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Research Article

Characterization and regulation of a CHO cell line stably expressing human serotonin N-acetyltransferase (EC 2.3.1.87)

G. Ferry [a], J. Mozo [a], C. Ubeaud [a], S. Berger [a], M. Bertrand [b], A. Try [a], P. Beauverger [a, c], C. Mesangeau [d], P. Delagrange [e], J. A. Boutin [a, *]

[a] Pharmacologie Moléculaire et Cellulaire, Institut de Recherches SERVIER, 125 chemin de Ronde, 78290 Croissy-sur-Seine (France), Fax +33 (0) 1 55722810, e-mail: jean.boutin@fr.netgrs.com
[b] Technologie SERVIER, 25/27 rue Eugène Vignat, 45007 Orléans Cedex (France)
[c] Present address: Département Cardiovasculaire Thrombose Sanofi-Synthelabo Recherche 1, Avenue Pierre Brossolette, 91385 Chilly-Mazarin Cedex (France)
[d] Institut de Chimie Pharmaceutique, 3, rue du Professeur Laguesse, 59045 Lille Cedex (France)
[e] Institut de Recherches Internationales SERVIER, 6, place des Pléïades, 92415 Courbevoie Cedex (France)

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Abstract. Current melatonin research is essentially based on the finding of new molecular tools, including synthetic or natural agonists and antagonists for the melatonin receptors and synthetic inhibitors of the enzymes involved in its biosynthesis. Indeed, the use of these compounds will improve our understanding of some of the numerous mechanisms of action of melatonin. The present report deals with the establishment and description of a new cell line expressing in a stable manner human arylalkylamine-N-acetyltransferase (AANAT, E.C.2.3.1.87). This new cellular system permits one to check the capacity of newly discovered inhibitors to penetrate the cell and reach their target. Some emphasis is put on inhibitors of the bromoacetyltryptamine family since these precursor compounds form in situ bisubstrate inhibitors with strong affinity for the human enzyme. AANAT is known to undergo complex and rapid regulation by a subtle balance between extremely fast catabolism and protection against it, both due to serine phosphorylation. In the present report, this phosphorylation is shown to occur in vitro after incubation with several kinases (rho-kinase, chk-1, protein kinase A) but not with protein kinase C. Phosphorylation enhances the specific activity of the enzyme by a factor of two to five. This phosphorylation is also shown to occur after treatment of the cell with compounds such as forskolin and rolipram that enhance or protect the intracellular pool of cAMP or the cell-permeable cAMP analogue, dioctanoyl-cAMP. The specificity of the cellular model was assessed using a series of substrates and inhibitors of AANAT already described in the literature, and the characteristics of this cellular system are shown to correspond with those reported for the purified enzyme. This cell line was used to screen libraries of compounds in a living system and led to the discovery of several potent specific and non-toxic AANAT inhibitors.

Key words. Melatonin; serotonin N-acetyltransferase; cellular model; inhibition; N-bromoacetyltryptamine.

* Corresponding author.

Melatonin, a neurohormone synthesised in the pineal gland during the dark period [1], has been proposed as an internal synchroniser of circadian rhythms which transmits photoperiodic information through the general circulation to all the structures expressing melatonin-binding sites. Its chronobiotic effect has been studied extensively [for a review, see ref. 2]. Its biosynthesis is mediated by a series of enzymes of which the activity of arylalkylamine-N-acetyltransferase (serotonin N-acetyltransferase, AANAT, E.C. 2.3.1.87) is the limiting step according to its circadian rhythm [3, 4]. Ovine AANAT has been purified, cloned and characterised [5, 6]. Furthermore, detailed analysis of its structural features by crystallography [7] provided precise descriptions of its catalytic mechanism of action [8]. Far less is known about the human form of AANAT, although it has been partially purified, pharmacologocially characterised in terms of its substrate and inhibitor specificity [9] and used in a medium throughput screening assay of several inhibitor families [10]. Several observations identified cAMP as one of the partners involved in the activation of the enzyme [11–13]. To better understand the role of melatonin, one needs to investigate all the possible mechanisms of its actions, either through its downstream targets, such as the two G protein-coupled receptors, MT_1 and MT_2, or its newly described atypical MT_3-binding site, a quinone reductase, or at the level of its synthesis or catabolism. Melatonin catabolism is believed to be mediated by two routes: indoleamine breakdown [14], still poorly explored, and through sulphate and glucuronide conjugations [15]. In addition to other research efforts described elsewhere [16, 17], we chose to investigate the inhibition of melatonin synthesis through the search for compounds able to impair the activity of AANAT with high potency and selectivity. Indeed, such inhibitors could be used to perform pharmacological pinealectomy which would have the advantage of being reversible, avoiding surgery and maintaining intact the physiological function of the pineal gland. Among the necessary tools to complete our investigations, we felt that a reliable and easy-to-use cellular model was still lacking. The rare cellular models currently described in the literature include the PGTβ cell line [18], the Y79 retinoblastoma [19], the SKMEL188 melanoma cell line [20] and the 1E7 cell line, derived from COS cells [21]. Clonal cells derived from COS cells are not common in the literature [22–24], and tend to lose their transgenes during culture. We, therefore, established a stable cell line based on CHO, in which the human enzyme was expressed at fairly high levels. This model was anticipated to complement the COS-based one in which AANAT is less expressed but better mimics the rat nocturnal situation [25], while the present one with a higher enzyme activity level, would be closer to an ungulate (ovine) model.

In the present report, we describe the functional characteristics of this cell line. We also developed specific AANAT assays with tritiated enzyme substrates such as serotonin, phenylethylamine and methoxytryptamine. We also characterised variations in AANAT activity upon various cellular treatments, such as those modifying intracellular cAMP content (e.g. forskolin), or those directly activating protein kinase C (phorbol-myristate ester or cell-penetrating diacylglycerol derivatives). Since the sequence of the enzyme has been shown to possess several potential sites for serine phosphorylation [4], and since the in vivo activation by a serine/threonine protein kinase plays a key role in the general control of melatonin rhythm [13], we wondered if other kinases might phosphorylate and increase the activity of AANAT. Indeed, chk-1, a cell cycle-related kinase [26], as well as rho-kinase, a rho-associated protein kinase [27], were able to phosphorylate AANAT in vitro, and subsequently to increase its catalytic activity.

