MOLECULAR PHARMACOLOGY, 23:743–747

22-Ketocholesterol

A Potent Competitive Inhibitor of Cytochrome P-450$_{scc}$-Dependent Side-Chain Cleavage of Cholesterol

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Received May 20, 1982; Accepted January 4, 1983

SUMMARY

22-Ketocholesterol binds with high affinity to purified, phospholipid vesicle-reconstituted cytochrome P-450$_{scc}$. Binding, quantitated using reversal of cholesterol-induced absorbance changes in the Soret region of the enzyme, indicates an affinity 3–5 times greater than that for the normal substrate cholesterol. The ketosteroid cannot be hydroxylated at position 22 and thus acts as a potent inhibitor of cholesterol side-chain cleavage. Steady-state kinetics demonstrate competitive inhibition by this steroid and provide a K_I value several-fold lower than the cholesterol K_m. On the basis of recently proposed mechanisms for hydroxylation by cytochromes P-450, 22-ketocholesterol may exert its inhibitory effect by acting as a tightly bound analogue that resembles the enzyme-bound cholesterol from which a hydrogen has been abstracted from position 22.

INTRODUCTION

Cytochrome P-450$_{scc}$ catalyzes the oxidative side-chain cleavage of cholesterol to yield pregnenolone, the precursor of a variety of steroid hormones in both adrenal cortex and corpus luteum. Inhibition of this rate-limiting reaction has proven useful in the treatment of metastatic cancers in which the tumor has a steroid hormone growth requirement (1–3), but because of a relative lack of specificity of inhibitors presently in use, undesirable side effects can occur (1, 2, 4, 5). The cholesterol side-chain cleavage reaction occurs via an NADPH/O_2-dependent (6) three-step oxidation: the first hydroxylation appears to occur at position 22 R, the second at position 20 α, and a third oxidation results in scission of the 20–22 carbon-carbon bond, thus providing 22R-hydroxycholesterol and 20α,22R-dihydroxycholesterol as enzyme-bound intermediates of the side-chain cleavage reaction (7, 8). In a recent study (9), we utilized the relative binding affinities of these intermediates and other hydroxycholesterols to probe the structural requirements of the steroid binding site on cytochrome P-450$_{scc}$, and found that 22-ketocholesterol [5-cholesten-3β-ol-22-one] also interacted strongly with the enzyme, based upon its ability to reverse cholesterol-induced changes in the absorbance spectrum of the enzyme. In the present study this interaction has been quantitated and it has been demonstrated that this steroid is a very potent inhibitor of cholesterol side-chain cleavage. It is suggested that this steroid exerts its inhibitory effect by acting as a nonmetabolizable analogue, resembling the intermediate formed by an initial hydrogen abstraction from position 22 of cholesterol, prior to 22R hydroxylation.

EXPERIMENTAL PROCEDURES

Materials

Glucose-6-phosphate, egg phosphatidylcholine, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Company (St. Louis, Mo.), and NADPH was obtained from P-L Biochemicals (Milwaukee, Wisc.). [7-^{3}H]-Pregnenolone (5-pregnene-3β-ol-20-one) was a product of New England Nuclear Corporation (Boston, Mass.) and was purified by silicic acid chromatography. Cholesterol greater than 99% pure (by gas chromatography, thin-layer chromatography, and HPLC[2]) was purchased from Applied Science Laboratories (Waltham, Mass.), 22-ketocholesterol (5-cholesten-3β-ol-22-one) was obtained from Research Plus, Inc. (Bayonne, N. J.), and antipregnenolone antibody was from Radioassay Systems Laboratories (Carson, Calif.). Bacterial cardiolipin was from Supelco (Bellefonte, Pa.), and dioleoylphosphatidylcholine from Avanti Biochemicals (Birmingham, Ala.). All steroids and phospholipids were checked for purity by thin layer chromatography.

Methods

Protein purification. Cytochrome P-450$_{scc}$, adrenodoxin reductase, and adrenodoxin were purified from beef adrenal cortex mitochondria as described previously (9–12). The concentrations of adrenodoxin reductase and adrenodoxin were established using extinction coeffi-

This work was supported by National Institutes of Health Grant AM 27373 and American Cancer Society Grant IN-134.

