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TYROSINE HYDROXYLASE AND DOPAMINE β-HYDROXYLASE INHIBITING PROPERTIES OF A NEW SERIES OF PYRIDAZINYL HYDRAZONES*

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Abstract—New pyridazinyl hydrazones are described as a novel class of tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) inhibitors. A great number of these substances showed potent TH- or DBH-inhibiting capacities. The structure–activity analysis suggested an important role for the N^2 substitution of hydrazine in the inhibiting characteristics of the compounds. β-Substituted pyridazinyl hydrazones showed favourable TH- or DBH-inhibiting activities, whereas the free hydrazines were only slightly effective on tyrosine hydroxylation and noneffective on dopamine hydroxylation. The β-ketoester derivatives of 6-chloro-3-pyridazinyl hydrazones were characterized as potent DBH-inhibiting substances while the cyclohexylidene or the bicycloheptylidenepyridazinyl hydrazones were found to be marked TH- and DBH-blocking agents. Among the above substances, GYKI 11679 {chemically 1-(6-morpholino-3-pyridazinyl)-2-[(1-tertiary-butoxycarbonyl)-2-propylidene] hydrazine} has been found to be the most potent inhibitor of tyrosine hydroxylation both *in vitro* and *in vivo*. Its *in vitro* as well as its *in vivo* TH-blocking activity was comparable with that of α-methyltyrosine. The most effective representative of this new series on dopamine and tyramine hydroxylations *in vitro* and *in vivo*, compound GYKI 11473 {chemically 1-(6-chloro-3-pyridazinyl)-2-[(1-ethoxycarbonyl)-2-propylidene] hydrazine}, has been characterised as a reversible and competitive inhibitor of DBH with dopamine and a noncompetitive inhibitor of DBH with tyramine as substrate at concns of 10^{-5}–10^{-6} M. The compound has also been found to inhibit the neuronal and the vesicular uptake of dopamine in hypothalamic slices. GYKI 11473 showed a greater effect in the heart than in the brain and its *in vivo* efficiency has been established as being more potent than those of fusaric acid in both tissues. The noradrenaline (NA) determinations suggested similar, but not equal, and exclusive contributions of TH and DBH in the maintenance of NA pools in these tissues.

L-Tyrosine hydroxylase (TH)§ (tyrosine 3-monooxygenase) (EC 1.14.16.2) and DBH (3,4-dihydroxyphenylethylamine, ascorbate:O_2 oxidoreductase) (EC 1.14.17.1) are essential enzymes which catalyse the hydroxylation of tyrosine and DA the rate-limiting step of NA biosynthesis [1, 2]. Both enzymes occur in the brain as well as in peripheral noradrenergic nerves and the adrenal medulla [3, 4]. The inhibition of either enzyme led to a decreased synthesis and level of NA [5–8]. A great number of compounds have been reported to inhibit either TH [9–11] or DBH [12–15]. The present paper describes a new class of synthesis inhibitors showing either TH- or DBH-inhibiting characteristics. A few of these new substances produced marked inhibitions of both enzymes *in vitro*. Some pyridazinyl hydrazones have been described as potent antihypertensive agents [16, 17] and in a few cases a close parallelism was observed between the antihypertensive action and the peripheral DBH-inhibiting potencies of the compounds [18]. This paper summarizes the results of a structure–activity relationship study, and describes the *in vitro* and *in vivo* enzyme-inhibiting characteristics of these new substances.

MATERIALS AND METHODS

Materials

The synthesis of these novel pyridazinyl hydrazones has been described elsewhere [17, 18]. Compounds were relatively soluble in water but some were dissolved in 1% Triton-X-100 solution and 0.1 ml of this suspension was used for *in vitro* studies. The Triton-X-100, at final concns of 0.1–0.2%, did not influence DBH activity but produces an increased TH activity. In this latter case, 0.1–0.2% Triton-X-100 was also added to the reaction mixtures in the vol. used for the test compounds. For *in vivo* measurements, a homogeneous suspension, containing 1–4% of Tween 80, was prepared; and 100–150 mg amounts of the compound were suspended in 10 ml of physiological saline containing 1–4% of Tween 80. 0.2–0.5 ml of these suspensions were given to animals intraperitoneally. Controls received an equal quantity of 1–4% Tween 80 solution via the same route and at the same time. The compounds

* Parts of this study were presented at the Third Catecholamine Meeting, Asilomar, September 1978, and at the Third Meeting of the European Neurochemical Society, Bled, September 1980.

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§ Abbreviations: TH, tyrosine hydroxylase; DBH, dopamine β-hydroxylase; NA, noradrenaline; DA, dopamine; CA, catecholamine; TCA, trichloroacetic acid; THO, tritiated water.

were administered in a single dose to rats 1–3 hr prior to decapitation, or in double doses, 20 and 3 hr prior to the experiment, as indicated in Results. Radioactive compounds, L-[3,5-^{3}H]tyrosine (41 Ci/mmole), DL-[7-^{3}H]NA hydrochloride (19 Ci/mmole) and [ethylamine-1,2-^{3}H]DA hydrochloride (2.7 Ci/mmole), were obtained from the Amersham Radiochemical Centre (Amersham, U.K.), and, with the exception of L-[3,5-^{3}H]tyrosine, were used without further purification. The [^{3}H]tyrosine was purified by the method of Ikeda *et al.* [19].

