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The Use of the Substrate–Heme Complex Approach in the Design, Synthesis, Biochemical Evaluation, and Rationalization of the Inhibitory Activity of a Range of Azole Compounds against Cholesterol Side Chain Cleavage Enzyme

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Here, we report the synthesis and biochemical evaluation of a number of compounds as potent inhibitors of cytochrome P450 enzymes, such as aromatase (AR), *but* not cholesterol side chain cleavage (CSCC). This is a crucial enzyme in the steroidal cascade and its inhibition results in major side-effects, as a result, the ability of compounds to specifically inhibit enzymes such as AR *but not* CSCC would be an important factor in the drug design process. © 2000 Academic Press

The enzyme Cholesterol Side Chain Cleavage (CSCC) mediates the conversion of cholesterol to pregnenolone and isocapraldehyde (1) (Fig. 1) and requires both NADPH and oxygen in the sequential oxidative steps of the side chain. This is the first step in the steroidal cascade leading to the biosynthesis of the sex hormones, glucocorticoids and mineralocorticoids. It is thus of vital importance to recognise any potential of compounds to inhibit this enzyme when designing inhibitors of enzymes further down in the cascade, for example, aromatase (AR).

Although extensively studied, little information is known about the active site of this enzyme as no crystal structure for it exists, although a theoretical homology based structure has been proposed. We have previously reported studies (2, 3) of related cytochrome P450 enzymes where the nature of the active site has been determined. Using the substrate-heme complex (SHC) approach for alternative P450 enzymes, we have successfully rationalised the activity, or lack of activity, of several different types of inhibitors, in particular enantiomers of known inhibitors, such as ketoconazole (4, 5). We now report the use of the SHC for CSCC in the design, synthesis and biochemical evaluation and thus the rationalisation of inhibitory activity of a range of compounds against CSCC.

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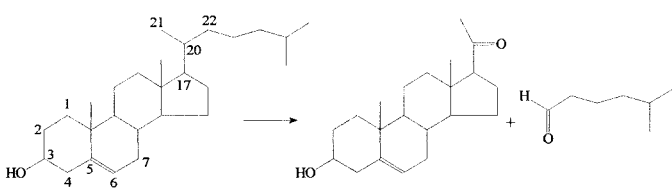
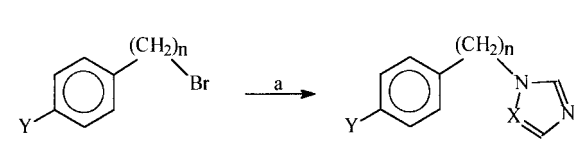


FIG. 1. Action of CSCC on cholesterol.

EXPERIMENTAL

Synthesis of inhibitors. The synthesis of the compounds to evaluate the hypotheses resulting from the molecular modelling are described below using Scheme 1. No major problems were encountered and the synthesis of *N*-(2-phenyl) ethyl imidazole (1) is given as an example.

N-(2-Phenyl) ethyl imidazole. (1) Imidazole (15.4 g, 0.23 mol) was added to anhydrous potassium carbonate (88.3 g, 0.64 mol) and tetrahydrofuran (THF) (50 ml). The mixture was stirred at room temperature for 10 min before (2-bromo ethyl) benzene (35 ml, 0.26 mol) was added. The mixture was then stirred at 40–60°C for a further 6 h. After filtration, the THF was removed under vacuum to leave a clear yellow oil. The oil was dissolved in dichloromethane (DCM) and the salt of the product extracted into hydrochloric acid solution (1 M). The acid layers were combined and then neutralised



SCHEME 1. Synthesis of potential inhibitors of CSCC (a = K_2CO_3/THF/Δ) [n = 2 & 3; Y = H, NH_2, NO_2; X = C (imidazole) or N (triazole)].

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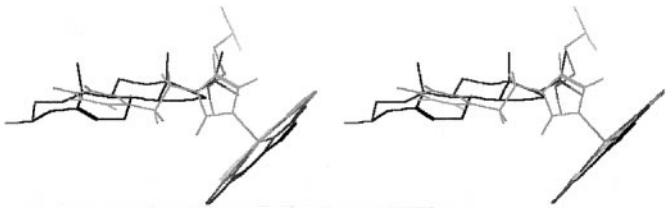

FIG. 2. The mode of binding of phenyl ethyl imidazole to the SHC of CSCC.

with sodium bicarbonate and the organic product extracted into DCM. The combined organic layer was washed with water and dried over magnesium sulfate. The DCM was removed under reduced pressure, to give **1** as a clear straw coloured oil (14.2 g, 36% yield). (Found C, 76.38; H, 7.18; N, 15.97. $C_{11}H_{12}N_2$ requires C, 76.71; H, 7.02; N, 16.22%).$\nu_{(max)}$ (Neat): 3100, 3050, 3020 (Ar C-H), 2920, 2850 (C-H), 1600 (Ar C═C) cm^{-1}. δ (300 MHz, $CDCl_3$) 7.1 (8 H, br. m, Ph, IM), 3.9 (2 H, t, J = 7 Hz, C$\underline{H}_2$-IM), 2.8 (2H, t, J = 7 Hz, C$\underline{H}_2$-Ph).

