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The interaction of temelastine with cytochrome P_{450} mixed-function oxidase enzymes *in vivo* and *in vitro*

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Temelastine, 2-[4-(5-bromo-3-methylpyrid-2-yl)butylamino]-5-[(6-methylpyrid-3-yl)-methyl]pyrimidin-4(1H)-one, is a drug which has been shown to have selective histamine H_1-receptor antagonist activity in a number of *in vitro* and *in vivo* tests in animals and also in man [1]. It has been shown not to penetrate the blood–brain barrier of the rat to any appreciable extent [2]. It is currently undergoing development at SK&F Research Ltd, The Frythe, Welwyn, as a potential non-sedating antihistamine compound for use in the treatment of allergic rhinitis, allergic skin disorders etc., in man. The presence of substituted pyridine rings in temelastine suggests the potential of the molecule to bind to the mixed-function oxidase enzymes referred to as cytochrome P_{450}. The binding constants of a number of substituted pyridines to this hemoprotein have been compared and ranged between 1 and 2250 μM [3]. The most potent of these compounds, metyrapone, induces a type II binding spectrum with liver microsomes and is a stronger ligand for phenobarbital-induced forms of cytochrome P_{450} than for those forms of cytochrome P_{450} induced by polyaromatic hydrocarbons [4]. The studies described in this investigation were designed to evaluate the interaction of temelastine with cytochrome P_{450} *in vitro* and *in vivo* in the rat.

Materials and methods

Temelastine (Batch 24) was supplied by the Department of Pharmaceutical Development, SK&F Research Ltd. [*N*-methyl-^{14}C]-Antipyrine was obtained from Amersham International plc (30–50 mCi/mmol).

NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were supplied by Sigma (Poole, Dorset, U.K.), ethylmorphine hydrochloride by May and Baker (Dagenham, Essex, U.K.), and 7-ethoxycoumarin and 7-hydroxycoumarin by Aldrich Chemical Company (Dorset, U.K.).

Animals. Male and female albino rats (SK&F Wistar Strain, 7 weeks) were supplied by the Department of Laboratory Animal Science, SK&F Research Ltd. Animals had unrestricted access to food and water for the duration of the study and were housed in groups of 3 or 5 on grade 6 greenwood granules.

Study design. The *in vitro* interaction of temelastine with cytochrome P_{450}-mediated reactions was investigated with 9000 *g* supernatant prepared separately from 4 male rats.

The induction of cytochrome P_{450}-mediated activity by temelastine was investigated by administering the drug at two target dose levels (300 mg.kg^{-1} and 1000 mg.kg^{-1}, p.o., 6 rats in each group). At 1000 mg.kg^{-1} the dosing was continued on a daily basis for 3 days. At 300 mg.kg^{-1} the dosing was continued on a daily basis for 30 days. Twenty-four hours after the last dose animals were sacrificed and the livers removed and cytochrome P_{450} levels, ethylmorphine *N*-demethylation and ethoxycoumarin *O*-deethylation activity measured in 9000 *g* supernatants. The drug metabolism activities were compared against values from the appropriate control animals treated with vehicle.

Rats were dosed once a day with temelastine for 14 days (100 mg.kg^{-1}, p.o. daily) and antipyrine half-life ($t_{1/2}$) was estimated 3 days prior to dosing, on day 1 and on day 14 of dosing, and three days subsequent to dosing. Antipyrine and temelastine were administered simultaneously to rats which had been starved of food overnight.

Preparation of dose solution of SK&F 93944. Solutions for treatment were prepared daily or weekly by dissolving the compound in 5 M HCl (1.355 ml.g^{-1}).

Measurement of cytochrome P_{450} enzyme activities. Untreated and dosed rats were killed by cervical dislocation. The livers were excised and the liver removed and homogenised. The suspension was centrifuged (20 min, 9000 *g*, 4°, Sorvall RC5-B centrifuge, SA-6000 rotor). The 9000 *g* supernatant was diluted (1:1 v/v) with KCl (0.154 M) and used directly. For the determination of cytochrome P_{450} 9000 *g* supernatant (300 μl) was added to a solubilizing buffer (2.7 ml) described by Warner *et al.* [5] except that Renex 690 (Atlas Chemicals, Surrey, U.K.) was substituted for Emulgen 911. Cytochrome P_{450} content was measured by the technique of McLean and Day [6] utilizing a Hewlett-Packard 8450A diode array spectrophotometer.

