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Structure–activity relationships for substrates and inhibitors of pineal 5-hydroxytryptamine-*N*-acetyltransferase: preliminary studies

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Abstract

Tryptamine, (1-naphthyl)ethylamine and phenethylamine derivatives were tested as substrates of ovine pineal serotonin-*N*-acetyl transferase (5-HT-NAT), a key enzyme involved in the synthesis of melatonin. Almost all of the indole derivatives possessed affinity similar to that of tryptamine (K_m = 0.05 mM), while the substituted naphthalene and phenyl derivatives were less potent. However, the K_m values seem be influenced by the steric hindrance and polar properties of the substituent. V_{max} values for the naphthyl and phenyl derivatives were generally 10–20-fold higher than those of the indole derivatives and no clear structure–activity relationship was observed. Melatonin and several bioisoteric derivatives were shown to be inhibitors of 5-HT-*N*-acetyltransferase. Preliminary data suggested that over the 5–50-μM concentration range, melatonin was a competitive inhibitor (IC_{50} = 10 μM) with a concentration-dependent inhibitory effect on its own synthesis in the pineal gland. However, the bioisosteric naphthalene derivatives were characterized instead as mixed inhibitors. (1-Napthyl)ethylacetamido, a putative melatoninergic antagonist, was also shown to be an inhibitor of 5-HT-*N*-acetyltransferase (IC_{50} = 8 μM) and is a promising tool for the regulation of melatonin synthesis and the understanding of its role.

Keywords: Melatonin; Pineal gland, ovine; 5-HT (hydroxytryptamine, serotonin)-*N*-acetyltransferase; Tryptamine

1. Introduction

Melatonin is a pineal hormone which modulates a variety of endocrinological, neurophysiological and behavioural functions in vertebrates (Reiter, 1991a). It is implicated in the reproduction of photoperiodic species and the regulation of circadian rhythms (Reiter, 1993). It also plays a key role in a number of disorders, such as seasonal depression (Partonen, 1994) and delayed sleep-phase syndrome (Palm et al., 1991). More recently, an influence of melatonin on the responsiveness of the immune system has been described (Maestroni, 1993). The synthesis of melatonin occurs mainly in the pinealocytes of the pineal gland where it reaches its maximum level during the night (Reiter, 1991a). On the other hand, it is also synthesized within retina where it acts locally. Therefore, melatonin has been described as 'the chemical expression of darkness' (Reiter, 1991b; Utiger, 1992). It exerts its effect through receptors which have been characterized using binding assays with 2-[^{125}I]iodomelatonin (Krause and Dubocovich, 1991; Dubocovich, 1995). Recently, several new melatonin receptor agonists (Yous et al., 1992; Copinga et al., 1993; Depreux et al., 1994; Garrat and Vonhoff, 1994a,b, Garrat et al., 1995; Langlois et al., 1995) have been described and they constitute important tools for studying the physiological role of this neurohormone. However, the availability of melatonin receptor antagonists and inhibitors of its synthesis is essential for the characterization of melatonin receptors and to understand the role of melatonin in the events described above. For this purpose, the inhibitors of the enzymes implicated in the melatonin synthesis in the pineal gland could constitute very useful pharmacological tools. To date, no compound possessing either of these properties has been described.

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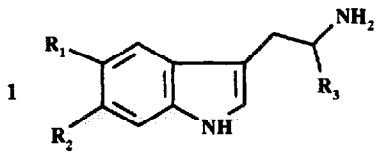
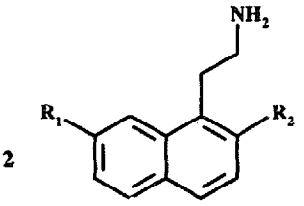
Table 1
Kinetic parameters for the acetylation of the tryptamine derivatives **1** by 5-HT-NAT



Compound	R_1	R_2	R_3	K_m (mM)	V_{max} (nmol/min/mg protein)
5	H	H	H	0.05±0.002	0.20±0.002
6	H	H	Me	0.05±0.002	0.05±0.001
7	F	H	H	0.01±0.001	0.20±0.003
8	Cl	H	H	0.01±0.001	0.07±0.004
9	Me	H	H	0.01±0.002	0.05±0.0013
10	BzO	H	H	0.25±0.030	0.17±0.013
11	MeO	H	H	0.01±0.001	0.06±0.0025
12	OH	H	H	0.04±0.002	0.02±0.001
13	OH	H	Me	0.02±0.002	0.05±0.005
14	$CONH_2$	H	H	Inactive	
15	H	MeO	H	0.29±0.026	0.08±0.005
16	H	F	H	0.05±0.004	0.30±0.02

Sheep pineal supernatant preparations were preincubated with pargyline (1 mM) and [^{14}C]acetyl-CoA for 10 min at 37°C. Six different amine concentrations were added in a total volume of 100 μl of 100 mM sodium phosphate buffer (pH 6.8) and the mixture was incubated for 10 min (37°C). The reaction was terminated by the addition of 1 ml $CHCl_3$. The amount of ^{14}C-acetylated compound was measured in a scintillation counter. The assay was done in triplicate and the kinetic constants (K_m and V_{max}) were estimated using a linearization adjustment with Eadie–Hofstee representation.

