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Interaction of stanozolol with cytochrome P-450

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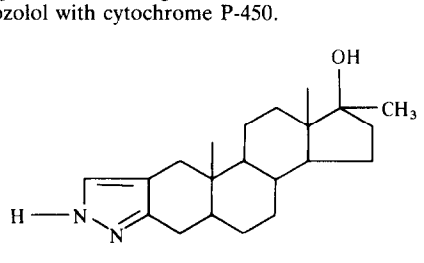
The biotransformation of drugs catalysed by cytochrome P-450 requires interaction of the drug with the enzyme as a substrate. This substrate-type interaction causes spectral changes of the oxidized form of cytochrome P-450 characterized by a trough at 420 nm and a peak at about 390 nm. The ligand type of interaction with cytochrome P-450, characterized by a trough at about 400 nm and a peak between 420 nm and 430 nm, has been found with lipophilic compounds possessing nitrogen or other hetero-atoms in the molecule which can coordinate the heme iron. Depending on binding affinity, compounds interacting as ligands to cytochrome P-450 can be strong inhibitors of the enzyme [1]. In our previous papers [2–5], we have shown that inhibition of the drug-metabolizing activity *in vivo* observed with H_2-receptor antagonist cimetidine can be attributed to its ligand binding properties caused by the structural features of the compound, i.e. by the presence of cyano and imidazole groups in the molecule [2, 4]. Studies of the interaction of drugs with cytochrome P-450 can provide information about the nature and mechanisms of the binding processes and give useful information when studying structural features of particular compounds.

Stanozolol, an anabolic steroid used for the treatment of aplastic anaemia [6], is also misused alone or in combinations by sportsmen, and the International Olympic Committee consider it a doping agent which is to be controlled in sport [7]. The very limited information on its *in vivo* and *in vitro* metabolism available as well as its interesting structural feature possessing, namely, the pyrazole ring as a potential ligand to cytochrome P-450 prompted us to investigate the nature of the interaction of Stanozolol with cytochrome P-450.



Stanozolol

Materials and methods

Preparation of liver microsomal fractions. The liver microsomal fractions from Sprague–Dawley rats (100–150 g) were prepared by differential centrifugation as described by Frommer *et al.* [8]. Phenobarbital was administered orally (1 mg/ml of drinking water) for one week.

Determinations of spectra. Difference spectra were recorded on a Shimadzu UV-300 spectrophotometer using 10 mm cells at temperature 295 K. The microsomal suspension contained 1.85 μM cytochrome P-450 and 0.98 mg protein/ml in 0.01 M Tris–HCl buffer, pH 7.5. Stanozolol dissolved in ethanol was added from 5 mM and 50 mM stock solutions. The corresponding volume of ethanol was added to the reference cell. The maximal concentration of ethanol in the cells was 0.12%. Final concentrations of the compound are given in the legend of the figure. Reduction of cytochrome P-450 was performed by addition of few grains of solid $Na_2S_2O_4$ to both cells.

The spectral dissociation constant was obtained by double reciprocal plot of the absorbance difference between the peak and trough in each difference spectrum. The intercept on the abscissa is equivalent to $-1/K_s$.

Determination of cytochrome P-450. Cytochrome P-450 was determined by the method of Omura and Sato [9].

Determination of protein. Protein concentrations were determined according to Gornall *et al.* [10].

Electron paramagnetic resonance (EPR). EPR spectra were measured at temperature 90 K with a Varian E-9 spectrometer connected to a digital computer (Data General Nova 820) which performed the integration and superposition of the spectra. The *g*-values were calculated using the magnetic field readings from the "Varian Fieldial". Diphenylpicrylhydrazyl was used as a *g*-marker.

Chemicals. Stanozolol was obtained as a gift from Sterling Winthrop Group Ltd, Guildford, Surrey, U.K., and was used without further purification.

