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Antithyroid Action of Ketoconazole: In-vitro Studies and Rat In-vivo Studies

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Abstract—Inspection of the chemical structure of ketoconazole indicates that it may have antithyroid activity. The antithyroid action of this drug was demonstrated in-vitro and in-vivo. In-vitro, it was found to form a complex with iodine (formation constant K_c 141 L mol^{-1}), and to inhibit lactoperoxidase (IC50 2×10^{-4} M). Its effects in-vivo in the rat were assessed by assay of circulating-thyroxine, and from the histological appearance of the thyroid gland. Thyroid gland weight was increased in rats treated with ketoconazole.

We have previously reported on the secondary antithyroid action of numerous drugs of different chemical structures from various therapeutic categories (Raby et al 1990; Lagorce et al 1992). The antithyroid action of ketoconazole was demonstrated in-vitro from its complex formation with iodine and from its activity on thyroid peroxidase. In-vivo, its action was demonstrated by assay of L-thyroxine (T_4) and from the histological appearance of the thyroid glands of rats treated chronically with the drug. Ketoconazole is a relatively new drug that is now widely used in the treatment of fungal infections. Its chemical structure comprising several heterocyclic rings and electron donor groups, indicated that it would possess antithyroid activity (Fig. 1). Kitching (1986) has reported several cases of hypothyroidism after treatment with ketoconazole, although De Pedrini et al (1988) did not detect any antithyroid activity after administration of ketoconazole to five hypothyroid patients and ten healthy subjects.

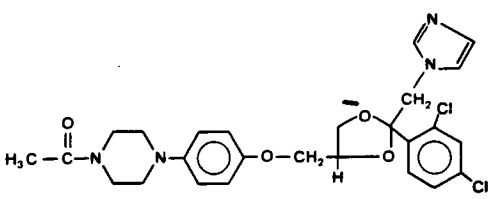

FIG. 1. Chemical structure of ketoconazole.

Materials and Methods

Complexation with iodine

Iodine was bisublimed (Merck Suprapur 4763, Nogent sur Marne, France), and chloroform was spectroscopic grade (Merck). The absorption spectra were recorded on a double beam UV-vis spectrophotometer (Kontron 930, Saint-Quentin, France). Complexes of electron donors n (ketoconazole, Sigma, La Verpillère, France) with the electron acceptor σ (iodine) were studied.

Mulliken (1952) proposed the theory of intermolecular charge transfer to account for the forces within molecular complexes. Mixture of a solution of ketoconazole with a solution of iodine leads to alterations in their individual spectra. The solution of iodine was prepared just before use by accurate weighing, and the various solutions of ketoconazole were prepared by dilution of a stock solution made up in $CHCl_3$. The formation constant K_c of the ketoconazole-iodine complex was calculated using the method described by Lang (1968) from spectra of various solutions of the complex recorded in the visible region. In the UV region, we demonstrated the existence of a charge transfer band which was characteristic of the particular complex with iodine.

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Enzyme kinetic study

Lactoperoxidase from bovine milk (E.C. 1.11.1.7, 80 units (mg solid)$^{-1}$) and 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) were obtained from Sigma (La Verpillère, France), and spectroscopic grade hydrogen peroxide (H_2O_2) obtained from Fluka (Mulhouse, France). The buffer solution (66·7 mM) was prepared from KH_2PO_4 and Na_2HPO_4 (Prolabo Normapur, Paris, France). All compounds and solutions were kept at 4°C. A small amount of dimethylsulphoxide (Sigma) was used to solubilize the ketoconazole in the buffer solution.

Enzyme kinetics were determined from the absorption spectra recorded in a Kontron UV-vis 860 spectrophotometer. Deme et al (1985) have shown that lactoperoxidase has the same activity as thyroid peroxidase on oxidation of iodide, and that it can be employed as a model of thyroid peroxidase. Similar behaviour of lactoperoxidase to that of thyroid peroxidase on the L-mono-iodotyrosine (MIT)-L-di-iodotyrosine (DIT) coupling reaction, giving rise to thyroid hormones L-tri-iodothyronine (T_3) and T_4, has also been reported (Taurog et al 1974; Edelhoch et al 1979). Our results on lactoperoxidase can thus be extrapolated to thyroid peroxidase.