[1] Recipient of a Research Career Development Award from the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases.

[2] The abbreviations used are: HPLC, high-pressure liquid chromatography; DNPH, 2,4-dinitrophenylhydrazine.

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cients of 10.9 mM^{-1} cm^{-1} at 450 nm (13) and 11 mM^{-1} cm^{-1} at 414 nm (14), respectively. Cytochrome P-450_{scc} was quantitated from the carbon monoxide-induced difference spectrum of the reduced form, using the difference extinction coefficient of 91 mM^{-1} cm^{-1} for A_{450} minus A_{490} (15). Absorbance spectra were recorded using a Varian 219 spectrophotometer for optically clear vesicle preparations and an Aminco DW2a spectrophotometer in the split-beam mode for difference spectra of microsomes.

Preparation of phospholipid vesicles. Small unilamellar vesicles were prepared as described previously (12). Solutions of phospholipids, cholesterol, and 22-ketocholesterol in chloroform were added to test tubes (13 × 100 mm) to give the ratios of components indicated in the figures, and solvent was removed by blowing under a gentle stream of dry nitrogen. Buffer [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2)/0.1 M NaCl] was added to each tube, and tubes were then sealed under N_2 and sonicated for 10 min using a Beuhler Ultramet III bath-type sonicator. Cytochrome P-450_{scc} was reconstituted into vesicle membranes as described previously (12), by addition of the cytochrome (0.2–0.3 μM final concentration) to preformed vesicles, followed by incubation at 25° for at least 10 min, or at 37° for 5 min prior to spectrophotometry or catalytic assays.

Assay of pregnenolone formation. Cholesterol side-chain cleavage to yield pregnenolone was assayed by a radioimmunoassay as described previously (9–16). The assay mixture consisted of 0.2 μM cytochrome P-450_{scc} and 667 μM phospholipid [30% (w/w) bacterial cardiolipin and 70% (w/w) dioleoylphosphatidylcholine]. The high ratio of phospholipid to hemoprotein and the presence of cardiolipin result in a low K_m for cholesterol and saturation can be achieved (9), as in Fig. 4. Included in an assay volume of 200 μl were 0.5 μM adrenodoxin reductase, 10 μM adrenodoxin, 2 mM neutralized glucose-6-phosphate, and glucose-6-phosphate dehydrogenase (2 units/ml). At zero time, NADPH was added to give 50 μM final concentration, and 30-μl aliquots were taken at 0, 2, 4, 6, and 8 min and pipetted into 1 ml of hexane. The vials were then tightly capped, and the contents were mixed for 15 sec using a Vortex mixer; the vials were stored at −20° for subsequent analysis.

Analysis of possible metabolites of 22-ketocholesterol. HPLC was carried out using a Beckman Model 110 A HPLC equipped with a Hitachi variable wavelength UV-visible detector. Incubations of 22-ketocholesterol with the enzyme-vesicle system were carried out as described above, except that a 20-fold higher concentration of vesicles, cytochrome P-450, and inhibitor were used in a 1-ml incubation volume, and incubations of 0, 1, or 2 hr were carried out to allow accumulation of possible metabolites. The reactions were quenched and steroids extracted by pipetting the incubation mixture into 5 ml of hexane. Following evaporation of the organic solvent, the steroids were either redissolved in mobile phase (acetonitrile/propanol, 9:1 or 8:2) or suspended in derivitization solvent. In order to increase our ability to detect 22-keto derivatives, two types of steroid derivatives were prepared. The dinitrophenylhydrazone derivative was prepared by reaction of steroid (either an unknown or a standard) with DNPH (Aldrich Chemical Company, Milwaukee, Wisc.), 1 mg/ml in methanol containing 1 N HCl. Pregnenolone reacted quantitatively when heated (50°) for 5 min with this reagent. However, 22-ketocholesterol was poorly derivatized; refluxing for 36 hr allowed production of sufficient quantities of DNPH-steroid to allow qualitative analysis of products. Another steroid derivative was prepared by reaction with *p*-toluenesulfonylhydrazine (Aldrich Chemical Company) [reagent (1 mg/ml) in methanol]. Two hours of reflux (80°) resulted in quantitative formation of the tosylhydrazones of both pregnenolone and 22-ketocholesterol.