Table 2
Kinetic parameters for the acetylation of the naphthalene derivatives **2** by 5-HT-NAT



Cpd	R_1	R_2	K_m (mM)	V_{max} (nmol/min/mg protein)
17	H	H	0.02±0.001	0.11±0.002
18	MeO	H	0.54±0.06	0.07±0.003
19	H	MeO	0.24±0.024	0.26±0.015
20	H	EtO	0.51±0.02	0.24±0.006
21	MeO	MeO	0.61±0.03	0.46±0.017

See footnotes for Table 1.

We present here our initial results on the structure–activity relationships for substrates and inhibitors of serotonin-*N*-acetyltransferase (5-HT-NAT) (arylalkylamine *N*-acetyltranferase, EC 2.3.1.87; NAT), a key enzyme in the synthesis of melatonin (Deguchi, 1992). It is synthesized by the consecutive actions of two enzymes: 5-HT-*N*-acetyltransferase which acetylates serotonin and hydroxyindole-*O*-methyltransferase which catalyzes the process of *O*-methylation of the indole ring to provide melatonin (Fig. 1).

5-HT-*N*-acetyltransferase is present in the retina (Zawilska and Nowak, 1992) and pineal gland (Rudeen et al., 1975) of various vertebrate species. The amount of melatonin produced depends essentially upon the activity of 5-HT-*N*-acetyltransferase (Reiter, 1993; Humlova and Illnerova, 1992) which is triggered by the onset of darkness and suppressed by light. Several mechanisms of control have been implicated in the regulation of melatonin synthesis. For instance, the release of dopamine involving the D_4 receptor subtype (Zawilska and Nowak, 1994) seems to control the decrease of melatonin synthesis in the retina and stimulation of α- and β-adrenoceptors by norepinephrine regulates the enzymatic activity of 5-HT-*N*-acetyltransferase in pinealocytes (Klein et al., 1983). Information on the molecular recognition by 5-HT-*N*-acetyltransferase of structurally closely related molecules mimicking serotonin or melatonin is essential to evaluate the specificity of structures derived from the indole moiety for this enzyme and for the design of inhibitors. We report, herein, the first results for the structure–activity relationships obtained with various substituted indoles **1**, (1-naphthyl)ethylamines **2** and substituted phenethylamines **3** which were tested as amines (Tables 1–3) or acetylated derivatives **4** (Table 4). Several papers have reported the synthesis of potent ligands for melatonin receptors derived from naphthalene (Yous et al., 1992; Depreux et al., 1994; Langlois et al., 1995) which possess good bioisosteric

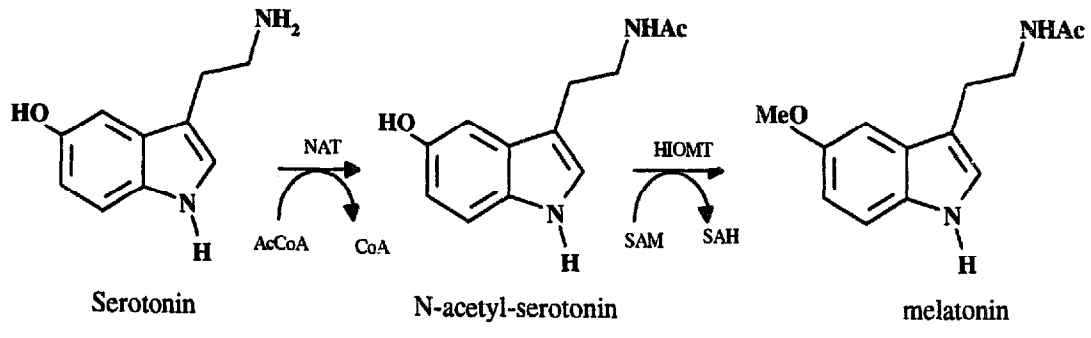
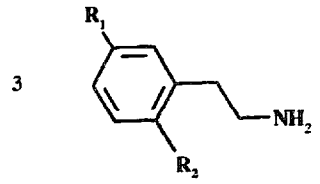


Fig. 1. Synthetic pathway for melatonin in the pineal gland.

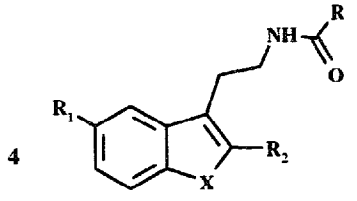
Table 3
Kinetic parameters for the acetylation of the phenethylamine derivatives **3** by 5-HT-NAT



Cpd	R_1	R_2	K_m (mM)	V_{max} (nmol/min/mg protein)
22	OH	H	inactive	
23	F	H	0.56±0.09	0.26±0.03
24	Me	MeO	0.53±0.04	0.43±0.02
25	Et	MeO	0.64±0.08	0.45±0.03
26	MeO	MeO	0.33±0.03	0.16±0.008
27	EtO	MeO	0.73±0.04	0.36±0.013

See footnotes for Table 1.