The activity of peroxidase (6·67 μg mL^{-1}) was determined at pH 7, 20°C by measuring the initial rate of reaction of oxidation of ABTS (1·94 mM) by H_2O_2 (1·15 mM). This reaction is catalysed by peroxidase. The effect of ketoconazole on this enzyme was assessed by adding it during the reaction, and the kinetics of the oxidation reaction were monitored by spectrophotometry, as described previously. Absorption was measured at 411 nm, the characteristic absorption band of oxidized ABTS. The initial rate of the reaction was determined from the slope of the tangent at time

zero, of the plot of optical density against time. The initial rates of reaction for different concentrations of ketoconazole enabled calculation of the percentage inhibition of peroxidase:

$$(V_{ox} - V_o) \times 100/V_o$$

where V_{ox} is the initial rate in the presence of ketoconazole, and V_o the initial rate of the control reaction.

The concentration leading to 50% reduction in enzyme activity (IC50) was determined.

In-vivo study

Male Wistar rats obtained from Janvier (Saint-Berthevin, France) were housed five per cage. Before treatment with ketoconazole, they were given a special diet for three weeks consisting of maintenance chow (AO-4, UAR Essonne, France), (10·75 μg iodine day^{-1}/rat), and drinking water supplemented with potassium iodide (7·5 mg L^{-1}). They were then given orally 50 mg ketoconazole (kg body weight)$^{-1}$ then given orally ketoconazole (50 mg kg^{-1}) day^{-1} for a period of three weeks. A control group received a placebo solution, and a reference group received an antithyroid agent (methimazole) at a dose of 50 mg kg^{-1} day^{-1} for three weeks.

At the end of the treatment period, the rats were killed and weighed. The body weights were expressed as a percentage of their initial weight, and compared with those of the controls. Thyroid glands were removed and weighed, and compared with the weights of glands from the controls. Cross-sections of thyroid gland were cut and examined histologically after suitable fixation and staining. The activity of the gland was indicated by the appearance of the cells, and by the presence or absence of colloids. Hyperfunction was scored from + to + + + + as a function of the ratio of cylindrical to cubical cells.

Blood samples were also taken for assay of T_4 by fluorescence polarization (Abbott, Rungis, France). The concentration of unsaturated thyroxine binding proteins (T_3 test) was also determined by the same method. The free thyroxine index (ITL = T_4/T_3 test) was calculated. Hypothyroidism is indicated by a fall in this ratio.

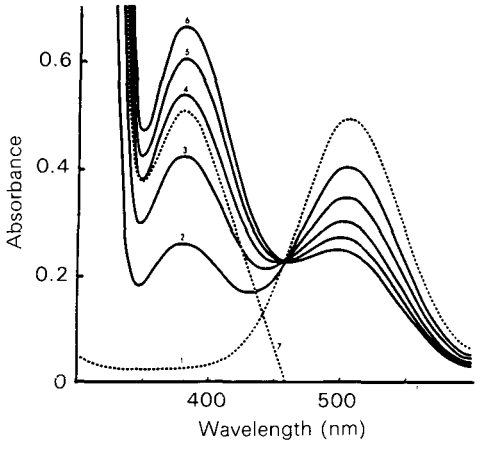

FIG. 2. Visible absorption spectra of the ketoconazole-iodine complex in $CHCl_3$ at 20°C. The concentration of iodine is $5{\cdot}61 \times 10^{-4}$ M. Concentrations (M) of ketoconazole are: 1 0, 2 $1{\cdot}92 \times 10^{-3}$, 3 $3{\cdot}85 \times 10^{-3}$, 4 $5{\cdot}77 \times 10^{-3}$, 5 $7{\cdot}70 \times 10^{-3}$, 6 $9{\cdot}62 \times 10^{-3}$, 7 visible absorption band of complex, calculated for solution 4.