Liquid chromatography was carried out using a Beckman Ultrasphere-ODS (reverse phase) column (4.6 × 25 cm). Isocratic chromatography utilized the following solvent systems and wavelengths: (a) underivatized steroids; acetonitrile/propanol (9:1 and 8:2), 210 nm; (b) DNPH-steroids; acetonitrile/propanol (4:1) and acetonitrile/propanol/acetic acid (90:9:1), 350 nm and 260 nm; (c) tosylhydrazone derivatives; acetonitrile/propanol/acetic acid (90:9:1) and acetonitrile/propanol/water/acetic acid (78:8:13:1), 230 nm. Each of these solvent systems was capable of separating cholesterol, pregnenolone, and 22-ketocholesterol as well as a variety of hydroxycholesterols including 22R-hydroxycholesterol, 22S-hydroxycholesterol, 20α-hydroxycholesterol, and 25-hydroxycholesterol.

Expression of concentrations. In the present studies, we have utilized the convention, developed in our previous work (9, 12, 17), of expressing substrate or inhibitor concentrations as a molar ratio of steroid to phospholipid rather than the more conventional molarity (calculated on the basis of total aqueous volume). We have previously demonstrated that the steroid binding site on cytochrome P-450_{scc} is in communication with the vesicle membrane rather than the aqueous environment (12), and that both cholesterol and hydroxy cholesterols are dissolved in the phospholipid vesicle membrane and do not partition appreciably into the aqueous phase (12, 17). Thus, the K_d and K_m values for substrates can vary if expressed as moles per liter of aqueous volume, but an invarient binding constant is obtained when values are expressed as moles substrate per mole of phospholipid.[3] One criterion for nonpartitioning of ligands from the lipid into the aqueous phase involves binding studies carried out at two different enzyme-to-phospholipid ratios (see Fig. 1). As discussed in ref. 17, if the same K_d value (expressed as steroid-to-phospholipid ratio) is obtained at the two ratios then no net partitioning into the aqueous volume has occurred.

RESULTS

Binding of 22-ketocholesterol to cytochrome P-450_{scc}. Cytochrome P-450_{scc} as purified contains 1–2 moles/mole of bound cholesterol and is in the predominantly high-spin form, characterized by a Soret peak at 392 nm (12). Incorporation of the hemoprotein into phospholipid vesicle membranes containing no additional cholesterol results in dilution of bound cholesterol into the phospholipid with resultant conversion to the low-spin (414 nm-absorbing) form, characteristic of substrate-free enzyme. Increasing the cholesterol content in the membrane leads to increasing fractions of hemoprotein in the high-spin form, and absorbance changes allow calculation of a binding constant for cholesterol (in units of cholesterol-to-phospholipid ratio (see Methods and refs. 12 and 17). When 22-ketocholesterol was incorporated in place of cholesterol in phospholipid vesicles, the enzyme became entirely low-spin even at high membrane content of this steroid, and the spectrum appeared to be identical with that for substrate-free cytochrome. However, when cholesterol was included, along with 22-ketocholesterol, the latter had the ability to inhibit the cholesterol-induced high-spin absorbance change. Thus, as shown in Fig. 1, at a given cholesterol concentration the high-spin to low-spin absorbance changes induced by various concentrations of ketosteroid could be used to determine an apparent binding constant (K_{obs}) for this steroid. When expressed as a ketosteroid-to-phospholipid ratio, the K_d value determined was independent of both phospholipid and cytochrome P-450 concentrations (see Fig. 1), thus indicating that there is no appreciable partitioning of this steroid from phospholipid into the aqueous environment (see Methods).

K_{obs} values obtained in Fig. 1 and in four other experiments (data not shown) were replotted in Fig. 2 as a function of the vesicle cholesterol-to-phospholipid ratio. The true K_d for 22-ketocholesterol binding to cytochrome P-450_{scc} is obtained by extrapolation to zero cholesterol (*y*-intercept). This yields a value of 0.002 mole/mole (steroid-to-phospholipid ratio). The binding constant for

[3] "Moles" of phospholipid rather than volume of phospholipid is used because of the uncertainties and assumptions required to calculate the internal volume of the lipid phase.