Table 4
Inhibitory activity of amido derivatives of compounds **4** for 5-HT-NAT



Cpd	X	R_1	R_2	R	IC_{50} (μM)[a]	Binding assay (K_i, nM)[b]
28	NH	H	H	Me	88±5.4	494±32
29	"	MeO	"	"	9.9±0.97	0.67±0.04
30	"	"	I	"	5.5±0.7	0.032±0.003
31	"	HO	H	"	19.9±7.8	1640±180
32	"	Cl	"	"	1.4±0.4.8	119±46
33	CH=CH	H	"	"	8±0.69	321±33
34	"	MeO	"	"	36±10	0.54±0.03
35	"	H	MeO	"	5±0.7	2.68±0.3
36	"	"	"	Et	7.8±60.8	0.67±0.05
37	"	"	"	n-Pr	>5000	1.37±0.22
38	"	"	H	c-C_3H_5	1600±590	19.4±2.7
39	"	MeO	MeO	Me	139±44	0.1±0.04

[a] Inhibition of 5-HT-NAT was performed with tryptamine (0.05 mM) as the substrate and 3 concentrations of the compound (10^{-6}, 10^{-5}, 10^{-4} M) under test. After preincubation with pargyline (1 mM) and [^{14}C]acetyl-CoA (1 mM) for 10 min at 37°C, the pineal supernatant was incubated with tryptamine and the test compound for 12 min in a final volume of 100 μl of buffer (pH 6.8, 37°C). The assay was done in triplicate and the inhibition curves were analysed with a computer-assisted curve-fitting program (Lotus or Graphpad).

[b] K_i values were evaluated in vitro in binding assays using 2-[^{125}I]iodomelatonin (0.05 nM) and chicken brain membranes (25°C, 60 min). Seven concentrations of the compound under test were used and each assay was performed in triplicate. K_i values are expressed in nM±S.E.M. and were calculated using the Cheng–Prussof equation (Hulme and Birdsall, 1992) and from IC_{50} values obtained from the competition curves: the data are the results of one or two separate determinations in triplicate.

properties for the melatonin receptor with regard to the indole ring. Therefore, it was considered worthwhile as the first step of our study to compare the molecular recognition of those different chemical structures by the enzyme (Tables 1 and 2) and to evaluate, in this preliminary search for 5-HT-*N*-acetyltransferase inhibitors, the potential inhibitory properties of melatonin and of several structurally related amidic compounds **4** (X = NH and CH = CH). The IC_{50} values for the amido compounds were compared to their affinities for the melatonin receptor evaluated by binding assays in chicken brain membranes. The compounds were prepared according to methods recently reported (Langlois et al., 1995) for the synthesis of new non-indolic melatoninergic agents.

In almost all animal species, 5-HT-*N*-acetyltransferase activity decreases dramatically during the light phase, the lowest activity being in the middle of this phase (Reiter, 1993). However, the size of the diurnal variation is species-dependent; thus, for the rat, variation can be up to 60 times greater during darkness than during light (Rudeen et al., 1975). In contrast, the variation is less marked in sheep (Namboodiri et al., 1987) and the gland is relatively larger. For these reasons, the ovine pineal gland was selected for the enzymatic assay. The enzymatic activity of 5-HT-*N*-acetyltransferase was determined, using a pineal supernatant preparation from sheep slaughtered at 04:00–05:00 h, according to the modified method of Deguchi (Deguchi and Axelrod, 1972) where acetylation of the arylalkyl amines in the presence of [^{14}C]acetyl CoA (coenzyme A) gave the [^{14}C]acetyl-arylalkylamines which were extracted with chloroform.

2. Materials and methods

2.1. Preparation of the enzyme

Pineal glands were collected from sheep of mixed age and sex (Cellu-Bio, France) slaughtered at 04:00–05:00 h from a local abattoir. The brains were dissected, the pineal glands were removed and quickly frozen on dry ice. They were stored at −80°C until use. Tissues were weighed and homogenized (Polytron) at 4°C in sodium phosphate buffer pH 6.8 (1 mg wet tissue/10 μl buffer solution). The homogenate was centrifuged for 30 min at 10000×*g* (0–2°C) and the supernatant was used as the enzyme source. It was stored in 50-μl aliquots at −80°C. They were used within 2 months.

2.2. Protein estimation

Protein content was measured using a dye-binding method (Bradford, 1976) with bovine serum albumin as the standard.

2.3. 5-HT-N-acetyltransferase activity assay

2.3.1. Kinetic characterization of the substrates

A modification of Deguchi and Axelrod's method (1972) was employed using the different arylalkyl amines as acceptors and [^{14}C]acetyl CoA as the acetyl donor. A 50-μl aliquot of the enzyme preparation was incubated with pargyline and [^{14}C]acetyl CoA in 1mM final concentration in 100 mM sodium phosphate buffer (pH 6.8) at 37°C for 10 min. The amino derivatives over a concentration range of 10^{-6}–10^{-3} M were then added and incubated for 10 min in a final volume of 100 μl. The reaction was terminated by the addition of 1 ml of water-saturated chloroform at 4°C and the *N*-[^{14}C]acetyl amine was extracted. The organic layer was separated by centrifugation and an 800-μl aliquot was placed in a scintillation vial and evaporated overnight under a ventilated hood. 4 ml of scintillation cocktail (Ready-Safe) was added and, after a 24-h equilibration period, the amount of the radioactivity of the *N*-[^{14}C]acetyl amine present was determined using a liquid scintillation β spectrometer (Beckman). Blank assays was carried out under similar conditions by omitting the amine and the non-specific activity represented 40% of the total enzymatic activity. Each assay was done in triplicate.

The kinetic constants (K_m and V_{max}) were calculated by a linearization adjustment with Eadie-Hofstee representation (Price and Stevens, 1989) using Lotus and Graphpad. Data are the results of one or two determinations.

