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Biochemical Pharmacology 61 (2001) 1369–1379

Biochemical Pharmacology

Comparative pharmacological studies of melatonin receptors: MT1, MT2 and *MT*3/QR2. Tissue distribution of *MT*3/QR2

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Received 19 October 2000; accepted 4 December 2000

Abstract

The neurohormone melatonin is the central switch of the circadian rhythm and presumably exerts its activities through a series of receptors among which MT_1 and MT_2 have been widely studied. The third binding site of melatonin, MT_3, has been recently characterized as a melatonin-sensitive form of the quinone reductase 2 (QR_2, EC 1.6.99.2). In the present work, we showed that the binding of melatonin at MT_3/QR_2 was better described with 2-[^{125}I]-iodomethoxy-carbonylamino-N-acetyltryptamine (2-[^{125}I]-I-MCA-NAT) and, most importantly, that it was measurable at 20° while it has been initially described and thoroughly studied using 2-[^{125}I]-iodomelatonin at 4°. Under these novel conditions, binding to MT_3 could be traced without cross-reactivity with MT_1 and MT_2 receptors and, moreover, under conditions similar to those used to measure MT_3/QR_2 catalytic activity. The pharmacology established here on hamster kidney samples using the reference compounds remained essentially as already described using other experimental conditions. A new series of compounds with nanomolar affinity for the MT_3 binding site and a high MT_3 selectivity versus MT_1 and MT_2 is reported. In addition, we further document the MT_3/QR_2 binding site by demonstrating that it was widely distributed among mammals, although inter-species and inter-tissues differences exist. The present report details new experimental conditions for the pharmacological study of melatonin-sensitive QR_2 isoforms, and suggests that, in addition to an already demonstrated inter-species difference, inter-tissues differences in QR_2 sensitivity to melatonin may exist in primates and, therefore, represent an original and interesting route of investigation on the effect of melatonin on MT_3/QR_2.

Keywords: Melatonion receptor; MT1; MT2; *MT3*; Quinone reductase; Tissue distribution; Inhibitors; Ligands

1. Introduction

Melatonin is an indole-derived neurohormone of long standing interest which is produced in the pineal gland and is derived from serotonin. The main feature of the pharmacodynamics of melatonin is its nocturnal synthesis and secretion. As a consequence, melatonin is suspected to relay the circadian rhythm and the information on the photoperiod to the peripheral organs for daily and seasonal physiological regulations. Furthermore, melatonin has a proven role in the sleep/wake cycle [1], and is involved in numerous physiological functions depending on the circadian rhythm, such as the immune [2] and the cardiovascular systems [3]. Many cellular targets of melatonin have been detected since 1986, after the synthesis of 2-iodomelatonin [4], a very potent melatonin agonist which was rapidly used as 2-[^{125}I]-iodomelatonin for labeling tissue sections and performing pharmacological studies [5,6]. Melatonin binding sites were initially detected in the central nervous system of rodents and chicken, and then, in numerous peripheral tissues of birds and mammals [6–8], suggesting their widespread distribution among tissues. Three melatonin receptor isoforms

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Abbreviations: MCA-NAT, methoxy-carbonylamino-N-acetyltryptamine, 2-[^{125}I]-I-MCA-NAT, 2-[^{125}I]-iodomethoxy-carbonylamino-N-acetyltryptamine; 2-IbMT, 2-iodo-N-butanoyl-5-methoxytryptamine; 4-P-PDOT, 4-phenyl-2-propionamido-tetraline; DH97, N-pentanoyl-2-benzyltryptamine; S20760, 5-methoxy-N-cyclopropanoyl-tryptamine; S24635, N-[2-(5-carbamoylbenzofuran-3-yl)ethyl]-acetamide; S25726, N-methyl-(3-{2-[(cyclopropylcarbonyl)-amino]ethyl}benzo[b]furan-5-yl)carbamate; S26553, N-methyl-{1-[2-(acetylamino)ethyl]-naphthalen-7-yl}carbamate.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00615-3

have been cloned to date. The Mel1a gene encodes the MT_1 receptor [9], the Mel1b gene encodes the MT_2 receptor [10] and the Mel1c was cloned from *Xenopus laevis* [11] but is not expressed in mammals [12]. The MT_1 and MT_2 receptors share a common seven-transmembrane predicted structure and the ability to transduce membrane signals *via* G-protein coupling [13,14]. These two receptors also share a close pharmacological profile, with the following order of affinities 2-iodomelatonin > melatonin > 6-hydroxymelatonin ≫ N-acetylserotonin ≫ prazosin [15]. The MT_1 and MT_2 receptors are also characterized by subnanomolar affinities for melatonin and 2-iodomelatonin.

In addition to these high affinity melatonin receptors, there is evidence for a nanomolar melatonin binding site in Hamster brain [15–17] and kidney [18,19], MT_3. The ligands known to date of MT_3 specificity over MT_1 and MT_2 include prazosin [17] and 5-methoxycarbonylamino-N-acetyltryptamine (MCA-NAT; [18]). Besides its original pharmacology, MT_3 has always displayed very fast kinetics of ligand association/dissociation [16, 18,19], raising difficulties for affinity measurements. Because of this property, MT_3 has always been studied at low temperature (0° or 4°) in order to impair the fast ligand dissociation kinetics. Despite major studies conducted on MT_3 binding sites, it remains that widely different experimental conditions may have occulted differences in MT_1, MT_2 and MT_3 ligand specificities, especially with the large utilization of 2-[^{125}I]-iodomelatonin as a radioligand, although 2-[^{125}I]-iodomethoxycarbonylamino-N-acetyltryptamine (2-[^{125}I]-I-MCA-NAT) was proven to be a more specific tool [18]. Further, our recent description of the MT_3 pharmacology [20] needs to be completed by a better assessment of the experimental conditions under which MT_3 is best described, i.e. with the specific radioligand 2-[^{125}I]-I-MCA-NAT and at 20°. The recent purification of MT_3 and its identification as the quinone reductase 2 (QR_2, EC 1.6.99.2), an enzyme related to the detoxifying enzyme quinone reductase 1, shed new lights on the pharmacological characterization of all the melatonin binding sites [20]. Indeed, melatonin binding sites can now be regarded both as a population of proteins encompassing genuine membrane receptor activity leading to intracellular signaling and, alternatively, as fugacious melatonin binding sites with yet unresolved function.

We describe in the present paper a new standard for the molecular pharmacology of MT_3/QR_2 receptor at higher temperature (as opposed to 4°), including comparisons with the MT_1 and MT_2 receptor pharmacology. Furthermore, we describe new compounds as potent as prasozin but more specific for the MT_3 binding site. We also discuss the relevance of the comparison of MT_3 binding and QR_2 enzymatic activity after determining the tissue distribution of the two signals in different species.

