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THE EFFECTS OF A NOVEL AND SELECTIVE INHIBITOR OF TRYPTOPHAN 2,3-DIOXYGENASE ON TRYPTOPHAN AND SEROTONIN METABOLISM IN THE RAT

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Abstract—The effects of a novel inhibitor 680C91 ((*E*)-6-fluoro-3-[2-(3-pyridyl)vinyl]-1*H*-indole) of the key enzyme of tryptophan catabolism tryptophan 2,3-dioxygenase (TDO) (EC 1.13.11.11), were examined on tryptophan catabolism *in vitro* and *in vivo* and on brain levels of tryptophan, serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA). 680C91 was a potent ($K_i = 51$ nM) and selective TDO inhibitor with no inhibitory activity against indoleamine 2,3-dioxygenase (EC 1.13.11.17), monoamine oxidase A and B, 5-HT uptake and $5\text{-HT}_{1A,1D,2A}$ and $_{2C}$ receptors at a concentration of 10 μM. 680C91 had no effect on the binding of tryptophan to serum albumin in plasma and inhibited TDO competitively with respect to its substrate tryptophan. 680C91 inhibited the catabolism of tryptophan by rat liver cells and rat liver perfused *in situ*. The catabolism of L-[ring-2-^{14}C]-tryptophan and a load dose of tryptophan (100 mg/kg) *in vivo* were inhibited by prior administration of 680C91. Administration of 680C91 alone produced marked increases in brain tryptophan, 5-HT and 5-HIAA. A load dose of tryptophan (100 mg/kg), producing increases in brain tryptophan 4-fold greater than that seen with 680C91, did not increase brain 5-HT and 5-HIAA to levels greater than those seen with 680C91 and produced a shorter-lasting increase in these parameters. These data therefore demonstrate the importance of TDO as a regulator of whole-body tryptophan catabolism and brain levels of tryptophan and 5-HT and suggest that a greater antidepressant efficacy might be achieved with inhibitors of TDO than tryptophan administration alone.

Key words: tryptophan 2,3-dioxygenase; tryptophan; serotonin; antidepressant

Over the last 30 years it has become clear that the effective treatment of depression is associated with an increased function of the serotonergic and noradrenergic pathways in the brain. The introduction of the selective serotonin re-uptake inhibitors has demonstrated that such drugs are as efficacious as non-selective serotonergic/noradrenergic modulators [1, 2]. However, the close interaction of the coerulean noradrenergic and mesencephalic raphe serotonergic pathways at both somatic and presynaptic sites [3–6] make it unlikely that the activity of either pathway can be selectively modified *in vivo*. Evidence supporting a causative role for 5-HT† in depression comes from a number of different areas and includes: (1) a lowered cerebrospinal fluid and brain level of the 5-HT metabolite, 5-HIAA, in depressed patients and in particular depressive suicides [7–10]; (2) an elevation of chronic serotonergic function with a wide range of clinically effective antidepressants [11]; (3) a precipitation of decreased mood or depressive relapse by depletion of the 5-HT precursor tryptophan [12–15]; (4) a marked disturbance in the serotonergic regulation of the hypothalamic–pituitary axis in depressed patients [16]; and (5) axons of the serotonergic pathway project widely from the raphe in the brainstem to many areas of the limbic brain thought to be involved in emotive function [17].

The controlling enzyme for 5-HT synthesis in brain serotonergic neurones, tryptophan hydroxylase, is unsaturated with its precursor, the essential amino acid tryptophan [18]. Raising the availability of brain tryptophan has been shown in many studies to increase the synthesis and levels of both whole-brain and brain extracellular fluid 5-HT in conscious animals [18–20] even under conditions of somatic autoreceptor inhibition of serotonergic pathway activity [21]. It is not surprising, therefore, that tryptophan has been used for a number of years as an antidepressant on its own or in combination with other antidepressants [18, 22]. However, its efficacy is limited and it has been hypothesized that this may be due to its rapid catabolism in the body. Studies in humans show that the large bolus doses of tryptophan used in the clinic (100 mg/kg) are rapidly metabolized and that tryptophan concentrations in the plasma are only elevated for a small proportion of the time between doses [23, 24]. Furthermore, clinical evidence suggests that the peak concentrations of tryptophan reached after large bolus doses of tryptophan may induce tryptophan catabolism [25], thereby further limiting its efficacy. The major site of tryptophan catabolism is the kynurenine pathway in the liver, and the key controlling enzyme in this pathway under normal conditions is tryptophan 2,3-dioxygenase (TDO) (EC 1.13.11.11) [26]. TDO, a haem-protein [27],

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† Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; TDO, tryptophan 2,3-dioxygenase.

is regulated by glucocorticoid availability [28] and is located exclusively in the liver [18].

Inhibition of TDO should decrease the catabolism of systemic tryptophan, raising its concentration not only in the blood but also in the brain. An increased availability of tryptophan will increase the activity of tryptophan hydroxylase, and therefore increase the synthesis of 5-HT and vesicular stores of 5-HT for subsequent release under certain conditions [18, 21, 29]. Studies *in vitro* have identified several inhibitors of TDO, but these have been found not to influence tryptophan levels in rats or in humans [25]. It is unlikely, therefore, that these compounds are effective inhibitors of TDO *in vivo*. The role of TDO in the regulation of brain tryptophan and 5-HT has not, therefore, been explored with a robust inhibitor. In this paper we describe the effects of a novel, selective and potent inhibitor of TDO on the catabolism of tryptophan *in vitro* and *in vivo* and on brain levels of tryptophan and 5-HT.