2.4. Inhibition of 5-HT-N-acetyltransferase activity

The assay was performed according to the conditions described above for the determination of the kinetic parameters of the amine substrates: the enzyme preparation was preincubated with pargyline and [^{14}C]acetyl CoA in sodium phosphate buffer (pH 6.8), followed by the addition of tryptamine (0.05 mM), used as the substrate and the test compound at 3 different concentrations (10^{-6}–10^{-4} M) in a final volume of 100 μl. The reference rate was determined by omitting the test compound. Data are the results of one or two determinations.

2.5. Data analysis

Estimation of the concentration inhibiting 50% of the reference rate (IC_{50}) was carried out using 3 concentrations of the potentially inhibitory compound and was calculated using Lotus and Graphpad.

Determination of the type of inhibition was made using Lineweaver–Burk and Dixon plots (Price and Stevens, 1989) based on measuring the initial velocity at 2 different substrate concentrations (tryptamine 0.05 mM and 1 mM) in the absence or the presence of 3 increasing concentrations of inhibitor (3, 10 and 30 μM). K_i value of melatonin was calculated from the Lineweaver–Burk and Dixon plots.

2.6. Determination of the melatonin concentration in the ovine pineal gland

Individual pineal glands were sonicated (30 s) in 0.2 M perchloric acid containing 0.1% of sodium metabisulfite and 0.1% of EDTA in 700 μl final volume using Vibra-cell microsonicator (20 kHz, 40 W). The homogenates were centrifugated for 5 min at 15000 × *g* (4°C) and the clear supernatant were stored at −80°C prior to the direct injection (20 μl) into the HPLC (high-pressure liquid chromatography) system.

The separation of melatonin was achieved by reverse-phase liquid chromatography, using a Beckman Ultrasphere C18 5 μm column (150 × 4.6 mm). The mobile phase (flow rate 1.2 ml/min) consisted of pH 4.70 water/acetonitrile (80/20) containing 0.01 mM EDTA, 0.1 mM KH_2PO_4 and 0.5 mM octane sulfonic acid (Pic B8, Waters). Electrochemical detection was performed using an EG&G model 400 amperometric detector at 0.80 V working potential (glassy carbon electrode) relative to an Ag/AgCl electrode. The sensitivity limit was 15 pg for a 3:1 signal:noise ratio. Melatonin was quantified from measurement of the peak height with regard to a single point standard. A linear response was obtained over the range of the observed peak heights. Protein content of the pineal gland was determined by the Bradford method (1977).

2.7. Melatonin receptor-binding assay

Chickens (Red Brook, 4 months, 3–4 kg; Cellubio, France) were decapitated at 12:00 h. The whole brains were quickly removed and stored at −80°C. They were homogenized (Polytron) in 10 vols. of ice-cold Tris–HCl buffer (50 mM, pH 7.4) and washed twice by centrifugation (44000 × *g*, 25 min, 4°C). The resulting pellet was resuspended in 10 vols. of the same buffer to a final concentration of 5 or 6 mg protein/ml determined by the method of Lowry et al. (1951). The membrane aliquots were stored at −80°C until subsequent use. Membranes aliquots (30 μl) were incubated in a total volume of 0.25 ml Tris–HCl buffer (50 mM, pH 7.4) with 0.05 nM of 2-[^{125}I]iodomelatonin and seven concentrations of the compound under test. Each binding assay was performed in triplicate. The incubation (25°C, 60 min) was stopped by the addition of 3 ml of ice-cold buffer and immediate vacuum filtration through glass fiber filters (GF/B Whatman strips) presoaked in 0.1% poly(ethyleneimine) using a Brandel cell harvester. The filters were washed (3 × 4 ml) with buffer, dried, and counted on a γ-counter (Crystal-Packard). Non-specific binding was defined with 10 μM 2-iodomelatonin and represented 10% of the total binding.

2.8. Chemical compounds

[^{14}C]Acetyl CoA (specific activity 50–60 μCi/mmol) was purchased from Amersham (Les Ulis, France) and 2-[^{125}I]iodomelatonin (specific activity 2000–2200 Ci/mmol) was purchased from NEN (Les Ulis, France) or Amersham. The different chemical compounds were obtained from the following source: melatonin, *N*-acetyl-5-hydroxytryptamine, tryptamine hydrochloride, DL-α-methyl-tryptamine, 5-methyl-tryptamine, 5-benzyloxytryptamine, 6-methoxytryptamine hydrochloride, DL-α-methylserotonin, 3-fluorophenylethylamine and 2,5-dimethoxyphenethylamine were purchased from Aldrich (Strasbourg, France). Acetyl CoA, pargyline, tryptamine 5-fluorotryptamine, 6-fluorotryptamine and serotonin creatine sulfate were purchased from Sigma Chemical Company (Strasbourg, France). The scintillation cocktail (Ready-Safe) was purchased from Beckman (Les Ulis, France). 5-methoxy-tryptamine hydrochloride, 5-carboxamidotryptamine and 3-hydroxyphenylethylamine were purchased from RBI (Strasbourg, France)

2-(2-methoxy-5-ethylphenyl)ethylamine, 2-(2-methoxy-5-methylphenyl)ethylamine, 2-methoxy-5-ethoxyphenethylamine, naphthylethylamine, 2-(2-methoxynaphthyl)ethyl amine, 2-(6-methoxynaphthyl)ethylamine, 2-(2-ethoxynaphthyl)ethylamine and 2-(2,6-dimethoxynaphthyl)ethylamine were prepared by the different methods reported recently (Depreux et al., 1994; Langlois et al., 1995).