2. Materials and methods

2.1. Materials

2-[^{125}I]-melatonin (2200 Ci/mmol) was purchased from NEN, 2-IbMT (2-iodo-N-butanoyl-5-methoxytryptamine), 2-iodomelatonin, 2-phenylmelatonin, 4-P-PDOT (4-phenyl-2-propionamidotetraline), 6-chloromelatonin, DH-97 (N-pentanoyl-2-benzyltryptamine), luzindole (N-acetyl-2-benzyltryptamine), MCA-NAT (5-methoxycarbonylamino-N-acetyltryptamine) and S20760 (5-methoxy-*N*-cyclopropanoyl-tryptamine) were purchased from Tocris and all other reagents were obtained from Sigma-Aldrich. S24635, S25726 and S26553 were synthesized according to Lesieur *et al*. [21].

2-[^{125}I]-MCA-NAT (2200 Ci/mmol) was synthesized by Iodine Ligand Development, Amersham Pharmacia Biotech, according to the following procedure. Iodo-Gen (100 μg in 100 μL of chloroform, Pierce) was coated on the reaction vial. Sodium [^{125}I]-iodide (1110 MBq in 170 μL, Amersham Pharmacia Biotech) and MCA-NAT (300 μg in 300 μL of 200 mM phosphate buffer, pH 6.0) were carefully added to the Iodo-Gen coated vial and allowed to react for 50 min. The reaction was terminated by transferring out the reaction mixture into a solution of sodium metabisulfite (200 μg in 200 μL). The reaction mixture was loaded onto a Vydac C-18 RP-HPLC column (250 × 4.6 mm) and [^{125}I]-I-MCA-NAT (74 TBq/mmol) was eluted using a linear gradient of water in methanol. The purified compound was diluted with ethanol and stored at 4°.

2.2. Preparation of tissue homogenates

Animal tissues were obtained either from Charles River Breeding Laboratories (rabbit, hamster, rat or mouse) as deep-frozen organs or from control animals (monkey, dog, pig, Yucatan (mini-pig) and guinea-pig) in toxicological or pharmacological studies approved by our Ethics Committee. Organs were carefully dissected, intensively washed in ice-cold PBS and snap-frozen in liquid nitrogen. They were maintained at −80° until further use.

2.3. Preparation of membrane extracts

Pellets of human embryonic kidney cell lines HEK293 stably expressing MT_1 or MT_2 human receptors were prepared as already described [19]. Male Syrian hamster kidneys and brains were obtained frozen from Charles River Breeding Laboratories. Membranes of MT_1- or MT_2-expressing HEK293 were prepared as described earlier [19] and membranes of hamster kidney and brain cells were prepared by the following procedure, all steps being performed at 4°. Tissues were thawed, chopped using a surgical blade and resuspended in 5 volumes of 50 mM Tris-HCl (pH 7.5) containing 250 mM sucrose, 1 mM $CaCl_2$ and protease inhibitors as a cocktail commercialized by Boehr-

inger Mannheim (one tablet of Complete™ in 50 mL). The cells were then gently disrupted by 10 strokes of a Dounce glass homogenizer. The nuclei and the unbroken material were pelleted by a spin of 10 min at 280 *g*. The supernatant was saved while the pellet was resuspended in half of the original volume and was subjected again to the Dounce and the centrifuge as described above. The two successive supernatants were pooled, five-fold diluted with 20 mM Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$ and protease inhibitors and were subjected to a 60-min centrifugation at 400,000 *g*. The membrane pellets were resuspended with a Dounce as a 2–4 mg/mL protein suspension, as measured by the method of Lowry [22] adapted for membrane proteins (DC Protein assay, BioRad) using BSA as a standard. Membrane preparations were flash-frozen in liquid nitrogen and were stored at −80° until use. Membranes from HEK293 cells expressing MT_1 or MT_2 and membranes from Syrian hamster kidney or brain are referred to as MT_1, MT_2 and MT_3 membranes respectively throughout the text.

2.4. Equilibrium binding assays

Binding experiments on MT_1 and MT_2 membranes were realized as described before [19]. Briefly, samples (10 μg of proteins) were incubated for 2 hrs at 37° with 25 pM (MT_1) or 170 pM (MT_2) 2-[^{125}I]-melatonin in the presence (non-specific binding) or not (total binding) of 10 μM melatonin and with varying concentrations of test drugs. Incubations were carried out in triplicates in 96-well microplates and were terminated by filtration through 96-well format glass-fiber plates (GF/B Unifilter, Packard) using a Filtermate (Packard) apparatus. Membranes were then washed three times with 2 mL of 50 mM Tris-HCl (pH 7.5) buffer before the addition of 30 μL per well of scintillation liquid (Microscint 20, Packard) and counting in a ß scintillation counter (TopCount NXT, Packard).

Binding experiments on MT_3 kidney membranes were performed on 100 μg of proteins in 20 mM Tris-HCl (pH 7.5) buffer containing 1 mM $CaCl_2$ (binding buffer) in a final volume of 150 μL. Filtrations were performed through 96-well glass-fiber supports (GF/B Unifilter, Packard) presoaked for two hours before use in 0.3 % (v:v) polyethyleneimine and rinsed extemporaneously three times with 200 μL per well of binding buffer. After sample filtration, the filters were rinsed once with 100 μL per well of binding buffer. The filtration plates were disposed directly onto a Multiscreen filtering apparatus (Millipore) connected to a vacuum pump, allowing rapid filtration after the samples were loaded using a 96-well pipetting device (Transtar, Costar). Radioactivity was measured as described above. Unless otherwise stated, incubations were performed for 30 min at room temperature (20–25°) using 2-[^{125}I]-I-MCA-NAT as the specific radioligand. In saturation experiments, 75 μL of membrane preparations (100 μg of proteins) were added to 75 μL of binding buffer containing 0 to 6 nM of 2-[^{125}I]-I-MCA-NAT supplemented (non-specific binding) or not (total binding) with 10 μM of MCA-NAT. Competition experiments were realized in similar conditions, in the presence of 200 pM 2-[^{125}I]-I-MCA-NAT and varying concentrations of drugs (8 points, most often spanning 10^{-11} to 10^{-4} M). Binding experiments on MT_3 hamster brain membranes were realized in the same conditions using 2-[^{125}I]-melatonin and melatonin as the labeled and "cold" specific ligands, respectively. The K_d and B_{max} were calculated from the saturation data using the Scatchard representation of the Graph Pad Prism 3.02 software. The K_i were obtained after Prism analysis of the competition data according to the method of Cheng and Prusoff [23]. All the binding experiments were conducted under a sodium light to reduce photolysis of drugs. Results are expressed as the specific binding, i.e. the total binding corrected for the non-specific binding.

