Biochemical Pharmacology, Vol. 22, pp. 1653–1659. Pergamon Press, 1973. Printed in Great Britain.

STEREOSELECTIVE INHIBITION OF STEROID 11β-HYDROXYLATION IN OX AND SHEEP ADRENOCORTICAL MITOCHONDRIA

COMPARISON OF METYRAPONE AND THE ENANTIOMERS OF 2-(4-AMINOPHENYL)-2-PHENETHYLAMINE (SKF 12185)

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(*Received* 1 *November* 1972; *accepted* 2 *January* 1973)

Abstract—The inhibitory potencies and difference spectra of the enantiomers of SKF 12185 and metyrapone were compared in ox and sheep adrenocortical mitochondrial suspensions. The SKF 12185 enantiomers induce a qualitatively identical type II difference spectrum to that of metyrapone (max at 420 nm; min at 390 nm) indicating that the enantiomers also interact with the haem iron of cytochrome P-450. (−)-SKF 12185 is a more potent inhibitor of 11β-hydroxylation than (+)-SKF 12185 at both 10 and 100 μM. Metyrapone is a more potent inhibitor than either enantiomer, giving greater inhibition at 10μM than the enantiomers did at 100 μM.

An inverse relationship exists between inhibitory potency and spectral dissociation constant (inhibitor concentration giving half-maximal type II spectral change); the greater the potency, the smaller the dissociation constant. No correlation exists between the magnitude of the spectral changes (at infinite inhibitor concentration determined by graphical extrapolation) and the inhibitory potency.

BOTH 2-methyl-1,2-di-3′-pyridylpropan-1-one (metyrapone) and 2-(4-aminophenyl)-2-phenylethyl-amine (SKF 12185) have been shown to be relatively specific inhibitors of adrenocortical steroid 11β-hydroxylation,[1–4,12] a cytochrome P-450 dependent reaction that occurs wholly within the adrenocortical mitochondria.[5] However metyrapone has been more fully investigated and adopted clinically usually to assess the functioning of the pituitary-adrenal axis.

Metyrapone has been shown to be a competitive inhibitor of steroid 11β-hydroxylase[6,7] and probably functions by interacting with the haem iron of the cytochrome P-450 component, thereby diminishing its rate of reduction.[8]

Similar to some other inhibitors of steroid 11β-hydroxylation, metyrapone induces a difference spectrum characterized by a maximum at 420 nm and a minimum at 390 nm when added to adrenocortical mitochondrial suspensions.[8,9] This is similar to the difference spectrum which metyrapone and a variety of other substances induce when added to liver microsomal suspensions and has been referred to as a type II,[10] or inhibitor type difference spectrum, in contrast to a type I[10,11] or substrate type difference spectrum characterized by a maximum at about 390 nm and a minimum at about 420 nm.

SKF 12185 exists in two enantiomeric forms but has been studied mainly using the racemic mixture as in the investigations of Saunders *et al*,[12] both *in vivo* and *in vitro*,

in guinea pigs and rats. These workers suggest that racemic SKF 12185 is a more potent inhibitor of steroid 11β-hydroxylation than metyrapone, since in rats racemic SKF 12185 in an oral dose of 1 mg/kg, inhibited the stress-induced rise in peripheral plasma corticosterone by about 50 per cent, whereas 40 mg/kg of metyrapone, orally, was required for similar inhibition (unpublished results cited by Saunders *et al.*[12]).

Clinical studies using the racemic mixture in patients with Cushing's syndrome[13] and primary aldosteronism[14] have been reported. An investigation has also been made using both the racemic mixture and the (+)-enantiomer in an unsuccessful attempt to ameliorate the severity of the diabetes in patients with juvenile-onset diabetes mellitus.[15] Studies using both enantiomers *in vivo* indicate that (−)-SKF 12185 is a more potent inhibitor of 11β-hydroxylation than (+)-SKF 12185 in rats and dogs[16] and in humans.[15] The enantiomers have not been compared *in vitro*.

The present investigation, *in vitro*, has a twofold purpose: (a) to provide further information on the mode of action of the SKF 12185 enantiomers with particular reference to the origin of their probable stereoselective inhibitory properties; (b) to compare their mode of action and inhibitory properties with metyrapone.

MATERIALS AND METHODS

Preparation of mitochondrial suspensions. Ox and sheep adrenals from freshly-slaughtered animals were stored in ice for about 1 hr prior to use.

