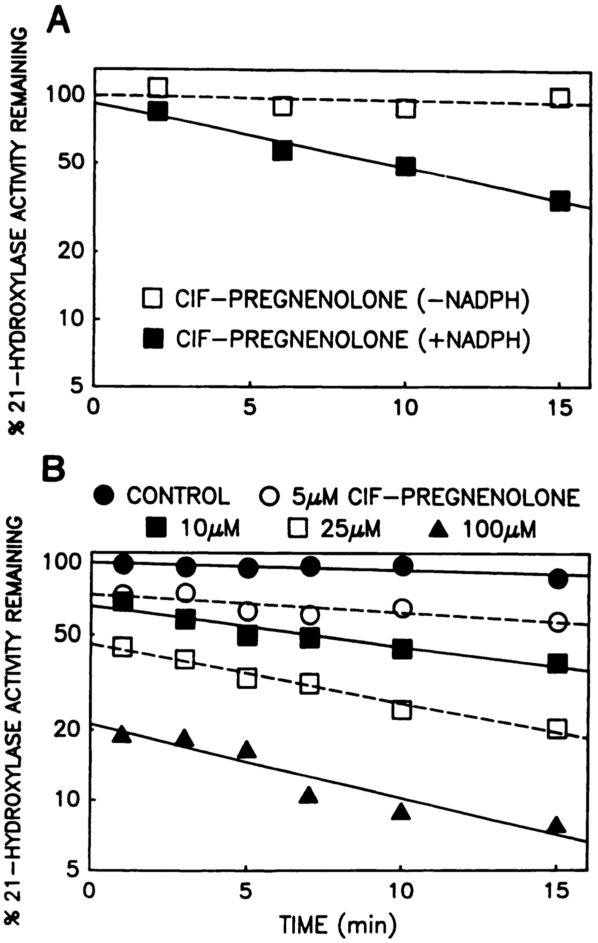

Fig. 4. Characterization of 21-chloro-21-fluoropregnenolone (**3a**) as a mechanism-based inactivator of P-450 1 in rabbit liver microsomes. A, NADPH requirement for time-dependent loss of activity. Microsomes (200 μg/ml) were preincubated with 25 μM compound at 37° in 50 mM HEPES buffer (pH 7.6), 15 mM $MgCl_2$, and 0.1 mM EDTA in either the absence or presence of 1 mM NADPH. After 2, 6, 10, or 15 min, 50-μl aliquots corresponding to 10 μg of microsomal protein were diluted into 450 μl of the same buffer containing 1 mM NADPH and 12.5 nmol of progesterone, which had been prewarmed to 37°. The incubations were allowed to proceed for 3 min and were quenched by the addition of 2 ml of methylene chloride and vortexing. The methylene chloride extracts were dried and subjected to thin layer chromatography for quantification of 21-OH-progesterone formation. In this experiment, the 100% value represents the extrapolated value at zero preincubation time for the 21-hydroxylase activity of the samples preincubated with inhibitor in the absence of NADPH. B, Effect of preincubation with increasing inhibitor concentrations on progesterone 21-hydroxylase activity in rabbit liver microsomes. The experiments were performed as described in the legend to Fig. 2.

dependent decrease in 21-hydroxylase activity caused by the compound required NADPH and increased as a function of the inhibitor concentration. Both of these properties are characteristic of mechanism-based inactivators. From the data in Fig. 4B, a maximal rate constant for inactivation of 0.09 min^{-1} and an apparent K_I for the inactivation process of 15 μM were calculated. These values can be compared with the corresponding ones reported previously (12) for **1a** (0.10 min^{-1} and 12 μM, respectively) and confirm that the chlorofluoro compound essentially retains the ability of the dichloro compound to inactivate P-450 1. In addition, **3a** was more selective than **1a**. Thus, as shown in Fig. 5A, **3a** in contrast to **1a**, caused no time-dependent decrease in 16α-hydroxylase in liver microsomes from inbred rabbits of the B/J strain. The selectivity of **3a** is summarized in Fig. 5B, where its effect on five different progesterone hydroxylases, reflecting the activity of four different cytochromes P-450, is illustrated. From these and similar experiments performed with 100 μM inhibitor, it is evident that 21-chloro-21-fluoropregnenolone inhibits four of the hydroxylases but inactivates only the 21-hydroxylase.