Finally, this cellular model was used to screen in situ several series of compounds and led to the discovery of several potent inhibitors.

Materials and methods

Preparation of an AANAT-expressing cell line

CHO-K1 cells were seeded (5×10^6/T75 cm^2 culture flask) and 24 h later transfected (10 µg of a pcDNA3.1-hAANAT plasmid) using lipofectamine (Life Technologies, GIBCO BRL). Isolated clones were picked following geniticin (0.8 mg/ml) selection, and amplified. AANAT activity in the homogenates was measured as described in the AANAT assay. A positive clone, CHO-hAANAT-8, was subcloned by limited dilution. This was followed by another round of subcloning before use in the experiments presented here. COS 1E7 cells were a kind gift of Drs Steve L. Coon and David C. Klein (NIH, Bethesda, Md.).

Analytical method for cellular AANAT

AANAT activity was measured with an HPLC technique. In brief, for clone selection and studies and for the lysate characterisation, 70 µl of incubation buffer (50 mM sodium phosphate, pH 6.8, containing 500 mM NaCl and 2 mM EDTA), containing 0.5% Tween 85 were added to each well of a 96-well plate containing subconfluent cells of each clone. Plates were mixed at room temperature for 30 min and each well of the plate was sonicated (setting, 40/100, probe 0.2 cm diameter) once for 5 s. Twenty microlitres of [^{3}H]acetyl-CoA (Amersham Pharmacia Biotech, 129 GBq/mmol), 1 mM final acetyl-CoA and 10 mM final serotonin were added to each well in a final volume of 100 µl. After incubation for 30 min at 37 °C, the reaction was stopped by the addition of 50 µl 10% trichloroacetic acid. Thirty microlitres of this solution was analysed by reverse-phase HPLC. The column was devel-

oped with a linear gradient of 5–35 or 0–100% acetonitrile in H_2O, 0.1% trifluoroacetic acid, at a flow rate of 1 ml/min for 15 or 30 min. The activity of CHO-hAANAT was measured in both the cell and the conditioned medium. CHO-hAANAT cells were seeded (one million cells per 96-well culture plate) and 72 h later were incubated with one of the tritiated substrates or product: [^{3}H]-serotonin, (NEN, 1 TBq/mmol), [^{3}H]-methoxytryptamine (Amersham Pharmacia Biotech, 3.03 TBq/mmol), [^{3}H]-phenylethylamine (PEA; Amersham Pharmacia Biotech, 2.78 TBq/mmol), [^{3}H]-N-acetyl-phenylethylamine (Nac-PEA; Amersham Pharmacia Biotech, 2.66 TBq/mmol), 10 µl in the incubation. Obviously, no co-substrate (acetyl-coenzyme A) of AANAT was added to the cell. Thirty microlitres of conditioned culture medium were injected in the HPLC, cells were lysed and analysed as described above. Separation conditions were as described above for AANAT activity.

Kinetic studies

All the measurements were done using the HPLC assay. Basically, the affinity data were collected using the classical process described by Segel [28] for bisubstrate enzymes. For the apparent K_m, the substrate concentrations were varied from 0.06 to 4 mM, while the co-substrate (acetyl-coenzyme A) was added at a concentration of 1 mM. For apparent co-substrate K_m measurement, a saturating concentration of 4 mM was used for the substrate (PEA or serotonin). Whereas the rule is that the saturating concentration should be 10 times the K_m [28], for most of the natural bioamine substrates of AANAT, this concentration could not be reached (ca 10 mM for serotonin, $K_m \sim 1$ mM). For real K_m determinations, experiments were also conducted according to the guidelines given by Segel [28]. At least five concentration points were routinely used and the experiments were repeated three times. Finally, for treatment of the data, the double reciprocal plot was used as the most accurate method when less than ten concentration points were available.

Synthesis of chemicals

The various chemicals used in the present studies were synthesised as follows: N-bromoacetyltryptamine (BAT) by Khalil and Cole [29]; the peptides S34461 and S 35117 were obtained as in Ferry et al. [9]; S 27481, an N-bromoacetylbenzothienyl derivative was synthesised by Beaurain et al [30]; S 23823 was synthesised according to Le Picard et al. [31], and S 28036 according to Lesieur et al. [32].

Phosphorylation of hAANAT

Purified human AANAT (16 µg) was phosphorylated by the protein kinase A catalytic subunit (0.05 units, Calbiochem) in 20 mM Tris-HCl, pH 7.5, or by the protein kinase C catalytic subunit (0.17 units, Calbiochem), in 20 mM Tris-HCl, pH 7.5 with $CaCl_2$ 0.5 mM, or by ROCK-II (0.1 units, Upstate Biotechnologies, Euromedex, Strasbourg, France) in 20 mM Tris-HCl, pH 7.2 with 1 mM dithiothreitol (DTT), 1 mM EGTA, 10 mM glycerophosphate or by chk-1 (5 mU, TEBU International, France) in 20 mM HEPES, pH 7.5 with 0.5 mM DTT, 100 mM NaCl. All reactions were conducted at 37 °C for 30 min in starting buffer containing 10 mM $MgCl_2$, 1 mM Na_3VO_4, 20 µM ATP, 2 µCi [^{32}P]γATP (Amersham). Fractions were separated in SDS-PAGE electrophoresis and evaluated by autoradiography. An aliquot of the incubation medium, before stop, was evaluated for AANAT catalytic activity, as described above.