Cortical tissue (4 g wet wt) was homogenized in 60 ml of 0·25 M sucrose solution at about 4° using a Waring Blendor. The homogenate was centrifuged at 1000 *g* for 5 min in an International Equipment Company refrigerated centrifuge (4°) to remove nuclei and cellular debris. The supernatant was then centrifuged at 8000 *g* for 10 min to sediment mitochondria which were washed by resuspension in the cold sucrose solution and recentrifugation at 1900 *g* for 10 min. The mitochondria were finally suspended in 0·1 M Sorensen's phosphate buffer, pH 7·4, to give a mitochondrial protein concentration of about 7 mg/ml as determined by the method of Lowry *et al.*[17]

Measurement of mitochondrial difference spectra. All spectra were recorded using a Unicam SP 800 double-beam spectrophotometer in conjunction with an SP 20 extension recorder.

The carbon monoxide difference spectra of dithionite-reduced mitochondria were obtained essentially as described by Omura and Sato[18] and the cytochrome P-450 content of the mitochondria determined from the absorbance difference between 450 and 490 nm using the extinction coefficient of 91 mM^{-1} cm^{-1} as determined by these authors.

The inhibitor-induced difference spectra using non-reduced mitochondria were studied thus: 2·5 ml mitochondrial suspension (1 mg/ml protein concentration) were transferred to the sample and reference cuvettes and a baseline obtained between 380 and 450 nm. Then inhibitor solutions were added from a microsyringe to the sample cuvette. The maximum volume added was only 12 μl and it was found unnecessary to add corresponding volumes of solvent to the reference cuvette in order to prevent distortion of the spectra. The absorbance difference (ΔA) between the maximum and minimum in the 380–450 nm region was measured for each addition of inhibitor from 0·4 to 15 μM for the enantiomers and from 0·2 to 2 μM for metyrapone. The

enantiomers, as dihydrochlorides,* were added in phosphate buffer solution (0·1 M), pH 7·4; the metyrapone was added in ethanol solution.

Graphs of $1/\Delta A$ against, $1/C$ were plotted, C denoting the inhibitor concentration. From each graph, the value of ΔA at infinite inhibitor concentration (ΔA_{max}) and the spectral dissociation constant (K_s) were determined. ΔA_{max} values and K_s values were the reciprocals of intercepts obtained by extrapolation of the line to the vertical and horizontal axes, respectively.

Inhibition of mitochondrial 11β-hydroxylation of deoxycorticosterone (DOC). ^{14}C-DOC labelled in the 4-position of the steroid nucleus was obtained from New England Nuclear Chemicals, Frankfurt.

At incubation, the flasks contained the following: ^{14}C-DOC (sp. act. 54·3 mCi/mmole), 0·37 μM; adrenocortical mitochondria equivalent to 0·55 μM cytochrome P-450; inhibitors at 10 μM or 100 μM for the SKF 12185 enantiomers and 10 μM for metyrapone (in flasks acting as controls no inhibitor was added); NADPH, 2·0 mM; Sorensen's phosphate buffer (pH 7·4), 0·1 M; in a total volume of 2 ml. The incubation media minus NADPH were prepared at 0–4°. The NADPH was added to the incomplete media after 5 min preincubation. Subsequent incubation was for 3 min.

After termination of the incubation by addition of 5 ml of a 5:1 v/v mixture of diethyl ether and chloroform to each flask, the flask contents were transferred to glass-stoppered tubes, each containing 20 μg of unlabelled DOC and 20 μg of unlabelled corticosterone which subsequently served as chromatographic markers, facilitating the detection of the chromatographic loci under u.v. light.

Solvent extraction with the ether–chloroform mixture was performed three-times and the extracts completely evaporated in separate tubes at 50° under air-blowers. The residue was dissolved in a few drops of chloroform and transferred to Whatman No. 1 paper chromatography strips impregnated with formamide.

Descending chromatography was performed for 1 hr with benzene as the mobile phase. This separated the DOC, which ran close to the benzene front, from corticosterone and other more polar substances which remained at, or near, the origin. Rechromatography in the same system for 2 hr separated corticosterone from still more polar substances remaining at the origin.

The positions of the DOC and corticosterone were detected on the dried strips by viewing under u.v. light. The proportions of radioactivity associated with the DOC and corticosterone in relation to the total radioactivity present were determined by a Nuclear Chicago Actigraph II radioactive strip scanner fitted with an electronic integrator and print-out system. A routine check on this procedure was provided by elution of the radioactive loci using methanol followed by liquid scintillation spectrometry. Close correspondence of results was observed.

The conversion of DOC to corticosterone was calculated as the ratio of the radioactivity corresponding to corticosterone to the total radioactivity on the strips and expressed as a percentage.

Treatment of results. Apart from the graphically derived spectral data, all data were expressed as the arithmetic mean values with the standard errors of the means. Where appropriate, the difference between means was analysed for significance using Student's *t*-test.[19]

* $[\alpha]_D^{20}$ + 11·2° and − 10·2° (c = 1, water) for the (+) and (−)-SKF 12185 salts, respectively.