Progesterone hydroxylation by rat liver microsomes. Previous results indicate that the formation by male rat liver microsomes of two hydroxylated progesterone metabolites (2α- and 21-OH) each largely reflects the activity of a single form of cytochrome P-450, termed P-450h and P-450k (PB-C), respectively (16, 28). Furthermore, the formation of a third major metabolite, 6β-OH-progesterone, appears to be catalyzed by P-450p (29) and/or a closely related member of the P-450III gene family (13, 16, 30). Liver microsomes from PB-treated male rats constitute a convenient source for monitoring progesterone 6β- and 21-hydroxylase activities and, although 2α-hydroxylase activity is decreased by PB, the activities are still sufficiently high to be readily monitored (13, 16). In such microsomes, 16α-OH progesterone is also a major metabolite, although its formation does not appear to be diagnostic for the activity of a particular cytochrome P-450 form.

Inactivation and inhibition of rat liver progesterone hydroxylases. In a previous investigation, we found that preincubation of liver microsomes from PB-treated male rats with **1a** or **1b** caused a time-dependent decrease in progesterone 6β- and 21-hydroxylase but not in 2α- or 16α-hydroxylase activities (13). The rate constants for inactivation of the 6β-hydroxylase were approximately the same for the two compounds, whereas **1b** was the better 21-hydroxylase inactivator. In the present investigation, the eight additional compounds tested in the rabbit liver microsomes were also assessed for their ability to inhibit and inactivate progesterone hydroxylases in liver microsomes from PB-treated male rats.

Fig. 6 shows the inhibition and inactivation of the progesterone 21- and 6β-hydroxylases caused by compounds **1a**, **3a**, **4a**, **1b**, and **4b** at a concentration of 25 μM. The results of these and similar experiments performed at inhibitor concentrations

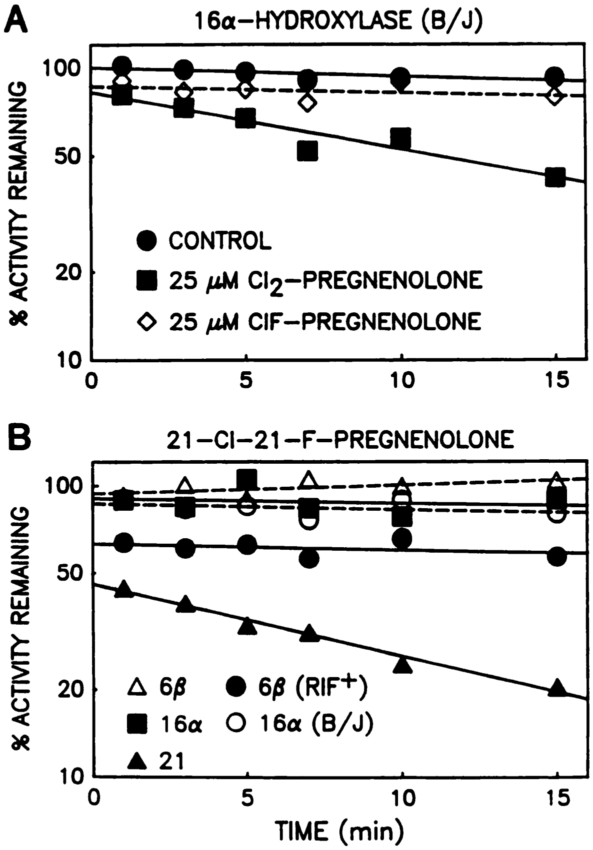

Fig. 5. Selectivity of 21-chloro-21-fluoropregnenolone. A, Effect of preincubation with 21,21-dichloro- and 21-chloro-21-fluoropregnenolone on progesterone 16α-hydroxylase activity in liver microsomes from untreated rabbits of the inbred B/J strain. The experiments was performed essentially as described in the legend to Fig. 2 except for the use of 30 instead of 20 μg of protein per assay. The 100% value for the activity was 0.82 nmol of 16α-OH progesterone/min/mg of protein. B, Effect of preincubation with 25 μM 21-chloro-21-fluoropregnenolone on selected hydroxylase activities from inbred and outbred rabbits. Activities refer to liver microsomes from NZW 21H rabbits unless otherwise indicated. Experiments were performed essentially as described in the legend to Fig. 2, except for the use of 30 instead of 20 μg of protein for the incubations with the microsomes from the untreated B/J rabbits. *Rif*+ refers to liver microsomes from a B/J rabbit that had been injected with 50 mg/kg rifampicin once daily for 4 days. The experiments with these microsomes were performed in 50 mM potassium phosphate buffer (pH 7.5) containing no EDTA or $MgCl_2$. In the phosphate buffer, the 100% value for the 6β-hydroxylase activity was 3.00 nmol/min/mg, compared with a value of 0.33 in the HEPES buffer.