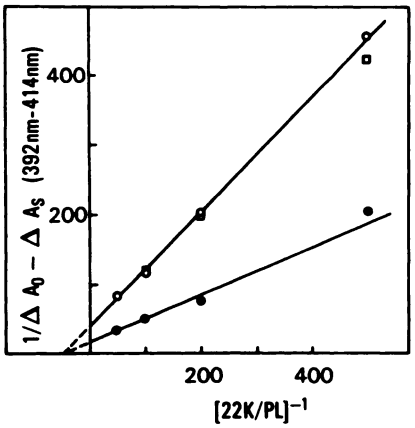

FIG. 1. *Apparent binding of 22-ketocholesterol to cytochrome P-450$_{scc}$*

The values of A_{392} minus A_{414} were measured in phospholipid vesicle preparations containing a cholesterol-to-phospholipid molar ratio of 0.06 and varying contents of 22-ketocholesterol (expressed as the molar ratio of ketosteroid to phospholipid, [22K/PL]. Phospholipid consisted of 30% (w/w) cardiolipin and 70% dioleoylphosphatidylcholine, and both reference and sample cuvettes contained equal concentrations of the same vesicle preparations in order to eliminate any absorbance due to the vesicle preparation. In an initial experiment (□), 0.3 μM cytochrome and phospholipid (0.08 mg/ml) were used. In two subsequent experiments the ratio of cytochrome P-450 to phospholipid was varied either by doubling the phospholipid vesicle concentration (○) or tripling the cytochrome P-450 concentration. ΔA_0 is the absorbance difference in the absence of added ketosteroid; ΔA_s is that in the presence of added ketosteroid.

cholesterol obtained from the x-intercept (0.005) is in reasonably good agreement with the value of 0.010 determined previously (9). Thus, 22-ketocholesterol binds to cytochrome P-450$_{scc}$ 2.5–5 times more tightly than does cholesterol.

Inhibition of pregnenolone synthesis by 22-ketocholesterol. In incubations of cytochrome P-450$_{scc}$ with 22-ketocholesterol (as described under Experimental Procedures), no conversion of this steroid into pregnenolone

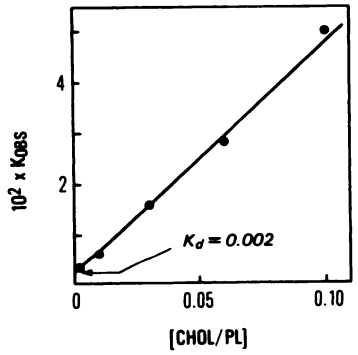

FIG. 2. *Determination of the K_d for 22-ketocholesterol binding to cytochrome P-450$_{scc}$*

Apparent K_d values for 22-ketocholesterol binding were determined as in Fig. 1, and four similar experiments (data not shown) measuring the ability of the ketosteroid to reverse the high-spin absorbance changes induced in cytochrome P-450$_{scc}$ by a given amount of cholesterol. Extrapolation of observed K_d values (K_{obs}) obtained at each cholesterol concentration to zero cholesterol yields the true K_d for binding of the ketosteroid.

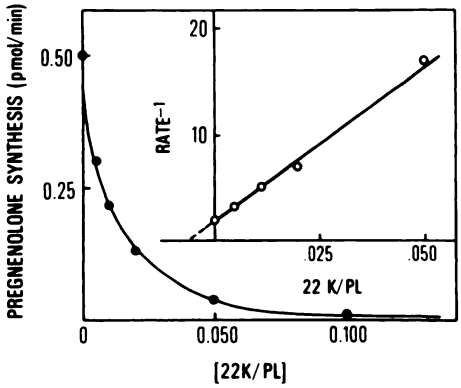

FIG. 3. *Effect of 22-ketocholesterol on pregnenolone synthesis by cytochrome P-450$_{scc}$*

Rates of pregnenolone synthesis were measured as described under Experimental Procedures. Phospholipid vesicles consisted of 15 mole % bacterial cardiolipin and 85 mole % dioleoylphosphatidylcholine, and the cholesterol-to-phospholipid molar ratio was 0.03; the 22-ketocholesterol-to-phospholipid molar ratio was varied as indicated. In the *inset*, the increase in the rate of pregnenolone synthesis is plotted versus 22-ketosteroid-to-phospholipid ratio. The rate expressed represents picomoles produced in a 2-μl aliquot of the hexane extract, and corresponds to a turnover number of approximately 1.2/min.