RESULTS

Mitochondrial difference spectra. The addition of the SKF 12185 enantiomers to ox or sheep adrenocortical mitochondrial suspensions gave type II difference spectra qualitatively identical to the metyrapone-induced difference spectrum (max at 420 nm; min at 390 nm).

Figure 1 shows $1/\Delta A$ plotted against $1/C$ for (+)- and (−)-SKF 12185 and for metyrapone using ox adrenocortical mitochondria. The plots appear essentially linear. Similar results were obtained using sheep adrenocortical mitochondria. Table 1 gives the K_s and ΔA_{max} values (derived from the double-reciprocal plots) for the three inhibitors with corresponding cytochrome P-450 levels for ox and sheep adrenocortical mitochondria. For both species the K_s values for the (+)-enantiomer are about 30–50 per cent greater than the corresponding values for the (−)-enantiomer, but four- to thirteen-times greater than the corresponding values for metyrapone. The ΔA_{max} values for the SKF 12185 enantiomers are almost identical but are two- or three-times greater than the ΔA_{max} values for metyrapone. The between-species differences for cytochrome P-450 levels and ΔA_{max} values for each inhibitor are small.

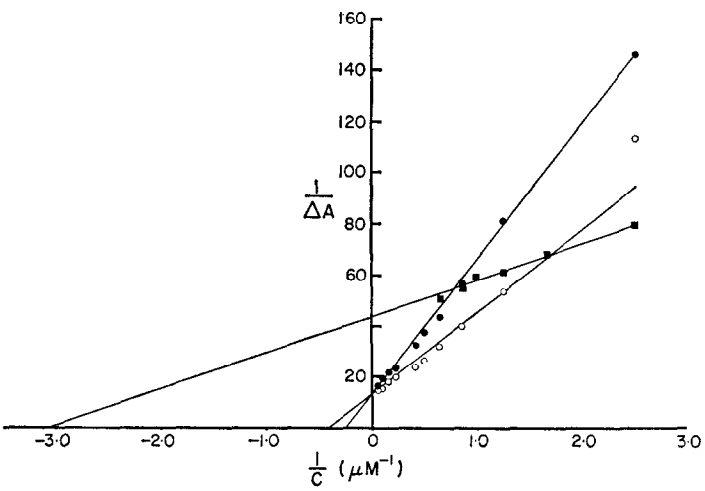

FIG. 1. Type II spectral data for (+)-SKF 12185 (●), (−)-SKF 12185 (○) and metyrapone (■) plotted $1/\Delta A$ against $1/C$ where C is the inhibitor concentration. Ox adrenocortical mitochondria were used at a protein concentration of 1 mg/ml. Each point is the mean of not less than three values for metyrapone and not less than eight values for the enantiomers.

Inhibition of steroid 11β-hydroxylation. Table 2 shows the effect of each inhibitor on the conversion of DOC to corticosterone at the stated concentrations using ox adrenocortical mitochondria at constant cytochrome P-450 concentration (0·55 μM).

It is seen that (−)-SKF 12185, compared with (+)-SKF 12185, is a more potent inhibitor of the 11β-hydroxylation of DOC by ox adrenocortical mitochondria both at 10 μM and at 100 μM, the greater potency being more marked at 100 μM. Metyrapone is apparently a more potent inhibitor than either of the enantiomers, since at 10 μM it inhibits the 11β-hydroxylation of DOC to a greater extent than (−)-SKF 12185 does at 100 μM.

TABLE 1. K_s AND ΔA_{max} VALUES DERIVED FROM TYPE II SPECTRAL DATA USING OX AND SHEEP ADRENOCORTICAL MITOCHONDRIA*

Species	Value†	SKF 12185 (+)	SKF 12185 (−)	Metyrapone
Ox‡	K_s	3·8 ± 0·2	2·6 ± 0·2	0·3 ± 0·1
	ΔA_{max}	0·073 ± 0·003	0·079 ± 0·003	0·028 ± 0·002
Sheep§	K_s	2·0 ± 0·1	1·5 ± 0·1	0·5 ± 0·1
	ΔA_{max}	0·077 ± 0·003	0·088 ± 0·004	0·036 ± 0·002

* Values are means ± S.E. obtained from ten duplicate determinations at a mitochondrial protein concentration of 1 mg/ml.

† Derived from double-reciprocal plots. Units are μM for K_s values and absorbance units per milligram protein per millilitre for ΔA_{max} values.

‡ Mitochondrial cytochrome P-450 content is 0·8 ± 0·1 nmole/mg (mean ± S.E. from ten duplicate determinations).

§ Mitochondrial cytochrome P-450 content is 0·9 ± 0·1 nmole/mg (mean ± S.E. from ten duplicate determinations).