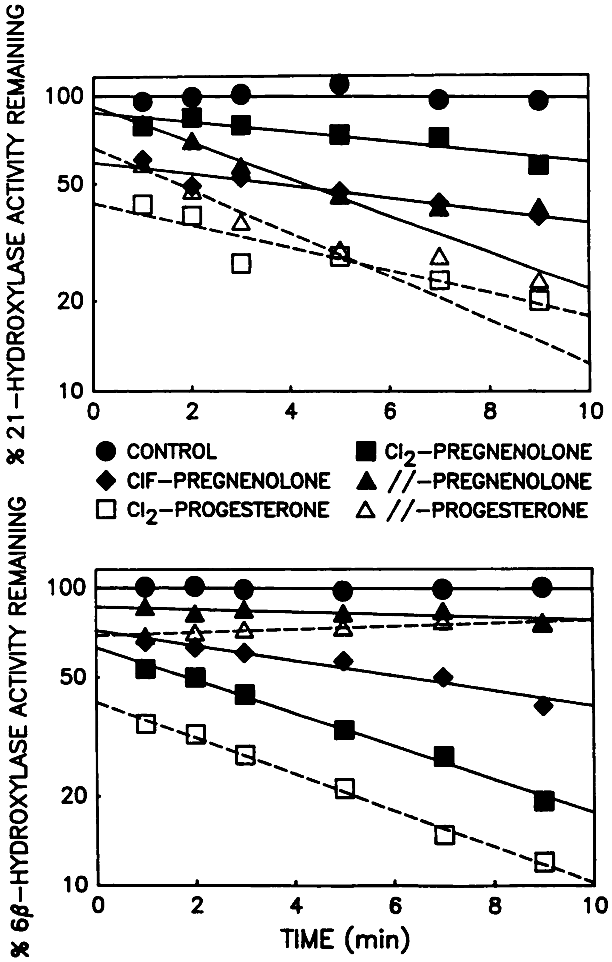

Fig. 6. Effect of preincubation with selected 17β-substituted steroids on progesterone 21- and 6β-hydroxylase activities in liver microsomes from PB-treated rats. The *lines* shown were drawn by linear regression analysis of the natural logarithm of the residual activity as a function of time. Rate constants for inactivation are derived from the negative slope of the lines. Samples were incubated with or without inhibitors (25 μM) for 2 min at 37°. Reactions were started by the addition of NADPH and were allowed to proceed for the times indicated, at which point 80-μl aliquots were removed and added to 20 μl of [^{14}C]progesterone in buffer. The reactions were allowed to proceed for an additional 1.5 min and were quenched with 50 μl of THF. The concentrations of the various components of the incubation mixture after addition of the progesterone were 500 μg/ml microsomal protein, 25 μM progesterone, 1 mM NADPH, 50 mM HEPES buffer (pH 7.6), 15 mM $MgCl_2$, and 0.1 mM EDTA. The inhibitors were added from methanol stock solutions, and the concentration refers to the preincubation period before addition of the progesterone. The 100% values for the activities, in nmol of product formed/min/mg of protein, were 21-OH, 0.32; and 6β-OH, 2.86.

of 25 and 100 μM are tabulated in Table 1. The major findings can be summarized as follows. 1) Among the halogenated compounds, inactivation of the 6β-hydroxylase was partially suppressed by replacement of chlorine by fluorine but, of the five halogenated compounds that caused significant inactivation of the 21-hydroxylase (**1a, 2a, 3a, 1b,** and **3b**), none was totally selective. 2) As with the rabbit liver enzymes, of the halogenated steroids, the difluoro compounds were the best 21-hydroxylase inhibitors. For example, at an inhibitor concentration of 20 μM and a progesterone concentration of 25 μM, the per cent inhibition by **1a, 3a,** and **2a** was 12, 40, and 60%, respectively. 3) Within each series (i.e., pregnenolone or progesterone derivatives) the vinyl compounds (**4a** and **4b**) were the poorest inhibitors but the best and most selective inactivators of the rat liver 21-hydroxylase. Thus, at an inhibitor concentration of 20 μM, the per cent inhibition caused by **4a** and **4b** was 4 and 32%, respectively. This is in contrast to the situation in the rabbit liver microsomes, in which the vinyl compounds were among the best inhibitors but were either poor or nonselective inactivators. 4) The ethynyl compounds (**5a** and **5b**) were nonselective, although at a concentration of 25 μM they preferentially inactivated the 6β-hydroxylase.

Further characterization of pregn-4,20-diene-3-one (4b). The effect of compound **4b**, at a concentration of 100 μM, on the four rat liver progesterone hydroxylase activities is summarized in Fig. 7. From these and similar data, it is clear that the vinyl compound inhibits all four hydroxylases in a time-*independent* manner but causes a time-*dependent* loss

TABLE 1

Rate constants for inactivation of rat hepatic progesterone 6β- and 21-hydroxylases by 17β-substituted pregnenolone and progesterone derivatives

Experiments were performed and rate constants were calculated as described in the legend to Fig. 6. The values given represent the mean of duplicate experiments.