was seen.[4] In addition, no other steroid products could be detected by HPLC analysis of native or derivatized incubation (see Methods).[5] When cholesterol was maintained constant and the concentration of ketosteroid was varied, the rate of pregnenolone synthesis was seen to decrease as the concentration of 22-ketocholesterol was increased (Fig. 3). If competitive inhibition is assumed (see below) a replot of the inverse of the rate of pregnenolone synthesis versus the concentration of inhibitor (*inset*, Fig. 3) allows calculation of the inhibition constant, K_I, as follows (X_i represents the value of the x-intercept):

$$K_I = \frac{X_i}{1 + \frac{(S)}{K_m}}$$

The value obtained from such an analysis is 0.004.

The competitive nature of the inhibition is demonstrated in Fig. 4, in which the inhibitor concentration is held constant and the membrane cholesterol content is varied. For comparison, the same concentration of a known inhibitor of cholesterol side-chain cleavage, aminoglutethimide, was tested in the assay system. K_I values obtained from analysis of the X-intercept were 0.006 and

[4] This is in contrast to a previous report (18) in which a low rate of conversion was reported. In our experiments we see a small burst of pregnenolone synthesis, the size of which correlates with the presence of a small amount of endogenous cholesterol which is bound to the purified enzyme. However, there is no detectable rate of product formation following this burst. Thus, erroneous results may occur if only a single time point is obtained. Rates reported herein represent linear rates obtained by sampling every 2 minutes for a total of 8 minutes.

[5] Although this technique is sufficiently sensitive to detect major metabolites, the production of trace quantities of 22-ketocholesterol metabolites cannot be ruled out. Nevertheless, these studies suggest that appreciable metabolic conversion of the ketosteroid does not occur.

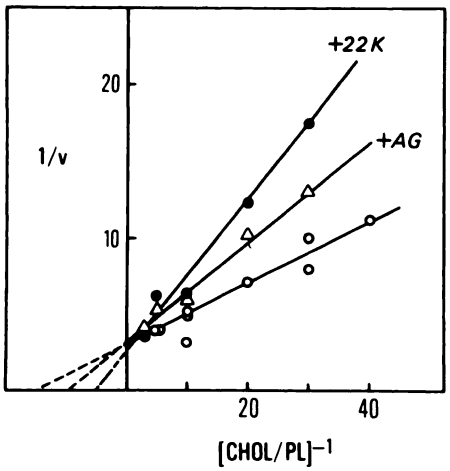

FIG. 4. *Competitive inhibition of pregnenolone synthesis by 22-ketocholesterol and aminoglutethimide*

Incubations were carried out as in Figure 3, except that the inhibitor-to-phospholipid ratio was maintained at 0.013 mole/mole (● for 22-ketocholesterol and △ for aminoglutethimide), and the cholesterol-to-phospholipid ratio was varied as indicated. ○, Rates with no inhibitor present. The V_m value obtained from the *y*-intercept corresponds to a turnover number of approximately 10/min. Rates of pregnenolone synthesis were measured as described under Experimental Procedures.

0.024, respectively, for 22-ketocholesterol and aminoglutethimide, in excellent agreement with the value obtained in Fig. 3 for 22-ketocholesterol. Thus, in this assay system 22-ketocholesterol is about 4 times more potent than aminoglutethimide as a competitive inhibitor of cholesterol side-chain cleavage.