MATERIALS AND METHODS

Animals. Male Wistar rats (Charles River, U.K.), weighing 200–250 g, were housed in a 12 hr light–dark cycle with free access to food and water, except where stated.

Cell free TDO activity. Rat liver was homogenized in 20 vol. 50 mM potassium phosphate buffer, pH 7.0, containing 1 mM L-tryptophan and 1.36 mg/10 mL met-haemoglobin at 4°. After centrifugation (100,000 *g*, 30 min, 4°), the supernatant was incubated ± 680C91 for up to 1 hr at 37°; reactions were stopped by addition of 0.1 vol. 3.07 M perchloric acid. After centrifugation (12,000 *g*, 2 min, 4°), 50 μL of the supernatant was injected onto a 8 cm × 4 mm Zorbax ODS HPLC column. L-Kynurenine was eluted with 100 mM ammonium acetate, pH 4.5, 1% methanol, flow rate 2 mL/min; profiles were monitored at 350 nm. L-Kynurenine eluted at approximately 1.5 min.

Indoleamine 2,3-dioxygenase (EC 1.13.11.17) activity. Indoleamine 2,3-dioxygenase was assayed according to the method described in [30] and [31]. Briefly, rat duodenum was removed and washed in 0.9% NaCl (4°) and then homogenized in 50 mM potassium phosphate (pH 7.0) containing 100 μg/mL met-haemoglobin and centrifuged (12,000 *g*, 5 min, 4°). The supernatant was assayed for 10 min at 37° in 50 mM potassium phosphate (pH 7.0), 10 mM sodium ascorbate, 25 μM methylene blue, 20 μM L-tryptophan, 50000 d.p.m. of L-[ring-2-^{14}C]-tryptophan/mL, and 100 units of catalase/mL. The reaction was terminated by addition of 0.1 vol. 3.07 M perchloric acid. After centrifugation (12,000 *g*, 2 min, 4°), unreacted tryptophan in the supernatant was adsorbed by the addition of 1 vol. charcoal suspension (Norit GSX, 10 mg/mL) and after centrifugation (12,000 *g*, 2 min, 4°), the ^{14}C-labelled non-aromatic products in the supernatant were determined by scintillation counting.

Tryptophan hydroxylase activity. Tryptophan hydroxylase activity was determined according to the method described in [32] by measuring the production of 5-hydroxytryptophan from extracts of whole rat brain removed 4 hr after administration of 680C91 (15 mg/kg, p.o.) or vehicle (H_2O, 5 mL/kg).

Monoamine oxidase activity. Monoamine oxidase was assayed according to the colorimetric assay method described in [33] using a crude mitochondrial fraction from whole rat brain. Tryptamine and benzylamine were used as substrates for monoamine oxidase A and B, respectively and 0.1 μM clorgyline and 0.1 μM deprenyl were used as selective inhibitors of monoamine oxidase A and B, respectively.

5-HT$_{1A,1D,2A}$ and $_{2C}$ receptor binding. Guinea pig hippocampus (5-HT$_{1A}$), calf caudate (5-HT$_{1D}$), rat prefrontal cortex (5-HT$_{2A}$) or guinea pig cortex (5-HT$_{2C}$) were homogenized in 10 vol. 5 mM Tris (pH 7.4) and centrifuged (48,000 *g*, 20 min, 4°). Pellets were washed and resuspended two times before final resuspension in 50 mM Tris, 4 mM $CaCl_2$, 0.1% ascorbic acid, 10 μM pargyline and 25 μg/mL tris(2-butoxyethyl)phosphate. The final concentrations of tissue in the assays were 2, 10, 0.5 and 10 mg/mL, respectively. For the 5-HT$_{1A}$ and $_{1D}$ binding assays, tissue was incubated for 30 min at 27° in the presence of 1.3 nM [^{3}H]-8-OH DPAT or in the presence of 0.1 μM mesulergine, 0.1 μM 8-OH DPAT and 3 nM [^{3}H]-5-HT, respectively. For the 5-HT$_{2A}$ binding assay, tissue was incubated for 90 min at 27° in the presence of 0.5 nM [^{3}H]-ketanserin. For the 5-HT$_{2C}$ binding assay tissue was incubated for 120 min at 27° in the presence of 0.63 nM [^{3}H]mesulergine and 0.1 μM spiperone. At the end of the incubation 5 mL of 50 mM Tris (pH 7.4) was added to the incubate and the resulting mixture was vacuum filtered using a 24-well Brandel™ cell harvester. Filters were washed by vacuum filtering a further 5 mL of buffer after which the filters were punched into vials containing scintillant and the ^{3}H determined by scintillation counting.