Compound	Steroid Nucleus	17β-Substituent	Concentration	$k_{inactivation}$	
				6β	21
			μM	*min⁻¹*	
1a	Pregnenolone	$CHCl_2$-CO	25	0.12	0.04
			100	0.12	0.08
3a	Pregnenolone	CHClF-CO	25	0.06	0.06
			100	0.06	0.08
2a	Pregnenolone	CHF_2-CO	25	0.02	0.05
			100	0.04	0.06
4a	Pregnenolone	Vinyl	25	0.02	0.11
			100	0.04	0.12
5a	Pregnenolone	Ethynyl	25	0.16	0.04
			100	0.20	0.09
1b	Progesterone	$CHCl_2$-CO	25	0.11	0.08
			100	0.13	0.14
3b	Progesterone	CHClF-CO	25	0.00	0.05
			100	0.05	0.08
2b	Progesterone	CHF_2-CO	25	0.00	0.01
			100	0.00	0.02
4b	Progesterone	Vinyl	25	0.00	0.17
			100	0.01	0.23
5b	Progesterone	Ethynyl	25	0.08	0.04
			100	0.07	0.08

only of 21-hydroxylase activity. In fact, it is noteworthy that the 21-hydroxylase is actually the least susceptible of the four activities to inhibition by the compound. The time-dependent loss of 21-hydroxylase activity caused by **4b** in the microsomes appeared to be biphasic. In order to investigate whether this biphasicity might be due to differential effects on two 21-hydroxylases in the microsomes, experiments were also carried out with P-450 PB-C in a reconstituted system. As seen in Fig. 7B, biphasic behavior was observed with the purified enzyme as well, whereas no evidence of biphasicity was observed with compound **1b** in the reconstituted system (Fig. 7B) or microsomes (Fig. 6). These data suggest that the apparent biphasicity may be inherent in the mode of action of **4b**.[3] Based on a series of three experiments, rate constants for inactivation of purified PB-C by 5 μM **1b** and **4b** were 0.22 and 0.34 min^{-1}, respectively. These values are approximately 50% higher than the largest rate constants determined in the microsomes, possibly reflecting the presence of greater amounts of P-450 reductase and cytochrome b_5 in the reconstituted system. However, it is of interest that, in both the reconstituted system and the microsomes, the rate constant for 21-hydroxylase inactivation by **4b** is 1.5–2-fold higher than the rate constant for **1b**, which is consistent with the identification of PB-C as the target 21-hydroxylase in the microsomes.

Inactivation of rat liver androstenedione hydroxylases. The selectivity of compound **4b** was examined further using androstenedione as a substrate, because this allowed us to monitor the inactivation of two additional cytochromes P-450, PB-B and UT-F, by measuring androstenedione 16β- and 7α- hydroxylase activities, respectively (31–33). As shown in Table 2, all four major androstenedione hydroxylases were inhibited by **4b** in a concentration-dependent manner. No inactivation of the 7α- or 6β-hydroxylases was observed, whereas slow inactivation of the 16β- and 16α-hydroxylases occurred only at the highest concentration of inhibitor.[4] Both of these latter effects can be attributed to inactivation of P-450 PB-B in liver microsomes from PB-induced rats (32).