N-acetyltryptamine, *N*-acetyl-5-chlorotryptamine, *N*-acetyl-5-benzyloxytryptamine, *N*-acetyl-6-methoxytryptamine, *N*-propionyl-6-fluoro-5-methoxytryptamine, *N*-cyclo-propylcarbonyl-6-fluoro-5-methoxytryptamine, *N*-acetyl-naphthylethylamine, *N*-(2-(6-methoxynaphthyl-ethyl) acetamide, *N*-(2-(2-methoxynaphthy)ethyl) acetamide, *N*-(2-(2-methoxynaphthy)ethyl) propionamide, *N*-(2-(2-methoxynaphthy)ethyl) butyramide, *N*-(2-(2-methoxynaphthy)ethyl) cyclopropylformamide, *N*-(2-(2-7-dimethoxy-naphthy)ethyl) acetamide and *N*-(2-(2-7-dimethoxy-naphthy)ethyl) propionamide were prepared by the different methods of acylation reported recently (Langlois et al., 1995) for the preparation of melatoninergic ligands.

3. Results

The data reported in Table 1 demonstrate the weak influence of substituents on the 5 position of the indole ring, such as chlorine and fluorine atoms, or OMe, OH and Me groups (compounds **7**, **8**, **9**, **11** and **12**) on the K_m values which were equipotent to that of tryptamine. The K_m values calculated for tryptamine, 5-MeO-tryptamine and 5-HT were identical to those reported in a previous study (Voisin et al., 1984). On the other hand, a bulky group, such as benzyl (compound **10**), or the polar carboxamido group (compound **14**) brought about a decrease or a dramatic drop in the affinity, respectively. It is also worthwhile noting the equipotency of the branched compounds **6** and **13** with tryptamine and serotonin, although these structural modifications were shown to be unfavourable for recognition by the melatonin receptor (Langlois et al., 1995).

Conflicting results were obtained with the substituent on the 6 position as a clear drop in affinity was observed with compound **15**, which was clearly less potent than 5-methoxy-tryptamine, while substitution of a fluorine in this position affected the affinity of compound **16** to a lesser extent with regard to **7**. The efficiency of the enzyme (V_{max}) depended upon the nature of the substituent and, for almost all the compounds, substitution on the indole ring decreased the V_{max} value, except for the fluorine atom (compounds **7** and **16**) and the benzyloxy group (compound **10**) which were acetylated at the same rate as tryptamine.

The kinetic parameters of the 1-naphthylethylamine derivatives **2**, precursors of the new potent melatoninergic ligands described recently, are reported in Table 2. The data show, in contrast to the indole derivatives, a marked decrease in affinity with the methoxy or ethoxy derivatives with regard to the unsubstituted compound **17** which possessed an affinity similar to that of tryptamine. In particular, compound **18** was 50-fold less potent than 5-MeO-tryptamine **11**, indicating that the molecular recognition properties of 5-HT-*N*-acetyltransferase for substrates are dramatically different from those of the melatonin receptor. On the other hand and, in contrast to the indole derivatives, a remarkable increase in the rate of acetylation was observed. In particular, the dimethoxy derivative **21** was characterized by a high V_{max} value compared to its low affinity (K_m = 0.61 mM).

The design of new melatoninergic ligands was initially based on the structural analogy of serotonin and melatonin, and 2–5 dimethoxyphenylethylamine, a moiety well-known for its serotoninergic properties (Glennon et al., 1986), was used initially in the development of potent melatoninergic ligands. Consequently, we examined the properties of a number of arylethylamines **3** as substrates for 5-HT-*N*-acetyltransferase and the results are reported in Table 3. No clear relationship between the nature of the substituent and the affinity (K_m) was seen and the compounds were poor substrates with regard to tryptamine or 1-naphtylethylamine. On the other hand, the efficiency of the acetylation reaction was similar to that observed with the substituted naphtylethyl amines **2**.

Enzyme inhibitors can be designed from substrates either by using the transition state analogy (Lindquist, 1975; Frick and Wolfenden, 1989) during the enzymatic reaction or by using enzymatic properties to alter molecules in reactive species leading to irreversible inhibition (Shaw, 1989; Tipton, 1989). However, in the initial studies reported herein, we examined whether melatonin **29** could regulate its own synthesis by exerting a concentration-de-

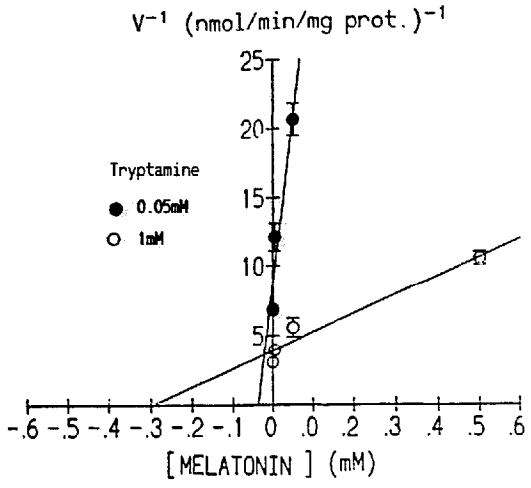


Fig. 2. Inhibition of 5-HT-*N*-acetyltranferase in ovine pineal gland (0.05 mM and 1 mM tryptamine as the substrate) by different concentrations of melatonin (0–500 μM). Results are represented as Dixon plots. The acetyl-CoA concentration was 1 mM. Each assay was performed in triplicate.