Prediction of intestinal absorption

Caco2 cells were cultured for 3 weeks on the filter of a 24-well, two compartment device (Transwell 24 HTS, Costar) up to complete maturation. Assuming no possible limitation due to solubility, absorbed fractions (Abs %) were estimated using permeability measurements through this Caco2 cell monolayer model. Briefly, compounds were incubated on Caco2 monolayers (apical compartment) at 20 µM and concentration versus incubation time appearance in the basal compartment was quantified by LC-MS-MS after 30–120 min incubation. The unchanged compound in the apical compartment at the start and end of the incubation period was also quantified by LC-MS-MS, making possible the recovery control (to quantify possible loss of unchanged compound by plastic adsorption, by cellular metabolism or by intracellular concentration). Apparent permeability (P_{app}) was calculated and then extrapolated to a human absorption prediction (Abs Man) using a calibration curve based on the P_{app} of a series of compounds measured in the same conditions, for which the data of absorption in humans have been reported from the clinic, namely mannitol (16%), atenolol (50%), cimetidine (70%), propanolol (90%) and antypyrine and testosterone (100%). The P_{app} of mannitol, a compound absorbed via a paracellular mechanism only, was determined as a control of the monolayer integrity and tight-junction quality (a mannitol P_{app} of about 0.1×10^{-6} cm/s is considered to reflect very good tight-junction quality). A P_{app} for the tested compounds above 10×10^{-6} cm/s is considered a good predictor of excellent absorption and of a transcellular absorption mechanism. This method has been shown by us and others to give a good in vitro-in vivo correlation [33].

Results

Our slightly modified previous HPLC assays [9, 10] were used to measure AANAT activity in cells. We decided to test several AANAT substrates on the cell, namely, serotonin, PEA and methoxytryptamine, all in their tritiated

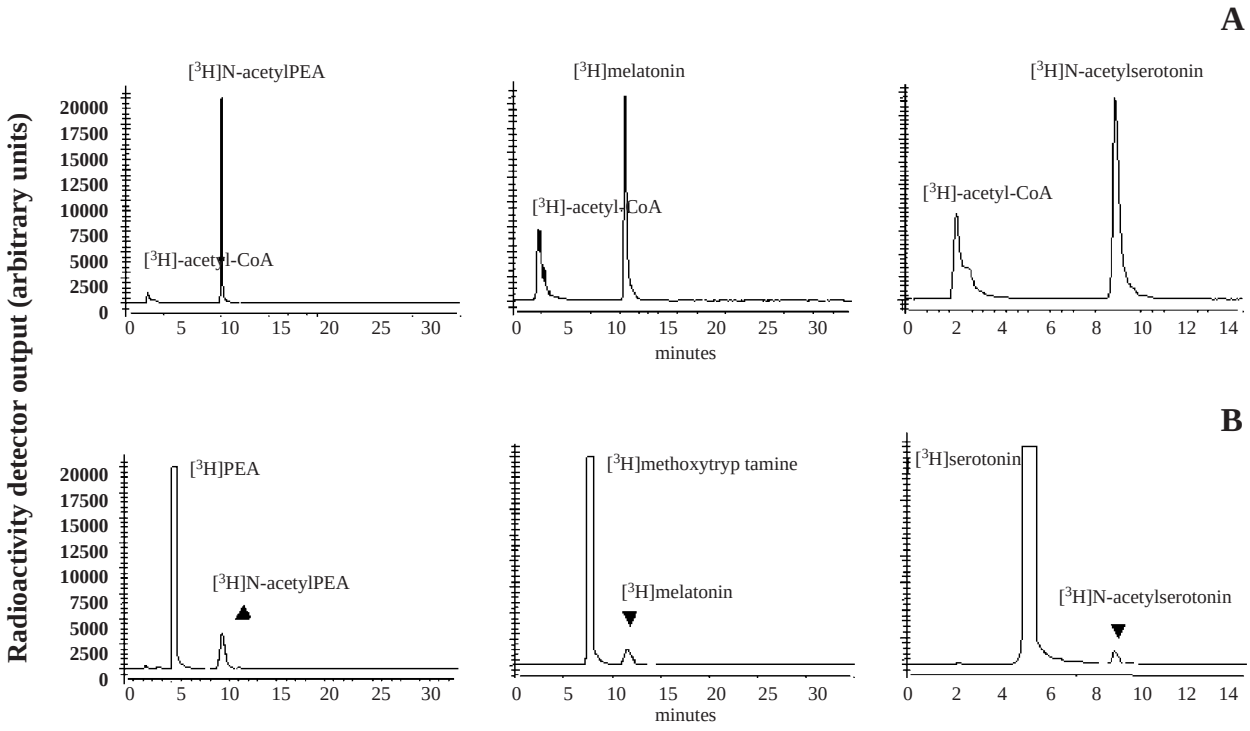

Figure 1. N-acetyltransferase activity, as estimated from the amount of tritiated product. Purified recombinant human enzyme (*A*) and transgenic CHO-supported activity (*B*) were measured in a similar manner. (*A*) Enzyme was incubated for 30 min with cold substrate, tritiated acetyl-CoA and stopped by 10% trichloroacetic acid, and an aliquot was injected in an HPLC analytical system. (*B*) Cells were incubated with tritiated substrates, but without added acetyl-CoA, the conditioned media were then injected in an HPLC system, as described in Materials and methods. Chromatograms presented are representative of many experiments.

form. Figure 1 presents three chromatograms of these compounds after enzymatic (fig. 1A, with the tritiated co-substrate) or cellular (fig. 1B, with the tritiated substrates) incubations. As expected, the substrates and the products of the reaction could be clearly separated and quantified by this technique. The production of the N-acetylated products was estimated after 1, 3 and 6 h incubation (fig. 2). Two sets of data were obtained, either by measuring the amount of product present in the conditioned medium (fig. 2A) or inside the cells (fig. 2B). The latter results were obtained after removing the culture medium by aspiration, and breaking the cells by sonication in the well. This particular technique was delicate, leading to relatively high levels of experimental variation. In both cases, however, PEA was rapidly acetylated, while N-acetylserotonin and melatonin were produced at much slower apparent rates. The rates of production increased over time, leading to a measurable amount of all the products after 6 h. Nevertheless, the sensitivity of the assay is limited by the detection system. Indeed, radioactivity detection on-line is known to have a yield of about 35% or less, depending on the quality of the detector. The quantitative difference is very important, because there are ten times more reaction products outside the cells than inside (note the difference in the y-axis units in the two cases; fig. 2). The concentration of the reaction products remained approximately constant between 3 and 6 h.