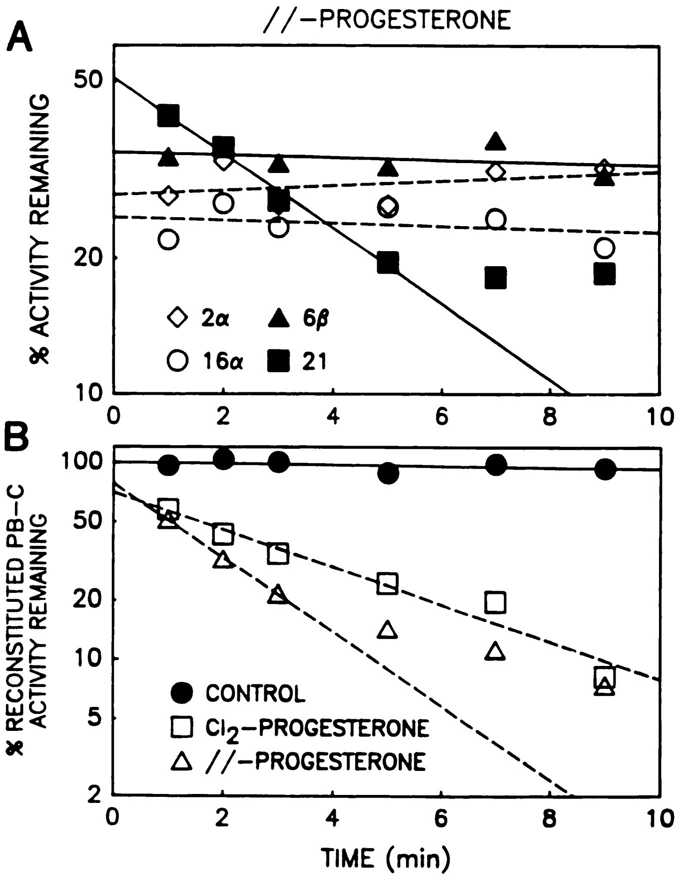

Fig. 7. Inactivation of rat liver cytochrome P-450 PB-C in intact liver microsomes and a reconstituted system. A, Effect of preincubation with 100 μM pregn-4,20-diene-3-one (//-progesterone, **4b**) on progesterone hydroxylase activities of liver microsomes from PB-treated rats. The experiment was performed as described in the legend to Fig. 6. The 100% values for the activities, in nmol of product formed/min/mg of protein, were 2α-OH, 0.42; 6β-OH, 2.86; 16α-OH, 1.29; and 21-OH, 0.32. B, Effect of preincubation with 5 μM 21,21-dichloroprogesterone or pregn-4,20-diene-3-one on the progesterone 21-hydroxylase activity of purified cytochrome P-450 PB-C in a reconstituted system. The experiment was performed essentially as described in the legend to Fig. 6 except that instead of 50 μg of microsomal protein, each 80-μl aliquot of the preincubation mixture contained a reconstituted system consisting of 10 pmol of PB-C, 0.06 units of NADPH-cytochrome P-450 reductase, and 20 pmol of cytochrome b_5. The 100% value for the 21-hydroxylase activity was 0.79 nmol of 21-OH progesterone/min/nmol of P-450.

Discussion

Several previous studies have used derivatized steroids as potential active site-directed reversible (34) or irreversible inhibitors (9, 35) of extrahepatic cytochromes P-450, such as aromatase (35) or cholesterol side-chain cleavage enzyme (9,

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[3] We have previously observed biphasic kinetics for the inactivation by certain dichloroacetamides of two other purified rat liver cytochromes P-450, the reasons for which remain obscure (10, 11).

[4] In a similar experiment in which inactivation of the microsomal progesterone 21-hydroxylase was monitored at **4b** concentrations of 25, 50, and 100 μM, the rate constant for inactivation was found to be essentially maximal at 50 μM inhibitor. At that concentration, no inactivation of the androstenedione 16β-hydroxylase is observed.

TABLE 2

Rate constants for inactivation of rat hepatic androstenedione hydroxylases by pregn-4,20-diene-3-one (4b)

The experiments were conducted essentially as described in the legend to Fig. 6 except for the use of androstenedione (25 μM) instead of progesterone as the substrate to monitor residual hydroxylase activities. The final protein concentration was 250 μg/ml during the assays of androstenedione hydroxylase activity. Androstenedione metabolites were separated and identified by thin layer chromatography as described previously (33). The values given represent the means of two or three experiments. The numbers in parentheses represent the relative activity at zero preincubation time and are a measure of the extent of inhibition caused by the compound. The 100% values, in nmol of product formed/min/mg of protein, were 7α-OH, 0.90; 6β-OH, 3.53; 16β-OH, 18.5; and 16α-OH, 3.65.

Concentration	$k_{inactivation}$ 7α	6β	16β	16α
μM		min^{-1}		
0	0.01 (100)	0.01 (100)	0.01 (100)	0.01 (100)
25	0.00 (50)	0.02 (84)	0.00 (57)	0.00 (50)
50	0.00 (48)	0.01 (72)	0.01 (34)	0.00 (27)
100	0.01 (26)	0.02 (42)	0.04 (25)	0.03 (23)

34). The focus in these studies has been on the potency and effectiveness of the compounds as inhibitors of the target enzyme. Not surprisingly, however, some of the compounds later proved to be inhibitors of other extrahepatic, as well as hepatic, cytochromes P-450 (24, 36). In contrast to these earlier studies, the focus of the present investigation has been on the selectivity, as well as effectiveness, of a number of pregnenolone and progesterone derivatives as inactivators of rabbit and rat hepatic cytochromes P-450 responsible for progesterone 21-hydroxylation. The emphasis on selectivity has meant that non-target enzymes as well as the target 21-hydroxylase have received considerable attention.

In both rabbit and rat liver microsomes, the judicious combination of 17β side-chain and steroid nucleus resulted in a compound that was an effective inactivator of the progesterone 21-hydroxylase, while causing negligible inactivation of non-target progesterone hydroxylases. However, in each species, only one of the ten compounds tested possessed the desired specificity. Moreover, the most specific rabbit liver 21-hydroxylase inactivator, 21-chloro-21-fluoropregnenolone, was of only moderate selectivity in the rat, whereas the most specific rat liver 21-hydroxylase inactivator, pregn-4,20-diene-3-one, was of only moderate selectivity in the rabbit. These results indicate that even a rational approach to the design of selective cytochrome P-450 inactivators must encompass a considerable element of empiricism.