DISCUSSION

The term cytochrome P-450 refers to a large class of heme-containing, oxygen-activating enzymes which are involved in the NADPH-dependent oxidative metabolism of a variety of hydrophobic molecules, including both normal biological compounds (e.g., steroids and fatty acids) and xenobiotics (e.g., many drugs and carcinogens). Several relatively nonspecific inhibitors of this class of enzymes have been studied. Carbon monoxide inhibits cytochromes P-450 noncompetitively with respect to substrate by binding to the ferrous heme iron, which normally binds oxygen. Many compounds containing a nitrogen base are also known to be effective inhibitors of cytochromes P-450, although—like carbon monoxide—most of these show some degree of nonspecificity. Metyrapone [2-methyl-1,2-bis(3-pyridyl)-1-propanone], for example, is an excellent inhibitor of adrenal mitochondrial cytochrome P-450$_{11\beta}$ (19), but it also inhibits other cytochromes P-450, including those in liver microsomes and the bacterial camphor hydroxylase. This inhibitor exhibits noncompetitive inhibition with respect to substrate and appears to bind to the heme iron (19). Another inhibitor of this class, aminoglutethimide [3-(4-aminophenyl)-3-ethyl-2,6-piperidinedione], is a potent inhibitor of the cholesterol side-chain cleavage enzyme (20, 21). Although it has no obvious structural similarity to cholesterol, it must not only interact with the heme, but must also occupy at least a portion of the substrate binding site, since inhibition is competitive with respect to substrate (ref. 20; Fig. 4). This compound has sufficient specificity to have achieved use as a pharmacological agent (1–5), but like others in this class aminoglutethimide can also inhibit other cytochromes P-450 (22) and for unknown reasons appears also to have adverse effects on thyroid function (4).

Design of a more specific inhibitor for a given cytochrome P-450 should take into account both the inhibitory properties of specific groupings on the compound and the structural requirements of the substrate binding site. While this may be a difficult task for many of the liver microsomal enzymes because of their rather broad substrate specificities, several cytochromes P-450 (including the side-chain cleavage enzyme) exhibit strict structural requirements, and rational design of inhibitors may be successful. In the present studies, 22-ketocholesterol has been shown to be a potent competitive inhibitor[6], of cholesterol side-chain cleavage, and in our assay system it is 4–6 times more potent than aminoglutethimide, the most potent and specific inhibitor yet described for this enzyme. The following explanations for this potent inhibitory effect are provided in order to aid in the design of other specific inhibitors for other cytochromes P-450.

The side-chain cleavage enzyme normally catalyzes a triple hydroxylation of cholesterol, beginning with hydroxylation at position 22R. The currently accepted hydroxylation mechanisms require an initial abstraction of a hydrogen (possibly as a hydrogen atom) from the position to be hydroxylated. Not only cholesterol, but also both 22R- and 22S-hydroxycholesterol (both of which contain an abstractable hydrogen at position 22) are readily metabolized by cytochrome P-450$_{scc}$ into pregnenolone. In 22-ketocholesterol, however, no hydrogen abstraction is possible, and further oxidation at this position cannot occur. The efficiency of this compound as an inhibitor is further enhanced by its relatively tighter binding to enzyme compared with cholesterol. We have previously provided evidence for an enzyme grouping near position 22 of the bound steroid which can hydrogen-bond with hydroxyl of 22R- and 20α-hydroxycholesterols, thus accounting for the enhanced binding of these metabolizable steroids (9). Such an explanation cannot be offered for 22-ketocholesterol since no hydrogen is present for bonding. However, if the hydroxylation mechanism does indeed require an initial hydrogen abstraction from the tetrahedral carbon 22, a planar, trigonal transition state is expected for this portion of the reaction. The inhibitor 22-ketocholesterol has such a trigonal arrangement of bonds at carbon 22, and this structure may contribute to the several-fold enhanced binding of this compound compared with cholesterol. Thus, 22-ketocholesterol and related substrate analogues with a carbonyl function at the hydroxylated position may prove useful

[6] That this inhibitor is not only potent but also specific is suggested by the finding that addition of up to 10 μM 22-ketocholesterol to rat liver microsomes (2 mg of protein per milliliter) produces no detectable change in the spin state of the microsomal cytochromes P-450, as measured by difference spectroscopy (see Methods). (Addition of higher concentrations produces increased turbidity due to precipitation of the poorly soluble steroid.)

as pharmacological agents for the inhibition of specific cytochrome P-450.

ACKNOWLEDGMENTS

I wish to thank Ms. Wendy Lou for expert technical assistance in performing HPLC analyses, and Nancy Linsey and Khaleelah Muwwakkil for typing the manuscript. I also wish to thank Dr. Dean Jones for reviewing the manuscript.

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