The core of the assay is the free penetration of the substrates of the reaction and the free release of the product of the reaction in the culture medium. Since, for sensitivity reasons, we decided to use PEA as the substrate to measure the enzyme activity, we had to verify first that both the substrate (PEA) and the product of the reaction (NacPEA) were able to penetrate the cell as freely as one can measure it. The widely described Caco2 cell model [33], which predicts the bioavailability of candidate drugs (table 1), was used to verify this parameter. The results show that the compounds (substrates and products of the N-acetyltransferase reaction) move freely inward as well as outward. We decided, therefore, to keep the conditioned medium analysis as the reference for all subsequent experiments, since it is reasonable to assume that the amount of reaction product in the conditioned medium reflects well the situation inside the cell line. To be sure that the amount of product in the cell medium did not influence the apparent enzymatic rate (inhibition by excess reaction product), the measurement was also done in the presence of 100 μM cold N-acetylphenylethylamine (NacPEA). This had no effect on the apparent rate of cellular enzy-

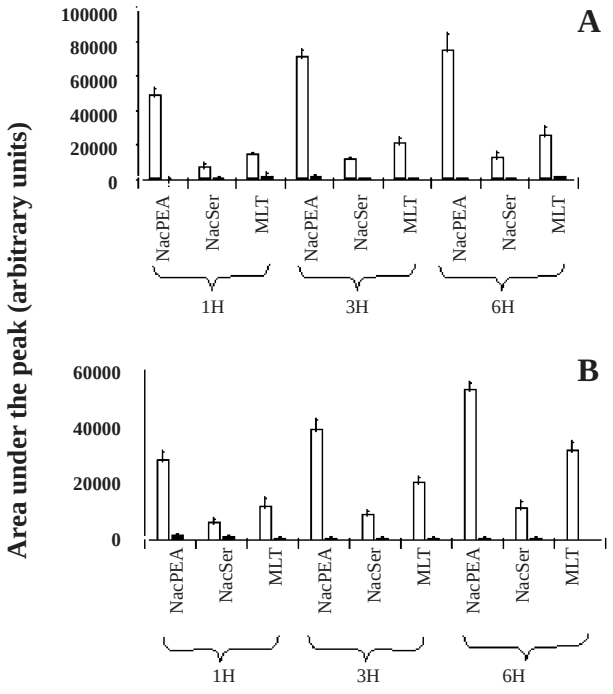

Figure 2. N-acetyltransferase activity after incubation of transgenic CHO cells, stably expressing the human serotonin N-acetyltransferase (EC 2.3.1.87), as a function of incubation time: (*A*) conditioned media were analysed; (*B*) the cells were collected, and broken. The amount of radioactive products of the reactions was analysed by HPLC. White bars, activity supported by transgenic cells; dark bars, activity supported by the native CHO cells. Results are presented as means ± SE of three independent determinations. NacPEA, N-acetyl-phenylethylamine; NacSer, N-acetylserotonin; MLT, melatonin (N-acetyl-5-methoxytryptamin).

matic activity (data not shown). Furthermore, a 6-h incubation of [^{3}H]-NacPEA with the cell line or with a cell homogenate did not change the amount of compound, as followed by HPLC (data not shown), thus excluding noticeable metabolism during the experiment.

Finally, previous literature has shown that the substrates used by AANAT are arylethylamines, including natural compounds such as PEA, tryptamine and serotonin, and synthetic compounds such as naphthylethylamines [9]. By using one or the other of these substrates under ad hoc conditions, one can measure equally well the activity of the enzyme. To further demonstrate this particular point, we used the reference bromo-inhibitor, BAT, in an enzymatic assay with the three known natural substrates. A comparison of the IC_{50} of BAT using three different substrates, namely PEA, serotonin and methoxytryptamine, in the same cellular system, confirmed that the measured IC_{50}s were identical (table 2). For technical reasons due to the lack of sensitivity of the method, we had to use PEA as the best substrate (i.e. the one giving the highest amount of product) in the cellular assay, it being a far better substrate for the human enzyme than either serotonin or tryptamine, a feature that we stressed in our initial human AANAT characterisation work [9]. We measured a K_m for PEA of 86 (± 12) μM, which compares well with the value we reported with the pure enzyme (173 ± 17 μM), and with the K_m measured with the COS cell-based enzyme (550 ± 90 μM) [21]. Due to the methodology (intact cell assay), no co-substrate (acetyl-CoA) was added, thus representing more accurately the metabolism in vivo. Under these conditions, the maximal velocity for any of the substrates used could not be measured.

Tween 85 is a detergent reported to activate AANAT activity in vitro [9]. As shown in figure 3, the detergent can increase the enzyme activity (measured with serotonin as substrate) by a factor of at least ten (at 100 min) as well as the activity observed with the human AANAT stably expressed in COS cells [25]. The same figure shows that the cellular clone (CHO cell line) is much more potent in terms of activity than the COS cell line both before and after the detergent treatment.

One of the striking features of AANAT is the way it is active at night and inactive during the day. The process involved in this phenomenon has been dissected by several authors [13, 21, 25]. It involves a phosphorylation step via protein kinase A, the cAMP-regulated serine/threonine kinase. We wanted to check that this post-translational pathway was still effective in the present cell line. As shown in figure 4, the enzyme activity of AANAT is notably enhanced by treatment of the cells with 10 μM forskolin. This compound is known to activate adenylate cyclase, leading to a sustained level of cAMP inside the cells. To our surprise, however, another protein kinase activator, phorbol myristate acetate (PMA), which specifically acti-

Table 1. Determination of cell permeability (P_{app}) of phenylethylamine and N-acetylphenylethylamine using the Caco2 cell model.

