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Biochemical Pharmacology, Vol. 37, No. 11, pp. 2291–2293, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Interaction of civetone with rat liver microsomal cytochrome P-450 and steroidogenic enzymes

(*Received* 3 *November* 1987; *accepted* 7 *January* 1988)

Civetone (9-cycloheptadecen-1-one) is a component of the animal secretion, civet. The characteristic musky odour of civetone becomes pleasant at very dilute concentrations, hence its use in the perfumery industry. This 17-membered macrocyclic ketone contains one ethylenic double bond, therefore it can exist as either the *cis* or *trans* isomer. Naturally occurring civetone has been identified as the *cis* isomer and bears some structural resemblance to a steroid [1]. The biological properties of civetone have not been characterised.

Another naturally occurring macrocyclic ketone is muscone (3-methylcyclopentadecanone), which is responsible for the odour of musk. Unlike civetone, there is some data about the biological properties of muscone. Pretreatment of rats with muscone causes induction of rat liver microsomal cytochrome P-450. In addition, a Type I binding spectrum was produced on addition of muscone to microsomes [2]. In this study, we report the interaction of civetone with rat liver microsomal cytochrome P-450 and assess its inhibitory activity *in vitro* against the aromatase, cholesterol side chain cleavage (CSCC) and steroid 5 α-reductase enzymes. Inhibitors of such steroidogenic enzymes may provide leads in the development of useful therapeutic agents for the treatment of hormone dependent breast and prostate tumours.

Materials and methods

Chemicals. NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADPH were obtained from Boehringer (Mannheim, F.R.G.). All non-radioactive steroids were purchased from Sigma Chemical Co. (St Louis, MO). [26-^{14}C]Cholesterol (specific activity 56 mCi/mmol) and [1,2-^{3}H]androstenedione (specific activity 48 Ci/mmol) were from New England Nuclear (Boston, MA). [4-^{14}C]Testosterone (specific activity 50–60 mCi/mmol) was from Amersham International (U.K.). Aminoglutethimide was a gift from Ciba Geigy Ltd. (Horsham, U.K.). Samples of optically pure *cis* and *trans* iso-

mers of civetone were gifts from Firmenich U.K. Limited (Hayes Road, Middlesex UB2 5NN, U.K.).

Purity of civetone samples. Purity of the *cis* and *trans* isomers of civetone was checked by gas chromatography. A Pye-Unicam 204 gas chromatograph was used with flame ionisation detection and an OV 17 column (5.5 m × 2 mm) packed with Gas Chrom Q. A column temperature of 100° was held for 1 min followed by an increase to 280° at 12°/min. Under these conditions, a sample of mixed isomers gave one peak with a retention time of 13 min. The individual isomers gave identical results confirming the purity of the samples.

Spectral interactions with microsomal cytochrome P-450. Phenobarbitone induced rat liver microsomes and human placental microsomes were obtained as described previously [3, 4]. Binding spectra were run at room temperature with a Pye-Unicam SP8-150 spectrophotometer in the wavelength range of 360–520 nm. Microsomal pellets were diluted with 0.1 M potassium phosphate buffer (pH 7.4). A final protein concentration of 2 mg/ml was used for liver microsomes and 7 mg/ml for placental microsomes. Each isomer of civetone was dissolved in dimethyl sulphoxide and equal volumes of solvent were added to the reference and sample cuvettes. Spectra were recorded at 1 nm/sec with a slit width of 2 nm and a full scale absorbance of 0.1. K_s values (average of three determinations) were calculated from Lineweaver–Burk plots of reciprocal absorbance difference against reciprocal concentration.

Assays of steroidogenic enzymes. The aromatase enzyme was obtained from the microsomal fraction of human placental tissue [4]. Activity was monitored by measuring the ^3H_2O formed from [1,2-^{3}H]androstenedione during aromatization [5]. The mitochondrial fraction of bovine adrenal cortex provided the source of the cholesterol side chain cleavage (CSCC) enzyme [6]. We assayed the enzyme using [26-^{14}C]cholesterol as substrate and measuring the [^{14}C]isocaproic acid released [6, 7]. Experimental details of both assays are described elsewhere [8]. The K_i value for each inhibitor was the average of three determinations, all ±10%.