Preparation of tissues

Male Sprague–Dawley rats (100–200 g) were killed by cervical dislocation. For enzyme activity measurements, the tissue was homogenized in 3–10 vols (w/v) of ice-cold buffer with a Janke–Kunkel tissue homogenizer. For CA determinations, the brain or heart was homogenized in 10% TCA, centrifuged at 4000 *g* for 60 min and the supernatant used for the assay.

Preparation of enzymes

Beef adrenal DBH was partially purified through the second sulphate step as described by Duch *et al.* [20].

Enzyme assays in vitro

TH activity. This was measured by the method of Nagatsu *et al.* [9], as modified by Levitt *et al.* [21]. The standard incubation mixture contained 10.8 mM phosphate buffer (pH 6.1), 316 mM acetate buffer (pH 6.1), 0.15 μmoles NSD-1055 (decarboxylase inhibitor), 300 U. catalase, 0.9 μmoles $DMPH_4$, 1.25 μmoles ascorbic acid, 0.05 μmoles unlabeled tyrosine and 40–60 mg tissue in a final volume of 0.6 ml. The test compounds were added in 60–120 μl to the reaction mixture and incubated at 37° for 5 min without substrate. The reaction was started with the addition of 0.2 μCi [^{3}H]tyrosine and the incubation lasted for 10 min. After separation on a Dowex 50 WX8 column, the THO formed from L-[3,5-^{3}H]tyrosine was measured. The enzyme activity was calculated as nmoles THO formed from labeled tyrosine per g tissue per hr and found to be 1085 ± 58 nmoles/g tissue/hr for rat adrenal TH and 217 ± 16 nmoles/g tissue/hr for rat hypothalamic TH.

DBH activity. Measurements were carried out by using the method of Nagatsu *et al.* [22], which is based on the enzymatic conversion of tyramine to octopamine. The octopamine, formed during the reaction, was oxidized to *p*-hydroxybenzaldehyde and determined photometrically. The standard reaction mixture consisted of 800 mM acetate buffer (pH 5.5), 10 μmoles fumaric acid, 10 μmoles ascorbic acid, 300 U. catalase, 20 μmoles *N*-ethylmaleimide and 10 μmoles tyramine in a final vol. of 1 ml. Each tube contained an enzyme preparation (1.5 mg protein) obtained from bovine adrenals. In a number of experiments, DA was used as substrate and the NA formed during the reaction was isolated on a Dowex 50 WX8 column and measured fluorimetrically [23]. The partially purified beef adrenal DBH activity was determined to be 780 ± 52 nmoles/mg protein/hr with tyramine, and 520 nmoles/mg protein/hr with DA as substrate.

Calculation of the inhibiting potency in vitro

The IC_{50} values were determined graphically. The test compounds were added at four or five different concns to the reaction mixtures and the percentages of inhibition obtained were plotted against the logarithms of inhibitor concns. The inhibitor concn required to produce 50% inhibition was determined from the assay curves.

Uptake measurements

Neuronal uptake of CA. This was determined as previously described [24].

The hypothalamic slices were incubated in a Krebs–Ringer bicarbonate buffer, containing ascorbic acid, EDTA and pargyline. Test compounds were added in a vol. of 0.05 ml. After the slices had been preincubated for 15 min in an oxygenated medium at 37° in the presence of the test compounds, the reaction was started by the addition of varying amounts of labeled CA. After 10 min incubation, uptake was stopped by placing the tubes into ice. Slices were separated from the medium by rapid filtration and the tissue was homogenized in 5% TCA for radioactive determinations.

Vesicular uptake of DA. For this determination the method of Seidler *et al.* [25] was used. Vesicles were prepared according to the modified Philippu–Beyer method [26].

CA synthesis measurements in vivo

Measurements in the hypothalamus or the total brain were performed by injecting labeled tyrosine or DA intracerebroventricularly (i.c.v.) in a 20 μl solution into the third ventricle of the animals. Controls received saline in an equal volume and via the same route of administration. Injections were checked by using a methylene blue dying test. The test compounds were given in a single dose 1–3 hr prior to decapitation, or in double doses, 20 and 3 hr prior to the experiment, as indicated in Results. The radioactive CA and their deaminated metabolites were collected on aluminium oxide (Woelm, Eschwege, F.R.G.) and eluted with 2 N perchloric acid and 1 N hydrochloric acid respectively as previously described [24]. Determinations in the heart were carried out by injecting [^{14}C]DA in to rats pretreated with pargyline 20 hr and the test compound 24 and 3 hr prior to the injection of the labeled amine, and measuring the [^{14}C]NA content in the tissue. DA and NA were isolated on a Dowex 50 WX8 column and eluted with 1.4 and 3 N hydrochloric acid as described by Nybäck *et al.* [27].

CA assays

Rats were treated with various dosages of the compounds as indicated in Results. They were killed at the specified times by decapitation, the heart was immediately removed, cut open and rinsed in an isotonic saline solution. The brain was placed on ice and dissected. The tissue or the tissue fractions were weighed, homogenized in 5% TCA and centrifuged for 1 hr at 5000 *g*. The supernatant was processed on alumina according to the method of Anton and Sayre [28]. The endogenous concns of DA and NA were assayed spectrofluorimetrically. The fluorescent

product of DA was developed by the method of Atack [23]. NA was estimated according to Merrils [29].