Despite the species differences, certain structure-activity relationships were apparent that may allow some prediction as to how steroids or other molecules derivatized with the functional groups used in the present study might behave, from the standpoint of effectiveness and selectivity as cytochrome P-450 inhibitors and inactivators. Thus, among the compounds containing a dihalomethylketo side-chain, the difluoro compounds were the best inhibitors of the 21-hydroxylase but, with the exception of 21,21-difluoropregnenolone in the rat liver microsomes, the difluoro compounds were ineffective as 21-hydroxylase inactivators. In contrast, the chlorofluoro compounds, although poorer inhibitors than the difluoro steroids, retained much of the reactivity of the dichloro compounds towards the 21-hydroxylase and were more selective inactivators. A similar disparity between the ability to inactivate, as opposed to inhibit, both the target and non-target enzymes was observed with the vinyl-substituted derivative of progesterone (**4b**). Thus, of all the progesterone derivatives, **4b** was the least potent inhibitor of the rat liver 21-hydroxylase and, in addition, caused less inhibition of the target enzyme than it did of three non-target progesterone hydroxylases. Nonetheless, **4b** was both the most effective and the most specific rat liver progesterone 21-hydroxylase inactivator. These and other findings suggest that conversion of the enzyme-inhibitor complex to products that inactivate the enzyme, rather than reversible binding of the inhibitor, is the key event in determining the selectivity of the inactivation process.

In a previous report, we commented on how much more rapidly 21,21-dichloroprogesterone (**1b**) inactivated the rat compared with the rabbit liver progesterone 21-hydroxylase, despite the 15–20-fold greater activity of the latter enzyme towards the parent compound progesterone (13). Similar results were obtained in the present study with several of the other progesterone and pregnenolone derivatives. For example, the largest rate constant observed for inactivation of the 21-hydroxylase in rabbit liver microsomes by the vinyl compound **4b** was only half that observed in the rat liver microsomes. In fact, the rate constants for inactivation of rat liver PB-C in a reconstituted system by 5 μM **4b** or **1b** are only 2–4-fold lower than the turnover number of the enzyme for progesterone. It should be noted that the rate constants for inactivation reflect not only the rate of metabolism of the inactivator but also the number of turnovers per inactivation event, the so-called partition ratio (5, 9). We have not yet measured the partition ratios for the 17β-substituted steroids and can therefore only infer that a relatively low rate of inhibition metabolism or an unfavorable partition ratio accounts for the large discrepancy between progesterone 21-hydroxylation and P-450 1 inactivation, whereas a relatively high rate of metabolism or a favorable partition ratio accounts for the efficiency of PB-C inactivation.

Although acetylenic-based compounds have proven very useful as selective inactivators of a number of hepatic and extrahepatic cytochromes P-450 (8, 9), the two ethynyl compounds tested in the present study were too nonselective to be useful as 21-hydroxylase inactivators. In fact, in rat liver microsomes, **5a** preferentially inactivated the progesterone 6β-hydroxylase. This is of interest in light of the recent report by Guengerich (37) that another acetylenic compound, 17α-ethynylestradiol, inactivates the human ortholog of the rat liver cytochromes P-450 responsible for progesterone 6β-hydroxylation. Although a number of 17α-ethynyl compounds have previously been shown to cause a loss of spectrally detectable cytochrome in liver microsomes from PB-treated rats, the precise forms of the cytochrome destroyed have to our knowledge not strictly been identified (38). The results of the present investigation, as well as those of Guengerich, implicate enzymes of the cytochrome P-450III family as potential targets for 17-ethynyl steroids.

Acknowledgments

We thank Dr. Eric Johnson, Research Institute of Scripps Clinic, La Jolla, CA, for providing the rabbit liver microsomes and the purified P-450 1, Joanne Alfandre and Gina Escobar for technical assistance, and Charleen Prytula for help in preparation of the manuscript.

References

1. Nebert, D. W., M. Adesnik, M. J. Coon, R. W. Estabrook, F. J. Gonzalez, F. P. Guengerich, I. Gunsalus, E. F. Johnson, B. Kemper, W. Levin, I. R. Phillips, R. Sato, and M. R. Waterman. The P450 gene superfamily: recommended nomenclature. *DNA* **6**:1–11 (1987).
2. Waxman, D. J. Rat hepatic cytochrome P-450: comparative study of multiple isozymic forms, in *Cytochrome P-450—Structure, Mechanism, and Biochemistry* (P. R. Ortiz de Montellano, ed.). Plenum Press, New York, 525–539 (1986).