Two cytochrome P_{450} mediated activities were measured in rat liver 9000 *g* supernatant. Ethylmorphine *N*-demethylation (EM) was measured by the liberation of formaldehyde from ethylmorphine [7, 8] and 7-ethoxycoumarin *O*-deethylation (ECOD) was measured by the formation of 7-hydroxycoumarin from 7-ethoxycoumarin [9].

The Michaelis–Menten kinetic parameters for each of these enzyme activities had been determined previously in 9000 *g* from male rat livers. The apparent K_m for EM was 363 μM whereas the value for ECOD was 186 μM. Each enzyme activity was measured at K_m substrate concentration and at $5 \times K_m$ substrate concentration (83% saturation). No apparent K_m determination had been made in livers from female rats and therefore substrate concentrations appropriate for male rat 9000 *g* were used with the livers from female rats.

Measurement of antipyrine half-life ($t_{1/2}$). Antipyrine $t_{1/2}$ determinations were performed essentially as described by Bakke *et al.* [10]. Male rats (200 g) were dosed with antipyrine (15 mg.kg^{-1} antipyrine, 7.5 μCi.kg^{-1} [*N*-methyl-^{14}C-] antipyrine, i.p.). Blood samples (>200 μl) were taken from a tail vein every 40 min for 5 hr into Sarstedt capillary tubes (heparin coated). The regression of log concentration upon time was calculated by least squares and the $t_{1/2}$ calculated from the slope.

Treatment of data. The initial rate data for ethylmorphine *N*-demethylation and ethoxycoumarin *O*-deethylation were analysed by various computer curve fitting routines. The Michaelis–Menten kinetic parameters K_m^{app}, V_{max}^{app} were determined by a rectangular hyperbola iterative fit programme from Wilkinson [11]. K_i (inhibitor dissociation constant) was determined by plotting inhibitor concentration (i) against K_m/V_{max}. The following equation for inhibition was assumed to apply:

$$V = \frac{V_{max}}{1 + \frac{K_m}{S}\left(1 + \frac{i}{K_i}\right) + \frac{i}{\alpha K_i}}$$

where $\alpha = K_m'/K_m = K_i'/K_i$ and i = inhibitor concentration.

Results

(i) *Inhibition studies* in vitro. The effect of different concentrations of temelastine on EM activity in 9000 *g* supernatant from a single rat liver is shown in Fig. 1a. In four separate rats temelastine caused a concentration-dependent inhibition of *N*-demethylation resulting in a marked increase in the apparent K_m for ethylmorphine and a marked decrease in the apparent V_{max}. The apparent K_i for the inhibition of EM activity was calculated for each rat by the technique of secondary plots. The average K_i was calculated to be 5.1 μM (range 2.1–10 μM) with an average α value of 592 indicating a competitive mechanism of enzyme inhibition.

The effect of temelastine on ECOD in 9000 *g* supernatant from a single rat liver is shown in Fig. 1b. In all four rats temelastine caused a concentration dependent inhibition of *O*-deethylation. Rat 1 demonstrated no effect on the apparent K_m in the presence of temelastine at either 50 μM or 100 μM whereas the other rats all demonstrated increases in the apparent K_m ethoxycoumarin in the presence of temelastine.

In contrast, the apparent V_{max} for ECOD activity only demonstrated a concentration-dependent decrease in rat 1. This may reflect real differences between animals or may simply reflect the complex kinetics arising from the contribution of multiple cytochrome P_{450} species to the metabolism of this substrate which give rise to non-linear Lineweaver–Burke plots [9]. The average K_i value for ECOD was estimated to be 119 μM (range 57–218 μM) with an α value of 11.2 which would indicate a predominantly competitive inhibition.

(ii) *Temelastine administration* in vivo: *effects on cytochrome P_{450} mixed-function oxidase activities measured* in vitro. The effects of multiple oral dosing of temelastine to male and female rats (300 mg.kg^{-1} and 1000 mg.kg^{-1}) on various parameters of hepatic drug metabolism were investigated. The effects on liver weight, cytochrome P_{450} levels, EM activity and ECOD activity were determined 24 hr after the last dose of drug. Total liver weight was increased by 35% ($P < 0.05$) in the case of female rats dosed with temelastine for 30 days at 300 mg.kg^{-1}. In contrast, cytochrome P450 tended to increase in all dosed groups but only achieved statistical significance in female rats treated for 3 days at 1000 mg^{-1}.kg^{-1} ($P < 0.05$, 31% increase). EM activity was decreased in male rats treated with temelastine after 3 days and after 30 days dosing whereas EM activity was increased in female rats particularly when treated with temelastine for 3 days at 1000 mg^{-1}.kg^{-1} (Fig. 2a).

