Mechanism-Based Inactivation of Human Liver Microsomal Cytochrome P-450 IIIA4 by Gestodene

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A series of 17α-acetylenic steroids was examined with regard to ability to inactivate human liver microsomal cytochrome P-450 (P-450) IIIA4, an enzyme involved in the oxidation of a number of drugs, carcinogens, and steroids, including estrogens and progestogens. Of the eight compounds tested, gestodene was found to be particularly active as a mechanism-based inactivator of P-450 IIIA4. Inhibition of both microsomal nifedipine oxidation and 17α-ethynylestradiol (EE) 2-hydroxylation was dependent upon NADPH and gestodene concentration. Rates of inactivation were pseudo first order—values of $k_{\text{inactivation}} = 0.4 \text{ min}^{-1}$ and $K_i = 46 \mu M$ and a partition ratio of 9 were calculated. The $k_{\text{inactivation}}$ is ~50-fold greater than estimated for EE and is one of the highest reported for P-450 mechanism-based inactivators. Spectrally detectable P-450 was also destroyed in microsomes, but several experiments indicate that little covalent binding to amino acid residues of P-450 IIIA4 occurs. Microsomal inactivation of P-450 could be blocked by the presence of other P-450 IIIA4 substrates, and several activities catalyzed by other P-450s were not inhibited under conditions in which >90% of P-450 IIIA4 was inactivated. Consideration of structure/activity relationships among the 17α-acetylenic steroids examined indicates that the Δ15 double bond is critical but is not in itself sufficient for the inactivation process, which is postulated to result from attack of P-450 on the substituted acetylenic carbon and lead to porphyrin N-alkylation. The effectiveness of this mechanism-based inactivator may account for reports of increased estrogen and steroid levels in some women using gestodene in oral contraceptives.

Many compounds cause the mechanism-based inactivation of P-450s (for reviews see refs 16 and 17). The list contains a great variety of chemicals, including olefins (16–19), acetylenes (20–26), strained cycloalkylamines (27, 28), 4-alkylidihydropyridines (29–31), and several other types of compounds. Information is now available concerning details of how the heme of P-450 is modified during some of these inactivations (16, 17, 25), but insight into the modification of amino acid residues and the unusual cross-linking of heme and apoprotein is lacking (32, 33).

Acetylene moieties, with the potential for inactivation of P-450s, are found in a number of synthetic steroids (17). In particular, a 17α-acetylenic group has been included in many steroids, especially those used in oral contraceptives. The major estrogenic component in most formulations is EE, the 17α-acetylenic derivative of 17β-estradiol (34). EE was previously found to be a mechanism-based inactivator of P-450 in human liver microsomes in this laboratory (35).

The major oxidative reaction that EE undergoes, 2-hydroxylation, was shown to be catalyzed by P-450 IIIA4 (35). Several lines of evidence indicate that levels of P-450 IIIA4 are elevated in individuals administered rifampicin or barbiturates (4, 14, 15, 35, 36), and taken together, these observations can explain the increased rates of EE clearance in women taking rifampicin or barbiturates (37, 38) and the decreased therapeutic effectiveness of EE, resulting in some reports of unanticipated pregnancies (36, 38–41).

In experimental animals several 17α-acetylenic steroids have been shown to inactivate P-450 both in vitro and in vivo (20, 21, 42–44). While EE is commonly used as the estrogenic component in many oral contraceptives, the

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1 Abbreviations: P-450, liver microsomal cytochrome P-450; EE, 17α-ethynylestradiol; TAO, trocleandomycin; HPLC, high-performance liquid chromatography. The trivial names of the steroids are defined as follows: 17α-ethynylestradiol, 19-nor-17α-pregna-1,3,5(10)-tien-20-yn-3,17-diol; gestodene, 13-ethyl-17α-ethynyl-18(R)-19-nor-17α-pregna-4,15-dien-20-yn-3-one; desogestrel, 11-methylene-13-ethyl-17α-ethynyl-18(R)-19-nor-17α-pregna-4,15-dien-20-yn-3-one; levonorgestrel, 13-ethyl-17β-ethynyl-18(R)-19-nor-17α-pregna-4,15-dien-20-yn-3-one; norethisterone, 17α-hydroxy-19-nor-17α-pregna-4,15-dien-20-yn-3-one; and 11-CH2-13α-norcortestosterone, 11-methylene-17α-hydroxy-19-nor-17α-pregna-4,15-dien-20-yn-3-one. The P-450s are referred to here according to the nomenclature