Compound	P_{app} (10^{-6} cm/s)	SD (n = 2)	Abs Man* (%)	P_{app} mannitol (10^{-6} cm/s)	Recovery with cell monolayer (%)	Recovery with filter alone (%)
N-acetylphenylethylamine	89.4	25.6	100	0.09	95	118
Phenylethylamine	14.8	0.7	100	0.09	48	111

Caco2 cells were cultured for 3 weeks on the filter of a 24-well, two compartment device (Transwell 24 HTS, Costar) up to complete maturation. The tested compounds (20 μM) were incubated in the upper (apical) compartment and the amount of unchanged compound appearing in the lower (basal) compartment was quantified after 30–120 min by LC-MS-MS.

* Absorption of mannitol.

Table 2. Inhibition of serotonin N-acetyltransferase by N-bromoacetyl tryptamine, using phenylethylamine, methoxytryptamine and serotonin as substrates.

Substrates	IC_{50} (μM) ± SE	
	enzyme	cells
Phenylethylamine	1.43 ± 0.7	1.85 ± 1.1
5-Methoxytryptamine	1.1 ± 0.2	3.2 ± 0.8
Serotonin	1.32 ± 0.3	not determined*

Experiments were conducted using the purified enzyme. Concentrations for BAT ranged from 1 nM to 100 μM. In the enzyme experiments, the substrates were used at a concentration of 4 mM. In the cellular experiments, 5 μM of the substrates was used. Experiments were conducted three times independently and results are presented as the mean ± SE.
* The results with serotonin are below the sensitivity of the method.

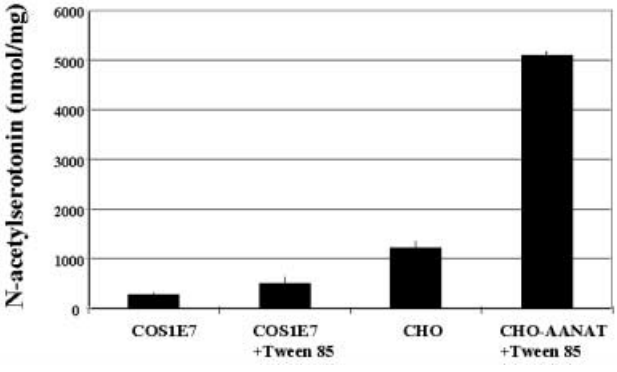

Figure 3. Detergent activation of broken cell activities supported by CHO and COS cells stably expressing human serotonin N-acetyltransferase activity. The two cell pellets were broken, treated with Tween 85 and incubated for 100 min with tritiated serotonin and cold acetyl-CoA. Controls were performed without detergent pre-treatments. Results are presented as means ± SE of three independent determinations.

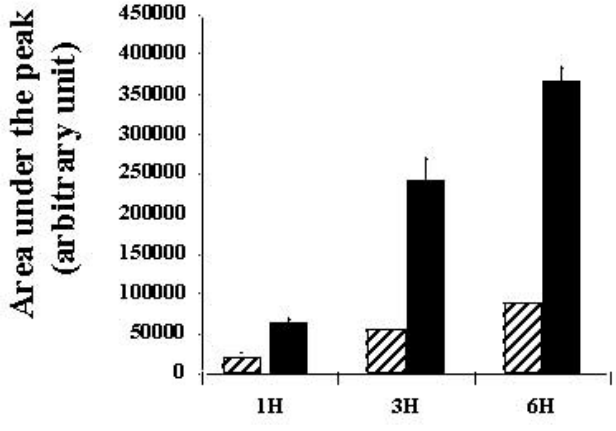

Figure 4. Effect of forskolin on CHO-transfected human serotonin N-acetyltransferase activity. CHO cells stably expressing the human recombinant serotonin N-acetyltransferase were incubated with 10 μM (dark bars) or without (hatched bars) forskolin. The activity was measured as described, with [^{3}H]-phenylethylamine as substrate. Results are presented as means ± SE of three independent determinations.

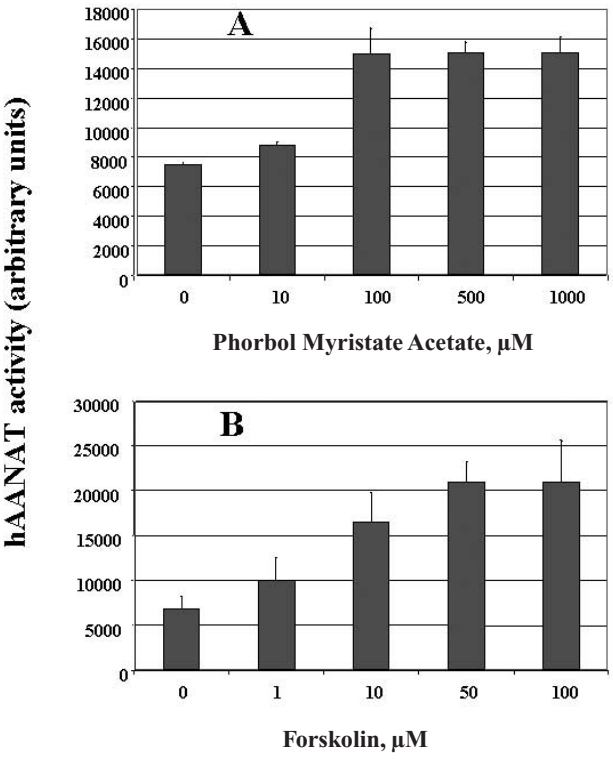

Figure 5. Dose effects of forskolin and PMA on the activity of the CHO expressing human serotonin N-acetyltransferase activity. The compounds were tested from 0.01 to 1 μM (PMA) (*A*) and from 1 to 100 μM (forskolin) (*B*) in the presence of [^{3}H]-phenylethylamine, with the cells for 100 min, after which a sample of the conditioned medium was analysed by HPLC. Results are presented as means ± SE of three independent determinations.

vates protein kinase C, the calcium/lipid-regulated serine/threonine kinase, was also able to enhance significantly the level of AANAT activity (fig. 5). The effects of both compounds (forskolin and PMA) on the enzyme activity were measured as a function of their doses. PMA was tested between 10 and 1000 nM, while forskolin was tested between 1 and 100 μM, in line with the concentration reported for these two products in the literature. The effect was enhanced as a function of the increased compound concentration but seemed to plateau around 50 μM for forskolin and 500 nM for PMA.