2.5. Kinetic binding assays

Association kinetics were performed on MT_3 membranes in the conditions described above, with increasing times of incubation before filtration. Dissociation kinetics were realized after a previous 30 min-equilibration of membranes with 200 pM 2-[^{125}I]-I-MCA-NAT supplemented (non-specific binding) or not (total binding) with 10 μM of MCA-NAT. 100 μL of the reaction medium (150 μL in total) was then mixed with 10 μL of 110 μM MCA-NAT. The samples were then incubated for increasing periods of times before filtration. Due to the rapidity of the kinetics involved, the MT_3 membranes were not rinsed after filtration. The kinetic parameters were obtained after data analysis in the Graph Pad Prism 3.02 software.

2.6. QR_2 enzymatic activity

The measurement of QR_2 quinone reductase activity was performed as previously described [20]. Briefly, the QR_2 activity was measured in 20 mM Tris-HCl pH 8.0, 1 mM *n*-octylglucoside, 100 μM menadione, 100 μM dihydrobenzylnicotinamide (BNAH) and 100 μM dicoumarol to ensure QR_2 specificity over QR_1. The incubations were realized at 25° in 200 μL of reaction medium, and the data were collected by measurement of the fluorescence of the benzylnicotinamide produced (excitation at 340 nm and emission at 440 nm, PolarStar 96-wells plate reader, BMG).

3. Results

3.1. Determination of binding conditions on MT_3 membranes

Using an original technique, based on the distribution of samples directly onto 96 well filter plates under constant vacuum, the filtration of samples and the subsequent rinsing of the filters lasted clearly less than one second, partly

overcoming the rapidness of ligand dissociation from the MT_3 binding sites. It was then possible to perform comparative binding assays on MT_1, MT_2, and MT_3 membranes using 2-[^{125}I]-iodomelatonin and 2-[^{125}I]-I-MCA-NAT at different temperatures. Fig. 1 shows the high specificity of 2-[^{125}I]-I-MCA-NAT for MT_3 vs MT_1/MT_2, which were barely detected. We can also note that 2-[^{125}I]-I-MCA-NAT binding on MT_3 was about twice as important as 2-[^{125}I]-iodomelatonin binding. Because 2-[^{125}I]-iodomelatonin and 2-[^{125}I]-I-MCA-NAT were used at the same concentration in both experiments, this result suggests a higher affinity of 2-[^{125}I]-I-MCA-NAT for MT_3. Interestingly, the binding performed at 20° on MT_3 membranes was about 50% of the signal measured at 4°, demonstrating the possibility of performing the pharmacological study of MT_3 at room temperature. Similar values of specific binding were obtained when MCA-NAT or melatonin were used for the determination of non-specific binding. MT_1 and MT_2 receptors were less sensitive to temperature fluctuations of 2-[^{125}I]-iodomelatonin binding than MT_3, and, as expected from their known pharmacological properties, higher specific binding was obtained when melatonin was used for the determination of non-specific binding. These results set up new conditions for specific and relevant experimental conditions for MT_3 pharmacological characterization. Namely, 2-[^{125}I]-iodomelatonin/melatonin were subsequently used for the classical binding on MT_1 and MT_2 membranes at 37°, while 2-[^{125}I]-I-MCA-NAT/MCA-NAT was used for the binding on MT_3 membranes at 20°.

3.2. Characterization of 2-[^{125}I]-I-MCA-NAT binding sites at 20°

The kinetics of association and dissociation of 2-[^{125}I]-I-MCA-NAT on MT_3 kidney membranes were determined at 20° and, as expected, the results showed extremely rapid exchange rates (Fig. 2), with saturation reached after one minute and complete dissociation after 20 sec. Indeed, 2-[^{125}I]-I-MCA-NAT displayed rapid kinetic association and dissociation constants [$k_{+1} = 0.00054 \pm 0.00015$ sec^{-1}. pM^{-1} and $k_{-1} = 0.303 \pm 0.059$ sec^{-1}, respectively (half life 2.36 ± 0.44 sec)]. Hence, the deduced affinity constant was $k_{-1}/k_{+1} = 561$ pM.

Saturation experiments and Scatchard analysis (Fig. 3) revealed a single 2-[^{125}I]-I-MCA-NAT binding site in Syrian hamster kidney membranes (linear regression, $R^2 = 0.995$). The calculated affinity constant was 549 pM, in agreement with the value calculated from the kinetic parameters (561 pM) and close to the value determined on Syrian hamster brain membranes at 4° (164 pM; [18]). The total number of binding sites was estimated to 173 fmol/mg proteins, which provided comfortable binding conditions for further studies on hamster kidney membranes.