RESULTS

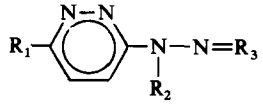
Structure–activity relationship studies

Twenty-five new pyridazinyl hydrazones were compared for their relative inhibitions of TH and DBH *in vitro*. Six compounds were shown as almost equally potent inhibitors of both enzymes. Among these, the most potent substances were GYKI 11468 [chemically 1-(3-pyridazinyl)-2-cyclohexylidene hydrazine], GYKI 11461 [chemically 1-(6-chloro-3-pyridazinyl)-2-cyclohexylidene hydrazine] and GYKI 11295 [chemically 1-(3-pyridazinyl)-2-(1,7,7-trimethyl-2-bicyclo[2.2.1]heptylidene) hydrazine]. These compounds inhibited rat adrenal TH and beef adrenal DBH at concns of 10^{-5} M–10^{-6} M (Table 1). The β-substitution of the free hydrazines appears to be extremely important with regard to the inhibiting efficiency of the compounds. Free hydrazines such as GYKI 11493 did not show a remarkable TH-inhibiting potency and did not even cause a weak inhibition of DBH. The substitution of N^2 hydrogen of hydrazine by a cyclohexylidene group led to an increased effectiveness of these compounds similar to that resulting from the introduction of a propylidene or ethoxycarbonylpropylidene group into the molecule (Table 1). The cyclohexylidene and heptylidene derivatives were found to be potent inhibitors of TH and DBH, whereas the propylidenes were characterized mainly as DBH inhibitors. Among these latter pyridazinyl hydrazones however the 6-morpholino derivatives were prominent TH-inhibiting agents. The compound GYKI 11679 {chemically 1 - (6 - morpholino - 3 - pyridazinyl) - 2 - [(1 - tertiary-butoxycarbonyl)-2-propylidene] hydrazine} produced a 50% inhibition of rat adrenal TH activity at a concn of 6×10^{-5} M (IC_{50}) and an equal inhibition of the hypothalamic enzyme at a concn of 8×10^{-5} M. The compound did not show any DBH-inhibiting characteristics in the wide concn range studied. The TH-inhibiting potency of this substance was comparable with that of α-methyltyrosine (Table 1).

The β-ketoester hydrazones of 6-chloro-3-pyridazine were found to be potent DBH inhibitors. The highest *in vitro* and *in vivo* DBH-blocking capacity was shown by GYKI 11473 {1-(6-chloro-3-pyridazinyl) - 2 - [(1 - ethoxycarbonyl) - 2 - propylidene] hydrazine}. Its IC_{50} value was calculated as 4×10^{-6} M for adrenal DBH; and this value was close to that obtained with fusaric acid (1×10^{-7} M) (Table 1). The TH-blocking capacity of the substance was

Table 1. Inhibition of tyrosine hydroxylase and dopamine–β-hydroxylase by a new series of pyridazinyl hydrazones—structure–activity relationship studies: (1) substitutions of the hydrazine N^2 hydrogen atom, and (2) substitutions at the 6-position of the pyridazine ring

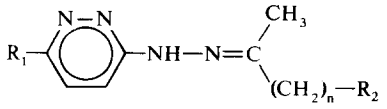
Chemical structure

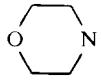
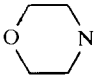
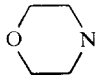
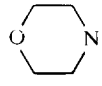


Compound	Substituents R_1	R_2	R_3	IC_{50} (M)* TH	DBH
GYKI 11468	H	H	Cyclohexylidene	4×10^{-6}	1×10^{-5}
GYKI 11295	H	H	1,7,7-Trimethyl-2-bicyclo[2.2.1]heptylidene	1×10^{-5}	5×10^{-5}
GYKI 11461	Cl	H	Cyclohexylidene	6×10^{-5}	1×10^{-5}
GYKI 11595	Cl	H	(2-Carboxy)-cyclohexylidene	6×10^{-5}	4×10^{-5}
GYKI 11679		H	(1-Tertiary-butoxy-carbonyl)-2-propylidene	6×10^{-5}	No inhibition
GYKI 11563	H	H	(1-Ethoxycarbonyl)-2-propylidene	1×10^{-5}	1×10^{-5}
GYKI 11640	Cl	H	(1-Ethoxycarbonyl)-2-butylidene	1×10^{-4}	2×10^{-6}
GYKI 11473	Cl	H	(1-Ethoxycarbonyl)-2-propylidene	1×10^{-4}	4×10^{-6}
GYKI 11529	Cl	H	2-Propylidene	6×10^{-5}	1×10^{-5}
GYKI 11493	Cl	CH_3	H_2	1×10^{-4}	No inhibition
Reference compounds					
α-Methyltyrosine				1×10^{-5}	No inhibition
Fusaric acid				No inhibition	1×10^{-7}

* Double or triple experiments were carried out and the test compounds were added at four or five different concns. The percentages of inhibition were plotted against the logarithms of inhibitor concns and the IC_{50} values were determined graphically as indicated in Materials and Methods. No remarkable alterations in the IC_{50} values, determined in individual experiments, have been demonstrated.