The effects of other compounds acting at the level either of protein kinase A, such as the stable cAMP analogue, dioactanoyl-cAMP (do-cAMP), or of protein kinase C, such as the glycerol derivatives didecanoyl-rac-glycerol (rac-dag) and dioctanoyl-sn-glycerol (sn-dag) were tested. As shown in figure 6, do-cAMP and sn-dag increased hAANAT activity by 50 and 35%, respectively, at the maximal concentration used. The effect of rac-dag, however, was less marked, probably due to penetration and/or stability reasons. To complete the characterisation of the post-translational regulation of AANAT, we finally tested the effect of rolipram, an

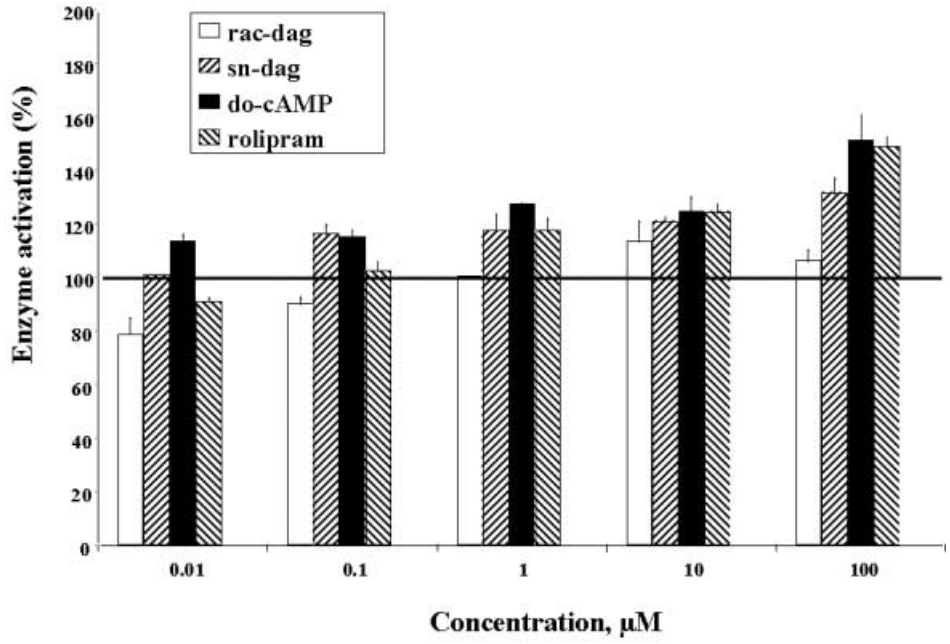

Figure 6. Dose effects of diacyl-rac-glycerol (rac-dag), diacyl-sn-glycerol (sn-dag), dioctyl-cAMP (do-cAMP) and rolipram on the activity of the CHO expressing human serotonin N-acetyltransferase activity. The compounds were tested on the cells from 0.01 to 100 µM in the presence of [^{3}H]-phenylethylamine for 3 h, after which a sample of the conditioned medium was analysed by HPLC. Results are presented as means ± SE of three independent determinations.

inhibitor of phosphodiesterase that impairs the catabolism of cAMP in cells (fig. 6). The effect of rolipram was almost linear up to 100 µM, at which concentration the activity was enhanced by 50%, compared to untreated cells.

Furthermore, since several protein kinases seemed to recognise AANAT as a substrate, we tested the capacity of protein kinase A, protein kinase C, chk-1 and rho-kinase to phosphorylate in vitro recombinant human AANAT. Protein kinase C did not phosphorylate the enzyme, whereas AANAT was phosphorylated by the other three protein kinases. Subsequently, AANAT specific activity was enhanced by a factor of two to four, as shown in figure 7.

We then characterised the molecular pharmacology of the AANAT activity in the stable CHO cell line by testing a series of inhibitors. Some of these inhibitors have been described in a previous report on an enzymatic test [9, 30]. In brief, the cells were treated with the compounds at concentrations ranging from 0.1 nM to 100 µM. The compounds were dissolved in DMSO, and the effect of the solvent was assessed in control experiments. This effect at the highest dose used (1%) was negligible. The cells were treated 3 h before measurement of the AANAT products. In the case of forskolin pre-treatments, the drugs were added at the same time, after having verified that this did not influence the forskolin-induced response. We compared in table 3, the results obtained in the CHO cell line.

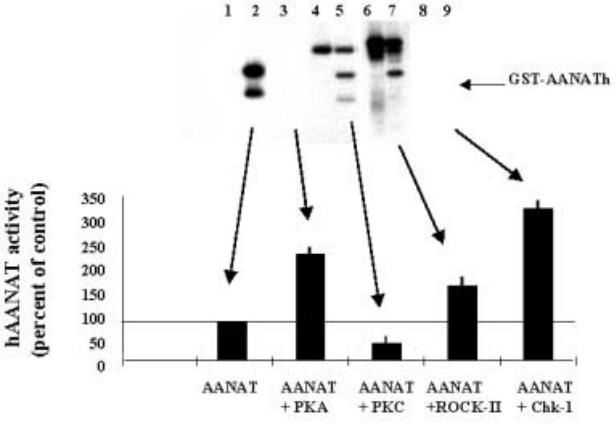

Figure 7. In vitro phosphorylation of human recombinant GST-AANAT with various serine/threonine protein kinases (PKs). Purified fusion protein GST-AANAT was incubated with various amounts of kinase in the presence of [^{32}P]-γ ATP. After incubation for 30 min, the reaction was divided into two parts: one stopped by adding 25 µl of SDS sample buffer, boiled for a minute and separated by electrophoresis (*A*) and the other used for hAANAT activity determination (*B*). (*A*) The resulting gel was dried, and analysed by a PhosphoImager (FujiFilm). Lane 1, molecular-weight standards (not labelled); lane 2, AANAT; lane 3, PKA and AANAT; lane 4, PKC; lane 5, PKC and AANAT; lane 6, rho-kinase; lane 7, rho-kinase and AANAT; lane 8, chk-1; lane 9, chk-1 and AANAT. (*B*) The hAANAT activity was quantified by the standard assay, using serotonin as substrate; the activity is represented as percentage of control (no phosphorylation) activity.