5-HT uptake. Rat cerebral cortex was dissected from whole brain and chopped at 0.1 mm intervals using a McIlwain tissue chopper. A second series of 0.1 mm-spaced cuts were made perpendicular to the first to produce 0.1 mm × 0.1 mm cortical slices. Slices were resuspended in 35 mL of Krebs–Henseleit buffer (containing 1.96 mg/mL pargyline, 200 mg/mL ascorbic acid and 191 mg/L $CaCl_2.2H_2O$) and centrifuged (300 *g*, 5 min) three times before final resuspension in 14 vol. 100 μL of tissue suspension was added to 840 μL of buffer, incubated for 10 min at 37° in shaking water bath. 50 μL of unlabelled 5-HT and 5-[1,2-^{3}H]HT (final concentration 100 nM, 0.5 μCi) were added and the mixture was incubated for a further 5 min then vacuum filtered using a Brandel™ cell harvester. The filters were washed three times with 5 mL of 0.9% NaCl (4°) and processed for scintillation counting as described above. 680C91 or uptake inhibitor standard (fluvoxamine) were added before tissue addition.

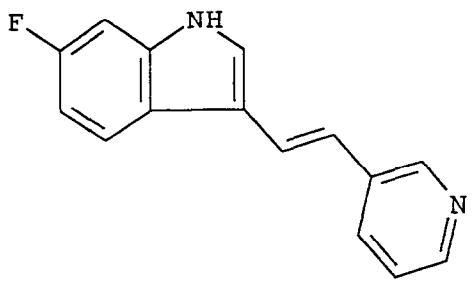
L-[ring-2-^{14}C]-tryptophan catabolism in isolated rat liver cells. Preparation of the cells and assays were carried out as described previously [34]. Briefly, rats (starved for 24 hr) were anaesthetised (60 mg/kg, sodium pentobarbitone, i.p.) and after loss of the flexor response a dorsal laparotomy was performed. The inferior vena cava and hepatic portal vein were canulated and the liver was perfused (25 mL/min)

in situ in the physiological direction with 150 mL of Ca^{2+}-free Krebs–Henseleit buffer (118 mM NaCl, 4.74 mM KCl, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4, 25 mM $NaHCO_3$ all pregassed with 95:5 O_2:CO_2 to give a pH of 7.4) containing 0.1 mM EGTA at 37°. A further 50 mL of Krebs–Henseleit buffer containing 1.25 mM $CaCl_2$ was perfused to waste before recirculating 100 mL of the same buffer containing 50 mg of collagenase (type XI, Sigma) for approximately 10 min. The liver was then removed and cut up gently in fresh Ca^{2+}-containing Krebs–Henseleit buffer (at room temperature), filtered through a 150 μm-pore size nylon mesh and filtrate centrifuged (50 *g*, 2 min). The pellet was resuspended in the same buffer and centrifuged a further two times. The liver cell pellet was resuspended in the same buffer to a final concentration of 2 mg protein/mL. Incubations were carried out at 37° in sealed vials under an atmosphere of 95%:5% O_2:CO_2. The combined concentration of the L-[ring-2-^{14}C]-tryptophan and non-labelled L-tryptophan used in the assays was 20 μM and was added either with or without 680C91 at time zero. Incubations were stopped by the addition of 0.1 vol. 3.07 M perchloric acid and tryptophan catabolic flux was determined by absorption of $^{14}CO_2$ onto 2-phenylethamine-soaked filter paper and charcoal separation of ^{14}C-labelled non-aromatic products from L-[ring-2-^{14}C]-tryptophan in the aqueous phase [34].

L-*Tryptophan catabolism in whole-liver* in situ. After cannulation of the hepatic portal vein and inferior vena cava under anaesthesia (sodium pentobarbitone, 60 mg/kg, i.p.), the liver was perfused (25 mL/min) *in situ* in the physiological direction. Blood was washed from the liver by perfusing 200 mL of Krebs–Henseleit buffer, pH 7.4 (containing 1.25 mM $CaCl_2$ and 10 μM L-tryptophan at 37°) to waste after which 100 mL of perfusion buffer was recirculated through the liver for 30 min. Every 3 min 100 μL of recirculating perfusate was removed, deproteinized by the addition of 10 μL of 3.07 M perchloric acid, centrifuged (12,000 *g*, 2 min) and the supernatant measured for tryptophan as described below. 680C91 (final concentration 10 μM) was added to the perfusate 15 min after recirculation.

Serum albumin binding studies. Rats were bled from the inferior vena cava into a heparinized syringe under sodium pentobarbitone anaesthesia (60 mg/kg i.p.). After centrifugation of the blood (2,000 *g*, 5 min) 1 mL of the plasma supernatant was added to a 10 mL plastic tube, the tube was sealed (Subaseals, BDH) and the air space was gassed (O_2:CO_2 95:5) for 2 min. Unlabelled L-tryptophan and 100,000 d.p.m. L-[ring-2-^{14}C]-tryptophan (20 μM final concentration) were added to the tube through the seal and incubated at 37° for 10 min. 680C91 or vehicle (0.9% NaCl) was then added to the tube through the seal and incubated for a further 20 min. The mixture was then transferred to a Centrisart I tube (cut-off MW20KDa, Sartorius AG, Germany), the air space was gassed briefly with O_2:CO_2 (95:5), centrifuged (2,000 *g*, 2 min), and the resulting filtrate was mixed with scintillation fluid and counted for ^{14}C-products. Only unbound tryptophan was contained in the filtrate.