Results

Iodine complex formation

Mixtures of ketoconazole solutions ($1{\cdot}92 \times 10^{-3}$–$9{\cdot}62 \times 10^{-3}$ M) with a solution of iodine ($5{\cdot}61 \times 10^{-4}$ M in $CHCl_3$) led to a shift in the visible band of iodine (507 nm). This new band attributed to halogen in the complex was shifted to a shorter wavelength (hypsochromic shift) with a peak at 382 nm (Fig.

Table 1. Formation constants (K_c) and molar extinction coefficients (ε_c) for ketoconazole-iodine complex studied in chloroform at 20°C.

λ (nm)	ε_c	K_c ($L\ mol^{-1}$)
380	1912	152·54
385	1932	149·36
390	1913	146·12
395	1859	141·90
400	1769	138·15
405	1662	133·58
410	1541	128·23

λ, wavelength.

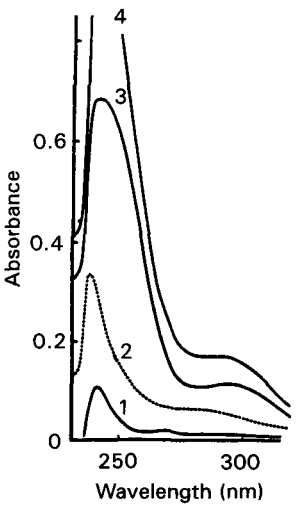

FIG. 3. UV absorption spectra of ketoconazole-iodine complex in chloroform at 20°C. Concentrations (M) are: 1 iodine $5{\cdot}61 \times 10^{-4}$, 2 calculated absorption band of complex, 3 iodine $5{\cdot}61 \times 10^{-4}$ and ketoconazole $4{\cdot}81 \times 10^{-5}$, 4 ketoconazole $4{\cdot}8 \times 10^{-5}$.

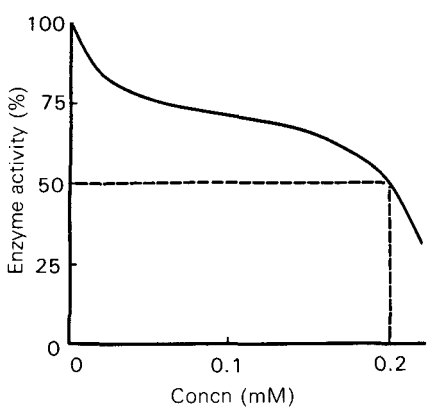

FIG. 4. Inhibiting activity of ketoconazole on lactoperoxidase. Concentration for 50% inhibition: IC50 2×10^{-4} M. At concentrations greater than $2{\cdot}2 \times 10^{-4}$ M ketoconazole, the enzymatic reaction is not possible because of the appearance of a precipitate.

Table 2. Summary of biological (T_4, ITL) and histological (thyroid epithelium, antithyroid activity) studies.

	T_4 (μg dL^{-1})	ITL	Epithelium	Antithyroid activity
Control	4·30±0·42	14·40±1·13	Cubical	−
Ketoconazole	3·55±0·16**	11·83±1·28	Cylindrical	++
Methimazole	0·56±0·04*	1·24±0·04	Cylindrical	++++

Control = untreated animals. ITL = free thyroxine. T_4 = total L-thyroxine. Rating of activity (ratio of cylindrical to cubical cells): − = normal activity (< 5% of cylindrical cells), ++ = clear activity (> 50 to 75% of cylindrical cells), ++++ = extra-strong activity (90% of cylindrical cells). * $P < 0.001$; ** $P < 0.01$, Student's t-test.

Table 3. Weight gain and weight of thyroid gland of rats treated with ketoconazole and methimazole for three weeks.

Treatment	Weight gain (g/100 g)	Thyroid gland (mg/100 g body weight)
Control	99·9±8·0	3·98±0·35
Ketoconazole	88·7±11·3	4·56±0·25
Methimazole	131·1±6·0*	27·72±3·68**

* $P < 0.01$, ** $P < 0.001$ compared with control values.

2). The curves crossed at a single isosbestic point at 466 nm. The values of the formation constant K_c of the ketoconazole-iodine complex were determined, and the values of the molar extinction coefficients were calculated for seven neighbouring wavelengths of the halogenated complex (Table 1). The value of K_c (141·41 L mol^{-1}) indicated an interaction between ketoconazole and iodine.