Table 3. Inhibitory potency of hAANAT inhibitors.

Compound	Inhibitor type	IC_{50} (μM)					
			without forskolin			with forskolin	
		Pure hAANAT	cell lysate	cells	conditioned medium	cells	conditioned medium
BAT	bisubstrate	1.43 ± 0.7 (>10)*	2 ± 0.4 (5)	1.78 ± 0.32 (5)	1.3 ± 0.5 (8)	1.85 ± 1(5)	0.2 ± 0.05 (>10)
S 27481-1	bisubstrate	0.18 ± 0.002 (3)	0.24 ± 0.04 (3)	0.045 ± 0.007 (3)	0.03 ± 0.004(3)	0.78 ± 0.4 (3)	0.44 ± 0.2 (3)
S 28036	bisubstrate	5.6 ± 0.42 (3)	4.6 ± 1.9 (3)	1.9 ± 0.3 (3)	0.7 ± 0.2 (3)	2 ± 0.9 (3)	0.21 ± 0.1 (3)
S 23823-1	substrate	5.1 ± 1 (>10)	4.6 ± 0.7 (3)	24.8 (1)	2.8 ± 0 (3)	13.4 (1)	13.8 ± 3.8 (4)
S 34461-1	mixed inhibition	4.3 ± 1.3 (4)	57.9 ± 1 (3)	no inhibition (2)	no inhibition (2)	no inhibition (2)	no inhibition (2)
S 35117-1	mixed inhibition	18.7 ± 3 (4)	49.3 ± 0.6 (3)	no inhibition (2)	no inhibition (2)	No inhibition (2)	no inhibition (2)

The compounds were used at concentrations ranging from 0.1 nM to 100 μM, in 1% DMSO aqueous solution for the highest concentration, either with purified hAANAT or cells expressing hAANAT, as described in Materials and methods and Results sections. The amount of reaction product (N-acetylphenylethylamine) was quantified either in the incubation medium (purified enzyme experiments) or in the conditioned medium (cellular experiments) by HPLC. The products of the reaction were either [^{3}H]-acetylphenylethylamine (purified enzyme experiments) or acetyl-[^{3}H]-phenylethylamine (cellular experiments). These two compounds have the same retention times in HPLC (see fig. 1). Cell lysate experiments were conducted after cell treatment, sonication of the cell pellet. Inhibitor type refers to the enzymological determination of the inhibition type [9]. Appropriate controls (including incubations without drugs, with 1% DMSO, as well as viability experiments at 100 μM drug concentrations) were ran either in parallel or separate experiments (viability). None of the treatments showed any difference with the absolute control (untreated cells). Number of experiments is given in parentheses. Data are the mean ± SE when more than one experiment.

As expected, the most active compounds on the purified enzyme fell into two categories: on the one hand, those (BAT, S 27481, S 28036 or S 23823) presenting a measurable inhibitory potency on the cellular enzyme activity, and on the other, the peptides, discovered by combinatorial library deconvolutions [9], unable to inhibit cellular AANAT activity. These differences can be easily attributed to differences in cell penetration of the various inhibitors, and therefore poor bioavailability, particularly for the complex bisubstrate derivative and the peptides. The structures of all the compounds are shown in figure 8. Finally, a tenfold difference was observed between the IC_{50}s of S 27481 before and after forskolin treatment, an intriguing effect not seen with any of the other compounds. Such behaviour, though, might be due to differences in the accessibility of the compound to the hAANAT/14.3.3 protein complex, considering that this binding depends on the phosphorylation status of hAANAT, on the one hand, and, on the other, that this particular compound, bearing a sulphur analogue of the indole core (benzothiazine) might behave differently, in this context, than the other analogues of the series such as S 28036. S 27481 could have more affinity for free hAANAT – in its unphosphorylated state – than for the complex formed after hAANAT became phosphorylated (after forskolin treatment) and associated with 14.3.3.

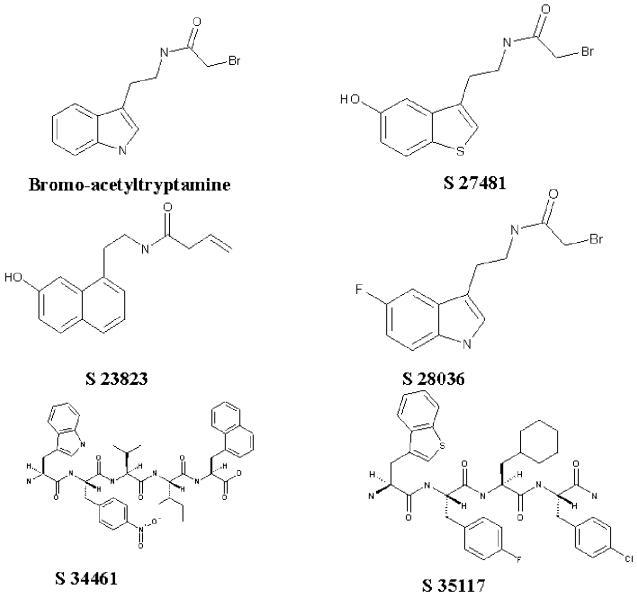

Figure 8. Structures of the inhibitors tested in the present report.