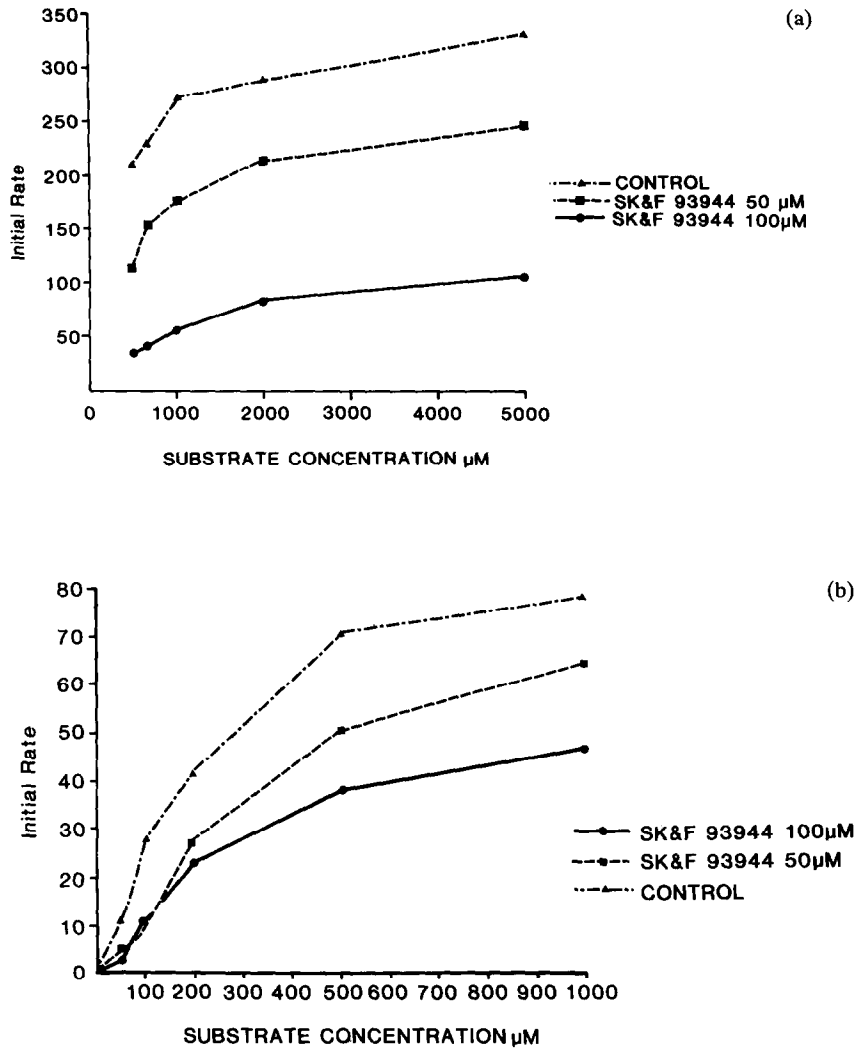

Fig. 1. The effect of temelastine on EM (a) and ECOD (b) activity. Each data point is the mean of quadruplicate determinations. The K_i for EM was calculated to be 5.1 ± 3.4 μM with an α value of 592 ± 205. The K_m for ECOD was calculated to be 119 ± 70 μM with an α value of approximately 11 (mean ± 1 SD).

The effect of temelastine on ECOD activity also demonstrated small increases in enzyme activity at 3 days (Fig. 2b). Thus, at these high dosage levels temelastine demonstrates only modest indications of enzyme induction and then only in female rats.

(iii) *Antipyrine* $t_{1/2}$. Temelastine was administered p.o. simultaneously with the dosing of antipyrine. Analysis of variance suggests that changes in antipyrine $t_{1/2}$ occurred during the 14 day dosing period which were significant statistically ($P < 0.05$, Table 1). Simultaneous dosing (100 $mg.kg^{-1}$, p.o.) caused a 24% increase in antipyrine $t_{1/2}$ on day 1 which returned to control values by day 14. However, by day 17 the antipyrine $t_{1/2}$ had reduced to values below that observed at day −3 suggesting the possibility of some enzyme induction by temelastine, although this change was not statistically significant.

The contrast between the potent inhibition of EM activity observed *in vitro* and the limited inhibition seen *in vivo* may have a number of explanations. The substantial difference in the determined K_i for EM and ECOD activities may imply that the cytochrome P_{450} enzymes responsible for metabolizing antipyrine have a high K_i for temelastine.