3. Conney, A. H. Induction of microsomal cytochrome P-450 enzymes: the first Bernard B. Brodie lecture at Pennsylvania State University. *Life Sci.* **39**: 2493–2518 (1986).
4. Guengerich, F. P., and D. C. Liebler. Enzymatic activation of chemicals to toxic metabolites. *CRC Crit. Rev. Toxicol.* **14**:259–307 (1985).
5. Rando, R. R. Mechanism-based enzyme inactivators. *Pharmacol. Rev.* **36**:111–142 (1984).
6. Abeles, R. H. Suicide enzyme inactivators, in *Enzyme-Activated Irreversible Inhibitors* (N. Seiler, M. J. Jung, and J. Koch-Weser, eds.). Elsevier/North Holland Biomedical Press, Amsterdam, 1–12 (1978).
7. Ortiz de Montellano, P. R., and N. O. Reich. Inhibition of cytochrome P-450 enzymes, in *Cytochrome P-450—Structure, Mechanism, and Biochemistry* (P. R. Ortiz de Montellano, ed.). Plenum Press, New York, 273–314 (1986).
8. Ortiz de Montellano, P. R., and N. O. Reich. Specific inactivation of hepatic fatty acid hydroxylases by acetylenic fatty acids. *J. Biol. Chem.* **259**:4136–4141 (1984).
9. Nagahisa, A., R. W. Spencer, and W. H. Orme-Johnson. Acetylenic mechanism-based inhibitors of cholesterol side-chain cleavage by cytochrome P-450scc. *J. Biol. Chem.* **258**:6721–6723 (1983).
10. Halpert, J., C. Balfour, N. E. Miller, and L. S. Kaminsky. Dichloromethyl compounds as mechanism-based inactivators of rat liver cytochromes P-450 *in vitro*. *Mol. Pharmacol.* **30**:19–24 (1986).
11. Miller, N. E., and J. Halpert. Analogs of chloramphenicol as suicide substrates of rat liver cytochromes P-450: modification of the propanediol side-chain, the p-nitro group, and the dichloromethyl moiety. *Mol. Pharmacol.* **29**:391–398 (1986).
12. Halpert, J., J.-Y. Jaw, C. Balfour, E. A. Mash, and E. F. Johnson. Selective inactivation by 21-chlorinated steroids of rabbit liver and adrenal cytochromes P-450 involved in progesterone hydroxylation. *Arch. Biochem. Biophys.* **264**:462–471 (1988).
13. Halpert, J., J.-Y. Jaw, L. Cornfield, C. Balfour, and E. A. Mash. Selective inactivation of rat liver cytochromes P-450 by 21-chlorinated steroids. *Drug Metab. Disp*, in press.
14. Johnson, E. F., and K. J. Griffin. Variations in hepatic progesterone hydroxylase activity reflect differences in the microsomal concentration of rabbit cytochrome P-450 1. *Arch. Biochem. Biophys.* **237**:55–64 (1985).
15. Reubi, I., K. J. Griffin, J. L. Raucy, and E. F. Johnson. Three monoclonal antibodies to rabbit microsomal cytochrome P-450 1 recognize epitopes that are shared to different degrees among other electrophoretic types of cytochrome P-450. *J. Biol. Chem.* **259**:5887–5892 (1984).
16. Swinney, D. C., D. E. Ryan, P. E. Thomas, and W. Levin. Regioselective progesterone hydroxylation catalyzed by eleven rat hepatic cytochrome P-450 isozymes. *Biochemistry* **26**:7073–7083 (1987).
17. Graves, P. E., L. S. Kaminsky, and J. Halpert. Evidence for functional and structural multiplicity of pregnenolone 16α-carbonitrile-inducible cytochrome P-450 isozymes in rat liver microsomes. *Biochemistry* **26**:3887–3894 (1987).
18. Halpert, J., N. E. Miller, and L. Gorsky. On the mechanism of the inactivation of the major phenobarbital-inducible isozyme of rat liver cytochrome P-450 by chloramphenicol. *J. Biol. Chem.* **260**:8397–8403 (1985).
19. Kissman, H. M., A. M. Small, and M. J. Weiss. Fluorinated steroids. II. The reaction of perchloryl fluoride with alkoxalylated steroids: synthesis of certain 2α-fluoro and 21,21-difluorosteroids. *J. Am. Chem. Soc.* **82**:2313–2317 (1960).
20. Krubiner, A. M., N. Gottfried, and E. P. Oliveto. The synthesis of 17-deoxy-17-α and -17β-20-pregnynes and −20-pregnenes. *J. Org. Chem.* **34**:3502–3505 (1969).