Results and discussion

The optical difference spectra resulting from interaction of Stanozolol with liver microsomal fractions at different concentrations of the compound indicate the ligand type of binding to cytochrome P-450. The spectra obtained with oxidized cytochrome P-450 poses a peak at 429 nm, a trough at 394 nm, and an isosbestic point at 416 nm. After reducing the microsomes with dithionite, the peaks at 444 nm and 425 nm, and the trough at 403 nm were produced. These results show that Stanozolol, in addition to substrate and O_2, binds to both Fe(III) P-450 and Fe(II) P-450 and may act as an inhibitor to cytochrome P-450.

The K_s-value derived from the double reciprocal plot (Fig. 1), obtained by plotting the spectral change vs concentration of the compound, indicate the high affinity of ligand interaction of Stanozolol to cytochrome P-450 (apparent $K_s = 1.1$ μM).

EPR can detect changes in the ligand field of the iron in cytochrome P-450 and thus reveal a ligand interaction [11]. After addition of Stanozolol to microsomal suspensions, peaks at $g = 2.47$ and 1.89 appeared with concomitant decrease of the signal of the native cytochrome P-450 at $g = 1.92$ and $g = 2.40$ (Fig. 2, curves A to C). This change in EPR spectrum is compatible with the nitrogen atom as a coordinative ligand to the iron [11], and shows that only distinct native species participate in the binding process. At saturating concentration of Stanozolol 60–80% of cytochrome P-450 bond Stanozolol. The total integrated intensity of the signal before and after binding was not changed significantly (curve A = 6.82, B = 7.38, C = 6.76 int. units) within experimental error (±10%). This finding indicated no change in the spin equilibrium at 90 K and is different from the optical spectra obtained at 295 K where, due to the temperature dependence, the spin equilibrium of native cytochrome P-450 is shifted to higher spin.

Figure 3 shows that Stanozolol is a strong inhibitor of a substrate mono-oxygenation reaction *in vitro*, and this is in agreement with a ligand complex formation with cytochrome P-450. As a model reaction 7-ethoxyocoumarin *O*-dealkylation was investigated. With the concentration of 7-ethoxycoumarin at 10^{-3} M, an IC_{50}-value of about 4 μM was found. The inhibition of 7-ethoxycoumarin *O*-dealkylation with Stanozolol is in agreement with the finding [15] that the ligand type of binding leads to an inhibition which, in the case of blocking of oxygen binding through bond formation with ferrous iron, is the non-competitive type.

These results show that Stanozolol interacts with cytochrome P-450 by forming a high-affinity ligand complex. The resulting EPR and optical difference spectra are compatible with the nitrogen atom from the pyrazole ring as a ligand to cytochrome P-450. As a consequence of such a high-affinity ligand complex inhibition of substrate mono-oxygenation catalysed by cytochrome P-450 occurred.

EPR allowed the estimation that 60–80% of cytochrome P-450 interacted with Stanozolol. Hence Stanozolol interacted with major form(s) induced by phenobarbital in rat liver. Furthermore Stanozolol inhibited the deethylation of

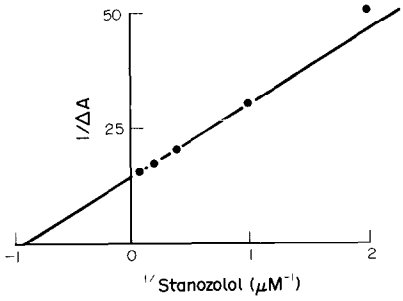


Fig. 1. Apparent spectral dissociation constant, K_s, of Stanozolol and microsomal cytochrome P-450. Double reciprocal plot of the absorbance difference between the peak at 425 nm and trough at 394 nm.