L-[*ring*-2-^{14}C]-*Tryptophan catabolism* in vivo. Rats



680C91

Fig. 1. The chemical structure of 680C91.

(starved for 24 hr) were given L-[ring-2-^{14}C]-tryptophan (1 μCi in 0.9% NaCl 5 mL/kg, i.v.) at zero time 1 hr after administration of 680C91 (10 mg/kg free base equivalent, p.o.) or vehicle (H_2O, 5 mL/kg). At various times (0. 30, 60, 90 and 120 min) rats were anaesthetised with (60 mg/kg, i.p.) sodium pentobarbitone and bled (by cannulating the inferior vena cava and collecting approximately 4 mL of blood) into a heparinized syringe. Blood was centrifuged (2,000 *g*, 5 min) and the plasma supernatant was deproteinized by addition of 0.1 vol. 3.07 M perchloric acid and centrifuged (12,000 *g*, 2 min, 4°). The ^{14}C-labelled non-aromatic fraction was isolated from the supernatant by mixing it with an equal volume of charcoal suspension (10 mg/mL; Norit GSX) as described [34], centrifuged (12,000 *g*, 2 min) and the resulting supernatant was mixed with scintillation fluid and counted for ^{14}C-products.

L-*Tryptophan load catabolism* in vivo. Rats (starved for 24 hr) were given L-tryptophan (100 mg/kg in 0.9% NaCl 10 mL/kg, i.p.) at zero time, 30 min after administration of 680C91 (2, 5 or 10 mg/kg free base equivalent, p.o.) or vehicle (H_2O, 5 mL/kg). Groups of rats were bled (as described above) at 0, 10, 30, 60, 90, 120, 180, 240 and 300 min after tryptophan administration and plasma was isolated and deproteinized as above. Supernatants were measured for tryptophan as described below.

680C91 *and* L-*tryptophan administration and brain tryptophan, 5-HT and 5-HIAA.* At various times after administration of 680C91 (p.o. in 5 mL/kg H_2O) or L-tryptophan (100 mg/kg, i.p. in 10 mL/kg 0.9% NaCl), rats (starved for 24 hr) were decapitated, the whole-brain freeze-clamped in liquid N_2 and stored at −70° until assay.

Brain and plasma tryptophan, 5-HT and 5-HIAA measurement. Whole brains were homogenized and deproteinized in 10 vol. of 0.307 M perchloric acid. Plasma was deproteinized by the addition of 0.1 vol. of 3.07 M perchloric acid. Acidified extracts were then centrifuged (12,000 *g*, 2 min, 4°) and 50 μL of each supernatant was injected onto a 25 cm × 4.6 mm Waters μ Bondapak ODS HPLC column and eluted with 100 mM ammonium acetate, pH 4.5, 7% methanol (flow rate 1 mL/min). Peaks were monitored by fluorescence, excitation 285 nm, emission 340 nm, and typically eluted at 5.5 min (5-

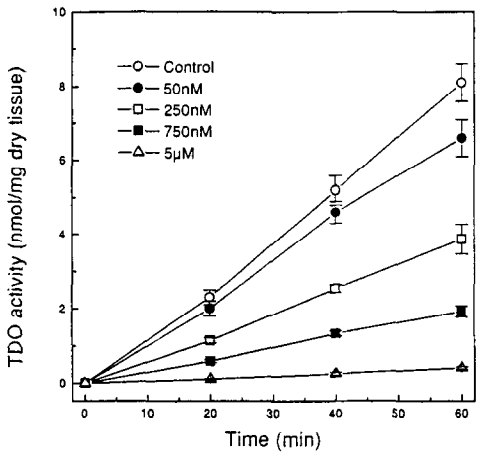


Fig. 2. The effect of 680C91 on cell-free rat liver TDO activity. TDO was assayed by measuring the production of L-kynurenine from L-tryptophan using HPLC (see Materials and Methods). 680C91 was added at time zero. Rules are means ± SEM (N = 3).

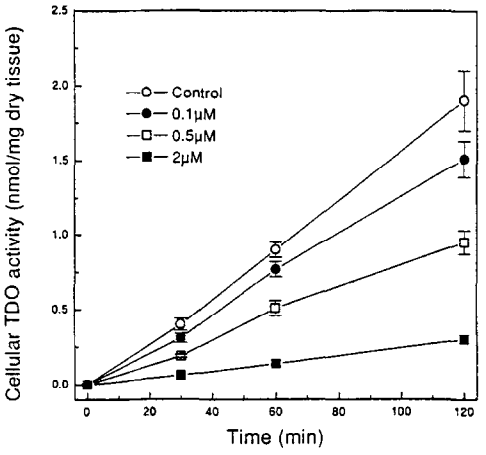


Fig. 4. The effect of 680C91 on TDO flux in isolated rat liver cells. TDO flux was determined by measuring the release of $^{14}CO_2$ and ^{14}C-labelled non-aromatic products from L-[ring-2-^{14}C]-tryptophan. 680C91 and tryptophan were added at zero time. Results are means ± SEM (N = 3).