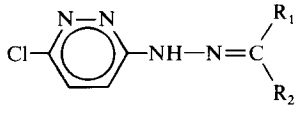
Table 2. Inhibition of dopamine β-hydroxylase by pyridazinyl hydrazones: structure–activity relationship studies with the aliphatic ketone derivatives of the 6-substituted 3-pyridazinyl hydrazones



Compound	Substituents R_1	R_2	n	IC_{50} (M)*
GYKI 11473	Cl	$COOC_2H_5$	1	4×10^{-6}
GYKI 11563	H	$COOC_2H_5$	1	1×10^{-5}
GYKI 11686	OH	$COOC_2H_5$	1	No inhibition
GYKI 11636		$COOC_2H_5$	0	No inhibition
GYKI 11662	Cl	$COOC_3H_7$	1	5×10^{-6}
GYKI 11741	CH_3	$COOC_4H_9^t$	1	3×10^{-5}
GYKI 11702	$CONH_2$	$COOC_4H_9^t$	1	8×10^{-5}
GYKI 11679		$COOC_4H_9^t$	1	No inhibition
GYKI 11663	Cl	$COOC_8H_{17}$	0	2×10^{-5}
GYKI 11687	CH_3	COOH	1	1×10^{-4}
GYKI 11792		COOH	2	6×10^{-5}
GYKI 11744		COOH	3	3×10^{-5}

* Double or triple experiments were carried out and the test compounds were added at four or five different concns. The IC_{50} values were determined graphically as indicated in Materials and Methods. No remarkable alterations in the IC_{50} values, determined in individual experiments, have been demonstrated.

Table 3. Inhibition of dopamine β-hydroxylase by pyridazinyl hydrazones: structure–activity relationship studies with the aliphatic ketone derivatives of the Cl-substituted hydrazones

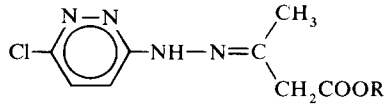


Compound	Substituents R_1	R_2	IC_{50} (M)*
GYKI 11595	CH_3	$COOC_2H_5$	5×10^{-5}
GYKI 11473	CH_3	$CH_2COOC_2H_5$	4×10^{-6}
GYKI 11628	CH_3	$(CH_2)_2COOC_2H_5$	8×10^{-5}
GYKI 11633	CH_3	$(CH_2)_3COOC_2H_5$	4×10^{-5}
GYKI 11659	CH_3	$(CH_2)_4COOC_2H_5$	8×10^{-5}
GYKI 11640	C_2H_5	$CH_2COOC_2H_5$	2×10^{-6}

* Double or triple experiments were carried out and the test compounds were added at four or five different concns. The IC_{50} values were determined graphically as indicated in Materials and Methods. No remarkable alterations in the IC_{50} values, determined in individual experiments, have been demonstrated.

Table 4. Inhibition of dopamine β-hydroxylase: structure–activity relationship studies with various aliphatic ketoester derivatives

Chemical structure



Compound	R	IC_{50} (M)*
GYKI 11616	CH_3	2×10^{-5}
GYKI 11473	CH_2—CH_3	4×10^{-6}
GYKI 11662	$(CH_2)_2CH_3$	5×10^{-6}
GYKI 11652	$CH(CH_3)_2$	2×10^{-5}
GYKI 11653	$C_4H_9^t$	8×10^{-5}
GYKI 11663	C_8H_{17}	2×10^{-5}

* Double or triple experiments were carried out and the test compounds were added at four or five different concns. The IC_{50} values were determined graphically as indicated in Materials and Methods. No remarkable alterations in the IC_{50} values, determined in individual experiments, have been demonstrated.

Table 5. Reversibility of DBH inhibition produced by GYKI 11473

Sample	Enzyme activity (nmoles octopamine/g tissue) Non-dialysed Activity value	% of control	Dialysed Activity value	% of control
Beef adrenal DBH*	760 ± 5.6	100	456 ± 3.2	100
Beef adrenal DBH incubated with 4×10^{-6} M GYKI 11473	387 ± 2.9	51	460 ± 3.6	100

* Partially purified beef adrenal DBH was incubated at 37° for 15 min in the absence and the presence of 4×10^{-6} M GYKI 11473, and aliquots were taken for enzyme activity measurements. Other portions of this enzyme preparation were dialysed against 0.02 M phosphate buffer (pH 5.5) at 4° for one night and enzyme activity determinations were carried out with these dialysed portions of enzyme preparation.

very low and could not be demonstrated *in vivo* (unpublished observation).

Because a great number of these new pyridazinyl hydrazones showed more potent DBH- than TH-inhibiting potencies, a more detailed structure–activity relationship study was carried out in order to determine the influence of various substituents on the DBH capacity of these compounds (Tables 2–4). With the ester derivatives, the Cl substitution at the 6-position by a hydroxy or morpholino group led to the total loss of the DBH-inhibiting potency (Table 2). The compounds GYKI 11686 [1-(6-hydroxy-3-pyridazinyl)-2-(1-ethoxycarbonyl)-2-propylidene) hydrazine] and GYKI 11636 {1-(6-morpholino - 3 - pyridazinyl) - 2 - [(ethoxycarbonyl)-2-ethylidene] hydrazine} did not show any DBH-inhibiting activity. The Cl substitution at the 6-position by a methyl or carbamoyl group also resulted in a decreased effectiveness (Table 2). Generally, the ketoester derivatives showed more favourable DBH-inhibiting characteristics than those of the free acids but the introduction of a morpholino group into the molecule of these acids led to an increased DBH-inhibiting potency as in the cases of GYKI 11792 and GYKI 11744.