The protein was dialyzed versus 0.1 M ammonium acetate (pH 8.5 at 23°C) before use in HPLC solvents. Trifluoroacetic acid was distilled from CrO3 and then used without further purification, and [6,7-3H]EE was purchased from Du Pont-New England Nuclear (Boston, MA) and judged to be >97% radiochemically pure by HPLC (35). Other steroids were provided by Dr. H. Kuhl, J. W. Goethe-University (Frankfurt, FRG). A different sample of gestodene was provided from Schering (Berlin, FRG) through Dr. D. Back (Liverpool, U.K.), and similar results regarding the inhibition of microsomal activities were found with this preparation. [9,11-3H]Gestodene (0.82 mCi/mmol) was also provided by Dr. H. Kuhl; the material yielded a single A400 peak on HPLC analysis (vide infra), and >98% of the injected radioactivity was recovered in this fraction. TAO was a gift of Dr. D. J. Waxman, Harvard University (Boston, MA).

Enzyme Sources and Purification. Human liver samples were obtained from organ donors through Tennessee Donor Services (Nashville, TN) and denoted with the initials "HL" (human liver) and a number assigned in chronological order. One of the samples (FH 80) was obtained from Dr. P. H. Beaune, CHU Necker (Paris, France). Samples were stored at -70°C, and microsomes were prepared and stored as described (47, 48). Other studies with some of the preparations cited here have been published elsewhere (35, 49-51).

P-450 IIIA4 was expressed from a pAAH5/NF25 cDNA plasmid vector (3) in Saccharomyces cerevisiae and the protein was purified using slight modifications of a method presented elsewhere (2). Rabbit NADPH-P-450 reductase was purified essentially as described elsewhere (52, 53).

Human liver microsomes were incubated with [3H]gestodene, and P-450 IIIA4 was purified to electrophoretic homogeneity by using a method described in detail elsewhere (2).

**Assays.** Protein concentrations were estimated by using Pierce bicinchoninic (BCA) reagents (Pierce Chemical Co., Rockford, IL). P-450 concentrations were measured spectrally as described by Omura and Sato (54). The basic procedures, as used in this laboratory, were described elsewhere for the assay of nifedipine oxidation (2), EE 2-hydroxylation (35, 55), N,N-dimethyl-\(N\)-demethylase (56), phenactin O-deethylation (56), bufuralol 1'-hydroxylation (49), and mephenytoin 4'-hydroxylation (53). The general methods for the study of inactivation of P-450s presented elsewhere (35). Briefly, enzyme was incubated with the compound under consideration and a NADPH-generating system in a small volume (~50 μL) for a fixed time and then diluted to do the catalytic assay (57). When P-450 spectra were to be measured, reactions were quenched by the addition of cold buffer containing detergents (35). In all cases the NADPH-generating system consisted of final concentrations of 10 mM glucose 6-phosphate, 0.5 mM NADP+, and 2 IU of yeast glucose-6-phosphate dehydrogenase/mL. The rate of gestodene metabolism was estimated by incubating gestodene (20 μM) with human liver microsomes and quantifying the residual substrate after extraction (into CH2Cl2) and HPLC, using a 6.2 × 80 mm Zorbax octadecasyl (C18) column (Mac-Modd, Chadds Ford, PA) with a mixture of CH3OH/H2O (64:36 v/v) as the eluent. Gestodene was detected at 240 nm (ε240 = 14.3 mM-1 cm-1) and eluted with tR ~11 min at a flow rate of 1.0 mL/min.