A solution of $4{\cdot}81 \times 10^{-5}$ M ketoconazole in $CHCl_3$ was mixed with the solution of iodine. The spectrum of the ketoconazole-iodine complex exhibited a charge transfer band at 239 nm (Fig. 3).

The presence of a single complex of 1:1 stoichiometry between ketoconazole and iodine is demonstrated by the existence of the formation constant K_c, by a single isosbestic point and by a matrix verification procedure using the method described by Liptay (1961).

Enzyme kinetics

The concentrations of ketoconazole in the reaction medium ranged from 10^{-5} to 5×10^{-3} M. The initial rates of reaction were obtained from the plots of optical density against time. There was a decrease in initial rate with rise in concentration of ketoconazole, indicating an inhibition of lactoperoxidase. The IC50 was determined from a plot of the activity of lactoperoxidase against ketoconazole concentration (Fig. 4). A value of 2×10^{-4} M was obtained.

In-vivo results

The results of the in-vivo study (Table 2) indicated that ketoconazole has antithyroid activity, albeit less than that of methimazole. With ketoconazole, T_4 35·5 μg L^{-1} and ITL (11·8) were lowered compared with controls (43·0 μg L^{-1} and 14·4, respectively). The hypothyroid state of the rats treated with ketoconazole was indicated by hyperfunction of the thyroid gland as indicated by the presence of cylindrical thyrocytes. In control animals, the epithelium was mostly cubical cells (Table 2). The weight gains were expressed with respect to the initial body weights, and the thyroid gland weights were expressed with respect to the final body weights of the animals (Table 3). The results were compared using Student's t-test.

Discussion

The results show that ketoconazole has antithyroid activity. Its formation constant K_c for complex with iodine (141 L mol^{-1}) was above 100 L mol^{-1}, a value regarded as a threshold for antithyroid activity (Buxeraud et al 1985). For comparison, K_c for the antithyroid agent methimazole, is 23 194 L mol^{-1}. We also determined the IC50 with respect to lactoperoxidase. The value of 2×10^{-4} M can be compared with that of $2{\cdot}1 \times 10^{-6}$ M for methimazole.

The action of ketoconazole on the thyroid gland can be accounted for by an action on iodine and by inhibition of peroxidase (Fig. 5).

The consequences of this action were evaluated in-vivo. After treatment of rats with ketoconazole, T_4 and ITL were significantly lower than in controls, which is indicative of hypothyroidism. The animals react by attempting to increase production of thyroid hormones, which is generally shown by an increase in both thyroid gland weight and body weight of the treated rats. No increase in body weight was observed in our animals. The 6% fall in body weight with respect to controls may have been due to a loss of appetite induced by the drug. With methimazole, the most powerful antithyroid drug known, only a 16% gain in weight is commonly observed.

The small in-vivo effects of ketoconazole are perhaps not due to the direct effects on thyroid peroxidase, presumed in

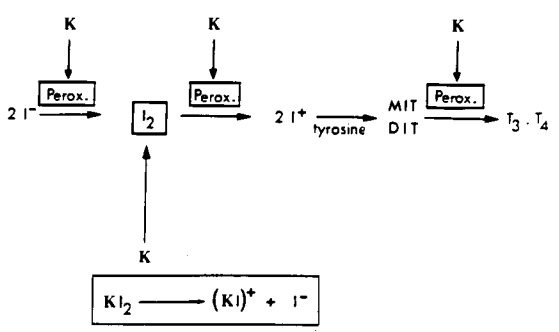

FIG. 5. Ketoconazole action on thyroid hormone biosynthesis. (K = ketoconazole, MIT = L-mono-iodotyrosine, DIT = L-di-iodotyrosine.)

our experiments, but to the influence of the drug on hepatic metabolism of thyroxine. Ketoconazole could inhibit 5′-des-iodase which is responsible for T_3 and T_4 des-iodation, so keeping T_4 levels higher than expected.

Our results indicate that hypothyroidism might be encountered in patients treated with this antifungal agent or exposed to residues in food from animals treated with the drug.

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