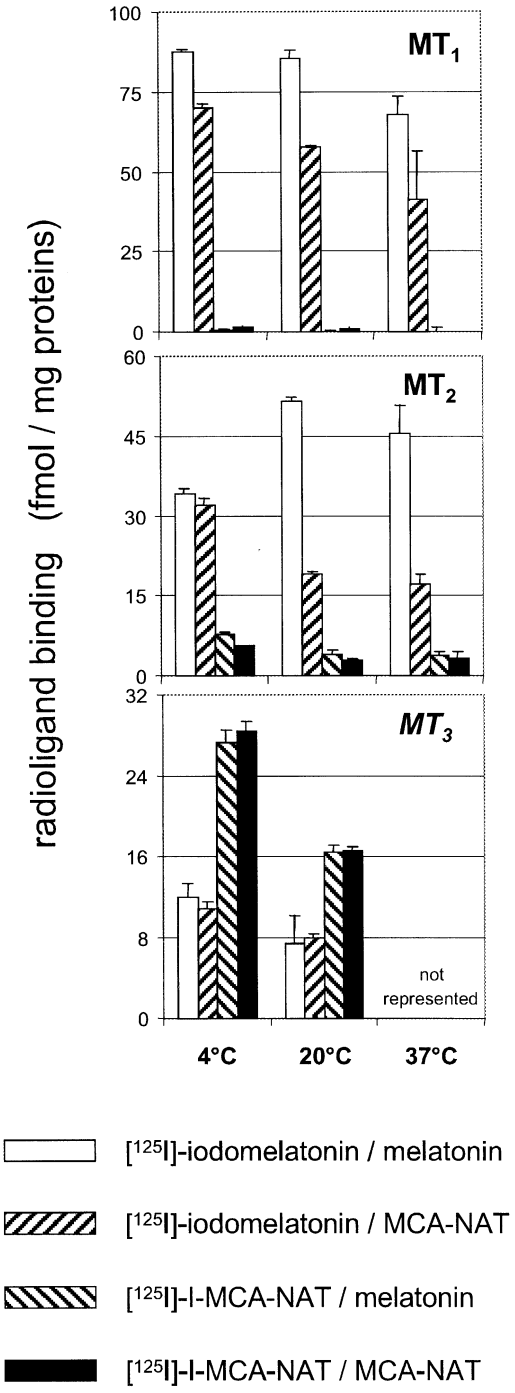


Fig. 1. Binding of 2-[^{125}I]-melatonin and 2-[^{125}I]-I-MCA-NAT to HEK-MT_1 (MT_1), HEK-MT_2 (MT_2) and Syrian hamster kidney (MT_3) membranes at different temperatures. Membranes (MT_1 and MT_2 10 μg, MT_3 100 μg) were incubated for 3 hr with 2-[^{125}I]-melatonin or 2-[^{125}I]-I-MCA-NAT, and the non-specific binding (NS) was determined in the presence 10 μM melatonin or MCA-NAT as follows: open bars, 2-[^{125}I]-melatonin (NS 10 μM melatonin); upward hatched bars, 2-[^{125}I]-melatonin (NS 10 μM MCA-NAT); downward hatched bars, 2-[^{125}I]-I-MCA-NAT (NS 10 μM melatonin); solid bars, 2-[^{125}I]-I-MCA-NAT (NS 10 μM MCA-NAT). The radioligand concentrations were 25 pM for MT_1, 170 pM for MT_2, and 200 pM for MT_3. Incubations were performed at 4°, 20° or 37°, but filtrations were performed at 4°, 20°, and 20°, respectively, as detailed in section 2. Each point represented is the mean of triplicates and experiments were repeated twice with similar results.

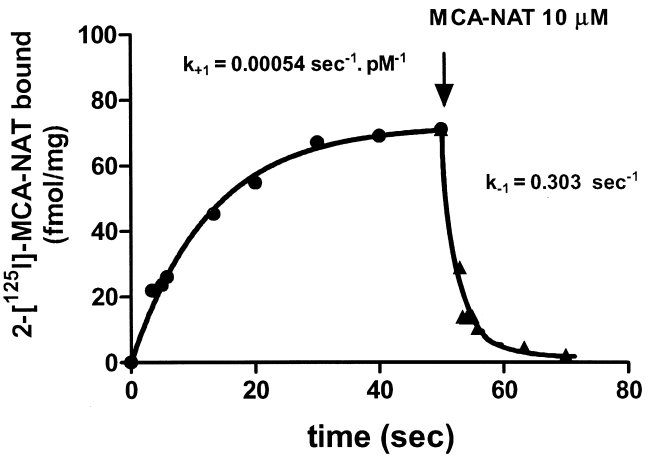


Fig. 2. Kinetics of association and dissociation of 2-[^{125}I]-I-MCA-NAT with Syrian hamster kidney (MT_3) membranes at 20°. Association kinetics were determined by incubation of Syrian hamster kidney membranes (100 μg) with 200 pM 2-[^{125}I]-I-MCA-NAT for varying periods of time before filtration. Dissociation kinetics were determined on membranes pre-equilibrated for 30 min with 2-[^{125}I]-I-MCA-NAT and were initiated by the addition of 10 μM final MCA-NAT. The filtration procedure was performed as described in section 2 and resulted in filtration times of much lower than one second. The experiments were conducted at 20°, and each point represented is the mean of three values. Each experiment was repeated twice with similar results.

3.3. Pharmacology

The previous MT_3 pharmacological profiles reported in the literature were performed at 4° with the ligands 2-[^{125}I]-iodomelatonin [17,19] or 2-[^{125}I]-I-MCA-NAT [18]. Here, in order to lay a standard for the pharmacology of this binding site more in line with its enzymatic nature (as reported by Nosjean *et al*. [20]), we determined the MT_3 pharmacological profile at 20° using 200 pM 2-[^{125}I]-I-MCA-NAT. These data were compared with the results obtained from binding experiments using 2-[^{125}I]-iodomelatonin on MT_3 at 4°, and on MT_1 and MT_2 at 37°, respectively. Hence, all melatonin binding sites have been studied in their most appropriate conditions. The compounds were chosen as representatives of MT_2, MT_1 + MT_2, and MT_3 families of ligands, and were presented according to this classification in Table 1, by decreasing order of affinity. Our study included novel commercially available ligands (DH 97 or N-pentanoyl-2-benzyltryptamine, 2-IbMT or 2-iodo-N-butanoyl-5-methoxytryptamine and S 20760 or 5-methoxy-N-cyclopropanoyltryptamine) as well as several compounds synthesized at the Institute (Fig. 4). The data obtained are in overall good agreement with previous reports on MT_1, MT_2, and MT_3. The K_i of the ligands already described in the literature with the same radioligand [18] do not differ by a factor greater than four, underlining the univocal interpretation of 2-[^{125}I]-I-MCA-NAT pharmacology on MT_3 membranes. Furthermore, the pharmacological profile of MT_3 obtained with 2-[^{125}I]-iodomelatonin in previous studies (2-iodomelatonin > 6-chloro-melatonin > MCA-NAT = prazosin = N-acetylserotonin = melatonin, [19]) closely resembles our classification (2-iodomelato-