P-450 IIIA4 was isolated from an incubation of human liver microsomes with gestodene and an NADPH-generating system, and tryptic peptides were isolated as described elsewhere (4, 58), with the exception of the following changes. Iodoacetic acid was recrystallized from hexane before use, and the carbosymethylated protein was dialyzed versus 0.1 M ammonium acetate (pH 8.5 at 23°C) before trypsin treatment (done in this buffer at 37°C for 24 h). Trifluoroacetic acid was distilled from CrO3 and then alumina before use in HPLC solvents (59). Radioactivity in individual fractions collected from the separation of the tryptic peptides was measured by liquid scintillation spectrometry.

**Results.**

**Inhibition of Microsomal Nifedipine Oxidation.** Initial studies were done with nifedipine inhibition because of the ease of assay and the knowledge that this substrate is oxidized primarily by P-450 IIIA4 (2). A series of eight 17α-acetylenic steroids (Chart I) was examined for inhibition of nifedipine oxidation in microsomes isolated from a human liver sample known to be high in P-450 IIIA4 linked catalytic activities (35). TAO inhibits P-450s in the IIIA family after oxidation of the amine to a nitroso group, which forms a tight σ complex with the ferrous iron (60, 61), and was used for comparison. When the steroids were tested at an initial concentration of 100 μM [based on the EE work done previously (35)] and a fixed amount of nifedipine in the subsequent assay, gestodene was clearly the most inhibitory of the steroids tested (Figure 1). Essentially no inhibition was seen with any of the compounds when the NADPH-generating system was not present during the initial incubation (with the steroid).

**Inhibition of Microsomal EE 2-Hydroxylation.** Initial studies on EE 2-hydroxylation were done with sample HL 110 liver microsomes, known to be high in P-450 IIIA4 and this catalytic activity (4, 35). Of the steroids examined, gestodene was clearly the most effective in the inactivation of this activity in human liver microsomes (Figures 2 and 3). As found in the case of nifedipine oxidation, little inhibition was seen in the absence of NADPH.

Gestodene and EE were examined as inactivators of EE 2-hydroxylation in other liver samples, including several with rather low rates of catalytic activity (4). In samples with low activity, the inhibition seen with gestodene was not always as dramatic as in the case of a liver...
Figure 1. Inhibition of nifedipine oxidation by TAO and various steroids. Incubations were done with sample HL 110 liver microsomes at 37 °C. TAO was present at 20 μM, and all steroids were added at 100 μM. The initial incubation period was 30 min in the absence (solid bars) or presence (hatched bars) of an NADPH-generating system. The incubation was then diluted 20-fold into 0.1 M potassium phosphate buffer (pH 7.4) containing 200 μM nifedipine and an NADPH-generating system. After 10 min of incubation, the oxidized product was measured by using HPLC. The values presented represent means of triplicate experiments. EE 2-hydroxylation occurring during the first incubation period was measured in separate experiments and used to correct the values indicated for EE.

Figure 2. Inhibition of EE 2-hydroxylation activity following incubation of human liver microsomes with NADPH and gestodene. Human liver microsomes (sample HL 110, 10 μM P-450 in 50 μL of 0.1 M potassium phosphate buffer, pH 7.4) were incubated with (A) an NADPH-generating system, (B) 100 μM gestodene, or (C) 100 μM gestodene plus an NADPH-generating system for 30 min at 37 °C. Incubations were diluted 20-fold with 0.1 M potassium phosphate buffer (pH 7.4) containing 100 μM EE, 1 mM ascorbate, and the NADPH-generating system for 10 min (at 37 °C), and 2-hydroxy-EE was determined by HPLC. The arrows indicate the 2-hydroxy-EE peak (tR ~1.95 min).

Figure 3. Inhibition of EE 2-hydroxylation by TAO and various steroids. Incubations were done with sample HL 110 liver microsomes at 37 °C. TAO was present at 20 μM, and all steroids were added at 100 μM. The initial incubation period was 30 min in the absence (solid bars) or presence (hatched bars) of an NADPH-generating system. The incubations were then diluted as described in the legend to Figure 2, and EE 2-hydroxylation activity was determined. The results are shown as means ± SD of triplicate experiments. EE 2-hydroxylation occurring during the first incubation period was measured in separate experiments and used to correct the values indicated for EE.