The steroid 5α-reductase enzyme catalyses the reduction of [^{14}C]testosterone into [^{14}C]5α-dihydrotestosterone. The preparation and assay of the rat prostatic 5α-reductase were as described [9]. After incubation and extraction, the steroids were applied to Merck Kieselgel 60 F_{254} (Art. 5729) 20 × 20 cm TLC plates and developed in dichloromethane:ethyl acetate (9:1). Radioactivity on the plates was scanned and quantified by a Berthold TLC analyzer. The only radioactive product was 5α-dihydrotestcrone. Compounds were added to the 5α-reductase assay to give final concentrations of 1, 2, 5 and 10 μM.

Results and discussion

Spectral interactions of civetone. The *cis* and *trans* isomers of civetone elicited strong binding spectra with rat hepatic microsomal cytochrome P-450 (Table 1). In both cases, a Type I binding spectrum was produced with a trough at 420 nm and a peak at 385 nm. The K_s value for *cis* civetone (3.6 μM) increased to 5.2 μM when the binding spectrum was determined in the presence of 50 μM SKF 525 A, a compound that produces a well characterised Type I binding spectrum. The resulting Lineweaver–Burk plot was of a competitive nature confirming that *cis* civetone causes a Type I spectrum with hepatic cytochrome P-450. This is the same type of spectral interaction that was observed with muscone [2] and suggests that civetone is also a substrate for the cytochrome P-450 system causing a transition of the heme group to a high spin state.

Civetone was examined for its reaction with human placental microsomal cytochrome P-450. Unlike the hepatic system, no binding spectrum was obtained with *trans* civetone (Table 1). However, the *cis* isomer produced a Type II spectral change with a peak at 435 nm and a trough at 410 nm. The resulting K_s value was 37 μM.

Table 1. Interaction of *cis* and *trans* civetone with microsomal cytochrome P-450

Compound	Rat hepatic microsomes K_s (μM)	Rat hepatic microsomes Binding spectrum	Human placental microsomes K_s (μM)	Human placental microsomes Binding spectrum
cis Civetone	3.6	Type I	37	Type II
trans Civetone	6.6	Type I	none	none

Table 2. Effect of *cis* and *trans* civetone on the aromatase and CSCC enzyme assays

Compound	Aromatase K_i (μM)	CSCC enzyme K_i (μM)
Aminoglutethimide	0.6	14
cis Civetone	1.2	none
trans Civetone	none	none

Effect of civetone on in vitro *steroidogenic enzyme assay.* The demonstration that civetone binds to placental cytochrome P-450 and the steroidal nature of the *cis* isomer prompted us to test the two isomers against three steroidogenic enzymes. The aromatase enzyme is a NADPH and cytochrome P-450 dependent monoxygenase that catalyses the conversion of androgens to estrogens [4]. Another NADPH and cytochrome P-450 dependent system is the CSCC enzyme, which converts cholesterol to pregnenolone and is the initial step in steroid hormone biosynthesis [6]. Finally, steroid 5α-reductase requires NADPH, but is not cytochrome P-450 dependent and catalyses the formation of dihydrotestosterone from testosterone within prostatic tissue [9].

The assays were carried out as described in the Materials and Methods. The results for the aromatase and the CSCC enzymes are shown in Table 2. For comparison, aminoglutethimide, an established inhibitor of both enzymes, was tested under identical conditions. The *trans* isomer of civetone has no significant activity towards either enzyme. Although *cis* civetone does not inhibit the CSCC enzyme, it is a potent inhibitor of the aromatase system with an inhibition constant (K_i) of 1.2 μM. Aminoglutethimide has a K_i value of 0.6 μM under similar conditions. The Dixon plots (not shown) of the reciprocal velocity of reaction against the concentration of inhibitor for both inhibitors are of a competitive nature. No activity was displayed by either *cis* or *trans* civetone towards the steroid 5α-reductase enzyme up to a final concentration of 10 μM. The assay results confirm that *cis* civetone interacts with placental cytochrome P-450 and is a potent inhibitor of the aromatase enzyme. It has no activity towards the other steroidogenic enzymes. The *trans* isomer of civetone is inactive in all the assay systems.

In conclusion, we have demonstrated that both isomers of civetone exhibit strong Type I spectral interactions with hepatic microsomal cytochrome P-450. The *cis* isomer is a potent specific inhibitor of the *in vitro* aromatisation of androgens to estrogens.