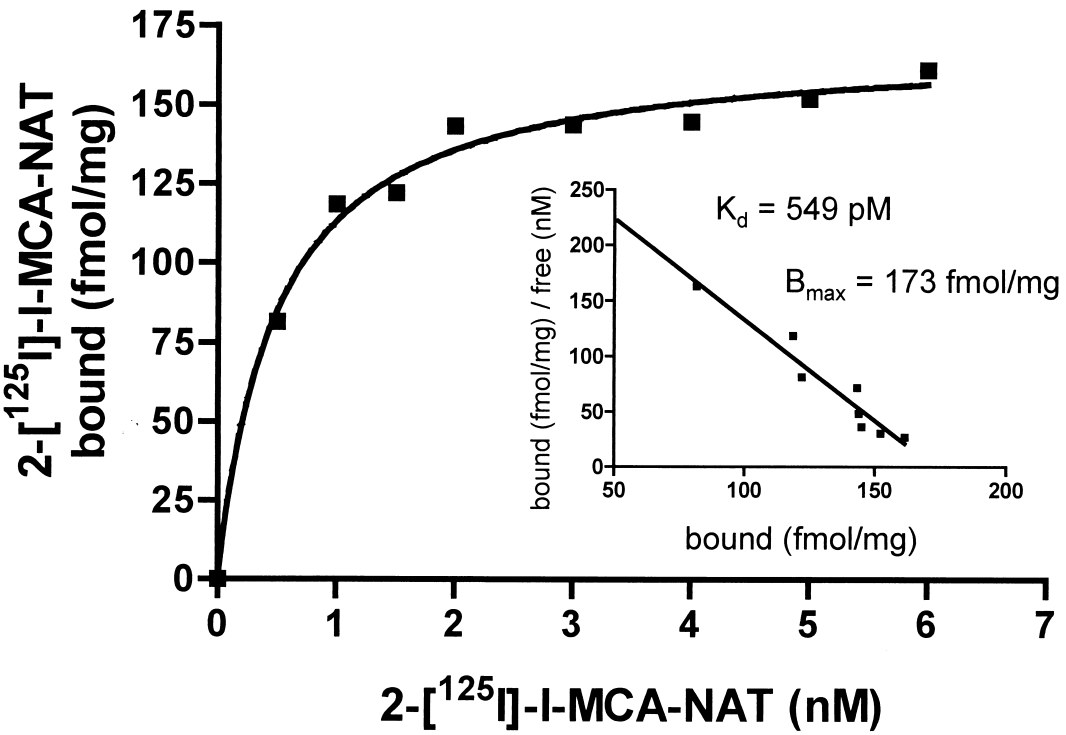


Fig. 3. Saturation and Scatchard analysis of 2-[^{125}I]-I-MCA-NAT specific binding of on Syrian hamster kidney (MT_3) membranes. MT_3 membranes (100 μg) were incubated for 30 min at 20° with 0 to 6 nM 2-[^{125}I]-I-MCA-NAT and were further proceeded as described in section 2.

Table 1
Comparison of the pharmacological profiles of MT_3 binding site with MT_1 and MT_2 melatonin receptors

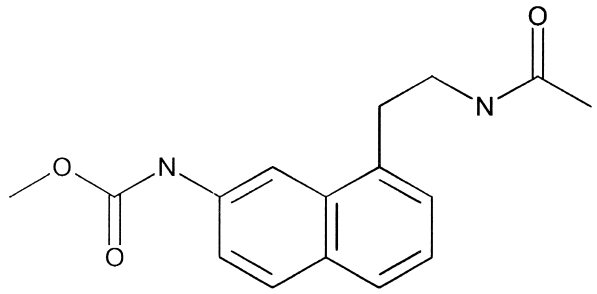
	K_i (nM)				K_i ratios
	HEK-MT_1 (MT_1 membranes)	HEK-MT_2 (MT_2 membranes)	Hamster brain (MT_3 membranes)	Hamster kidney (MT_3 membranes)	MT_3/MT_1; MT_3/MT_2
Radioligand	2-[^{125}I]-iodomelatonin	2-[^{125}I]-iodomelatonin	2-[^{125}I]-iodomelatonin	2-[^{125}I]-I-MCA-NAT	
MT_1 + MT_2 ligands					
2-iodomelatonin	0.013 ± 0.002	0.2 ± 0.03	3.7 ± 0.53	8.35 ± 2.3¶	640; 40
2-IbMT	0.051 ± 0.0013	0.163 ± 0.0113	8.92 ± 0.62	17.3 ± 5.1	340; 110
2-phenylmelatonin	0.020 ± 0.003	0.090 ± 0.008	33 ± 1.6	31 ± 11.6	1,550; 340
melatonin	0.12 ± 0.02	0.31 ± 0.05	56.9 ± 0.4	277 ± 22	2,300; 890
6-chloromelatonin	0.60 ± 0.14	0.24 ± 0.04	9.9 ± 0.11	35 ± 5.9¶	60; 150
S20760	1.55 ± 0.13	5.55 ± 0.61	77 ± 10	209 ± 64	134; 40
MT_2 ligands					
4-P-PDOT	220 ± 42	1.04 ± 0.30	4,022 ± 256	6,333 ± 2,375	30; 6,100
luzindole	474 ± 9.2	23.3 ± 0.5	1,381 ± 100	1,157 ± 304¶	2; 50
DH97	1,100 ± 135	252 ± 44	1,862 ± 310	4,430 ± 2,325	4; 20
MT_3 ligands					
S26553	73 ± 7.5	26 ± 11	0.26 ± 0.05	3.0 ± 1.0¶	0.04; 0.11
prazosin	539 ± 70	5,303 ± 998	3.3 ± 0.15	56 ± 2.3	0.10; 0.01
S25726	>10,000	>10,000	16.0 ± 1.1	62 ± 17	<0.006
MIA	>10,000	>10,000	15.2 ± 0.49	64 ± 17	<0.006
S24635	>10,000	>10,000	54 ± 3.6	82 ± 10	<0.008
MCA-NAT	667 ± 167	3,500 ± 2,500	65 ± 1.7	81 ± 19¶	0.12; 0.02
acridine orange	>10,000	>10,000	16.4 ± 2.7	95 ± 19.7	<0.009
rolipram	>10,000	>10,000	70 ± 2.3	537 ± 11	<0.05

Competition studies were performed using the specified radioligand and eight concentrations of drugs. K_i were calculated from the IC_{50} values using the method of Cheng and Prusoff (Cheng and Prusoff, 1973). Values represent the means of two to four independent experiments performed in triplicate.
¶ These data are part of a previous report [20].

nin > 6-chloromelatonin = prazosin = MCA-NAT = melatonin), although absolute values vary. Furthermore, a program of medicinal chemistry was set up and led to potent chemical structures with high MT_3 specificities. Among them, a series of MCA-NAT analogues were obtained, particularly a naphthyl derivative (S 26553) with a K_i of 3.0 ± 1.0 nM but a poor selectivity (MT_3/MT_1 = 0.04 and MT_3/MT_2 = 0.11, see Table 1). To the contrary, benzo-furanyl analogues such as S 25726 and S 24635 were synthesized with high potencies towards MT_3 (16 and 53 nM, respectively) and deprived of any affinity for MT_1 and MT_2 (K_i > 10 μM), leading to selective compounds (MT_3 versus MT_1 or MT_2) over a hundred fold. MT_3 has some other ligands with affinity in the nanomolar range, but the selectivity of these compounds for MT_3 over the other melatonin receptors is poor (e.g. 2-iodomelatonin, 2-phenylmelatonin, 2-IbMT and 6-chloromelatonin). The pharmacology of MT_1 and MT_2 receptors confirms previous data [24–26,19], where it can be observed that, firstly, substituted melatonin analogues are equally potent for MT_1 and MT_2, and that, secondly, subtype specificity is achieved through structures very loosely related to the melatonin core such as 4-P-PDOT.