Biochemical evaluation of inhibitors. The bovine mitochondrial suspension was thawed under cold running water, and sonicated, on ice, using a probe sonicating disintegrator, at 25 microns, for 3 × 15 s, interspersed with 30 s cooling periods. The final incubation assay mixture (0.5 ml) consisted of incubation buffer (pH 7.4, 315 μl), cholesterol as substrate (12 μM), NADPH generating system (50 μl) and inhibitor (200 μM). The tubes were warmed to 37°C for 5 min and the assay was commenced by addition of sonicated enzyme (2 mg/ml) which had been preincubated for 2 min at 37°C. The reaction was arrested by the addition of aliquots (300 μl) in triplicate, to cold alkaline buffer-glycine, sodium hydroxide and mercuric chloride (1.7 ml, pH 9.5) and storage of the mixture on ice. The alkaline buffer mixture was then filtered through microcolumns of alumina. Filtration was generally complete within 24 h. Aliquots (1 ml) of the filtrate were dispersed in scintillation fluid (3 ml) and counted for 5 min each for ^{14}C.

Molecular modelling. The general approach to the construction of the substrate–heme complex has been previously described (2–5) and will therefore not be detailed here. In general, however, the structures of cholesterol and heme were constructed, minimised and optimised (using molecular mechanics followed by MOPAC using PM3 parameters) within the molecular modelling program CaChe. The proposed inhibitors were constructed, minimised and optimised within Alchemy III. Binding studies were also undertaken within Alchemy.

RESULTS AND DISCUSSION

Figures 2 and 3 show the design process, where the constructed inhibitors were bound to the SHC for CSCC and the imidazole N to heme Fe bond was rotated so as to determine the distances between the steroid C(3)-βOH group and the inhibitor hydrogen bonding group. As can be observed, the phenyl propyl based compounds would be expected (due to the smaller distances for any hydrogen bonding) to produce more potent inhibitors if, as we suggest, the mimicking of the steroid C(3)-βOH group is important in the inhibition process.

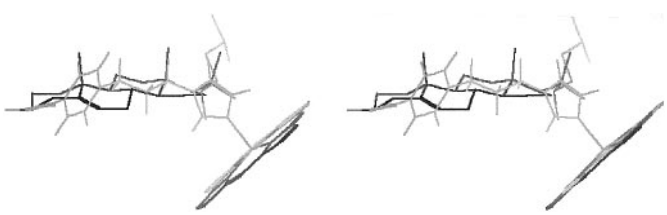

FIG. 3. Mode of binding of 4-nitrophenyl propyl imidazole to the SHC of CSCC to clearly show potential mimicking of the C(3)-βOH group.

TABLE 1

Inhibitory Activity for a Number of Compounds against CSCC

Compound	% inhibitory activity
n = 2; X = H	0
n = 2; X = NO_2	0
n = 2; X = NH_2	0
n = 3; X = H	25
n = 3; X = NO_2	37
n = 3; X = NH_2	28

Note. ([I] = 200 μM and the inhibitory data represents the mean of three values, i.e., n = 3).

Table 1 shows the inhibitory data obtained for the inhibition of CSCC by the synthesised compounds. The biochemical evaluation of the synthesised compounds, clearly show that the longer chain compounds (n = 3) possessed inhibitory activity, however, the phenyl ethyl compounds possessed *no* inhibitory activity. The results obtained therefore add further support to the initial model for the SHC derived model as a representation of the CSCC active site.

The compounds described above have also undergone biochemical evaluation against AR, and have been shown to possess highly potent inhibitory activity against this cytochrome P450 enzyme. In particular, the 4-nitrophenyl ethyl imidazole was found to be some 125 times more potent than aminoglutethimide, but possessed no inhibitory activity against CSCC.

In conclusion, the SHC approach allows the derivation of a simplified, yet relatively accurate, representation of the CSCC active site which can be used in the design of novel (and specific) inhibitors of alternative cytochrome P450 enzymes.

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