The R_1 and R_2 substituents influenced only slightly the DBH-inhibiting potency of the compounds examined (Table 3). GYKI 11473 {1-(6-chloro-3-pyridazinyl) - 2 - [(1 - ethoxycarbonyl - 2 - propylidene)] hydrazine} and GYKI 11640 {1-(6-chloro-3-pyridazinyl) - 2 - [(1 - ethoxycarbonyl) - 2 - butylidene] hydrazine} were found to be almost equally potent DBH inhibitors (Table 3).

The length of the aliphatic chain of the esters does not alter the DBH-inhibiting capacity to a remarkable extent; the ethoxycarbonyl group was however more effective than the propoxy-, butoxy- or methoxycarbonyl groups (Table 4).

Mechanisms of enzyme inhibition in vitro

The *in vitro* TH-inhibiting potency of GYKI 11679 has already been described and compared to that of α-methyltyrosine in the previous section. GYKI 11468 and GYKI 11679 elicited non-competitive inhibitions of TH, whereas the inhibition of TH by α-methyltyrosine was found to be competitive with tyrosine [30].

For a detailed *in vitro* analysis of DBH inhibition by these new pyridazinyl hydrazones, one of the most effective substances (GYKI 11473) was selected and studied in detail. The kinetic analysis of experimental data indicated a reversible and competitive type of inhibition with DA and a non-competitive type of inhibition with tyramine as substrate in the presence of this compound (Table 5, Figs 1 and 2). The apparent K_i values were calculated as 3×10^{-5} and 4×10^{-6} M respectively. In a previous communication [24] we reported that GYKI 11473 inhibits the vesicular and synaptosomal uptake of DA in rat hypothalamic slices. In further studies, these results were confirmed and the apparent K_i values were found to be 3×10^{-5} and 1×10^{-4} M respectively. Inhibition was competitive with DA in both cases (Fig. 3).

Enzyme inhibition studies in vivo

A 50 mg/kg (0.16 nmoles) dose of GYKI 11473 and a 75 mg/kg dose (0.20 nmoles) of GYKI 11679 were injected into male 100-g rats i.p. in a 4% Tween

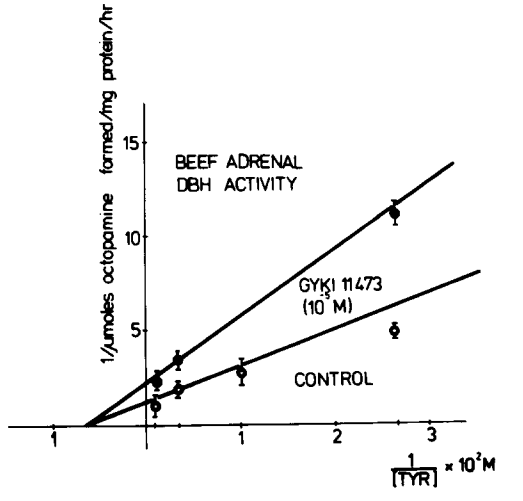


Fig. 1. Non-competitive inhibition of tyramine hydroxylation by GYKI 11473 using partially purified beef adrenal DBH as enzyme source.

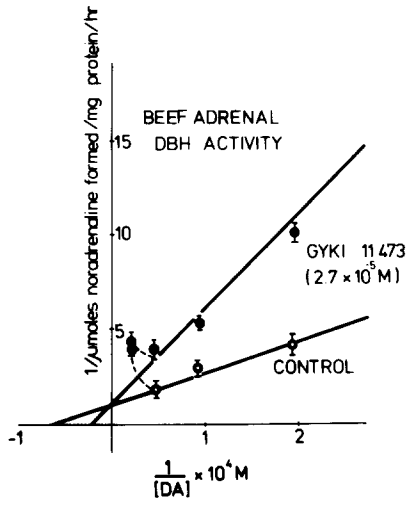


Fig. 2. Competitive inhibition of dopamine hydroxylation by GYKI 11473 using partially purified beef adrenal DBH as enzyme source.

80 suspension 1 hr prior to L-[3,5-^{3}H]tyrosine administration. Another group of animals received, as standard, 50 mg/kg (0.20 nmoles) α-methyltyrosine methylester. Controls were treated with 0.5 ml of 4% Tween 80 solution via a similar route and at the same time. The radioactive tyrosine was administered i.c.v. as indicated in Materials and Methods at a dose of 24 ng (6 μCi). One hour after tyrosine administration the [^{3}H]CAs as well as their metabolites were determined (Table 6).