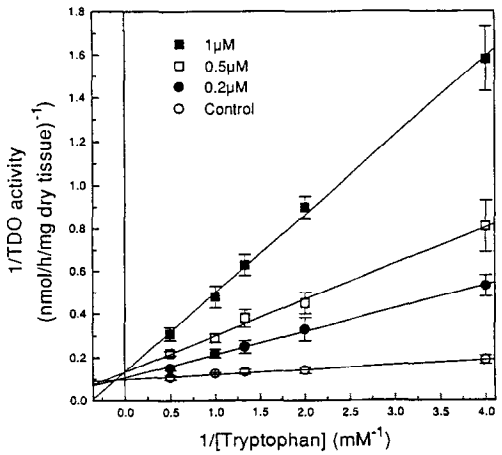


Fig. 3. Lineweaver–Burk plot of the effect of 680C91 on cell-free rat liver TDO activity. Results are means ± SEM (N = 3). TDO activity was determined as in Fig. 1, and was measured over 1 hr.

HT), 6.9 min (tryptophan) and 8.6 min (5-HIAA). Recoveries were complete.

Chemicals. The following drugs were used: 680C91 ((*E*)-6-fluoro-3-[2-(3-pyridyl)vinyl]-1*H*-indole, Fig. 1), methanesulphonate (Wellcome Foundation Limited, Beckenham, U.K.) and L-[ring-2-^{14}C]-tryptophan (CEA, France). 680C91 was synthesized by condensing 6-fluoroindole-3-carboxaldehyde (obtained by Vilsmeiyer formylation of 6-fluoroindole) with pyridine-3-acetic acid in the presence of piperidine. Other chemicals were from Sigma (U.K.).

RESULTS

680C91 (chemical structure shown in Fig. 1) inhibited TDO in cell-free rat liver extracts rapidly and completely (Fig. 2), and competitively with respect to its substrate tryptophan (Fig. 3) with a K_i of 51 ± 8 mM (N = 3; determined from the 0–60 min TDO activity rates in Fig. 2). 680C91 inhibited the catabolism of a mixture of 20 μM unlabelled L-tryptophan and L-[ring-2-^{14}C]-tryptophan in liver cells (Fig. 4) with an IC_{50} of 492 ± 65 nM (N = 3; determined from the linear 30–90 min TDO activity rates in Fig. 4). *In vitro* studies demonstrated that 680C91 (tested up to a concentration of 100 μM) had no effect on the binding of L-[ring-2-^{14}C]-tryptophan to serum albumin in plasma (data not shown). 680C91 (up to concentration of 10 μmM) had no effect upon the uptake of 5-HT into cerebral cortical slices, monoamine oxidase A and B activity, indoleamine 2,3-dioxygenase activity or binding to the 5-HT_{1A} and $_{1D}$ autoreceptors or to the 5-HT_{2A} and $_{2C}$ receptors (data not shown).

The effect of 680C91 on tryptophan concentrations in a recirculating perfusate of rat liver *in situ* was examined. The concentration of L-tryptophan added to the perfusion buffer was not significantly changed by recirculation of the buffer through the liver for 15 min (Fig. 5). Addition of 10 μM 680C91 after 15 min of recirculating perfusion produced a marked increase in the concentration of tryptophan in the perfusate with significant increases observed as quickly as 3 min after 680C91 addition (Fig. 5). Higher concentration of 680C91 did not increase tryptophan levels further (data not shown).

Metabolism of L-[ring-2-^{14}C]-tryptophan by TDO generates unlabelled aromatic L-kynurenine and ^{14}C-labelled non-aromatic products (such as ^{14}C-formate). Measurement of the production of ^{14}C-labelled non-aromatic products in the plasma of rats previously administered L-[ring-2-^{14}C]-tryptophan will, therefore, predominantly represent

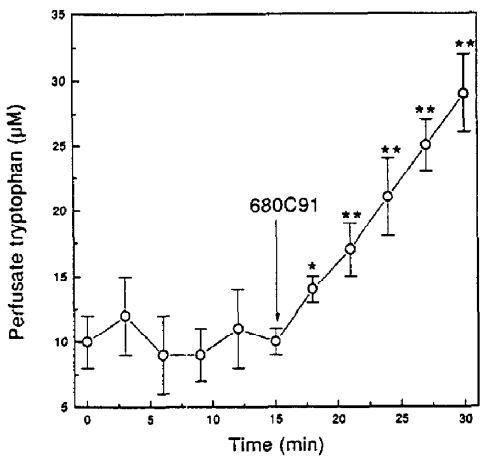


Fig. 5. The effect of 680C91 on the concentration of tryptophan in the perfusate from *in situ* perfused rat liver. Samples (100 μL) of perfusate were removed every 3 min and measured for tryptophan. 680C91 (final concentration in the perfusate 10 μM) was added after 15 min. Results are means ± SEM (N = 3). Differences between means were assessed by Student's *t*-test: P (versus mean of points in absence of 680C91) * < 0.05, ** < 0.005.