Figure 4. Inhibition of EE 2-hydroxylation following incubation with EE or gestodene in various human liver samples. Different human liver samples were incubated with an NADPH-generating system alone (solid bars) or plus 100 μM EE (open bars) or 100 μM gestodene (hatched bars) for 30 min at 37 °C, and EE 2-hydroxylation was subsequently determined as described in the legend to Figure 2. The results are shown as means ± SD of triplicate experiments. 2-Hydroxy-EE formed during the first incubation period was measured in separate experiments and used to correct the values indicated for EE 2-hydroxylation.

Figure 5. Inhibition of nifedipine oxidation activity in human liver microsomes as a function of gestodene concentration and time. Human liver microsomes (sample HL 110) were incubated at 37 °C in the presence of NADPH for various periods of time and with the indicated concentrations of gestodene; nifedipine oxidation was subsequently measured as described in the legend to Figure 1 and under Experimental Procedures. The arrows mark the peak for the product [2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester], which eluted at approximately 1.5 min in all cases. (A) No gestodene, 0-min incubation; (B) no gestodene, 20-min incubation; (C) 2 μM gestodene, 1-min incubation; (D) 2 μM gestodene, 20-min incubation; (E) 10 μM gestodene, 20-min incubation; (F) 20 μM gestodene, 20-min incubation; (G) 30 μM gestodene, 20-min incubation; (H) 60 μM gestodene, 20-min incubation; (I) 100 μM gestodene, 20-min incubation. For further analysis see Figures 6 and 7.

sample known to contain a high level of P-450 IIIA4 (Figure 3). The explanation might be due to catalysis of EE 2-hydroxylation by other P-450s in some of these samples. However, the extent of inhibition was not dependent (Figures 1-3). Inactivation of P-450 IIIA4 related activities by gestodene had already been shown to be NADPH-dependent (Figures 1-3). Inactivation of microsomal nife-
under conditions known to inactivate nifedipine oxidation in P-450 IIId4 was incubated with gestodene and NADPH. The experiment is described in the legend to Figure 5. The points shown are for individual data. The concentrations of gestodene present during the initial incubation period (37 °C, prior to addition of excess nifedipine) were zero (top ●), 2 μM (○), 5 μM (●), 10 μM (△), 20 μM (●), 30 μM (△), 60 μM (△), and 100 μM (bottom ●). See Figure 5 for examples of data. Inset: This plot was derived after fitting each curve to a semilogarithmic plot and then plotting reciprocals of the estimated first-order rates of inactivation versus the reciprocal of the gestodene concentration. Values of $k_{inactivation} = 0.39 \text{ min}^{-1}$ and $K_i = 46 \mu M$ were estimated. See also Figure 5.

Figure 6. Dependence of inhibition of human liver microsomal nifedipine oxidation upon time and gestodene concentration. The experiment is described in the legend to Figure 5. The points shown are for individual data. The concentrations of gestodene present during the initial incubation period (37 °C, prior to addition of excess nifedipine) were zero (top ●), 2 μM (●), 5 μM (○), 10 μM (●), 20 μM (○), 30 μM (△), 60 μM (△), and 100 μM (bottom ●). See Figure 5 for examples of data. Inset: This plot was derived after fitting each curve to a semilogarithmic plot and then plotting reciprocals of the estimated first-order rates of inactivation versus the reciprocal of the gestodene concentration. Values of $k_{inactivation} = 0.39 \text{ min}^{-1}$ and $K_i = 46 \mu M$ were estimated. See also Figure 5.