GYKI 11473 (50 mg/kg) caused a 55% whereas GYKI 11679 (75 mg/kg) produced a 48% decrease in the amount of labeled CAs formed in the hypothalamus from the radioactive tyrosine. A 50 mg/kg i.p. dose of α-methyltyrosine methylester resulted in a similar percentage inhibition of the CA formation. The amounts of the labeled metabolites of CA in the hypothalamus of GYKI 11679 or α-methyltyrosine treated rats were less than that in the control animals. In contrast, GYKI 11473 treatment produced a slightly increased accumulation of these metabolites. This latter observation points to a decreased uptake of DA into the vesicles and indicates a partial contribution from uptake inhibition in the total *in vivo* effect of GYKI 11473. In similar experiments, 100 mg/kg of GYKI 11468 and GYKI 11295 caused only slight decreases in the newly synthetized CA content of the total brain.

The direct *in vivo* DBH-blocking capacity of GYKI 11473 was studied in 160- or 100-g male rats by measuring the [^{14}C]NA content in the heart and hypothalamus 1 hr after [^{14}C]DA injection. GYKI 11473 and fusaric acid in 0.5 ml of 4% Tween 80 suspension were administered to rats i.p. 24 and 3 hr prior to decapitation. Controls received 0.5 ml of 4% Tween 80 solution via a similar route and at the same time. Pargyline (100 mg/kg) (a potent inhibitor of MAO) was given to each animal 20 hr prior to the [^{14}C]DA injection. Rats were starved during the experimental period. The radioactive DA was injected i.p. or i.c.v. into the animals and 1 hr after the injection the radioactive NA content was determined either in the heart or in the hypothalamus as indicated in Tables 7 and 8. GYKI 11473 produced a marked reduction in the cardiac and a moderate decrease in the hypothalamic [^{14}C]NA content. An equal dose of fusaric acid blocked the [^{14}C]NA formation less significantly in both tissues (Tables 7 and 8). GYKI 11473 as well as fusaric acid showed higher efficiency in the periphery than in the brain.

The effects on endogenous NA content

It has previously been described that 3–10 mg/kg i.p. doses of GYKI 11679 produce marked 40–60% decreases in the endogenous NA level and a significant increase in NA turnover in the hypothalamus of rats [31]. This effect however reflects an increased release of this amine. The NA-releasing capacity of GYKI 11679 was demonstrated *in vitro* at a concn of 10^{-5}–10^{-6} M [31].

In our hands, 100 mg/kg of α-methyltyrosine resulted in an approximately 50% reduction in the cerebral NA content (Table 9), and this was comparable to those described by others [5, 32]. A

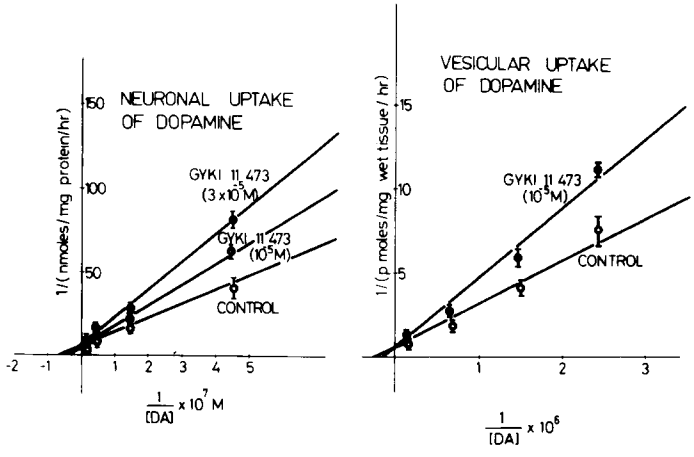


Fig. 3. Competitive inhibition of the neuronal and the vesicular uptake of dopamine by GYKI 11473 in rat hypothalamic slices.

Table 6. Inhibition of the formation of [^{3}H]catecholamines (CAs) from [^{3}H]tyrosine by GYKI 11473, GYKI 11679 and α-methyltyrosine methylester in rat hypothalamus

Treatment	Dose (mg/kg) (nmoles/kg)	[^{3}H]Tyrosine (dpm/mg tissue)	[^{3}H]CAs formed (dpm/mg tissue)	Total (%)	% of control	Deaminated [^{3}H]CA metabolites (dpm/mg tissue)	Total (%)	% of control
Control	—	1906 ± 38*	430 ± 8.6	22.46 ± 0.34	100	39.64 ± 1.50	2.08 ± 0.10	100
GYKI 11473	50 (0.16)	2439 ± 24	246 ± 2.4	10.10 ± 0.08‡	45	61.21 ± 1.80	2.51 ± 0.08†	121
GYKI 11679	75 (0.20)	1884 ± 94	222 ± 11.1	11.83 ± 0.68†	52	29.76 ± 1.21	1.58 ± 0.06‡	76
α-Methyltyrosine methylester	50 (0.20)	1961 ± 117	230 ± 13.8	11.73 ± 0.73†	52	31.77 ± 1.61	1.62 ± 0.09‡	78

* Mean ± S.E. Each group contained six animals.
† The difference from the control value is significant ($P < 0.001$).
‡ The difference from the control value is significant ($P < 0.01$).

100 mg/kg i.p. dose of GYKI 11295 causes only a slight (25%) decrease in the endogenous NA level. This effect and those obtained by the *in vivo* studies on NA formation show a relatively weak *in vivo* efficiency for this compound.