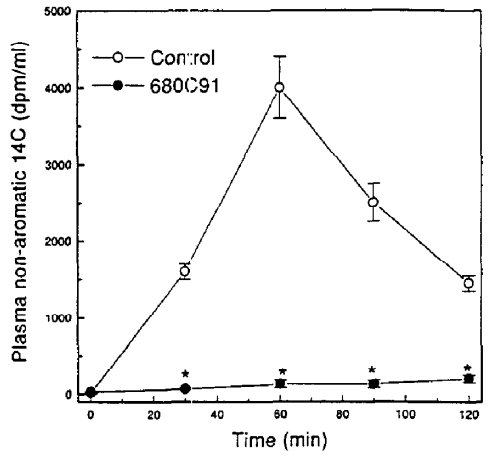


Fig. 6. The effect of 680C91 (10 mg/kg free base equivalent, p.o.) on the production of ^{14}C-labelled non-aromatic products from L-[ring-2-^{14}C]-tryptophan *in vivo*. L-[ring-2-^{14}C]-tryptophan (1 μCi) was administered (i.v. in 0.9% NaCl, 5 mL/kg) at zero time 1 hr after administration of 680C91 or vehicle (H_2O, 5 mL/kg). Blood (approximately 4 mL) was removed under sodium pentobarbitone (60 mg/kg, i.p.) and plasma was isolated and processed as described in Materials and Methods. Results are mean ± SEM (N = 3). Differences between means were assessed by Student's *t*-test: P (versus control) * < 0.001.

TDO activity *in vivo*. Figure 6 shows a pronounced and time-dependent increase in the plasma levels of ^{14}C-labelled non-aromatic products after administration of L-[ring-2-^{14}C]-tryptophan. When 680C91 was administered (10 mg/kg free base equivalent,

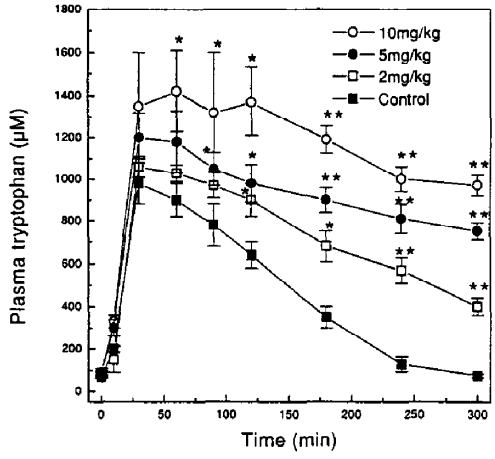


Fig. 7. The effect of 680C91 (2, 5 or 10 mg/kg free base equivalent, p.o.) on the plasma concentration of tryptophan after tryptophan administration. L-Tryptophan (100 mg/kg) was administered (i.p. in 5 mL/kg 0.9% NaCl) at zero time, 30 min after administration of 680C91 or vehicle (H_2O, 5 mL/kg). Blood (approximately 4 mL) was removed under sodium pentobarbitone anaesthesia (60 mg/kg, i.p.) and plasma was isolated and processed as described in Materials and Methods. Results are mean ± SEM (N = 3). Differences between means were assessed by Student's *t*-test: P (versus control) * < 0.05, ** < 0.005.

p.o.) 1 hr before L-[ring-2-^{14}C]-tryptophan, only modest increase in plasma ^{14}C-labelled non-aromatic products could be seen.

Load doses of tryptophan, given as antidepressant therapy, have been shown in man to produce large but short-lasting increases in plasma tryptophan [23, 24]. We therefore investigated the effect of 680C91 on the disposal of such a load dose of tryptophan in the rat. As expected, a large dose of tryptophan produced marked increases in plasma tryptophan levels but for only a relatively short period of time (Fig. 7). Prior administration of 680C91 (2, 5 or 10 mg/kg, p.o.) decreased the rate of tryptophan removal from the plasma but only slightly increased the maximal concentration reached compared to the control animals.

Administration of 680C91 alone produced marked and dose-dependent increases in brain tryptophan, 5-HT and 5-HIAA (Fig. 8). Administration of vehicle had no effect on these parameters (data not shown). The maximally effective dose of 680C91 (15 mg/kg) increased tryptophan and 5-HT to 240% and 125% of control values, respectively. The increased levels of the 5-HT metabolite, 5-HIAA, that were seen indicate that an increase in the breakdown of 5-HT accompanied the increase in its synthesis. Administration of 680C91 produced increases in brain contents of tryptophan, 5-HT and 5-HIAA up to 10 hr after administration, with maximal elevation seen between 4 and 6 hr (Fig. 9). Administration of 680C91 (15 mg/kg, p.o.) 4 hr before sacrifice had no effect on the activity of brain tryptophan hydroxylase activity measured *ex vivo* (data not shown). The duration of elevation of brain