Figure 7. Destruction of human P-450 following incubation with gestodene and an NADPH-generating system. Human liver microsomes (sample HL 110) were incubated with an NADPH-generating system and 100 μM gestodene, and the reaction was quenched by the addition of an equal volume of cold 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA, 40% glycerol (v/v), 0.5% sodium cholate (w/v), and 0.4% Emulgen 913 (w/v). P-450 spectra (Fe2+-CO versus Fe2+) were measured as described by Omura and Sato (54). The incubation times are indicated in the figure. See also Figure 8.

dipine oxidation activity was clearly shown to be dependent upon incubation time and the concentration of gestodene (Figures 5 and 6), and the inactivation can even be seen at a gestodene concentration of 2 μM in Figure 5. The individual curves in Figure 6 were fitted to semilogarithmic plots, and the estimated rates of inactivation at individual concentrations were used to derive $K_i = 46 \mu M$ and a value of $0.39 \text{ min}^{-1}$ for $k_{inactivation}$, the extrapolated maximum rate of inactivation (57).

The rate of disappearance of gestodene from a similar microsomal incubation (with the same sample) was examined at a single initial substrate concentration of 20 μM. The apparent initial rate of oxidation, based upon total P-450, was 0.9 min$^{-1}$. Division of this rate by that of inactivation at the same gestodene concentration yields an estimate of 9 for the partition coefficient, the number of times the substrate is transformed per inactivating event (57). This number may be compared to a value of 110 for EE estimated in this work (vide infra).

Destruction of P-450 Related to Gestodene Oxidation. When a human liver microsomal preparation high in P-450 IIIId4 was incubated with gestodene and NADPH under conditions known to inactivate nifedipine oxidation and EE 2-hydroxylation activities (Figures 1, 3, 5, and 6), spectrally measured P-450 was also found to be decreased (Figure 7). The rate at which P-450 was lost was similar to that seen for the decrease in nifedipine oxidation (Figures 6 and 8), and the extent of loss was never greater than ~60%. As expected, EE also decreased the level of P-450 in microsomes (35), although the rate was far less. With the assumption that only 60% of the P-450 can be destroyed during oxidation of gestodene or EE, rates can be estimated for inactivation by the two compounds. The apparent inactivation rates are 0.009 min$^{-1}$ for EE and 0.46 min$^{-1}$ for gestodene (at concentrations of 100 μM). The rate measured with gestodene is comparable to the $k_{inactivation}$ estimated by use of loss of nifedipine oxidation (0.39 min$^{-1}$, Figure 6) and is approximately 50 times greater than that for EE. The inactivation rate for EE coupled with an EE 2-hydroxylation rate gives a partition ratio of 110 for this liver sample, consistent with the value of 120 reported previously (35).

Selectivity of Inactivation of P-450s. The loss of spectrally detected P-450 in microsomes could be seen even at gestodene concentrations of 10 μM (Table I). This loss could be partially blocked by the presence of the alternative P-450 IIIId4 substrates nifedipine and quinidine (2, 62). Testosterone, another substrate (2, 63), was not effective—this result is unexpected and may be related to...
Figure 9. Specificity of gestodene for inactivation of human P-450s. Human liver microsomes were incubated with 100 μM gestodene in the presence of an NADPH-generating system for 20 min at 37 °C. Relative values for the oxidation of indicated substrates are shown for incubations done in the absence (solid bars) and presence (hatched bars) of 100 μM gestodene during the initial incubation period. The values are means of triplicate experiments ± SD. The following human liver samples were used for the various measurements (with absolute rates of product formation indicated for samples devoid of gestodene): (S)-mephenytoin 4'-hydroxylation, HL 124, 0.47 nmol/(min·nmol of P-450); N,N-dimethylmethylnitrosamine N-demethylation, HL 105, 3.4 nmol/(min·nmol of P-450); phenacetin O-deethylation, HL 99, 1.45 nmol/(min·nmol of P-450); (4)-bufuralol 1'-hydroxylation, FH 80, 2.8 nmol/(min·nmol of P-450); and nifedipine oxidation, HL 110, 10.8 nmol/(min·nmol of P-450).