Discussion

The biochemical characteristics of AANAT from various species have been described in several reports over the last decade [9, 34–36] indicating clear differences in terms of amino acid sequence and substrate specificity. From a purely technical point of view, the availability of a reliable enzyme assay is crucial. Our HPLC method was based on following the appearance of a tritiated peak at a retention

time corresponding to the genuine [^{3}H]-acetylated product of the reaction [9]. The advantage of this particular method over the Deguchi extraction technique [37] is the actual visualisation of the reaction product. In the cellular-based assay, however, the adaptation of this particular acetyl-CoA assay was difficult, if not impossible. Therefore, use of labelled substrates facilitated the measurement of the enzyme reaction, as described in the present work. Methods that would involve the chromatography of a non-labelled AANAT substrate cannot apparently ensure a reasonable throughput. Similarly, no fluorescence-based assay, which would require the synthesis of a fluorescent or a fluorescence-generating substrate, has been developed so far.

There is an urgent need for pharmacological tools to study in more depth the mechanisms by which melatonin acts on many different living systems [38–42]. Our understanding of some of those systems, such as the melatonin receptors MT_1 and MT_2, are based on results obtained with agonists and antagonists of various structures [43–49], but also on the discovery of the molecular nature and function of the putative receptor MT_3 [16, 17, 50]. Furthermore, we contributed to the characterisation of the enzymatic properties of human AANAT, the enzyme responsible for the main step of melatonin synthesis [9]. We also assessed the tools to screen compounds, in the search for potent and specific inhibitors [10, 30]. One next strategic point was to set up the necessary tools to characterise the potency of the discovered inhibitors in a living system. The need for a cell line expressing the enzyme was obvious, since the natural systems described in the literature are either difficult to manipulate or to obtain [18, 19]. Although the 1E7 cell line model, derived from COS cells, has many features amenable to testing, the need for a cell line derived from either HEK or CHO seemed important, since few stable transgenic cell lines have been established from COS so far [23, 24]. The stable cellular cell line described here certainly represents an improvement in the presently available screening tools.

The catabolism of AANAT in situ has been well documented and described in detail by Ganguly et al. [25] and Coon et al. [21]. The elevation in cAMP and the subsequent activation of cAMP protein kinase that, in turn, specifically phosphorylates a particular sequence in AANAT, namely RRH**T**LPAN, promotes the association of this protein with 14-3-3 proteins. The formation of the AANAT/14-3-3 complex protects AANAT from dephosphorylation and probably from subsequent proteolysis, maintaining a high level of melatonin production. Much more needs to be explored to understand these regulation pathways. The 14-3-3 protein is also implicated in many different systems [51, 52], and has a key role in apoptosis promotion [53, 54], although the specificity of this pathway is not yet clear. In this context, a cell line expressing hAANAT without any translational regulatory features such as the pineal regulatory element [55] would permit study of its post-translational regulation. This mechanism has been scrutinised in different models, in particular, stably transfected COS cells. We provide here new data on the first step of this mechanism, by showing that in CHO cells, the increase in cAMP by forskolin treatment led to a similar, massive increase in AANAT activity, a feature mentioned by Coon et al. [21]. By using a pharmacological, non-toxic treatment with rolipram, a potent inhibitor of phosphodiesterase, we showed that the loss of cAMP catabolism leads to a similar, sustained enhanced AANAT activity. Furthermore, we suggest unexpected alternative routes for this enhancement, by showing that several activators of protein kinase C, the calcium-, diacylglycerol-activated kinase, led to a similar enhancement in AANAT catalytic activity, strongly suggesting that the mechanism by which melatonin production is regulated in cells is not limited to a cAMP route but also follows a protein kinase C activation pathway. The fact that protein kinase A phosphorylates hAANAT in vitro strongly strengthens the hypothesis that hAANAT is regulated by this pathway. In contrast, the fact that protein kinase C does not phosphorylate hAANAT in vitro suggests that activation by protein kinase C is an indirect process. Indeed, protein kinase C activates a series of pathways, some of which lead to the activation of PKA [56, 57]. Interestingly, both serine/threonine kinases, rho-kinase and chk-1, are able in vitro to phosphorylate and to subsequently activate hAANAT. The extent of this activation is higher with chk-1 (1.5-fold) than with protein kinase A, leading to a possible additional regulation of hAANAT activation and/or catabolism. Indeed, whereas the level of hAANAT activity in 1E7 (COS cell based) is similar to that of rat pineal gland at night, the level of hAANAT activity in the CHO-derived cell line was more than ten-fold higher at night, and better mimicked the activity of the nocturnal ungulates, which have a circadian rhythm similar to humans'.

Considering the CHO cell line-derived model for the study of hAANAT inhibition, the first results reported here clearly indicate that more studies and compound screening are needed before obtaining the right compounds to inhibit the enzymatic activity in vivo. In short, the bisubstrate compound (acetyl-CoA-tryptamine) reported by De Angelis et al. [58] cannot penetrate the cell. The bisubstrate precursors [59], such as BAT, S 28036 and S27481, are potent compounds which inhibit enzyme activity by producing a potent inhibitor, via an *in situ* mechanism at the catalytic-site level. Finally, the peptide inhibitors derived from combinatorial libraries did not score positively in the cellular assay, and we confirmed in the present report that they are unable to penetrate the cell. Further synthetic compounds, belonging to several other chemical classes, are being developed at our institute, either derived from the initial medium-throughput screen-

ing experiments reported elsewhere [10] or from melatonin structure [60].

In summary, in the present report, we describe the cellular activities of known inhibitors of hAANAT, as well as the presence in this particular cell line of some of the post-translational mechanisms that may regulate the function of this particular enzyme. Most of the tools are now ready to open new routes towards new inhibitors acting on hAANAT in vitro, and to identify compounds that are also active in a living organism. Furthermore, two interesting compounds, S 28036 and S27481, both potent inhibitors of AANAT in situ, have been identified for further testing in an animal model.

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