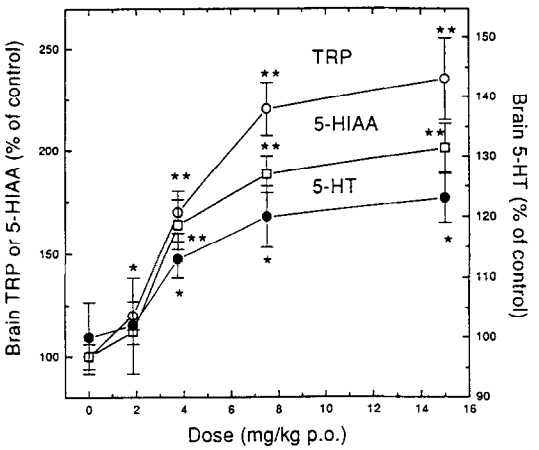


Fig. 8. Dose–response of 680C91 (free base equivalent, p.o.) on whole brain levels of tryptophan, 5-HT and 5-HIAA. 680C91 or vehicle (H_2O, 5 mL/kg) were administered at zero time, 4 hr before sacrifice and removal of the brain. Levels of brain tryptophan, 5-HT and 5-HIAA from vehicle-treated controls were 19.60 nmol/g, 3.10 nmol/g and 1.65 nmol/g, respectively. Results are means ± SEM (N = 3). Differences between means were assessed by Student's *t*-test: P (versus vehicle-treated controls) * < 0.05, ** < 0.005.

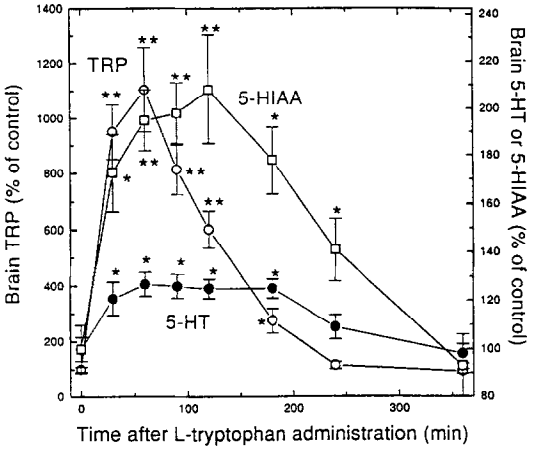


Fig. 10. The effect of tryptophan (100 mg/kg, i.p.) on brain levels of tryptophan, 5-HT and 5-HIAA. Tryptophan was administered (10 mL/kg in 0.9% NaCl) at zero time. Levels of tryptophan, 5-HT and 5-HIAA in brains from zero time controls were 22.65 nmol/g, 2.81 nmol/g and 1.49 nmol/g, respectively. Results are means ± SEM (N = 3). Differences between means were assessed by Student's *t*-test: P (versus zero time controls) * < 0.05, ** < 0.005.

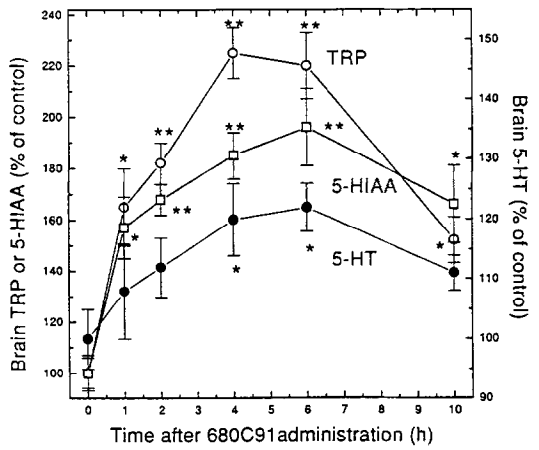


Fig. 9. Time-course of 680C91 (7.5 mg/kg free base equivalent, p.o.) on brain levels of tryptophan, 5-HT and 5-HIAA. 680C91 was administered (5 mL/kg in H_2O) at zero time. Levels of tryptophan, 5-HT and 5-HIAA in brains from zero time controls were 20.50 nmol/g, 3.11 nmol/g and 1.66 nmol/g, respectively. Results are means ± SEM (N = 3). Differences between means were assessed by Student's *t*-test: P (versus zero time controls) * < 0.05, ** < 0.005.

tryptophan, 5-HT and 5-HIAA by 680C91 compares favourably with the effect of a large load dose of L-tryptophan (100 mg/kg) on these parameters (Fig. 10). The effect of this dose of L-tryptophan on brain tryptophan, 5-HT and 5-HIAA could not be seen at times greater than 4 hr after administration (Fig. 10). Administration of vehicle had no effect on brain tryptophan, 5-HT and 5-HIAA (data not shown). Although a higher peak concentration of tryptophan was achieved with the L-tryptophan load dose, when compared with that seen with 680C91, there was no further increase in 5-HT or 5-HIAA.

DISCUSSION

The present results demonstrate that 680C91 is a potent inhibitor of TDO and is selective with respect to a number of other enzymes and receptors modulating serotonergic function. 680C91 is a potent inhibitor not only at the cell-free level but also in more complex systems *in vitro* and *in vivo*. Since 680C91 is a robust inhibitor of TDO *in vitro* and *in vivo*, we have been able to explore the role of this enzyme in the regulation of tryptophan and 5-HT metabolism in the body.

Tryptophan is known to bind to serum albumin and under normal conditions in plasma the majority is in the bound state [35]. Increases in the free-to-bound ratio of tryptophan in plasma have been shown to increase brain levels of both tryptophan and 5-HT [35–37]. Alternatively, decreases in the free-to-bound ratio of tryptophan produced by a reduction in the blood concentration of the competing unesterified fatty acids have been shown to decrease brain tryptophan, 5-HT and 5-HIAA [38]. It has been suggested that this mechanism may precipitate a decrease in mood in people consuming a low-fat diet [39]. 680C91, however, does not displace tryptophan from its serum albumin binding sites and the increases in brain tryptophan (and 5-HT) observed in this study can therefore be attributed to TDO inhibition alone. This assumption is supported

by the selectivity of 680C91 against other systems likely to modulate brain levels of tryptophan, 5-HT or 5-HIAA.