The higher rate of testosterone 6α-hydroxylation relative to disappearance of the other two substrates (6, 63).2

Although gestodene was able to destroy a large portion of the total P-450 present in a liver sample high in P-450 IIIA4, it does not appear to inactivate other P-450s. Liver samples known to be high in other P-450s were selected for the various measurements (with absolute rates of product formation indicated for samples devoid of gestodene): (S)-mephenytoin 4'-hydroxylation, HL 124, 0.47 nmol/(min·nmol of P-450); N,N-dimethylmethylnitrosamine N-demethylation, HL 105, 3.4 nmol/(min·nmol of P-450); phenacetin O-deethylation, HL 99, 1.45 nmol/(min·nmol of P-450); (4)-bufuralol 1'-hydroxylation, FH 80, 2.8 nmol/(min·nmol of P-450); and nifedipine oxidation, HL 110, 10.8 nmol/(min·nmol of P-450).

The values are means of triplicate experiments ± SD. The following human liver samples were used for the various measurements (with absolute rates of product formation indicated for samples devoid of gestodene): (S)-mephenytoin 4'-hydroxylation, HL 124, 0.47 nmol/(min·nmol of P-450); N,N-dimethylmethylnitrosamine N-demethylation, HL 105, 3.4 nmol/(min·nmol of P-450); phenacetin O-deethylation, HL 99, 1.45 nmol/(min·nmol of P-450); (4)-bufuralol 1'-hydroxylation, FH 80, 2.8 nmol/(min·nmol of P-450); and nifedipine oxidation, HL 110, 10.8 nmol/(min·nmol of P-450).

P-450 IIIA5 is another protein that is 86% identical with P-450 IIIA4 in its primary amino acid sequence. It shows some, but not all, of the activities catalyzed by P-450 IIIA4. The nifedipine oxidation activity of this purified enzyme was inhibited to the same degree as P-450 IIIA4 (results not shown; it is of interest to point out that P-450 IIIA5 does not appear to catalyze EE 2-hydroxylation).3

Covalent Binding of Gestodene to P-450 IIIA4. Acetylenic compounds can inactivate P-450s by either of two established mechanisms, porphyrin N-alkylation or modification of amino acid residues (25, 65). The latter possibility was examined in detail, for information concerning the active site might be obtained if a specific residue were labeled.

Human liver microsomes (sample HL 110) were incubated with tritiated gestodene to labeled P-450 IIIA4. The purified protein only contained radioactivity at the level of 0.06 nmol (of gestodene)/nmol of P-450 IIIA4.

Finally, human liver microsomes (sample HL 110, 250 nmol of P-450) were incubated with NADPH and 60 μM tritiated gestodene for 60 min at 37 °C. P-450 IIIA4 was purified to electrophoretic homogeneity (2) and estimated to contain 0.12 adduct per P-450 IIIA4. The sample (10 nmol) was used to prepare tryptic peptides, which were separated by HPLC and analyzed for radioactivity. Of the peptides, four or five contained similar levels of radiolabel and cumulatively accounted for only 0.01 adduct per P-450 IIIA4.

Discussion

The results presented in this paper argue that gestodene is a mechanism-based inactivator of P-450 IIIA4. The inhibition of two characteristic activities of this enzyme, nifedipine oxidation and EE 2-hydroxylation, showed appropriate characteristics of dependence upon cofactor, time, and inhibitor concentration (57). The prosthetic group was apparently destroyed, at least as judged by the loss of the P-450 spectra (Figure 7), and this process was blocked by other substrates. The inactivation was selective for one P-450 (or a group of closely related P-450s).

The mechanism of inactivation is postulated to involve destruction of the porphyrin as opposed to modification of critical nucleophiles in the apoprotein, for little binding to the protein could be found. Thus, in Scheme I, path a should be preferred, where the substituted carbon of the

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[Scheme I. Postulated Mechanisms of Oxidation of Acetylenes to (a) Alkylated Porphyrins and (b) Carboxylic Acids and Protein Adducts*]

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1.45 nmol/(min·nmol of P-450); (6R)-bufuralol 1'-hydroxylation, FH 80, 2.8 nmol/(min·nmol of P-450); and nifedipine oxidation, HL 110, 10.8 nmol/(min·nmol of P-450).

References:


Scheme I. Postulated Mechanisms of Oxidation of Acetylenes to (a) Alkylated Porphyrins and (b) Carboxylic Acids and Protein Adducts*
Acetylenic group is attacked in preference to the terminal carbon (25).