Using sheep adrenocortical mitochondria, inhibition by the SKF 12185 enantiomers and metyrapone was less marked, although metyrapone was still the most potent inhibitor. The difference in potencies at 10 μM for (+)- and (−)-SKF 12185 was not apparent, although (−)-SKF 12185 still exhibited its greater potency at 100 μM.

TABLE 2. INHIBITORY EFFECT OF THE SKF-12185 ENANTIOMERS AND METYRAPONE ON THE CONVERSION OF DEOXYCORTICOSTERONE (DOC) TO CORTICOSTERONE*

Inhibitor	Inhibitor concn (μM)	Conversion of DOC to corticosterone (%) (mean ± S.E.)
None	0	44·7 ± 2·7
(+)-SKF 12185	10	38·7 ± 1·1†
(−)-SKF 12185	10	33·9 ± 1·5‡
(+)-SKF 12185	100	24·5 ± 0·5
(−)-SKF 12185	100	14·8 ± 2·1‡
Metyrapone	10	5·7 ± 0·4

* Each mean was from at least five determinations using ox adrenocortical mitochondria (range of protein concentrations was 0·6–1·0 mg/ml with a mean value of 0·7 mg/ml) at constant cytochrome P-450 concentration (0·55 μM).

† Significantly different from conversion where no inhibitor was added ($P < 0·01$).

‡ Significantly different from conversion with (+)-SKF 12185 at the same concentration ($P < 0·01$).

DISCUSSION

As with metyrapone, the enantiomers of SKF 12185 induce a type II spectral change suggesting a similar, if not identical, mode of action, namely probable interaction with the haem prosthetic group of cytochrome P-450 associated with diminished rate of reduction of the cytochrome.

The K_s value for metyrapone is of the order of ten less than those for the enantiomers. This may indicate a greater affinity between metyrapone and cytochrome P-450 compared with the enantiomers of SKF 12185 and cytochrome P-450. In accord with this, our incubation results indicate that metyrapone is a more potent inhibitor of steroid 11β-hydroxylation *in vitro* compared with the enantiomers of SKF 12185. Furthermore (−)-SKF 12185 has a K_s about three-quarters that of (+)-SKF 12185, which correlates with a greater inhibitory potency *in vitro* for the (−)-enantiomer. Thus our results show a correlation between K_s values derived from type II mitochondrial difference spectral data and the respective inhibitory potencies of (+)- and (−)-SKF 12185 and metyrapone; the smaller the K_s values, the greater are the inhibitory potencies.

The ΔA_{max} values are apparently unrelated to inhibitory potencies since the ΔA_{max} values for the enantiomers are essentially identical.

Our finding that metyrapone is a more potent inhibitor of steroid 11β-hydroxylation than the enantiomers of SKF 12185 using ox and sheep adrenocortical mitochondria contrasts with the unpublished work cited by Saunders *et al*,[11] which indicates that in rats (*in vivo*) racemic SKF 12185 is more potent than metyrapone. Such a contrast could have arisen as a result of differences in metabolism and elimination of the two drugs *in vivo*, although a species difference between rat and ox at the level of adrenocortical mitochondrial cytochrome P-450 is also a possibility. Support for the latter is provided by the work of Sprunt and Hannah[20] from this laboratory, who found that (+)-SKF 12185 is a more potent inhibitor of steroid 11β-hydroxylation than metyrapone in rat adrenal homogenates but is less potent than metyrapone in ox adrenal homogenates.

It seems likely that a stereoselective inhibitory interaction at adrenocortical mitochondrial cytochrome P-450 can account for the difference in the inhibitory potencies of the SKF 12185 enantiomers *in vitro* and to some extent at least, the difference observed in laboratory animals *in vivo*[21] and in patients.[14] However, the contribution of other factors *in vivo* is possible, such as differences in distribution, metabolism and elimination of the SKF 12185 enantiomers.

A number of investigations have been reported using enantiomeric drug substrates giving difference spectra with liver microsomes.[21–23] In one case enantiomers (amphetamine) giving type II difference spectra were studied.[23] It was suggested from type II spectral data that (+)-amphetamine had a greater affinity for the haem iron of hepatic microsomal cytochrome P-450 than (−)-amphetamine and that this greater inhibitory interaction could account for the lower rate of microsomal deamination of (+)-amphetamine. The present study indicates that a similar stereoselectivity is associated with the haem iron of adrenocortical mitochondrial cytochrome P-450 and that this can contribute to the different inhibitory potencies of the SKF 12185 enantiomers.

Acknowledgements—We would like to thank Mr. G. Wilfon, Medical and Scientific Services, Smith, Kline & French Overseas Co., 1500 Spring Garden St., Philadelphia, Pennsylvania 19101, U.S.A., for the gift of the enantiomers of 2-(4-aminophenyl)-2-phenethylamine.

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