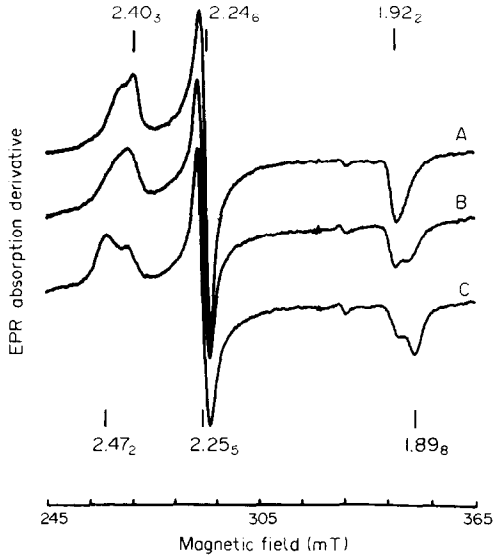


Fig. 2. Interaction of rat liver microsomes with Stanozolol as detected by EPR spectra. Traces A—native rat liver microsomes (151 μM cytochrome P-450 and 80 mg protein/ml). Traces B—after addition of Stanozolol in ethanol, final concentration 0.5 mM. Traces C—after addition of saturating amounts of the solid compound. EPR was measured at 9.18 GHz microwave frequency, 40 mW microwave power, 2.5 mT modulation amplitude, a gain of 2000 and temperature 90 K.

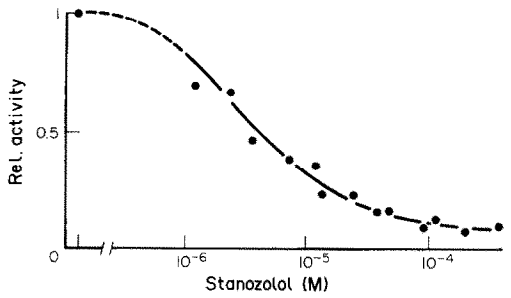


Fig. 3. Inhibition of *O*-dealkylation of 7-ethoxycoumarin in rat liver microsomes with Stanozolol. Stanozolol concentrations were in the range from 10^{-6} M to 5×10^{-4} M, 7-ethoxycoumarin was 10^{-3} M, cytochrome P-450 was 0.37 μM and protein 0.20 mg/ml. The enzymatic activity without Stanozolol was 2 nmol/min.

7-ethoxycoumarin to more than 90% at saturating concentration. This demonstrated interaction with the form(s) which catalyzed this monooxygenation in livers from rats pretreated with phenobarbital.

The data available shows differences in the tissue distribution when drug was administered subcutaneously to calves [13] in comparison to oral administration to rats [14]. In the first case only low level of the drug was found in liver (0.03–0.04 μg/g of tissue or 0.6% of dose in total organ). In rat liver, however, high tissue level was found (5.3 μg/g or 5% of dose). Although there are preliminary results showing that Stanozolol does not affect the microsomal activity in humans and does not influence the cytochrome P-450 content in rat liver *in vivo* [15], our data allow us to propose that Stanozolol may reduce the substrate interaction of other drugs, especially with such compounds which bind with low affinity to the enzyme. Further studies should be performed to determine the existence of species differences in the interactions with other forms of cytochrome P-450 and with the purified enzymes.

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Enzyme induction produced by *N*-(3,5-dichlorophenyl)succinimide (NDPS) in rats*

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N-(3,5-Dichlorophenyl)succinimide (NDPS) is an *N*-(haloaryl)succinimide which is active against many pathogenic plant fungi [1, 2]. NDPS-induced nephrotoxicity, however, limits the agricultural use of this fungicide. The mechanism of NDPS-induced nephrotoxicity is presently undetermined. Renal damage may be mediated by a toxic metabolite of NDPS generated by the hepatic mixed-function oxidase system. Previous work showed that deuterium labeling of the succinimide ring reduces NDPS nephrotoxicity [3]. These data suggest that oxidation of the carbon–carbon bridge is essential in the generation of nephrotoxic NDPS metabolites.

Many compounds present in the environment have the capacity to alter the activity of the mixed-function oxidase system. This paper focused on the capacity of NDPS to induce hepatic mixed-function oxidase enzymes. The following studies also determined if multiple exposures to NDPS may induce microsomal enzyme activity and modify NDPS nephrotoxicity.

Materials and methods

Male Fischer 344 rats (240–360 g) were maintained on a 12-hr light cycle at 23–25°. Animals were individually housed in metabolism cages to monitor daily urine output and food and water consumption [4]. Urine was semiquantitatively analyzed daily for protein, glucose, ketones and blood.

Rats (four/group) received a single intraperitoneal (i.p.)

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