An N-alkylated porphyrin would be expected, and although a search was not made in this case, Ortiz de Montellano and his associates have characterized such compounds in the case of other acetylenic steroids, including norethisterone, the analogue of gestodene devoid of the $\Delta^{18}$ double bond (16, 17, 20, 21, 24). However, it should be pointed out that acetylenic steroids, like several other mechanism-based P-450 inactivators, have also been demonstrated to produce covalent heme–apoprotein cross-links in vitro (32), and the mechanism and relevance of this phenomenon are still under investigation (33, 36). Finally, it should be pointed out that if the pathway (b) leading to the ketene were followed in Scheme I, then the formation of the carboxylic acid would be expected (25, 26)—such a gestodene metabolite has not been found as of yet (67).

In these studies gestodene was found to be a selective inhibitor of P-450 IIIA4 and did not inhibit P-450s in other gene families (Figure 9). However, we did find that P-450 IIIA5 was inhibited to a similar extent. At this time it appears that there may be at least two other human P-450s in the P-450 IIIA family, P-450 IIIA3 and P-450 IIIA6. It is not clear yet that either of these proteins is expressed often in adult liver, and since no substrates are yet known for these enzymes that are not oxidized by P-450 IIIA4,4 it is difficult to determine if gestodene will inhibit these enzymes. Related P-450s are found in rats and rabbits (IIIA1, IIIA2, IIIA7), but the effect of gestodene on these enzymes has not been determined.

The structure–activity relationships of the steroids for mechanism-based inactivation of P-450 IIIA4 are interesting, but a clear picture of the structural requirements required for inactivation is not available as of yet. The importance of the $\Delta^{18}$ double bond is clearly shown by comparison of the results obtained with gestodene and levonorgestrel (Figures 1 and 3 and Chart I). Although attack at the substituted carbon of an olefin has been shown to produce mechanism-based heme inactivation (68), attack at the terminal carbon of an olefin is much more common and inactivation through the $\Delta^{15}$ position seems unlikely (17). It should be pointed out that all of the 17α-substituted steroids examined caused some degree of NADPH-dependent inactivation of P-450 IIIA4 in one or more catalytic assays (Figures 1 and 3). The $\Delta^{15}$ bond does not in itself seem to be sufficient to cause mechanism-based inactivation, for the addition of an 11-methylene group to gestodene appears to block the inactivation (compare results seen with gestodene and 11-CH$_2$, $\Delta^{15}$-norethisterone in Figures 1 and 3). Thus, it would appear that the interaction of the bonds of gestodene with P-450 IIIA4 (IIIA5) somehow favors attack in the manner leading to inactivation. Such a favorable transition state might be facilitated by puckering of the D ring due to the double bond. The $\Delta^{13}$ bond does not need to be necessarily avoided in the design of new 17α-acetylenic steroids, if other features such as the 11-methylene can compensate.

The clinical significance of the mechanism-based inactivation of gestodene is somewhat controversial. Wide differences in the rates of clearance of EE are seen among women (69–72), and this variation is not surprising in light of the demonstrated variation in levels of P-450 IIIA4 in people (4, 14, 35). There are several indications that continued administration of EE and other 17α-acetylenic steroids to women results in decreased rates of elimination of EE (see refs 20, 21, 69, 70, and 72 and references therein). Kuhl and Jung-Hoffmann (73, 74) have found that gestodene is considerably more pronounced than other progestogens in slowing the elimination of EE in individual women, and their work argues that simple displacement (of EE) is not the basis. The increased estrogen load has been considered as a possible factor in occasional incidences of thrombosis. The increase in EE levels occurring after the onset of gestodene administration is rapid, and this and other results argue that it cannot be attributed to protein induction but is most likely due to inhibition of metabolism. Women using gestodene show not only elevated levels of EE but also 17β-estradiol, cortisol, and gestodene itself. Such observations would be in accord with inhibition of P-450 IIIA4, which is a primary enzyme involved in the oxidation of EE (35), 17β-estradiol (2), and cortisol (75).