Acknowledgements—We thank Professor A. B. Foster for his advice and support and Firmenich U.K. Limited, for supplying the civetone samples. This work is supported by grants from the Cancer Research Campaign and the Medical Research Council.

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Biochemical Pharmacology, Vol. 37, No. 11, pp. 2293–2297, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Interactions of cardiac glycosides with cells and membranes. Therapeutic and toxic doses of ouabain acting on sodium and calcium pumps in plasma membranes

(*Received* 17 *November* 1987; *accepted* 7 *January* 1988)

According to the currently accepted mechanism for cardiac glycoside action, theses agents bind to and inhibit the action of the sodium pump [1–3]. Following several reports of data that are inconsistent with sodium pump inhibition mechanism, attention has been directed toward alternative explanations [4–6]. One possible mode is via the sarcolemmal calmodulin-regulated calcium pump, which has been implicated in the control of cellular Ca^{2+} [7–12]. The present communication describes the biphasic response to ouabain of two membrane-bound enzymes, Na^+–K^+-ATPase and Ca^{2+}-Mg^{2+}-ATPase in erythrocyte membrane vesicles, which serve as a good model system. Our data suggest that nanomolar concentrations of ouabain stimulate both Na^+–K^+-ATPase activity and calmodulin-mediated Ca^{2+}–Mg^{2+}-ATPase with concomitant inhibition of the "basal" Ca^{2+}–Mg^{2+}-ATPase activity. The possible involvement of the calcium pump mediated via cardiac glycosides in the regulation of intracellular Ca^{2+} concentrations is proposed.

Materials and methods

Human erythrocyte membrane vesicles were prepared by hemolysis in either (a) hypotonic media without Ca^{2+} [13] or in the presence of 1 mM EGTA [14], or (b) isotonic media containing 0.3 g/l saponin, 0.2 mM EGTA and Ca^{2+}. Whereas at $(Ca^{2+})_f < 0.1\ \mu M$ only "basal" Ca^{2+}–Mg^{2+}-ATPase could be detected, during hemolysis, at $(Ca^{2+})_f > 1\ \mu M$, Ca^{2+}–Mg^{2+}-ATPase activity that was stimulated by calmodulin was found [14, 15]. ($(Ca^{2+})_f$ is the concentration of free unbound Ca^{2+}.)

Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase activities were assayed by measuring the release of inorganic phosphate [16] in an isotonic medium containing 1 mM EGTA ± $CaCl_2$ (1 mM Ca^{2+} in the presence of EGTA yielded $(Ca^{2+})_f = 14\ \mu M$ [17]); and ± ouabain in increasing concentrations. The effects of endogenous or exogenous calmodulin (derived from bovine brain) were measured by adding either 100 μg/ml compound 48/80 [18] or, 0.5 μM calmodulin and 10–100 μM Ca^{2+}. The enzyme was preincubated with increasing concentrations of ouabain either in the absence or presence of calmodulin. A similar procedure was followed with compound 48/80. Incubations at 37° for up to 60 min gave a linear response. The reaction was terminated by placing the tubes into liquid air. The P_i released was determined by the malachite green–molybdate complex assay, measuring color development at 1° during a 30 min period [19]. The activities of both enzymes were calculated according to Raess and Vincenzi [16] and expressed in mU/mg protein. Protein content was determined in the presence of 0.01% sodium dodecyl sulfate according to Lowry *et al.* [20].

The values are presented as means ± SEM. Significance of the differences between means was checked by Student's *t*-test, and gave values of less than 0.05.

Results and discussion

Upon treatment with calmodulin, vesicles prepared from human erythrocyte membranes exhibited a bimodal response to ouabain: nanomolar concentrations of ouabain stimulated Na^+–K^+-ATPase and inhibited "basal" Ca^{2+}–Mg^{2+}-ATPase, whereas micromolar concentrations had the opposite effect (Fig. 1). Calmodulin (0.5 μM) led to a fivefold increase in the "basal" Ca^{2+}–Mg^{2+}-ATPase activity. Nanomolar concentrations of ouabain further stimulated this activity by 160%. Micromolar concentrations of ouabain seemed to inhibit this "calmodulin-activated" Ca^{2+}–Mg^{2+}-ATPase (Fig. 2). Na^+–K^+-ATPase did not respond to calmodulin at these concentrations. Compound 48/80, 0.1–0.4 μM [18], a potent and specific antagonist of "calmodulin-dependent" cellular activities,