pendent inhibitory effect on the enzyme. We were encouraged by the marked inhibitory effect observed with melatonin itself (IC_{50} = 10 μM) using tryptamine as substrate to investigate the potential inhibitory properties of a number of amido derivatives designed as potential melatoninergic ligands (Depreux et al., 1994; Langlois et al., 1995). The IC_{50} values for inhibition of the enzyme by the different compounds were compared to their affinity values (K_i) for melatonin receptors which were evaluated in binding assays in chicken brain membranes using 2-[^{125}I]iodomelatonin as the radioligand (Table 3). The results show that almost of all the compounds possessed inhibitory activity in the 1–100-μM range, except for the naphthalene derivatives **37–39**. Then, we compared the inhibitory activity of the potent melatoninergic ligands **34** and **35** (IC_{50} = 5 and 36 μM, respectively) and the putative melatoninergic antagonist **33** (Brémont et al., 1994) to melatonin. Preliminary determinations of the inhibition profiles by the Lineweaver–Burk plot showed that a concentration-dependent decrease of V_{max} value was observed with compounds **33–35**, suggesting a profile of mixed inhibitory activity for these compounds. On the other hand, the V_{max} value was not affected by melatonin in the 5–50-μM concentrations range and a competitive profile was confirmed by the Dixon plot where the lines intersect above the abscissa (Fig. 2). Melatonin concentration in the pineal gland was determined by reverse-phase liquid chromatography and was found to be 17.2 ± 4.2 (n = 4) ng/mg protein corresponding to 3.1 ± 0.6 μM concentration.

4. Discussion

The results reported in the present study demonstrate that 5-HT-*N*-acetyltransferase recognizes the indole ring better than other ring systems as almost all of the indolic derivatives studied as substrates possessed a higher affinity for this enzyme than did the bioisosteric amines derived from naphthalene or benzene ring. This is an important finding for the identification and design of specific compounds, such as a specific inhibitor of 5-HT-*N*-acetyltranferase capable of interacting with the melatoninergic system in the pineal gland. On the other hand, we also showed a clear drop in the V_{max} value with the introduction of a substituent on the 5 position of the indole ring indicating a decrease in enzymatic efficacy which could be due to the acetylation product having good affinity for the enzymatic site compared to that of *N*-acetyl-tryptamine. In particular, the K_m value of 40 μM for 5-HT (compound **12**) is similar to that of tryptamine **5** (K_m = 50 μM) and indicates the weak influence of the hydroxy group on the binding of the substrate to the enzymatic site. However, the V_{max} value was 10-fold lower for 5-HT than for tryptamine. These data could explain the recent report on the inhibition of 5-HT-*N*-acetyltransferase activity by 5-HT in frog retina (Alonso-Gomez et al., 1995) where 5-HT found to act as a strong mixed inhibitor with tryptamine as the substrate. The kinetic parameters of the fluoro derivatives **7** and **16** had similar values to those of tryptamine and, in particular, the similar V_{max} values should be noted. These data suggest that the values for the kinetic parameters could be related to the size of the substituent on the aromatic ring because it is well-known that the steric hindrance of the fluorine atom is similar to that of hydrogen and, consequently, compounds **7** and **16** could mimic the structure of tryptamine.

The inhibitory effect of melatonin on 5-HT-*N*-acetyltransferase suggests that it could regulate its own synthesis by exerting a concentration-dependent inhibitory effect on this enzyme. Nevertheless, the IC_{50} value of 10 μM for melatonin is about 4 orders of magnitude less potent than it is with melatonin receptors. Endogenous melatonin concentration in the pineal gland was measured by reverse-phase chromatography and was 3.1 ± 0.6 μM. Thus, the competitive inhibition of 5-HT-*N*-acetyltransferase by melatonin in the 5–50-μM range seen in this first study where a K_i value of 5 μM was calculated could be relevant biologically. The data reported for the indolic derivatives suggests that their inhibitory activity could not be related to their affinity for the melatonin receptor. Although 2-iodo-melatonin **30** was one of the most potent inhibitory compounds, while *N*-acetyl-tryptamine **28**, a ligand with affinity in the micromolar range, was clearly less active, 5-chloro-*N*-acetyl-tryptamine **32**, a ligand with low affinity for melatonin receptors (K_i = 119 nM) was found to be the most potent inhibitor (IC_{50} = 1.45 μM). In the naphthalene series, the unsubstituted compound **33** was a more potent inhibitor of 5-HT-*N*-acetyltransferase than the melatonin receptor bioisostere **34**. Nevertheless, the melatoninergic ligands **34** and **35** did inhibit 5-HT-*N*-