There is, however, a difficulty with the view that gestodene has a potent inhibitory effect on P-450 IIIA4 in clinically relevant situations. The normal dose of gestodene is $\sim 75$ μg/day, and in making some comparisons it will be assumed that all of this is oxidized in the liver every day. This amount is $\sim 250$ nmol. Typically, an adult human liver might be expected to contain about 7500 nmol of P-450 [1.5 kg of liver $\times (10$ mg of microsomal protein/g of liver) $\times (0.5$ nmol of P-450/mg of protein)]. Let us assume that the P-450s turn over with a half-lives of about 24 h, using animal models as a guide (76). Therefore, a human liver could synthesize several thousand nanomoles of new P-450 every day. The fraction of total P-450 attributable to P-450 IIIA4 varies considerably, probably 40-fold (4, 14, 35)—the results presented in Figure 8 can be used to argue that in this particular liver as much as 60% of the P-450 is P-450 IIIA4 or closely related forms inactivated by gestodene. Therefore, in such a liver more than 1000 nmol of P-450 IIIA4 should be synthesized each day, and the fraction that would be destroyed by consumption of 250 nmol of gestodene should be small. If the estimated partition rate of 9 is reliable in vivo, then only about 30 nmol of P-450 IIIA4 should be destroyed. However, some individuals might contain a small amount of P-450 IIIA4 and lack other P-450s that could catalyze reactions such as EE 2-hydroxylation. A further possibility to consider is that only a small population of this P-450 is actually involved in EE 2-hydroxylation and is destroyed by gestodene, but this view seems unlikely in light of the demonstrated ability of the P-450 IIIA4 expressed in yeast (and therefore having the known primary sequence) to catalyze EE 2-hydroxylation. One explanation for the situation may involve the amounts of endogenous substrates (vide infra) that occupy the enzyme binding site under physiological conditions—the presence of these substrates might reduce the effective enzyme concentration. A hypothesis to consider is that some of the individuals with low levels of EE 2-hydroxylation activity (e.g., samples HL 100, HL 114, and HL 129 in Figure 4) that can be inhibited by gestodene should be susceptible to in vivo inhibition by the 7b-μg dose of gestodene. Obviously, there are still some unresolved issues about the pharmacological effects a given dose of gestodene can have in vivo, and the relationship between plasma estrogen levels and thrombosis incidence is also unclear.

Nevertheless, the in vitro inactivation of P-450 IIIA4 by gestodene is interesting itself. P-450 IIIA4 appears to be one of the most versatile catalysts in the P-450 superfamily—indirect evidence has been provided that the enzyme is the major one in human liver catalyzing nifedipine oxidation (2, 6), quinidine N-oxygenation and 3-hydroxylation (62), EE and 17β-estradiol 2-hydroxylation (2, 35), testosterone and cortisol 6-hydroxylation (2, 63,
75), and the bioactivation of the carcinogens aflatoxin B1, aflatoxin G1, sterigmatocystin, 7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene, 3,4-dihydroxy-3,4-dihydro-7,12-dimethylbenz[a]anthracene, 9,10-dihydroxy-9,10-dihydrobenzo[b]fluoranthene, 6-aminochrysene, and tris(2,3-dibromopropyl)phosphate (77–79). Recently, we have expressed P-450 IIIA4 in S. cerevisiae and demonstrated all of these catalytic activities, plus ergothionein N-demethylation and the 16a-hydroxylation of dehydroepiandrosterone 3β-sulfate. (6) We have also found that P-450 IIIA4 expressed in Hep G2 mammalian cells catalyzes nifedipine oxidation, the 6β-hydroxylation of testosterone, androstenedione, and progesterone, the hydroxylation of cyclosporin at three different positions, the 1- and 4-hydroxylation of midazolam (80), and the N-deethylation of lidocaine (81). Other substrates for P-450 IIIA4 include TAO and gestodene, which are oxidized to testosterone 6β-hydroxylation. J. Biol. Chem. 264, 10388–10395.


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