The DBH-blocking agents caused moderate decreases in the endogenous NA content (Table 10). Repeated administrations of 50 mg/kg GYKI 11473 produced a 35% decrease in the cardiac NA level and a 25% decrease in the cerebral NA content. An equal dose of fusaric acid resulted in an approximately 25% decrease in both tissues. Disulfiram (100 mg/kg) did not alter the endogenous NA concn significantly.

DISCUSSION

Pyridazinyl hydrazones represent a novel class of NA synthesis inhibitors since neither their structure nor their inhibiting properties relate to those of other TH or DBH blockers. Until now, TH inhibitors were characterized by three classes of compounds: (1) divalent metal-chelating agents which complex with ferrous ions, (2) di- or triphenolic compounds which compete with the reduced pteridine cofactor for the oxidized enzyme, and (3) α-methylated or halogenated analogues of several L-amino acids which compete with tyrosine for the reduced enzyme [10]. The inhibition of tyrosine hydroxylation by these pyridazinyl hydrazones showed a reversible but non-competitive type of inhibition. The *in vitro* efficiencies of the most active substances were found to be greater than that of α-methyltyrosine, the most useful representative of the inhibitors.

Until now, the dithiocarbamates [11], the aromatic and alkyl thioureas [12], the homopiperazinyl disulphides [13], the picolinic acids [14] and the imidazolines [15] have been described as potent inhibitors of DBH, the enzyme which converts DA to NA. Most of these inhibitors are thought to mediate their effect on the enzyme by chelating the cupric ion [15]. It seems that the inhibiting characteristics of these newly synthesised pyridazinyl hydrazones also differ from those described for the above compounds. GYKI 11473 showed a reversible and competitive type of inhibition with DA as substrate (Fig. 1) and a non-competitive type of inhibition with tyramine as substrate (Fig. 2). DA and tyramine are thought to be converted to NA and octopamine by the same enzyme. These observations however suggest different binding sites for these amines. Besides this inhibiting effect, the compound has been found to block both the neuronal and the vesicular uptake of DA in hypothalamic slices and these effects increased with decreasing concns of amine, showing a competitive type of inhibition (Fig. 3). The similarities observed in the inhibiting capacities and characteristics of GYKI 11473 in three different systems might indicate some similarities in the active site of DBH and the carriers of the neuronal and vesicular uptake systems. GYKI 11473 also appears to be a useful tool for this kind of study.

In spite of the fact that there are serious differences between TH and DBH in their structures and also in their coenzymes [10, 32, 33], a number of pyridazinyl hydrazones showed remarkable effects on

Table 7. Effects of GYKI 11473 and fusaric acid on the ^{14}[C]dopamine–[^{14}C]noradrenaline transformation in the heart of rats

Treatment	Dose (mg/kg)	Total activity (dpm/mg tissue)	% of control	[^{14}C]Noradrenaline (dpm/mg tissue)	% of control
Control	—	119 ± 25.0*	100	30.24 ± 5.7	100
GYKI 11473	2 × 50	125 ± 5.6†	105	10.29 ± 0.7‡	34
Fusaric acid	2 × 50	124 ± 9.2†	104	13.22 ± 2.1‡	44

* Mean ± S.E. Each group contained six animals.
† The difference from the control values is not significant.
‡ The difference from the control value is significant ($P < 0.001$).

Table 8. Effects of GYKI 11473 and fusaric acid on the [^{14}C]dopamine–[^{14}C]noradrenaline transformation in the hypothalamus of rats

Treatment	Dose (mg/kg i.p.)	Total activity (dpm/mg tissue)	% of control	[^{14}C]Noradrenaline (dpm/mg tissue)	% of control
Control	—	4041 ± 348*	100	502.7 ± 40.1	100
GYKI 11473	2 × 50	3007 ± 239	74	311.6 ± 32.7†	62.5
Fusaric acid	2 × 50	4253 ± 425	105	437.3 ± 15.9‡	87.6

* Mean ± S.E. Each group contained six animals.
† The difference from the control value is significant ($P < 0.01$).
‡ The difference from the control value is not significant.

Table 9. The effect of GYKI 11295 and α-methyltyrosine methylester on the cerebral noradrenaline content

Treatment	Dose (mg/kg i.p.)	Noradrenaline (μg/g tissue)	% of control
Control	—	0.49 ± 0.02* (12)§	100
GYKI 11295	100	0.37 ± 0.05† (6)	75
α-Methyltyrosine methylester	100	0.26 ± 0.03‡ (6)	53

* Mean ± S.E.
† The difference from the control values is significant ($P < 0.025$).
‡ The difference from the control value is significant ($P < 0.001$).
§ Number of animals in parentheses.

both enzymes *in vitro* (Table 1). It seems reasonable that steric factors which affect penetration to the active sites of the enzymes play the main role in these inhibitions.