Alternatively, it has been shown that temelastine exhibits marked protein binding to plasma proteins which may reduce the concentration of free drug to levels where the inhibition of cytochrome P_{450} is modest. Administration of ^{14}C-temelastine by gavage to male Wistar rats at a target dose of 100 $mg.kg^{-1}$ resulted in maximum blood concentrations of total radioactivity of 17.9 ± 3.8 (N = 3) μM equivalents, and therefore plasma concentrations of parent drug must be below this value. Determination of the degree of protein binding measured by equilibrium dialysis showed that the proportion of ^{14}C-temelastine bound to rat serum proteins lay between 99.35% and 98.46% over the equilibrium concentration range 0.042–100 μM. Thus, the calculated maximum free drug concentration would be less than 0.1 μM and therefore protein binding would provide an adequate explanation to account for the limited effects

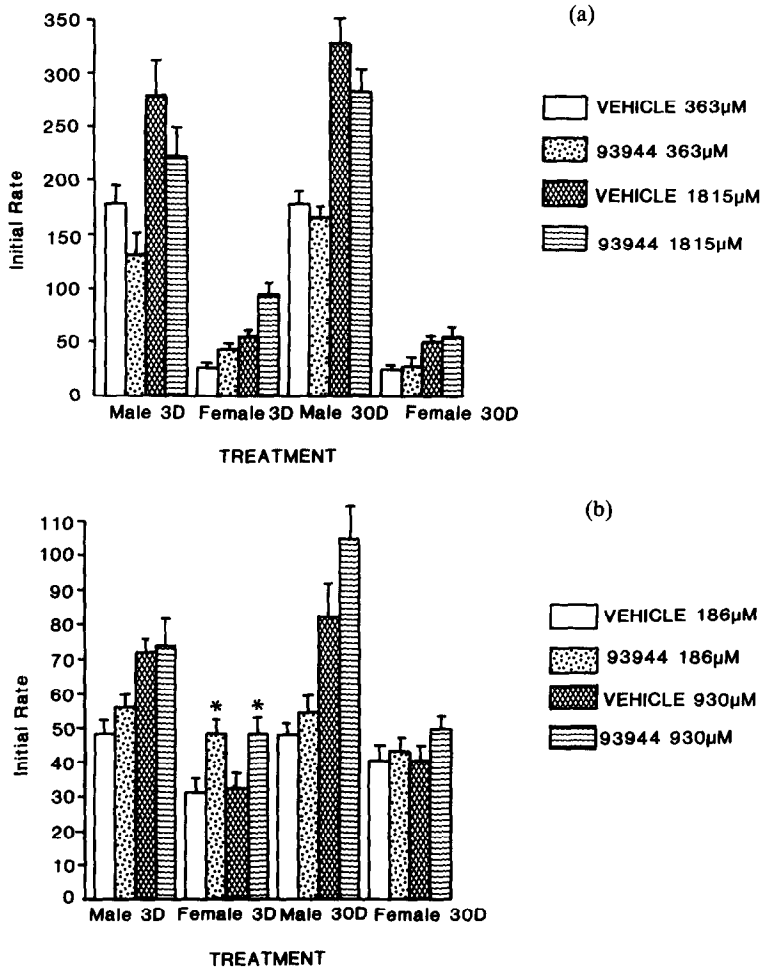

Fig. 2. The effect of dosing temelastine to male and female rats on liver EM (a) and ECOD (b) activity. Rats were given daily doses of 1000 mg.kg^{-1} temelastine for 3 days or 300 mg.kg^{-1} temelastine for 30 days as described in Materials and methods. * Denotes $P < 0.05$. Each bar represents the mean and standard deviation from six rats.

Table 1. The effect of SK&F 93944 (100 mg.kg^{-1}, p.o.) on antipyrine half-life during a 14-day dosing period

	$t_{1/2}$ (min)			
Rat	Day −3	Day 1	Day 14	Day 17
1	54.9	83.9	59.8	57.2
2	90.7	102.8	67.5	65.2
3	77.3	89.2	66.7	51.8
4	71.4	94.4	72.3	58.1
5	63.6	78.8	63.2	55.7
6	68.6	78.8	79.7	69.7
Mean±	71.1±	*88.0±	68.2±	59.7±
SD	12.2	9.5	7.0	6.7

* $P < 0.05$ compared to control.
One-way analysis of variance $P < 0.01$.
Control −3 days prior to the initiation of dosing.

of temelastine at 100 mg.kg^{-1} on antipyrine metabolism. However, it should be emphasised that for a true comparison the free drug concentration in the *in vitro* inhibition studies should also be monitored to provide a K_i determination for unbound temelastine.