21. Ruschig, H. Zur Darstellung von 21-Estern des pregnen- (5)-diol-(3β.21)-ons-(20) aus pregnen-(5)-ol-(3β)-on-(20). *Chem Ber.* **88**:878–883 (1955).
22. Eng, R. R., L. A. Spitznagle, and W. F. Trager. Preparation of radiolabeled pregnenolone analogs: 21-fluoropregnenolone-21-^{18}F, 21-fluoropregnenolone-3-acetate-21-^{18}F, 21-fluoropregnenolone-7-^{3}H, and 21-fluoropregnenolone-3-acetate-7-^{3}H. *J. Labelled Compd. Radiopharm.* **20**:63–72 (1983).
23. Julian, P. L., E. W. Meyer, and H. C. Printy. Sterols. IV. Δ^{20}-Pregnenes from bisnorsteroid acids. *J. Am. Chem. Soc.* **70**:887–892 (1948).
24. Johnson, E. F., G. E. Schwab, J. Singh, and L. E. Vickery. Active site-directed inhibition of rabbit cytochrome P-450 1 by amino-substituted steroids. *J. Biol. Chem.* **261**:10204–10209 (1986).
25. Johnson, E. F., M. Finlayson, J. Raucy, H. Barnes, G. E. Schwab, and K. Griffin. Intraspecies individuality for the metabolism of steroids. *Xenobiotica* **17**:275–285 (1987).
26. Schwab, G. E., J. L. Raucy, and E. F. Johnson. Modulation of rabbit and human hepatic cytochrome P-450-catalyzed steroid hydroxylations by α-naphthoflavone. *Mol. Pharmacol.* **33**:493–499 (1988).
27. Schwab, G. E., and E. F. Johnson. Two catalytically distinct subforms of P-450 3b as obtained from inbred rabbits. *Biochemistry* **24**:7222–7226 (1985).
28. Waxman, D. J. Rat hepatic cytochrome P-450 isoenzyme 2c: identification as a male-specific, developmentally induced steroid 16α-hydroxylase and comparison to a female-specific cytochrome P-450 isoenzyme. *J. Biol. Chem.* **259**:15481–15490 (1984).
29. Wrighton, S. A., P. Maurel, E. G. Scheutz, P. B. Watkins, B. Young, and P. S. Guzelian. Identification of the cytochrome P-450 induced by macrolide antibiotics in rat liver as the glucocorticoid responsive cytochrome P-450p. *Biochemistry* **24**:2171–2178 (1985).
30. Halpert, J. Multiplicity of steroid-inducible cytochrome P-450 isozymes in rat liver microsomes. *Arch. Biochem. Biophys.* **263**:59–68 (1988).
31. Waxman, D. J., G. A. Dannan, and F. P. Guengerich. Regulation of rat hepatic cytochrome P-450: age-dependent expression, hormonal imprinting, and xenobiotic inducibility of sex-specific isoenzymes. *Biochemistry* **24**:4409–4417 (1985).
32. Waxman, D. J., D. P. Lapenson, S. S. Park, C. Attisano, and H. V. Gelboin. Monoclonal antibodies inhibitory to rat hepatic cytochromes P-450: P-450 form specificities and use as probes for cytochrome P-450-dependent steroid hydroxylations. *Mol. Pharmacol.* **32**:615–624 (1987).
33. Stevens, J. C., and J. Halpert. Selective inactivation of four rat liver microsomal androstenedione hydroxylases by chloramphenicol analogs. *Mol. Pharmacol.* **33**:103–110 (1988).
34. Sheets, J. J., and L. E. Vickery. Active-site directed inhibitors of cytochrome P-450scc: structural and mechanistic implications of a side chain-substituted series of amino-steroids. *J. Biol. Chem.* **258**:11446–11452 (1983).
35. Marcotte, P. A., and C. H. Robinson. Design of mechanism-based inactivators of human placental aromatase. *Cancer Res.* **42** suppl.:3322s–3326s (1982).
36. Sheets, J. J., M. X. Zuber, J. L. McCarthy, L. E. Vickery, and M. R. Waterman. Discriminatory inhibition of adrenocortical 17α-hydroxylase activity by inhibitors of cholesterol side chain cleavage cytochrome P-450. *Arch. Biochem. Biophys.* **242**:297–305, 1985.
37. Guengerich, F. P. Oxidation of 17α-ethynylestradiol by human liver cytochrome P-450. *Mol. Pharmacol.* **33**:500–508 (1988).
38. Ortiz de Montellano, P. R., and K. L. Kunze. Self-catalyzed inactivation of hepatic cytochrome P-450 by ethynyl substrates. *J. Biol. Chem.* **255**:5578–5585 (1980).

Send reprint requests to: Dr. James Halpert, Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona, Tucson, AZ 85721.