The structure–activity analysis of several 6-substituted 3-pyridazinyl hydrazones showed that relatively great TH- and DBH-inhibiting effects could be observed with the N^2 hydrogen of hydrazine is substituted. The hydrogen substitution with either a cyclohexylidene or a bicycloheptylidene group strengthens the TH-inhibiting capacity of the compounds whereas the introduction of an aliphatic ketoester into the molecule results in a relatively strong DBH-inhibiting effect. Among the β-substituted aliphatic ketoesters of pyridazinyl hydrazones, the 6-chloro derivatives produced marked inhibition of dopamine hydroxylation while the 6-morpholino derivatives showed a potent TH-blocking capacity. The compound GYKI 11473 {chemically 1-(6-chloro-3-pyridazinyl)-2-[(1-ethoxycarbonyl)-2-propylidene] hydrazine} showed a smaller *in vitro* DBH-inhibiting potency but a higher *in vivo* activity than that of fusaric acid. A slight modification in the ketoester group or the alkyl chain led to relatively small changes in the inhibiting potency. In contrast, the 6-substitution of the pyridazinyl ring could influence markedly the inhibiting characteristics of the compounds. The introduction of a group such as a morpholino group into the molecule at the 6-position (especially in the ester derivatives) results in a total loss of DBH-inhibiting capacity. In this group, a number of morpholino derivatives showed a potent TH-inhibiting effect such as compound GYKI 11679. The β-substituted aliphatic acids of these pyridazinyl hydrazones showed a favourable DBH-inhibiting potency *in vitro* even in the case when a morpholino group was introduced into the pyridazine ring at the 6-position (Table 2). These morpholino derivatives might bind to the copper of the enzyme and therefore these substances might inhibit DA hydroxylation to some extent. It seems however that the inhibition by these compounds, like that obtained for the ketoester derivatives, is not due primarily to the formation of chelates with the copper of DBH.

The *in vivo* studies reflected relatively weak absorptions of these pyridazinyl hydrazones into the

Table 10. The effect of GYKI 11473, fusaric acid and disulfiram on the cardiac and cerebral noradrenaline contents

Treatment	Dose (mg/kg i.p.)	Noradrenaline (μg/g tissue) Brain	% of control	Heart	% of control
Control	—	0.53 ± 0.03	100	1.06 ± 0.08*	100
GYKI 11473	50	0.39 ± 0.01†	75	0.69 ± 0.07†	65
Disulfiram	100	0.42 ± 0.02†	79	0.84 ± 0.07‡	79
Fusaric acid	50	0.41 ± 0.01†	77	0.75 ± 0.06†	71

* Mean ± S.E. Each group contained six animals.
† The difference from the control value is significant ($P < 0.01$).
‡ The difference from the control value is not significant.

tissues and therefore their *in vivo* effects were much smaller than those observed *in vitro*. There were however some exceptions. GYKI 11679 caused a marked inhibition of tyrosine hydroxylation both *in vitro* and *in vivo* (Table 6). Its NA-lowering effect was found to be even higher than that calculated on the basis of its *in vitro* TH-inhibiting potency. This discrepancy however could be solved if we consider that this effect reflects both the TH-inhibiting and the NA-releasing capacities of GYKI 11679 [31]. GYKI 11473 also caused remarkable effects *in vivo*. The inhibition of DA hydroxylation by the compound has been found to be higher in the periphery that in the brain; a 66% reduction in the cardiac and a 38% decrease in the hypothalamic NA formation have been observed after an injection of labeled DA in GYKI 11473 pretreated rats. When radioactive tyrosine was injected into animals, the reduction in the hypothalamic CA formation was greater than that in the earlier experiments, but in this case the inhibition of the vesicular uptake of DA by the compound modifies the total effect (Table 6).

The NA depletion studies showed smaller effects when DBH inhibitors were administered to animals than when TH inhibitors were injected. Unfortunately, the compounds showed TH- as well as DBH-inhibiting potencies *in vitro*, and have been found to be ineffective or slightly effective *in vivo*, and therefore we were unable to establish the proportion of the participation of TH and DBH in the maintenance of the cardiac and hypothalamic NA pools. Experiments with GYKI 11473, a new potent inhibitor of DBH, reflected a slight but direct effect on the cerebral NA content and suggested a secondary but non-negligible role of DBH in NA synthesis. The relative insensitivity of cardiac NA towards the inhibitors [either TH (unpublished observation) or DBH] confirms the relatively smaller role of synthesis in the maintainance of these pools. In this case, the uptake of NA into the tissue is much more important than the synthesis of this amine [34].

Both GYKI 11473 and GYKI 11679 [32] were shown to produce hypotension [17, 18]. The antihypertensive effect of GYKI 11473 showed a close parallelism with its peripheral DBH-inhibiting capacity. A 50 mg/kg i.p. dose of the compound caused an approximately 70% inhibition of the DA–NA transformation in the heart, and resulted in an approximately 35% decrease in the cardiac NA level. The same dose of the compound elicited a remarkable antihypertensive effect in spontaneously hypertensive rats [18]. GYKI 11679, a novel substance with a remarkable TH-inhibiting capacity, lowered the hypothalamic NA level at relatively low (3–10 mg/kg) oral doses [31]. The same doses produced marked reductions in the blood pressure of spontaneously hypertensive rats [17, 18]. The central depletion of the NA stores, produced by low doses of the compound, is probably the consequence of an increased NA release which leads to a concomitantly increased turnover of hypothalamic NA; and this increased sympathetic activity might result in a decreased peripheral vascular tone and a lowered blood pressure. Alterations of the central NA synthesis might be involved differently in blood pressure regulation. Both an increased and a decreased turnover of NA might produce a lowered blood pressure depending on its localization [35, 36]. It seems that in the periphery however the NA synthesis inhibition produces hypotension in all cases [37–39].

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