3.4. Tissue distribution of MT_3/QR_2

Several tissues were tested for the presence of the MT_3 melatonin binding site. Data were collected from binding experiments performed on total proteins of tissues from different origins, using 2-[^{125}I]-I-MCA-NAT as the radioligand. The same samples were also assayed for QR_2 enzymatic activity, in order to confirm or not the inter-species differences previously observed between hamster and human MT_3/QR_2 [20]. In Fig. 5A, hamster data are represented by decreasing order of MT_3 binding in the different tissues, with the highest amounts in liver and kidney, modest amounts in brain, heart, brown adipose tissue, and low amounts in skeletal muscle and lung. The QR_2 followed the same distribution, except proportionally higher values in lung. Mouse tissues (Fig. 5B) displayed a similar pattern of distribution of MT_3 and QR_2 signals in tissues. In dog (Fig. 5C), the few organs tested also showed an overall good correlation of MT_3 and QR_2 data, with highest values in brain (MT_3 data) or brain, kidney and liver (QR_2 data). Interestingly, in monkey—*Macaca fascicularicus* (Fig. 5D)—the distribution among organs showed a similar MT_3 pattern as described above, but a surprisingly different pattern of distribution of the QR_2 signal. The most striking feature of this discrepancy may be the difference observed in the skeletal muscle sample, where the MT_3 binding was within the background level, whereas the QR_2 activity was the highest among the tissues tested. Furthermore, in addition to the comparison of the overall pattern of distribution of MT_3 and QR_2 signals among tissues, it is noteworthy that the absolute values of MT_3 binding sites and QR_2 enzymatic activity varied in a wide range between species. MT_3 bind-



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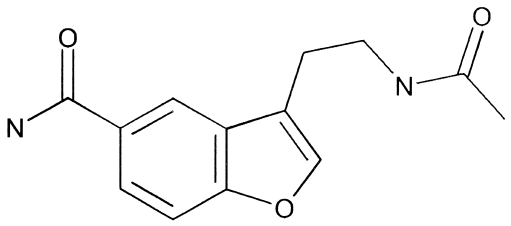
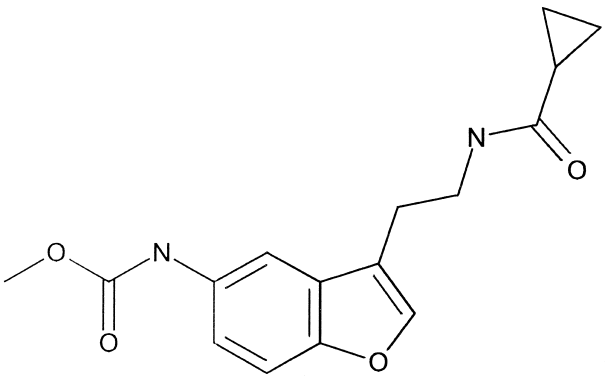
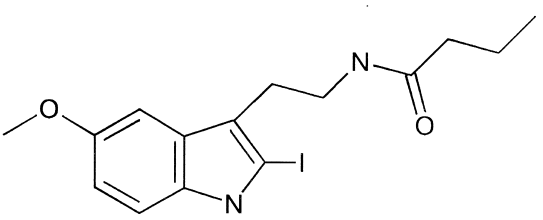


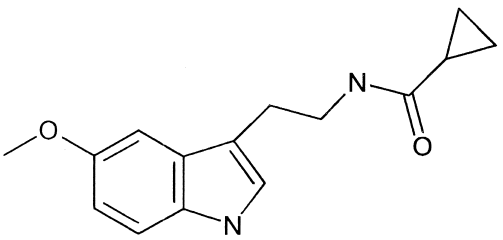
S 24635



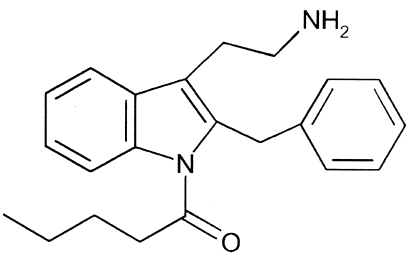
S 25726



2-IbMT



S20760



DH97

Fig. 4. Chemical structure of some of the synthetic compounds included in this study. 2-IbMT: 2-iodo-*N*-butanoyl-5-methoxytryptamine; DH97: *N*-pentanoyl-2-benzyltryptamine; S20760: 5-methoxy-*N*-cyclopropanoyltryptamine; S24635: *N*-[2-(5-carbamoylbenzofuran-3-yl)ethyl]-acetamide; S25726: *N*-methyl-(3-{2-[(cyclopropylcarbonyl)amino]ethyl}benzo[b]furan-5-yl)carbamate; S26553: *N*-methyl-{1-[2-(acetylamino)ethyl]naphthalen-7-yl}carbamate.

ing sites were best detected in CD-1 mouse (1 to 13 fmol/mg) and hamster (1 to 8 fmol/mg) but in lower amounts in dog and monkey (below 2 fmol/mg). QR_2 was detected in similar quantities in all but one animals (1 to 8 nmol/min/mg), and in lower amounts in monkey (1 to 4 nmol/min/mg).

4. Discussion

In addition to the now well documented seven transmembrane domains G protein-coupled receptors, MT_1 and MT_2, several melatonin binding sites are presented as putative