In conclusion temelastine is a relatively potent inhibitor of cytochrome P_{450}-mediated reactions *in vitro* but appears to exhibit only weak interactions *in vivo*.

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Prevention by antioxidants of oxidative damage to rabbit kidneys subjected to cold ischaemia

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Cold storage of kidneys while awaiting transplantation into a suitable recipient involves deprivation of the blood supply to the organ and concomitant starvation of oxygen. This period of ischaemia results in gradual deterioration of the organ and eventually to irreversible loss of renal function. That the damage becomes more manifest when the organ is transplanted and reperfused with oxygenated blood has led to the hypothesis that oxygen-derived free radicals (reactive species containing an unpaired electron) may be involved in the pathogenesis of this condition [1].

One consequence of an unchecked burst of free radical activity is the peroxidation of membrane-bound polyunsaturated fatty acids which can result in the loss of membrane integrity and dysfunction of intracellular organelles. We have previously shown that storage of rabbit kidneys at 0° results in significant increases in the rate of lipid peroxidation in both the medulla and cortex [2, 3] and that further substantial increases are observed when the organs are replanted and reperfused *in vivo* [2]. The levels of markers of lipid peroxidation were inversely correlated to the known physiological function of the kidney transplants [2] and could be reduced by administration of free radical scavengers and the chelation by desferrioxamine of iron [4] which catalyses the conversion of the superoxide anion ($O_2^{\cdot-}$) to the highly reactive hydroxyl radical ($OH^{\cdot}$).

Other studies have suggested that altered cell calcium homeostasis leading to increased cytosolic free calcium levels during storage of kidneys plays a role in mediating oxidative membrane damage [5]. This may be the result of stimulation of calcium-activated phospholipases which remove fatty acids, in particular arachidonic acid, from the membrane. Subsequent enzymic peroxidation of free arachidonic acid by cyclooxygenase results in the formation of prostaglandins including thromboxane (a vasoconstrictor) and prostacyclin (a vasodilator). Ischaemia has been reported to upset the delicate balance between these two compounds (e.g. [6]) which results in vasoconstriction and may lead to blockage of the vascular bed on reperfusion. In addition, oxidation of arachidonic acid by lipoxygenase leads to the production of leukotrienes some of which cause vasoconstriction, are chemotactic and enhance capillary permeability and have been implicated in ischaemic/reperfusion damage [7].

In view of the possibility that several mechanisms may be working in consort to cause membrane damage, cell necrosis and vascular injury in stored organs, we have investigated the effect of a number of agents which inhibit both non-specific and enzyme-catalysed peroxidation of polyunsaturated fatty acids, on the production of markers of lipid peroxidation during *in vitro* incubation of homogenates of stored kidneys.

Materials and methods

Rabbit kidneys were harvested, flushed with and stored in isotonic saline solution for 24 hr at 0° as previously described [3]. After division into cortex and medulla, homogenates (10% w/v) were prepared in phosphate-buffered saline (40 mM KH_2PO_4: K_2HPO_4; pH 7.4) and incubated, with shaking, at 37° in the presence of various test compounds. Aliquots were taken at 0 and 60 min incubation and the rate of lipid peroxidation quantitated by measuring the formation of Schiff bases and thiobarbituric acid (TBA)-reactive material, as previously described [3]. The amount of TBA-reactive material was quantitated using malonaldehyde tetraethylacetal and the results were corrected for the protein content of the homogenates determined by the method of Lowry *et al.* [8].

The compounds tested were silymarin (Legalon®, a gift from Dr. Madaus & Co., Koln, F.R.G.) a flavonoid isolated from the milk thistle which is a mixture of three isomers including silibinin (Fig. 1); chlorpromazine, a psychotrophic drug; propyl gallate (Fig. 1) a water soluble antioxidant; quercetin (Fig. 1) a flavonoid; and the antioxidant nordihydroguaiaretic acid (NDGA) (Fig. 1).

Statistical analysis was performed using a paired *t*-test modified according to the method of Bonferroni for simultaneous multiple comparisons as described by Wallenstein *et al.* [9].

Results and discussion

We have previously shown that homogenates of kidneys undergo lipid peroxidation when incubated at 37° in an aerobic environment and that the rate of this process is very significantly greater when the kidney has undergone a period of cold storage [2, 3]. Addition of compounds with antioxidant properties to the incubation medium allows the investigation of the mechanisms of lipid peroxidation in homogenates of stored kidneys and provides a rapid indication of whether a compound may have possible therapeutic value in preventing free radical induced damage to ischaemic kidneys *in vivo*. Two markers of lipid peroxidation were measured: TBA-reactive material includes