acetyltransferase, their potency depending upon the position of the substituent methoxy on the naphthalene ring. Thus, the potent melatoninergic ligand **35** (Langlois et al., 1995) ($K_i = 2$ nM), possessing a methoxy group in the ortho positon of the side-chain, was more potent than the 7-methoxy isomer **34** ($IC_{50} = 8$ and 36 μM, respectively). The data reported in Table 4 also demonstrated that the affinity for the enzymatic site is closely related to the length of the acyl chain because the inhibitory activity decreased greatly with the C_3 chain as we had observed, but less so, for the melatoninergic receptor (Langlois et al., 1995). Preliminary determinations of the inhibition profiles of the naphthalenic derivatives **33**, **34** and **35** suggested a mixed inhibitor profile over the whole concentration range which differentiated them from melatonin. These results indicate, as was observed with the amines, a difference in the molecular recognition of the indolic and the naphthalenic moieties by the enzyme. However, additional data are necessary to draw definitive conclusions, in particular, more tryptamine concentrations used and the study of the inhibition profile against acetyl-CoA the other substrate of this enzyme explored. The inhibition of 5-HT-*N*-acetyltransferase by compounds **32** and **33** were also interesting as, while they have moderate affinity for melatonin receptors, they were as potent inhibitors as the naphthalenic ligand **35** which has nanomolar affinity for these receptors. These results indicate that it is possible to obtain an inhibitor of 5-HT-*N*-acetyltransferase with moderate affinity for melatonin receptors. Moreover, interest in **33** was highlighted in the binding experiments where its affinity, in contrast to those of melatonin or naphthalenic derivatives, such as **27**, was hardly affected by the addition of GPT-γ S and Mn^{2+} (Brémont et al., 1994; Hamon et al., 1983), suggesting an antagonist profile for this compound. An antagonist for melatonin receptors, with inhibitory properties regarding the major synthetic enzyme in the melatonin pathway would constitute a useful tool for the understanding of the physiological role of melatonin.

In summary, this work has demonstrated the influence of the substituent in the 5 position of tryptamine on the kinetic parameters of 5-HT-*N*-acetyltransferase. In particular, the decrease in the efficacy of the enzyme without modification of the affinity indicates the potential inhibitory activity of these compounds and, consequently, provides a suitable way to design the first potent and selective inhibitors of this enzyme. On the other hand, the relatively potent inhibitory activity of melatonin itself on 5-HT-*N*-acetyltransferase, which was demonstrated to be competitive in the 5–50-μM range and the determination of the melatonin concentration in the micromolar range in the pineal gland suggest autoregulatory control of its own synthesis. The molecular recognition by 5-HT-*N*-acetyltransferase of the acetamido naphthalenic derivatives was approximately similar to that of the corresponding indolic derivatives, indicating fairly good bioisoteric properties for both series of compounds, regardless of the inhibitory potency. Further experiments are in progress to complete the enzymatic profiles of the different compounds, in particular by studying their inhibitory properties against Acetyl-CoA. This study highlighted compounds **32** and **33** as important leads for the design of more potent inhibitors devoid of melatonin receptor agonist properties and additional experiments are in progress to confirm their interest, in particular, by studying the inhibition of 5-HT-*N*-acetyltransferase in vivo.

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References

Alonso-Gomez, A.L., A.I. Valenciano, M. Alonso-Bedate and M.J. Delgado, 1995, Differential characteristics and regulation of arylamine and arylalkylamine *N*-acetyltransferase in the frog retina (*Rana perezi*). Neurochem. Int. 26, 223.

Brémont B., S. Shen, S., M. Langlois, I. Serraz, J. Andrieux and A. Poncet, 1994, Binding studies on the melatonin receptor in the chicken brain and characterization of new specific agonists or antagonists by modulation of affinity with GTP and Mn^{2+}. XIIIth International Symposium on Medicinal Chemistry (Paris), p. 160.

Bradford, M., 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72, 248.

Copinga, S., P.G. Tepper, C.J. Grol, A.S. Horn and M.L. Dubocovich, 1993, 2-amido-8-methoxytetralins, a series of nonindolic melatonin-like agents, J. Med. Chem. 36, 2891.

Deguchi, T., 1992 Physiological and molecular biology of arylamine *N*-acetyltransferases, Biomed. Res. 13, 231.

Deguchi, T. and J. Axelrod, 1972, Sensitive assay for serotonin *N* acetyltransferase activity in rat pineal, Anal. Biochem. 50, 174.

Depreux, P., D. Lesieur, H.A. Mansour, P. Morgan, H.E. Howell, P. Renard, D.-H. Caignard, B. Pfeiffer, P. Delagrange, B. Guardiola-Lemaitre, S. Yous and J. Andrieux, 1994, Synthesis and structure–activity relationships of novel napthalenic and bioisosteric related amidic derivatives as melatonin receptor ligands, J. Med. Chem. 37, 3231.

Dubocovich, M.L., 1995, Melatonin receptors, are there multiples subtypes?, Trends Pharmacol. Sci. 16, 50.

Frick, L. and R. Wolfenden, 1989, Substrate and transition state affinity, in: Design of Enzyme Inhibitors as Drugs, ed. M. Sandler and H.J. Smith (Oxford University Press, London) p. 19.

Garrat, P.J. and S. Vonhoff, 1994a, Mapping the melatonin receptor. 1. The 5-methoxy group of melatonin is not an essential requirement for biological activity, BioMed. Chem. Lett. 4, 1555.