The IC_{50} of 680C91 against liver cell catabolism of tryptophan by TDO was significantly higher than the K_i against the isolated enzyme. This is unlikely to be due to a higher substrate concentration in the liver cells compared to the cell-free assays as the extracellular concentration of tryptophan used is only a tenth of the K_m of TDO for tryptophan [35] and it is known that an inward concentration gradient of tryptophan is not maintained by liver cells [40]. The high control coefficient of TDO for tryptophan catabolism in isolated rat liver cells [26] predicts that a similar degree of inhibition of cell-free TDO and liver cell tryptophan catabolism would be seen. The difference may be explained, however, by the observation that 680C91 itself is metabolized by isolated rat liver cells (M. Salter and J. Salmon, unpublished results) and that liver cell intracellular concentrations of 680C91 may therefore be substantially lower than extracellular concentrations of 680C91. This would explain the apparent decrease in potency of 680C91 in liver cells compared to cell-free TDO.

The results obtained with the liver perfused *in situ* (Fig. 5) suggest that in the absence of 680C91 the rate of tryptophan removal (via TDO) and tryptophan supply (probably via proteolysis) are balanced. There is no net change, therefore, in perfusate tryptophan levels. After addition of 680C91 to the perfusate, the removal of tryptophan (by TDO) is inhibited and the production of tryptophan from proteolysis is unmasked leading to an increase in perfusate tryptophan.

Studies with L-[ring-2-^{14}C]-tryptophan (Fig. 6) and L-tryptophan load dose (Fig. 7) administration *in vivo*, demonstrate that 680C91 is an effective inhibitor of TDO *in vivo* and that TDO is the major site of whole-body tryptophan catabolism. This is in contrast with earlier studies where inhibitors of TDO *in vitro* were without effect on tryptophan concentrations when administered *in vivo* [25]. This may be explained by the lower potency and/or availability of these compounds *in vivo*. From Fig. 7 it can be seen that 680C91 will enhance the duration of brain 5-HT elevation after tryptophan administration by inhibiting the removal of tryptophan from the body and may therefore potentiate its antidepressant effect.

This study has demonstrated that an inhibitor of TDO will not only sustain supraphysiological levels of tryptophan after tryptophan administration but will also elevate endogenous tryptophan and consequently brain 5-HT and 5-HIAA. Although maximally effective doses of 680C91 could only elevate brain tryptophan to much lower levels than the peak concentrations achieved by exogenous tryptophan administration (compare Figs 8, 9 and 10), no further increase in brain 5-HT (or 5-HIAA) were seen with tryptophan administration. This suggests that the maximal elevation of brain tryptophan produced by 680C91 is sufficient to saturate tryptophan hydroxylase, supporting evidence of an approximate 50% saturation of this enzyme with tryptophan *in vivo* [18, 25]. This statement is supported by the observation that administration of 680C91 had no effect on tryptophan hydroxylase activity measured *ex vivo* (data not shown) and therefore the increases in brain 5-HT and 5-HIAA observed with 680C91 in this study are due only to the increased availability of tryptophan in the brain. Furthermore, increases in 5-HT, produced by 680C91, are sustained for a greater length of time compared to those produced by exogenous tryptophan.

This work has demonstrated the key role of TDO in the homeostasis of systemic tryptophan catabolism and central nervous system levels of tryptophan and 5-HT. The role of TDO in the regulation of brain 5-HT synthesis, is of significance considering that the majority of patients suffering from depression have elevated cortisol levels [16] and that glucocorticoids are potent inducers of TDO activity; the artificial glucocorticoid, dexamethasone, has been shown to increase TDO by up to 10-fold in rats [28]. Such an increase in TDO activity is likely to decrease the concentration of tryptophan levels in the blood and brain and consequently decrease brain 5-HT. Tryptophan depletion in both normal people [12] and depressed patients in remission [13–15] has been shown to lower mood and cause a relapse of depressive symptoms, respectively. Because tryptophan hydroxylase is approximately 50% saturated with tryptophan *in vivo* [18], decreases in tryptophan will have a far greater effect on 5-HT than increases in tryptophan. Increases in cortisol may therefore play a role in depression by decreasing the supply of tryptophan to the brain for 5-HT synthesis.

L-Tryptophan has been used alone and in combination with other antidepressants for over 30 years to treat depression with varying degrees of success. One property of tryptophan that may explain its modest efficacy is its rapid catabolism by TDO in the liver. This study has shown that an inhibitor of TDO, 680C91, can elevate brain tryptophan and 5-HT without resorting to the administration of large doses of tryptophan. Furthermore, the superior (i.e. longer-lasting) duration of action of 680C91 compared to tryptophan, should enhance the therapeutic efficacy of this 5-HT-precursor-based approach in both mono- and combination antidepressant therapy.

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