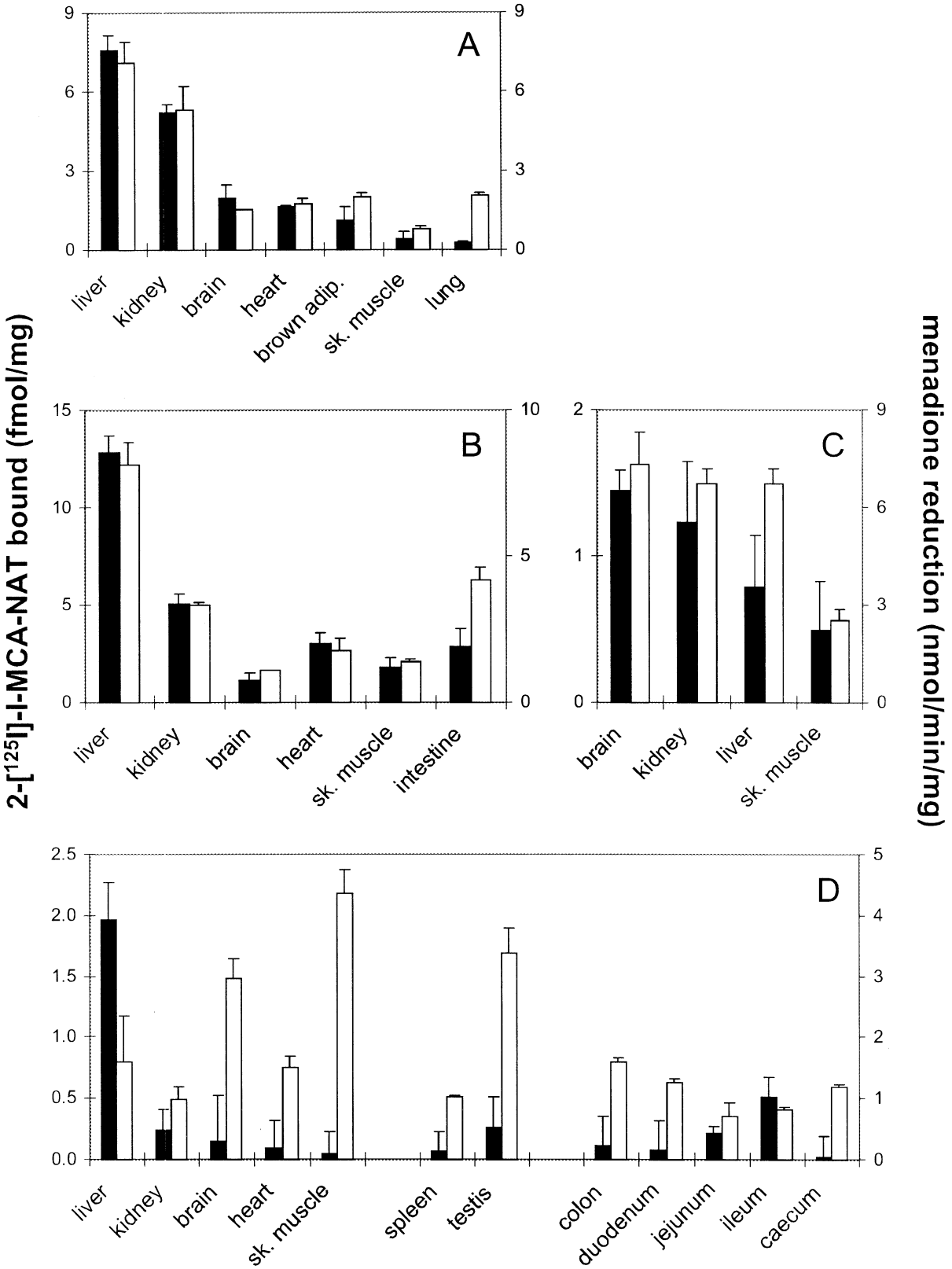


Fig. 5. MT_3 binding (solid bars, left vertical axes) and QR_2 activity (open bars, right axes) in cell homogenates from different tissues. MT_3 binding: 0.2 nM 2-[^{125}I]-I-MCA-NAT was incubated at 20° with 100 μg of proteins from total cell lysates according to the procedure described in section 2. QR_2 activity: menadione reduction was followed by the concomitant oxydation of dihydrobenzylnicotinamide into its fluorescent product, benzylnicotinamide as described in Section 2. Tissues were obtained from Syrian hamster (A), from CD-1 mouse (B), from dog (C) and from *Macaca fascicularicus* (D). Data represented are the mean of two independent experiments performed in triplicate.

melatonin receptors. Among these are the MT_3 binding site, the nucleus-associated site identified by 2-[^{125}I]-melatonin binding [27–29], a site on adipocytes [30] and several other binding sites with original 2-[^{125}I]-iodomelatonin binding properties (discussed in [19]). The MT_3 melatonin binding site was originally described by Pickering and Niles [17] and was confirmed by other pharmacological studies [15, 19]. A specific ligand, 2-[^{125}I]-I-MCA-NAT, was synthesized to discriminate this site from other melatonin-binding sites [18]. Our recent identification of MT_3 as the quinone reductase 2 [20] confirmed previous suspicions that this binding site was in fact an enzyme, particularly based on extremely rapid association/dissociation kinetics of the ligands (at the scale of the second: [16,18,19]). This finding was accompanied by a series of studies in which the melatonin pharmacology of quinone reductase 2 was achieved with pharmacological as well as enzymatic methodologies (i.e., competition with 2-[^{125}I]-I-MCA-NAT binding and inhibition of menadione enzymatic reduction, respectively). The data obtained correlate the potency of melatoninergic compounds to inhibit MT_3 binding with their potency to inhibit QR_2 enzymatic activity. Thereafter, we now view the 2-[^{125}I]-I-MCA-NAT binding as a tracer of melatonin-sensitive QR_2, until a full assessment of the functional role of melatonin towards QR_2 is reached.

To achieve this, specific binding conditions must be set up, in which the rapid ligand dissociation can be overcome to perform room temperature studies. Indeed, the question of the temperature used for MT_3 pharmacological studies has always been a hint in the design and interpretation of the experiments. Our temperature study demonstrated that, with an appropriate filtering apparatus, pharmacological experiments can be performed on MT_3 at room temperature, ultimately allowing comparison between binding and enzymatic data obtained in similar conditions. The MT_3 binding at 37° was difficult to realize, mainly because the rapidity of ligand exchange necessitates to operate all steps at the desired temperature. In this respect a culture room, a feature not available to us, would have been necessary. Supporting this assumption, the MT_3 binding results obtained after incubation at 20° or 37° were similar when rinsing was performed at 20° (data not shown). Conversely, binding experiments at 4° were realized in a cold room, providing constant temperature over the length of the experiment, in particular during filtration and rinsing steps. In the light of our results, 2-[^{125}I]-iodomelatonin binding at 4° cannot be any longer considered as optimal MT_3-specific conditions, since a significant binding can be obtained in these conditions on MT_1 and MT_2 receptors, thus yielding to ambiguous binding data when investigating melatonin-sensitive MT_3/QR_2 in tissues. Our kinetics of ligand association/dissociation from MT_3 further refined the kinetic constants determined previously [16,18,19], with a value of half-dissociation constant of 0.3 sec^{-1}. Although very fast kinetics of ligand exchange have been previously described with dopamine receptors [31], we follow here the ligand binding and release into or close to the active site of the QR_2 enzyme, which may be analyzed later differently in the context of the complex ping-pong catalytic mechanism of QR_2 enzymatic activity. Therefore, despite legitimate questioning at the time, the physiological relevance of the melatonin/MCA-NAT binding on MT_3 at 4° is now confirmed by our experiments at 20°. The Scatchard analysis of saturation studies also confirms that, in addition to its high specificity, 2-[^{125}I]-I-MCA-NAT is a good ligand for MT_3, with an affinity constant $K_d = 549$ pM, below the Kd of 2-[^{125}I]-iodomelatonin, 1,900 pM [19].