Garrat, P.J. and S. Vonhoff, 1994b, Mapping the melatonin receptor. 2. Synthesis and biological activity of indole derived melatonin analogues with restricted conformations of the C-3 amidoethane side-chain, BioMed. Chem. Lett. 4, 1559.

Garrat, P.J., R. Jones and D.A. Tocher, 1995, Mapping the melatonin receptor. 3. Design and synthesis of melatonin agonists and antagonists derived from 2-phenyltryptamines, J. Med. Chem. 38, 1132.

Glennon, R.A., J.D. McKenney, R.A. Lyon and M. Titeler, 1986, 5-HT_1 and 5-HT_2 binding characteristics of 1-(2,5-dimethoxyphenyl)-2-aminopropane analogues, J. Med. Chem. 29, 194.

Hamon, M., C. Goetz and H. Gozlan, 1983, in: CNS Receptor Subtypes,

Pharmacology, Significance and Clinical Implications, ed. P. Mandel and F.V. DeFeudis (Raven, New York) p. 349..

Hulme, E.C. and J.M. Birdsall, 1992, in: Receptor–Ligand Interactions, A Practical Approach, ed. E.C. Hulme (IRL, Oxford) p. 63.

Humlova, M. and H. Illnerova, 1992, Entrainment of the rat circadian clock controlling the pineal *N*-acetyltransferase rhythm depends on photoperiod, Brain Res. 584, 226.

Klein, D.C., D. Sugden and J.L. Weller, 1983, Postsynaptic α-adrenergic receptors potentiate the β-adrenergic stimulation of pineal *N*-acetyltransferase, Proc. Natl. Acad. Sci. USA 80, 599.

Krause, D.N. and M.L. Dubocovich, 1991, Melatonin receptors, Annu. Rev. Pharmacol. Toxicol. 31, 549.

Langlois, M., B. Brémont, S. Shen, A. Poncet, J. Andrieux, S. Sicsic, I. Serraz, M. Mathé-Allainmat, P. Renard and P. Delagrange, 1995, Design and synthesis of new naphthalenic derivatives as ligands for 2-[^{125}I]-iodomelatonin, J. Med. Chem. 38, 2050.

Lindquist, R.N., 1975, The design of enzyme inhibitors. Transition state analogs, in: Drug Design, Vol. V, ed. E.J. Ariens (Academic Press, New York) p. 23.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951, Protein measurements with the Folin reagent, J. Biol. Chem. 193, 265.

Maestroni, G.J.M., 1993, The immunoneuroendocrine role of melatonin, J. Pineal Res. 14, 10.

Namboodiri, M.A., M.J. Browstein, P. Voisin, J.L. Weller and D.C. Klein, 1987, A simple rapid method for the purification of ovine pineal arylalkylamine *N*-acetyltransferase, J. Neurochem. 48, 580.

Palm L., G. Blennnow and L. Wetterberg, 1991, Correction of non-24 hour/sleep/wake cycle by melatonin in a blind retarded boy, Ann. Neurol. 29, 336.

Partonen, T., 1994, Involvement of melatonin and serotonin in winter depression, Med. Hypoth. 43, 165.

Price, N.C. and L. Stevens, 1989, An introduction to enzyme kinetics, in: Fundamentals of Enzymology (Oxford University Press, London) p. 136.

Reiter, R.J., 1991a, Pineal melatonin, cell biology of its synthesis and of its physiological interactions, Endocr. Rev. 12, 151.

Reiter, R.J., 1991b, Melatonin, the chemical expression of the darkness, Mol. Cell. Endocrinol. 79, C153.

Reiter, R.J., 1993, The melatonin rhythm, both a clock and a calendar, Experientia 49, 654.

Rudeen, P.K., R.J. Reiter and M.K. Vaughan, 1975, Pineal serotonin-*N*-acetyl-transferase activity in four mammalian species, Neurosci. Lett. 1, 225.

Shaw, E., 1989, Active-site-directed irreversible inhibitors, in Design of Enzyme Inhibitors as Drugs, ed. M. Sandler and H.J. Smith (Oxford University Press, London) p. 49.

Tipton, K.T., 1989, Mechanism-based inhibitors, in: Design of Enzyme Inhibitors as Drugs, ed. M. Sandler and H.J. Smith (Oxford University Press, London) p. 70.

Utiger, R.D., 1992, Melatonin – the hormone of darkness, New Engl. J. Med. 327, 1377.

Voisin, P., M.A. Namboodiri and D.C. Klein, 1984, Arylamine *N*-acetyltransferase and arylalkylamine *N*-acetyltransferase in: the mammalian pineal gland, J. Biol. Chem. 259, 10, 913.

Yous, S., J. Andrieux, H.E. Howell, P.J. Morgan, P. Renard, B. Pfeiffer, D. Lesieur and B. Guardiola-Lemaitre, 1992, Novel naphthalenic ligands with high affinity for the melatonin receptor, J. Med. Chem. 35, 11844.

Zawilska, J.B. and J.Z. Nowak, 1992, Regulatory mechanisms in melatonin biosynthesis in retina, Neurochem. Int. 20, 23.

Zawilska, J.B. and J.Z. Nowak, 1994, Dopamine receptor regulating serotonin *N*-acetyltransferase activity in chick retina represents a D_4-like subtype, pharmacological characterization, Neurochem. Int. 24, 275.