Using the conditions described above, we determined the pharmacological profile of MT_3/QR_2 with 2-[^{125}I]-I-MCA-NAT at 20°, and compared our data with the standard MT_3 pharmacology carried out with 2-[^{125}I]-iodomelatonin at 4°, as well as with the 2-[^{125}I]-iodomelatonin pharmacology of MT_1 and MT_2. No MT_1-specific ligand is available to date with a MT_1/MT_2 ratio over 10-fold, while some MT_2-specific compounds have been described, such as 4-P-PDOT and luzindole. Surprisingly, DH97, although slightly MT_2-selective, did not show the high affinity for MT_2 that has been previously reported ($K_i = 8.03$ nM; [32]). There are several non-discriminating MT_1 and MT_2 ligands with subnanomolar affinity for both receptors. 2-IbMT [33] and S 20760 [34] are interesting synthetic compounds, 2-IbMT for its high affinity and S 20760 for its original naphthalenic structure. Except for S 26553, no MT_3 compound displayed submicromolar affinities on MT_1 and MT_2, confirming the distinct pharmacological properties of the MT_3 melatonin binding site. Surprisingly, the pharmacological characterization of MT_3 at 20° with reference ligands did not bring any major discrepancy when compared to published results, as the inhibition constants were in good agreement with the values obtained on MT_3 at 4° using 2-[^{125}I]-I-MCA-NAT [18]. Furthermore, the MT_3 pharmacology at 4° using 2-[^{125}I]-iodomelatonin confirmed our previous results [19], and showed no marked difference with the pharmacology obtained with 2-[^{125}I]-I-MCA-NAT. It is noteworthy that MT_3 ligands were hardly in the nanomolar range, and that several MT_1 and MT_2 ligands displayed K_i on MT_3 in the range of the K_i of MT_3 compounds (20–200 nM). These findings support the idea of a broad specificity of MT_3, and explain the difficulty in finding specific molecules. The family of MT_3-specific ligands comprises a set of compounds of widely different structure and pharmacological background. Indeed, although these molecules are all built around heteropolycyclic structures, the number of cycles and the nature of their substituents varies greatly, as does the known pharmacological implication of some of these molecules: prazosin is an α_1-adrenergic blocker [35], acridine orange is a dye, MIA (methyl isobutyl amiloride) is an inhibitor of Na^+/H^+ antiport [36] and rolipram is an inhibitor of cyclic AMP phosphodiesterase [37]. Therefore, it was of particular interest to develop new ligands for MT_3, with affinity constants in the nanomolar range and good specificity *versus* MT_1/MT_2.

To investigate and characterize the tissue distribution of MT_3/QR_2, we assayed the 2-[^{125}I]-I-MCA-NAT binding as well as the enzymatic activity in a collection of tissues from different species. In this respect, we primarily focused on hamster tissues, well known for expressing high amounts of MT_3 binding sites. We observed in this animal, as well as in mouse and dog tissues, an overall good correlation between MT_3 binding and QR_2 activity. Hamster MT_3 and QR_2 signals were highest in liver and kidney, while mouse levels peaked in liver only, in agreement with recently published messenger RNA data [38]. MT_3 binding and QR_2 activity were in the same range in the two rodents, while MT_3 binding was much lower in dog, suggesting in this animal the presence of a form of QR_2, which is less sensitive to 2-[^{125}I]-I-MCA-NAT binding. Surprisingly, monkey tissues showed important discrepancies between MT_3 and QR_2 results, without any apparent correlation between the two sets of data. Interestingly, skeletal muscle and brain displayed high levels of QR_2 activity, in agreement with the detection of messenger RNA from human libraries [39]. Besides, liver, kidney and heart also expressed high levels of this mRNA, and gave here modest amounts of QR_2 activity. Therefore, there is an interesting aspect of MT_3/QR_2 properties to be investigated: the tissue-specific distribution and/or regulation of MT_3/QR_2. We can speculate that differences of QR_2 ability to bind 2-[^{125}I]-I-MCA-NAT binding traces a potential regulation by melatonin or melatonin-derived molecules, with potential physiological implications with regards to melatonin peripheral effects. Rodents, such as Syrian hamster and CD-1 mouse, have circadian and seasonal physiological regulations by melatonin, for nocturnal activity and for reproduction, respectively. It may be for this reason that they share a good MT_3/QR_2 correlation, which is distinct from the situation observed in dog and monkey. Indeed, monkeys have a diurnal activity and a poor seasonal reproduction cycle and, thereby, certainly exhibit marked differences with rodents regarding they central and peripheral regulations by melatonin. This could explain why, according to our results presented here and in a previous report [20], monkey and human QR_2 are not regulated by melatonin derivatives as strongly as Syrian hamster QR_2 is.

There is now an open field of investigation for the identification of the most powerful regulators and/or substrates of QR_2 in various species, especially in rodents and primates, and their relationship to indoleamines. In this respect, one can speculate that the reduction of the products of radical oxidation of melatonin by QR_2 would be of considerable importance. This enzyme is so far believed to be, by analogy to QR_1, a detoxifying enzyme, and its relationship to natural compounds is undocumented. For instance, its known substrate is menadione, a synthetic quinone, and its cosubstrate, dihydrobenzylnicotinamide [20], or N-methyldihydronicotinamide, a natural hydrolytic breakdown product of NAD [40]. Furthermore, investigating melatonin regulation of MT_3/QR_2 may have as much interest as finding highest levels of binding sites, as, for instance, in the rat suprachiasmatic nucleus, the density of melatonin binding site does not exceed 2 fmol/mg protein although it is highly responsive to melatonin [41].

In conclusion, we brought new pharmacological evidence about the physiological relevance of the melatonin binding to MT_3/QR_2, although extremely rapid kinetics of exchange are implicated (half life in the second range). We also confirm that a specific pharmacology of MT_3 can be developed. The experimental conditions are now set up for the investigation of the implication of melatoninergic compounds in the regulation of MT_3/QR_2 in various tissues and species. We suggest here to consider MT_3 as a nanomolar melatonin binding form of QR_2, which can be discriminated from other non-melatonin sensitive forms of QR_2 by the use of 2-[^{125}I]-I-MCA-NAT. It remains to be determined if melatonin binding to MT_3/QR_2 is significant of an inhibition of QR_2 oxydo-reductive catalytic activity or, alternatively, if melatonin or some of its metabolic derivatives are involved with MT_3/QR_2 at the catalytic level, as substrate or cosubstrate.

Acknowledgment

The authors are indebted to Sophie Lallier for technical assistance.

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